ANTIFUNGAL ANTIBIOTICS

and

STUDIES ON 8-SUBSTITUTED HOMOPHTHALIMIDES

A Thesis presented by

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to

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for the

Degree

of

Doctor of Philosophy

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To my mother, father and Eve.

CONTENTS

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Part 1.

Studies on 8-substituted homophthalimides

INTRODUCTION

Section 1.1

The addition of amines to acetylenes has been the subject of much study.[1] Even at the turn of the century it was observed[2] that addition occurs smoothly and 1:1 adducts with the amino group β to the activating group are obtained from acetylenic esters, and similar amine additions to acetylenic ketones and sulphenes to give products of the type(1),have later been demonstrated.[3-7]

More recently, the stereochemistry of the enamines resulting from amine addition to acetylenes has been studied using the sophisticated tools available to the modern-day chemist.[5-8]

It has been found that the stereochemistry of the enamine depends on whether the amine nucleophile is primary or secondary.[5-8] With the addition of secondary amines the situation is straightforward with the trans-isomer, sterically and thermodynamically most favoured, being the sole product formed. This has been demonstrated[5-8] by the addition of secondary amines to various activated acetylenes as shown in Table I.

High resolution NMR analysis has proved to be an extremely useful method for discriminating between cis- and trans-isomers, and evaluation of the cis-trans ratio, since the coupling constants between cis- and trans-

TABLE 1.

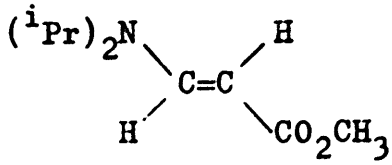
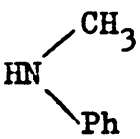
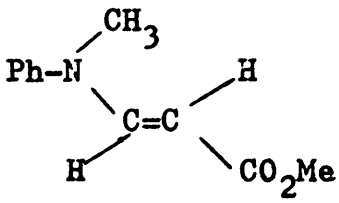
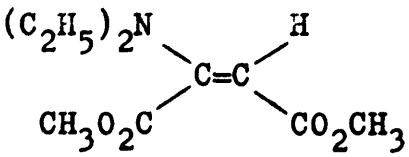
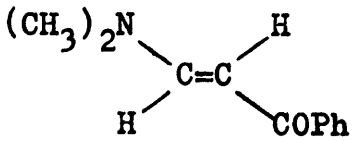
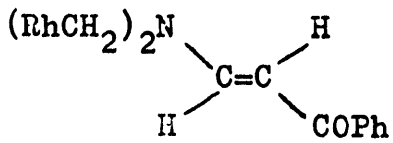
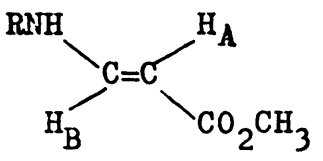
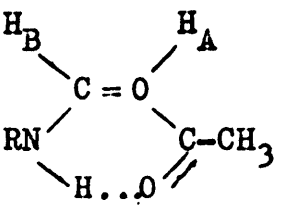
Activated Acetylene	Secondary Amine	Trans Product	Cis Product
$HC{\equiv}C{\cdot}CO_2CH_3$	$HN(^iPr)_2$	 100%	- 0%
$HC{\equiv}C{\cdot}CO_2CH_3$		 100%	- 0%
$CH_3O_2C{\cdot}C{\equiv}C{\cdot}CO_2CH_3$	$HN(C_2H_5)_2$	 100%	- 0%
$HC{\equiv}C{\cdot}COPh$	$HN(CH_3)_2$	 100%	- 0%
HC C·COPh	$HN(CH_2Ph)_2$	 100%	- 0%

TABLE 2.

trans				cis			
							
R	H_A	H_B	J_{AB}	R	H_A	H_B	J_{AB}
n-C_4H_9-	5·44	2·61	13·3	n-C_4H_9	5·66	3·47	8·0
$(CH_3)_2CH$-	5·43	2·67	13·4	$(CH_3)_2CH$-	5·64	3·44	8·1
$(CH_3)_3C$-	5·3	2·59	13·0	$(CH_3)_3C$-	5·60	3·27	8·1

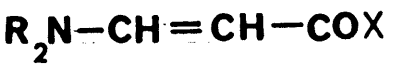
Chemical shift in τ; coupling constants in cycles/sec.

TABLE 3 .

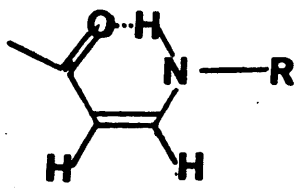
Acetylene	Amine	NMR Solvent	cis (%)	trans (%)
$PhCO \cdot C \equiv CH$	$PhCH_2NH_2$	$CDCl_3$	100	0
		DMSO (5·5 hrs)	50	50
$CH_3O_2C \cdot C \equiv CH$	$PhCH_2NH_2$	$CDCl_3$	70	30
		DMSO (24 hrs)	20	80
$CH_3O_2C \cdot C \equiv CH$	$(CH_3)_3C \cdot NH_2$	$CDCl_3$	100	0
$PhSO_2 \cdot C \equiv C \cdot CH_3$	$PhCH_2NH_2$	$CDCl_3$	65	35

olefinic protons, are markedly dissimilar, as is the chemical shift of a proton cis- or trans- to the activating group. The examples shown in Table 2 clearly illustrate this point. Whereas the addition of a secondary amine to an activated acetylene invariably produces the trans-isomer, the addition of primary amines to acetylenes however results in equilibrium mixtures of enamines where considerable amounts of cis-isomers appear in the mixtures. Examples of this are shown in Table 3 . Stabilisation of the cis-isomer in these cases has been ascribed to hydrogen bonding[6,7] as in (2), and this has been borne out by IR and NMR studies. It has been observed during NMR studies[7] that a change of solvent has a marked effect on the cis-trans-ratio. In chloroform, little hydrogen bonding to the solvent can occur and the intramolecularly bonded cis-forms are thus favoured. Dimethyl sulphoxide, by contrast, forms strong hydrogen bonds and in this solvent a greater degree of intermolecular bonding is expected and so displacement of the equilibrium in favour of the trans-isomer occurs (c.f. Table 3).

In all cases so far reported the adducts obtained with primary amines have the enamino structure (3) and (3a) not the imino structure (4) but enamine-imino tautomerism is an added complication which has recently

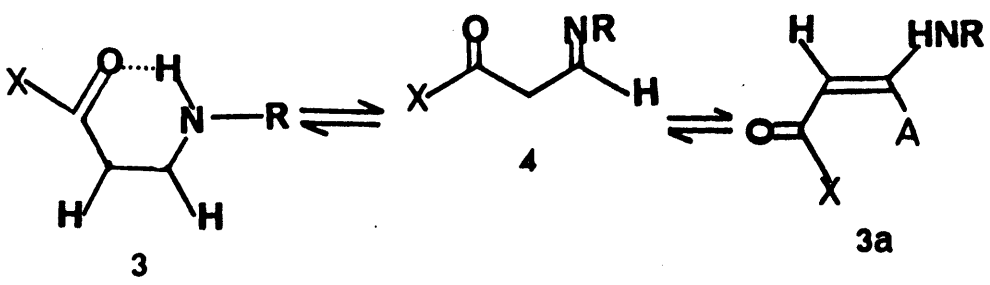


1



2

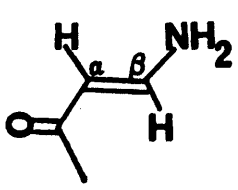
SCHEME 1



3

4

3a



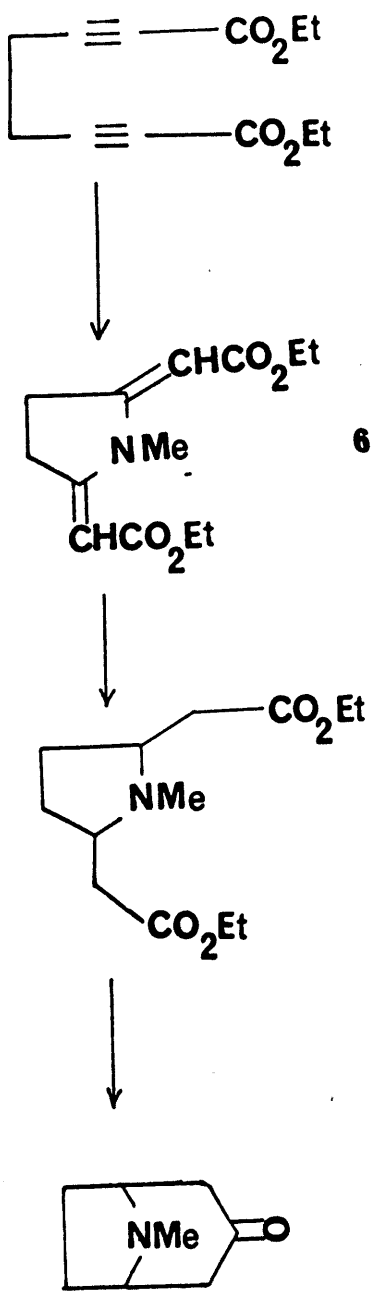
5

been implicated in the cis-trans-isomerisation of enamines.[9] (Scheme I). Despite the fact that the imino form has not been observed experimentally after many attempts to detect it using different techniques, and although it is energetically the most unfavourable, the existance of very small amounts of this form cannot be completely excluded. It has been postulated[9a] that the activation energy of cis-trans-isomerisation is low, in the order of 15 Kcals/mol, so that isomerisation occurs spontaneously at room temperature resulting in the continuous exchange of hydrogen between the nitrogen and α carbon if the mechanism in Scheme I were correct. This has been elegantly borne out by storing the β-amino vinyl ketone (5), in an excess of deuterium oxide for three days at room temperature. NMR examination of the ketone after this treatment revealed a decrease in intensity of the Hα proton (4·9 τ, d) and the transformation of the Hβ sextet to a broad singlet because of incomplete exchange of the α hydrogen and the amino hydrogens giving a superposition of diverse multiplets. Integration fixes the deuterium incorporation at 65%. Further evidence was obtained by keeping the αβ dideutero compound with water, NMR analysis of the product indicating the loss of about 70% of the α deuterium.

These workers note the fact that the rate of exchange is slow, whereas it has been shown that isomerisation is fast;[9a] thus one must assume the existence of other mechanisms for cis-trans-isomerisation as well as that proceeding via enamine-imino tautomerism. No further explanation was offered, but it may be noted that isomerisation can only occur when there is free rotation about the Cα and Cβ bond of the ketone (5). That is, the isomerisation might be due to a 1,4 addition - elimination sequence involving another mole of β-amino vinyl ketone or even ammonia, traces of which might be generated by hydrolysis. Such a scheme would account for cis-trans-isomerisation with no incorporation of deuterium. Other factors affecting the cis-trans-ratio are the structures of the activating group and the amine, as the larger and more bulky these groups are, the less likelihood is there of obtaining the cis-isomer because of non-bonded interactions.[7]

Thus the evidence suggests that the adduct obtained from primary amine addition to an acetylene prefers the hydrogen bonded cis-configuration if this is sterically favourable.

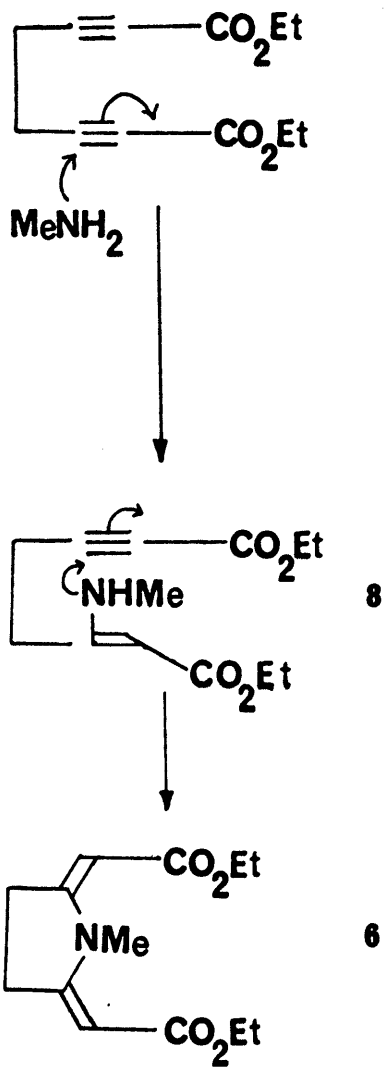
SCHEME 2



6

7

SCHEME 3



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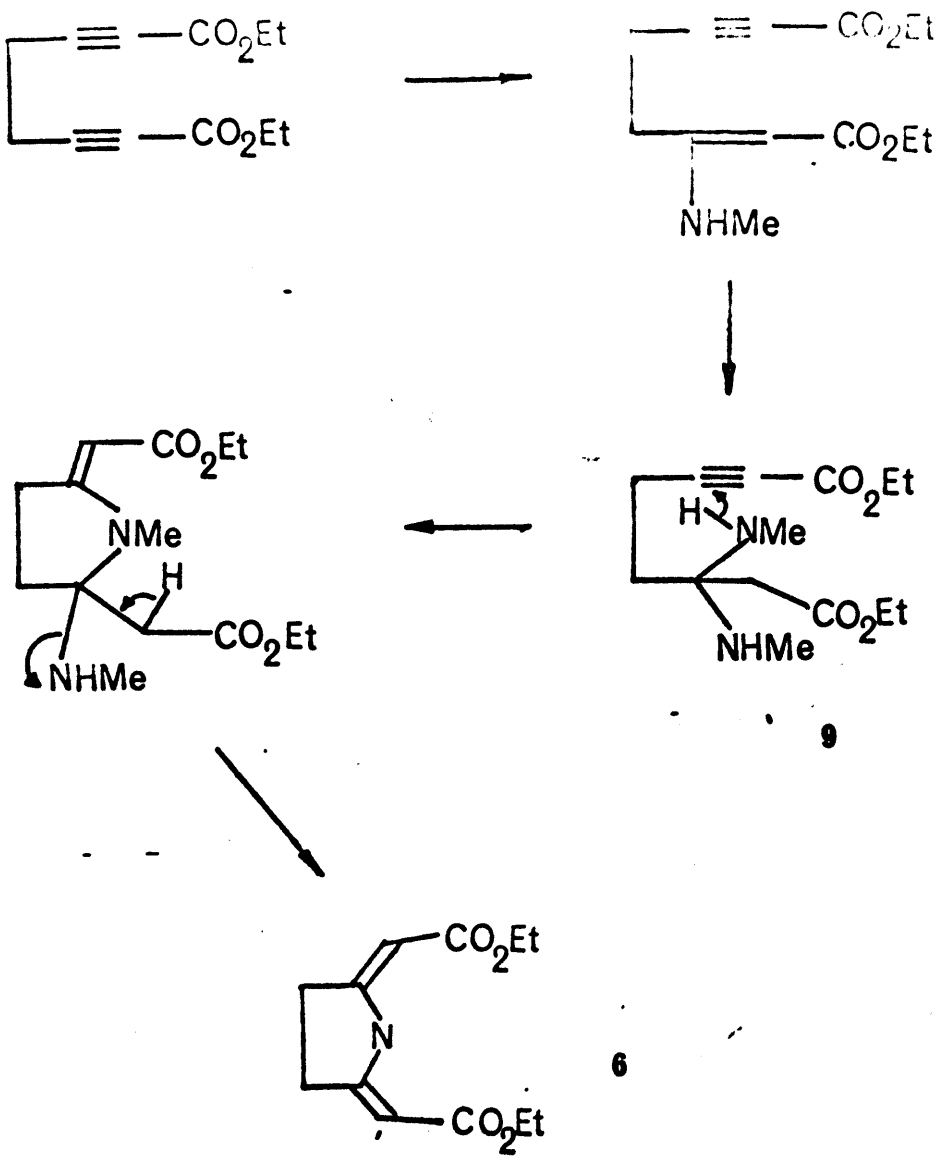
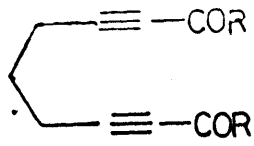

CO2Et
CO2Et
CO2Et
CO2Et
NHMe
CO2Et
H
NMe
CO2Et
NHMe
CO2Et
NMe
H
CO2Et
NHMe
CO2Et
N
CO2Et

9

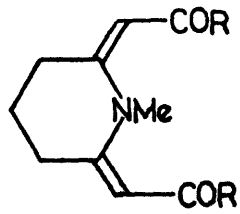
6

In 1959, the addition of methylamine to a di-acetylenic diester was exploited[10,11] in a synthesis of tropinone (7) via the pyrollidine diester (6) as outlined in Scheme 2. This could involve interaction of the acetylenic mono-emamine (8) to give (6), (Scheme 3) or it is also possible that methylamine adds to one acetylenic bond, the resulting di-enamine (9) undergoing internal addition as shown in Scheme 4. However application of this reaction to the homologous ester diethyl nona-2,7-diyne-1,9 dioate (10, R = OEt) did not lead to an analogous product.

Although an oily compound, E, whose analysis was consistent with the desired piperidine, (11, R = OEt) could be obtained by the action of hot ethanolic methylamine only one double bond of this could be reduced even after prolonged hydrogenation in ethanol, while in acetic acid, catalytic hydrogenation gave a product formulated as the pyran (12). Both reduction products afforded the same derivatives with Brady's reagent.[10,11] The mechanisms which were proposed[11] to account for these transformations are shown in Scheme 5. An analogous product F, tentatively formulated as (11, R = NH_2), was produced in the same way from the diaimide (10, R = NH_2).[10]

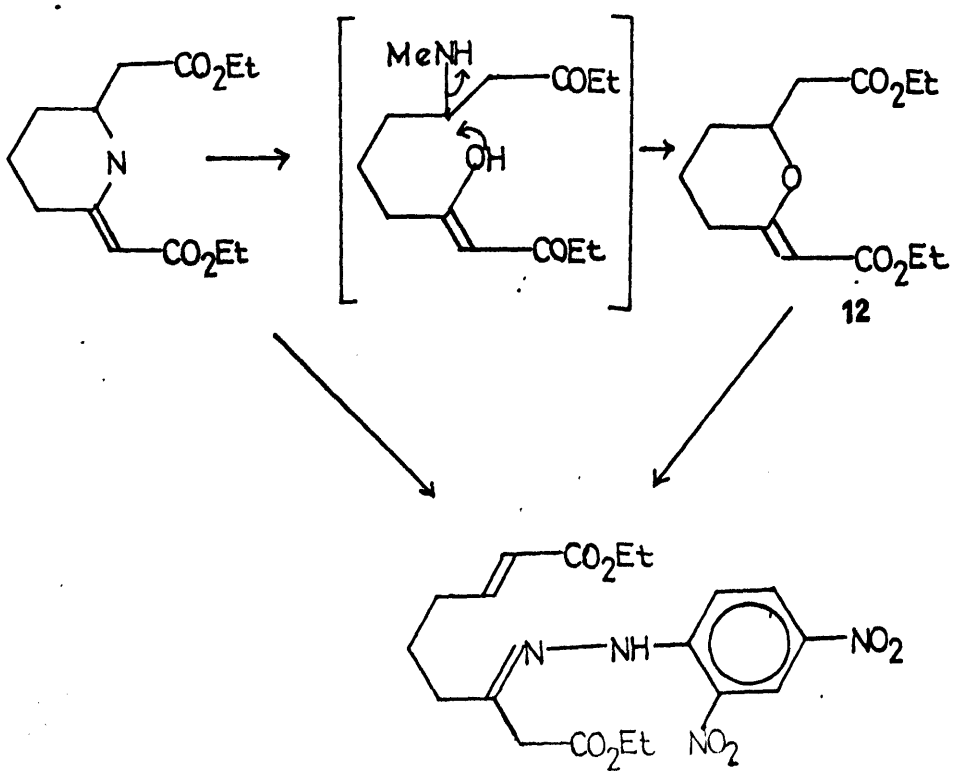


10



11

SCHEME 5

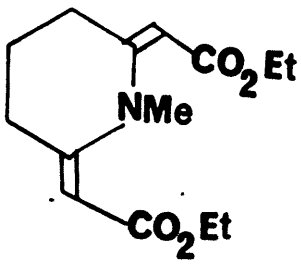


Treatment of the diethyl ester, (10, R = OEt), with cold aqueous ammonia or methylamine afforded the corresponding diamides. With hot aqueous methylamine however, the dimethyl or diethyl ester (10, R = OMe, or OEt) gave a compound, A, m.p. 160-162·5°c which absorbed 1 mole of hydrogen and was chiefly remarkable for its high wavelength UV absorption at 348 mμ (log ε 4·51); 284 mμ (log ε 3·32); 240 mμ (log ε 3·91). A compound B m.p. 277-279°c (d), whose properties (IR, UV, response to hydrogenation) were closely analogous to those of A, was obtained by the action of hot aqueous methylamine on the diamide (10, R = NH_2).[10]

From these facts it was evident that addition of methylamine to the diacetylenic diester (10, R = OEt or NH_2) had in this case taken an unexpected course which is elucidated in the following section.

DISCUSSION

Section 1.2



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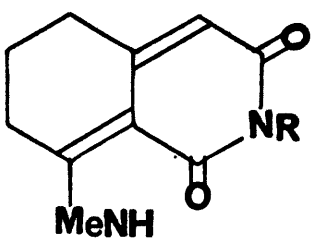

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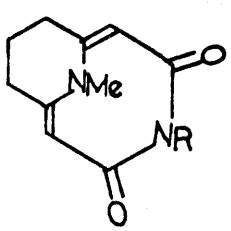

Following a re-examination of the action of methylamine on the diacetylenic diacid derivatives (10, R = OEt or NH_2) it became apparent that the compounds A and B were closely related.[12] This was fully borne out by analytical data, and by spectral information. From this and from the following evidence, structures (14, R = Me) and (14, R = H) were advanced for A and B respectively.[12]

Since A is derived by the action of hot aqueous methylamine on compound E,[13] which had been formulated as (11), a possible structure assignment for A and B might seem to be the bicyclo imides (15, R = Me or H). (The bridgehead double bonds of these structures do not contravene Bredt's rule since they are in an eight-membered ring and models do not show undue strain in the molecules.) However these structures were clearly excluded by the NMR spectra of these two compounds (Table 4 and Fig. 1) which each exhibited the resonance of only one vinyl proton. In addition A and B respectively exhibited doublets at 6·90 τ (3H, J = 5·4 c.p.s. deuterochloroform) and 6·53 τ (3H, J = 5·4 c.p.s. trifluoracetic acid) betraying the presence of an -NHMe grouping,[14] and A (14, R = Me) also displayed a singlet peak at 6·68 τ attributed to the N-methyl grouping of the imide system, a feature which

Table No. 4. NMR data of the compounds A, B, C and D.

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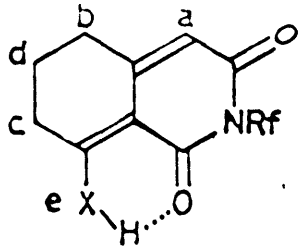
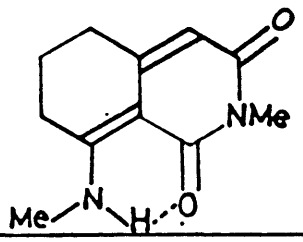
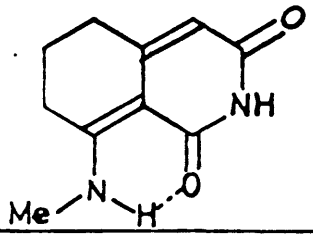
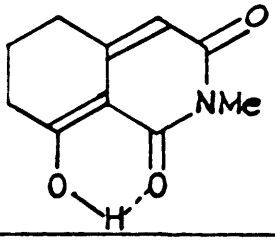
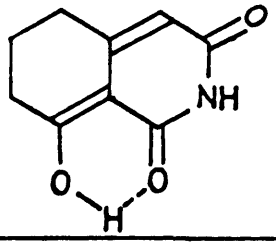


COMPOUND	NMR SOLVENT	τ_a	τ_b, τ_c	τ_d	τ_e	τ_f	τ_g
	$CDCl_3$	4·37 b.s.	7·36 and 7·43 sup.t J~6 c/s	8·1 m	6·9 d J=5·4 c/s	6·68 s	-2·1 b
	CF_3CO_2H	3·68 b.s.	6·99 sup.t J~6 c/s	7·79 m	6·53 d J=5·4 c/s		
	$CDCl_3$	4·16 b.s.	7·37 sup.t J~6 c/s	7·99 m	-	6·68 s	*
	$CF_3.CO_2H$	3·57 b.s.	6·98 and 7·08 sup.t J~6 c/s	7·74 m	-		

* not found $< -10\ \tau$

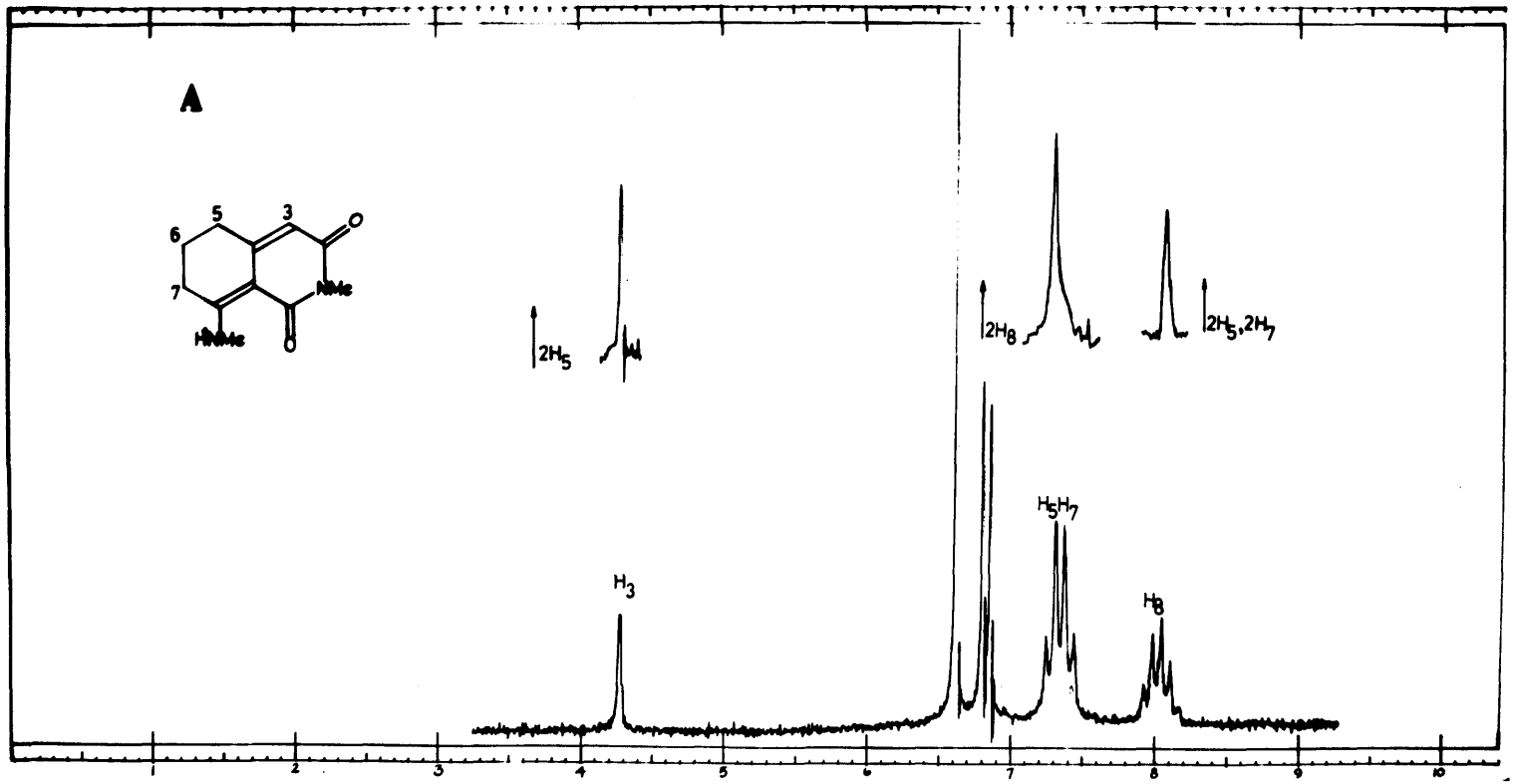


Fig. 1

Table 5. IR data of the compounds A B C and D

COMPOUND	SOLID SOLN.	$\nu_{C=O}$	ν_{OH} or NH
A	NUJOL	1655 $cm.^{-1}$	
	$CHCl_3$	1650 $cm.^{-1}$	3225 $cm.^{-1}$
B	NUJOL	1655 $cm.^{-1}$	
	-		
C	NUJOL	1660 $cm.^{-1}$	
	CCl_4	1687 $cm.^{-1}$	2900-3050 $cm.^{-1}$
D	NUJOL	1660 $cm.^{-1}$	
	-		

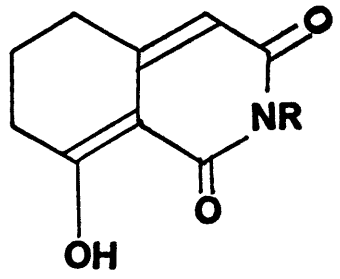
Table 6. UV data of A, B, C, D and related compound

Compound	$\lambda_{max.}^{EtOH}$ (mμ)	log ε	$\lambda_{max.}^{EtOH-NaOH}$ (mμ)	log ε	$\lambda_{max.}^{on\ reacid^{n}}$ (mμ)	log ε
A	350	4·38	350	4·42	350	4·38
	286	3·23	281	3·48	286	3·23
	236	3·72	257	3·70	236	3·72
B	349	4·44	352	4·39	349	4·44
	284	3·32	282	3·61	284	3·32
	235	3·79	257	3·88	235	3·79
C	320	4·26	329	4·35	320	4·26
	240	3·89	280	3·64	240	3·89
			211	3·33		
D	319	4·20	334	4·37	319	4·20
	233	3·89	278	3·57	233	3·89
Decevinic acid	325	4·25				

is absent in the spectrum of B (14, R = H). The spectra of both compounds showed the presence of a methylene group (multiplet at 8·1 τ) coupled to four allylic protons (multiplet at 7·4 τ).

This was demonstrated in the present work by irradiation at 8·1 τ (Fig. 1), when the multiplet centred at 7·4 τ collapsed to a broad singlet and upon irradiation at 7·4 τ the multiplet centred at 8·1 τ similarly collapsed to a broad singlet. Allylic coupling between the methylene protons and the olefinic proton was also demonstrated since irradiation at 7·4 τ considerably sharpened the singlet at 4·37 τ.

The IR spectra of these compounds display strong carbonyl absorption (Table 5) and their UV spectra show evidence of an extensively conjugated system (Table 6). These data are effectively accommodated by the structures (14, R = Me) and (14, R = H) for A and B respectively. It will be noted that these structures represent vinylogous amides which accounts for the principle features of the chemistry of these compounds, including their failure to absorb more than one mole of hydrogen (c.f. Introduction).



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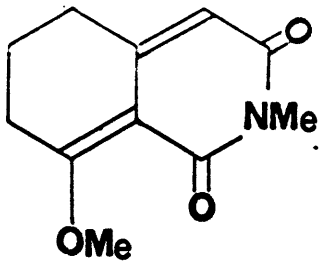
It was also reported[13] that treatment of A and B with refluxing sodium ethoxide solution yields respectively the acidic compounds C ($C_{10}H_{11}NO_3$) and D ($C_9H_9NO_3$). From the evidence discussed below the compounds C and D were formulated as the vinylogous acids (16, R = Me) and (16, R = H) respectively.

The NMR spectra (Table 4) of these compounds differ from those of A and B only in that the doublets displayed by A and B and attributed to the -NHMe grouping are absent.

Both the -NHMe and -OH groupings in these respective compounds are part of stable chelate systems. However while A (14, R = Me) gives a broad signal at -2·1 τ (Table 4) which can be attributed to the N-H proton, its low field position suggesting that it is participating in strong hydrogen bonding, the corresponding hydrogen bonded hydroxyl proton expected in C (16, R = Me) cannot be detected above -10 τ. This might be due to the involvement of the proton in a rapid tautomeric shift between the two atoms to which it is bonded where the rate of exchange is sufficiently fast (at 37°c) to prevent the detection of a signal corresponding to either extreme position, yet not fast enough to allow the spectrometer to locate the proton in only one average position.[15]



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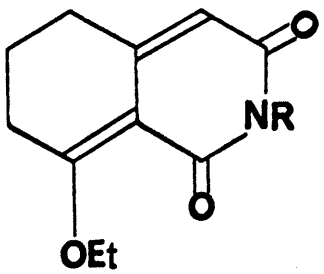

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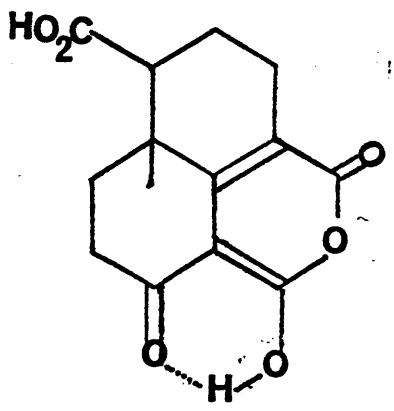

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Further evidence of the chelate system is furnished by the IR spectra (Table 5) and the formation by C (16, R = Me) and D (16, R = H) of stable insoluble green complexes on treatment with cupric acetate in acetone, and by the production of deep red-purple colourations by all four compounds when treated with ethanolic ferric chloride. As might be expected the methyl ether of C (17) is extremely labile being rapidly hydrolysed at room temperature to the stable chelate system.

This tends to confirm the observation that ethoxide treatment of the vinylogous amides (14, R = Me) and (14, R = H) results not in the respective vinylogous ethyl esters (18, R = Me) and (18, R = H) but in the vinylogous acids C (16, R = Me) and D (16, R = H). The alternative explanation that the acids are produced by $B_{AL}2$ hydrolysis of the esters may well be complementary to this.

Like A (14, R = Me) and B (14, R = H), the UV spectra of C (16, R = Me) and D (16, R = H), both display an extensively conjugated system (Table 6). The bathochromic shifts in alkali displayed by C and D (Table 6) are in accord with their enolised β-keto imide structures. A useful analogy is provided by decivinic acid[16] (19) which absorbs at 325 mμ (log ε 4·25) compared with

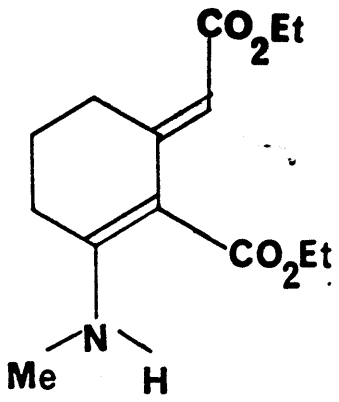

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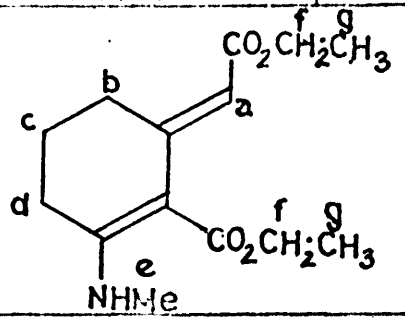
Table 7. NMR data of compound E (20).

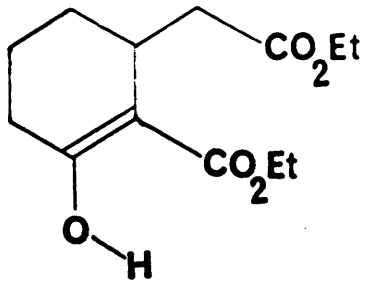


COMPOUND	NMR SOLVENT	τ_a	τ_b	τ_c	τ_d	τ_e	τ_f	τ_g
E	$CDCl_3$	3·87 s	6·9 m	8·25 m	7·6 m	7·05 d J=5·4 c/s	5·9 q J=6·6 c/s	8·67 and 8·7(sup' J=6·(c/s

values of 320 mμ (log ε 4·26) and 319 mμ (log ε 4·20) for C (16, R = Me) and D (16, R = H) respectively, - a very reasonable correspondence in view of the small variations which can be expected in comparing an anhydride with its imide counterpart.

In view of the fact that compound A (14, R = Me) can be readily obtained from compound E on treatment with aqueous methylamine[13] the structure of the latter (formerly given as 11) was re-examined[12] and revised to (20) on NMR evidence (Table 7). The spectrum of E shows a three proton doublet at 7·05 τ (J = 5·4 c.p.s.) due to an -NHMe grouping and a single unsplit proton at 3·87 τ. The resonance of one of the allylic methylene groups occurs as a multiplet at 6·9 τ which is somewhat lower than the value observed for the allylic methylene groups in A (14, R = Me) (7·4 τ cf. Table 4). This indicates that one methylene group in E is deshielded by the carboethoxyl group and hence suggests that the double bond geometry is as shown in (20).

This structure satisfactorily accounts for the uptake of only one mole of hydrogen observed in hydrogenation, for the high UV absorption ($\lambda_{max.}$ 322 mμ) and for the ready conversion to A.



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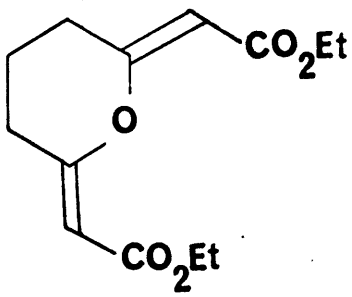

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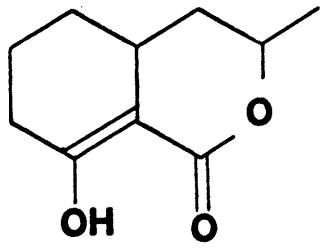

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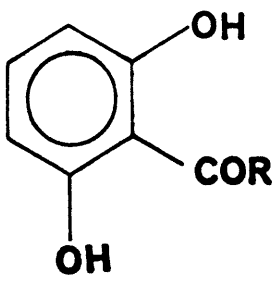
The product obtained by catalytic reduction of E (20) in acetic acid, which was previously formulated as (12), can now be understood to be the vinylogous acid (21). The NMR spectrum no longer exhibited a vinyl proton resonance, but a complex multiplet at ca. 7·65 τ due to the resonance of 5 protons. (Two methylenes α to carbonyl and one allylic methine.) The resonance due to the remaining methylene groups appeared as an ill defined multiplet at 8·4 τ, and the signals at 5·85 τ (4H, q, J = 7 c.p.s.) and 8·75 τ (6H, t, J = 7 c.p.s.) were ascribed to the two ethoxyl groups.

The UV spectrum of the reduction product (21) indicated the presence of a less extensive chromophore (255 mμ, log ε 3·79) than that of E (20) (322 mμ log ε 4·32). The IR showed intense bands at 1725 and 1650 $cm.^{-1}$ typical for a normal ester group and a β-keto ester system.[17]

In order to prove conclusively the nature of the carbon skeleton, as well as rendering these compounds more accessible, the synthesis of compounds like A, B, C, D, or E was studied. It was also thought that some of these compounds might be useful as possible intermediates in the synthesis of various naturally occurring isocoumarins, the reduction product (21) obtained from E (20) for example, being a potential intermediate in the synthesis of the antibiotic ramulosin (22).[18]



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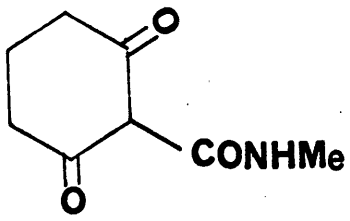

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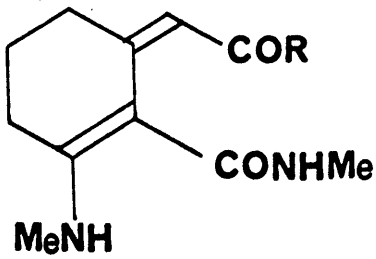

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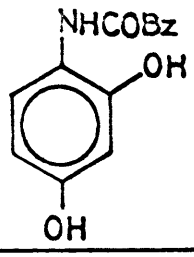
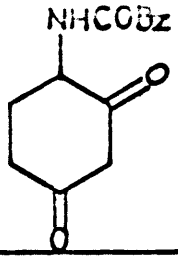
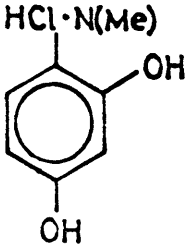
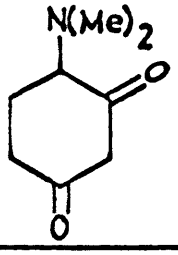
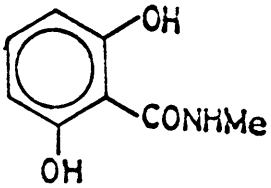
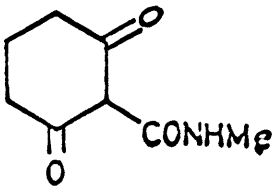
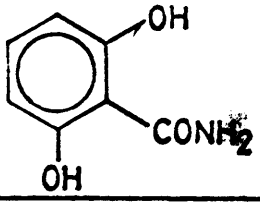
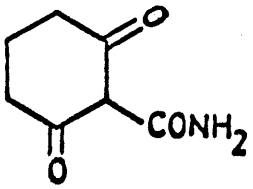
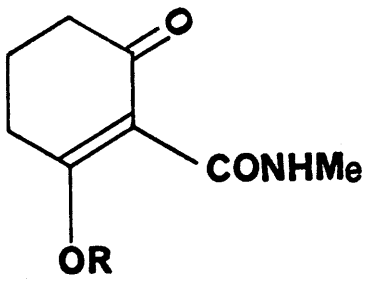
Table 6.

COMPOUND	REACTION MEDIUM CATALYST	REDUCTION PRODUCT
	0·22N NaOH Raney Ni (8 hrs.)	
	0·28N NaOH 10% Pd/C.	
	Aqueous pH 9·2 10% Pd/C	
	Aqueous pH 9·2 Raney Ni	

The key intermediate required in one proposed synthesis (24 → 27 → 14) was the substituted cyclohexane-1,3-dione (24). This type of compound has been prepared by reduction of substituted resorcinols[19] (Table 8 gives some examples of these), and this method was applied to the synthesis of 2-N-methylamido-cyclohexane-1,3-dione (24). Thus treatment of the silver salt of 2,6 dihydroxy benzoic acid (23, R = OAg) with methyl iodide selectively gave the methyl ester (23, R = OMe) which on treatment with aqueous methylamine gives the N-methylamide derivative (23, R = NHMe). This was smoothly reduced to the required dione (24).

The NMR spectrum of the dione (24) exhibited a three proton doublet due to the -NHMe moiety (7·08 τ, J = 5·4 c.p.s.), two triplets almost superposed at ca. 7·4 τ (J = 6 c.p.s. in each case) corresponding to the resonance of the two methylene groups α to the carbonyl groups, while the broad triplet at 8·08 τ (J = 6 c.p.s.) was ascribed to the remaining methylene group β to the two carbonyl groups. The resonance due to the N-H proton appeared at 0·25 τ and disappeared slowly on deuteration, accompanied by collapse of the methyl doublet to a singlet at 7·1 τ. The IR spectrum was in complete agreement with the structure (24) showing no



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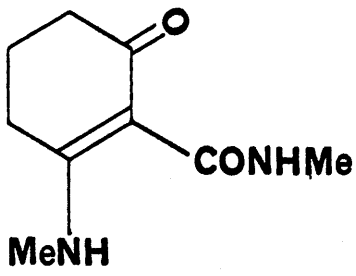
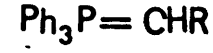


26

evidence of aromatic character but displaying intense broad bands in the region of 1650 - 1530 $cm.^{-1}$ typical of β tri-ketones.[20] The IR spectrum also showed evidence of a hydrogen bonded enolic β-dicarbonyl system confirmed by an immediate and conclusive colouration with methanolic ferric chloride.

It was hoped that one of the carbonyl groups of the dione (24) could be converted into a carbethoxy-methylene group, and attempts were made to inactivate the remaining carbonyl group by formation of an enol ether (25, R = alkyl) but repeated attempts to prepare this under a variety of conditions were unsuccessful. However upon refluxing the dione (24) with ethanolic methylamine a crystalline product was obtained.

The NMR spectrum was in good accord with the enamine structure (26) displaying two N-methyl doublets at 6·98 τ (J = 5·4 c.p.s.) and 7·16 τ (J = 5 c.p.s.). The resonance due to the methylene group α to the remaining carbonyl group and the allylic methylene group both appeared at the same value of 7·4 τ, and the 2H multiplet at 8·05 τ was ascribed to the remaining methylene group. The two broad signals at -0·1 τ and -2·3 τ were assigned to the two hydrogen bonded N-H protons, the chelate system also being indicated by colouration with



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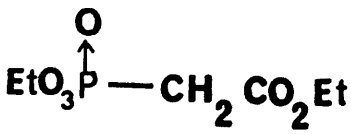

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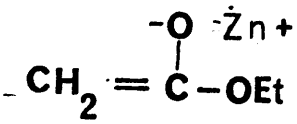

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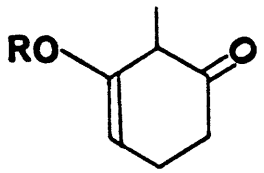
methanolic ferric chloride, and by the carbonyl absorption of the β-keto amide system which appeared as intense broad bands at 1618 - 1580 $cm.^{-1}$. The enamine absorbed at 294, 245 mμ rather higher wavelengths than the dione and gave a mass spectral fragmentation pattern in accord with structure (26).

The next step in the synthesis then, required the condensation of one C_2 unit with the ketonic carbonyl group of (26) to give a carboxymethylene derivative of the type (27) which it was hoped might react with hot aqueous methylamine to give A (14, R = Me). Three unsuccessful approaches to the required product were investigated.

The first approach involved attempts to condense the β keto amide (26) with malonic acid derivatives hoping that even if an aldol product (e.g. 27) was formed reversibly, ring closure might intervene to force the reaction to completion. However neither direct reaction with malonic acid under Knoevenagel conditions or prolonged reaction with dimethyl sodiomalonate gave any of the desired product. Secondly the enamine was subjected to the Wittig reaction. Although ylids of the type (28, R = H or Me) are powerful nucleophiles, ylids with a stabilizing α-substituent as with (29, R = OEt, OMe)



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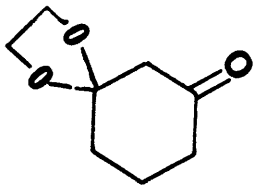

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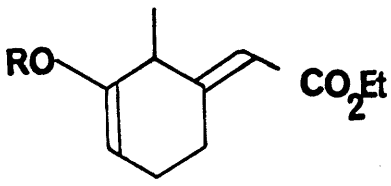

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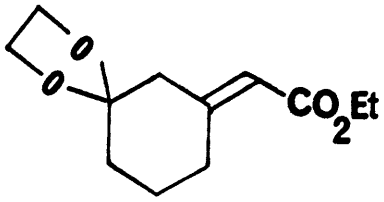

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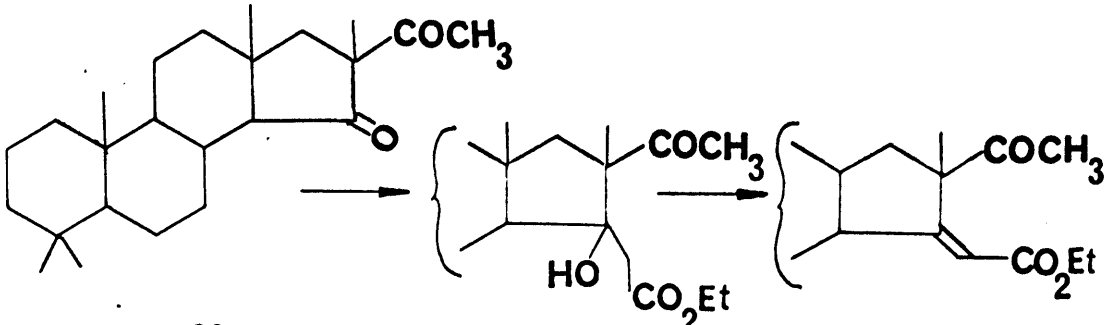


36 37 38

are less reactive, but under forcing conditions[21,22] react with ketones to yield the corresponding condensation product. Refluxing the ylid (29, R = OEt) with the β-keto amide (26) in benzene or toluene solutions for long periods failed to produce any of the desired product.

Even when the β-keto amide (26) was treated with the more reactive phosphonate (30) in an aprotic solvent such as benzene, dimethyl formamide a diglyme with one mole of sodium hydride[23] there was no reaction.

Finally, the Reformatsky reaction was attempted using ethyl bromoacetate. There are several reports[24,25] in the literature on the application of the Reformatsky reaction to such cyclohexane-1,3-dione derivatives as (32, R =alkyl) and (33) giving the respective aldol products (34, R =alkyl) and (35), and also to the relatively hindered ketone (36)[26] the resulting β-hydroxy ester (37) being subsequently dehydrated to the αβ unsaturated ester (38). However no condensation product from the β-keto amide (26) could be detected whether the usual Reformatsky conditions were employed or the enolate anion (31) was preformed.[27]

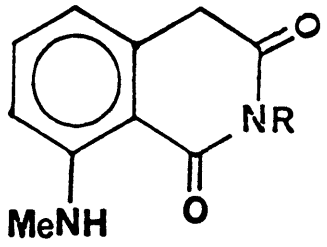
In all these experiments the starting material was recovered quantitatively and identified by IR, UV and TLC.

This lack of success may result from the reduced reactivity of the ketonic carbonyl group of the β-keto amide (26) due to hydrogen bonding, while steric hindrance to nucleophilic attack caused by the N-methylamide grouping could be another factor.

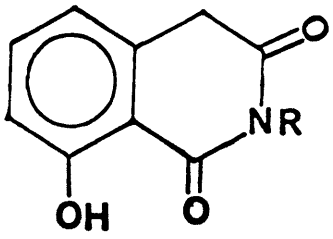
However the β-keto amide (26) proved to be a useful model compound. It will be observed that there is much similarity between the main skeletal features of the enamine (26) and A (14, R = Me), a fact borne out by comparison of their NMR spectra. As discussed before A shows 4H and 2H multiplets at 7·4 τ and 8·1 τ respectively; the synthetic enamine (26) showing signals which were almost identical.

The above synthetic studies having provided a limited degree of support for the structures of the products obtained by the action of methylamine on the diacetylenic ester (10, R = OEt), attention was turned to some aromatic derivatives of these products.

These aromatic compounds were readily obtained from A, B, C or D by dehydrogenation with 10% palladium-on charcoal in refluxing diphenyl ether for periods ranging from two minutes to one hour to give the corresponding 8-substituted homophthalimides.[12]



39



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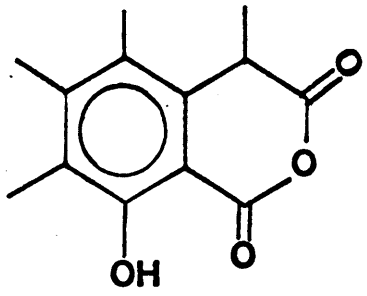
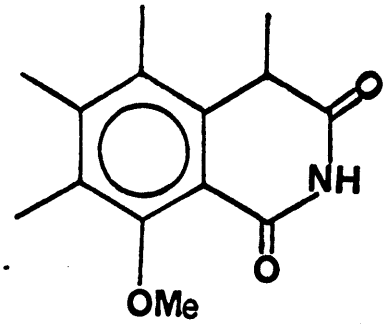
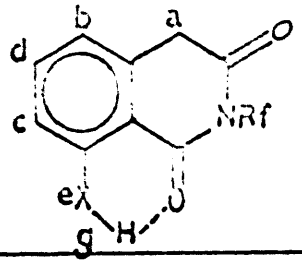


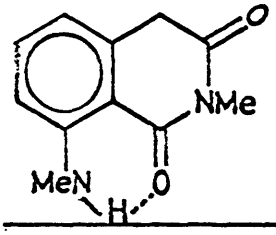
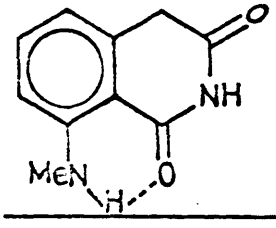
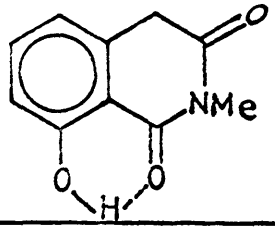
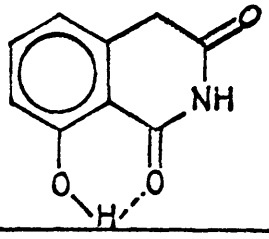
41



42

Table 9. NMR data of the dehydro compounds.

KEY:- 

COMPOUND	NMR SOLVENT	τ_a	τ_b, τ_c	τ_d	τ_e	τ_f	τ_g
	$CDCl_3$	5·98 s	3·42 d 3·6 d J=8 c/s	2·66 q J=8· c/s	7·06 s	6·70 s	2·30 b
	$CF_3.CO_2H$	5·69 s	2·15 complex		6·59 s	*	*
	$CDCl_3$	5·97 s	3·06 d 3·24 d J=8 c/s	2·5 q J=8· c/s	-	6·66 s	-1·75 s
	$CF_3.CO_2H$	5·58 s	2·80 d 2·90 d J=8 c/s	2·20 q J=8· c/s	—	*	*

* : not sought.

The NMR spectra of dehydro-A (39, R = Me) and dehydro-B (39, R = H) (Table 9) are both in good accord with their respective structure assignments. The spectrum of dehydro-A for example shows resonance due to three aromatic protons at 2·5-3·5 τ. First order analysis suggests that this is an AMX system where H_6 is a double doublet at 2·66 τ (J = 8 c.p.s. in each case), and H_5 and H_7 are doublets (J = 8 c.p.s.) at 3·6 τ and 3·42 τ respectively. The spectrum also shows a singlet at 5·98 τ due to a benzylic methylene group α to an imide carbonyl group.

However the N-methyl group of both bases occurs as a sharp singlet at 7·06 τ (dehydro-A, deuterochloroform) and 6·59 τ (dehydro-B, trifluroacetic acid). The absence of spin-spin coupling of the methyl group is an apparent anomaly but it has been reported[28] that while the occurrence of a methyl doublet is excellent evidence for a hydrogen atom on the methyl-bearing nitrogen atom, the absence of coupling does not lead with any certainty to the converse conclusion. This is owing to the fact that when the energy barrier for hydrogen exchange between oxygen and nitrogen becomes sufficiently low, (i.e. exchange becomes rapid) spin-spin coupling is eliminated irrespective of the length of time spent by the proton on any one site.

Evidently exchange of the -NH proton in dehydro-A (39, R = Me) is sufficiently fast to prevent spin-spin coupling. One way of retarding this exchange would be to cool the NMR solution. This indeed occurred since the NMR spectrum of dehydro-A in deuterochloroform at -52^{o}c now showed a 3H doublet at 7·05 τ (J = 5 c.p.s.). Complementary to this the NMR spectrum also showed the resonance of the NH proton as a quartet at 1·47 τ (J = 5 c.p.s.)

Both dehydro-C (40, R = Me) and dehydro-D (40, R = H) behave like typical phenols being insoluble in saturated aqueous sodium bicarbonate but soluble in dilute sodium hydroxide from which they are re-precipitated by acid.

The NMR spectra of the compounds dehydro-C (40, R = Me) and dehydro-D (40, R = H) are shown in Table 10. The presence of the hydrogen bonded hydroxyl group is demonstrated by the appearance in the spectrum of a sharp singlet at - 1·75 τ. The corresponding peak in dehydro-D (40, R = H) is presumably obscured by the solvent trifluoroacetic acid. Further, the spectra of dehydro-C and dehydro-D show resonances due to the benzylic methylene group at 5·97 τ and 5·58 τ respectively, and the aromatic protons comparable to those observed

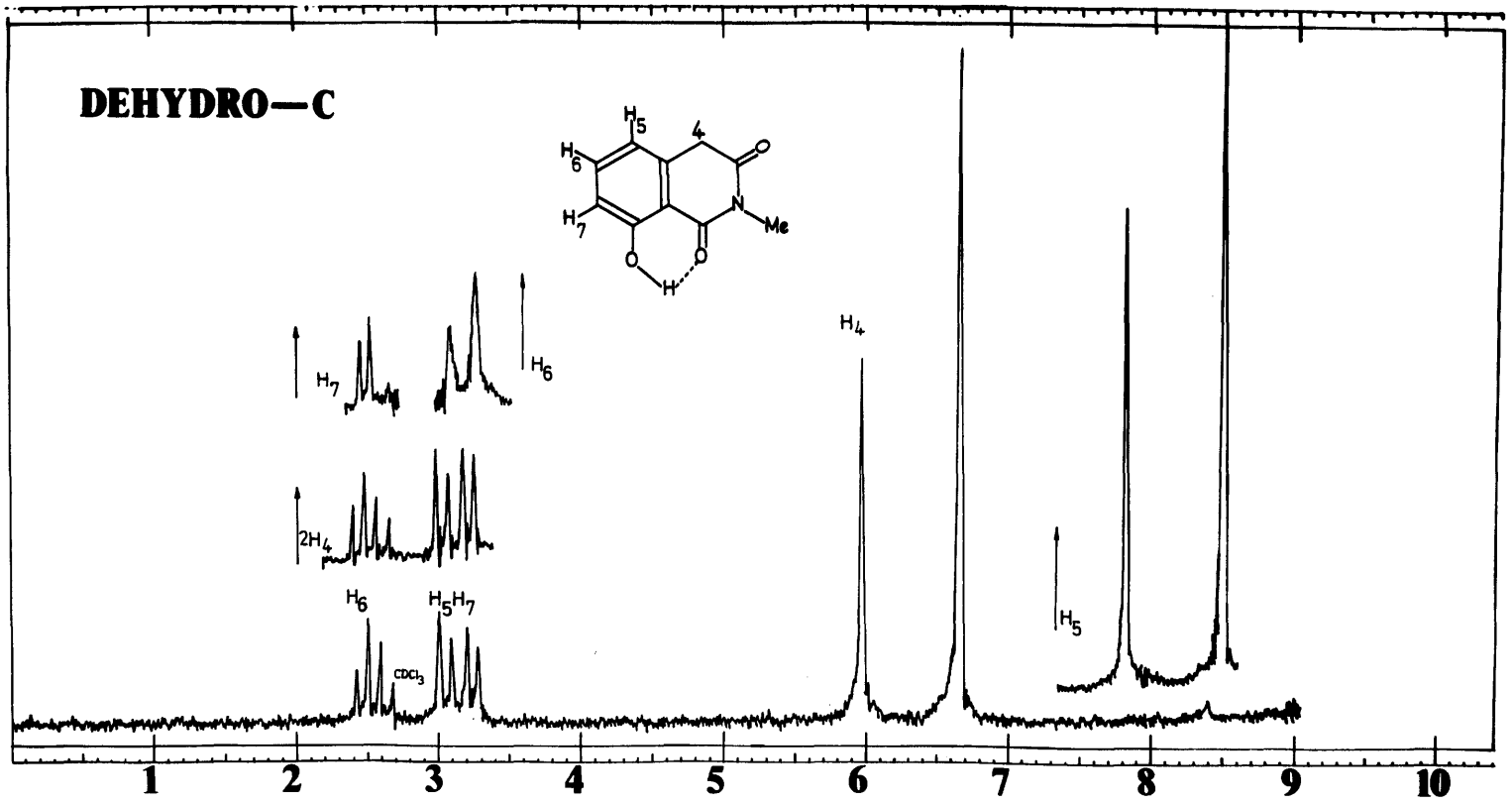

DEHYDRO—C
H5
4
H6
H7
O
N
Me
O
H
H7
H6
2H4
H6
H5H7
CDCl3
H4
H5
1
2
3
4
5
6
7
8
9
10

Fig. 2

for dehydro-A (39, R = Me) and dehydro-B (39, R = H). In the case of dehydro-C (40, R = Me) it was shown that the double doublet at 2·5 τ (J = 8 c.p.s. in each case) due to the proton on H_6 collapsed to a doublet (J = 8 c.p.s.) upon irradiation in the vicinity of the doublet at 3·06 τ (H_7) (Fig. 2). Conversely upon irradiation in the vicinity of 2·5 τ (H_6), the doublets at 3·24 τ and 3·06 τ assigned to H_5 and H_7 respectively, collapsed to broad singlets . The width of these peaks evidently indicates that both protons H_5 and H_7 are involved in additional long range couplings. (The occurrence of an irradiation point at one side of these peaks invalidates comparison of peak heights.) Spin decoupling also revealed a degree of long range coupling between the aromatic protons and the benzylic methylene group, since irradiation at 3·24 τ sharpened the singlet at 5·97 τ, and conversely irradiation at 5·97 τ noticeably increased the intensity of the doublet at 3·24 τ, the intensity of the other signals remaining essentially the same. This suggests that the doublet at 3·24 τ corresponds to the resonance of the proton H_5. [However it may be noted that calculated values[29] appear to suggest that H_7 should be at higher field (3·00 τ) than H_5 (2·95 τ).]

Table 10. UV data of Dehydro Compounds

Compound	$\lambda^{EtOH}_{max.}$ (mμ)	log ε	$\lambda^{EtOH-NaOH}_{max.}$ (mμ)	log ε	$\lambda^{EtOH\ HCl*}_{max.}$ (mμ)	log ε
Dehydro-A	375	3·77	396	3·91	295+	2·94
	260	3·95	313	3·96	283	3·08
	242	4·01	287	4·13	248	3·88
	222	4·05	250	4·20	235+	3·80
			226	4·18	210	4·01
Dehydro-B	378	3·71	404	3·83	294+	3·04
	261	3·89	313	3·90	284	3·18
	243	3·92	289	4·03	247	3·89
	223	3·90	251	4·03	235	3·84
			229	4·00	217	3·76
Dehydro-C	316	3·58	392	3·73		
	257	3·88	309	4·14		
	222+	3·95	299	4·09		
	214	4·01	264	3·99		
			234	4·13		
Dehydro-D	318	3·58	391	3·82		
	253	3·93	306	4·22		
	220+	4·11	296+	4·16		
	212	4·21	264	4·04		
			227+	4·32		
			221	4·33		

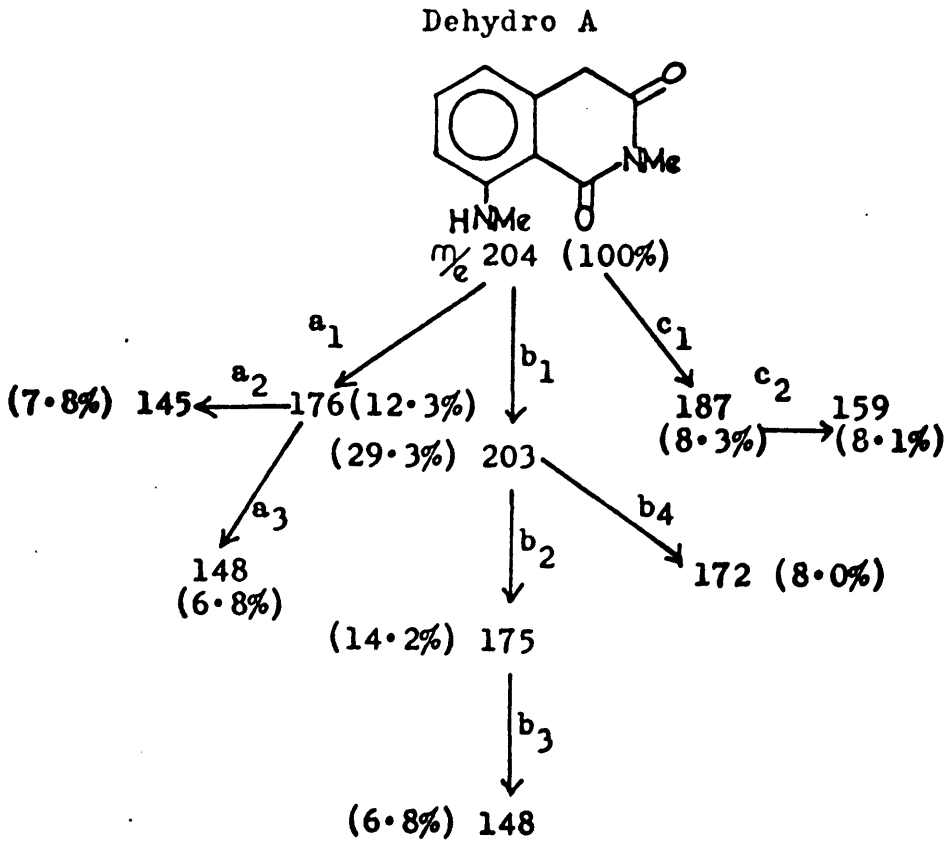
\+ Inflection

* Measured in 50% aqueous ethanol hydrochloric acid (3N)
In the presence of smaller concentrations of acid, intermediate spectra were obtained.

The long wavelength peaks of the bases dehydro-A and dehydro-B are higher by 60 mμ than those of the phenols dehydro-C and dehydro-D (Table 10). However the bathochromic shifts on basification of the phenols are much greater (ca. 75 mμ) than those of the bases (ca. 24 mμ) and so all four compounds in basic solution have very similar UV spectra. The calculated value for the major electron transfer band[30] of the bases (39, R = Me) and (39, R = H) was 269 mμ, which is in fair agreement with the observed values of 260 mμ and 261 mμ respectively. A similar treatment applied to the phenols (40, R = Me) and (40, R = H) resulted in a calculated value of 256 mμ, in good accord with the observed values of 257 mμ and 253 mμ respectively.

The IR spectra of the four aromatised compounds show the twin carbonyl absorption bands characteristic of an imide,[31] namely at 1700 $cm.^{-1}$ and 1660 $cm.^{-1}$(Nujol). The latter peak is somewhat lower than the corresponding peak in homophthalimide (1684 $cm.^{-1}$, Nujol)[31] presumably because of intramolecular hydrogen bonding. This is supported by the solution spectra of dehydro-C (40, R = Me), which shows the hydroxyl absorption as a broad peak centred at 3,100 $cm.^{-1}$. The carbonyl frequencies in this case appeared at 1655 $cm.^{-1}$ and 1716 and 1728 $cm.^{-1}$,

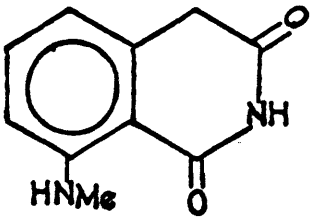
Table 12. Proposed mass spectral cracking pattern for dehydro-A (39, R = Me)



	Δm/e	m*
a_1	28	151·8
a_2	31	-
a_3	28	124·5
b_1	1	202·0
b_2	28	150·9
b_3	27	125·2
b_4	31	145·7
c_1	17	171·4
c_2	28	135·2

Table 13. Proposed mass spectral cracking pattern for dehydro-B (39, R = H).

Dehydro B



190 (100%)

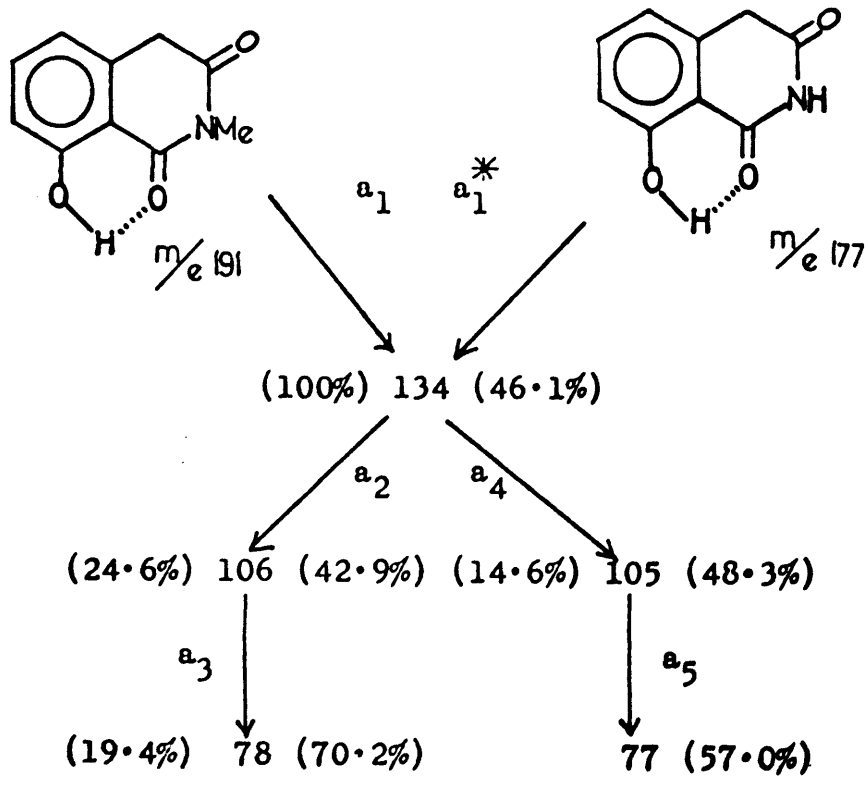
a_1 ↓ , b_1 →

173 (9·2%)

162 (12%) ---- a_2 ⟵ 119 ⟵ 118 (31·5%)

	Δm/e	m^*
a_1	28	138·1
a_2	1	117·0
b_1	17	157·5

Table 14. Proposed mass spectral cracking patterns for dehydro-C (40, R = Me) and dehydro-D (40, R = H)



	Δm/e	m*
a_1^*	43	-
a_1	57	94·0
a_2	28	83·8
a_3	28	57·4
a_4	29	82·3
a_5	28	56·5

Table 11. Spectral comparisons of Dehydro-C and Dehydro-D with Sclerin and its Homophthalimide Derivative

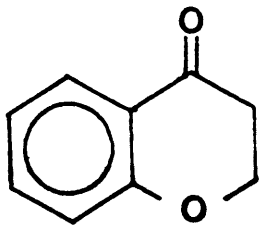
COMPOUND	Carbonyl Absorption ($cm.^{-1}$)	$\lambda_{max.}$ (C_2H_5OH) mμ
Dehydro-C (4C, R = Me)	1728, 1716, 1655 (SOLN.)	214 222 257 316
Dehydro-D (4O, R = H)	1700, 1660 (NUJOL)	212 220 253 318
Sclerin (41)	1800, 1690 (SOLN.)	215·5 263 333
8-methoxy-homophthalimide (42)	1700, 1660 (NUJOL)	210 224 (inf.) 270 339

the splitting of the higher band possibly being due to the contribution of free and hydrogen bonded forms.

The mass spectra (Tables 12 - 14) of the four dehydro compounds show certain similarities to one another and are in good agreement with their assigned structures.[32]

Recently a plant growth stimulant isolated from Sclerontinia libertiania and designated sclerin was shown to have the structure (41).[33] The reported UV spectrum of this compound was very similar to that of the two 8-hydroxyhomophthalimides dehydro-C (40, R = Me) and dehydro-D (40, R = H) (Table 11). The corresponding homophthalimide (42) obtained from sclerin (41) was also described and this has very similar UV and IR absorption to those of dehydro-C (40, R = Me) and dehydro-D (40, R = H). Table 11 provides a summary of these comparisons.

The isolation of sclerin (41) is of further significance since it presents the interesting possibility that 8-hydroxyhomophthalimides may well occur in nature, and further that 8-substituted homophthalimides may be of physiological importance. To our knowledge no physiologically active 8-substituted homophthalimides have been reported, and time has prevented the execution of such an assay although it is anticipated that such a scheme will be initiated in the future.



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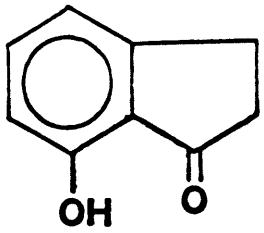

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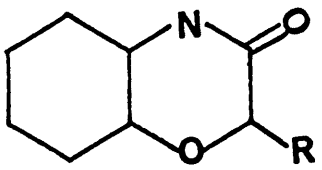

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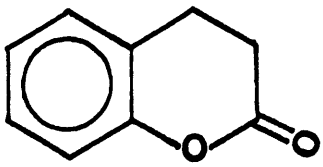
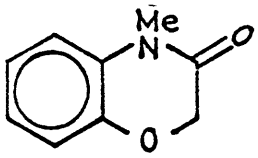
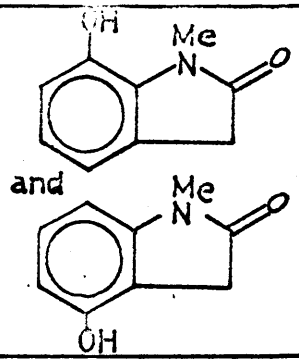
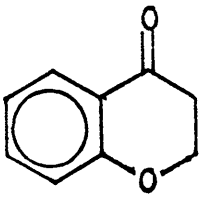
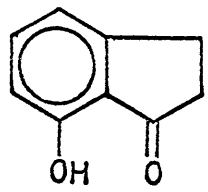
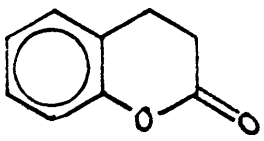
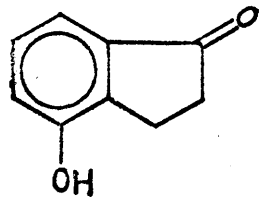


46

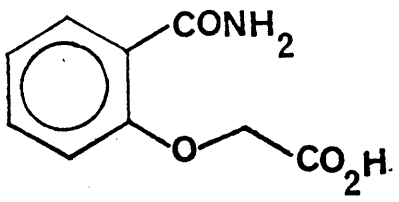
Table 15.

COMPOUND	REACTION CONDITIONS	PRODUCT(S)
	$AlCl_3$ 220°c 1 hr.	
	$AlCl_3$ 180-190°c (20 min.) 200°c (15 min.)	
	$AlCl_3$ 170-180°c 3 hr.	

In the present work an unambiguous synthesis of dehydro-C (40 R = Me) or dehydro-D (40, R = H) was sought in order to confirm the skeletal structure of the whole series of compounds, A, B, C, D, and their dehydro derivatives. The possible occurrence and/or physiological activity of 8-hydroxyhomophthalic anhydride or 8-hydroxy-homophthalimides could add significance to such synthetic studies.

6-Substituted 2-hydroxybenzoyl derivatives are somewhat inaccessible. One way of overcoming this orientation problem was suggested by a facile synthesis of 7-hydroxyindan-1-one(44) by Loudon et. al[34] via aluminium chloride rearrangement of chromanone (43). These workers also illustrated that this type of rearrangement might have wider application by applying the reaction to 2,3-dihydro-3-oxabenz-1,4-oxazines (45, R = H or Me) [35] and to dihydrocoumarin (46)[34]. The products of these aluminium chloride rearrangement reactions are shown in Table 15.

In the first line of approach to the synthesis of dehydro-D (40, R = H) it was hoped that aluminium chloride rearrangement of tetrahydrobenzo-1,4-oxazepine-3,5-diene (48, R = H) might lead directly to the required homo-phthalimide dehydro-D (40, R = H), whose formation should be detectable by TLC analysis and UV spectroscopy.



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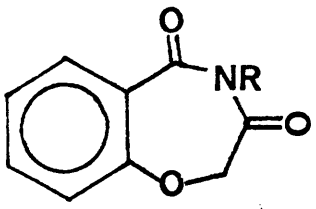
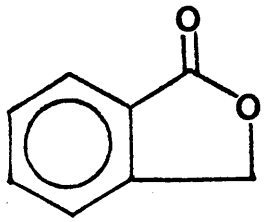


48



49

A synthesis of the required tetrahydrobenzo-1,4-oxazepine-3,5-dione (48, R = H) from the aryloxy-acetic acid derivative (47) had in fact been claimed,[36] although the original reference (Patent literature) was inaccessible. The information available from abstracts was sketchy but little difficulty was anticipated.

Surprisingly some difficulty was experienced in the preparation of the aryloxyacetic acid derivative (47) from salicylamide. However this was overcome by careful control of the conditions, particularly the proportion of reagents used and so the desired aryloxy-acetic acid was obtained in excellent yield.

The reported method[36] of cyclising this compound is by refluxing in acetic anhydride, but in our hands this led to much charring and low yields of cyclised product. However when the aryloxyacetic acid derivative was refluxed in diphenylether for 1·5 hours, the oxazepine (48, R = H) was formed in high yield. It was also found that brief treatment of the aryloxyacetic acid derivative (47) with oxalyl chloride at room temperature effected the same result.

Consistent with its assignment to structure (48, R = H) the product exhibited resonance in trifluoracetic acid at 1·8 - 3 τ due to four aromatic protons and a sharp 2H singlet at 5·2 τ ascribed to the methylene attached to an oxygen atom and α to an imide carbonyl group.

Carbonyl absorption in solution occurred at 1688 cm.$^{-1}$, 1712 cm.$^{-1}$ (inflexion) and 1721 cm.$^{-1}$ these bands being typical of an imide.[37] The UV spectrum was representative of an O-alkoxybenxamide[38] showing maxima at 211, 243, and 295 mμ.

The mass spectral cracking pattern (Table 16) is also in agreement with this structure[32] (vide infra).

After fusion of the oxazepine (48, R = H) with aluminium chloride at 180-210°c for 45 minutes, the product was examined by TLC and found to consist of one major product and two minor ones neither of which had the same R_f value as dehydro-D. Nevertheless the mixture was separated by preparative TLC and all three components were extracted and their UV spectra examined. The two less polar components showed little else than absorption at 210 mμ and 230 mμ (inflexion) and were not investigated further. The major more polar band yielded on extraction a white solid m.p. 140°c which exhibited $\lambda_{max.}$ at 215,

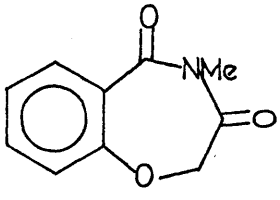
236, and 302 mμ. This compound was subsequently identified as salicylamide by IR, UV and mixed m.p.

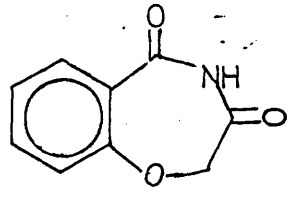
Altering the conditions of the rearrangement by lowering the reaction temperature or varying the reaction time did not result in any of the desired homophthalimide, and in each case salicylamide was the major product.

The mechanism of these aluminium chloride rearrangements appears to involve cleavage of the $-O-CH_2-$ bond, this probably being facilitated by the presence of the carbonyl function in the ortho position (*cf.* the ready cleavage of aromatic methoxyl groups ortho to a carbonyl function by MgI_2).[39,40] Internal Friedel-Crafts type alkylation by the resulting carbonium ion would give the desired product but evidently preferential cleavage occurred under the reaction conditions.

It was thought that the presence of a free -NH proton might be interfering with the course of the aluminium chloride rearrangement and so the crystalline N-methyl derivative (48, R = Me) of the oxazepine was prepared using methyl iodide and potassium carbonate.[41]

Table 16. Proposed mass spectral cracking pattern for the oxazepines (48, R = Me or H)





m/e 191 (100%) m/e 177 (81%)

a_1^* a_1

(20·1%) 134 (25·6%)

a_2

(95·4%) 105 (100%)

	Δm/e	m^*
a_1	43	101·4
a_1^*	57	94·0
a_2	29	82·3

The NMR spectrum showed the N-methyl resonance as a sharp 3H singlet at 6·58 τ, a 2H singlet at 5·2 τ ($ArOCH_2CO-$) and complex resonances in the region 1·7-3 τ due to four aromatic protons.

The methylation of the nitrogen atom is reflected in the lower carbonyl frequencies of the imide bands[37] which appear at 1654 and 1711 $cm.^{-1}$ (chloroform solution), and the absence of associated forms at intermediate frequencies.

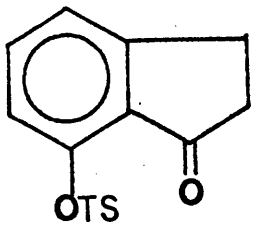
Comparison of the mass spectra of the N-methyl and unmethylated oxazepines (48, R = Me and H respectively), (Table 16), showed that while the parent ion of the former (48, R = Me) appeared at 14 mass units higher, the main fragment ions at m/e 134 and m/e 105 were the same. The ion at m/e 134 could arise by losses of $CH_3N{=}C{=}O$ and $HN{=}C{=}O$ from the respective oxazepines (48, R = Me) and (48, R = H). The benzofuranone (49), a possible structure for the ion at m/e 134, loses 29 mass units as a principal fragmentation, giving rise to the ion at m/e 105.[32]

It was hoped that aluminium chloride rearrangement of this molecule (48, R = Me) might yield dehydro-C (40, R = Me) which again should be detectable by TLC and UV spectroscopy. However treatment with aluminium chloride gave a complex mixture of products, the phenolic

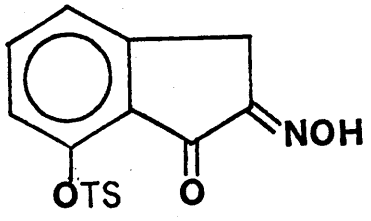
fraction of which was shown by TLC examination to be a mixture of six components. Separation by preparative TLC and examination of the UV spectrum of each band indicated that none of the desired homophthalimide dehydro-C (40, R = Me) had been formed, a result confirming the initial TLC examination. The major product had almost identical UV and IR spectral properties to that of salicylamide and in fact was shown to be N-methyl salicylamide. Again manipulation of the reaction temperature and reaction time did not lead to any of the desired product.

From these results then, it appeared that although the benzoxazine (45, R = H or Me) and chromanone (43) underwent facile rearrangement, the two oxazepines (48, R = H or Me) underwent a preferential cleavage reaction under the conditions for isomerisation.

In the other synthetic routes investigated it was decided to exploit the fact that 7-hydroxyindanone (44) (whose preparation by aluminium chloride rearrangement[34] was used as a model for the previous synthetic route) incorporated the desired 6-substituted 2-hydroxybenzoyl system.



50



51

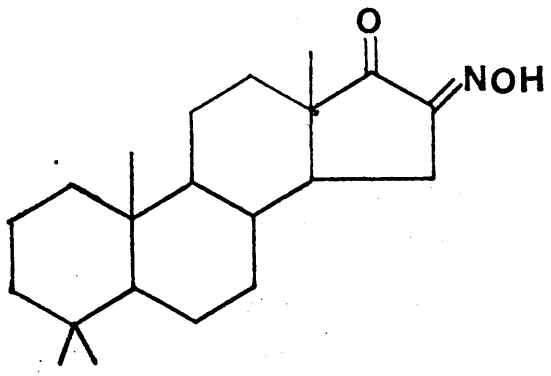
In order to protect the phenolic hydroxyl group the indanone was treated with tosyl chloride and pyridine to give the tosylate (50) in good yield.

The NMR spectrum of this produce showed resonance attributed to seven aromatic protons in the region between 2 τ and 3 τ, a sharp 3H singlet at 7·54 τ due to an aromatic methyl group and two triplets centred at 6·85 τ (J = 8 c.p.s.) and 7·40 τ (J = 8 c.p.s.) due to a methylene group α to a carbonyl and a benzylic methylene group respectively. Consistent with esterification of a phenolic hydroxyl group, the UV spectrum which showed peaks at 210, 228, and 292 mμ, with an inflexion at 245 mμ, did not alter in base. The IR spectrum of the tosylate (50) showed carbonyl absorption at 1715 $cm.^{-1}$ (Nujol) which as expected, was somewhat higher than the absorption (1680 $cm.^{-1}$, Nujol) found for the hydrogen bonded carbonyl of 7-hydroxyindanone (44). As found for other chelate systems like that of 7-hydroxyindanone (44) the tosylate (50) was more polar on TLC than the hydrogen bonded indanone and, as expected, was inactive to methanolic ferric chloride.

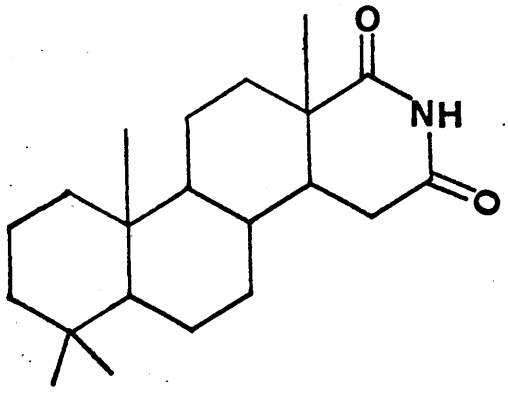
The isonitroso derivative (51) was prepared by treatment of the tosylate (50) with isoamyl nitrite in dry benzene, TLC of the product showing this to be homogeneous and more polar than the starting material.

The retention of the tosylate group in the product was indicated in the NMR spectrum by the presence of a 3H singlet at 7·52 τ (trifluroacetic acid) due to the aromatic methyl group. The benzylic methylene group appeared as a singlet at 6·0 τ (the width of the signal possibly being due to slight non-equivalence of the two protons) while the seven aromatic protons gave rise to a multiplet in the region 2 τ - 3 τ. The IR spectrum revealed the presence of a hydrogen bonded hydroxyl group (3,150 $cm.^{-1}$) and showed absorption at 1660 $cm.^{-1}$ and 910 $cm.^{-1}$ typical for an oxime grouping.[42] The carbonyl frequency (1720 $cm.^{-1}$) showed little change from that of 3-tosyloxyindanone (50) (1715 $cm.^{-1}$) since lowering to be expected from intramolecular hydrogen bonding is presumably countered by the effect of the imino system in the α position. The isonitroso compound showed a $\lambda_{max.}$ at 226, 273 mμ with a shift in base to 260 mμ and 335 mμ. On reacidification the original spectrum was developed. This parallels the reversible bathochromic shift in alkali which various 1,2 dione dioximes have recently been observed to undergo.[43]

The final step in the synthesis involved a Beckmann rearrangement of the isonitroso compound (51) employing the usual reagents to effect this type of rearrangement.

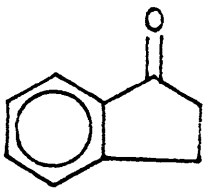
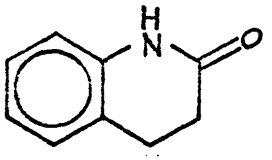
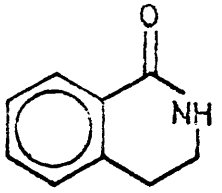
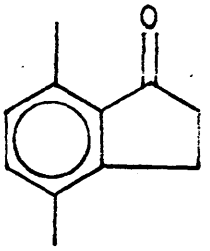
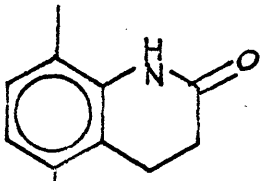
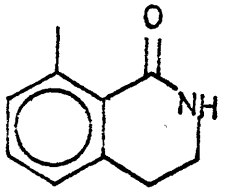
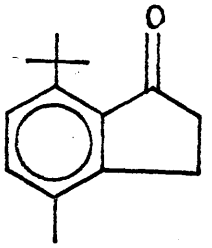
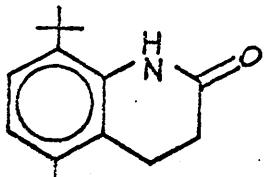
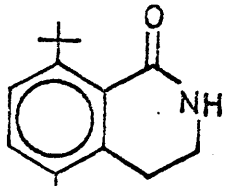


52



53

Table 17.

KETONE	% Axyl migration	%Alkyl migration	Overall Yield
	 90	 10	20%
	 34	 66	48%
 Br	 19 Br	 81 Br	25%

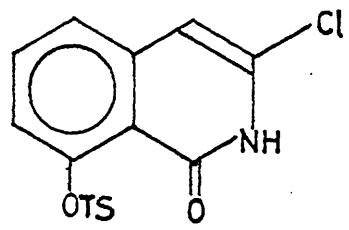
Much work has been done on the Beckmann rearrangement of indanone oximes[43,44] Table 17 gives a list of some starting compounds and their rearrangement products. From these studies it is concluded that a whole spectrum of mechanistic behaviour for oxime rearrangement can be expected, and that the generalisation of trans-stereochemistry must be used cautiously in predicting rearrangement products and assigning oxime configuration, especially in a compound liable to form iminium cations.

Although the Beckmann rearrangement appears to be fairly non-specific with numerous mechanistic pathways open, the formation of the imide (53) on treatment of the oxime (52) with thionyl chloride at ice temperature for one hour and then room temperature for another hour has been reported.[45]

The mild conditions used in this reaction appeared to be ideal for the present synthetic route and the isonitroso derivative (51) was subjected to a similar treatment with thionyl chloride, which afforded a crystalline solid m.p. 220-222°c.

The IR spectrum of this product showed strong peaks at 1655 and 1638 $cm.^{-1}$ (Nujol) characteristic of a lactam, rather than those expected for the desired imide. The mass spectrum (Table 18) showed a parent

Table 18. Proposed mass spectral cracking pattern for the compound (54)



m/e 349 (25%) 351 (10%)

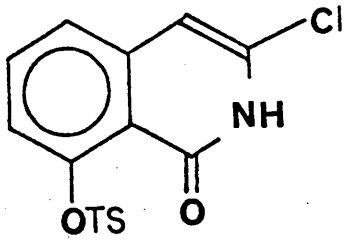
b_1 → (92·5%) 155 → b_2 → (100%) 91

a_1 → 195 (27·6%) 197 (10·2%)

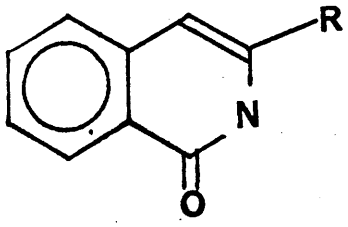
a_2 → 159 (24·8%) 161 (> 2%)

a_3 → 131 (10·4%)

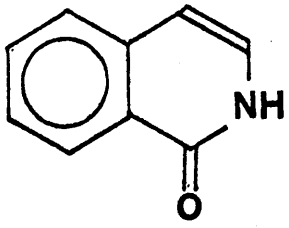
	Δm/e	m*
a_1	154	-
a_2	36	129·6
a_3	28	107·9
b_1	194	-
b_2	64	53·4



54



55



56

molecular ion at m/e 349, the large P+2 ion (abundance ca. 1/3 of the parent ion) indicating the presence of one atom of chlorine, and this was confirmed by a positive Beilstein test. The presence of a peak at m/e 155 and the loss of 155 mass units from the parent ion suggested the retention of the tosyl group in the product. These data together with microanalysis indicated the molecular formula $C_{16}H_{12}ClNO_4S$.

Solubility factors governed the use of dioxan as NMR solvent, which in fact, served admirably. The signal at 6·43 τ due to the dioxan resonance was used as a lock and the spectrum scanned above and below this signal. The 3H singlet at 7·81 τ (aromatic methyl group) confirmed the presence of the tosyl grouping. A series of signals between 2 τ and 3 τ were ascribed to the aromatic protons and it was significant that the peaks were superposable with those in the NMR spectrum of 7-tosyloxyindanone (50) indicating that the aromatic substitution pattern was the same in both molecules. The only remaining signal in the NMR spectrum of the product was a sharp 1H singlet at 3·56 τ.

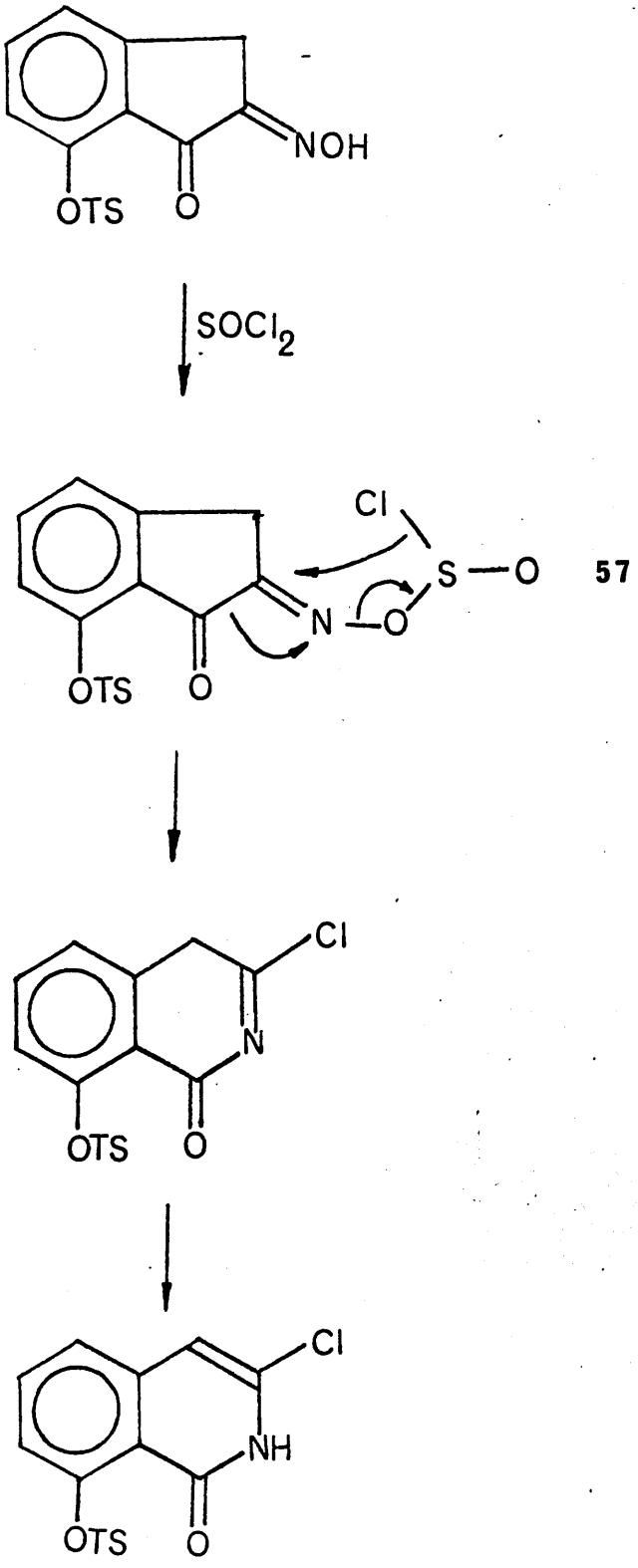
These spectral features are accommodated if the product is 3-chloro-8-tosyloxy-1-isoquinolone (54). Striking confirmation of this assignment was obtained by comparison with other 3-substituted isocarbostyrils.

Table 19. Comparison of UV data of the Beckmann product with various 1-quinolones

COMPOUND	$\lambda_{max.}$ (C_2H_5OH) mμ	$\lambda_{max.}$ (C_2H_5OH-OH) mμ
3-chloro-1-isoquinolone (54)	227, 250*, 285, 293, 325*, 337, 350*	319
1-isoquinolone	230, 280, 290*, 330, 340*	300
3-phenyl-1-isoquinolone (55, R = Ph)	232, 250*, 305, 335 350*	327

* inflexion.

SCHEME 6



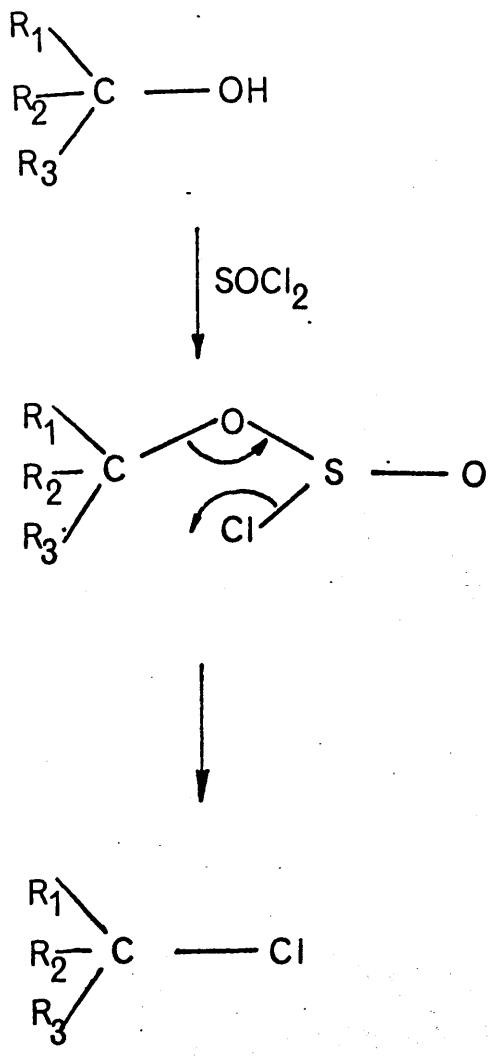
The NMR spectra of 3-ethyl isocarbostyril (55, R = Et) and 3-phenyl isocarbostyril (55, R = Ph) exhibit a sharp singlet at 3·65 τ and 3·17 τ respectively,[46] assigned to proton H_4, and comparable to the signal (3·56 τ) for the rearrangement product.

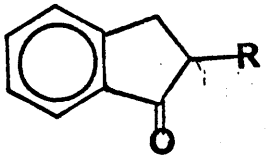
Inspection of the IR spectrum of 3-phenyl isocarbostyril (55, R = Ph) revealed that the carbonyl region was almost superposable with that for the rearrangement product.

The UV spectrum of the rearrangement product was similar to that of 3-phenylisocarbostyril (Table 19) especially in the longer wavelength regions, and both compounds displayed a reversible bathochromic shift in alkali of ca. 31 mμ. The UV spectrum of the product was also similar to that reported[47] for 1-isoquinolone (56) (Table 19) which also displays a bathochromic shift in alkali of about 20 mμ.

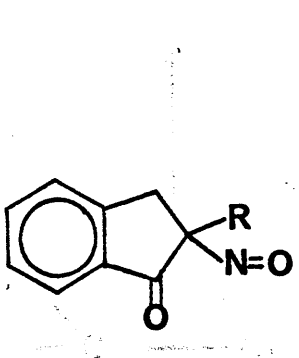
The formation of the chloroquinolone (54) may be accounted for as shown in Scheme 6. The postulated internal substitution mechanism involving the chlorosulphite ester (57) is analogous to the well known reaction whereby alcohols can be converted to their

SCHEME 7





58



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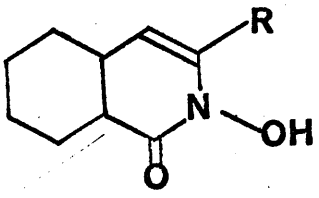


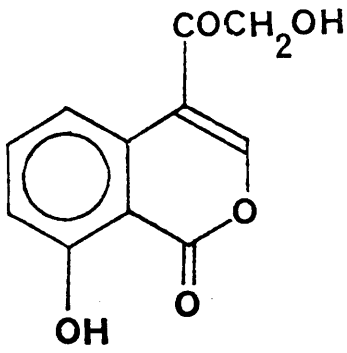
60

chlorides via a similar chlorosulphite intermediate[48] (Scheme 7).*

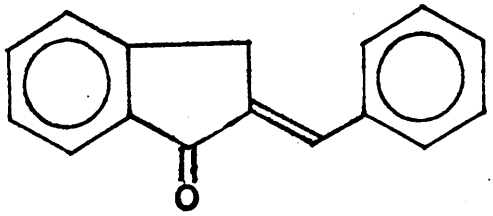
Imino chlorides have in fact been shown to arise as intermediates in Beckmann rearrangements[49] using reagents like phosphorous oxychloride, and are readily hydrolysed to the amides. That the chloroquinolone (54) is obtained rather than the imide is probably due to the known exceptional resistance of 3-halo-1-isoquinolones to hydrolysis.[50]

Further attempts at rearrangement of the iso-nitroso derivative (51) employing other Beckmann reagents like phosphorous pentachloride and phosphorous oxychloride each resulted in complex mixtures, one of the components of which could be identified by UV and TLC analysis as the 3-chloroisoquinolone (54). When sulphuric acid was employed, it too gave a complex mixture of products none of which could be cleanly separated.

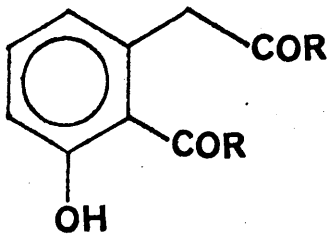
* It may be of interest to note that nitrosation under acidic conditions of 2-alkyl indan-1-ones (58, R = Me or Et) results in the 3 alkyl-2-hydroxyisoquinolones[51] (60, R = Me or Et) via the isolable intermediate (59, R = Me or Et). Reduction of (60, R = Me or Et) gives the known 3-alkyl isoquinolones.



61



62



63

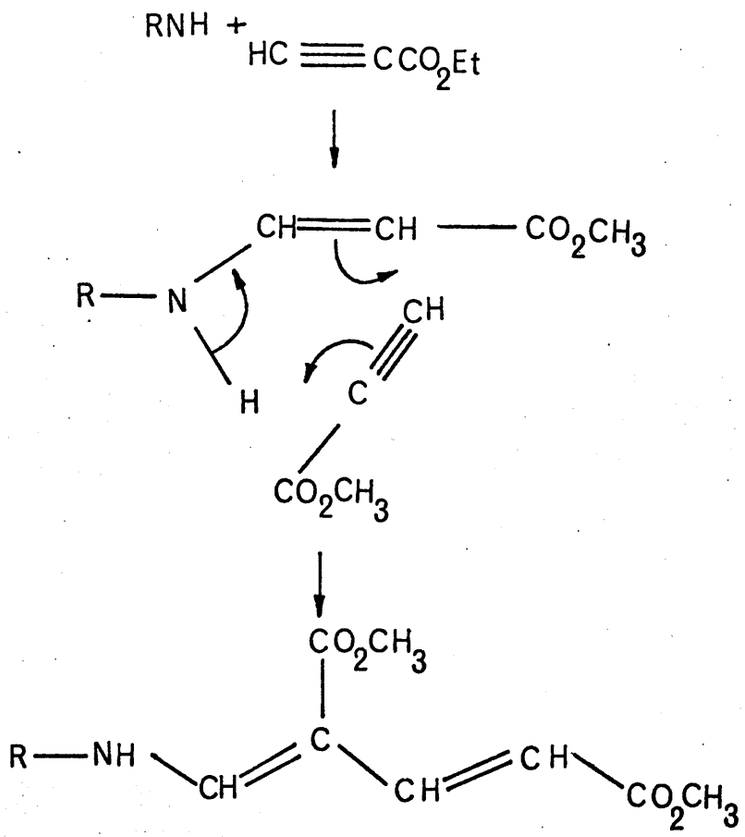
These studies were not continued further since an alternative approach to 8-hydroxyhomophthalimides via 7-hydroxyindanone was found to be successful. A key step in this synthetic sequence had in fact been recently carried out during the synthesis of the isocoumarin oosponol (61).[52] This key step involved ozonolysis of the benzylidine derivative (62) of 7-hydroxyindanone.

The resulting acid (63, R = OH) was methylated with diazomethane to give the dimethyl ester (63, R = OMe) as a pale yellow oil, TLC analysis indicating it to be a homogeneous, relatively non-polar, compound. This compound showed the expected carbonyl absorption bands at 1735 $cm.^{-1}$ and 1670 $cm.^{-1}$ due to a free and intramolecularly hydrogen bonded aromatic ester grouping.

Treatment of the diester (63, R = OMe) with hot aqueous methylamine for three hours afforded a crystalline product m.p. 140-141°c which had identical NMR, IR, UV, MS, and TLC with the homophthalimide dehydro-C (40, R = Me), and the m.p. was undepressed by a mixture of the two.

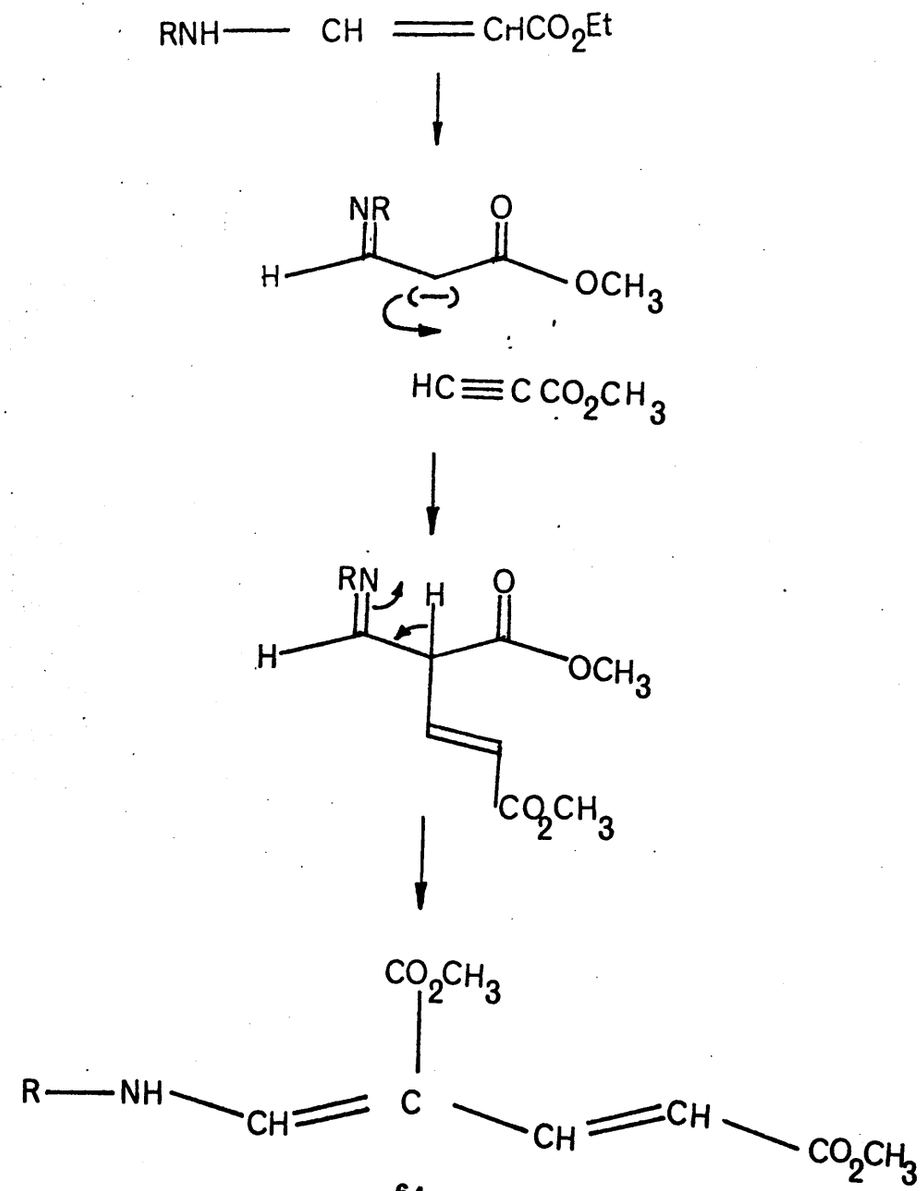
This synthesis provides confirmation of the structures of the dehydro derivatives of compounds C and D, thus in turn establishing the structures of C and D and of A and B.

SCHEME 8A



64

SCHEME 8B



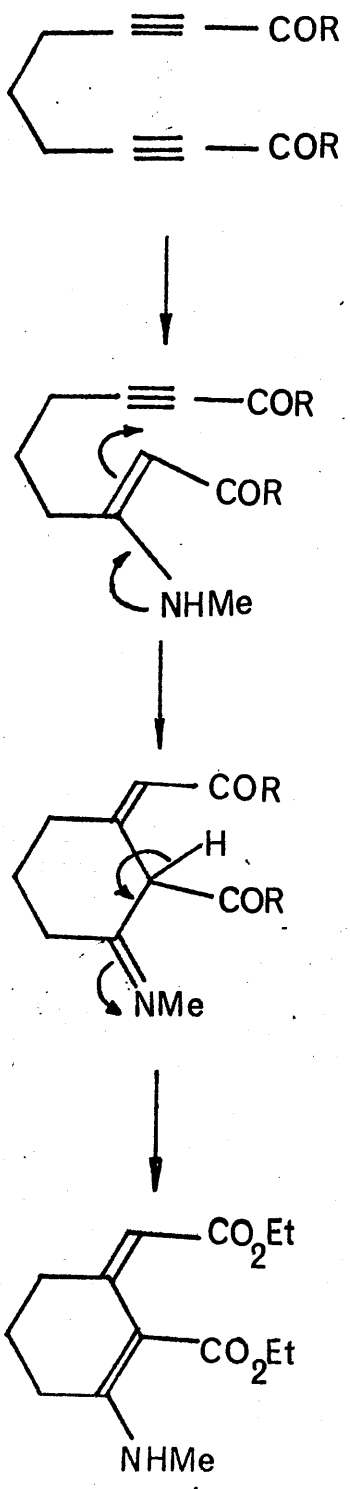
64

Thus it can be said that the addition of methylamine to the diacetylenic diester (10, R = OEt) appears to take an unusual course. The probable reasons and mechanisms for this are discussed below.

Very recently it has been reported[52a] that at higher temperatures the addition of primary amines to the activated acetylene methyl propiolate, results in the diadduct (64). Since the monoadduct is formed solely at lower temperatures and gives the diadduct when heated with an excess of methyl propiolate it is assumed that the monoadduct is formed first which then reacts with another mole of acetylenic ester to form the diadduct. The mechanism that was suggested for this sequence is shown in Scheme 8A and is essentially an enamine condensation. Another possible mechanism could involve condensation via the imino form, which has been shown to exist,[9] resulting in the desired product (Scheme 8B).

This is exactly analogous to what is observed in the reaction of methylamine with the heptadiyne diester (10, R = OEt). Thus instead of addition of one mole of amine across both triple bonds to give the piperidine (11), intramolecular condensation via the monoenamine (65, R = OEt) (Scheme 9A) or the dimino form (66, R = OEt) (Scheme 9B) predominates with the formation of compound E (20).

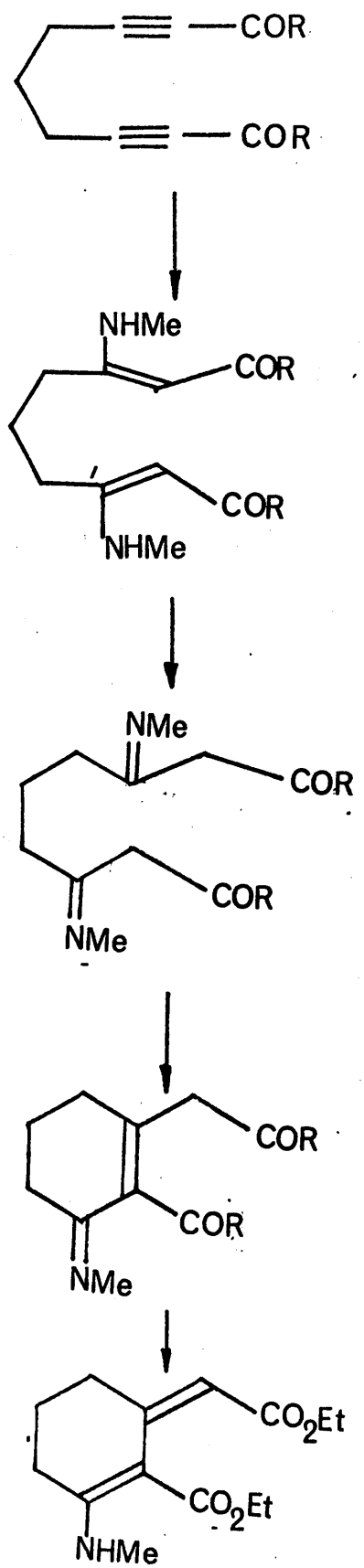
SCHEME 9A



65

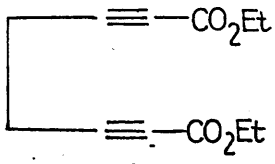
20

SCHEME 9B

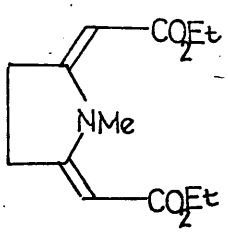


66

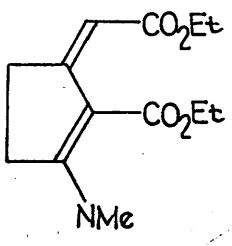
20



67



6



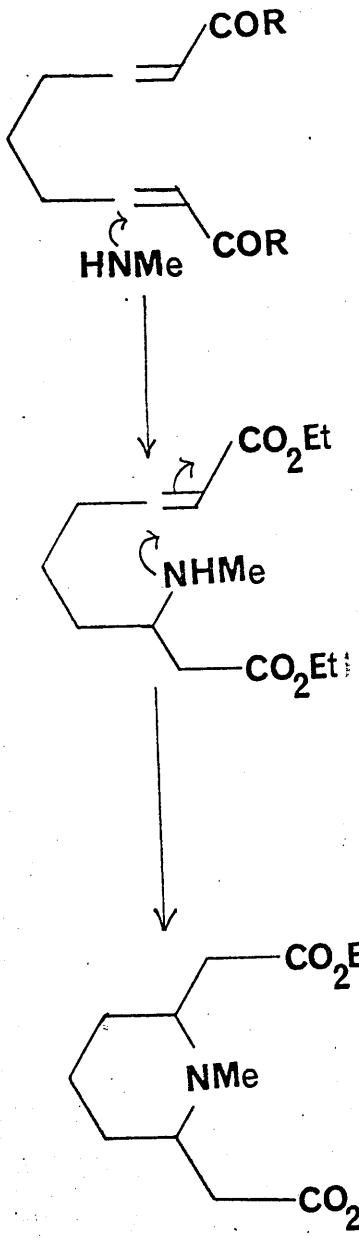
68

It is of some interest to note that this is the opposite of what happens in the reaction of methylamine with hexa-1,5-diyne (67) where the addition of one mole of amine across both triple bonds occurs to give the pyrollidine diester (6),[10] rather than intramolecular condensation to give a compound (68) analogous to compound E (20).

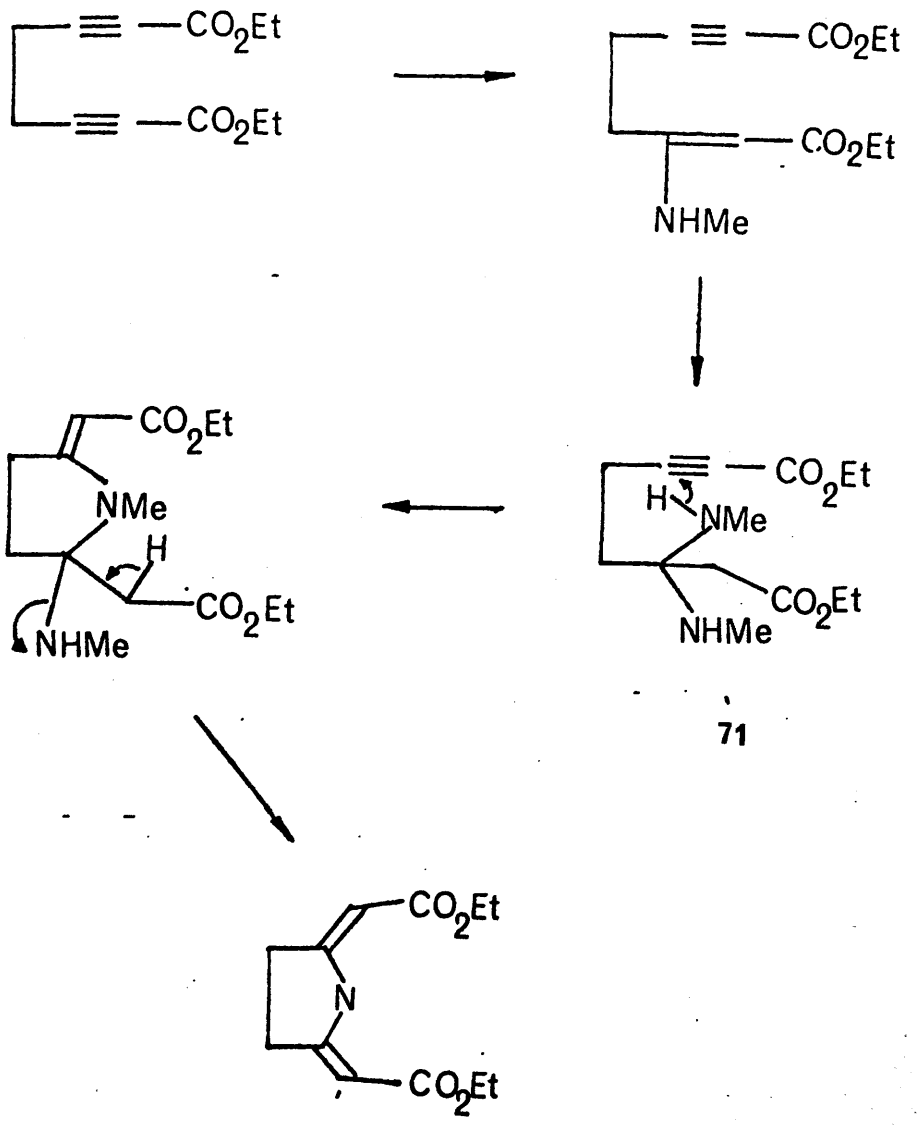
A priori, since the nucleophilicity of enamines and their reactivity towards electrophilic or activated double bonds is well known, it is reaasonable to suppose that enamine condensation or addition would be a more favourable process than addition of the enamine -NH group across another mole of acetylene. This would appear to be borne out by the formation of the diadduct (64) from methyl propiolate and ethanolic methylamine at high temperatures.

It may therefore be said that the formation of E (20) might be predicted on these grounds. Moreover E should be stabilized not only by conjugation (the chromophore being that of an amine dienoic acid) but also by hydrogen bonding between the amino -NH proton and the adjacent ester carbonyl. Models show that the ring system is not unduly strained. However models of the product (68) not formed by amination of hexa-1,5-diyne

SCHEME 10



SCHEME 11


CO2Et
CO2Et
CO2Et
CO2Et
NHMe
CO2Et
H
NMe
CO2Et
NHMe
71
CO2Et
NMe
H
CO2Et
NHMe
CO2Et
N
CO2Et

shows that in this case the ring would be fairly rigid, almost planar, and that the ring methylene groups would be eclipsed. It also appears that the -NH proton and the adjacent ester carbonyl group would be too far apart to allow strong hydrogen bonding. These considerations then, oppose the formation of this product (68).

Whereas addition of methylamine to the diendoic ester (69, R = OEt) to give the saturated piperidine diester (70)[10] is perfectly feasible mechanistically (Scheme 10), the same does not hold for nucleophilic attack by an enamine -NH group to give the unsaturated bis-carbethoxymethylene pyrollidine (6). This mechanistic difficulty can be circumvented if a second mole of amine is involved to give an intermediate of the type (71) (Scheme 11).

The above studies appear to throw some light on the course of addition of amines to diacetylenic diesters, and suggest that the scope of mechanism of these reactions merits further investigation.

EXPERIMENTAL

Section 1.3

General.

Instrumentation

Melting points were determined on a Kofler Hot-Stage apparatus and are uncorrected. Untraviolet spectra (UV) were obtained on a Unicam S.P.800 recording spectrophotometer while infra-red spectra (IR) were measured on a Unicam S.P.100 spectrophotometer (quantitative) and on a Perkin Elmer 237 spectrophotometer (qualitative). The large majority of nuclear resonance spectra (NMR) were determined on a Varian HA-100 spectrophotometer tetramethylsilane being used as internal standard. Mass spectra were obtained by direct insertion into an A.E.I. M.S.9 double focussing mass spectrometer; precise mass measurements were made relative to perflurotributylamine. Unless otherwise stated gas-liquid chromatography was performed on Pye Argon and Perkin Elmer F11 Chromatographs.

1,2,3,5,6,7-Hexahydro-2-methyl-8-methylamino-1,3-dioxo-isoquinoline (14, R = Me)

(a) Diethyl nona-2,7-diyne-1,9-dioate, (10, R = OEt) (4·3 g), was refluxed with 25% aqueous methylamine (25 ml) and ethanol (40 ml) for 2 hours. Evaporation and crystallisation of the residue from benzene gave the 2-methyl-8-methylamino compound, (14, R = Me), as prisms (2·5 g; 67%), m.p. 161-162°c, identical with previously prepared material.

$\nu_{max.}(CHCl_3)$: 3225, 1650 $cm.^{-1}$.

$\lambda_{max.}(C_2H_5OH)$: 236 mμ(log ε 3·72), 286 mμ (log ε 3·23) 350 mμ (log ε 4·38).

τau values ($CDCl_3$): 4·37 (1H, bs, J = 2 c.p.s.), 6·68 (3H, s), 6·9 (3H, d, J = 5·4 c.p.s.), 7·4 (4H, m), 8·1 (2H, m).

(b) The enamino diester (20, R = Me), (1 g), prepared as described below, was refluxed for 2 hours with aqueous alcoholic methylamine as in method (a). The 2-methyl-8-methylamino compound, (14, R = Me), obtained in this way was identical with a sample prepared as in (a).

1-Carbethoxy-6-carbethoxymethylene-2-methamicrocyclohex-1-ene (20)

The acetylenic diethyl ester (10, R = OEt) (4·72 g) was refluxed with 33% ethanolic methylamine (30 ml) and ethanol (20 ml) for 3 hours. The excess amine and solvent were removed in vacuo and the residue distilled, using as short a path as possible, giving 1-carbethoxy-6-carbethoxymethylene-2-methylaminocyclohex-1-ene (20) as a viscous yellow oil, (75-85% yield, depending on the scale of distillation), b.p. 170-174°c/ 0·06 m.m.

$\nu_{max.}$(thin film): 3150 (broad), 1695, 1644 1580 (broad) 1282, 1150 cm.$^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 322 mμ (log ε 4·32).

τau values ($CDCl_3$): 3·87 (1H, s), 5·9 (4H, q, J = 6·6 c.p.s.), 6·9 (2H, m), 7·05 (3H, d, J = 5·4 c.p.s.), 7·6 (2H, m), 8·25 (2H, m), 8·67 and 8·76 (6H, t, J = 6·6 c.p.s.)

2-Carbethoxy-3-carbethoxymethylcyclohexanone (21)

The methylamino diester (20) (1·34 g) in glacial acetic acid (10 ml) with platinum oxide (0·2 g) absorbed 118 ml. of hydrogen in 40 minutes. (Required for one double bond 115 ml). The catalyst was separated on glass paper and the filtrate neutralised with solid sodium

carbonate, with addition of water, and the product isolated with ether. The ethereal layer was washed thoroughly with saturated sodium chloride solution, dried ($MgSO_4$), and the solvent removed <u>in vacuo</u> to yield <u>2-carbethoxy-3-carbethoxymethylcyclohexanone</u> (21) as a colourless mobile oil. b.p. 118°c/0·02 mm.

$\nu_{max.}$(thin film): 3550 (broad), 1725, 1650, 1614, 1294, 1180 $cm.^{-1}$.

$\lambda_{max.}$(EtOH): 255 mμ (log ε 3·79).

$\lambda_{max.}$(EtOH-OH^-): 281 mμ (log ε 3·98).

τau values ($CDCl_3$): 5·85 (4H, q, J = 7· c.p.s.), 7·65 (5H, m), 8·4 (4H, m), 8·74 (6H, t, J = 7· c.p.s.)

<u>1,2,3,5,6,7-Hexahydro-2-methyl-8-hydroxy-1,3-dioxoisoquinoline (16, R = Me).</u>

The above 2-methyl-8-methylamino compound (14, R = Me), (0·54 g), was refluxed for several hours with a solution prepared from sodium (0·4 g) in ethanol (30 ml). After evaporating to dryness the residue was dissolved in water and acidified with dilute aqueous hydrochloric acid. The precipitate was crystallised from light petroleum to give the <u>8-hydroxy-2-methyl compound</u> (16, R = Me), as colourless plates (0·35 g; 69%) m.p. 95·5-96·5°c. Lit.m.p.[12] 95·5-96·5°c.

$\nu_{max.}(CCl_4)$: 3050-2900, 1687 $cm.^{-1}$.

$\lambda_{max.}(C_2H_5OH)$: 240 mμ (log ε 3·89), 320 mμ (log ε 4·26).

τau values ($CDCl_3$): 4·16 (1H, bs), 6·68 (3H, s), 7·37 (4H, m), 7·99 (2H, m).

2-N-Methylamidocarbonylcyclohexane-13,-dione (24)

2,6-Dihydroxy-N-methylbenzamide (4 g) was suspended in deionised water (60 ml), and sodium hydroxide (4H) (prepared with deonised water) added until the pH was adjusted to 9·2, measured by means of a pH meter. When the correct pH was recorded, 10% palladium-on-charcoal (2 g) was added and the mixture was hydrogenated at room temperature . After 20 hours ~ 580 ml of hydrogen was consumed. (Required for one double bond, 540 ml.) The catalyst was separated on glass paper and the filtrate cooled and acidified, and the resulting precipitate crystallised from ethyl acetate-light petroleum mixtures as colourless prisms (2 g; 50%) m.p. 51-52°c. Lit m.p.[19] 53°c. TLC examination (10% ethyl acetate in light petroleum as eluant) of the product showed one spot Rf 0·41 which gave a blood red stain with methanolic ferric chloride spray.

$\nu_{max.}$(Nujol): 3300 (broad), 1650, 1585, 1150 $cm.^{-1}$.

$\lambda_{max.}(C_2H_5OH)$: 261 mμ (log ε 4·05).

τau values ($CDCl_3$): 7·08 (3H, d, J = 5·4 c.p.s.), 7·45 (4H, m), 8·08 (2H, m).

Attempts to prepare an enol ether of 2-N-methylamido-carbonylcyclohexane-1,3-dione (24).

(1) The dione (24) (0·44 g) was gently refluxed with potassium carbonate (1 g) in acetone (10 ml), and isopropyl iodide (1 ml) or methyl iodide (1 ml) added dropwise. Heating was maintained for twelve hours and then the solution was cooled filtered and concentrated *in vacuo*. The resulting oil which solidified on standing was identified by IR, TLC and m.p. as starting material.

(2) A solution of the dione (24) (0·5 g) in sodium dried benzene (30 ml) with isobutyl alcohol (5 ml) (freshly distilled from calcium hydride) and a few crystals of p-toluenesulphonylchloride added, was refluxed for 20 hours in a soxhlet containing finely powdered calcium hydride.

The reaction mixture was poured into ice-cold bicarbonate solution and the organic material extracted with ether (25 ml). The ethereal extract was washed with water (2 x 10 ml) dried ($MgSO_4$), removal of the solvent *in vacuo* affording a solid identified by IR, TLC and m.p. as starting material.

2-N-Methylamidocarbonyl-3-methylamino-cyclohex-2-enone (27)

A solution of the cyclohexane-1,3 dione compound (24) (1·2 g) in ethanol (3 ml) with a drop of piperidine added, was refluxed with 33% ethanolic methylamine for four hours. Concentration of the reaction mixture in vacuo followed by cooling and trituration with light petroleum deposited the 2-N-methylamidocarbonyl-3-methylamino-cyclohex-2-enone (27) as an almost colourless solid (0·96 g), which crystallised from benzene-light petroleum mixtures as needles (70%) m.p. 129-130°c. (Found: C, 59·02%; H, 7·68%; N, 15·7%. M^+182. $C_{11}H_{14}N_2O_2$ requires C, 59·30%; H, 7·74%; N, 15·38%. MW 182).

$\nu_{max.}$(KBr): 3200 (broad), 1618-1580, 1289, 1191, 863 $cm.^{-1}$

$\lambda_{max.}$(C_2H_5OH): 294 mμ (log ε 4·25), 245 mμ (log ε 2·0).

τau values ($CDCl_3$): 6·98 (3H, d, J = 5·4 c.p.s.), 7·16 (3H, d, J = 5· c.p.s.), 7·4 (4H, m), 8·05 (2H, m).

Attempted Condensation of 2-N-methylamidocarbonyl-3-methylamino-cyclohex-2-enone (27) with malonic acid derivatives.

(a) The above β-keto amide (27) (0·7 g) was dissolved in dry pyridine (3 ml) and piperidine (0·4 ml) and malonic acid (0·5 g.) added. The solution was heated at

100°c with the exclusion of moisture for periods ranging from three to six hours. The cooled solution was carefully acidified with dilute acid and thoroughly extracted with chloroform (2 x 20 ml). The organic extract was washed with brine (2 x 10 ml) water (3 x 10 ml) dried ($MgSO_4$) and removal of the solvent resulted in a quantitative recovery of starting material identified by IR, TLC and m.p.

A similar experiment using potassium monomethyl malonate in place of malonic acid gave the same result.

(b) A solution of the β-keto amide (27) (0·44 g) in dry methanol (3 ml) was added slowly with stirring to a solution of sodium (0·03 g) and dimethylmalonate (0·66 g) in dry methanol (5 ml). The reaction mixture was refluxed for periods of 3 to 6 hours, or left at room temperature for 16 hours. After dilution with water and acidification the aqueous solution was extracted with chloroform (2 x 20 ml). The organic extract was washed with brine (3 x 20 ml) dried ($MgSO_4$) and the solvent removed <u>in vacuo</u> to yield a wet looking solid. Crystallisation from benzene-light petroleum mixtures to remove traces of dimethyl malonate resulted in a quantitative recovery of starting material, identified by IR, UV and m.p. TLC (benzene or chloroform eluant) showed no other product.

Attempted Wittig reactions on 2-N-methylamidocarbonyl-3-methylamino-cyclohex-2-enone (27)

(a) A solution of the β-keto amide (27) (0·22 g), in dry benzene (10 ml) or dry toluene (10 ml) was refluxed with carbethoxymethylenetriphenylphosphorane (29, R=OEt) (0·56 g), for periods of 1·5 to 7 days under an atmosphere of nitrogen. Removal of the solvent and TLC examination of the residue showed no product other than starting material, which was recovered quantitatively.

(b) Diethylethoxycarbonylmethylphosphonate (30) was prepared from triethyl phosphite and ethyl bromo-acetate *via* the Michaelis-Arbuzov reaction.[55]

The phosphonate (30) (0·56 g) was added dropwise to a slurry of sodium hydride (0·12 g) in dry 1,2-dimethoxyethane (5 ml) or dry dimethyl formamide (5 ml) and stirring continued for one hour. The solution quickly developed a yellow colour indicating formation of the phosphonate anion, and at this stage a solution of the β-keto amide (27) (0·42 g) in dry diglyme (3 ml) or dry dimethylformamide (3 ml) was added slowly and stirring continued overnight. The resulting solution was poured into an excess of ice-water and thoroughly extracted with chloroform (40 ml). The organic extract was washed with very dilute hydrochloric acid (2 x 20 ml) brine (3 x 20 ml)

and water (1 x 20 ml), dried ($MgSO_4$) and on removal of the solvent in vacuo, starting material was recovered quantitatively. (Identified by IR, UV and TLC.)

Attempted Reformatsky reaction on 2-N-methylamidocarbonyl-3-methylamino-cyclohex-2-enone (27)

(a) Zinc turnings (0·166 g) washed successively with dilute hydrochloric acid, water, alcohol, and ether and then dried and heated in vacuo with a crystal of iodine, were added to ethyl bromoacetate (0·2 g) in dry ether (2 ml) and the solution refluxed for two hours.

The coloured solution was decanted under nitrogen from the unreacted zinc, and the β-keto amide (27) (0·11 g) in dry ether (3 ml) was added slowly to this solution and refluxing resumed for five hours. Removal of the solvent and acidification of the residue followed by chloroform extraction gave only starting material and ethyl bromoacetate.

(b) To a stirred solution of clean dry zinc (1·25 g) and iodine (0·035 g) in dry benzene (15 ml) and dry ether (10 ml) was added ethyl bromoacetate (0·37 g) and the β-keto amide (27) (0·455 g) in dry ether (5 ml) and the reaction mixture was refluxed for 5·5 hours. During this time five more portions of zinc (1·25 g) with a

trace of iodine were added at 45 minute intervals while after 1·5 hours more ethyl bromoacetate (0·37 g) was added. Dilute acetic acid was added until the solution was acidic and this acidic aqueous layer was extracted with ether. The ethereal solution was thoroughly washed with ammonium hydroxide solution until the organic layer was clear. Concentration in vacuo of the dried ethereal solution gave only ethyl bromoacetate, identified by IR.

The combined aqueous extracts and washings were acidified with dilute hydrochloric acid and subjected to constant chloroform extraction. Removal of the solvent in vacuo gave only starting material, identified by IR, UV and TLC.

Dehydrogenations

Dehydrogenations were carried out by refluxing the compound in a metal bath with 20% by weight of 10% palladium-on-charcoal, in diphenyl ether (1 ml for 100 mg material) for varying periods. After refluxing and cooling for ca. 1 minute the reaction mixture was poured into a large excess of light petroleum (b.p. 40-60°c). After 1 hour the solid was filtered off and the product extracted from the accompanying catalyst by a suitable solvent.

8-Hydroxyhomophthalimide (40, R = H)

Prepared from 1,2,3,5,6,7-hexahydro-8-hydroxy-1,3-dioxoisoquinoline (16, R = H), by dehydrogenation during 1 hour. The product was extracted with hot ethanol which, on cooling, deposited 8-hydroxyhomophthalimide (40, R = H) as pale brown needles (74%) m.p. 195-215°c (d).

$\nu_{max.}$(Nujol): 1700, 1660, 1616, 1190, 810, 700 cm.$^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 212 mμ (log ε 4·21), 220 mμ (inf.) (log ε 4·11), 253 mμ (log ε 3·93), 318 mμ (log ε 3·58).

τau values (Trifluroacetic acid): 2·20 (1H, q, J = 8· c.p.s.), 2·80 (1H, bd, J ~ 8 c.p.s.), 2·90 (1H, bd, J ~ 8 c.p.s.), 5·58 (2H, s).

8-Hydroxy-2-methylhomophthalimide (40,R = Me).

Prepared from 1,2,3,5,6,7-hexahydro-8-hydroxy-2-methyl-1,3-dioxoisoquinoline (16,R = Me) by dehydrogenation during 15 minutes. The product was extracted from the catalyst by chloroform and crystallised from methanol to give the 8-hydroxyhomophthalimide (40, R = Me), as long almost colourless needles (57%) m.p. 140-141°c.

$\nu_{max.}$(Nujol): 1700, 1640, 1618, 800, 700 cm.$^{-1}$.

$\nu_{max.}$(1·44mM.$CHCl_3$): 3100 cm.$^{-1}$(broad), 1728 cm.$^{-1}$ (ε 292), 1716 cm.$^{-1}$ (ε 326), 1655 cm.$^{-1}$ (ε 950), and 1621 cm.$^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 214 mμ (log ε 4·01), 222 mμ (inf.) (log ε 3·95), 257 mμ (log ε 3·88), 316 mμ (log ε 3·58). τau values ($CDCl_3$): 2·50 (1H, q, J = 8 c.p.s.), 3·06 (1H, d, J = 8 c.p.s.), 3·24 (1H, d, J = 8 c.p.s.), 5·97 (2H, s), 6·66 (3H, s).

σ-Aminocarbonylphenoxyacetic acid (47)

To a solution of salicylamide (10 g) and sodium hydroxide (20 g) in water (40 ml) was added dropwise a 50% aqueous solution of chloroacetic acid until the alkaline solution had assumed a pale orange colour. When this was achieved the reaction mixture was heated at 100°c for 1 hour, and after cooling and acidification with dilute hydrochloric acid the aryloxyacetic acid (47) (12 g, 94%) was precipitated as a colourless solid m.p. 213-215°c Lit.m.p.[56] 213-215°c. The product was soluble in bicarbonate solution and reprecipitated on acidification.

Tetrahydrobenzo-1,4-oxazepine-3,5 dione (48,R = H)

(a) The aryloxacetic acid (47) (1 g) was refluxed with acetic anhydride (20 ml) and a drop of concentrated sulphuric acid for four hours, (after which time a fair amount of charring had occurred). Distillation removed most of the acetic anhydride and the somewhat charred residue was extracted with ether (100 ml). The ethereal solution was thoroughly washed with bicarbonate solution (3 x 50 ml) brine (3 x 50 ml) dried ($MgSO_4$) and solvent removal in vacuo yielded tetrahydrobenzo-1,4-oxazepine-3,5 dione (48, R = H) as a brown solid which crystallised from aqueous ethanol as long needles (0·22 g, 24·5%), insoluble in bicarbonate solution. M.p. 151-153°c. Lit.m.p.[36] 152-153°c.

(Found M^+ 177. $C_9H_7NO_3$ requires MW 177).

$\nu_{max.}$(KBr): 3200, 3155, 1726, 1669, 1613, 1344, 1270, 775 $cm.^{-1}$.

$\nu_{max.}$(1·7mM.$CHCl_3$): 1721 $cm.^{-1}$ (ε 598), 1712 $cm.^{-1}$(inf.) (ε 400), 1688 $cm.^{-1}$ (ε 647), and 1606 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 211 mμ (log ε 4·58), 243 mμ (log ε 4·36), 295 mμ (log ε 3·52).

τau values (Trifluroacetic acid): 1·8-3·0 (4H, m), 5·2 (2H, s).

(b) The aryloxyacetic acid (47) (3 g) was refluxed in diphenyl ether (40 ml) for 1·5 hours. After cooling for a few minutes the diphenyl ether solution was carefully poured into an excess of light petroleum. The oxazepine (48, R = H) was deposited as a brown powder (2·5 g) and had identical IR and m.p. as the oxazepine prepared by method (a). (Yield after crystallisation 91%).

(c) The aryloxyacetic acid (47) (1·7 g) in dry benzene (9 ml) was stirred with oxalyl chloride (9 g) for 1·5 hours at room temperature. The excess oxalyl chloride and solvent were removed in vacuo and the resulting gum crystallised from aqueous ethanol to give the oxazepine identical to that obtained in methods (a) and (b). (Yield 68%.)

Treatment of tetrahydrobenzo-1,4-oxazepine-3,5 dione (48, R = H) with aluminium chloride

The oxazepine (48, R = H) (1·5 g), was thoroughly mixed with fresh aluminium chloride (2·8 g), and the finely powdered mixture heated with frequent stirring at 180-210°c for periods of 0·5 to 1·5 hours. On cooling the solid was powdered and added to an excess of ice-hydrochloric acid. Constant chloroform extraction

of the aqueous solution for 1 or 2 days and removal of the solvent gave an oil which solidified on standing. TLC (10% methanol in chloroform as eluant) of the solid showed there was one major component and two minor ones. The mixture was separated by preparative TLC on five Kieselgel plates (200 x 200 x 0·7 mm) using 10% methanol-90% chloroform as developing solvent. The major band was extracted and identified as salicylamide by IR, TLC and mixed m.p. with an authentic sample. The two other components were also extracted but both displayed no UV absorption above 250 mμ and were not investigated further.

N-methyltetrahydrobenzo-1,4-oxazepine-3,5 dione (48, R = Me)

The oxazepine (48, R = H) (1·77 g) and finely powdered potassium carbonate (3·5 g) were gently refluxed in acetone (40 ml). Methyl iodide (2·13 g) was added to the refluxing solution and heating continued for 5 hours. The potassium carbonate was separated by filtration and removal of the acetone *in vacuo* gave N-methyltetrahydrobenzo-1,4-oxazepine-3,5 dione (48, R = Me) as a solid which crystallised from light petroleum as needles (1·6 g, 83·7%) m.p. 107°c. TLC (benzene as eluant) showed the compound to be homogeneous and less polar than the starting material.

(Found: C, 62·56%; H, 4·78%; N, 7·23%. M^+ 191. $C_{10}H_9NO_2$ requires C, 62·82%; H, 4·74%; N, 7·33%; MW 191). $\nu_{max.}$(KBr): 1705, 1655, 1610, 1320, 1232, 780 $cm.^{-1}$. $\nu_{max.}$(1·52 mM $CHCl_3$): 1711 $cm.^{-1}$ (ε 320); 1654 $cm.^{-1}$ (ε653); and 1606 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 212 mμ (log ε 4·6), 246 mμ (log ε 4·4), 300 mμ (log ε 3·55).

τau values ($CDCl_3$): 1·7-3·0 (4H, m), 5·2 (2H, s), 6·58 (3H, s).

Treatment of N-methyltetrahydro-1,4-oxazepine-3,5 dione (48, R = Me) with aluminium chloride

The N-methyloxazepine (48, R = Me) (1 g) was fused with fresh aluminium chloride (2 g) at 190-210°c for periods of 0·5 to 1 hour. After cooling the dark coloured solid was powdered and added to ice - hydrochloric acid and the aqueous solution extracted with chloroform. TLC (10% methanol in chloroform as eluant) of the organic solution revealed it to be a mixture of numerous components from which the phenolic material was extracted with dilute (2N) sodium hydroxide. The base soluble material (0·5 g) was shown by TLC to be a mixture of six components. A degree of separation was achieved by preparative TLC on five Kieselgel G plates (200 x 200 x 0·7 mm.) and 10%

methanol - 90% chloroform as developing solvent. Each component was extracted and the UV spectrum recorded.

Band 1, (R_f 0·93), $\lambda_{max.}$(C_2H_5OH): End Absorption only.

Band 2, (R_f 0·71), $\lambda_{max.}$(C_2H_5OH): 210 mμ, 239 mμ, 300 mμ, $\lambda_{max.}$(C_2H_5OH-OH^-), 327 mμ.

Band 3, (R_f 0·56), $\lambda_{max.}$(C_2H_5OH): End absorption only.

Band 4, (R_f 0·5), $\lambda_{max.}$(C_2H_5OH): 215 mμ, 237 mμ, 300 mμ, (no shift in base or acid).

Band 5, (R_f 0·3) and Band 6 (R_f 0·14). Both showed no absorption above 250 mμ, and no shift in acid or base.

The major component (Band 2) had similar IR and UV properties to that of N-methyl salicylamide [$\lambda_{max.}$ (C_2H_5OH): 210 mμ, 238 mμ, 301 mμ. $\lambda_{max.}$(C_2H_5OH-OH^-) 328 mμ] and like the other components which were examined by UV spectroscopy it was not further investigated.

7-Hydroxyindan-1-one (44)

7-Hydroxyindan-1-one (44) was prepared essentially by the method of Loudon et al., by aluminium chloride rearrangement of chromanone (43). The product was steam distilled from the reaction mixture and crystallised from methanol as long silky needles (55%) m.p. 110-111°c. Lit.m.p.[35] 111°c.

$\nu_{max.}$(Nujol): 3350 (broad), 1680, 1620, 1295, 800 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 220 mμ, 254 mμ, 315 mμ.

7-Tosyloxyindan-1-one (50)

A solution of 7-hydroxyindan-1-one (44) (3 g) and p-toluenesulphonylchloride (4·8 g) in dry pyridine (12 ml) was left at room temperature for 48 hours. Cooling on ice and trituration with ice-cold water afforded 7-tosyloxyindan-1-one (50) as a colourless powder, which crystallised from ethanol as plates (3·2 g, 52·5%) m.p. 119-120°c.

(Found: C, 63·7%; H, 4·84%; M^+ 302. $C_{16}H_{14}SO_4$ requires C 63·57%; H, 4·67%; MW 302.)

$\nu_{max.}$(Nujol); 1712, 1610, 1150, 855, 838, 779 $cm.^{-1}$

$\nu_{max.}$(1·52 mM $CHCl_3$): 1725 $cm.^{-1}$ (ε 626·5), 1614 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 210 mμ (log ε 4·41), 228 mμ (log ε 4·23), 245 mμ (inf.) (log ε 3·98), 292 mμ (log ε 3·44).

τau values ($CDCl_3$): 1·9-2·8 (7H, m), 6·85 (2H, t, J = 8 c.p.s.), 7·4 (2H, t, J = 8 c.p.s.), 7·54 (3H, s).

2-Oximino-7-tosyloxyindan-1-one (51).

A solution of 7-tosyloxyindan-1-one (50) (1·3 g) in dry benzene (12 ml) was heated at 50°c and isoamyl nitrite (1·85g) was added dropwise with stirring. Finally a drop of concentrated hydrochloric acid was added and the temperature maintained at 50°c for one hour. During the reaction the solution darkened and the oximino

compound (51) was precipitated as a fine powder which was collected, washed thoroughly with benzene, and crystallised from aqueous ethanol as needles (0·8 g, 56·6%) m.p. 202-205°c. TLC (10% methanol in chloroform as eluant) showed one spot (more polar than the starting material). (Found: C, 57·85%; H, 3·78%; N, 4·37%. $C_{16}H_{13}NO_5S$ requires, C, 58·01%; H, 3·96%; N, 4·23%.)

$\nu_{max.}$(Nujol): 3150 (broad), 1720, 1660, 1615, 1170, 910, 835, 785 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 226 mμ (log ε 4·4), 273 mμ (log ε 4·29).

$\lambda_{max.}$(C_2H_5OH-OH^-): 260 mμ (log ε 4·14), 335 mμ (log ε 4·31).

τau values (trifluroacetic acid): 2-3 τ (7H, m), 6·0 (2H, bs, J = ~ 1/2 c.p.s.), 7·52 (3H, s).

Beckmann rearrangement of 2-oximino-7-tosyloxyindan-1-one (51).

The isonitroso compound (51) (0·42 g) was suspended in dry benzene (3 ml) at 0°c and treated with thionyl chloride (9 ml) (distilled twice from linseed oil with the exclusion of moisture) with occasional shaking. The isonitroso compound was quickly consumed and the solution darkened with gas evolution. After a further hour at room temperature the solvent and excess

thionyl chloride were removed in vacuo and the residue taken up in chloroform (2 x 15 ml). The organic extracts were washed thoroughly with freshly prepared bicarbonate solution (2 x 10 ml) saturated brine solution (3 x 10 ml), water (2 x 10 ml) dried ($MgSO_4$) and concentrated in vacuo to yield an oil (0·5 g) which solidified on standing. Trituration with benzene and crystallisation from chloroform, or chloroform-light petroleum, or sublimation at 180°c/0·06 mm. all afforded fine colourless needles (0·15 g, 34%) m.p. 220-222°c, identified as 3-chloro-8-tosyloxy-1-isoquinolone (54).

Preparative TLC of the benzene washings on one Kieselgel HF_{254} plate (600 x 200 x 0·7 mm) employing 20% benzene - 80% chloroform as developing solvent, afforded more (ca. 5%) of the substituted isocarbostyril, identical with the material obtained above.
(Found: C, 54·63%; H, 3·25% M^+ 349. $C_{16}H_{12}NO_4SCl$ requires, C, 54·93%; H, 3·45% MW 349. Positive Beilstein test.)
$\nu_{max.}$(KBr): 3155 (broad), 1661, 1640, 1182, 1012, 831, 780, 754 $cm.^{-1}$
$\lambda_{max.}$(C_2H_5OH): 227 mμ (log ε 4·75), 250 mμ (inf.) (log ε 4·14), 285 mμ (log ε 4·2), 293 mμ (log ε 4·2), 325 mμ (inf.) (log ε 3·9), 337 mμ (log ε 3·99), 350 mμ (inf.) (log ε 3·69).

$\lambda_{max.}$(C_2H_5OH-OH^-): 350 mμ (inf.)(log ε 3·98), 319 mμ (log ε 4·29).

τau values (Dioxan): 2-3 (7H, m), 3·56 (1H, s), 7·81 (3H, s).

2-Benzylidene-7-hydroxyindan-1-one (62).

Treatment of 7-hydroxyindan-1-one (44) (3 g), (prepared as above) with freshly distilled benzaldehyde (8 g) in the presence of sodium ethoxide yielded 2-benzal-7-hydroxyindan-1-one (62) as pale yellow needles (3·1 g, 64·8%) m.p. 147-148°c. Lit.m.p.[52] 147-148°c.

(Found, M^+ 236. $C_{16}H_{12}O_2$ requires, MW 236).

$\nu_{max.}$(Nujol): 3350 (broad), 1675, 1620, 1295, 805, 760, 690 $cm.^{-1}$.

$\nu_{max.}$(1·48 mM.$CHCl_3$): 3460 (broad), 3356 (broad), 1684 (ε 527), 1628 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 227 mμ (log ε 4·15), 306 mμ (log ε 4·31), 317 mμ (log ε 4·36), 345 mμ (log ε 4·19).

2-Methyl-8-hydroxyhomophthalimide (40, R = Me) via dimethyl-3-hydroxyhomophthalate (63, R = OMe).

Ozonolysis of 2-benzylidene-7-hydroxyindan-1-one (62) (3 g) followed by hydrolytic work up afforded the 3-hydroxyhomophthalic acid as colourless crystals (1·1 g, 45%) m.p. 165-167°c. Lit.m.p.[52] 167-168°c. $\nu_{max.}$(Nujol): 3080 (broad), 1710, 1660, 1612, 1260, 766, 714 cm.$^{-1}$.

Treatment of the homophthalic acid (63, R=OH) (1g.) with diazomethane for two hours afforded a yellow oil on removal of solvent. The oil was dissolved in chloroform and thoroughly washed with freshly prepared bicarbonate solution and water, dried ($MgSO_4$) and the solvent removed *in vacuo* to yield dimethyl-3-hydroxyhomophthalate (63, R=OMe) (0·99 g) as an oil which solidified in the fridge. TLC (chloroform as eluant) of the semi-solid showed one spot (less polar than the starting acid).
$\nu_{max.}$(thin film): 3450, 3110 (broad), 1735, 1670, 1612, 1260, 1205, 1175, 787, 740 cm.$^{-1}$.
Dimethyl-3-hydroxyhomophthalate (63, R=OMe) (0·3g) was refluxed with 25% aqueous methylamine (5 ml) and ethanol (8 ml) for three hours. Evaporation and crystallisation of the residue from ethanol gave 2-methyl-8-hydroxyhomophthalimide (40, R=Me) as almost colourless needles (0·16 g,

62·6%) m.p. 140-141°c. This synthetic material had identical IR, UV, MS, TLC, and mixed m.p. with dehydro-C (40, R = Me).

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Part 2.

Antifungal Antibiotics

PREFACE

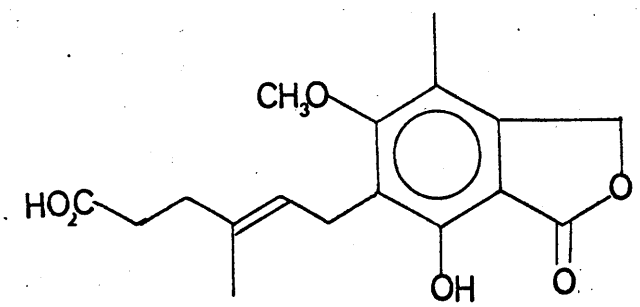
Section 1.1

Since the beginning of time, man's inherent curiosity and insatiable thirst for knowledge has led to the development of scientific research in innumerable fields. In particular the curiosity of the chemist and microbiologist has been aimed at obtaining understanding of organic reactions, a knowledge of biological systems and their function, and has resulted in an extensive library of biological metabolites.

The research on biological systems and their metabolites has offered unanticipated rewards and a striking example of this has been the isolation of products possessing antimicrobial properties and the realisation of their potential as therapeutic agents.

The earliest scientific expressions of the concept of antibiosis are credited to the fathers of the modern science of bacteriology, Pasteur and Joubert[1], who in 1877 observed the phenomenon of bacterial inhibition. In those days when sweeping prophecies and broad generalisations in science were popular, Pasteur's report contained the prophetic note:

> 'Tous ces faits autorisent peut-être les plus grande ésperances au point de vue theropeutique'.



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Several groups of workers in the following years made sporadic forays into the antibiotic field with little success. In 1896 Gosis isolated the first crystalline antibiotic substance from a fungus - (probably Penicillium brevi-compactum). This product was later named mycophenolic acid (1), but its limited antibiotic spectrum and toxic properties prevented its utilisation as a therapeutic agent. Pyocyanase (thus named because of the erroneous conclusion that activity was due to an enzyme) was the first commercial antibiotic product, and produced in Germany between 1901-1935, though subsequent investigation showed that most of the commercial preparations then available were inactive.

Thus early progress in the field of antibiotic research was slow and the discovery of penicillins was required to inspire a really serious exploration of antibiotics. The potential rewards in therapeutics were now able to justify the tremendous investment in terms of equipment and research effort which is essential for work in this field.

Since the term antibiotic originally introduced by Waksman encompasses a wide range of metabolites exhibiting for example, antifungal antibacterial or antitumor properties, to deal with all these antibiotics

would require an extensive compendium. However since this thesis is concerned with those products which exhibit antifungal behaviour the following introduction will be mainly concerned with antifungal antibiotics particularly those which have been isolated and characterised during the last decade.

INTRODUCTION

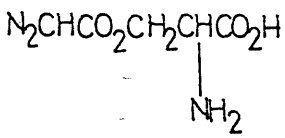
Section 1.2

Antibiotics are an extremely diverse group of compounds linked by a degree of similarity in one aspect of their biological activity. They are secondary metabolites since they are not essential to the normal growth process by which they are produced and different strains of the organism may flourish whether antibiotic production is maintained or not. The significance of antibiotic production in the economy of an organism remains a matter for speculation and only in isolated cases have antibiotics been attributed properties which aid in the survival or proliferation of the producing species.

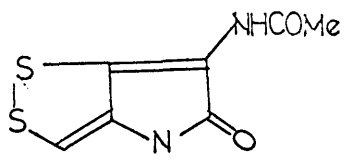
The production of antibiotics appears to involve the same type of enzymic processes and intermediary metabolites that are involved in primary metabolism. There are many antibiotics which bear interesting structural resemblances to primary metabolites, e.g., carbohydrates, proteins etc., and this may contribute to their biological activity. The antifungal antibiotics shown in Table 1, are grouped as polysaccharide, nucleic acid, cyclic polypeptide or amino acid according to their biogenetic origin and structural type. The Polymyxins (cyclic polypeptides) have been shown to interfere with the function of lypoprotein membrane[2] which may account

TABLE 1

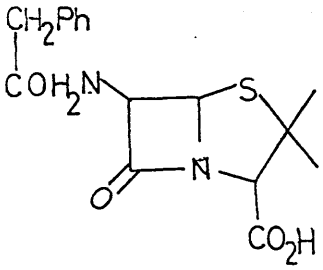
Amino Acid



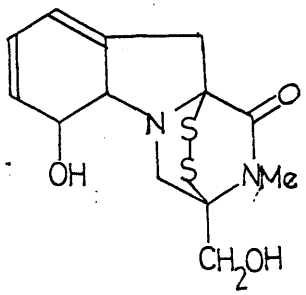
Azaserine 5



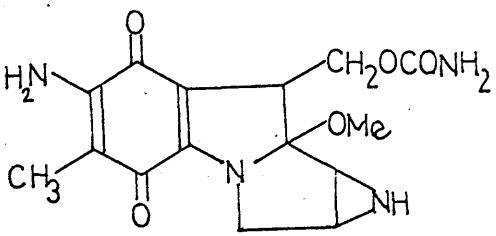
Thiolutin 6



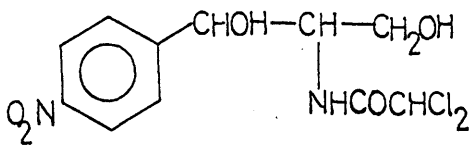
Penicillin G 7



Gliotoxin 8



Mitomycin C 9



Chloramphenicol 10

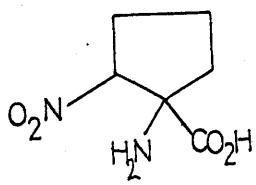


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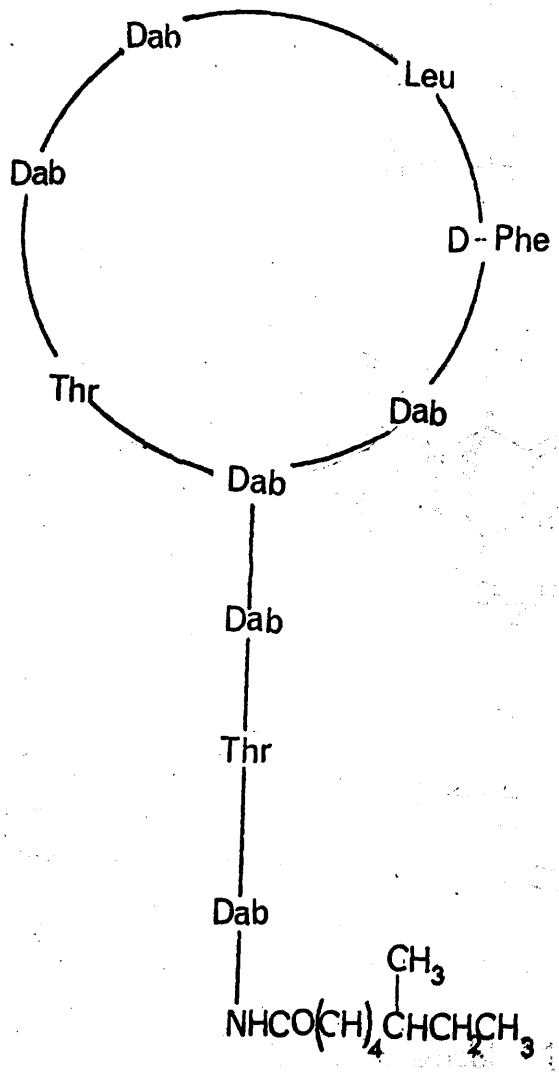
TABLE 1

Polypeptide



Polymyxin B 4

a Polymyxin A E

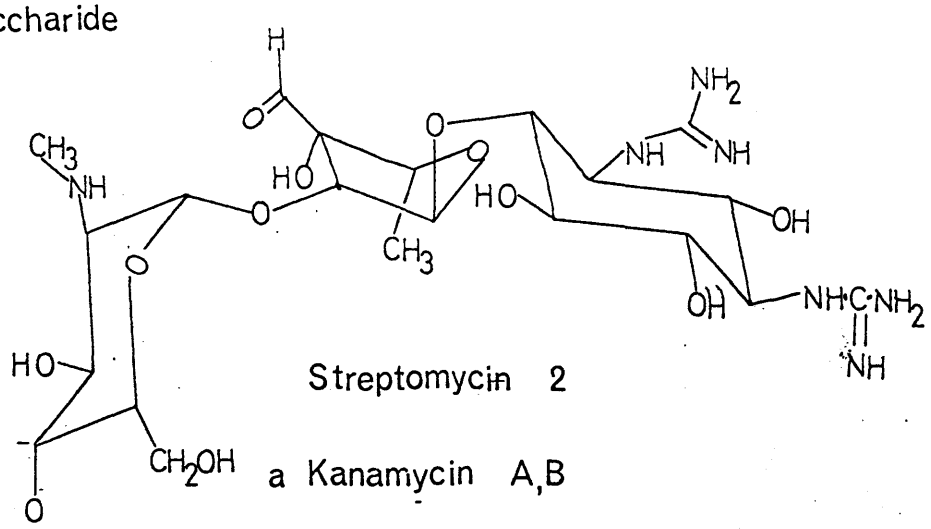
b Viomycin

c Vancomycin

d Tyrothricin

TABLE 1

Polysaccharide

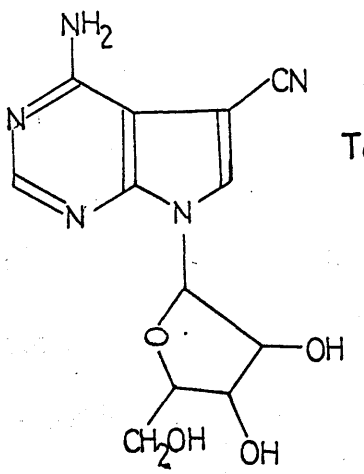


Streptomycin 2

a Kanamycin A,B

b Neomycin B

Nucleic Acid



Toyocamicin 3

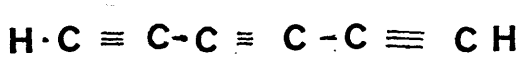
a Amiceticin

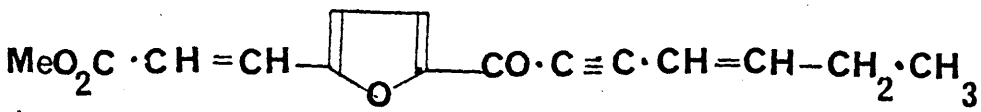
for their biological activity. The antifungal antibiotics in Table 1, derived from one or two amino acids, although relatively small in size, in several cases involve very novel structural features. For example the diazo grouping in azaserine (5), the cyclic disulphide systems in thiolutin (6), and gliotoxin (8) which also contains a 'hydrated benzene ring', the β-lactam system in Penicillin G (7), the aziridine ring in mitomycin C (9) and the nitro group and dichloracetamide system in chloroamphenicol (10).

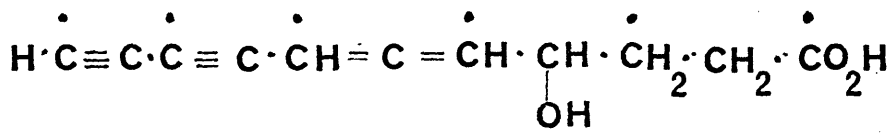
Although several of the antibiotics in Table 1, are very important therapeutic agents (Kanamycin, Neomycin Polymyxin and chloroamphenicol are in current use in Britain as antifungal agents[3]), it is beyond the scope of this introduction to discuss the details of their chemistry and biogenesis. It may also be noted that all of them with the exceptions of Penicillin G and the antibiotic (11) from Aspergillus wentii[4] (which is lysine derived) are produced by Actinomycetes or Bacteria rather than Fungi.

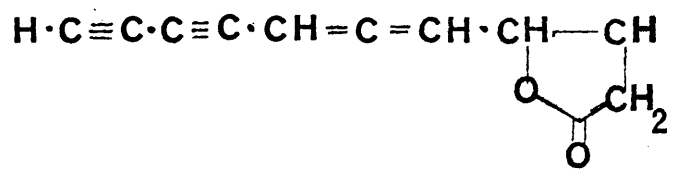
The remaining antifungal antibiotics to be discussed (including almost all the antifungal antibiotics of fungal origin) fall into two classes, polyketides and isoprenoid. The polyketides can all be formally derived

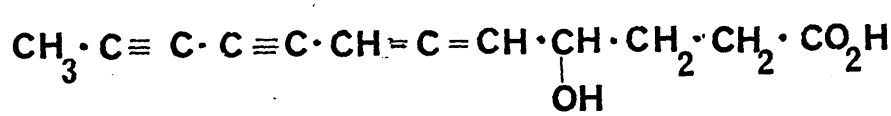
TABLE 2

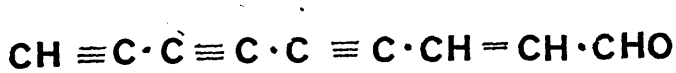
12 

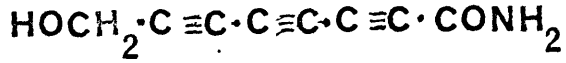
13 

14 

15 

16 

17 

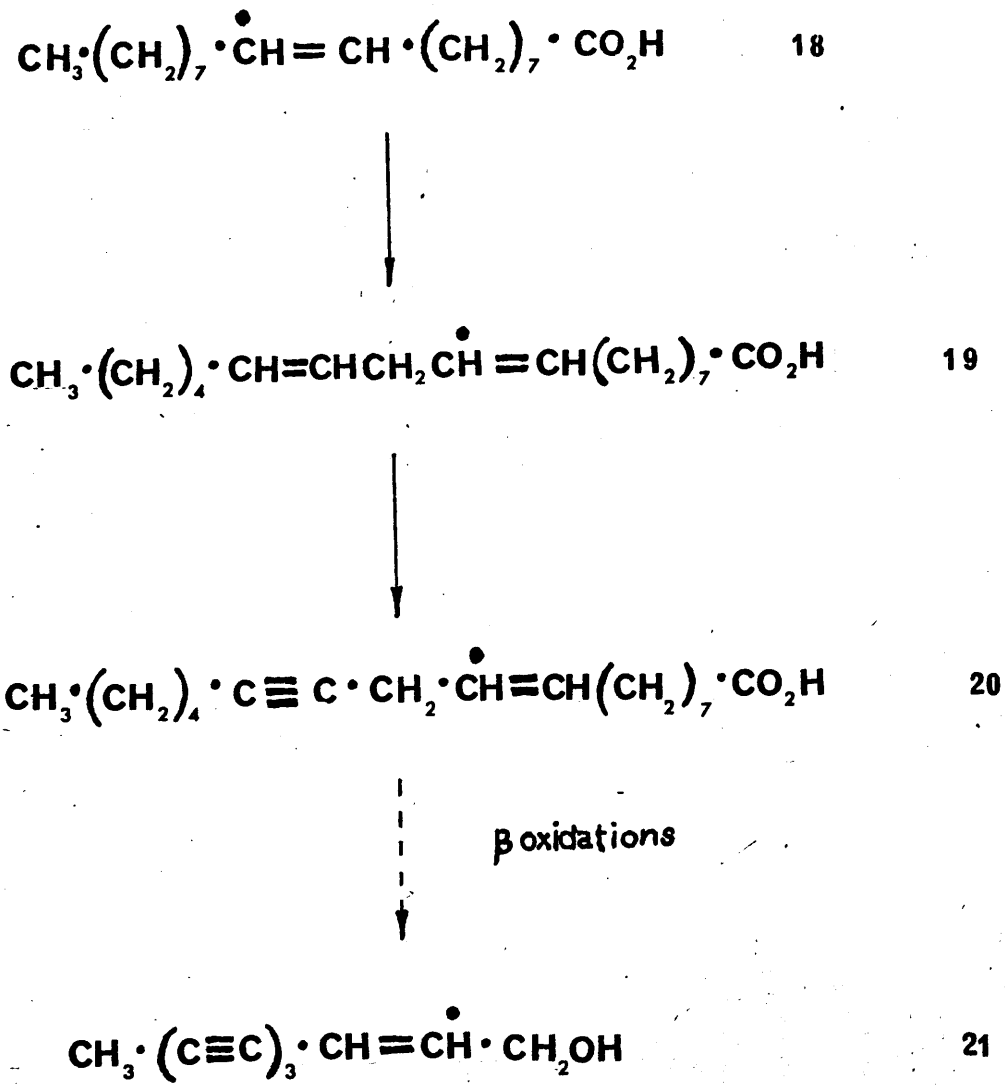
22 

from linear β-polyketide chains $\left(\text{CH}(\text{R})\text{-CO}\right)$ usually involving acetate units (R = H) and more rarely involving propionate units (R = Me). Biosynthetic studies on many different types of polyketide compounds suggests that as in fatty acid biosynthesis, the polyketide chain usually arises by successive addition of malonyl-CoA units (or where appropriate methylmalonyl-CoA) to an acyl-CoA 'starter group'.

Antifungal metabolites (which again may be closely related to primary metabolites) include many types of structurally unrelated compounds such as polyacetylenes, alicyclic compounds, phenols, polyene and other macrolides.

There have been extensive studies of polyacetylenic compounds during the last decade,[5,7] prompted in many cases by the existence of antibiotic activity in certain of these compounds.[6] Table 2 lists antifungal antibiotics of this type. Hexa-1,3,5-triyne (12) was identified as the substance responsible for the potent antifungal activity of the volatile emanation of the fungus Fomes annosus[8]. The inherent instability of the polynes (hexa-1,3,5-triyne is an extreme case since concentrations of this compound explode at room temperature)

SCHEME 1

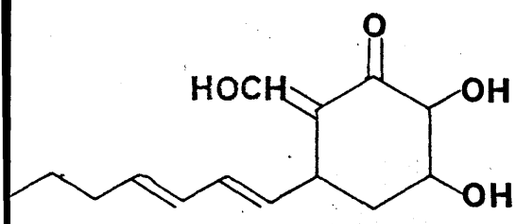

CH3·(CH2)7·CH=CH·(CH2)7·CO2H
18
CH3·(CH2)4·CH=CHCH2CH=CH(CH2)7·CO2H
19
CH3·(CH2)4·C≡C·CH2·CH=CH(CH2)7·CO2H
20
β oxidations
CH3·(C≡C)3·CH=CH·CH2OH
21

may account for the fact that they have not as yet found clinical application.

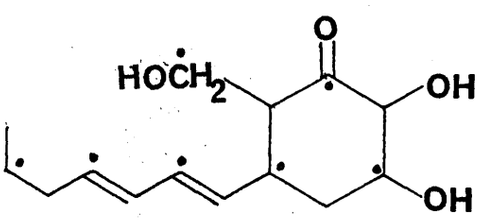
Theories of the biogenesis of these compounds are based partly on direct experimental evidence and partly on analogy. The assembly of a polyacetylene by an acetyl-CoA-malonyl-CoA type of pathway has been verified experimentally, and [1-^{14}C]-acetate was shown to be an efficient precursor (15% incorporation) of nemotinic acid (14),[9] the labelled carbon atoms being positioned as shown (heavy dots). Nemotinic acid (14) and its lactone (15) all have C_{11} chains which, by hypothesis, should contain six C_2 units, one carbon having been lost, and concordant with this view the C_{12} homologues odyssic acid (16) and lactone etc., are produced by the same cultures.

Another hypothesis has been put forward for the biogenesis of natural acetylene derivatives.[10] Briefly it was suggested that common taxonomically appropriate, fatty acids were the eventual precursors, into which double and triple bonds were introduced successively by specific dehydrogenations, or some equivalent mechanism, with reactions of chain shortening (e.g., by β oxidation), and of prototropic rearrangements, to give eventually, shorter chain polyacetylenic compounds.

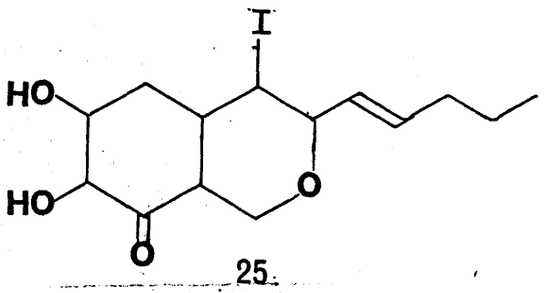
TABLE 3



Frequentin 23



Palitantin 24



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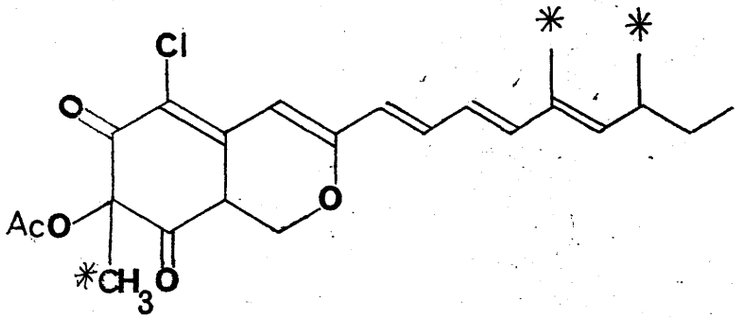
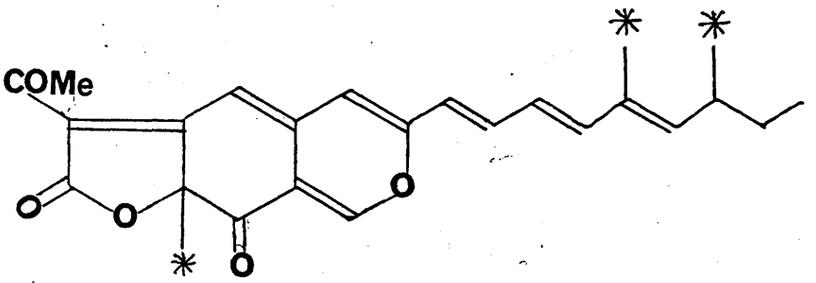
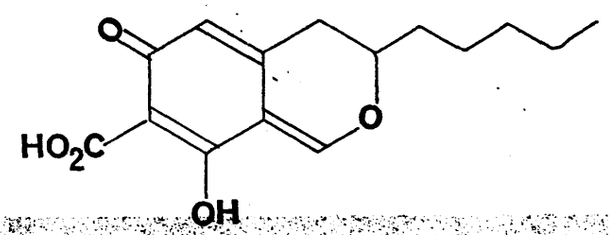


Sclerotiorin 26

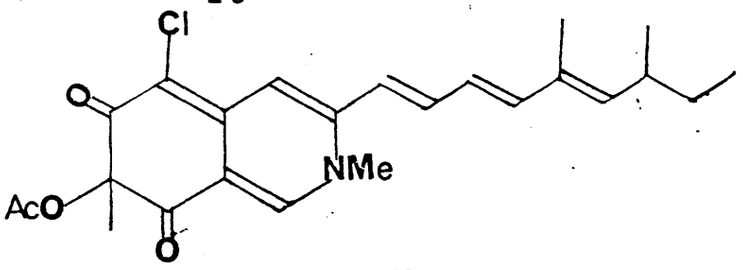


Rotiorin 27

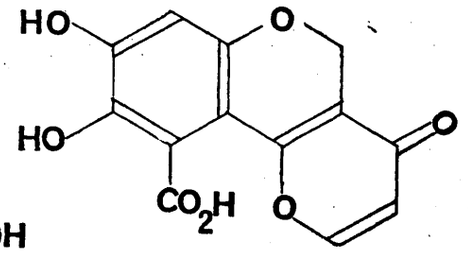


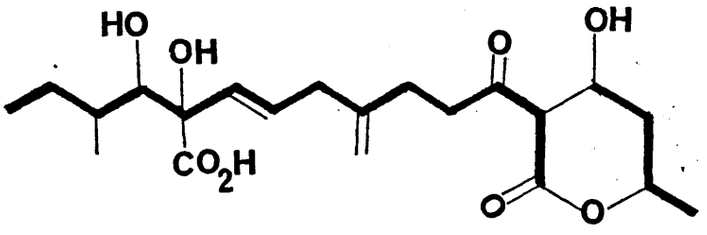
Pulvilloric Acid 28

29

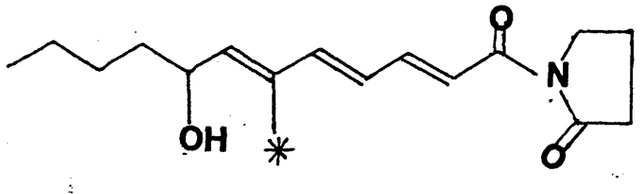


Citromycetin 31



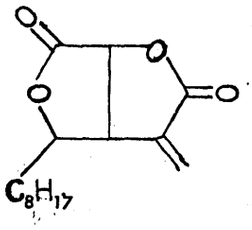


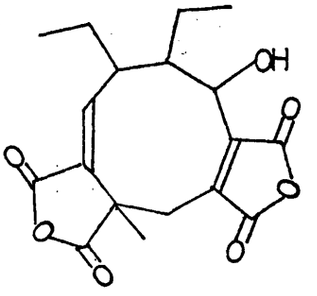
Alternaric Acid 30



Variotin 32

Avenaciolide 34





Glauconic Acid 35

Some degree of experimental proof of this hypothesis has been obtained[11] since the polyacetylene producing Tricholoma grammopodium converts [10-^{14}C]-oleic acid (18) into linoleic acid (19) and crepenynic acids (20) and eventually to dehydromatricarianol (21) labelled at position 2 (heavy dot) (Scheme 1).

On this theory the C_6 compound hexa-1,3,5-triyne (12)[8] would be ultimately derived from the C_{18} oleic acid by systematic breakdown. The C_8 acetylene agrocybin (22) could represent an intermediate stage.

It remains to be determined whether this 'desaturation' mechanism and subsequent breakdown is the universal pathway to polynes.

The second group of polyketide antifungal metabolites includes other aliphatic and alicyclic compounds (Table 3). The antifungal antibiotic frequentin (23)[12] produced by the fungi Penicillium frequentans and Penicillium palitans, is closely related to another metabolite designated palitantin (24).[13] Derivation from a linear polyacetate chain is strongly suggested by incorporation of [1-^{14}C]-acetate into palitantin (24) to the extent of 1·8%.[14] A comprehensive series of degradations established the position of the labelled carbon atoms shown (heavy dots) and it can be assumed that

frequentin (23) is derived in a similar fashion. Reaction of palitantin (24) with iodine[15] gives a cyclic ether (25) bearing a close resemblance to some other antifungal antibiotics (cf. 26 and 27). The basic skeleton of sclerotiorin (26)[17] and rotiorin (27)[16] (two metabolites of Penicillium multicolour) has been shown to be derived via the acetate-malonate pathway as shown in the formula.[18,19] Three extra carbon atoms (starred) in these molecules appear to be introduced via S-adenosylmethionine . Pulvilloric acid (28)[20] has the same type of skeleton and the 'extra' carboxyl group is probably again derived from methionine. The extra ring in rotiorin (27) appears to originate by acetoacetylation and condensation. Of special interest is the fact that this acetoacetate unit appears to arise by direct condensation of acetate units,[19] (analogous to the chain extension mechanism occurring in mitochondria), rather than via malonate.

Sclerotiorin (26) and rotiorin (27) are part of a group of compounds for which the name azaphilones has been coined because of a facile reaction with amines.[21] For example, treatment of sclerotiorin (26) with methylamine results in sclerotioramine (29).

A comprehensive series of degradations established the structure of the antifungal and phytotoxic metabolite alternaric acid (30)[22] obtained from Alternaria solani. Although presumably derived via the acetate-malonate pathway the complex structure does not yield to structural dissection as readily as others. However the skeleton could be derived by condensation of a heptaacetyl chain with a second C_4 chain as indicated (heavy lines).

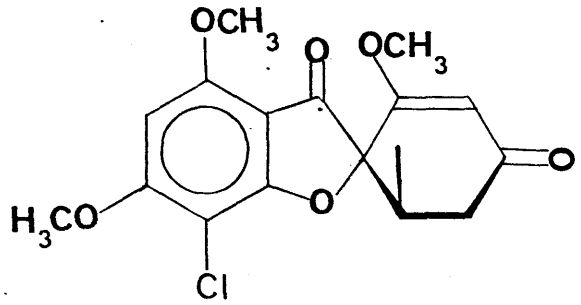
The occurrence of two different acetate chains has been indicated in the case of the fungal pigment citromycetin (31) by experiments using ^{14}C labelled malonate which located two different 'starter units'.[23]

The antibiotic variotin (32)[24] is partly derived from glutamic acid, but the main chain is polyacetate derived with one introduced or 'extra' carbon atom (Starred atom).[25]

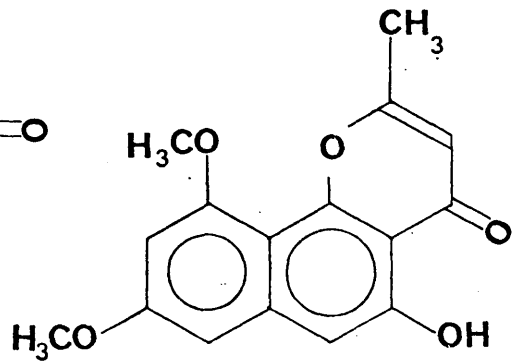
Two other antifungal antibiotics glauconic acid (33)[26] and avenaciolide (34)[27] which appear to be polyketides, will be dealt with in detail later in this section.

It may be noted at this point that there is no reason to exclude the possibility that all the foregoing antibiotics (Table 3) are derived in one way or another by elaboration of fatty acids, or C-methylated fatty

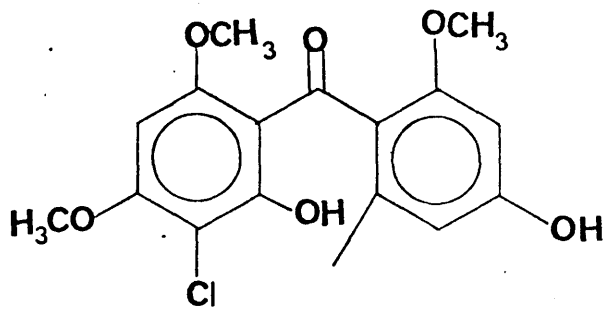
TABLE 4



Griseofulvin 35



Flavosperone 36



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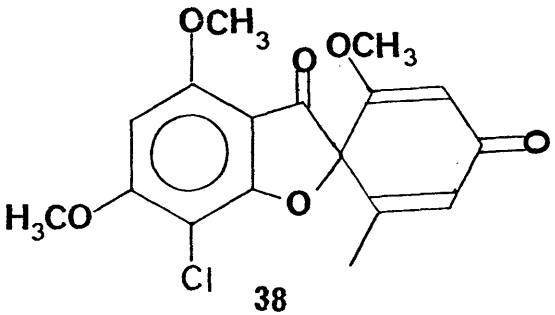

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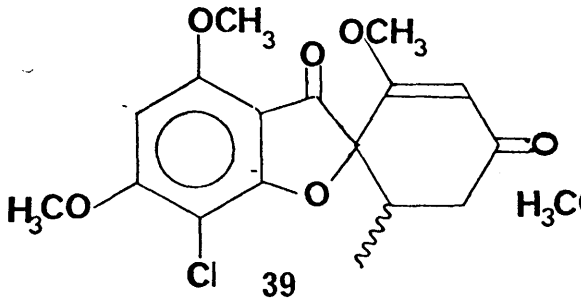

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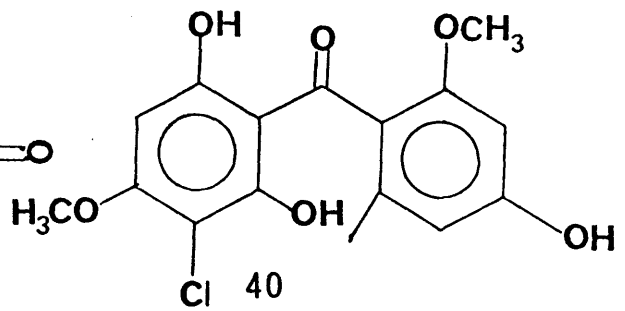

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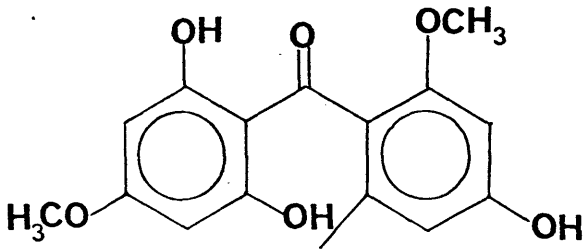

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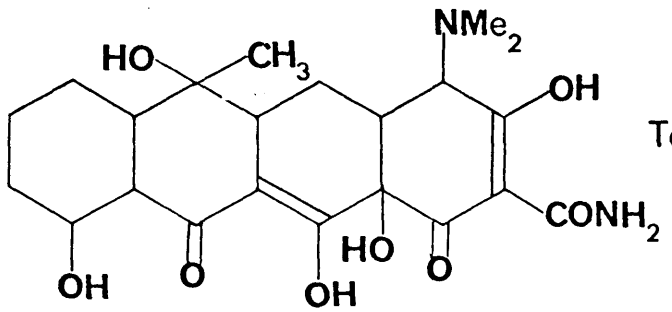
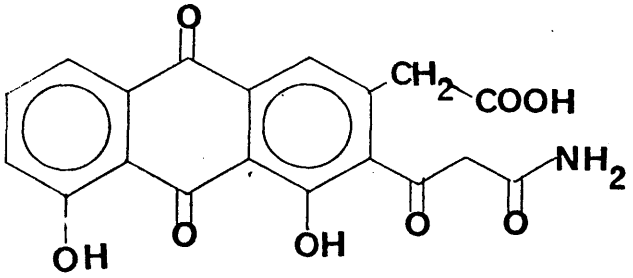


Tetracycline 42



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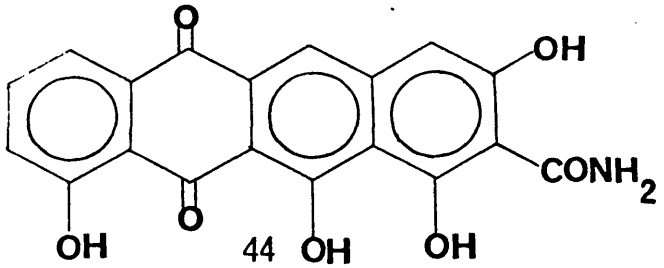

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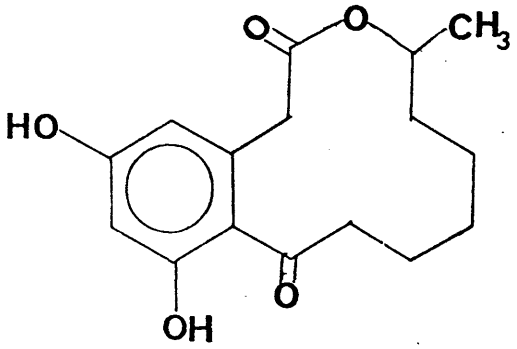
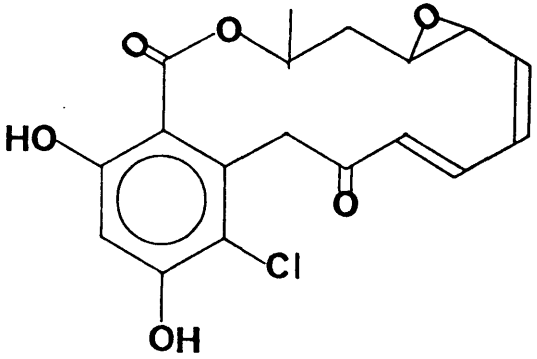
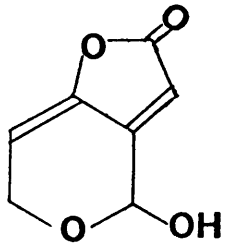


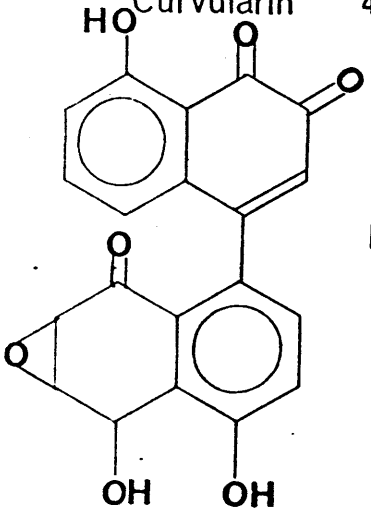
Curvularin **45**



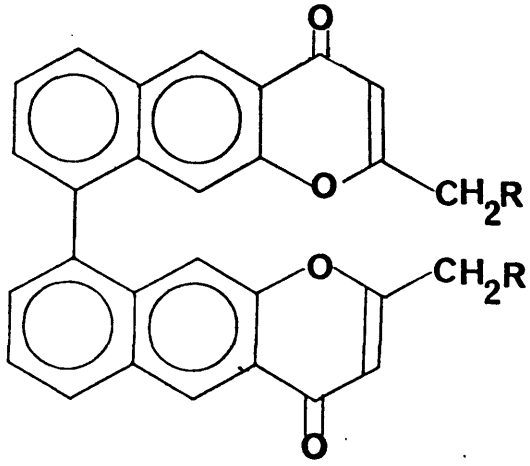
Monorden **46**



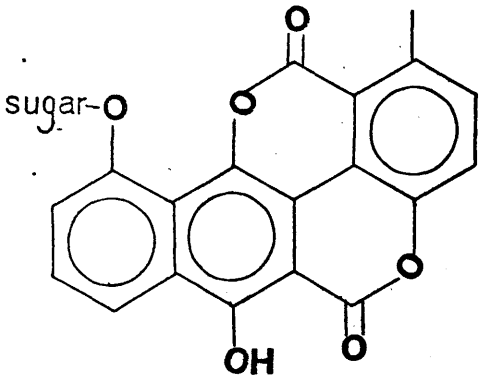
Patulin **47**



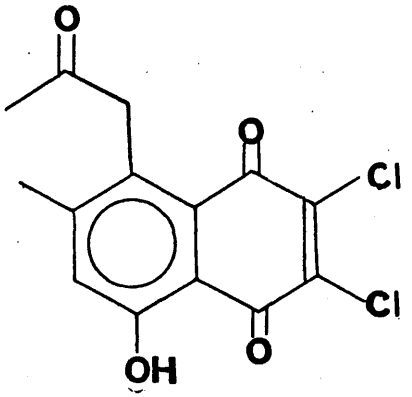
Mycochrysone **51**



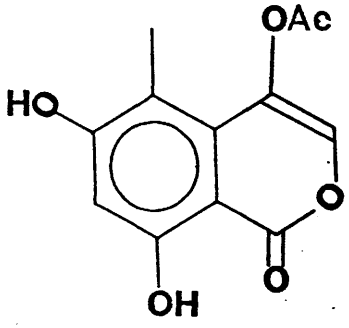
Ustilaginoidins A,B,C **52**



Chartreusin 50

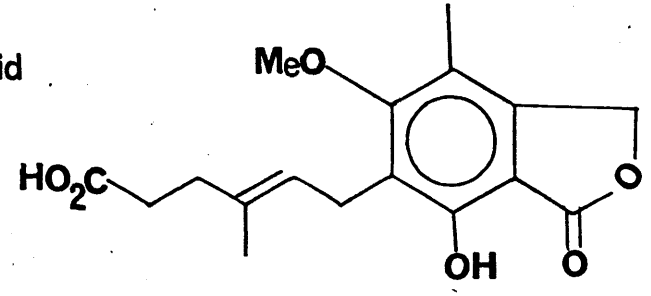


Mollisin 53



54

Mycophenolic Acid

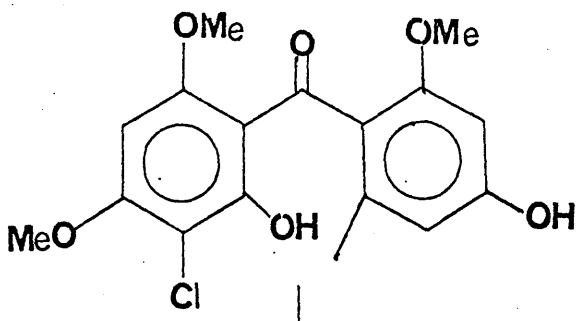


acids, since their biosynthesis is known to proceed with reduction at intermediate stages rather than via polyketonic intermediates.

In contrast, the next class of antifungal antibiotics we shall discuss are mainly aromatic compounds apparently derived by cyclisation of a preformed polyketonic chain (Table 4).

The antifungal antibiotics griseofulvin (35)[28] and flavosperone (36)[29] apparently illustrate two different ways in which a polyketide assembly derived from an acetate and six malonate units can cyclise. Griseofulvin (36) which is an important oral antifungal agent has been the subject of fairly intensive biosynthetic studies. Contrasting labelled acetate feeding experiments have been carried out,[30] the predicted labelling pattern being established in the case of 2-^{14}C - or 1-^{14}C -acetate by means of systematic chemical degradations and in the case of 2-^{13}C -acetate more elegantly by examination of the NMR spectrum.[31] The latter technique is only possible when the percentage incorporation of ^{13}C labelled substrates is greater than that of the natural abundance (1·1%) of ^{13}C.

SCHEME 2



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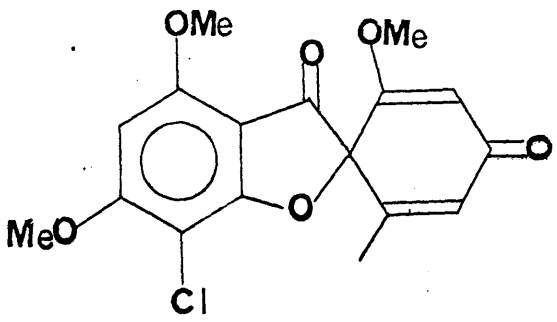

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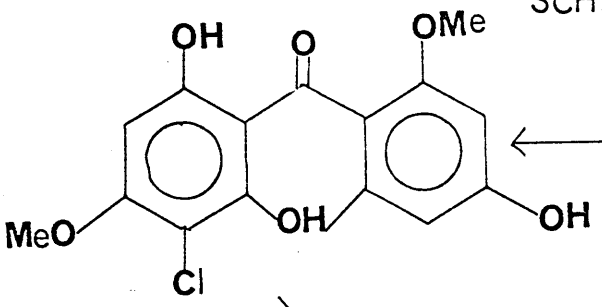
SCHEME 3



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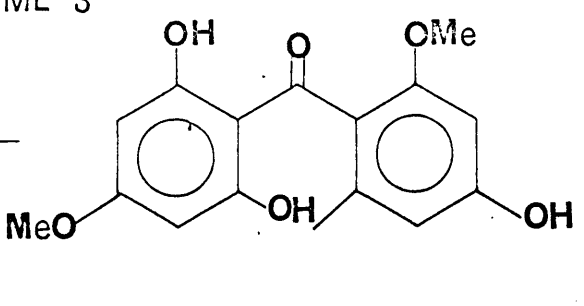

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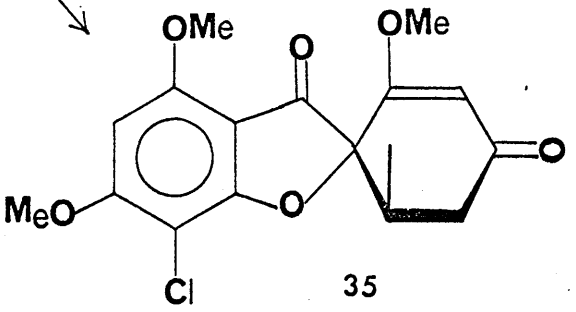

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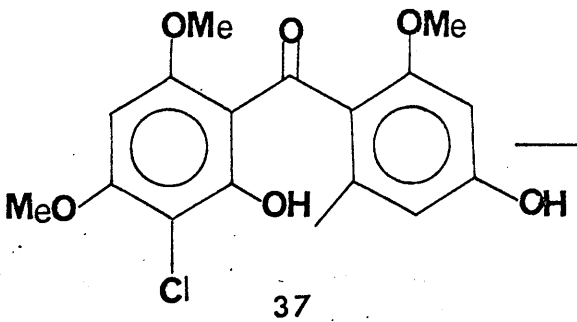

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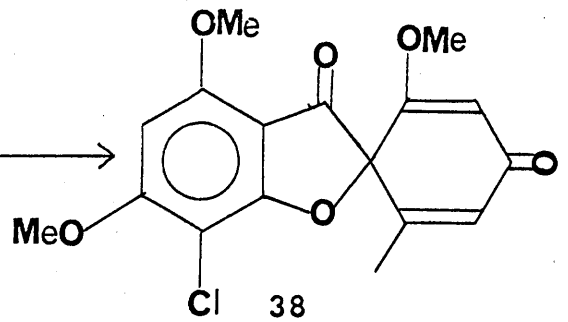
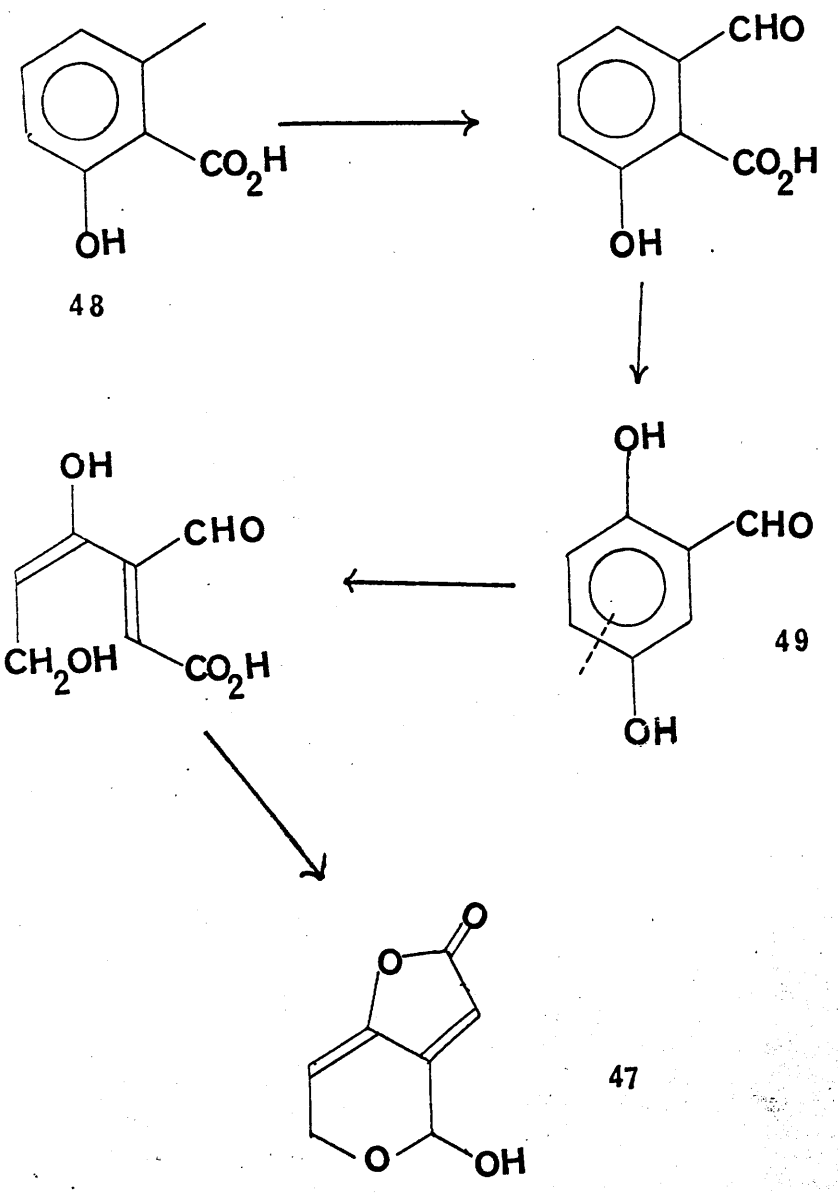


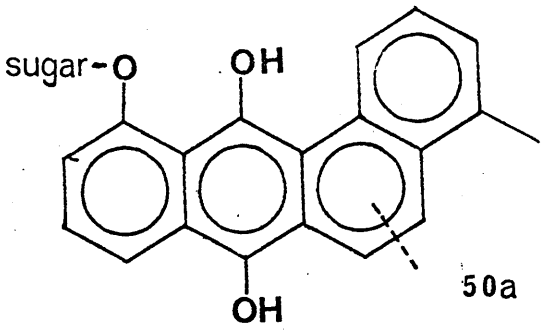
38

It was thought for some time that the isolation of griseophenone A (37) and dehydrogriseofulvin (38) from cultures containing griseofulvin (35) favoured a biogenetic pathway as in Scheme 3, in which these compounds were postulated as intermediates and this was supported by *in vitro* conversion of griseophenone A (37) to (±) griseofulvin (39) [32] (Scheme 2). However Rhodes and McGonigle[33] obtained mutants of *P. patulum* such that griseophenone B (40) and griseophenone C (41) were accumulated. These were shown to be true intermediates whereas griseophenone A (37) and dehydrogriseofulvin (38) were not. Evidently the phenolic group in griseophenone C(41) plays an important part in the biosynthesis of griseofulvin, perhaps as a point of attachment to an enzyme.

The highly reactive nature of even β-triketones and β-tetraketones suggests that some means of stabilization must be present for the larger β-polyketide chains such as are evidently involved in the biosynthesis of tetracycline (42),[34] a compound better known for its antibacterial properties than as an antifungal agent. This antibiotic appears to be formed from a polyketide chain possibly initiated by malonamic acid (though carbamoylation may occur at a later stage in the biosynthesis) and built from eight malonate units. A polyenolate enzyme

SCHEME 4





Chartreusin **50**

complex has been one suggestion for stabilization of the β-polyketone chain and chelation with a methyl ion another. An apparent blockage in the cyclisation process has been observed in one mutant of S. aureofaciens in that the anthraquinone (43) was obtained which can be cyclised in vitro by means of hydrogen bromide to give a tetracycline derivative (44).[34]

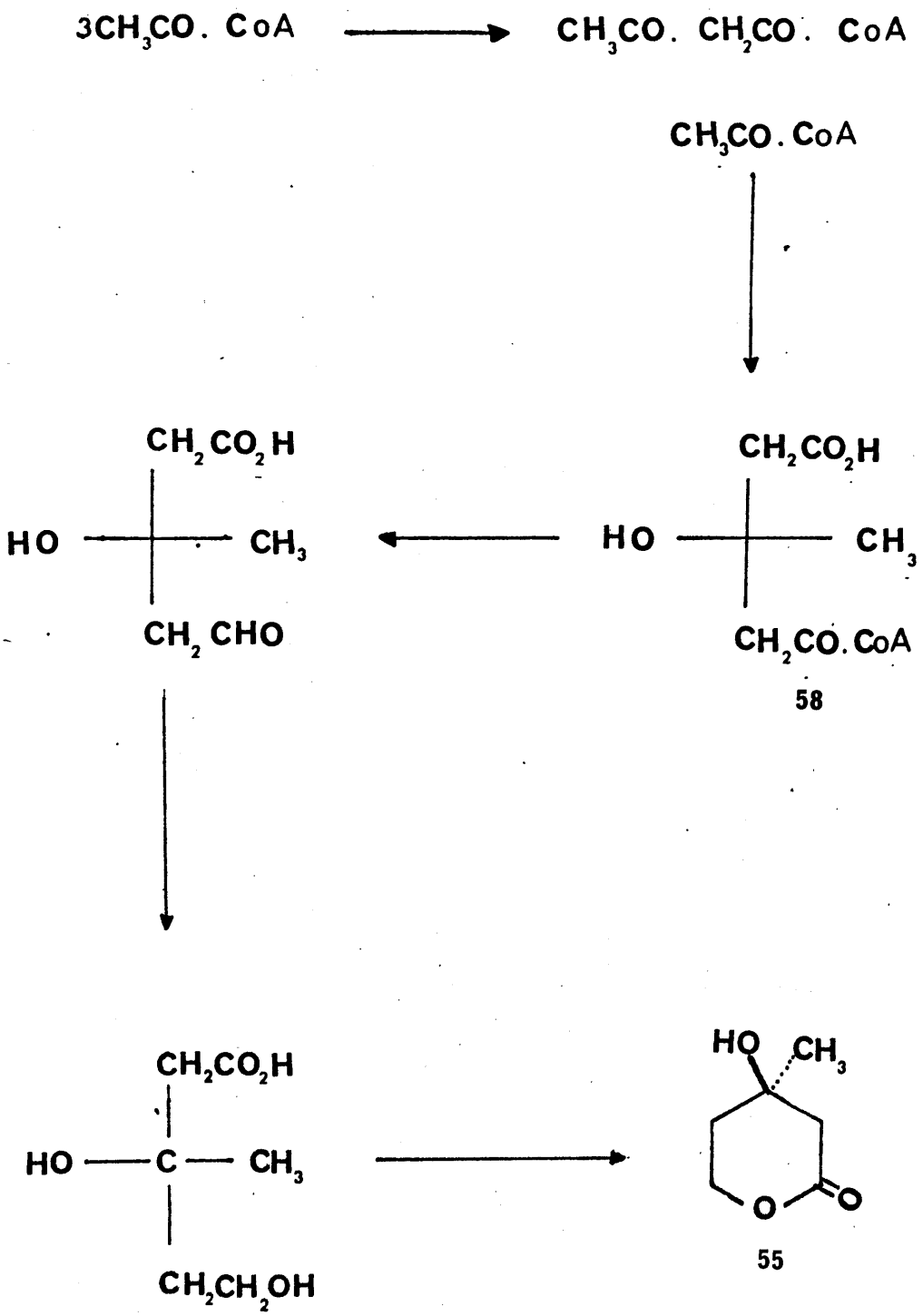
Partial cyclisation of a chain to give an aromatic system can also occur as exemplified by the antifungals curvularin (45)[35] and mon orden (46)[36,37] in which the remainder of the chain is stabilized by partial or complete reduction.

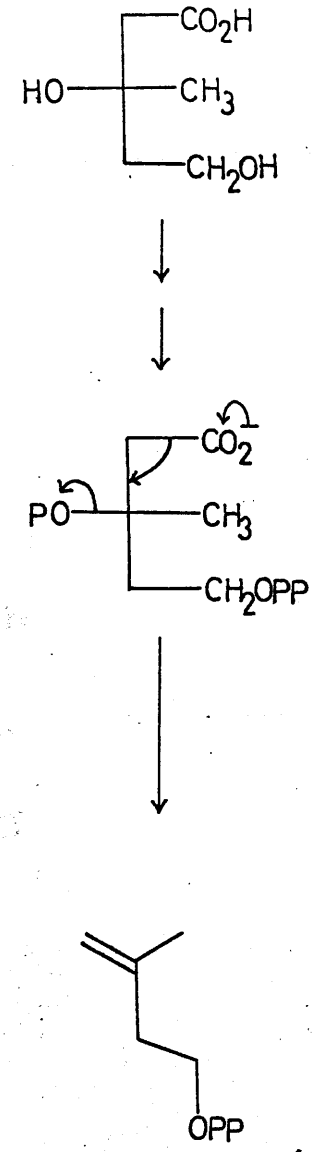
Several antifungal antibiotics result from various modifications of polyketides after stabilization, as for example in the biosynthesis of patulin (47) which involves oxidative fission of an aromatic ring. It has been shown that patulin (47)[38] is acetate derived and that the biosynthesis proceeds via 6-methylsalicylic acid (48) and gentisaldehyde (49), followed by the oxidative cleavage (Scheme 4). If the two carboxyl functions in chartreusin (50)[41] are originally adjacent carbon atoms in the tetraphene intermediate (50a), which is subsequently cleaved, then this antifungal metabolite could be derived from a linear polyketide chain of one acetate and 9 malonate units.

As in most classes of phenolic natural products dimeric types of compounds are known which could arise by phenolic oxidative coupling. Thus mycochrysone (51)[39] could be derived by oxidative coupling of two C_{10} napthols rather than from a C_{20} polyketide chain. However the red antibiotics ustilaginoidin A, (52, R =R'=H), B (52, R =H, R'=OH), and C (52, R=R'=OH),[40] could not be formally derived from a single polyketide chain. These compounds are unusual in that they are optically active, although there are no asymmetric centres and the system is fully aromatic. This property is due to restricted rotation about the bond linking the two naphthopyrone systems.

Finally the biogenesis of several aromatic antifungal products does not appear to involve only a linear polyketide chain. Different polyketide chains may combine to give mollisin (53).[42] The antibiotic (54) produced by Aspergillus viridinutans[44] could be derived in similar fashion or by C-methylation of a polyketide chain. Methionine has been shown to be the source of the C-Me group in mycophenolic acid (1)[43] and the biogenesis of this compound probably also involves alkylation of a polyketide aromatic framework with geranyl pyrophosphate.

SCHEME 5

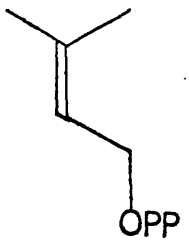

3CH3CO. CoA
CH3CO. CH2CO. CoA
CH3CO. CoA
CH2CO2H
HO
CH3
CH2CO. CoA
58
CH2CO2H
HO
CH3
CH2 CHO
CH2CO2H
HO
C
CH3
CH2CH2OH
HO
CH3
O
O
55



5 6

One process which competes with polyketide synthesis for the acetyl CoA available from breakdown of fat or carbohydrate, is the formation of terpenoids *via* mevalonic acid (55) and isopentenylpyrophosphate (56). The biosynthesis of lanosterol (57) and the steroids in particular has been extensively studied using specifically labelled substrates and purified enzyme systems.[46] A summary of the biosynthetic pathway is given in Scheme 5. The point of division from fatty acid biosynthesis appears to be the Claisen condensation of acetyl CoA with acetoacetyl CoA (or of the respective enzyme-bound species CH_3COS-Enz and CH_3COCH_2COS-Enz) to give the ester of hydroxymethylglutaric acid (58). Although this is a reversible process, subsequent reduction to mevalonic acid (55) is irreversible and mevalonate is a poor biosynthetic precursor of polyketides.

Condensation of varying numbers of isopentenyl units, derived from mevalonic acid as shown (Scheme 6), gives rise to geranyl pyrophosphate (C_{10}), farnesyl pyrophosphate (C_{15}) and geranylgeranyl pyrophosphate (C_{20}) etc., subsequent modifications of which give respectively mono-, sesqui-, and diterpenes.



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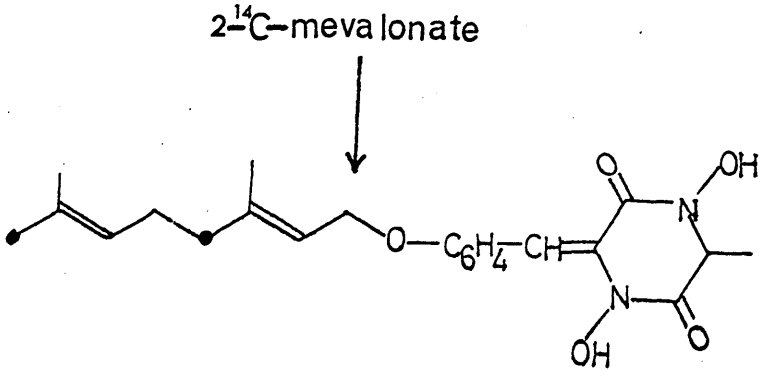

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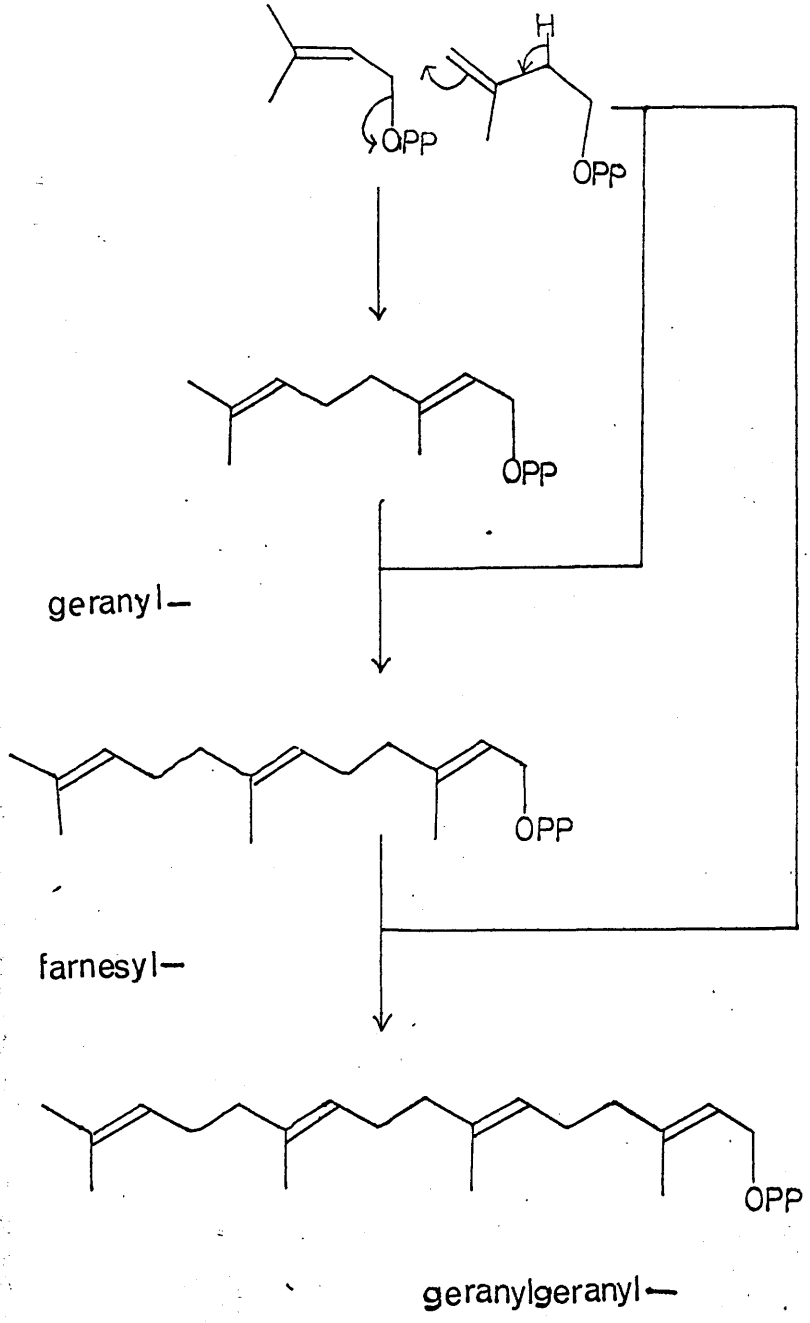
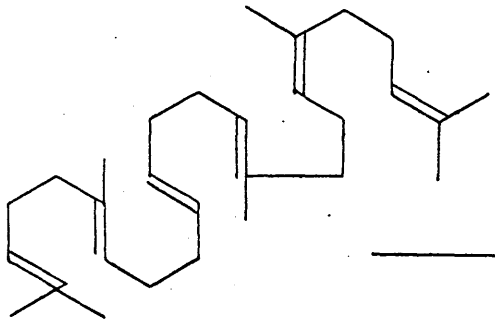
SCHEME 6



SCHEME 7



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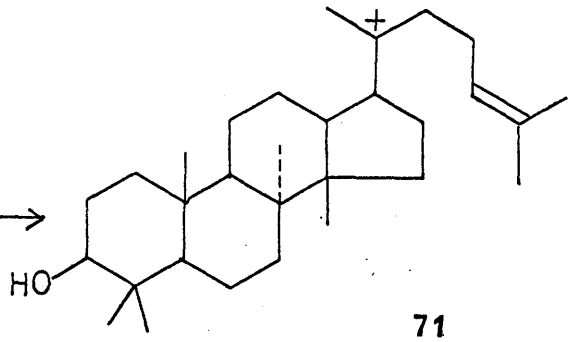

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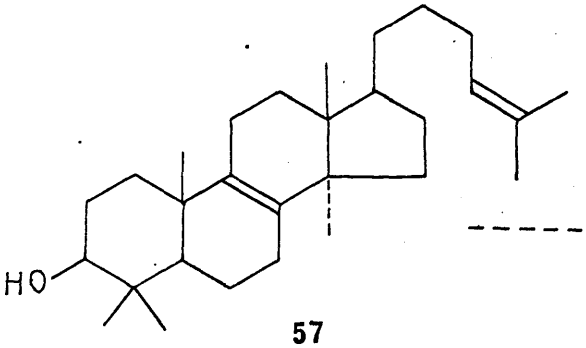

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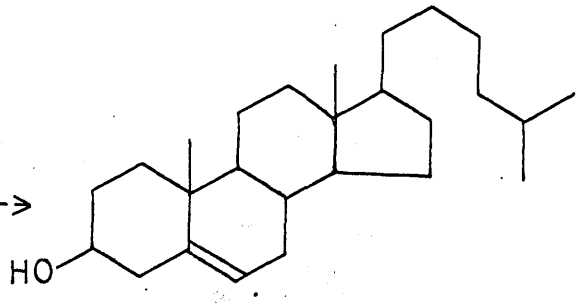

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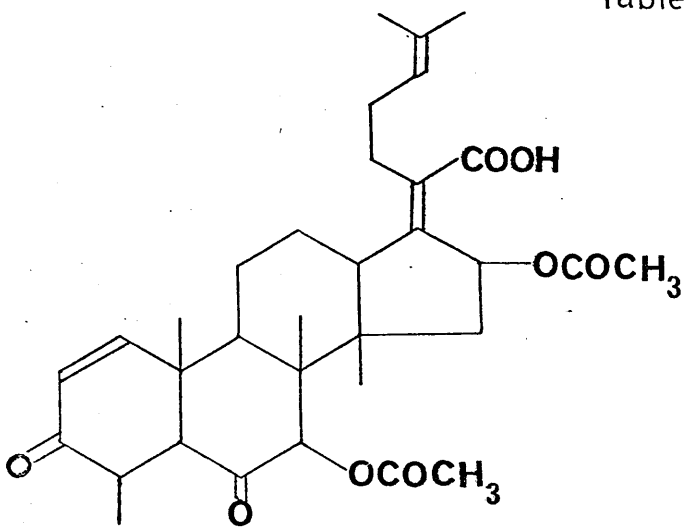
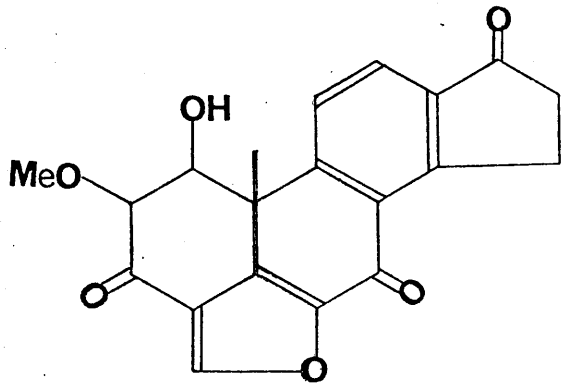
Table 5a



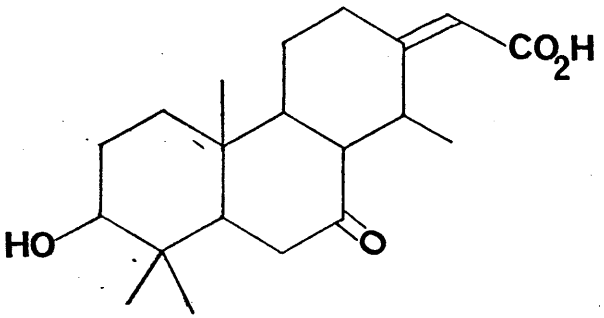
73

Helvolic Acid



74

Viridin

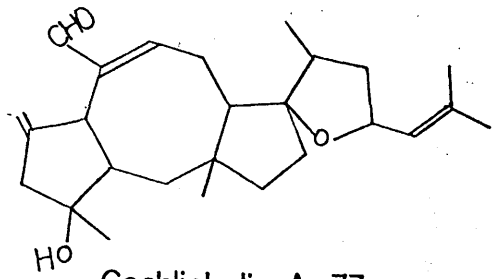


75

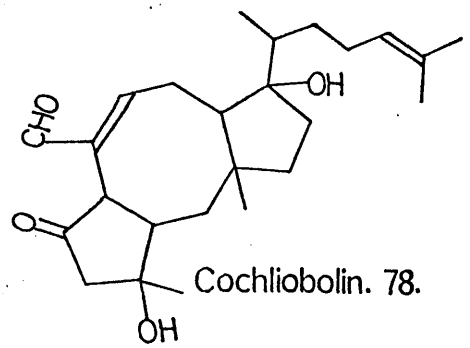
In spite of the apparent equivalence of the methyl groups in dimethylallyl pyrophosphate (59) which appears to initiate condensation of C_5 units, the distinction made in biological systems between the atoms derived from the C-2 and C-3 positions of mevalonic acid (55) has been demonstrated in a number of cases.[47] For example, this has been shown directly for the geraniol side chain of mycelianamide (60) where half the label from [2-^{14}C]-mevalonic acid appears in the terminal trans methyl group of the side chain.[48] Head-to-head linking of two farnesyl units by a process not fully understood[49-51] gives squalene (70) (C_{30}), from which triterpenoids are derived via a concerted cyclisation process[52] to give a tetracyclic carbonium ion (71) (Scheme 7), and a series of concerted stereospecific hydride and methyl shifts. The conversion of lanosterol (57) to cholesterol (72) involves oxidative removal of methyl groups.[53]

It will be seen that many processes similar to these could be involved in the biosynthesis of the terpenoid antifungal antibiotics. Loss of a proton from the intermediate tetracyclic carbonium ion (71), derived as above leads to the skeleton of the antibiotic helvolic acid (73).[54] The biogenesis of viridin (74)[55] a potent antifungal metabolite of Gliocladium virens, may be

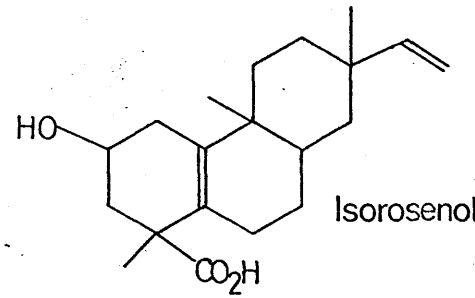
TABLE 5b.



Cochliobolin. A. 77.

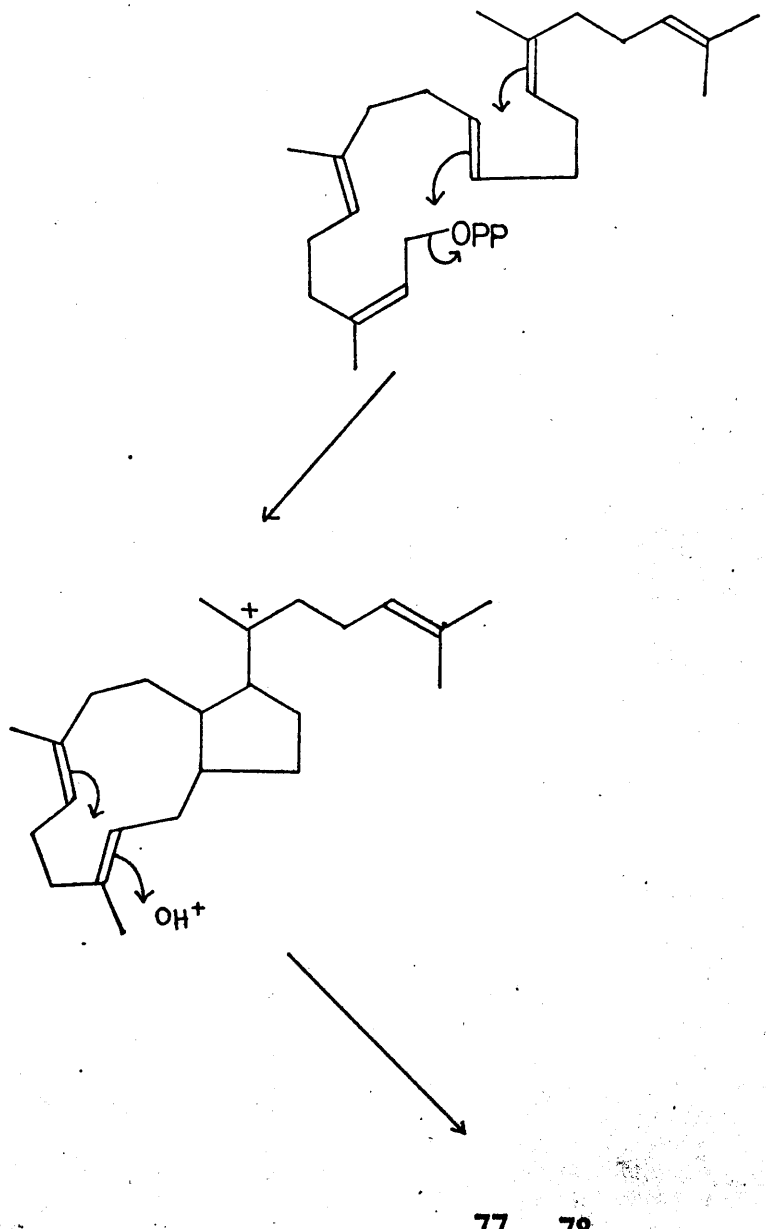


Cochliobolin. 78.



Isorosenolic acid. 79.

SCHEME 8

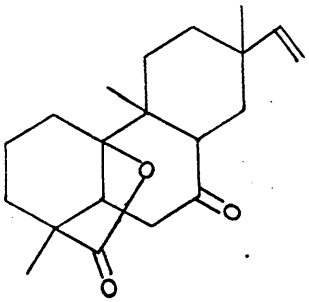


77, 78

presumed to follow in its early stages that of lanosterol (57) and to involve subsequent oxidations, decarboxylations, dehydrogenations etc. However, the possibility that viridin (74) is derived from a diterpenoid skeleton of cassaic acid type (75) has not yet been excluded. Preliminary biosynthetic studies have established that [2-^{14}C]-mevalonic acid is incorporated into viridin to a significant extent.

A concerted cyclisation (Scheme 8) of the hypothetical precursor geranylfarnesyl pyrophosphate (76) derived by head-to-tail linking of 5 isopentenyl units, has been suggested[56] for the biogenesis of a novel type of antifungal C_{25} terpenoid (a 'sesterterpene'), namely cochliobolin A (77) (Table 5b) isolated from Cochliobolin miyabeanus[56] and independently from Helminthosporium orizae[57] and Helminthosporium zizaniae[58]. The isoprenoid nature of this compound has been confirmed by incorporation of [2-^{14}C]-mevalonic acid, and it was also demonstrated that cochliobolin B (78), a co-metabolite of cochliobolin A (77) in cultures of H. orizae, was a precursor of the latter compound.[59]

These terpenoids whose basic skeleton is derived from four isoprene units are defined as diterpenes and these compounds are common among plant products. The



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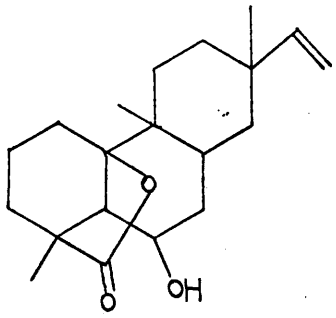

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SCHEME 9

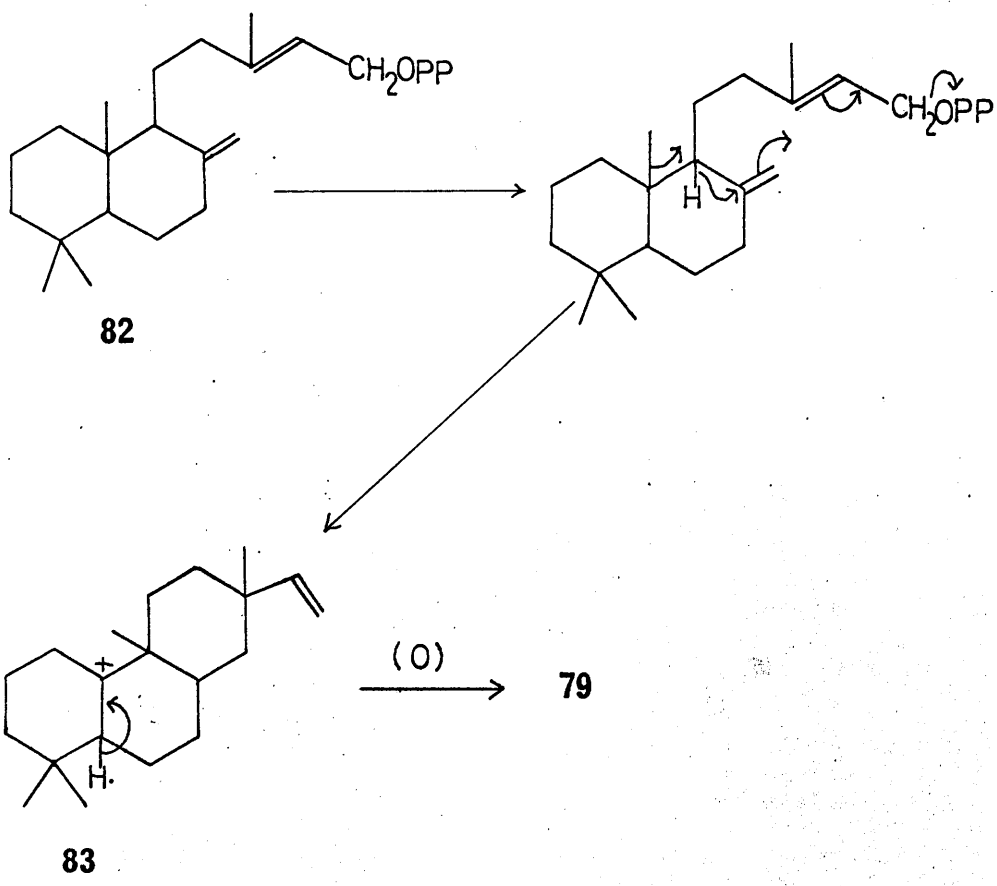

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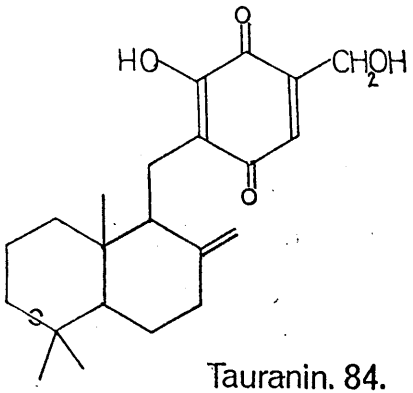
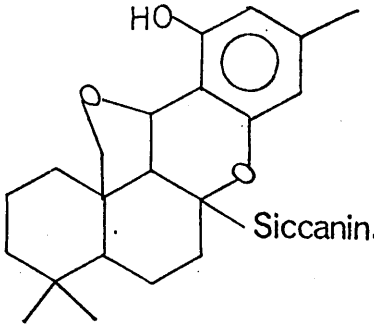
TABLE 5c

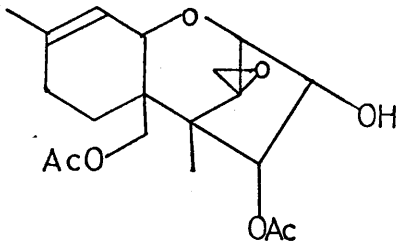


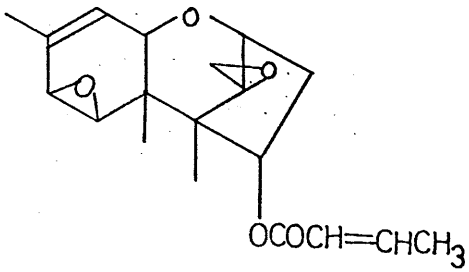
Tauranin. 84.



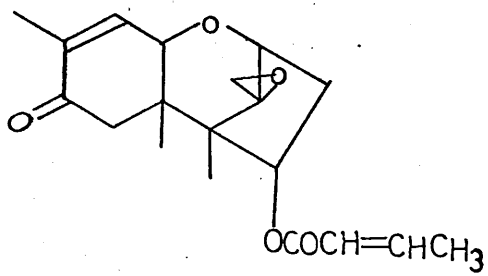
Siccanin. 85.

Diacetoxyscirpenol. 87.

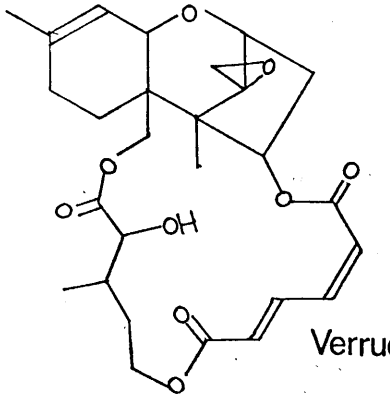




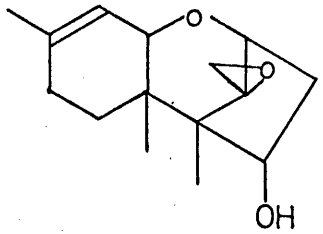
Crotocin. 88.



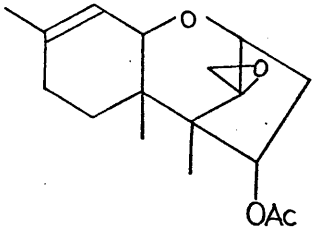
Tricothecin. 89.



Verrucarin. A. 90.

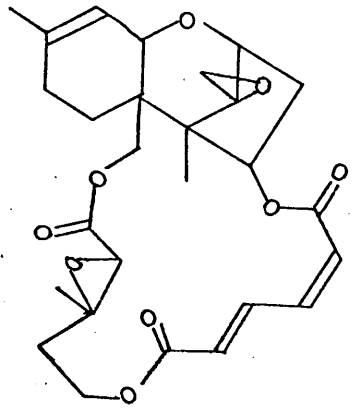


Roridin. 91.
C

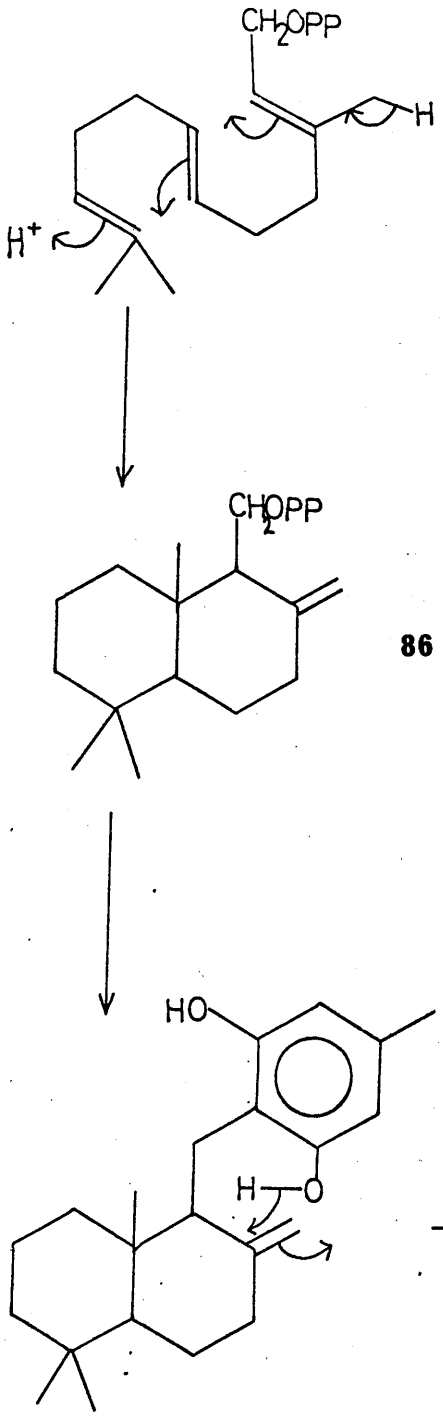


Trichodermin. 92.

Verrucarin. B. 96.



SCHEME 10

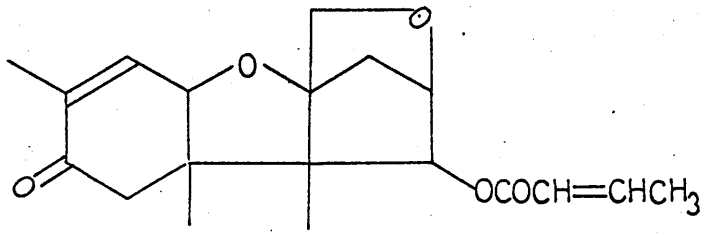


86

group is represented in the class of antifungal antibiotics by isorosenolic acid (79)[61] (Table 5b) which is closely related to the other diterpenoid metabolites of Trichothecium roseum, among them rosenonolactone (80) and resololactone (81).

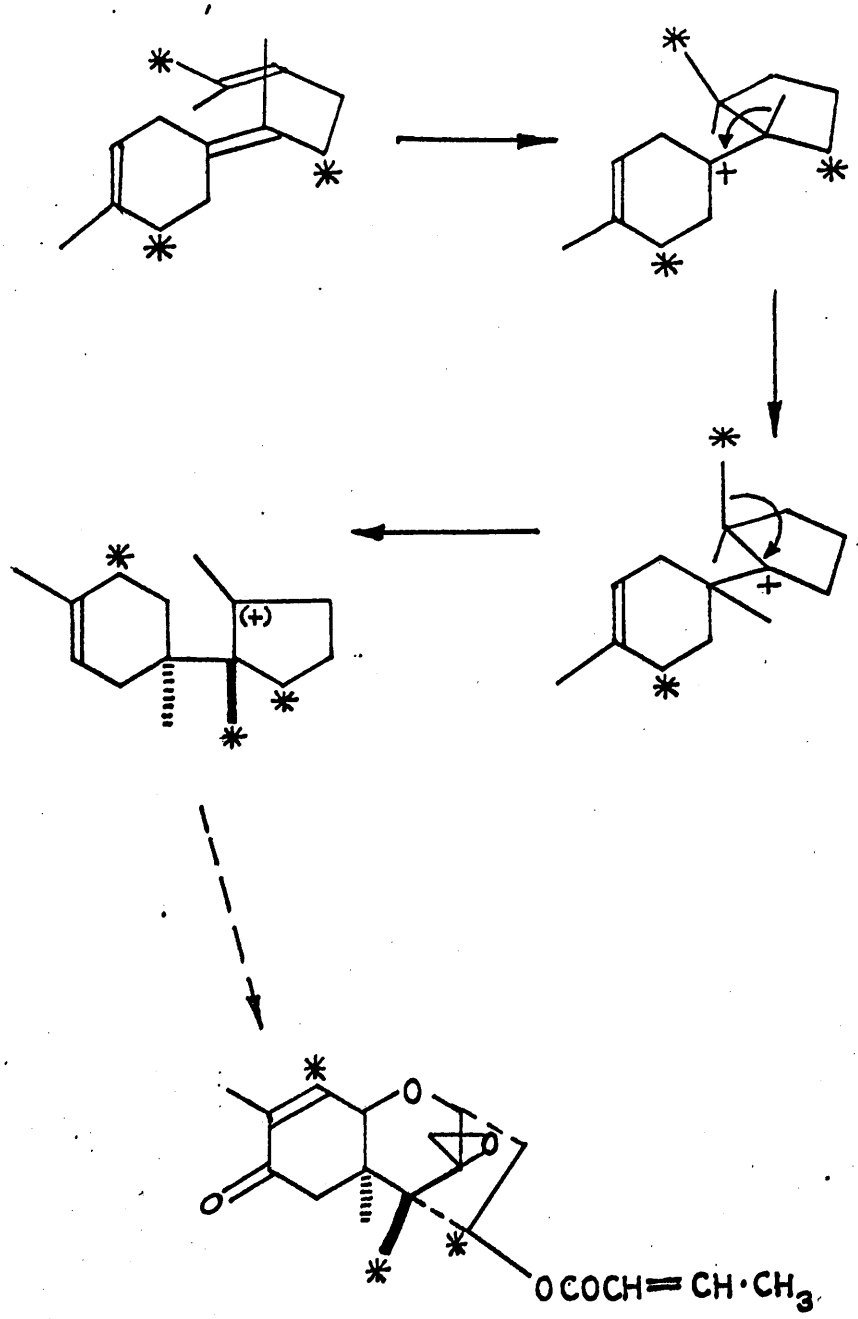
The biogenesis of these diterpenes is thought to proceed via the bicyclic precursor (82) (Scheme 9)[60] derived by cyclisation of geranylgeranyl pyrophosphate. This yields an allylic cation which cyclizes as shown (Scheme 9) with a 1,2 methyl shift, resulting in the tricyclic carbonium ion (83). A similar scheme involving deprotonation of the carbonium ion (83) and further oxygenation reactions can be envisaged for the biogenesis of the antibiotic isorosenolic acid (79).

The antifungal antibiotic tauranin (84) (Table 5c) isolated from Oospora aurantin[62] is partly isoprene and partly polyketide derived. A close relationship to the antifungal metabolite siccanin (85)[63] (from Helminthosporium siccans) is evident. These products can arise via a concerted cyclisation of farnesyl pyrophosphate as in Scheme 10, to give a drimane derivative (86) which may then alkylate orcinol, this being derived from acetyl-CoA and three moles of malonyl-CoA.

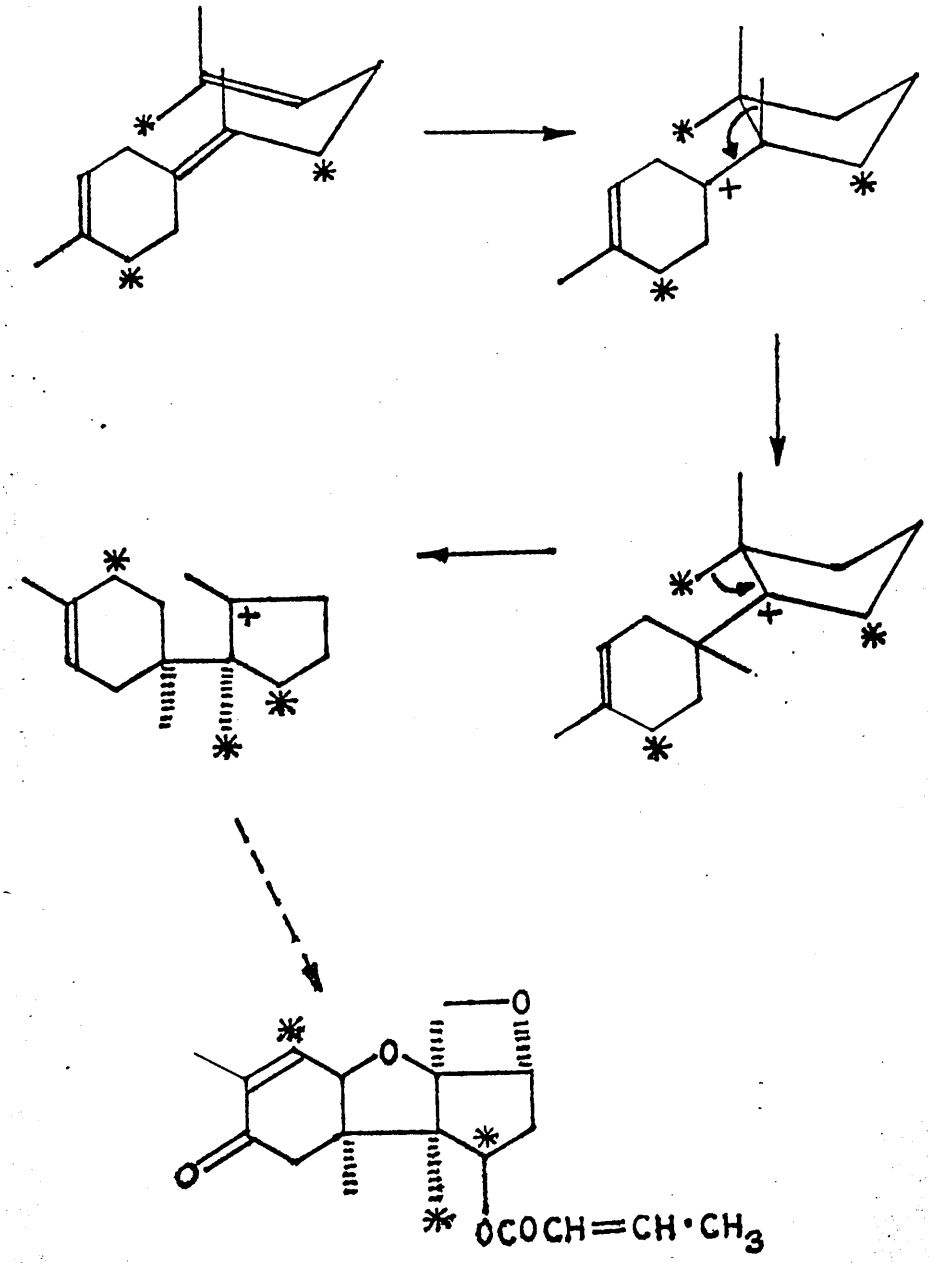


93

SCHEME 12



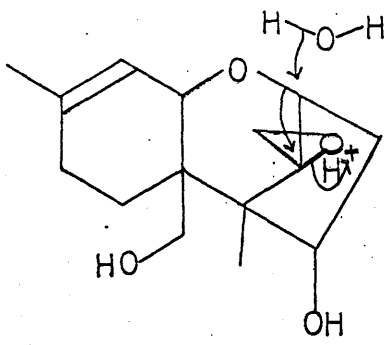
SCHEME 11



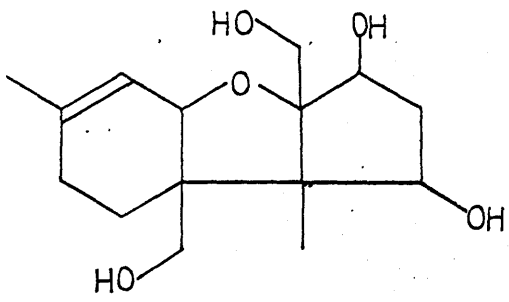
A different type of cyclisation of farnesyl-pyrophosphate, accompanied by methyl shifts is involved in the biogenesis of an expanding group of antifungal sesquiterpenoids (Table 5c). These compounds are produced by a variety of fungal species: Diacetoxyscirpenol (87) in several Fusarium species,[64] crotocin (88) in Cephalosporum crotocinigenus,[65] trichothecin (89) in Trichothecium roseum,[66] verrucarin A (90) in Myrothecium verrucaria,[66] Roridin C (91) in Myrothecium roridum,[66] trichodermin (92) in Trichoderma species.[67]

Before the structure of one member of the group trichodermin (92) had been conclusively established by chemical and X-ray studies,[67,68] the structure (93) had been advanced for trichothecin and a series of elegant tracer studies carried out using [1-^{14}C]-acetate and [2-^{14}C]-mevalonate.[69] These supported the proposed biogenesis (Scheme 11) including two 1,2 methyl migrations. However these results were equally significant in relation to the revised structure (89) for trichothecin, and the folding of the proposed intermediate γ bisabolene into a boat (as in Scheme 12) rather than a chair (as in Scheme 11) is the only significant change required to give a product of the correct stereochemistry and labelling pattern. It is interesting that acid catalysed rearrange-

Scheme 13



94



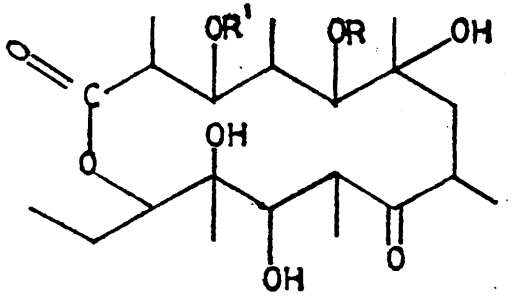
95

ment of this type of compound,[66] as shown in the case of verrucarol (94) (Scheme 13) gives rise to a product (95) with skeleton similar to that formerly proposed for trichothecin i.e. (93).

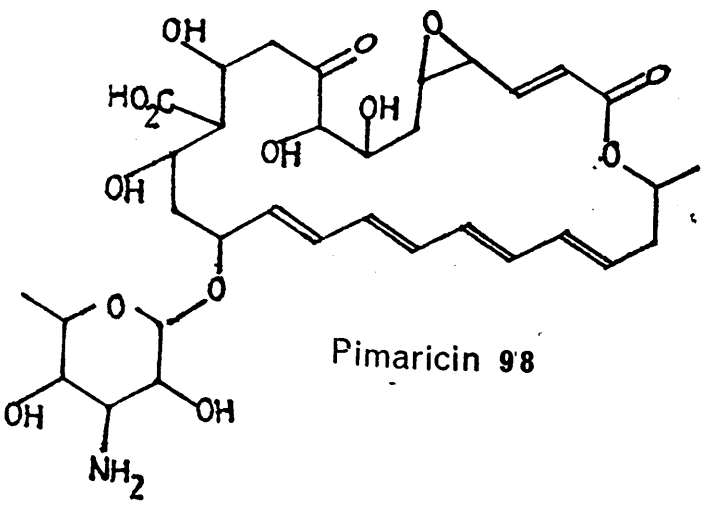
The conjugating acid in trichothecin (89) and crotocin (88) crotonic acid probably arising from linear condensation of 2 acetate units. The macro-ring of verrucarin A (90) is evidently built up from two acids, a C_6 diene diacid evidently of linear polyketide origin and a C_6 branched chain hydroxyacid $HO_2C.CH(OH)CHMeCH_2CH_2OH$ isomeric with mevalonic acid from which it could be derived. Verrucarin B(96) differs from verrucarin A (90) only in incorporating the epoxy acid $HO_2C.CH$(O)$C(Me).CH_2.CH_2OH$ instead of the latter.

One structural feature common to a surprising number of antifungal antibiotics listed in Tables 3, 4 and 5c is the presence of one or more secondary or tertiary methyl groups derived from methionine, (Tables 3 and 4) or as part of the isopentenyl unit (e.g. Table 5c). A third way in which methyl groups are derived is via methylmalonyl-CoA, as in an extensive group of antifungal antibiotics produced by Actinomycetes. These compounds possess a large or medium sized ring and are known collectively as macrolides.

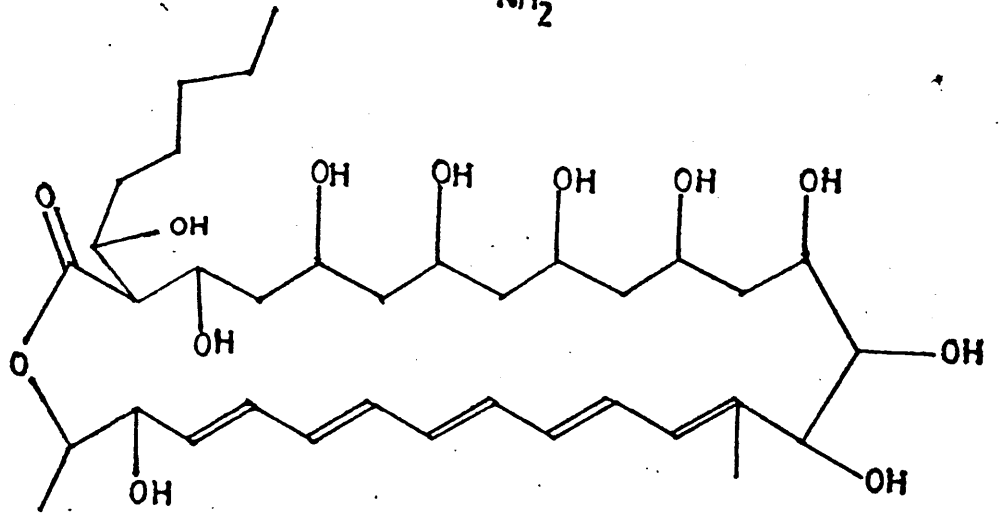
TABLE 6



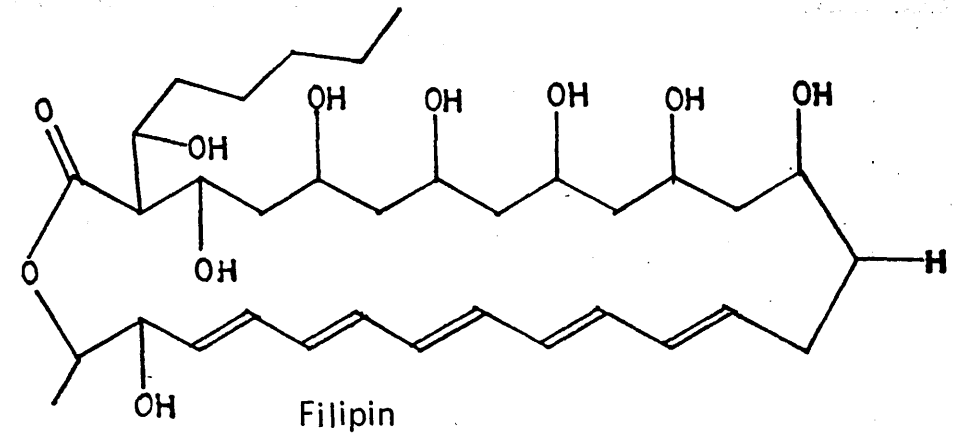
erythromycin **97**



Pimaricin **98**

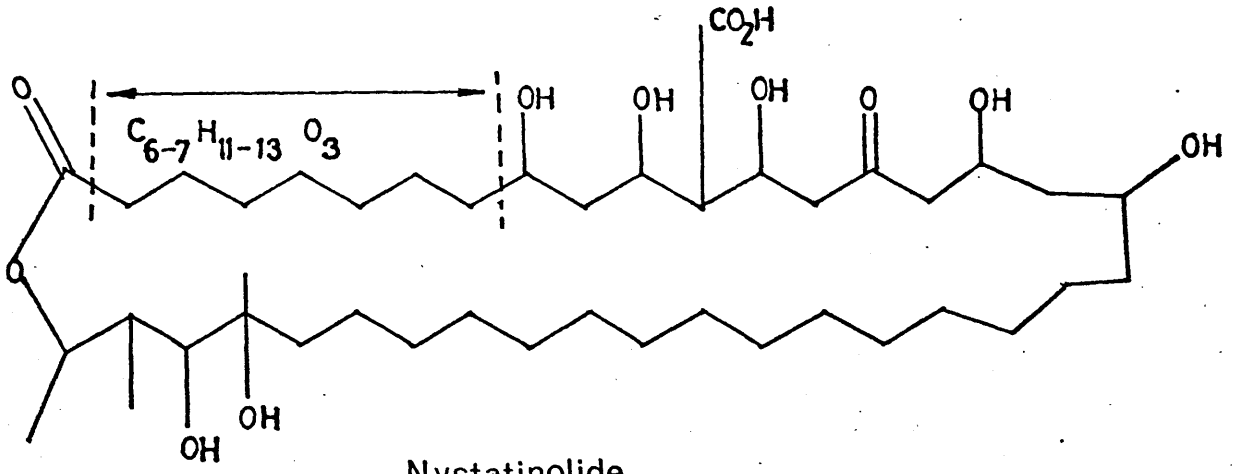


Fungichromin **99**



Filipin

100



Nystatinolide

101

Since the original discussion of these antibiotics as a group by Woodward,[70] many more substances have been characterised fully or tentatively as macrolides, and since most of the known macrolides exhibit antibiotic activity this property has probably been the main guide to their isolation.

The size of the lactone ring varies with the different antibiotics (Table 6) and indeed even the molecular weight has been difficult to assess in some cases (e.g. nystatin)[73] even by mass spectrometry, owing to the very high molecular weight and multiplicity of hydroxyl functions.

The biogenesis of macrolides has not been completely elucidated but several of the macrolides can be derived in principle from acetate and propionate units.[71]

Nystatin is an antifungal macrolide with a very large polyene - containing lactone ring with a sugar moiety attached, and the structure of the lactone ring, nystatinolide, has been tentatively assigned[72] as (101) ($C_{46}H_{71}NO_{18}$).

Biogenetic study of nystatin,[73] which has been useful in the actual structure analysis, demonstrated that the macrolide lactone arises mainly from acetate and propionate, since cultures of Streptomyces noursei effectively

Table 7. Summary of type of Antifungal Metabolites produced by Actinomycetes and Fungi

Actinomycetes	Fungi
Polyene macrolides	Amino acid
Macrolides	Polyketide (polyacetylene)
Polysaccharides	" (aliphatic or alicyclic)
Nucleic acid type	
Polypeptide	Aromatic polyketide
Amino acid type	Terpenoid
Polyketide (cycloheximide)	

use [1-^{14}C]-, [2-^{14}C]-, [3-^{14}C]- propionate (8-10% incorporation), and [1-^{14}C]-acetate (3-5% incorporation) for nystatin formation, while methyl labelled methionine, or [2-^{14}C]-mevalonic acid lactone are not utilised in the biosynthetic pathway. The biosynthesis of the lactone ring of another type of macrolide antibiotic, erythromycin (97, R = desosamine, R' = cladinose) has been shown to involve seven propionate or methylmalonate units.[74]

Finally it may be observed on comparison of the types of antifungal metabolites produced by Actinomycetes and Fungi (Table 7) that there is an apparent chemotaxonomic distinction between the two types of organism.

The metabolites of *P. canadense*

Section 2.1

One of the most fascinating aspects of investigation into members of the Vegetable Kingdom has been those studies on organisms possessing antimicrobial activity. From these studies many chemically interesting and therapeutically useful compounds have been isolated and characterised, and accordingly it was decided to investigate several fungi possessing antifungal activity, and if possible, to isolate and characterise the antibiotic responsible. As part of a general programme directed towards this end, the detailed chemical analysis of the fungus Penicillium canadense was undertaken. This fungus was originally isolated by M. Christensen from the soil in Shiwano County, Wisconsin,[75] but the cultures examined during this investigation were obtained from the Commonwealth Mycological Institute. The organism was screened for antifungal activity using the serial dilution spore germination test on the conidia of Botrytis alii[76] and it was shown that samples of the broth displayed antifungal behaviour. For large scale production Penicillium canadense was successfully grown on a synthetic medium for 10 days as a surface culture. Thereafter the mycelium was separated from the broth, dried, pulverised, and extracted with chloroform and methanol. The metabolites present in the broth were adsorbed onto charcoal which was

Table 8. Typical activity assay of the organic extracts of P. canadense.

Organic Extract	Assay
charcoal/acetone (broth)	11S12
methanol (mycelium)	4S8
chloroform mycelium	8S12

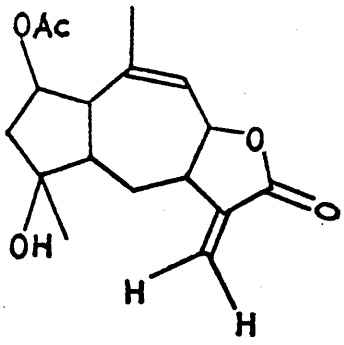
subsequently subjected to soxhlet extraction with acetone. Assays performed on these three extracts, namely methanol and chloroform extracts of the mycelium, and the acetone extract of the broth, asserted that antifungal activity was still present in the latter extracts and that a trace of antifungal activity existed in the chloroform extract of the mycelium. There was virtually no activity in the methanol extracts (Table 8).

The acetone extracts were dried by adding quantitites of dry methanol and concentrating in vacuo in a rotary film evaporator. The resulting gum was adsorbed on silica and separation of the metabolites achieved by means of column and thin layer chromatography. Throughout the separation procedure care was taken to avoid vigorous conditions which might lead to saponification, decomposition, rearrangement etc.

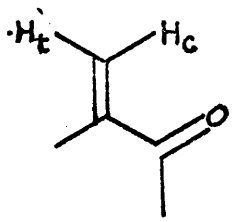
The source of antifungal behaviour was located by assaying each column fraction for activity, the active fractions being combined prior to thin layer chromatography. Using these techniques the antifungal antibiotic canadensiolide was isolated. Initially difficulty was experienced in separating the antibiotic from another slightly less polar metabolite,but it could be obtained pure after several preparative TLC operations. GLC

analysis of the mixture obtained after one preparative TLC operation (1% SE 30, column temp.125°c flow rate 50 mls./min.) showed only two components of retention times 10 and 13·5 minutes (Canadensiolide had the retention time of 13·5 min.) GCMS analysis of the mixture employing the same separation conditions revealed that the two compounds were closely related since the major ions found in the spectrum of canadensiolide were all 2 mass units less than the corresponding ions found in the spectrum of the second component. This suggested that this second component could be a dihydro derivative of canadensiolide. However with successive batches of Penicillium canadense the antifungal activity was found to increase to an apparent maximum, even though the growing conditions remained identical. Hand in hand with this was the disappearance of the metabolite suggested to be the dihydro-derivative of canadensiolide. This fortuitous circumstance greatly aided separation of the antibiotic which could now be fairly conveniently obtained from these later batches as a pure compound.

The nature of the antibiotic canadensiolide was established as follows.



102



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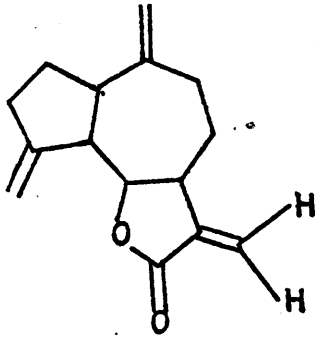

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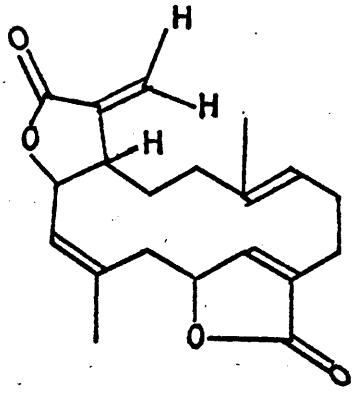

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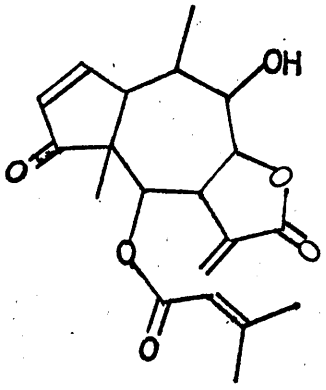


106

The molecular formula $C_{11}H_{14}O_4$ was assigned for canadensiolide on the basis of elemental analysis and mass spectrometry. The absorption band in the IR spectrum at 1775 cm.$^{-1}$ (ε 1320 $\Delta\nu_{\frac{1}{2}}$ 23 cm.$^{-1}$) suggested the presence of two γ-lactone carbonyl groups, while the absorption at 1663 cm.$^{-1}$ (ε 75) was indicative of a double bond exocyclic to a five membered ring.[77] That this double bond was in conjugation with a carbonyl group was shown by the high intensity end absorption (213 mμ, log ε 3·93) in the UV spectrum[78] although this appears to be contradicted in the IR spectrum by the high carbonyl frequency and lack of enhancement of the double bond intensity. The cytotoxic sesquiterpene lactone gaillardin (102),[79] for example, exhibits $\alpha\beta$ unsaturated γ lactone absorption at 1764 cm.$^{-1}$ and 1667 cm.$^{-1}$, the UV spectrum showing high intensity absorption at 209 mμ. The presence of the terminal methylene grouping was also indicated by ozonolysis of canadensiolide to give formaldehyde isolated as its dimedone derivative (m.p. 191^{o}c). The low yield obtained (10%) is apparently typical of compounds with an α methylene γ lactonic grouping.[80]

Two γ lactone functions and one exocyclic methylene group account for all the oxygen atoms and double bond equivalents if the calculated formula

($C_{11}H_{14}O_4$) for canadensiolide is correct. This is supported by the lack of hydroxyl, double bond, or other carbonyl absorption in the IR spectrum.

The NMR spectrum (Fig. 1) showed doublets due to the protons of the exocyclicmethylene group at 3·58 τ (H_A) (J ~ 2 c.p.s.) and 3·97 τ (H_B) (J ~ 3 c.p.s.). These values confirm that the double bond is conjugated to a carbonyl group. In such systems a proton cis to the carbonyl group will be deshielded by that group relative to a proton trans to the carbonyl group.[81] In gaillardin (102), for example, the exocyclicmethylene protons appear as doublets at 3·79 τ (cis, J = 3 c.p.s.) and 4·47 τ (trans, J = 3 c.p.s.), and in the antifungal antibiotic avenaciolide[27] (34), they appear as doublets at 3·3 τ (cis, J = 2·56) and 4·1 τ (trans, J = 2·17 c.p.s.).

The splitting of the terminal methylene signals in canadensiolide is due to coupling with an allylic proton. This was confirmed by double resonance experiments in which irradiation in the vicinity of a multiplet at ca. 6 τ reduced the doublets to sharp singlets (fig. 1). It is apparent from the latter that the geminal coupling is negligible.

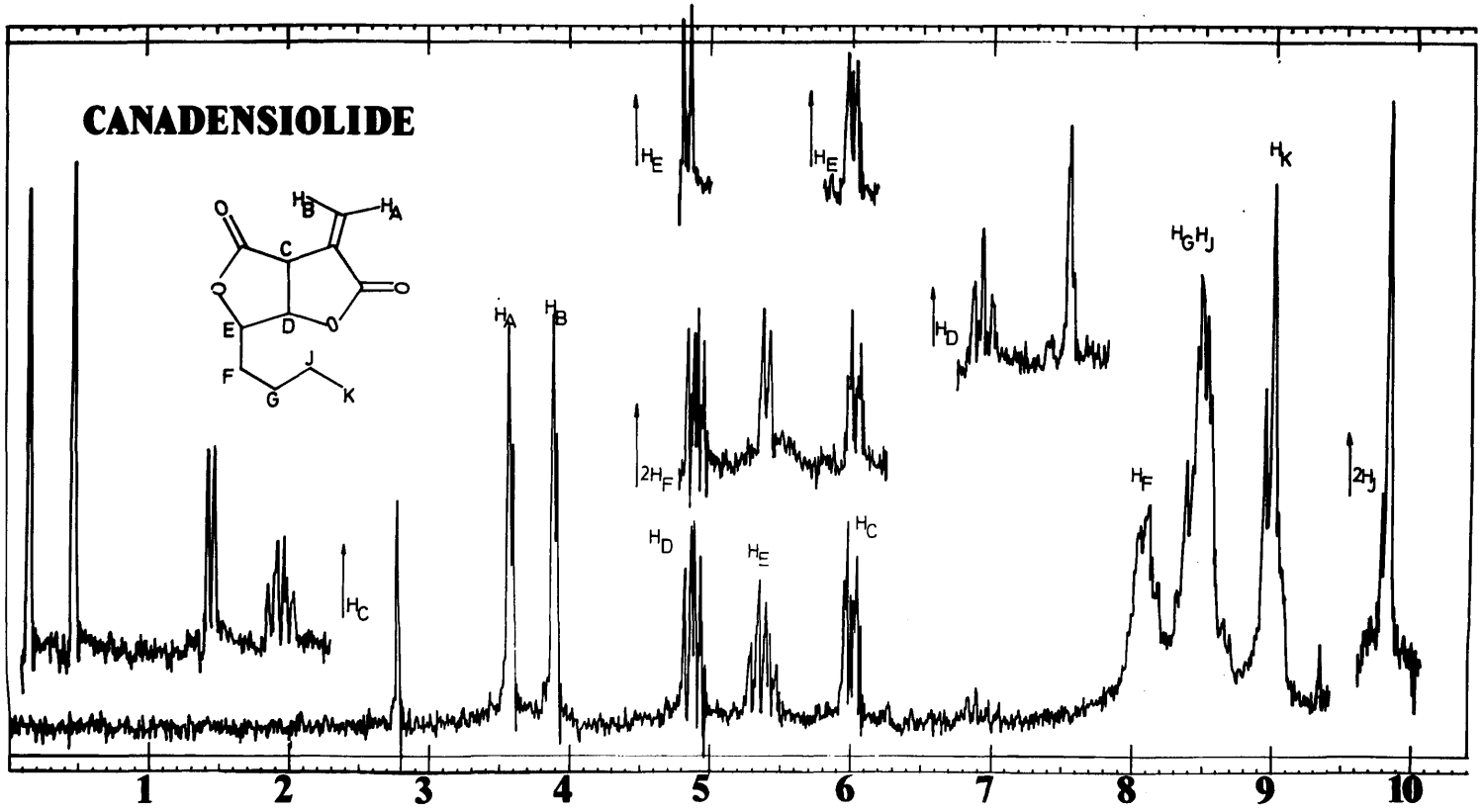
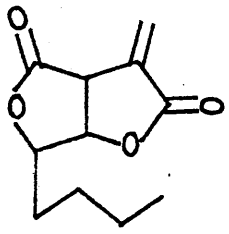

CANADENSIOLIDE
O
C
O
O
E
D
O
F
J
G
K
H_B
H_A
H_C
H_A
H_B
H_E
H_E
$2H_F$
H_D
H_D
H_E
H_C
H_F
$H_G H_J$
H_K
$2H_J$
1
2
3
4
5
6
7
8
9
10

Fig. 1

An apparent anomaly in the spectrum of canadensiolide is the positive slope in the downfield direction of each doublet although the methylene protons are coupled to a proton at higher field. This feature was also apparent in various published spectra of α methylene lactones and acrylic acids e.g., ovatodiolide (104),[82] dehydrocostus lactone (105),[83] fastigilin C (106)[84] and avenaciolide (34).[27] This effect has been attributed to 'saturation'[85] and is dependent on the speed and direction in which the spectrum is run.

The remaining low field protons in the spectrum of canadensiolide must be associated with the γ lactone functions, these being the only remaining functional groups liable to cause extensive deshielding. Thus a double doublet at 4·88 τ (H_E) and a double triplet at 5·38τ (H_F) were each assigned to protons geminal to an oxygen atom of the γ lactone functions. Spin decoupling studies established the sequence of vicinal couplings H_C to H_D (J_{CD} = 6·5 c.p.s.), and H_D to H_E (J_{ED} = 4·5 c.p.s.). That H_E was also coupled to a methylene group was shown by irradiation in the vicinity of the two proton multiplet at 7·1 τ ($2H_F$), which resulted in the transformation of the signal at 5·38 τ (H_E) to a doublet. This leads uniquely to the following partial structure



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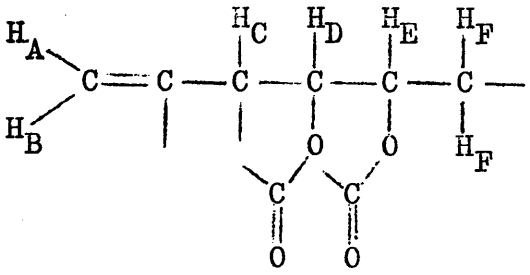


This accounts for all the C, H and O atoms in canadensiolide save a C_3H_7 moiety which must be an n-propyl group since the 3H triplet at 9·0 τ, corresponding to a methyl group, collapses to a singlet upon irradiation in the vicinity of the four proton multiplet at 8·5 τ. These experiments lead unambiguously to the structure (107) for canadensiolide.

Chemical evidence in accordance with this structure was obtained by treatment of canadensiolide with two equivalents of base to give a diol which was cleaved with sodium metaperiodate to yield valeraldehyde. This volatile product was removed from the reaction medium by steam distillation and characterised as n-valeraldehyde by GLC comparison with an authentic sample and by paper chromatography of the D.N.P. derivative. Borohydride reduction of the aldehyde gave n-pentanol which was also characterised by GLC comparison with an authentic sample. This degradation sequence provides chemical confirmation of the n-butyl side chain.

Scheme 14

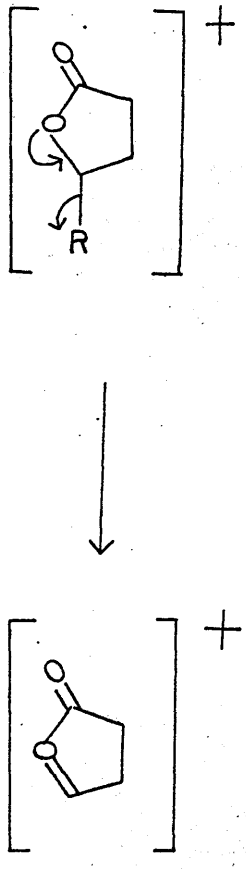
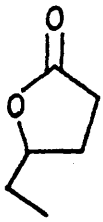
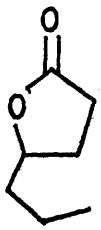
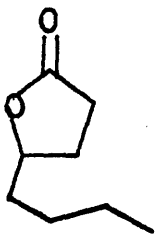


Table 9. MS data of 3-alkyl γ lactones.

COMPOUND	BASE PEAK	ORIGIN
 γ Caprolactone	m/e 75	M - 29
 γ Heptalactone	m/e 75	M - 43
 γ Octalactone	m/e 75	M - 57

SCHEME 15

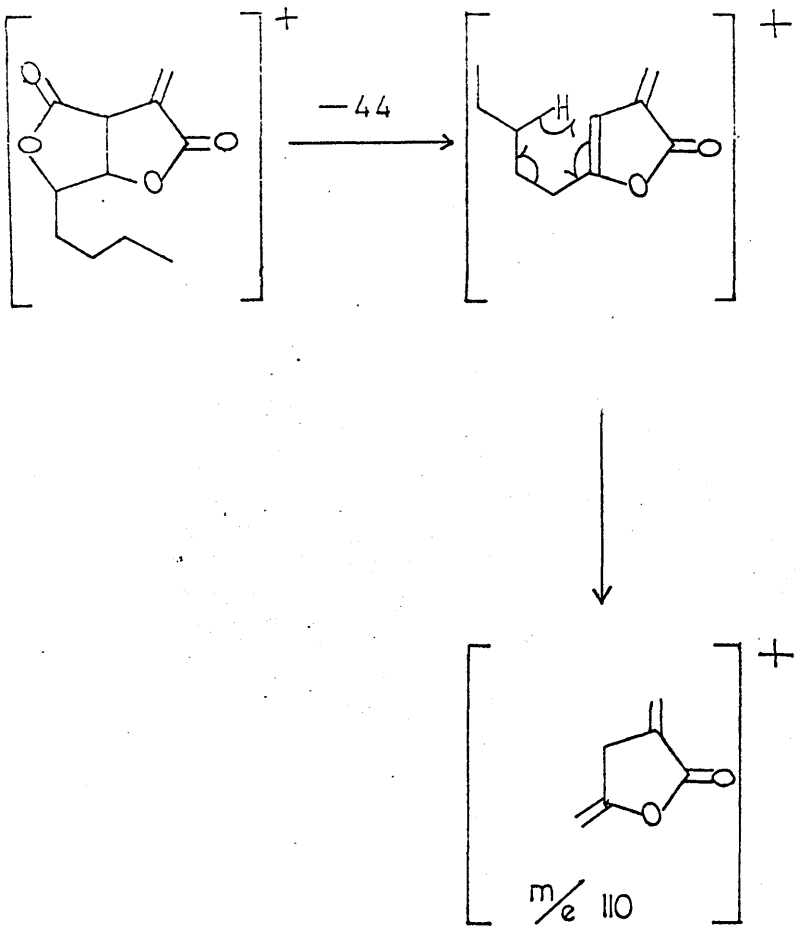


Table 10. MS data for canadensiolide and dihydrocanadensiolide

Interpretation	Canadensiolide		Dihydrocanadensiolide	
	m/e	% Abundance	m/e	% Abundance
M^+	210	0·5	212	2
M–28	182	1·5	184	1
M–57	153	2	155	4
M–44	166	3	–	–
–(44 + 15)	151	3	153	2
–(44 + 29)	137	7	139	2
–(44 + 43)	123	18	125	6
–(44 + 57)	109	19	111	5
See scheme 15	110	40	112	6
	96	100	98	100

Further corroboration was obtained from analysis of the mass spectrum. It has been reported[86,87] that for 3-alkyl substituted γ lactones, while the parent ion itself is generally of low abundance, an abundant and characteristic ion arises by loss of the side chain, and this has been demonstrated using several compounds with increasing length of side chain (Table 9) (Scheme 14). In agreement with this the mass spectrum of canadensiolide (107) (Table 10) shows an ion at m/e 153 (M-57) (loss of the n-butyl side chain), which is several times more abundant than the parent ion. Furthermore, there are losses of 57, 43 and 29 mass units from the ion at m/e 166 (M-44).

Since canadensiolide (107) possesses a very rigid bicyclic structure, and since NMDR experiments allow fairly accurate assessment of the various coupling constants, it is possible to propose the relative stereochemistry of the antibiotic with some degree of confidence.

Firstly it may be assumed that the two 5 membered rings are <u>cis</u>-fused, since models show that <u>trans</u> fusion would give a severely strained system, and that the dihedral angle between H_C and H_D would approach 180°, from which a large coupling constant (> 14 c.p.s.) would be expected,[88] more than double the observed value (6·5 c.p.s.).

Table 11. Application of Karplus equation to calculation of coupling constants.

Canadensiolide

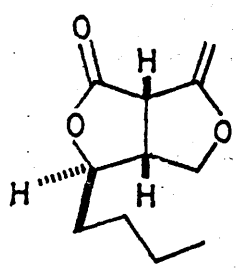
	θ_{cis}	J_{cis}	θ_{trans}	J_{trans}	$J_{obsv.}$
H_C/H_D	~25°	~8 c.p.s.	~170°	~14 c.p.s.	6·5 c.p.s.
H_D/E_E	~25°	~8 c.p.s.	~120°	~4 c.p.s.	4·5 c.p.s.

Dihydrocanadensiolide

	θ_{cis}	J_{cis}	θ_{trans}	J_{trans}	$J_{obsv.}$
H_B/H_C	~30°	~7 c.p.s.	~115°	~2 c.p.s.	<2 c.p.s.
H_C/H_D	~25°	~8 c.p.s.	~170°	~14 c.p.s.	6 c.p.s.
H_D/H_E	~30°	~7 c.p.s.	~120°	~4 c.p.s.	4 c.p.s.

Methyl isocanadensiolate

	θ_{cis}	J_{cis}	θ_{trans}	J_{trans}	$J_{obsv.}$
H_B/H_C	~25°	~8 c.p.s.	~120°	~4 c.p.s.	1/2c.p.s.



108

If the rings are cis-fused the dihedral angle appears from models to be less than 20° from which a coupling constant of ca.8 c.p.s. could be predicted. Since the presence of electronegative substituents on the carbon atoms in question is known if anything to reduce the coupling constant,[89] this is in much better agreement with the observed value (cf. Table 11). The low value of 4·5 c.p.s. for the vicinal coupling between the protons H_D and H_E suggests they are trans to each other. Models show that a dihedral angle is formed between H_E and H_F of ca. 120°, leading to a predicted coupling constant of ca. 4 c.p.s. close to the observed value. (A similar treatment applied to the two protons when in a cis-relationship results in a theoretical value for the coupling constant of ca. 8 c.p.s.) Thus the relative stereochemistry of canadensiolide can be depicted as (108) or its enantiomer.

A second metabolite slightly less polar (TLC) than canadensiolide, was also obtained from cultures of Penicillium canadense. At first this metabolite could be isolated in reasonable quantity from the original strain grown as surface cultures but later was not produced even though the growing conditions appeared to be identical. It is possible, however, that in the course of maintaining the fungus by subculturing every 2 weeks a mutant has

been obtained. This can be checked by comparison with a new sample of the original culture from the Commonwealth Mycological Institute. However a sufficient quantity of the metabolite had been isolated to permit chemical investigation.

Elemental analysis and mass spectrometry afforded the unique molecular formula $C_{11}H_{16}O_4$ for this compound. The IR spectrum showed absorption at 1783 $cm.^{-1}$ (ε 925) with a shoulder at 1789 $cm.^{-1}$ (ε 850) which was assigned to the carbonyl stretching frequencies of two γ lactones. Unlike canadensiolide (107) this compound showed no absorption due to an exocyclicmethylene group, or other unsaturation, a fact confirmed by the lack of absorption in the UV spectrum. However comparison of the rest of the IR spectrum with that of canadensiolide revealed a close similarity, which, combined with the analysis data suggested the possibility that the two compounds were closely related, and that the second metabolite was in fact the dihydro-derivative of canadensiolide.

This was fully borne out by inspection of the NMR spectrum (fig. 2). There were no signals corresponding to the resonance of two exocyclicmethylene protons but instead a 3H doublet occurred at 8·5 τ (J = 8 c.p.s.) corresponding to a secondary methyl group β to a carbonyl

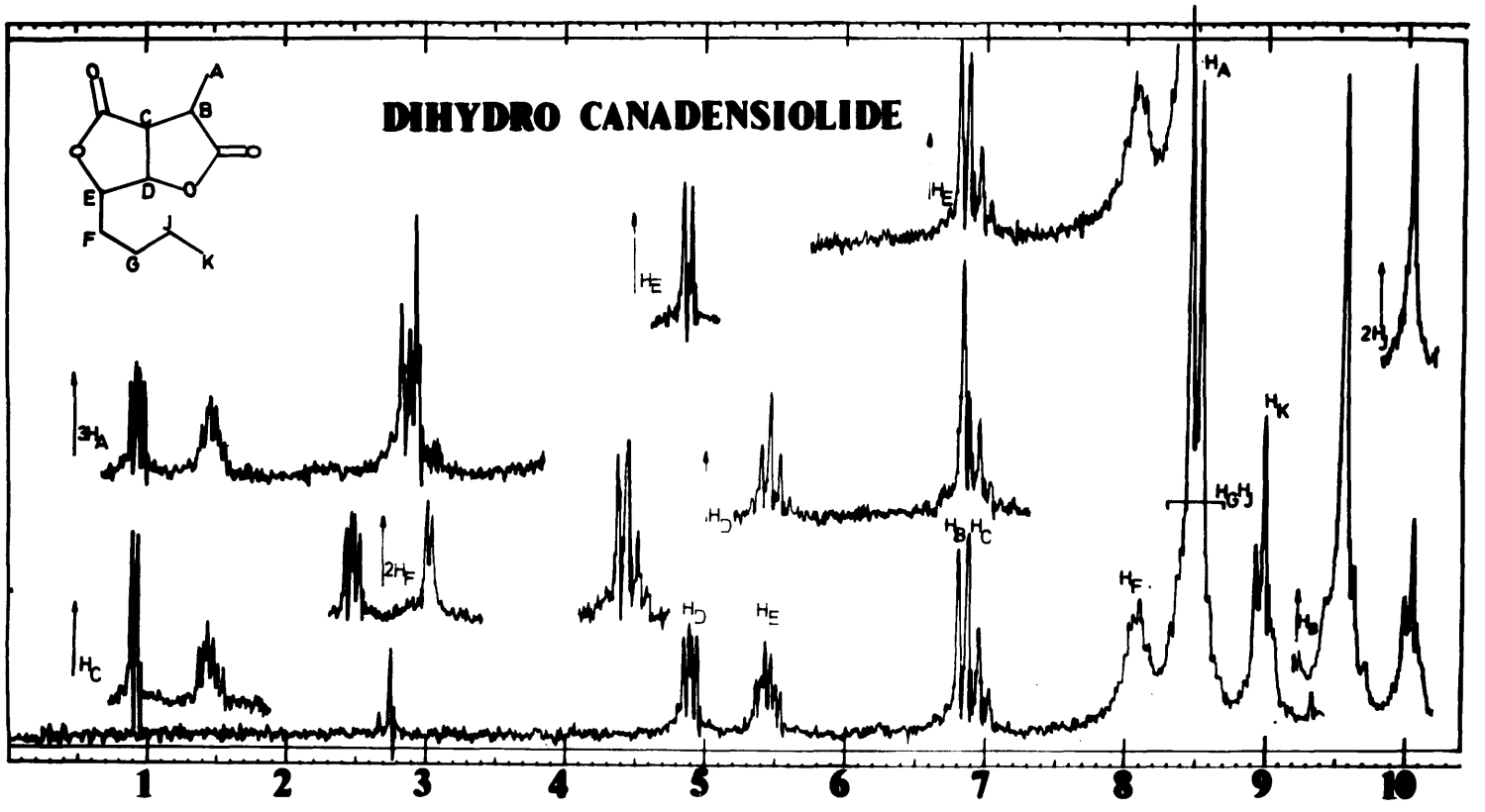
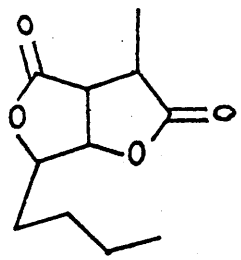

DIHYDRO CANADENSIOLIDE
A
B
C
D
E
F
G
J
K
O
O
O
O
3H_A
2H_F
H_C
H_D
H_E
H_E
H_A
H_K
H_G H_J
H_B H_C
H_F
2H_J
1
2
3
4
5
6
7
8
9
10

Fig. 2

group. Upon irradiation in the vicinity of 6·9 τ (H_B), the doublet at 8·5 τ (H_A) collapsed to a singlet, and conversely upon irradiation in the region of 8·5 τ the quartet at 6·9 τ collapsed to a fine doublet (J ca. 2 c.p.s.).

The 1H double doublet at 4·9 τ (H_D) and the 1H doublet of triplets at 5·44 τ (H_E) had almost identical appearance and chemical shift to the signals in the spectrum of canadensiolide ascribed to the protons geminal to the oxygen functions of the two γ lactone systems. Spin decoupling confirmed the vicinal coupling H_D to H_E (J_{ED} = 4 c.p.s.) and also the coupling H_D to H_C (J_{CD} = 6 c.p.s.). However on further comparing the two spectra the signal at 6·0 τ in the NMR spectrum of canadensiolide (107), ascribed to the allylic proton H_C α and β to the two carbonyl groups, is seen to be at higher field in the spectrum of the dihydro compound and appears as a doublet at 6·84 τ, superimposed on the H_B quartet. It was significant that the signals ascribed to protons H_B and H_C appeared as a well resolved quartet and doublet respectively, indicating practically no coupling between these protons a situation which can only be met by a <u>trans</u> relationship (<u>vide infra</u>). Finally, the presence of the n-butyl side chain was asserted by integration of



109

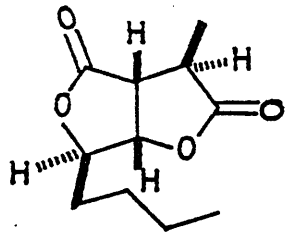
the region 8 - 8·8 τ (6H and secondary methyl group) and by irradiation in the vicinity of 8·5 τ when the 3H triplet at 9·0τ collapsed to a singlet. From this evidence and by analogy with canadensiolide (107), the structure (109) can be proposed for the second inactive metabolite, dihydrocanadensiolide.

The structure was further corroborated by the MS data. Dihydrocanadensiolide (109) showed a parent ion at m/e 212, (m/e 210, for canadensiolide), and the characteristic loss of side chain (M-57) resulted in an ion at m/e 155 (m/e 153 for canadensiolide). Furthermore, the abundant ions that appear at m/e 110 and m/e 96 in the spectrum of canadensiolide (107), now appear at m/e 112 and m/e 98 in the spectrum of dihydrocanadensiolide (109). The possible structure and derivations of these ions is shown in Table 10.

Models of canadensiolide suggest that hydrogenation might be fairly stereospecific leading to the introduction of a hydrogen atom trans- to the two bridgehead protons, i.e. in the same configuration as in natural dihydrocanadensiolide. Hydrogenation of canadensiolide overnight accomplished reduction of the exocyclicmethylene group, as shown by IR and NMR analysis, but apparently had also partially reduced the lactones to a mixture of

lactols as evidenced again by the IR spectrum which showed absorption at 3250 $cm.^{-1}$ (broad), and the NMR spectrum which showed a broad signal at 3·95 τ ascribed to the hydroxyl proton resonance. TLC also showed two spots more polar than the starting material. In an attempt to find conditions which would allow selective reduction of the double bond the reduction of model systems using various catalysts was studied. Reduction of the lactone group as well as the double bond in angelica lactone occurred when platinum oxide was used as at catalyst, but the desired selective reduction was achieved using 5% palladium-on-barium sulphate as catalyst. However this catalyst failed to effect reduction of canadensiolide (107) even after prolonged periods. This problem was successfully circumvented by oxidation of the lactols to lactones using Jones reagent. The crystals obtained after preparative TLC had identical IR, MS and TLC properties to those of naturally occurring dihydrocanadensiolide (109) and the m.p. of the synthetic product was not depressed by admixture with natural dihydrocanadensiolide.

Again, because of the rigid system, the NMR data allow the relative stereochemistry to be assigned. Since the coupling constants between H_C and H_D and between H_D and H_E are very similar to those in canadensiolide



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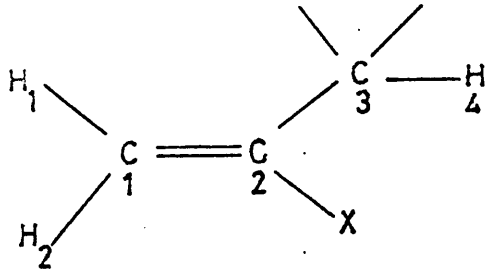
(cf. Table 11) it can be assumed that the stereochemistry about these centres in the two molecules is the same. The extra asymmetric centre in dihydrocanadensiolide can be assigned as in (110) (or its enantiomer), since the coupling constant between H_B and H_C is very small (< 2 c.p.s.) and these protons must be trans (cf. Table 11).

From the most recent batches of cultures a third compound was isolated that had not hitherto been observed in any significant amount. The compound was slightly more polar than canadensiolide but could be obtained free of antibiotic contaminant by repeated preparative TLC, and the compound crystallised from benzene-light petroleum mixtures as colourless needles m.p. 64-65°c. High resolution mass spectroscopy indicated a molecular weight of 242·1150 and hence the molecular formula as $C_{12}H_{18}O_5$. The polarity of this compound relative to canadensiolide was partly explained by the presence of a free hydroxyl group as indicated by IR absorption at 3590 $cm.^{-1}$ and a singlet in the NMR at 8·19 τ.

The IR spectrum also showed bands at 1763 $cm.^{-1}$ (ε 677) and 1715 $cm.^{-1}$ (ε 472), comparable with the presence of an αβ unsaturated γ-lactone grouping and another type of carbonyl group which could be ascribed as a saturated aliphatic ketone or 6-membered alicyclic ketone,



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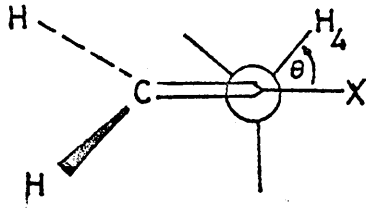

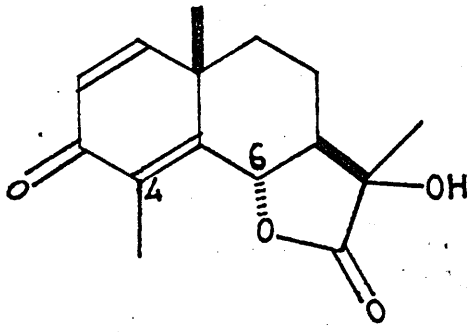

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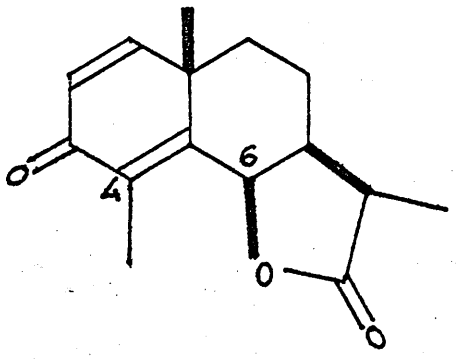

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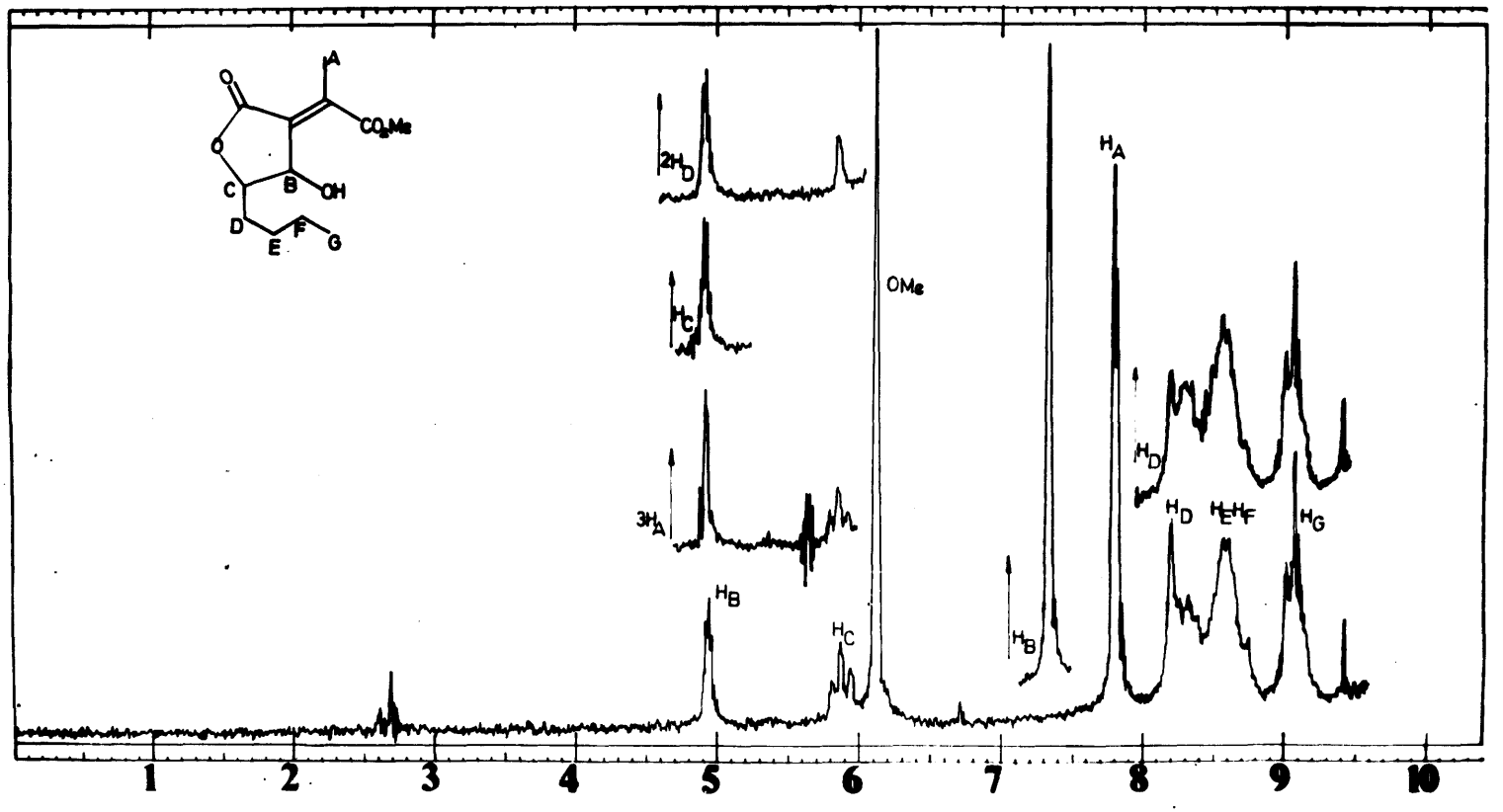
A
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OH
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HC
3HA
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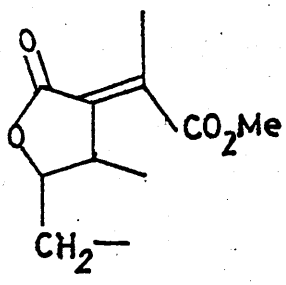
Fig. 3

or an αβ unsaturated alkoxycarbonyl system. The latter interpretation was favoured by the presence of a 3H singlet at 6·12 τ characteristic of a methoxyl group. The UV spectrum showed high intensity absorption at 229 mμ (log ε 4·17), suggesting that the conjugation extends from the carbomethoxyl group through the double bond to the lactone grouping. Since there was no double bond absorption in the IR spectrum it appeared that the double bond(s) was tetrasubstituted.

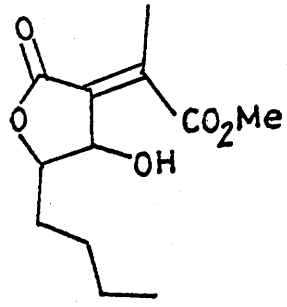
This fact was confirmed by the NMR spectrum which showed no resonance due to olefinic protons. However, the presence of a vinyl methyl group was indicated by the presence of a 3H doublet at 7·8 τ (J = 2 c.p.s.) which collapsed to a singlet upon irradiation in the vicinity of a finely split 1H multiplet at 4·94 τ assigned to an allylic proton (H_B) geminal to an oxygen function. This type of coupling through four bonds (homoallylic coupling) has been shown to depend on the magnitude of the θ^{90} [as shown in (111)], between the allylic C-H bond and the plane of the rest of the system, reaching a maximum of 2 - 3 c.p.s. as θ tends to 90°. In the santonin derivative (112), for example, the signal at 7·88 τ ascribed to the C-4 methyl group was observed as a doublet (J = 1·4 c.p.s.) due to homoallylic coupling with H_6.[90] Models



114

of this compound with ring B in the favoured chair conformation indicated a value of approximately 115° for θ.[90] In the 6-epi derivative (113) the C-4 methyl resonance appeared as a singlet at 7·93 τ and in this case the value for θ is approximately 25°.[90] In our case the observed coupling constant (ca. 2 c.p.s.) suggests a value for θ of close to 90°.

The rest of the spectrum was equally informative. The slightly broadened triplet at 5·86 τ (J = 6 c.p.s.) assigned to a proton (H_C) geminal to the oxygen atom of a lactone function collapsed to a broad singlet upon irradiation in the vicinity of the 3H multiplet at 8·3 τ ($2H_D$ + OH). In a similar fashion coupling between H_C and H_B was detected although the coupling constant was small (ca. 1 c.p.s.), permitting the partial structure (114) to be drawn. This accounts for all the C, H and O atoms except the hydroxyl group and a C_3H_7 moiety which, as in the case of canadensiolide (107) and dihydrocanadensiolide (109) appeared to be an n-propyl group as evidenced by the 3H triplet at 9·06 τ and the 4H multiplet at 8·58 τ. These features are accommodated in the structure (115) for this compound which was therefore named methyl isocanadensiolate.



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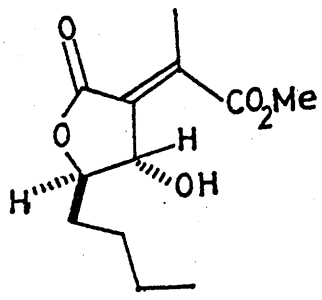
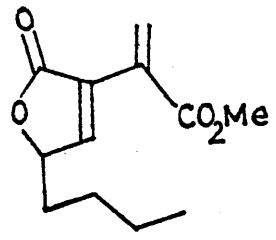


116

Although monocylcic, the inherent rigidity of the molecule as suggested by scale models, again permits application of the NMR data to predict the relative stereochemistry (Table 11). The extremely small vicinal coupling ($\sim$ 1/2 c.p.s.) of the protons H_B and H_C can only be accommodated by a trans relationship. Thus the relative stereochemistry for methyl isocanadensiolate can be shown as (116) or its enantiomer. As already discussed the size of homoallylic coupling between the vinylic methyl and H_B has been correlated to the magnitude of θ^{90} [cf (111)]. Scale models suggest that the value for θ in methyl isocanadensiolate is 60-70° which is in fair agreement with the correlation.

It was thought that this new compound may in fact be an artefact since methanolysis of the antibiotic canadensiolide (107) followed by double bond migration could conceivably give rise to methyl isocanadensiolate. Such a reaction could have occurred during the drying of the broth extracts by azeotropic distillation under reduced pressure with methanol.

In order to test this a small sample of canadensiolide was stored in methanol with a trace of acid for 7 days at room temperature. TLC (chloroform as eluant) of the resulting oil showed it to be a ternary



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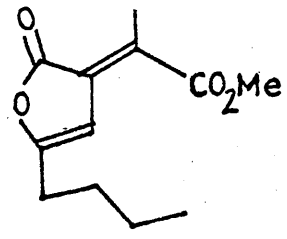


118

mixture, the major component (ca. 50%, R_f 0·3) being identified by TLC and IR as unreacted canadensiolide (107). A second more polar component (ca. 30%, R_f 0·25) has identical UV spectrum and R_f value as methyl isocanadensiolate (115). The minor component (ca. 15%) from its R_f value (0·7) and UV absorption (233 and 332 mμ), could be the diene (117) or (118) which might arise by dehydration of methyl isocanadensiolate in acidic medium, but unfortunately there was not sufficient material available for complete characterisation. It may be noted that the double bond migration not only extends conjugation but serves to relieve steric interaction between the side chain(carbomethoxy & terminal methylene group) and the hydroxyl function. However, it does appear from this experiment that methyl isocanadensiolate (115) could be an artefact arising by methanolysis of canadensiolide (107), but time has not allowed the isolation from fresh batches of culture to be attempted, avoiding methanol in the work up.

A fourth, acidic inactive metabolite, was isolated as a colourless compound from later fractions collected during chromatographic separation of the broth extracts. By elemental analysis and mass spectrometry the molecular formula for this fourth metabolite was calculated as $C_{11}H_{16}O_4$. The IR spectra confirmed the acidic nature of

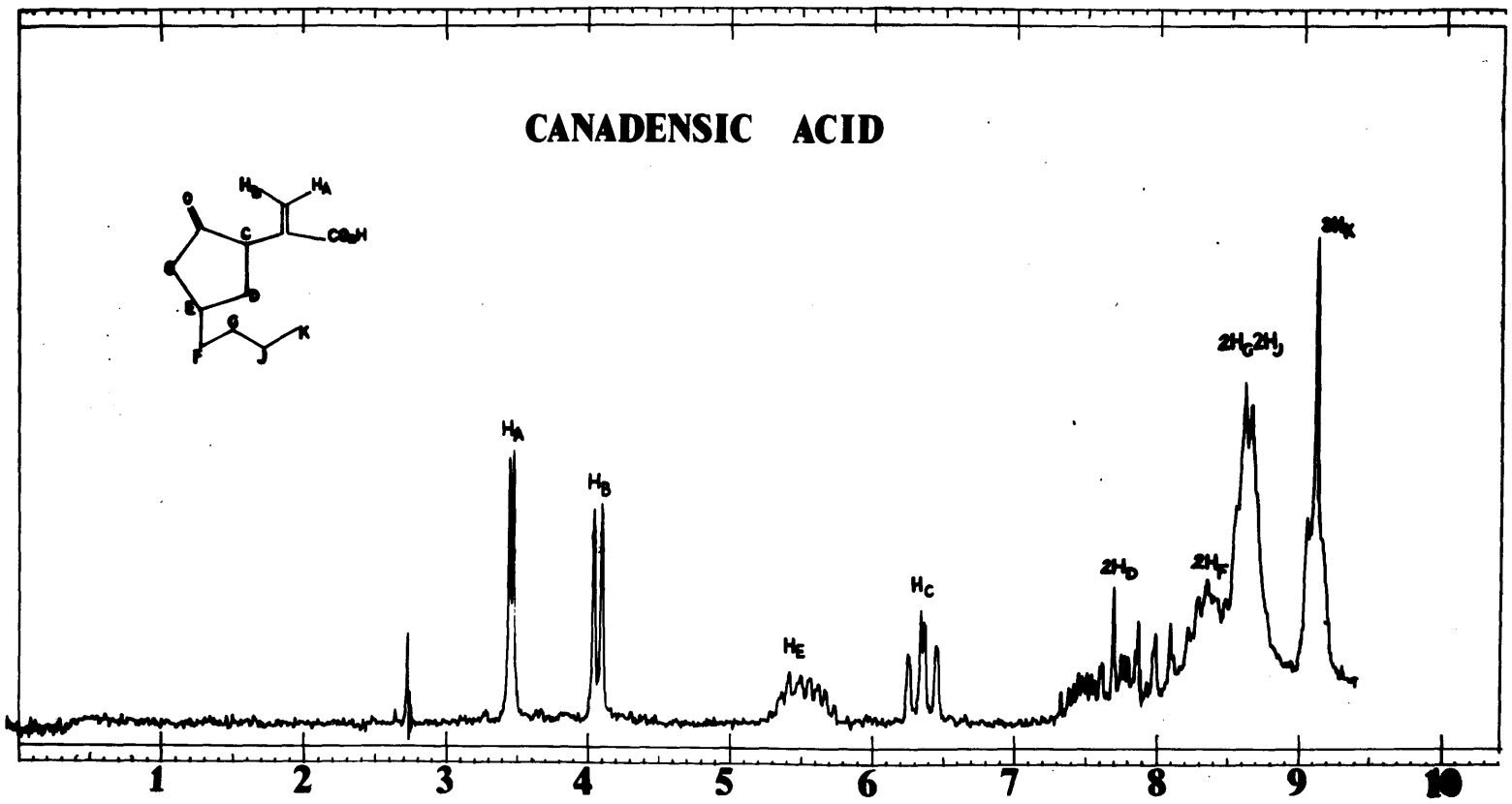
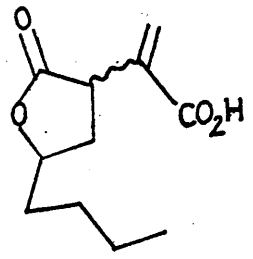


Fig. 4

this metabolite since it showed absorption at 3,500 $cm.^{-1}$ and 3,200-2800 $cm.^{-1}$, coupled with carbonyl absorption at 1700 $cm.^{-1}$ (ε 396 $\Delta\nu_{\frac{1}{2}}$ 20). Further, the spectra also exhibited absorption at 1768 $cm.^{-1}$ (ε 522 $\Delta\nu_{\frac{1}{2}}$ 28 $cm.^{-1}$) and 1630 $cm.^{-1}$ (ε 111) indicative of one γ lactone function and a conjugated terminal methylene group. The UV spectrum confirmed the conjugation showing high intensity absorption at 214 mμ.

From this data alone all the oxygen atoms and double bond equivalents have been accounted for. The NMR spectrum (fig. 4) showed the resonance of a proton (H_E) geminal to the oxygen of the lactone function and which appeared as a complex multiplet at 5·5 τ. (The corresponding signals found at 5·38 and 5·44 τ in the spectra of canadensiolide (107) and dihydrocanadensiolide (109) respectively were less complex and could be diagnosed as doublets of triplets). In the spectrum of the acid the double doublet at 6·38 τ (H_C) was assigned to a proton α to the carbonyl group of a γ-lactone and allylic, while the extremely complex region at ca. 7·6 τ was ascribed to a methylene group ($2H_D$) of the lactone ring the complexity being increased by a degree of non-equivalence between these protons. Double resonance established the vicinal couplings between H_E and 2 H_D and between 2 H_D



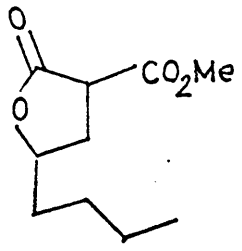
119

and H_C. The resonance of the terminal methylene protons appeared as doublets at 3·47 and 4·06 τ. (The multiplicity of these signals will be discussed later.) As with canadensiolide (107) and dihydrocanadensiolide (109) the presence of the n-butyl side chain was established by integration of the region 8·54–8·9 τ (6H), the 3H triplet at 9·1 τ (J = 6 c.p.s.), and the loss of the side chain (M–57) resulting in an ion at m/e 155 in the mass spectrum. This evidence permits the structure (119) to be proposed for this new metabolite hence designated canadensic acid.

It was observed in the 100 Mc spectrum of canadensic acid (119) that the coupling constant of the higher field doublet ascribed to one of the terminal methylene protons (H_B) was 5·7 c.p.s., i.e. too large for an allylic or homoallylic coupling. (There is no geminal coupling since the 'coupling constants' of the two doublets are different, and irradiation of one had no effect on the other.) Furthermore, in the 60 Mc spectrum of canadensic acid (119) it was observed that the separation between the peaks was 2·3 c.p.s. in the case of the low field pair and 3·5 c.p.s. for the high field pair, whereas in the 100 Mc spectrum the values were 3·8 c.p.s. and 5·8 c.p.s. respectively, exactly $^{10}/_{6}$ of the values observed in the 60 Mc spectrum. This shows

that these signals centred at 3·47 and 4·06 τ were doublets not by virtue of coupling but by superposition of two independent sets of signals from the terminal methylene groups in two slightly different magnetic environments thus accounting for the slight differences in chemical shift. Such a situation can be met by having a mixture of two rotamers, (the unsaturated carboxy side chain being held in two different positions by inter- or intra-molecular hydrogen bonding for example) or two epimers. The former explanation was excluded by high temperature NMR studies of canadensic acid in dioxan, which showed that the two doublets did not collapse to singlets even at 89°c. Thus it appears that canadensic acid is a 50:50 mixture of epimers (by integration), with the unsaturated carboxy side chain either α or β to the plane of the γ lactone ring.

From ozonolysis of the canadensic acid epimers (119) in glacial acetic acid, formaldehyde was removed from the reaction mixture by steam distillation and isolated as its bis-dimedone derivative in reasonable yield thus confirming the presence of the terminal methylene group. The residue left after steam distillation was shown by TLC (benzene : dioxan : acetic acid 35 : 5 : 1 as eluant) to be a mixture of four very polar components. Methylation with diazomethane afforded four less polar



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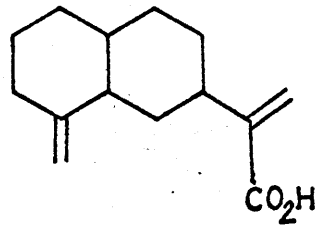

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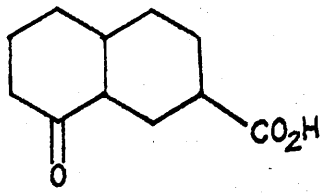
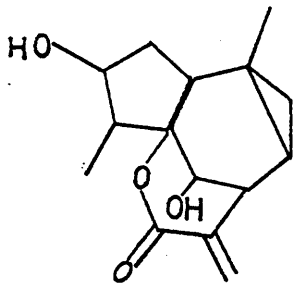


122

components which could not be satisfactorily separated by preparative TLC for subsequent characterisation. GLC (1% SE 30, 80°c) afforded a slightly better degree of separation and GCMS analysis revealed that the major component Rt. 13·6 min.) had a parent ion at m/e 200 and abundant ions at m/e 169 (M-31,-OCH_3) m/e 143 (M-57, n-butyl). These data (loss of methoxyl and n-butyl side chain) can be accommodated by structure (120) (MW 200) which is not unreasonable since ozonolysis of costic acid (121) under the same conditions affords the degraded acid (122).[91] The third component (Rt 11·2 min.) had a similar fragmentation pattern as that discussed above, while the spectra of the remaining components could not be satisfactorily recorded to permit an accurate determination of the data. The complex nature of the mixture inhibited any further chemical investigation, and the multiplicity of products may well be explained by the fact that canadensic acid (119) is a mixture in which H_C is cis or trans to the butyl side chain. Further attempts at oxidative cleavage of the carboxyl side chain using osmium tetroxide, osmium tetroxide/sodium metaperiodate,[92] and ruthenium tetroxide[93] were all unsuccessful, resulting either in recovery of starting material or intractable mixtures.

It was thought that esterification of the canadensic acid epimers (119) might afford a product whereby the epimers could be separated. Esterification with diazomethane was singularly unsuccessful resulting in a mixture of four products, all less polar (TLC, chloroform) than the starting material. The IR spectrum of this mixture showed no carbonyl absorption, but absorption at 1765 $cm.^{-1}$ (γ lactone) and 1740 $cm.^{-1}$. The latter absorption was too high for an unsaturated alkoxycarbonyl system, and this lack of conjugation was confirmed by further inspection of the IR spectrum which showed no absorption due to the terminal methylene group. These facts were further confirmed by the NMR spectrum which showed no resonance attributable to olefinic protons, although the typical sharp singlet at 6·25 τ for the resonance of a methoxyl group was present. Because of the complex nature of the mixture further structural assignments on the basis of NMR data were not possible.

Apparently diazomethane had esterified the carboxyl group, but had also reacted with the terminal methylene group. That this was an extremely facile and rapid reaction was demonstrated by leaving the acid epimers with ethereal diazomethane for one minute at ice-temperature, the resulting mixture being as complex as



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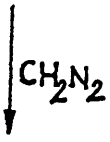
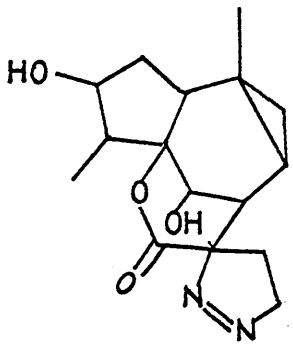
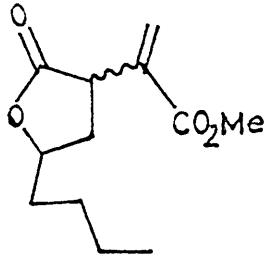




124

that obtained above. Anomalous reactions with diazomethane are known, and the formation of the pyrazoline ring system (124) by treatment of Zaluzanin A (123) with diazomethane has been reported.[94] Preparative TLC was unsuccessful in obtaining any reasonable amount of pure compound and GCMS analysis (1% SE 30, 150°c) was employed as an aid to characterisation of these products. The spectra of three components (Rt. of 9·25, 11·9, and 13·7 mins.)were satisfactorily recorded. All three components appeared to be isomeric and showed an ion at m/e 240 which appeared to be the parent ion (28 mass units higher than the parent ion for canadensic acid), and common ions at m/e 209 (M-31), m/e 183 (M-57) and m/e 126 (M-57+31) (base peak), corresponding to losses of a methoxyl group and the n-butyl side chain. These data did not confirm the presence of a heterocyclic ring system (though unrecorded loss of nitrogen is possible in the mass spectrum), but while they did not exclude several possible structures (derived by C-methylation,[95] cyclopropane formation etc.,), no definite conclusion could be drawn. It was, however, evident that this was an unsuitable method of esterification and a more satisfactory method was sought and found.



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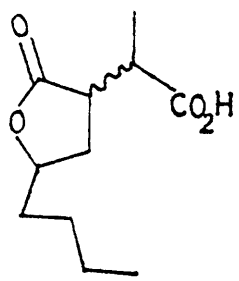

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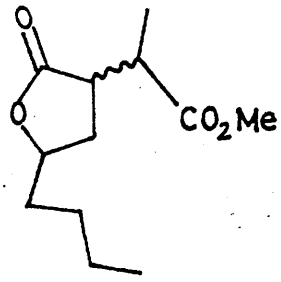

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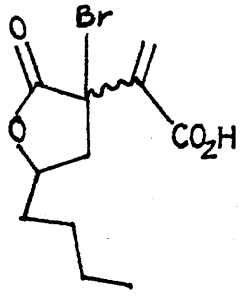

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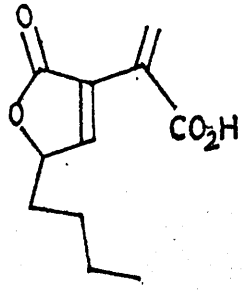


129

Refluxing the acid epimers (119) in a slurry of acetone, potassium carbonate and methyl iodide[96] for several hours afforded an oil which failed to crystallise. The compound was less polar than the canadensic acid epimers (119) (TLC) and the IR showed absorption at 1767 $cm.^{-1}$ (ε 540), 1721 $cm.^{-1}$ (ε 430) and 1633 $cm.^{-1}$ corresponding to a γ lactone system, an unsaturated alkoxycarbonyl group, and a terminal methylene group. This was corroborated by the NMR spectrum which showed a 3H singlet at 6·15 τ corresponding to the resonance of a methoxyl group, and the characteristic pairs of doublets at 3·4 and 4 τ due to the resonances of the two terminal methylene protons of the two epimeric esters. The MS data showed a parent ion at m/e 226 and ions at m/e 195 (M-31) and m/e 169 (M-57), corresponding to the anticipated losses of the methoxyl and side chain groups. These data afforded the unique solution that methyl canadensate and its epimer had been prepared and that the structure was (125). The methyl ester epimers (125) could also be prepared by storing the canadensic acid epimers (119) in methanol with a trace of acid at room temperature for seven days. The esters obtained in this way had identical IR, UV, NMR, GLC and TLC properties as those described above. However, these epimers failed to separate on TLC and by

GLC using the following columns; 1% SE 30, (125°c, Rt. 15·6 min.), 1% QF-1 (125°c Rt. 25 min.), and 5% APL (150°c, Rt. 48·4min).

Hydrogenation of the canadensic acid epimers for 50 minutes using platinum oxide afforded the dihydro-canadensic acid epimers (126) as a white powder which, by elemental analysis and mass spectrometry possessed the molecular formula, $C_{11}H_{18}O_4$. (As found with canadensiolide (107), 5% palladium-on-barium sulphate failed to effect hydrogenation.) The IR spectrum showed absorption at 1764 $cm.^{-1}$ (ε 712 $\Delta\nu_{\frac{1}{2}}$ 57·5), and 1711 $cm.^{-1}$ (ε 665 $\Delta\nu_{\frac{1}{2}}$ 38) consistent with there being a γ lactone group and saturated carboxyl group. The UV spectrum showed no absorption above 200 mμ. The NMR spectrum of the hydrogenation product, although showing no resonances due to olefinic protons, was more complex than that of the canadensic acid epimers. The 3H doublet at 8·77 τ (H_A) (J = 7·0 c.p.s.) was ascribed to a secondary methyl group β to a carbonyl group, and this collapsed to a singlet upon irradiation in the vicinity of the 2H multiplet at 6·92 τ (H_B and H_C), and conversely irradiation in the vicinity of 8·77 τ, resulted in a doublet (J = 6 c.p.s.) (H_B) superimposed on a multiplet (H_C) at 6·92 τ. These supply further proof as to the position ($\alpha\beta$ to a carbonyl)

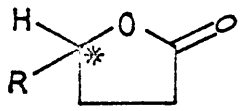
and type of unsaturation (terminal methylene group) present in the canadensic acid epimers (119).

In a further attempt to separate the epimers, the mixture of dihydrocanadensic acid epimers was esterified with diazomethane, which could now be successfully employed since the exomethylene group had been reduced, to give the methyl dihydrocanadensate epimers (127). The IR spectrum (thin film) showed absorption at 1768 $cm.^{-1}$, and 1740 $cm.^{-1}$, consistent with a γ lactone system and a saturated ester group. GLC analysis (5% APL, 125°c) showed two peaks that were just separable, but were not separable on 1% SE 30, or 1% QF-1. Thus it appears that the dihydro ester epimers were separable whereas the unsaturated esters were not.

An attempt was made to isomerise the double bond of the methyl canadensate epimers (125) using triethylamine. (Acidic conditions are evidently unhelpful since no isomeric product could be detected from esterification of the canadensic acid epimers (119) using methanol and concentrated sulphuric acid.) Similarly, however, triethylamine failed to isomerise the esters after refluxing for five hours. On the other hand, it was found during NMR studies that when the canadensic acid epimers were stored in deuterated DMSO for 24 hours isomerisation did occur.

The NMR spectrum now showed the resonance of a vinylic methyl group at 7·93 τ and that the resonances of the terminal methylene protons now appeared as singlets at 3·76 and 4·15 τ. The rest of the spectrum was similar to that of the canadensic acid epimers. The result can be interpreted as isomerisation of the canadensic acid epimers followed by equilibration of the isomerised acid with one epimer (presumably the more stable) of canadensic acid. The NMR spectra recorded at 2 and 4 day intervals showed little or no change confirming that equilibrium had been reached. Unfortunately, time has prevented any further investigation of this aspect but it is intended to carry out a complete and detailed examination of this isomerisation in the future.

Attempts were also made to introduce an allylic bromine α to the γ lactone carbonyl function. There were two objectives in this experiment. Firstly, a bromine atom in this position would greatly simplify the NMR spectrum of the canadensic acid epimers by removing a coupling (H_C to H_D) with the two non-equivalent protons H_D. Secondly, it was thought that the brominated compound (128) would be readily dehydrobrominated to give the diene (129) which could cyclise in acid to give the antibiotic canadensiolide (107). This would complete



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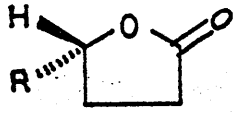

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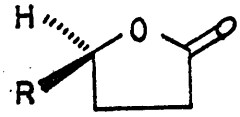
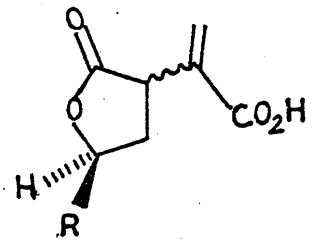


132

the interrelation of the four compounds isolated. However repeated attempts using N-bromosuccinimide failed to effect introduction of the halide atom. Again, unfortunately, time has prevented any further work on this promising synthetic route which will be pursued in the future.

The ORD curves for the canadensic acid epimers (119) and the dihydrocanadensic acid epimers (126) were measured in order to determine the absolute stereochemistry of these molecules and, by analogy, predict the absolute stereochemistry of the related metabolites canadensiolide (107) and dihydrocanadensiolide (109). An early attempt to relate the stereochemistry of a lactone to the sign of its optical rotation was made by Hudson[97] on the basis of studies on five- and six-membered lactones of the sugar series. Hudson's original rule was later extended by Klyne[98] such that Hudson's rule may be stated as follows.

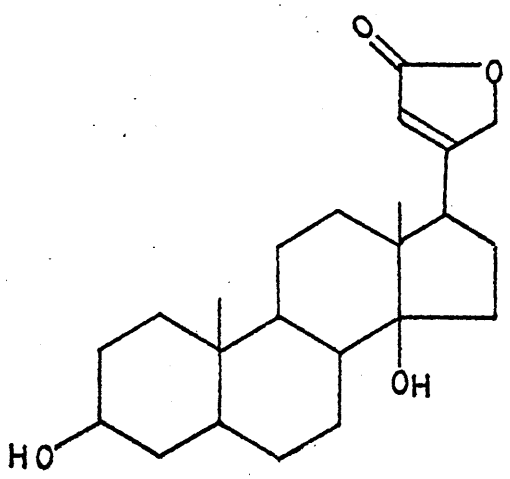
'If the hydrogen atom at the alkoxy-carbon (C^*) in (130) lies below the plane of the lactone ring then the compound is dextro-rotatory. Conversely if the hydrogen lies above the plane of the ring then the compound will have a negative rotation.'



134

This statement was fully borne out by experimental evidence[99] which showed that lactones of the configuration (131) had a negative rotation and that those possessing the configuration (132) had a positive rotation.

The canadensic acid epimers (and the dihydro derivatives) were extremely suitable for ORD measurements since if it is assumed that the effects of the epimeric centres cancel one another, the only effective asymmetric, centre is the 'alkoxy' carbon atom. The ORD curves of the canadensic acid epimers and the dihydrocanadensic acid epimers were plain positive curves, indicating that the hydrogen was α to the plane of the lactone ring, and hence that the n-butyl sidechain was β to the plane of the ring. Thus the configuration of the canadensic acid epimers can be expressed as (134). If the assumption is made that the configuration at the alkoxy carbon in the canadensic acid epimers will be the same for the metabolites of P. canadense, then from the basis of the NMR data and coupling constants already discussed the absolute stereochemistry for dihydrocanadensiolide and the antibiotic canadensiolide can be depicted as (110) and (108) respectively.



135

In conclusion, it may be noted that the occurrence of an unsaturated lactone ring in many physiologically active compounds has been reported and commented on.[100] The physiological activities of such compounds are very diverse ranging from antibiotic activity, as with the antifungal metabolite avenaciolide (34),[27] to cardiac activity exhibited by digitoxigenin (135).

EXPERIMENTAL

Section 2.2

General.

Instrumentation

The techniques and instruments used were the same as those indicated in p. 39.

Nomenclature used in describing activity assay results.

The code used for describing activity assay results is as follows: The first number refers to the dilution i.e., 1 is a dilution of $\frac{1}{2}$, 2 = 1/4, 3 = 1/8, etc. A fungistatic effect is indicated by the presence of a first figure which is the lowest dilution at which no germination or germination less than 1% is observed. The letter S indicates stunting and the following number the lowest dilution at which this occurs. Thus 3S5 indicates no germination at the lowest dilution of 1/8, and that stunting occurs at a dilution of 1/32.

Growth and Extraction of the Mould.

Penicillium canadense (Commonwealth Mycological Institute No. 95,493) was subcultured onto 2% malt agar slants and thence to agar seed bottles (15 x 9 cm.). A spore suspension, prepared from 12 such bottles and distilled water (2 l.), was used to innoculate 100 Roux surface culture bottles which had previously been sterilised (0·5 hours with steam at 242°F and 12 p.s.i.) and contained 200 ml. of the following medium.

Glucose	50 g.
ammonium tartrate	2·8 g.
dipotassium hydrogen phosphate	5·0 g.
magnesium sulphate	1·0 g.
sodium chloride	1·0 g.
Difco yeast extract	0·5 g.
$FeSO_4\ 7H_2O$ (0·1 g), $CuSO_4\ 5H_2O$ (0·015 g), $ZnSO_4\ 7H_2O$ (0·05 g), $MnSO_4\ 7H_2O$ (0·01 g), Na_2MoO_4 (0·01 g), 100 ml. distilled water.	1 ml.
distilled water to 1 litre.	

Cultures were allowed to grow undisturbed at 25°c and 70% relative humidity, artificial illumination being provided by Mazda fluorescent tubes for approximately 8 hours per day. After the prescribed period of growth

Table 12. Summary of the initial activity assays of the culture filtrate of P. canadense

Fungus	Days of Growth		
	6	12	18
P. canadense	589	487	184

had elapsed (10 days), the mycelial mats were separated from the broth, dried at 40°c, powdered in a mortar and subjected to soxhlet extraction with chloroform for 24 hours. The pH of the broth was adjusted to 7 (recorded on a pH meter) and extracted with activated charcoal (10 g. per litre of broth) at room temperature for 24 hours. Thereafter the charcoal was filtered off and extracted in a soxhlet apparatus for 24 hours with acetone.

ACTIVITY ASSAYS

Initial assays for antibiotic activity using the serial dilution spore germination test on the conidia of Botrytis allii were performed on replicate cultures of the fungus. After intervals of 6, 12 and 18 days growth, the broth of each medium was filtered through sterile No. 1 Whatman paper and the crude filtrates were used for the assays(Table 12). From these data it appeared that the optimum time for antibiotic production was 10 days and hence the cultures were harvested after this time.

On large scale production each group of cultures (100 Roux bottles) was assayed for antibiotic activity at intervals throughout the growth period of 10 days.

Table 13. Summary of activity assays carried out during a typical growth period of P. canadense (Large scale production)

Days of Growth		
3	6	10
3S9	5S10	5S11

The results from one of these assays, which is entirely typical, is shown in Table 13).

Assays carried out on the broth extracts indicated the retention of antifungal activity, while similar assays performed on the mycelial extracts revealed some activity though weak by comparison (Table 8).

CHROMATOGRAPHIC SEPARATION OF THE P. CANADENSE METABOLITES ON SILICA

The dry broth extracts (21·5 g.) were dissolved in methanol, silica added (60 g.) and the solvent removed on a rotary film evaporator. The silica with the absorbed organic material was allowed to dry thoroughly at room temperature, finely powdered and introduced onto a column of silica (1400 g., 5 x 90 cm). Separation was achieved by elution with benzene containing 0, 10, 20, 30 etc. % chloroform, using 1 column volume of each mixture successively. Fractions (25% column volume) were collected when the solvent composition had changed to 60% chloroform - 40% benzene. Each fraction was examined for antibiotic activity by assaying aliquots (5 mg.) in sterilised water (5 ml.) or 4% methanol-water (5 ml.). Table 14 summarises the fractionation produced.

Table 14. Summary of Chromatographic Separation of the *P. canadense* metabolites

Fraction	Weight (g.)	Antibiotic Activity	Eluant
1	0·02		
2	0·025		60% chloroform
3	0·03		in benzene
4	0·03		
5	0·03		
6	0·035		70% chloroform
7	0·1		in benzene
8	0·2		
9	0·25	+	
10	0·45	+	80% chloroform
11	0·5		in benzene
12	0·5		
13	0·7		
14	1·0		90% chloroform
15	1·05		in benzene
16	0·85		
17	0·7		
18	0·6		100% chloroform.
19	0·5		
20	0·35		

The fractions (9-10) which exhibited antifungal behaviour were combined and the mixture (0·7 g.) was separated by preparative TLC on two Kieselghur HF_{254} plates (500 x 20 x 0·7 mm.) using chloroform as eluant. The pure compound dihydrocanadensiolide was removed from the silica by elution with chloroform and was filtered through glass paper prior to crystallisation (112 mg.) Canadensiolide, contaminated to an extent of ca. 10% with the dihydro derivative (by GCMS analysis), was separated in a similar fashion. This binary mixture (272 mg.) (an oil) was further separated by preparative TLC on one Kieselghur HF_{254} plate (500 x 20 x 0·7 mm.), and elution with chloroform. A pure sample of canadensiolide (246 mg.) was obtained after several such plating operations.

The more polar fractions which exhibited no antifungal behaviour were crystallised from benzene-light petroleum mixtures. The yellowish solid obtained after several such crystallisations was dissolved in ice-cold freshly prepared aqueous sodium bicarbonate solution and then immediately reprecipitated with ice-cold dilute hydrochloric acid (2N). The acidic solution was filtered and the canadensic acid was washed thoroughly with water and allowed to dry prior to recrystallisation.

Canadensiolide (107).

The antibiotic was isolated as previously described as an oil which crystallised from benzene-light petroleum as colourless needles m.p. 46-47·5°c.

α_D -141·2° (c 0·86).

(Found, C 62·4%, H 7·18% M^+210: $C_{11}H_{14}O_4$ requires C, 62·8%, H 6·71% MW = 210.)

$\nu_{max.}$(KBr): 1764, 1666, 1185, 1060, 959, 918 908 cm.$^{-1}$

$\nu_{max.}$(1·46 mM $CHCl_3$): 1775 cm.$^{-1}$(ε 1320, $\Delta\nu_{\frac{1}{2}}$ 23 cm.$^{-1}$) 1663 cm.$^{-1}$ (ε 73)

$\lambda_{max.}$(C_2H_5OH): 213 mμ (log ε 3·93)

τau values ($CDCl_3$): 3·58 (1H, d, J = 2,5 c.p.s.), 3·97 (1H, d, J = 3 c.p.s.), 4·88 (1H, d.d, J =6·5and4·5c.p.s.), 5·38 (1H, m, J = 6·2 and 4·5 c.p.s.), 6 (1H, d.d, J = 6·5 and 2·0 c.p.s.), 8·08 (2H, m), 9·0 (3H, t, J = 6 c.p.s.).

Ozonolysis of Canadensiolide (107).

A stream of ozonised oxygen was passed through a solution of canadensiolide (60 mg.) in glacial acetic acid (3 ml.) for 2 hours and then the solution was left at room temperature for 2 hours. After treatment with 2N hydrochloric acid (2 ml.) followed by acetic acid (5 ml.) the mixture was left at room temperature overnight.

The solution was carefully steam distilled into an ethanolic solution of dimedone (50 mg./ml.). The dimedone solution was heated on a steam bath for a few minutes reduced in volume and the white solid obtained crystallised from water as needles m.p. 191°c (9 mg., 10%). Melting point unchanged on admixture with an authentic sample of formaldehyde dimedone.

Cleavage of Canadensiolide (107) with Sodium Metaperiodate

Canadensiolide (21 mg.) was treated with potassium hydroxide (13·5 mg.) in water (1 ml.) and gently warmed for a few minutes. Sodium metaperiodate (22 mg.) in the minimum volume of water was added dropwise with stirring. A white precipitate formed rapidly and stirring was continued for 4 hours. The aqueous solution was then filtered and the filtrate carefully steam distilled. The distillate was examined by GLC using an Aerograph 200 instrument and two separate columns. Column (1): 20% Carbowax 1500 on 60/80 Chromosorb W., 5' x 1/8". [Column temperature 60°c, detector temperature 170°c, injection temperature 120°c: Flow rate 20 mls./min. N_2 58 p.s.i. and H_2 18 p.s.i.]

Table 14A.

Column Phase Temp.	Degraded n-Valer-aldehyde	Synthetic Valer-aldehyde	Combined Injection	Product Reduced	n-Valer-aldehyde Reduced	n-Pentanol
D.N.P.* 60°c	Rt. 8·8 mins.	Rt. 8·8 mins.	Rt. 8·9 mins.	Rt. 23·1 mins.	Rt. 22·8 mins.	Rt. 23 mins.
Carbowax* 60°c	Rt. 2 mins.	Rt. 2 mins.	Rt. 2 mins.	Rt. 13·8 mins.	Rt. 13·8 mins.	Rt. 14 mins.

* For full details see text.

Column (2): 15% D.N.P. on 60/80 Chromosorb W., 6' x 1/8" (Column temperature 60°c, detector temperature 170°c, injection temperature 120°c: Flow rate 18 mls./min., N_2 58 p.s.i. and H_2 18 p.s.i.)

On both columns the n-valeraldehyde present in the distillate had the same retention time as an authentic sample (Table 14A).

The walls of a 1 ml. syringe were coated with a 50% aqueous solution of sodium borohydride. Vapour samples (0·2 ml.) from the distillate were introduced into the syringe and allowed to react with the borohydride for 10 minutes. Subsequent GLC examination of this vapour on the two columns showed one peak corresponding in retention time to n-pentanol (Table 14A). The vapour from an aqueous solution of an authentic sample of n-valeraldehyde was similarly treated with borohydride, GLC examination showing complete reduction to pentanol (Table14A).

Dihydrocanadensiolide (109).

This compound was isolated as described earlier and crystallised from benzene-light petroleum as colourless needles m.p. 94-94·5°c. $[\alpha]_D$ -31·13° (c 0·53). (Found C, 61·81%, 61·63%; H, 7·6%, 7·76%, M^+ 212 $C_{11}H_{16}O_4$ requires C, 62·25%; H, 7·6% MW = 212.)

$\nu_{max.}$(KBr): 1766, 1292, 1185, 1068, 958, 916, 908 $cm.^{-1}$

$\nu_{max.}$(0·78 mM $CHCl_3$): 1783 $cm.^{-1}$ (ε 925), 17899 $cm.^{-1}$ (shoulder) (ε 850).

$\lambda_{max.}$(C_2H_5OH): no absorption > 200 mμ

τau values ($CDCl_3$): 4·9 (1H, d.d, J = 6 and 4 c.p.s.), 5·44 (1H, m, J = 7 and 4 c.p.s.), 6·9 (2H, m. J = 6 and 8 c.p.s.), 8·08 (2H, m), 8·5 (3H, d, J = 8 c.p.s.), 9·0 (3H, t, J = 6·2 c.p.s.).

Hydrogenation of α angelica Lactone.

(a) Platinum oxide.

Angelica lactone (245 mg.) in ethyl acetate (20 ml.) was shaken with platinum oxide (170 mg.)(which had been pre-reduced) in an atmosphere of hydrogen for 50 minutes. Thence the solution was filtered (glass paper) and the solvent removed in vacuo to yield an oil (242 mg.). TLC of this oil showed a large diffuse spot more polar than the starting material.

$\nu_{max.}$(thin film): 3,400-2,600, 1760 (shoulder), 1710, 1200 $cm.^{-1}$.

(b) 5% palladium-on-barium sulphate.

Angelica lactone (500 mg.) was shaken with 5% palladium-on-barium sulphate in an atmosphere of hydrogen for 40 minutes. The catalyst was separated on glass paper and the filtrate was concentrated in vacuo to yield an oil (499 mg.) which had identical IR spectrum with butyrolactone.

$\nu_{max.}$(thin film): 1780, 1190, 955 $cm.^{-1}$

Catalytic reduction of canadensiolide (107) (dihydrocanadensiolide).

Canadensiolide (21 mg.) in ethanol (3 ml.) was shaken overnight with 5% palladium-on-charcoal (10 mg.) in an atmosphere of hydrogen. The catalyst was separated on glass paper and the solvent removed in vacuo to yield an oil (23 mg.) TLC employing chloroform as eluant showed two spots more polar than the starting material.

$\nu_{max.}$ (thin film): 3,600-3,200, 1780-1720 $cm.^{-1}$

τau values ($CDCl_3$): 3·95 (OH, b), 4·9 (1H, m), 5·4 (1H, m), 6-7 (2H, m).

Methyl doublets (J = 6 c.p.s.) of approximately equal relative intensity at 8·55, 8·65 and 8·68 τ. This mixture was treated with Jones reagent (0·2 ml.) for 20 minutes, diluted with water (2 ml.) and extracted with ether (2 x 10 ml.). The organic extract was washed twice with bicarbonate, water and dried ($MgSO_4$). Removal of the solvent in vacuo yielded an oil which was separated by preparative TLC on one Kieselghur HF_{254} plate (20 x 20 x 0·7 mm.) and elution with chloroform. The major band (Rf 0·45) was extracted from the silica by elution with chloroform, removal of solvent affording a colourless solid which crystallised from benzene-light petroleum mixtures as stout colourless needles m.p. 89-93°c, (4 mg., 20%). These needles had identical IR, MS and TLC properties as naturally occurring dihydrocanadensiolide (109).

Methyl Isocanadensiolate(115).

Crude broth extract (3·5 g.) obtained from cultures of P.canadense (50 Roux bottles) was absorbed on silica (20 g.) and introduced onto a column of silica(250 g. 60 x 3·5cm.). Separation was achieved using as eluant benzene-chloroform mixtures of increasing polarity as described previously (p.123). The fractions displaying antifungal behaviour were bulked (208 mg.) and separated by preparative TLC on one Kieselghur HF_{254} plate (500 x 20 x 20 mm.) using chloroform as eluant. The major band was extracted from the silica with chloroform, TLC analysis of the resulting oil (90 mg.) indicated this to be a binary mixture of canadensiolide and another compound which was the major component. This oil was separated on one Kieselghur HF_{254} plate employing a two-fold elution with chloroform. The major component was extracted from the plate in the usual manner, to yield an oil (40 mg.) which crystallised from benzene-light petroleum as colourless needles m.p. 65-66°c.

(Found: M^+ 242·1150 $C_{12}H_{18}O_5$ requires MW = 242·1154)

$\nu_{max.}$(Nujol): 3,500, 1760, 1710, 1235 $cm.^{-1}$.

$\nu_{max.}$(1·65 mM$CHCl_3$): 3590 $cm.^{-1}$, 1763 $cm.^{-1}$ (ε 677), 1715 $cm.^{-1}$ (ε 472).

$\lambda_{max.}$(C_2H_5OH): 229 mμ (log ε 4·17).

τau values ($CDCl_3$): 4·94 (1H, q, J ~ 2 c.p.s.), 5·86 (1H, t. J = 7 c.p.s.), 6·12 (3H, s), 7·8 (3H, d. J ~ 2 c.p.s.), 9·06 (3H, t, J = 7 c.p.s.).

Treatment of canadensiolide (107) with methanol and sulphuric acid.

Canadensiolide (21 mg.) was dissolved in methanol (3 ml.) and concentrated sulphuric acid (0·1 ml.) carefully added. The mixture was allowed to stand at room temperature for 7 days. After this period the organic solution was diluted with water (3 ml.) and the whole concentrated in vacuo and extracted with ethyl acetate (2 x 5 ml.). The organic extract was washed with freshly prepared bicarbonate solution (1 x 5 ml.), water (2 x 5 ml), and dried ($MgSO_4$). Removal of the solvent in vacuo yielded an oily solid which by TLC using chloroform as eluant was shown to be essentially a mixture of three components of R_f values 0·7, 0·3, 0·25. Preparative TLC on one Kieselghur plate (20 x 20 x 0·7 mm) afforded a degree of separation of these components which were extracted in the usual manner. The major component (R_f 0·3) (~ 50%) had identical R_f (0·3) and IR (1770, 1665 $cm.^{-1}$) with the starting material canadensiolide. The least polar band

(R_f 0·7) which was only present in small amounts ($\sim$15%) displayed maxima at 233 mμ and 322 mμ (concentrated solution). The more polar band (R_f 0·25) ($\sim$30%) was shown by UV (228 mμ) and TLC employing chloroform as eluant, to be methyl isocanadensiolate (R_f 0·25) contaminated with trace amounts of canadensiolide (R_f 0·3).

Canadensic acid and Epicanadensic acid (119).

Canadensic acid was isolated as described earlier and the acid crystallised from benzene-light petroleum as long colourless needles, m.p. 128·5-130·5°c (unchanged by further crystallisation) $[\alpha]_D$ +37·26 (c 1·06). (Found: C, 62·55%; H, 7·43%; M^+ 212. $C_{11}H_{16}O_4$ requires C, 62·25%; H, 7·6%, MW = 212).

$\nu_{max.}$(KBr): 3200-2500, 1761, 1692, 1630, 1210 1184 cm.$^{-1}$.

$\nu_{max.}$(1·22 mMCHCl$_3$): 3,500 cm.$^{-1}$, 3200-2800 cm.$^{-1}$, 1768 cm.$^{-1}$ (ε 522, $\Delta\nu_{\frac{1}{2}}$ 28 cm.$^{-1}$), 1737 cm.$^{-1}$ (shoulder) (ε 220), 1700 cm.$^{-1}$ (ε 396, $\Delta\nu_{\frac{1}{2}}$ 20), 1630 cm.$^{-1}$ (ε 111).

$\lambda_{max.}$(C_2H_5OH): 214 mμ (log ε 3·77).

τau values ($CDCl_3$): 3·45 ($\frac{1}{2}$H, s), 3·48 ($\frac{1}{2}$H, s), 4·04 ($\frac{1}{2}$H, s), 4·095($\frac{1}{2}$H, s), 5·5 (1H, m), 6·38 (1H, d.d, J = 9 c.p.s. in each case), 9·1 (3H, t, J = 6·0 c.p.s.).

Attempted methylation of canadensic acid with diazomethane

The canadensic acid epimers (21 mg.) dissolved in the minimum volume of ether were treated with an excess of ethereal diazomethane for 20 minutes. Removal of solvent yielded an oil which by GLC examination (1% SE30, column temperature 150°c, injection temperature 220°c, flow rate 37 mls./min.) was shown to be a mixture of four products. (R.T. 9·25, 11·9, 13·7 and 23·7 minutes). $\nu_{max.}$ (thin film): 1765, 1735 cm.$^{-1}$.
τau values ($CDCl_3$): 5·25 (m), 5·7 (m), 6·25 (s), 6·8-8 (m), 9·05 (t) (Relative integrals 2:2:3:3:6).
Cooling the reaction mixture on ice and immediate destruction of excess reagent failed to reduce the number of products.

Methyl canadensate (125) and methyl epicanadensate

(a) The canadensic acid epimers (21·2 mg.) were gently boiled in a slurry of potassium carbonate (35 mg.) and 'AnalaR' acetone (4 ml.). Methyl iodide (80 mg.) was added with stirring and under reflux, and heating continued for 4 hours. The reaction mixture was cooled, filtered (glass paper) and the solvent removed under reduced pressure. The residue was taken up in chloroform

(10 ml.) and washed with fresh aqueous sodium bicarbonate solution (2 x 10ml.), water (2 x 10 ml.), dried ($MgSO_4$) and concentrated in vacuo to give the methyl canadensate epimers (125) as an oil (21 mg., 93%) which failed to crystallise, and distilled at 115°c/0·3 mm.
(Found: C, 63·59%; H, 7·95%; M^+ 226. $C_{12}H_{18}O_4$ requires C, 63·7%; H, 8·02%; MW = 226.)
$\nu_{max.}$(thin film): 1765, 1716, 1635, 1195, 840 $cm.^{-1}$.
$\nu_{max.}$(1·48 mM$CHCl_3$): 1767 $cm.^{-1}$ (ε 540), 1721 $cm.^{-1}$ (ε 430), 1633 $cm.^{-1}$ (ε 80).
$\lambda_{max.}$(C_2H_5OH): 211 mμ (log ε 3·78).
τau values ($CDCl_3$): 3·4 (1H, d), 4 (1H, d), 5·4 (1H, m), 6·15 (3H, s), 6·2 (1H, d.d., J = 9 c.p.s.), 9·1 (3H, t, J =6·0c.p.s.).

(b) The canadensic acid epimers (22 mg.) were left in methanol (3 ml.) and concentrated sulphuric acid (0·1 ml.) for 7 days at room temperature. After this time the reaction mixture was diluted with water (3 ml) concentrated in vacuo and extracted with chloroform (2 x 5 ml.). The organic layer was washed thoroughly with aqueous sodium bicarbonate solution (2 x 10 ml.), water (2 x 10 ml.), and dried ($MgSO_4$). Removal of the solvent yielded an oil (20 mg.) which was identical with a sample of the mixtures of methyl canadensate epimers

as prepared in method (a), by comparison of IR and UV spectra and R_f (0·5) on TLC and retention time (15·6min.) on GLC (1% SE30).

Dihydrocanadensic acid (126).

The canadensic acid epimers (24 mg.) were shaken with platinum oxide (6 mg.) in ethanol (3 ml.) in an atmosphere of hydrogen for 50 minutes. The catalyst was separated on glass paper and the ethanol removed *in vacuo* to give the mixture of dihydrocanadensic acid isomers (126) as an oil (22 mg., 93·4%) which crystallised from benzene-light petroleum as a colourless powder, m.p. 103-105°c (softening at 90°c).

(Found: C, 61·45%; H, 8·29%; $(M-57)^+$ 157. $C_{11}H_{18}O_4$ requires C, 61·66%; H, 8·47%; MW-57 = 157).

$\nu_{max.}$(KBr): 3,300-2,600, 1755, 1690, 1195 $cm.^{-1}$.

$\nu_{max.}$(2·27 mM$CHCl_3$): 1764 $cm.^{-1}$ (ε 712, $\Delta\nu_{\frac{1}{2}}$ 57·5), 1711 $cm.^{-1}$ (ε 665, $\Delta\nu_{\frac{1}{2}}$ 38).

$\lambda_{max.}$(C_2H_5OH): No absorption > 200 mμ.

τau values ($CDCl_3$): 5·58 (1H, m), 6·92 (2H, m), 7·7 (2H, m), 8·77 (3H, d, J = 7·0 c.p.s.), 9·08 (3H, t, J = 7·0 c.p.s.).

Methyl dihydrocanadensate (127).

The dihydrocanadensic acid isomers (10 mg.), dissolved in the minimum volume of ether, were treated with excess ethereal diazomethane for 15 minutes. The reaction mixture was concentrated in vacuo and the residue taken up in chloroform and washed thoroughly with bicarbonate solution, water, and dried ($MgSO_4$). Removal of the solvent afforded the mixture of methyl dihydrocanadensate isomers (127) as an oil (10 mg.), R_f 0·51 on TLC employing chloroform as eluant. GLC analysis (5% APL, column temperature 125°c, injection temperature 210°c; flow rate 35 mls./min) showed two peaks of retention times 63·75 and 65·6 minutes. These peaks were not separable on 1% SE30 (Column temperature, 125°c; flow rate 35 mls./min.)R.T. 18·75 minutes or 1% QF-1 (Column temperature 125°c; flow rate 30 mls./min.) R.T. 29·4 minutes.

$\nu_{max.}$(thin film): 1768, 1740, 1180 $cm.^{-1}$.

Ozonolysis of canadensic acid (119).

A stream of ozonised oxygen was passed through a solution of the canadensic acid epimers (100 mg.) in glacial acetic acid (5 ml.) for 1·5 hours, and the

solution left at room temperature for 2 hours. After addition of 2N hydrochloric acid (3 ml.) followed by acetic acid (8 ml.) the mixture was left overnight at room temperature. Thereafter the solution was carefully steam distilled into an ethanolic solution of dimedone (50 mg./ml.) and the dimedone solution was heated on a steam bath for five minutes and allowed to cool. Formaldehyde dimedone was precipitated as long colourless needles m.p. 191°c (41·5 mg., 36·2%). Melting point unchanged by admixture with an authentic sample of formaldehyde dimedone.

The acidic aqueous solution left from steam distillation was treated with solid sodium bicarbonate until almost neutral and extracted thoroughly with chloroform. The organic layer was washed well with brine, dried ($MgSO_4$), and the solvent removed <u>in vacuo</u> to give an oil (60 mg.). Analysis of this oil by TLC using 10% methanol-chloroform or benzene-dioxan-acetic acid (35: 5 : 1) mixture as eluant indicated that at least four acidic components were present. A part of this oil (40 mg.) was subjected to preparative TLC or one Kieselghur HF_{254} plate (20 x 20 x 0·7 mm.) using benzene-dioxan-acetic acid as eluant. No component could be isolated satisfactorily in order to characterise it after three such preparative TLC operations.

Methylation of another part of the oily product (10 mg.) with diazomethane afforded four main, less polar components, which could not be satisfactorily separated by TLC, GLC analysis (1% SE30, column temperature 80°c, injection temperature 210°c, flow rate 40 mls./min.) showed four peaks with retention times of 2·8, 6·4, 11·2 and 13·6 mins. The major component had the retention time of 13·6 mins. and by GLC analysis showed a parent ion at m/e 200 with two major ions at m/e 169 (M-31) and m/e 143 (M-57).

Tetroxide-Periodate oxidations of canadensic acid (119).

(a) The canadensic acid epimers (21 mg.) were stirred in water (1 ml.) and dioxan (3 ml.) with osmium tetroxide (1 mole %) for 20 minutes. Sodium meta-periodate (210 mole %) was added over 30 minutes and the slurry stirred for a further 4 hours. The reaction mixture was filtered, the filtrate concentrated in vacuo and extracted with ethyl acetate. The organic layer was washed twice with brine, dried ($MgSO_4$), removal of solvent affording a solid having IR and R_f on TLC, using 10% methanol-chloroform as eluant, identical with the starting material.

(b) The canadensic acid epimers (21·2 mg.) in purified dioxan (10 ml.) (percolated through a grade 1 alumina column) were allowed to react with osmium tetroxide (26 mg.) for periods of 24–48 hours. The osmate ester was reduced in a stream of hydrogen sulphide (1·5 hours) and then nitrogen was bubbled through for a further 1·5 hours. The black solution was washed through celite using chloroform and the clear organic solution was concentrated in vacuo to afford a colourless solid (22 mg.) TLC analysis in 10% methanol-chloroform revealed the product to be a binary mixture, consisting of unreacted starting material (~30%) and a more polar component. Attempts to separate the two components by fractional crystallisation were unsuccessful as was preparative TLC since the more polar component could not be extracted from the silica. Storing the mixture with sodium metaperiodate for 48 hours followed by dilution with water and extraction with ethyl acetate afforded an oil which by TLC was shown to contain six components.

(c) An aqueous solution of sodium metaperiodate (10%) was added to a stirred solution of ruthenium dioxide (18 mg.) in acetone (5 ml.). The canadensic acid epimers (63 mg.) were added at intervals to this solution. Each time the reaction solution became

dark coloured, more periodate solution was added until a yellow colour developed and this equilibrium was maintained for three days. After this time isopropyl alcohol was added to destroy the excess reagent, and the mixture was filtered. Concentration of the filtrate *in vacuo* followed by dilution with brine and extraction with ethyl acetate yielded a complex mixture of products.

Attempted isomerisation of methyl canadensate.

The methyl canadensate epimers (7 mg.) were refluxed in dry benzene (3 ml.) with freshly distilled and dried triethylamine (3 ml.) for 5 hours. Removal of the solvent *in vacuo* afforded a solid which had identical IR, UV and TLC properties with these of the starting ester.

Attempted bromination of canadensic acid (119) using N-bromosuccinimide.

The canadensic acid epimers (42 mg.), and N-bromosuccinimide (84 mg.) were refluxed in dry carbon tetrachloride (8 ml.) for 2 hours. The cooled solution was filtered and concentrated *in vacuo*, and TLC analysis of the residue employing 10% methanol-chloroform as eluant

indicated that no reaction had occurred, confirmed by NMR analysis of the residue which crystallised from benzene-light petroleum mixtures as a colourless powder m.p. 127-132°c (softening at 80°c).

τau values ($CDCl_3$): 3·45 ($\frac{1}{2}$H, s), 3·5 ($\frac{1}{2}$H, s), 4·0 ($\frac{1}{2}$H, s), 4·05 ($\frac{1}{2}$H, s), 5·5 (1H, m), 6·4 (1H, m), 9·08 (3H, t).

Preliminary Studies on the Biosynthesis of Canadensic Acid.

Section 2.3

Once the structure of a natural product has been elucidated, a logical sequel is to examine its biosynthesis. Occasionally biosynthetic studies have been carried out on a structure only tentatively assigned and these studies have been important in gaining more knowledge about the structure as, for example, with the macrolide nystatin.

Usually the biogenesis of fungal metabolites has been proposed on the comparison of structures of related metabolites and on experiments with radioactive precursors such as acetate, malonate, mevalonic acid etc. Other biosynthetic studies directed towards gaining a knowledge of the individual biosynthetic steps by the use of mutants of a microorganism have been equally successful.[10]

In the present work this section will be concerned with preliminary studies into the biogenesis of canadensic acid and its epimer (119) using radioactive precursors. Inspection of the structure of the canadensic acid epimers (119) suggested that the molecule might be polyketide derived. Thus, the first experiments were incubations of the cultures with [2-^{14}C]-sodium acetate (50 μc), which was evenly spread as a sterile aqueous solution over 10 Roux bottles. The cultures were harvested after a further eight days and the canadensic

Table 15.

Substrate	Activity fed (μc)	Number of culture bottles	Activity isolated (μc)	% Incorporation
2-^{14}C sodium acetate	50	10	0·0834	0·134
2-^{14}C sodium acetate	50	50	0·0091	0·0182
1-^{14}C sodium acetate	45	5	0·18	0·4
2,3-^{14}C sodium acetate	40	10	0·012	0·03

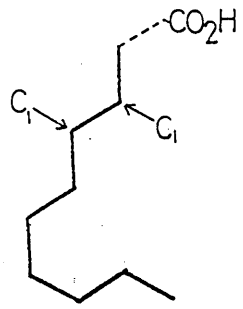
acid epimers were extracted with sodium bicarbonate. In this experiment it was found that the incorporation of radioactivity was 0·13% (Table 15). In a second series of experiments the cultures were incubated with [1^{14}C]-sodium acetate (45 μc) but in this case the radioactivity was spread over only five Roux bottles. Incorporation in this case was 0·4% (Table 15). Although the incorporations of radioactivity were not high they nevertheless appeared significant under the conditions of the experiments.

Time prevented an exhaustive series of experiments being carried out in order to determine the optimum time for the introduction of radioactive precursors. For these preliminary investigations samples of the broth of a growing culture of P. canadense were removed, worked up by extraction with ethyl acetate, and assayed for the canadensic acid epimers by TLC. The canadensic acid epimers were barely detectable after two days growth, but after three days growth there was a sharp increase in production and by the sixth day there were appreciable amounts of the acid epimers formed. Future experiments then, could involve the introduction of radioactive precursors on the second, third, fourth, etc., day of growth, and incubation of these cultures with the radio-

active substrates could be reduced to 48 hours. By these techniques it is possible that the incorporation of radioactivity would be increased.

However, the incorporation of radioactivity into the canadensic acid epimers was sufficiently good to make worthwhile an oxidative degradation of the molecule by ozonolysis, replicating the technique that had been used during the structure elucidation of the acid epimers. Thus it was possible to trap formaldehyde as its bis-dimedone derivative as well as isolate the residue. Ozonolysis of the canadensic acid epimers biosynthesised from [2-^{14}C]-sodium acetate resulted in the isolation of the formaldehyde dimedone derivative which contained 19·25% of the original radioactivity and the residue which contained 80·48%. Thus it could be stated that the $=CH_2$ group of the vinyl system was derived from the C-2 carbon of acetate. From this result it could be presumed unlikely that [1-^{14}C]-acetate would be incorporated in the aforementioned group. In fact ozonolysis of the canadensic acid epimers which had incorporated [1-^{14}C]-acetate afforded the dimedone derivative which was completely inactive.

It seems likely from the above evidence that the canadensic acid epimers are at least partly derived



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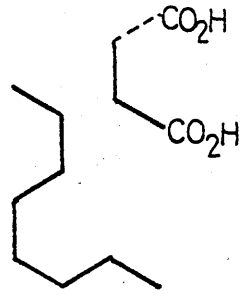

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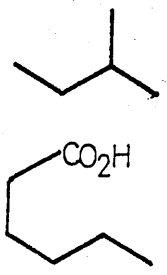

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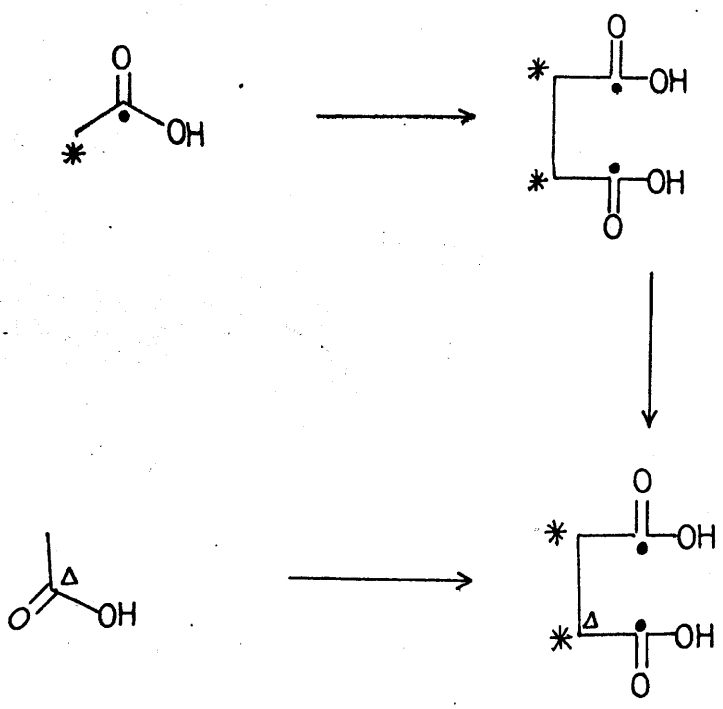
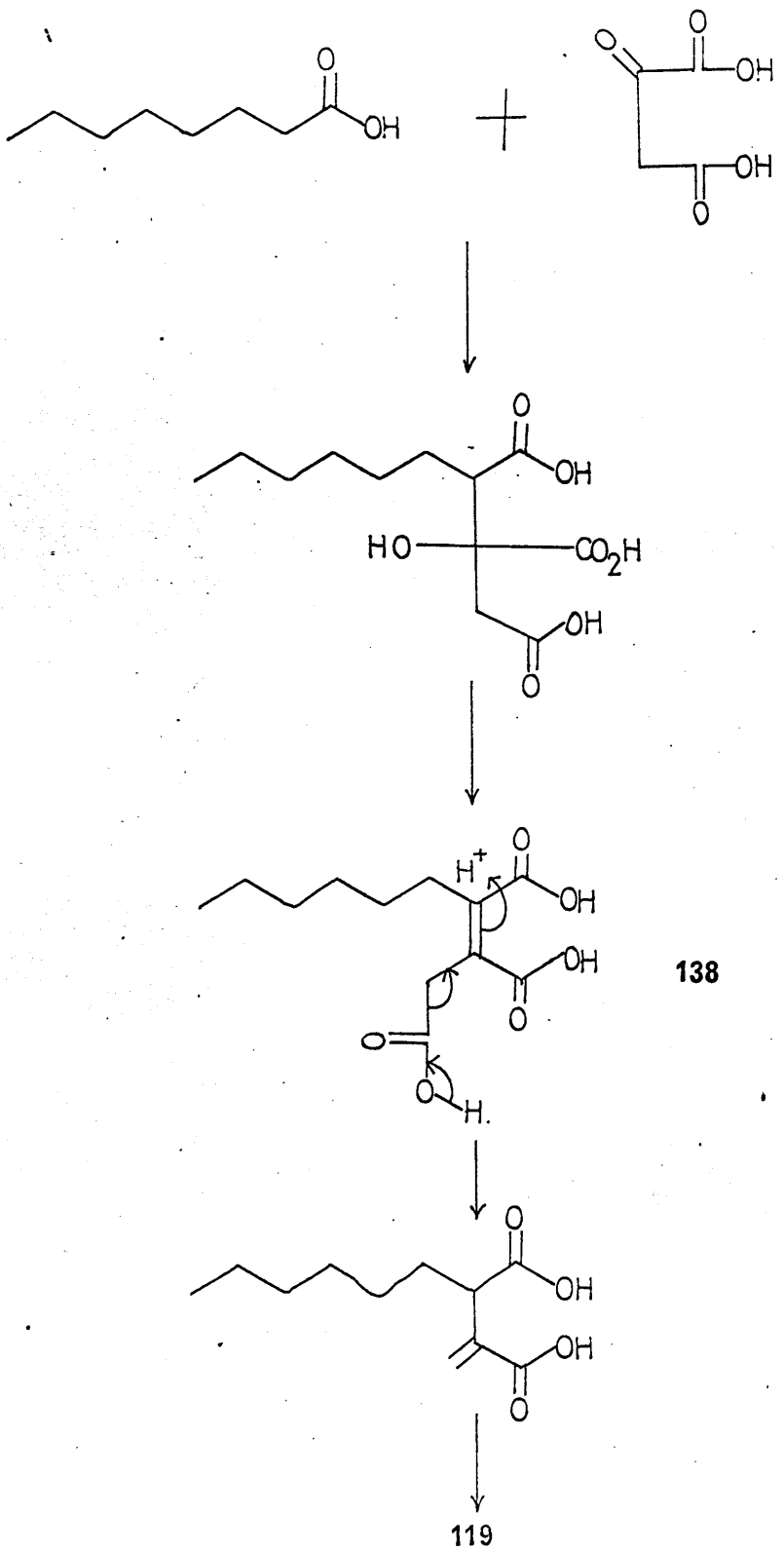
SCHEME 16



from a polyketide chain. The biosynthesis could involve (a) a C_{10} polyacetate chair + 2 C_1 units, (b) a C_8 polyacetate chain + a C_3 unit, or (c) a C_6 polyacetate chain + an isopentenyl unit. The terminal methylene group would be labelled in each case from [2-^{14}]-acetate incorporation in view of the mode in which [2-^{14}C]-acetate would label either mevalonic acid or any of the possible C_3 precursors succinate, oxaloacetate, or pyruvate[102] etc., (Scheme 16). Similarly [1-^{14}C]-acetate would not be expected to label the terminal methylene group if these routes were followed.

Structural comparison with two compounds favours biogenesis via (b). The antifungal antibiotic avenaciolide (34) bears a striking resemblance to canadensiolide and could be derived biogenetically by condensation of a C_{12} polyacetate chain with a C_3 unit. However, the carbon-carbon link is in the position β to the terminal carboxyl group of the polyacetate chain rather than α as suggested for the canadensic acid epimers. The C_9 anhydride which was found to be the biogenetic precursor of glauconic acid (an incorporation of 50% was reported) has in fact been shown to be derived by linking the α position of a polyacetate chain to a C_3 unit. It was suggested in this case that oxaloacetic acid was the

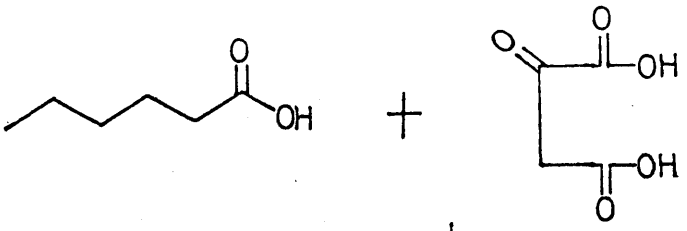
SCHEME 18

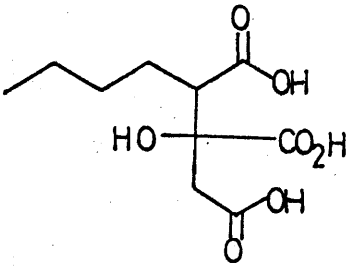


138

119

SCHEME 17





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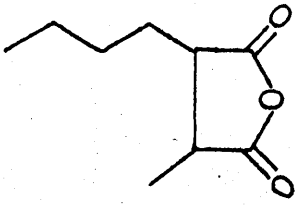

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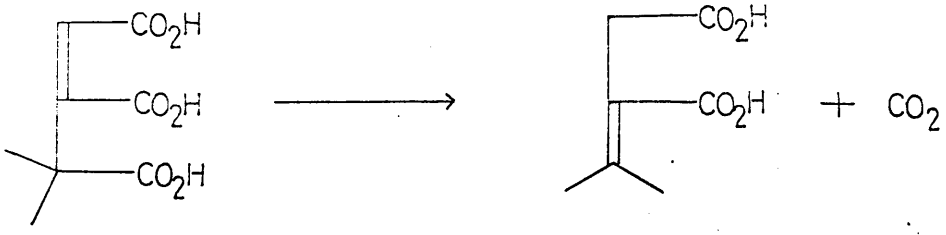
SCHEME 19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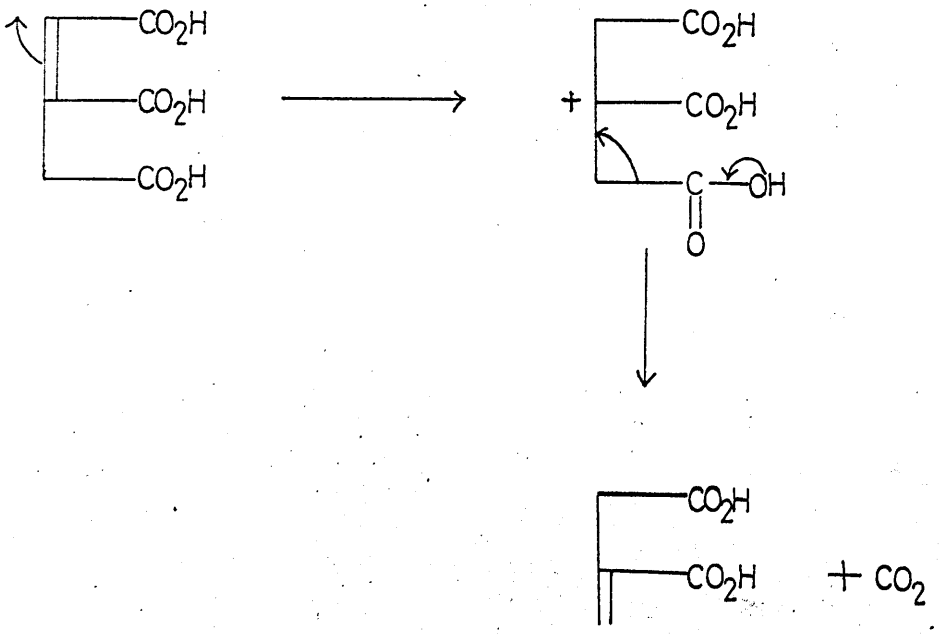
CO2H
+ CO2


CO2H
CO2H
CO2H
+
CO2H
CO2H
C
OH
O
CO2H
CO2H
+ CO2

actual precursor of the C_3 unit and that the polyketide chain condensed with this to give a citric acid like intermediate (136) which subsequently yielded the anhydride (137) (Scheme 17). A similar process could be involved in the biosynthesis of the canadensic acid epimers, the citric acid like intermediate in this case undergoing dehydration as in scheme 18 to give (138) (analogous to aconitic acid formation in the Krebs cycle). Decarboxylation to an itaconic acid derivative and subsequent cyclisation would then yield canadensic acid or a mixture of epimers.

The fact that canadensic acid is a mixture of epimers deserves special consideration. Reactions in biological systems such as hydrogenation, hydration etc., are usually specific, but in the suggested biogenesis (Scheme 18) epimerisation could occur during decarboxylation. This mechanism is based on that found to operate in the formation of various itaconic acids[104] (Scheme 19). It seems less likely that the centre in question could have epimerised in the culture medium (which is acidic) or during work up(which can be essentially under neutral conditions).

It is dangerous to predict the complete biogenesis of a metabolite on the minimum of evidence and the pro-

posals for the biogenesis of the canadensic acid epimers can only be regarded as a working basis for future experiments. It is anticipated that these experiments will include incubation with labelled pyruvate, and [2,3-^{14}C]-succinate in an effort to establish the biogenetic origin of the side chain, while other experiments will be directed at raising the incorporation of acetate. Another experiment might be the incubation of cultures with labelled canadensic acid epimers to establish whether the acid epimers are intermediates in the biogenetic pathway of canadensiolide (107) or whether they are by-products.

EXPERIMENTAL

Section 2.4

Growth and Extraction of Penicillium canadense.

Penicillium canadense with ^{14}C-labelled substrates was grown under identical conditions to those already described for normal cultures. Extraction procedures were also the same.

Radioactive Substrates and their Introduction into Cultures of P. canadense.

Radioactive materials used were 2-^{14}C sodium acetate of specific activity 464 μc/mg; and 1-^{14}C-sodium acetate of specific activity 485μc/mg. These radioactive precursors, in a sterilised medium, (0·5 hours steam at 242°F 12 p.s.i.) were spread evenly over 5-10 Roux bottles containing two-day old cultures of Penicillium canadense. These cultures were harvested after the normal 10 day growth period.

Isolation of ^{14}C -canadensic acid.

The dry broth extracts (2·5 g. obtained from cultures grown on ^{14}C-labelled substrates were taken up in the minimum volume of chloroform (250 ml.) and extracted with freshly prepared bicarbonate solution (2 x 125 ml.). The bicarbonate solution was cooled to 0°c and carefully acidified with ice-cold 6N hydrochloric

acid. The acidic aqueous solution was then extracted with chloroform (2 x 125 ml.) and the combined organic extracts were thoroughly washed with water (2 x 500 ml.) and dried ($MgSO_4$). Removal of the solvent in vacuo yielded a waxy solid which was recrystallised three times from benzene-light petroleum mixtures to afford canadensic acid as needles which were yellowish due to contamination with a trace amount of impurity. These needles were dissolved in ice-cold bicarbonate solution and canadensic acid was precipitated on acidification with cold 6N hydrochloric acid, separated by filtration and treated with animal charcoal prior to crystallisation. Canadensic acid was obtained as colourless needles (130 mg.) m.p. 128·5 - 130·5°c.

Assays for Radioactivity.

Radioactivity assays were carried out with a Packard Tri-Carb Liquid Scintillation Spectrometer (Series 3000). Efficiency for counting was ca. 79%. Samples were counted using a solution of 0·10 g. dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazalyl)-benzene) and 3·0 g. PPO (2,5-diphenyloxazole) in toluene (1 litre). Since most of the samples for counting were fairly polar a mixture of the above solution (9 ml.) with dry ethanol

(1 ml.) was used with a suitable quenching correction factor for the ethanol. The counting of radioactive samples was carried out for sufficient times to give a standard deviation of less than 2%.

The metabolites of *A. flaschentraegeri*

Section 3.1

Culture filtrates of the fungus Aspergillus flaschentraegeri show considerable inhibitory activity against Botrytis alii and it was decided to investigate the substance or substances responsible. The strain of Aspergillus used in the present investigation (obtained from C.M.I.) are reported to have been isolated in Egypt from the intestine of the larvae prodenia litura.

Fractions collected from chromatography of the dried acetone broth extracts were assayed for antifungal activity and the active fractions were bulked and separated using preparative TLC. The antibiotic traegerin was extracted from the silica with chloroform and crystallised from benzene-light petroleum mixtures as large colourless plates m.p. 160°c.

The antibiotic traegerin provided analytical data compatible with the empirical formula $C_{20}H_{26}O_5$ (MW 346), confirmed as the molecular formula by mass spectrometry (M^+ m/e 346).

In the NMR spectrum (fig. 5) the 1H quartet centred at 4·32 τ and the two 1H doublets at 5·01 and 5·14 τ formed a typical ABX spectrum. This suggested the presence of a vinyl grouping attached to a fully substituted carbon atom. The IR also showed absorption characteristic of a vinyl group at 1627, 986 and 906 $cm.^{-1}$.

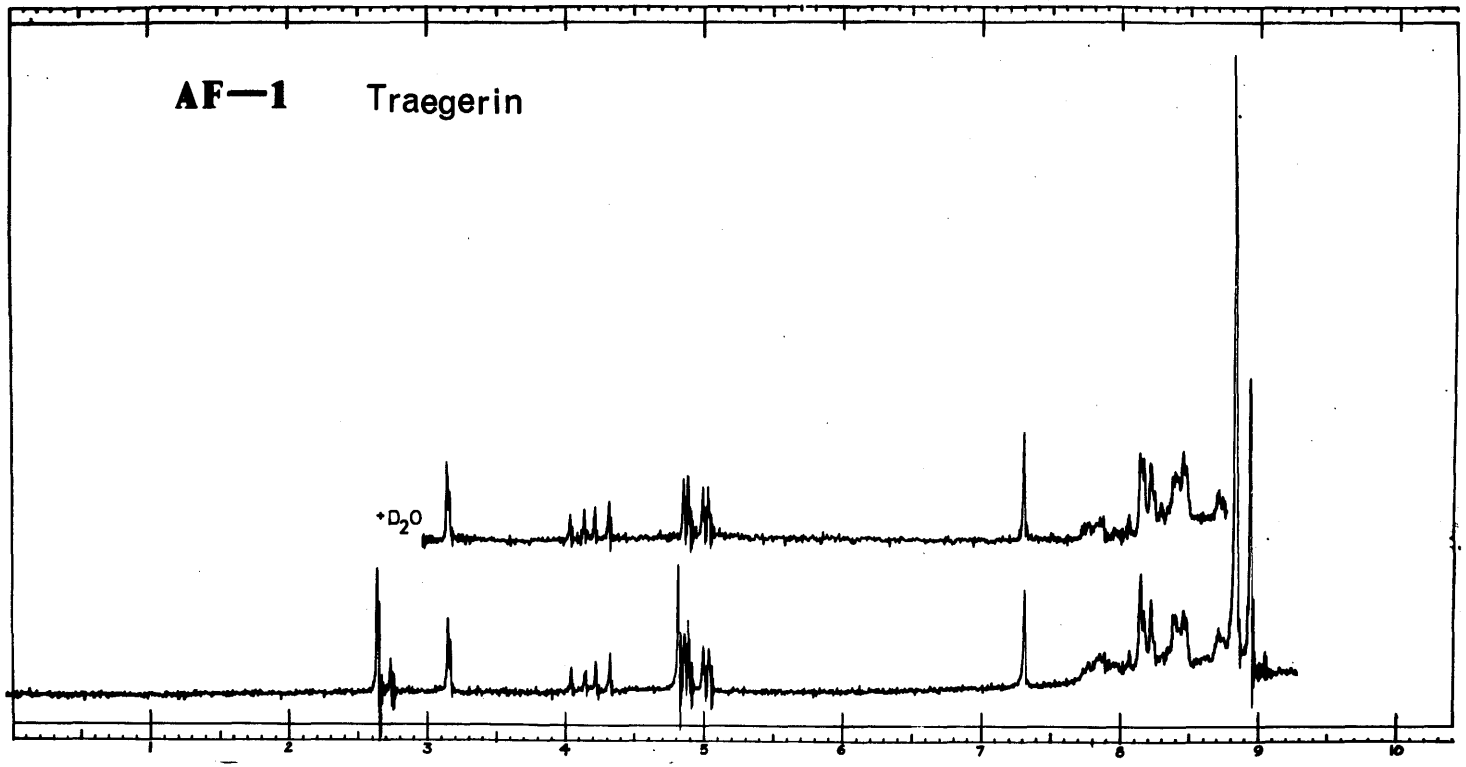
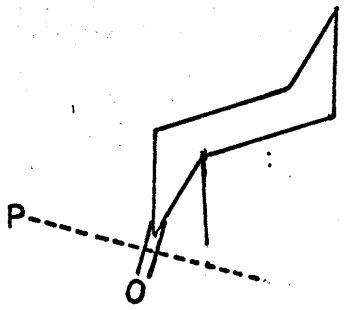


Fig. 5

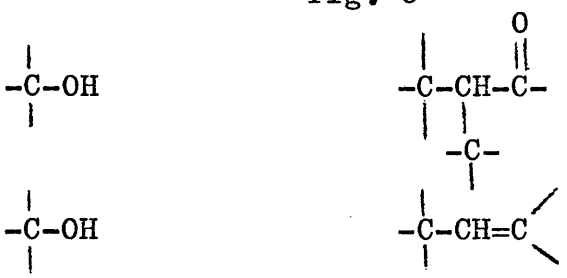
Vinyl groups occur in many diterpenes e.g., rosenonolactone (80) roselolactone (81), etc., as part of the system $CH_3-\overset{|}{\underset{|}{C}}-CH=CH_2$, and this system could be present in traegerin since it shows singlets in the NMR in the region of 9 τ (12 H). Two of these methyl groups appeared to be present as a gem-dimethyl grouping in view of IR absorption at 1390 and 1368 $cm.^{-1}$ typical of such systems.[105] A strong indication that the skeleton of traegerin was probably of terpenoid origin was shown by the incorporation of [2-^{14}C]-mevalonate into traegerin, and since traegerin is a C_{20} compound it may therefore be a diterpene.

From further inspection of the NMR spectrum the 1H singlet at 3·32 τ was ascribed to the resonance of an olefinic proton which by its height and breadth suggested a degree of allylic coupling. The sharp 1H singlet at 7·4 τ was interpreted to be the resonance of a proton α to a carbonyl group while the singlet at 4·83 τ and the broad singlet at 7·83 τ were ascribed to two hydroxyl protons since these signals disappeared on deuteration and since the IR showed absorption at 3458 and 3616$cm.^{-1}$. From the lack of further low field signals corresponding to the resonance of protons geminal to an oxygen atom it was concluded that the alcohols must be tertiary. Indeed from the lack of coupling in the aforementioned signals


P
O

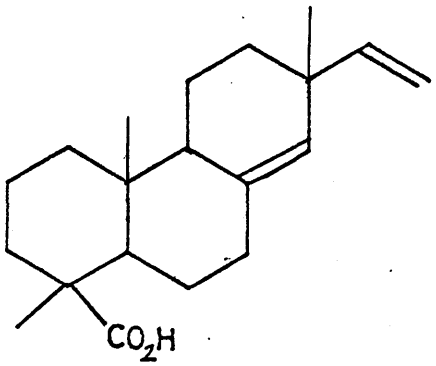
the following groupings (fig.6) can be written, which would, as drawn, require a maximum of 5 fully substituted carbon atoms, excluding those already attached to tertiary methyl groups.

"fig. 6"



Further evidence as to the nature of the methyl groups was obtained by examination of the NMR spectrum of traegerin in benzene. Many examples of benzene solvent shifts in the NMR spectra of ketones have been reported. An assessment of the mechanism of benzene-induced shifts has been made[106] in which it is suggested that benzene molecules form transient non-planar 1:1 collision complexes at each electron-deficient site in the solute molecule and that the orientation of the benzene molecules is goverened by a local dipole (rather than molecular dipole)-induced dipole interactions. Regardless of the actual mechanism, a useful empirical generalisation in the case of alicyclic carbonyl compounds is the so-called 'plane rule'[107,108]:

'If a reference plane (P) (139) is drawn through the carbon atom of the carbonyl group at right



(140)

angles to the C=O bond then protons close to (P) show very small shifts (in benzene); protons in front of (P), i.e., on the same side as the oxygen of the carbonyl, are deshielded while protons behind are shielded'.

Thus methyl groups can be shielded or deshielded depending on their position and in our case the information obtained from the NMR spectrum in benzene was extremely useful since two of the methyl groups (presumably part of the gem-dimethyl grouping) moved to lower field (8·55 and 8·67 τ) revealing the presence of a secondary methyl group at 8·91 τ (J ca.6c.p.s.). This cannot be accommodated by a 'normal' diterpene skeleton with a vinyl group geminal to a methyl group since all the methyl groups are tertiary e.g., pimarane group; pimaric acid (140), and the rosane group; rosenonolactone (80) .

Thus a proposed skeleton for traegerin must possess the following features:

a) enough methine positions to accommodate the features in fig. 6.

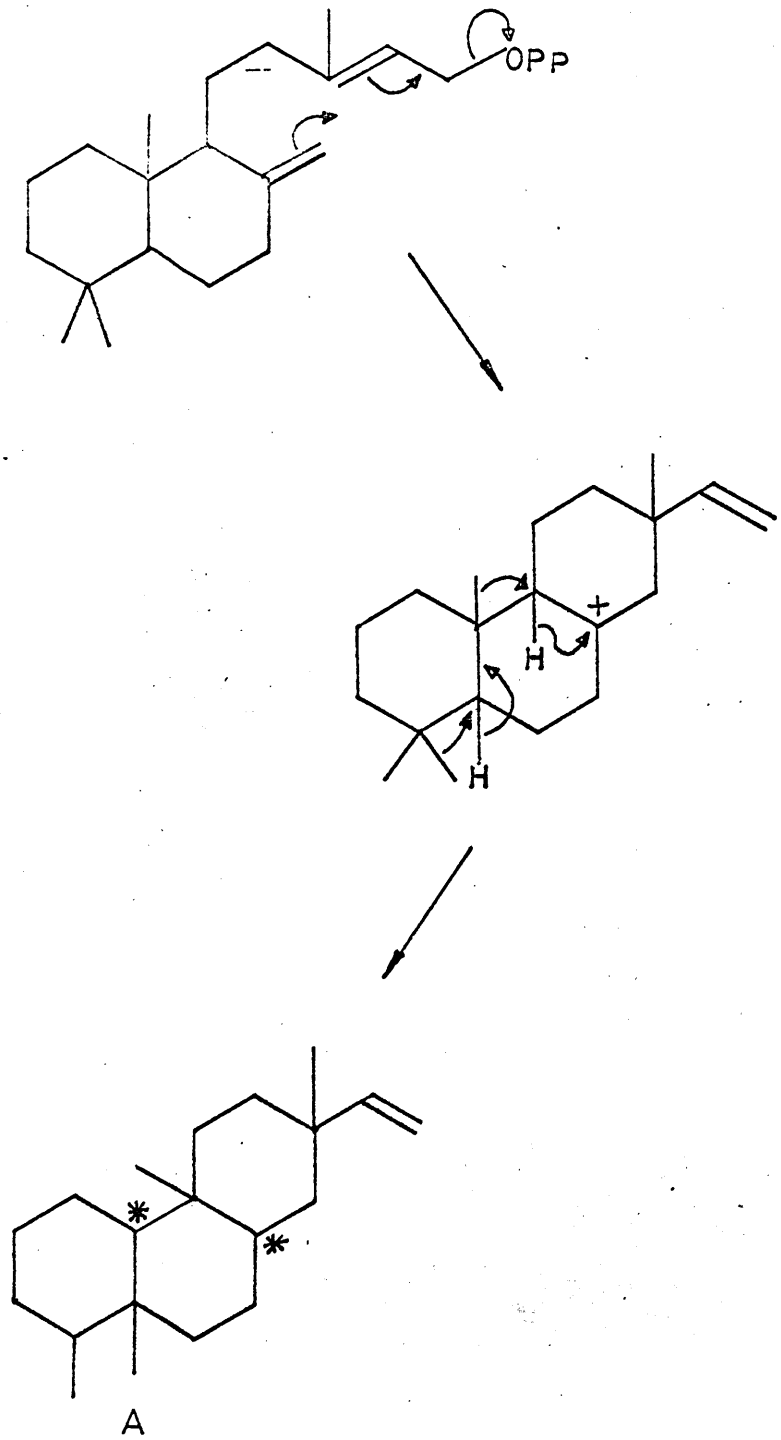
b) a secondary methyl group

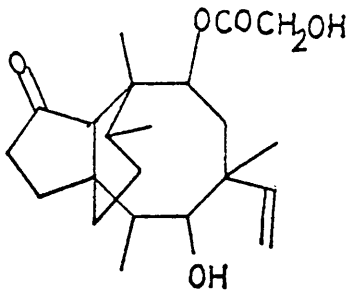
c) a gem-dimethyl grouping

d) (possibly) the grouping Me-C-CH=CH_2.

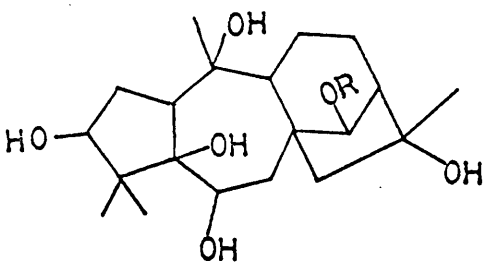
Three skeletons can be mentioned which have secondary methyl groupings. Skeleton (A) which could arise

SCHEME 20





141



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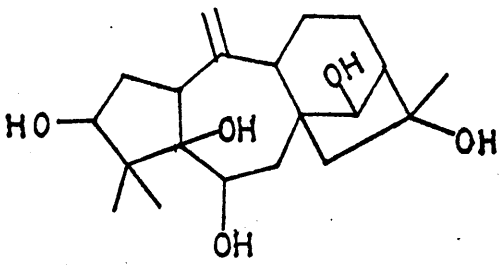
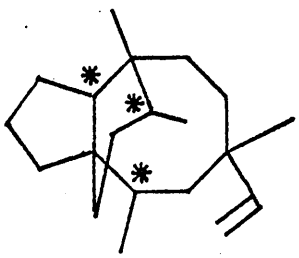
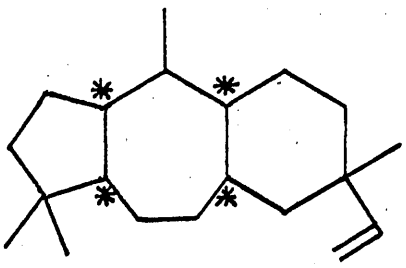


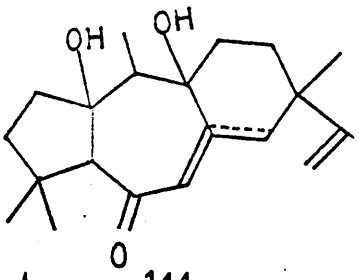
143



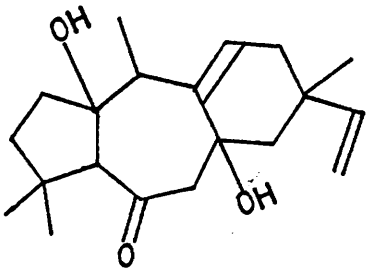
B



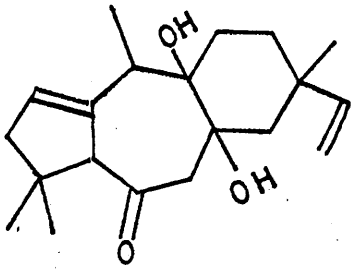
C



144



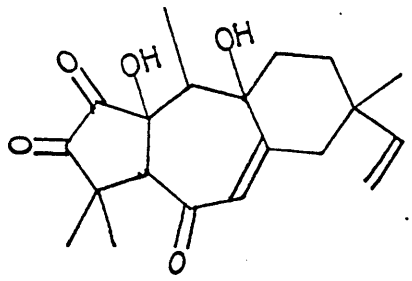
145



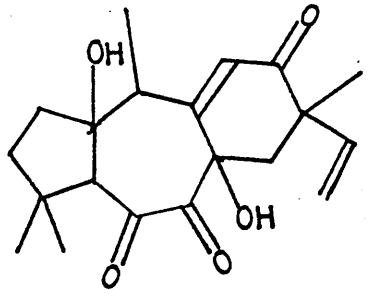
146

by two 1,2 methyl shifts and two 1,2 hydride shifts of the tricyclic carbonium ion (Scheme 20), skeleton (B) based on the structure of pleuromutulin (141) whose biosynthesis has been established,[109] and skeleton (C) based on a modification of the structure of the grayanotoxins GI (142, R = Ac), GII (143), and GIII (142, R = H) which possess a rearranged kaurene skeleton.[110]

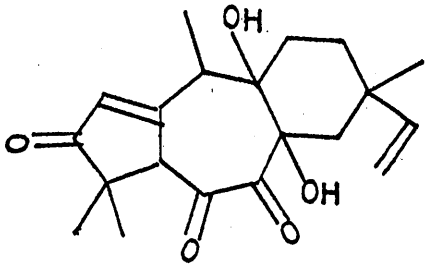
The first two skeletons can be discounted since although they possess a secondary methyl group, they do not incorporate a gem-dimethyl grouping and do not possess enough 'methine'positions to accommodate the features indicated in fig. 6 . Skeleton (C) however, would appear to be more satisfactory since apart from accommodating the methyl groups, etc., it has four methine positions (as shown starred). The most demanding of all the features in fig.6 is the methine α to a carbonyl and adjacent to two tertiary centres. This can only be accommodated by placing the carbonyl as in the partial structures (144), (145), or (146) with two tertiary hydroxyl groups arranged in any one of the three combinations shown. If the molecular formula $C_{20}H_{26}O_5$ is correct then we have 3 double bond equivalents and two oxygen atoms to account for. From the NMR data (1H, s, 3·3 τ) there is one trisubstituted double bond, which is conjugated as evidenced by the UV data (242 mμ, log ε 3·92). Thus the double bonds can only be positioned in the partial structures (144) (145)



147



148

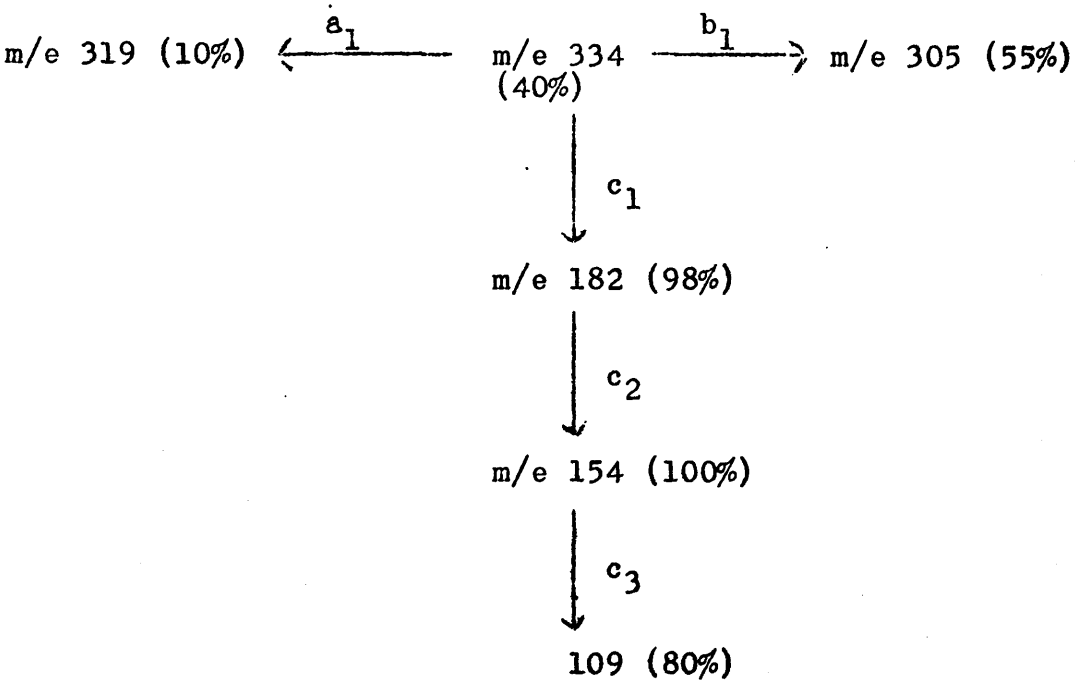


149

and (146) as shown. (An alternative position for the double bond is shown by a dotted line in partial structure (144) is excluded by later evidence.) Since two oxygen atoms and two double bond equivalents remain, and because of the complex nature of the carbonyl absorption it was reasonable to assume the presence of more than one carbonyl function. The two remaining oxygen atoms can be placed as carbonyl groups in the three ways indicated in formulae (147), (148) or (149) for traegerin, all of which accommodate the NMR data (one proton α to a carbonyl group etc.). It may be noted that a feature common to all three structures is the α diketone system and some evidence in favour of this system may be obtained by consideration of one of the products of hydrogenation. Hydrogenation of traegerin in ethanol using 5% palladium-on-charcoal resulted in an orange coloured solution. TLC indicated the presence of three relatively non-polar coloured components, a little unreacted starting material and a fifth component which gave a greenish blue colouration with methanolic ferric chloride, and was slightly less polar than the starting material. This ferric chloride active component was extracted and crystallised from carbon tetrachloride or benzene-light petroleum mixtures. A satisfactory elemental analysis could not be obtained for this hydrogenation product ('deoxy tetrahydrotraegerin') but the mass spectrum (Table 16) showed a parent ion at m/e 334,

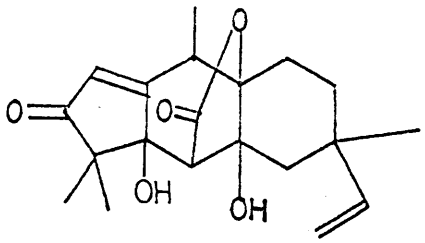
Table 16. Proposed mass spectral cracking pattern for Deoxytetrahydrotraegerin

Deoxytetrahydrotraegerin

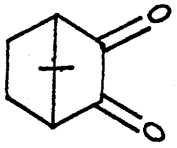


	Δm/e	m*
a_1	15	-
b_1	29	278·6
c_1	152	99·2
c_2	28	130·3
c_3	65	77·15

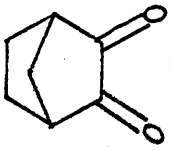
12 mass units lower than the parent ion of the antibiotic traegerin (M^+ 346), and can be accounted for by the reduction of two double bonds, one hydroxyl group being lost in the process by elimination or hydrogenolysis. Absorption in the IR spectrum at 1697 $cm.^{-1}$ (ε 496) and 1661 $cm.^{-1}$ (ε 430), coupled with the fact that the UV spectrum showed absorption at 280 mμ (log ε 3·82) ($\lambda_{max.}$ higher by 38 mμ compared with the starting material), and a reversible bathochromic shift of 50 mμ in alkali, suggested the presence of a diosphenol system. These data are in good agreement with those of other diosphenols that have been characterised.[111] The IR spectrum also showed a band at 1743 $cm.^{-1}$ characteristic of a 5-membered ring ketone. These data would suggest the presence of an α diketone system in traegerin which is either blocked or reluctant to enolise due to unfavourable steric interactions.[112] It may be noted at this point that in partial structure (144) the alternative position for the double bond (dotted line) would not allow the carbonyl groups to be placed such that there would only be 1 proton α to a carbonyl group.) However, the following chemical evidence suggests that (147) is the least likely of these three structures. Firstly, the antibiotic traegerin is slowly soluble in sodium bicarbonate solution (without



(150)



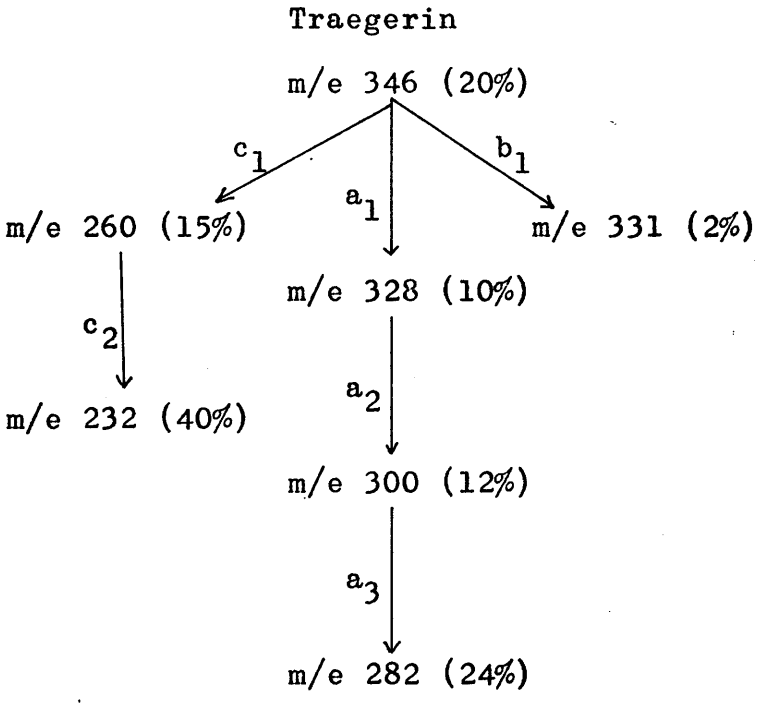
(151)



(152)

effervescence) resulting in a faint yellow solution. Reacidification affords a white solid which has identical IR and TLC characteristics to those of the starting material. Secondly, traegerin on a TLC plate slowly develops greenish blue colour (about 10-15 minutes) after spraying with methanolic ferric chloride seeming to indicate the presence of a slowly enolisable system (though, of course, the colouration may be due to oxidation). In structure (148) the αβ unsaturated six-membered alicyclic ketone would be expected to show absorption in the IR spectrum at ca. 1690 $cm.^{-1}$ rather lower than the absorption observed, 1714 $cm.^{-1}$ (chloroform solution) or 1725 $cm.^{-1}$ (KCl), these values being in better agreement with an αβ unsaturated five-membered ring ketone as in structure (149): (147) similarly would be expected to have absorption at a lower frequency than that observed. There are two features which are difficult to explain on the basis of any of these structures. Firstly, the low τ value of the olefinic proton and secondly, the fact that traegerin shows IR absorption at 1775 $cm.^{-1}$. Alternative structures like (150) containing a lactone ring were rejected since it is hard to see how a diosphenolic type chromophore could be developed on hydrogenation. It is not impossible that this high frequency absorption

Table 17. Proposed mass spectral cracking pattern for Traegerin



	Δm/e	m*
a_1	18	311·0
a_2	28	274·4
a_3	18	269·0
b_1	15	-
c_1	86	-
c_a	28	207

is due to the α-dicarbonyl system in structure(149), interaction between the two dipoles when in the cisoid conformation being known to give rise to similar high wavelength absorption. The bicyclic systems (151) and (152), for example, show twin absorption at 1776; 1760 $cm.^{-1}$ and 1771, 1760 $cm.^{-1}$ respectively.[113] Thus some uncertainty remains about these structures though of the three proposed structures,(149) is consistent with the most data. Analysis of the mass spectrum of traegerin (Table 17) apart from recording the metastabled losses of angular methyl, water, and carbonyl groups did not seem to favour any particular structure.

Similarly, the NMR spectrum of deoxytetrahydrotraegerin did not provide much assistance although reduction of the vinyl group was reflected in the absence of olefinic protons, and the presence of a 3H triplet at 9·05 τ (J ca. 6·5 c.p.s.). The spectrum also showed a 3H singlet at 9·17 τ, while the secondary methyl signal now appeared at slightly higher field, 9·07 τ (J ca. 6 c.p.s.). However, the rest of the spectrum was complex possibly due to the presence of α diketone and diosphenolic forms making further interpretations difficult.

The inherent reactivity of this molecule has hampered the chemical investigation which has been carried out on it so far. Thus many of its reactions were found to yield complex mixtures, many of the components being highly coloured. The hydrogenation of traegerin apart from affording the diosphenol deoxytetrahydrotraegerin also gave three highly coloured components which could not be satisfactorily separated by preparative TLC. The largest of these components, (AF-2), was eluted as a brown gum, which decomposed fairly rapidly at room temperature. The mass spectrum showed two parent ions at m/e 302 and m/e 300, so although appearing homogeneous on TLC it seemed to be a mixture. The IR spectrum showed absorption at 1660 and 1610 $cm.^{-1}$, indicating a highly conjugated carbonyl system. The UV spectrum was complex, showing high wavelength absorption at 320 mμ (OD 0·3), 334 mμ (inflexion)(OD 0·25) with the weaker absorption at 436 mμ (OD 0·1), and gave an irreversible shift in base to 320 mμ. The NMR spectrum of this mixture was remarkable in that there were no signals corresponding to olefinic protons, indicating that the conjugation may extend through tetrasubstituted double bonds. The 6H triplet at 9·15 τ was interpreted as a 3H singlet superimposed on a 3H triplet (J ca. 6 c.p.s.) corresponding to the ethyl group

of the reduced vinyl system. Little more information could be gained from this intimate unstable mixture.

In an attempt to methylate the latent diosphenol system suspected to be present in traegerin, the antibiotic was treated with methyl iodide and potassium carbonate. The product was an inseparable mixture of three coloured components all less polar than the starting material, and the two slightly less polar of these components gave a bluish green colouration with ferric chloride. The IR spectrum of the mixture showed absorption at 3,480 $cm.^{-1}$ (intramolecular hydrogen bonded hydroxyl), 1720 $cm.^{-1}$ (possibly a conjugated 5-membered ring ketone), 1690 and 1635 $cm.^{-1}$ (probably due to the diosphenol methyl ether system), and the UV spectrum showed maxima at 292 (inflexion) and 257 mμ. There was no shift in base. The NMR spectrum was very badly resolved but showed singlets at 6·25 and 6·35 τ corresponding to the resonance of two methoxyls which integrate (assuming the singlet at 9·1 τ is 3H), for 1·5 protons, indicating equal proportions of two O-methyl derivatives. Repeated attempts to separate this mixture failed, but from the evidence discussed (e.g., the unchanged nature of the UV spectrum in base) it would appear that the O-methyl derivatives were formed, 'freezing' the diosphenol system.

Another attempt to confirm the presence of an α-dicarbonyl system was made by treating the antibiotic traegerin with o-phenylenediamine. The resulting oil which failed to crystallise was shown by TLC to be a complex mixture of products.

It was hoped that treatment of traegerin with zinc and acetic acid would reduce the α ketol function and perhaps give another diosphenol system. However, attempts using two different methods resulted in a yellow coloured product of at least seven components in each case.

Another line of investigation was directed at dehydration of the molecule to give the characteristic tropone or tropolone nucleus which would then establish the presence of a seven-membered ring. To this end traegerin was treated with thionyl chloride and pyridine which on work up yielded a deep red coloured oil consisting of four products that could not be separated.

A small sample of the antibiotic was refluxed for 20 minutes in benzene with a crystal of iodine. The resulting brown oil which assumed a dark red colour in ethereal solution was shown by TLC to consist of one major non-polar coloured component and three minor more polar components. Unfortunately time prevented any further investigation of this reaction.

Preliminary attempts were also made to prepare a heavy atom derivative of traegerin that would be suitable for X-ray analysis. However care had to be taken as to the choice of reagent owing to the extreme sensitivity of the molecule. Treatment of traegerin with bromoacetyl bromide at room temperature afforded an inseparable quaternary mixture, one of the components being identified by TLC as unreacted starting material, while treatment with brosylhydrazine resulted in a yellow oil consisting of seven components. The antibiotic was also treated with N-bromosuccinimide in an effort to prepare the bromohydrin derivative of the vinyl group. The resulting green oil, consisting of four components, was subjected to preparative TLC but the separation was extremely poor and only one component could be extracted with any success. This yielded a small quantity of an oil which failed to crystallise and was not characterised.

It was unfortunate that time did not allow complete structural elucidation of this rather labile antifungal metabolite traegerin. Further attempts to prepare a derivative suitable for X-rays would seem to be called for.

EXPERIMENTAL

Section 3.2

Table 18. Typical assay carried out on growing cultures of A. flaschentraegeri

Fungus	No. of days			
	5	7	10	12
A. flaschentraegeri	2S11	3S11	5S10	5S11

Growth and Extraction of the Mould.

Aspergillus flaschentraegeri (Commonwealth Mycological Institute No. 101,651) was subcultured on 2% malt agar slants and seed grown on the same medium in Roux bottles (15 x 9 cm.). A spore suspension prepared from 12 of these bottles and distilled water (2 l.) was used to innoculate 100 Roux bottles, which had previously been sterilised, and contained 200 ml. of the same medium as used for cultures of Penicillium canadense (p. 123). The growth conditions were also similar to those for P. canadense and the cultures were harvested after 10 days. The extraction procedures were the same as used for P. canadense.

Activity Assays

Using the same techniques as described for P. canadense the optimum growth period with regard to antibiotic activity appeared to be 10 days. The result of a typical assay performed during the growth period for one group of cultures is shown in Table 18 . Again assays performed on the extracts indicated that the activity was still present in the broth extract etc.

Chromatographic Fractionation of the Metabolites of Aspergillus Flaschentraegeri.

Crude dry broth extract (4·5 g.) in methanol was adsorbed on silica (15 g.) air dried, finely powdered and introduced onto a column of silica (250 g., 70 x 3·5 cm.) Separation was achieved by elution with benzene containing 0, 10, 20, 30 etc., % chloroform using 2 column volumes of each mixture successively. Fractions (50% column volume) were collected from 100% benzene, and aliquots of each fraction were tested for antifungal activity, and fractions 37 - 44 (70% and 80% chloroform in benzene) were found to exhibit antifungal behaviour. These fractions were bulked (0·5 g.) and separated by preparative TLC on two Kieselghur HF_{254} plates (500 x 20 x 0·7 mm) employing 2% methanol-chloroform as eluant. The antibiotic traegerin (65 mg.) (R_f 0·3) was extracted from the silica by elution with chloroform.

Traegerin.

Traegerin was isolated as previously described and crystallised from benzene-light petroleum mixtures as colourless plates m.p. 160°c.

(Found C, 69·55%; H, 7·28%; M^+ 346. $C_{20}H_{26}O_5$ requires C, 69·34%; H, 7·28%; MW 346.)

$\nu_{max.}$(KBr): 3430, 3320 (broad), 1770 (inflexion), 1745, 1725, 1627, 1390, 1368, 986, 906 $cm.^{-1}$

$\nu_{max.}$(1·15 mM $CHCl_3$): 3616 $cm.^{-1}$, 3458 $cm.^{-1}$, 1775 $cm.^{-1}$ (ε 467), 1714 $cm.^{-1}$ (ε 398), 1623 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 242 mμ, (log ε 3·92).

$\lambda_{max.}$(C_2H_5OH-OH^-): 323 mμ (log ε 3·91), 430 mμ (log ε 3·44).

τau values ($CDCl_3$): 3·32 (1H, s), 4·32 (1H, q)

$J_{AX} + J_{BX}$ = 27 c.p.s.; 4·83 (1H, s, OH); 5·01 (1H, d), 5·14 (1H, d), $J_{AB} \sim 0$ c.p.s.; 7·40 (1H, s), 7·88 (2H, m), 8·9 (9H, s), 9·0 (3H, s).

Catalytic reduction of Traegerin.

Traegerin (100 mg.) in ethanol (11 ml.) was shaken with 5% palladium-on-charcoal in an atmosphere of hydrogen for 8 hours. The resulting orange solution was filtered twice (glass paper), removal of solvent in vacuo affording an orange coloured residue (98 mg.). TLC of the residue in 1% methanol or 2% methanol in chloroform showed this to be a mixture of five components, (R_f 0·93, 0·9, 0·8, 0·32, 0·25). Preparative TLC on one Kieselghur HF_{254} plate (20 x 20 x 0·7 mm.) in 1% methanol-charcoal afforded three of these components (R_f 0·8, 0·32, 0·25) these bands being extracted from the silica by elution with chloroform.

1) The component of R_f 0·8.

This was obtained as a brown coloured solid (20 mg.) which slowly decomposed although stored at 0°c under nitrogen.

$\nu_{max.}$(Nujol): 3500, 1720, 1660, 1620 cm.$^{-1}$

$\nu_{max.}$($CHCl_3$): 1730 w, (broad) 1660, 1610 cm.$^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 207 mμ, 320 mμ, 334 mμ (inflexion) 436 mμ(O.D. values 1·5, 0·3, 0·25, 0·1 respectively).

$\lambda_{max.}$(C_2H_5OH-OH^-): 215 mμ, 240 mμ (inflexion), 321 mμ.

τau values ($CDCl_3$): 7·6 (4H, m), 7·85 (2H, bs), 8·74 (6H, s), 9·15 (3H, s, and 3H, t, J ca. 6 c.p.s.).

The component of R_f 0·32 $FeCl_3$ active (Deoxytetrahydrotraegerin).

This was obtained as a colourless solid (30 mg. 31%) which crystallised from benzene-light petroleum mixtures as a colourless powder m.p. 160°c, + ve ferric chloride reaction.

(Found: M^+ 334, $C_{20}H_{30}O_4$ requires MW 334).

$\nu_{max.}$(KBr): 3425, 3200-2500, 1727, 1692, 1660, 1240 cm.$^{-1}$.

$\nu_{max.}$(1·7 mM $CHCl_3$): 3634 cm.$^{-1}$, 3480 cm.$^{-1}$ (broad), 1743 (ε 170), 1697 (ε 495), 1661 (ε 430).

$\lambda_{max.}$(C_2H_5OH): 280 mμ (log ε 3·82).

$\lambda_{max.}$(C_2H_5OH-OH^-): 330 mμ (log ε 3·99).

τau values ($CDCl_3$): Me resonances at 8·75, 9·07, 9·17 τ (singlets): 9·05 τ (tr.); complex resonances 7·3-7·9 τ.

Component 5 (R_f 0·25).

This was obtained as a colourless solid (21 mg.) which had identical IR, UV and TLC with traegerin.

Attempted methylation of Traegerin.

Traegerin (16 mg.) was gently refluxed in a slurry of potassium carbonate (24 mg.) and acetone (4 ml.). Methyl iodide (50 mg.) was added to the refluxing solution and heating continued for 5 hours. The reaction mixture was filtered (glass paper) and concentrated *in vacuo* to yield an oil (16 mg.). TLC employing chloroform as eluant showed the presence of three components, (R_f 0·68, 0·6, 0·5) all less polar than the starting material (R_f 0·1). The mixture could not be separated by preparative TLC.

$\nu_{max.}$(thin film): 3480, 1720, 1690, 1635 $cm.^{-1}$.

$\lambda_{max.}$(C_2H_5OH): 257 mμ, 292 mμ (inflexion).

τau values ($CDCl_3$): 6·25, 6·35 (ca. 1·5 H).

Methyl contour defined by peaks at 8·6 s, 8·73 s, 9·1 s τ.

Treatment of Traegerin with zinc and acetic acid.

(a) Traegerin (10 mg.) was dissolved in glacial acetic acid (2 ml.) and powdered zinc (50 mg.) was added. The reaction mixture was stirred and heated at 80°c for three hours. Thereafter the zinc acetate was separated on glass paper and the filtrate concentrated in vacuo. The acetic acid was removed by azeotropic distillation with benzene. TLC (in 1% methanol-chloroform) of the yellowish residue revealed the presence of at least 7 products.

(b) Traegerin (9 mg.) was dissolved in glacial acetic acid (2 ml.) with powdered zinc (40 mg.), and two drops of concentrated hydrochloric acid was added. The reaction mixture was stirred at room temperature with three additions of concentrated hydrochloric acid (1 drop) at 0·5 hour intervals. After a further 3·5 hours the reaction mixture was diluted with water and almost neutralised with solid sodium bicarbonate. This aqueous solution was extracted with chloroform and the extract washed with brine until free of acid. Evaporation gave a yellow oil which was shown by TLC (1% methanol-chloroform) to be a mixture of 7 or 8 components. This was not investigated further.

Treatment of Traegerin with thionyl chloride and pyridine.

Traegerin (22 mg.) in cold dry pyridine (1 ml.) was added to an ice-cold mixture of freshly distilled thionyl chloride (0·25 ml.) and dry pyridine (0·25 ml.), and the mixture left at 0^{o}c overnight. The reaction mixture was poured on ice-water and extracted with chloroform. The pink organic solution was washed with 2N hydrochloric acid, bicarbonate solution, brine solution and dried ($MgSO_4$). Removal of the solvent in vacuo afforded a red oil. TLC analysis in chloroform or 1% methanol in chloroform indicated the oil was a mixture of four products which could not be separated.

Reaction of Traegerin with iodine.

Traegerin (3·5 mg.) was refluxed in dry benzene (3 ml.) with a crystal of iodine for 20 mintes. Ether was added to the solution and the organic layer was washed with thiosulphite solution, water, dried ($MgSO_4$) removal of the solvent in vacuo affording a brown oil. TLC analysis employing chloroform elution showed the oil to consist of one major component (R_f 0·78) and three minor ones (R_f 0·65, 0·5, 0·16). Time prevented the repetition of this reaction on a larger scale.

Attempts to prepare a heavy atom derivative of Traegerin.

a) Using bromoacetylbromide.

Traegerin (40 mg.) in dry benzene (5 ml.) and pyridine (6 drops)was stirred with the acyl bromide (0·5ml.) at room temperature for 20 hours. Ice water was added and the solution extracted with ether. The ethereal layer was washed with 2N hydrochloric acid, bicarbonate solution, brine and dried ($MgSO_4$). The residue obtained by removal of solvent and which failed to crystallise was shown by TLC analysis in 4% methanol-chloroform to be a mixture of unreacted starting material and one more polar (R_f 0·1) and two less polar (R_f 0·86, 0·5) components none of which were cleanly separated.

b) Using brosylhydrazine.

Traegerin (15 mg.) was dissolved in the minimum volume of ethanol and brosylhydrazine (25 mg. 2·3 mole) was added. The solution was gently warmed for a few minutes and then allowed to stand at room temperature for 1·5 days. The solution was diluted with water and extracted with chloroform. The organic solution was washed with 2N hydrochloric acid, water and dried ($MgSO_4$). TLC of the resulting yellow oil showed it to be a mixture of seven components.

c) Using N-bromosuccinimide in an aqueous medium.

Traegerin (32 mg.) in a 50% aqueous solution of dioxan (24 ml.) was treated with N-bromosuccinimide (32 mg.) and the mixture stirred for 20 hours. The solution was concentrated in vacuo, the residue diluted with water and extracted with chloroform. The dried ($MgSO_4$) organic extract afforded a green oil which was resistant to crystallisation. TLC analysis employing 10% methanol-chloroform elution showed four products, two less polar and two more polar than the starting material Preparative TLC was unsuccessful in efficiently separating any of these components though the largest of the two less polar components was extracted (8 mg.) with relative success but failed to crystallise and was not characterised.

Radiotracer experiment with A. Flaschentraegeri.

[2-^{14}C]-mevalonate (40 μc) was fed to day-old cultures of A. flaschentraegeri (50 Roux bottles) which were harvested after the normal period of growth. These fractions were combined and traegerin separated by the same chromatographic procedures as used before. Radioactive assays showed that traegerin had incorporated [2-^{14}C]-mevalonate to the extent of ca. 0·01%.

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