THE ANTIBACTERIAL COMPOUND

ASPERGILLIC ACID

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A T H E S I S

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by

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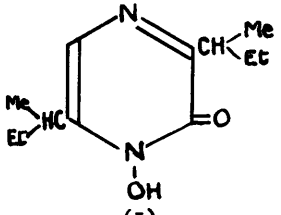
C O N T E N T S

THE ANTIBACTERIAL COMPOUND ASPERGILLIC ACID.

SUMMARY.

Aspergillic acid the antibacterial compound isolated by White in 1940 from culture filtrates of Aspergillus flavus was investigated by Dutcher and Wintersteiner who formulated it as (I) and its primary reduction product deoxyaspergillic acid as (II).



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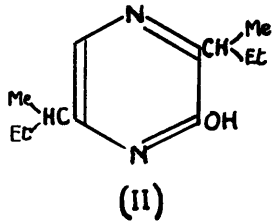
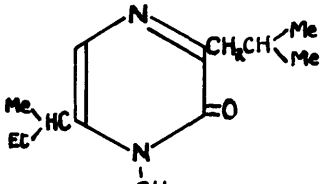


(II)

Deoxyaspergillic acid has been totally racemised by treatment with alkali at elevated temperature and the racemate is different from racemic 3-hydroxy-2:5-di-sec-butylpyrazine (II). By oxidation of aspergillic acid or deoxyaspergillic acid acetone is produced. Bromination of aspergillic acid followed by zinc and acetic acid reduction gives a 2:5-diketopiperazine. Hydrolysis of this diketopiperazine by hydrobromic acid gives a mixture of α-amino-acids which has been resolved and the components identified as leucine and isoleucine (and alloisoleucine). These reactions do not accord with the structure of aspergillic acid proposed by Dutcher and Wintersteiner and it is now

formulated as (III) or (IV). Growth of A. flavus on



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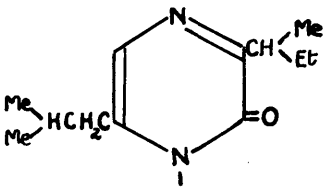


(IV)

a casein hydrolysate medium produces in addition to aspergillic acid another metabolite which was named flavacol, $C_{12}H_{20}ON_2$. It was identified by synthesis as 3-hydroxy-2:5-di-isobutylpyrazine. Kojic acid has been produced by growth of A. flavus on a casein hydrolysate medium to which brown sugar was added.

Synthetic approaches to compounds related to aspergillic acid and deoxyaspergillic acid have been examined.

INTRODUCTION

The phenomenon of microbial antagonism was recognised by Pasteur early in the development of micro-biological science. That this antagonism could be manifest in the production by one micro-organism of a substance inhibitory to another was recognised. The specific antagonistic action of Pseudomonas aeruginosa upon various pathogenic bacteria was found to be due to production of an active heat-resistant substance. By enrichment methods there was obtained from culture filtrates of this organism a preparation designated pyocyanase (1) which was responsible for the activity. Subsequently it was shown that fungi as well as bacteria are able to elaborate antibacterial agents, penicillic acid, active against both Gram positive and Gram negative organisms being isolated from culture filtrates of Penicillium puberulum (2).

In 1929 Fleming observed that growth of the mould Penicillium notatum on liquid media produced a substance which he named penicillin, which was active against Gram positive organisms (3). The isolation of the labile active principle by Chain and Florey and co-workers and the demonstration of its remarkable chemotherapeutic properties (4) stimulated interest in this field of

investigation. The outcome of the resulting intensive programme of research has been the recent discovery of a large number of antibacterial compounds, a few of which have been of clinical value.

The term antibiotic has been introduced by Waksman (5) to describe such antibacterial products and the term has been defined by Oxford (6). This last definition requires that the substance should show antibacterial activity at the arbitrary dilution of 1:20,000 against the selected test organism in vitro, that is in general, that the unenriched culture filtrate will have demonstrable antibacterial activity. The use of the term antibiotic is most generally limited to describing active metabolic products of bacteria, fungi and actinomycetes.

The production of antibiotic substances is not a fundamental metabolic process of the organism but is largely determined by the nutritional supply. It has, further, been pointed out that antibiotic production is not necessarily a manifestation of microbial antagonism as an organism may produce a substance which is active against other organisms with which it does not normally compete for existence (6). Besides nutritional factors environmental factors also influence formation of antibiotics and a rationale for investigation has been

evolved (7). Antibiotic production is not associated with strain specificity, different strains and even different genera producing the same substance, a fact which has resulted in a number of identical products bearing different names. Thus, for example, anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid has been variously named, patulin (8), clavacin (9), clavatin (10), claviformin (11), and expansin (12). On the other hand, under different conditions of growth the one species may produce more than one antibiotic.

By their mode of action antibiotic substances may be divided into two groups, those which are bacteriostatic in action and those which are bactericidal. The former although not destroying the organism, prevent, by interfering with some fundamental metabolic process, the proliferation of the organism. The latter are protoplasmic poisons and are destructive to animal as well as bacterial tissue. For an antibiotic to be useful, therefore, in the treatment of systemic infection its antibacterial action must be bacteriostatic (13).

Investigation of antibiotics necessitates methods of assaying the potency of culture filtrates and concentrates of culture filtrates. The most frequently used methods are based on the inhibition of growth and multiplication of the test organism. As the methods

are arbitrary, uniformity of results between different methods can only be obtained by correlation using standards. In the Agar Streak method (14) different dilutions of the active substance are mixed with a standard volume of nutrient agar medium and the solidified agar streaked with a number of test organisms. The dilution at which growth is inhibited under standard conditions gives a measure of the potency against the test organism. This method is generally used for investigation of antibiotic spectrum. In the Serial Dilution method (15) different volumes of the test medium are made up to a standard volume with different concentrations of the active substance. These solutions are inoculated with the test organism and incubated for a standard time. The highest dilution of the antibiotic giving complete inhibition of growth, as expressed by lack of turbidity of the medium, is taken as the end point. The Diffusion Cup method (16) consists of adding various dilutions of the active substance to the cup reservoir embedded in nutrient agar previously inoculated with the test organism. The active substance diffuses into the agar and sets up a zone of inhibition the diameter of which is a measure of the potency of the substance against the test organism.

Suggested classifications on the basis of chemical

structure have been made (6, 17), however, antibiotics whose structure has subsequently been elucidated do not necessarily fall into any of these general classes. Increasing knowledge of the structure of antibiotics merely shows the diversity in chemical nature of these substances.

Among the moulds the genus Aspergillus has frequently been reported as producing antibiotic substances. Aspergillic acid with which this investigation is concerned is produced by a member of this genus, A.flavus.

ASPERGILLIC ACID.

Isolation.

In an examination of culture filtrates of different species of the mould Aspergillus grown on liquid media it was observed by White (18) in 1940 that the antibacterial activity was very variable. A particular species which was tentatively identified as A. flavus, grown in suitable nutrient liquid media gave a filtrate which was highly active against both Gram positive and Gram negative bacteria. Later White and Hill (19, 20) established the species as A. flavus and examined its growth characteristics. It grew well on a variety of media, but heavy growth occurred on some

media without development of antibacterial activity. The most suitable medium consisted of tryptone or peptone and sodium chloride, the addition of glucose causing more rapid growth and giving higher antibacterial titres but increasing the difficulty of isolation. The filtrates were tested against a range of organisms, the titres being somewhat variable as was also the yield of active material. This inconsistency of results was due to variation of the mould, some of the variants being completely inactive. The active material because of its acidity was named aspergillic acid. The isolation procedure consisted of treating the culture filtrate at pH4 with absorbent charcoal. The air-dried charcoal was exhaustively extracted with ether, removal of the ether leaving a gum. Extraction of this gum with warm aqueous sodium hydrogen carbonate, followed by acidification of the extract gave crystalline aspergillic acid. The melting point of different samples fell within the range 84-96° although one sample had m.p.116°. Aspergillic acid showed amphoteric properties and on analysis and molecular weight determination was ascribed the formula $C_{12}H_{20}O_2N_2$. The yield of aspergillic acid averaged 5-6mg./litre of culture filtrate although it was obtained as high as 60mg./litre. Jones, Rake and

Hamre (21) showed that the strain of A. flavus employed by White and Hill consisted of a mixture of variants and isolated from it two highly active substrains. With these strains growth on a medium of 2% tryptone and 0·5% sodium chloride produced aspergillic acid in yields of 120-250mg./litre and with 2-4% added brown sugar the yield was as high as 400mg./litre of culture filtrate. These workers developed a rapid assay method for activity of culture filtrates based on the inhibition of the bioluminescence of Photobacterium fischerii. This assay, which could be carried out in thirty minutes, showed that maximal activity was reached in about seven days and in about half that time when mycelial mats from previous batches were reflooded with fresh medium. Menzel, Wintersteiner and Rake (22) have also isolated aspergillic acid from culture filtrates of a strain of A. flavus grown on tryptone medium. Pure aspergillic acid according to these workers has m.p.93° and is optically active ($[\alpha]_D$ +14°) and can be distilled in steam or in vacuo without loss of biological activity. These workers showed further that on a culture medium of tryptone-brown sugar a related product $C_{12}H_{20}O_3N_2$ of m.p.149°, $[\alpha]_D$ +42° was produced. This last product had only one tenth of the antibacterial activity of aspergillic acid. Menzel

and co-workers expressed the opinion that the active culture filtrate obtained by Glister (23) from growth of an unspecified species of Aspergillus owed its activity to the presence of aspergillic acid. Bush et.al.(24) by growth of A. flavus, Link strain, on peptone-lactose medium obtained culture filtrates active against Mycobacterium tuberculosis in vitro. The active material was separated by fractional crystallisation into two components, one of m.p. 96·5-97·5° and the other m.p.118-121°. Both components had molecular weight 230 but the high melting one had only 75% of the activity of the other. The lower melting component was identified as aspergillic acid. The highest yield of aspergillic acid which has been recorded is on a medium of 2% yeast extract and 1% glycerol. On this medium surface culture of A. flavus gave aspergillic acid, 800mg./litre of culture filtrate (25).

Moulds of the genus Aspergillus have been recorded as producing a number of other antibiotic substances. The production of penicillin - like antibacterials has been frequently reported (26, 27, 28, 29, 30). Waksman and co-workers (27) examining a number of strains of A. flavus observed that the activity of the culture filtrates resulted from the presence of two antibiotics,

aspergillic acid and flavicin. Production of the former was favoured by surface culture and the latter by submerged culture with aeration. Clavatin isolated from culture filtrates of A. clavatus (10) has been shown to be identical with patulin (10) produced by Penicillium patulum (8) as is clavacin also produced by A. clavatus (31, 32). Helvolic acid active against Gram positive organisms has been isolated from cultures of A. fumigatus mutant Helvola Yuill (33) and is identical with fumigacin also isolated from cultures of A. fumigatus (34, 35). Gliotoxin has also been isolated from A. fumigatus (35, 36) and aspergillin isolated from cultures of an unspecified species of Aspergillus (37) has been shown to be identical with gliotoxin (38). The pigment fumigatin is also produced by A. fumigatus (39).

Biological Properties of Aspergillic Acid.

Aspergillic acid acts as a powerful bactericide. At dilutions of 1:25,000 it was bactericidal in twelve hours to five of the six organisms tested, and at 1:50,000 dilution destroyed S. aureus in two hours (20). At much lower dilution it manifest bacteriostatic action, at a dilution of 1:400,000 having marked inhibitory effect on S. aureus (20). Aspergillic acid shows anti-tubercular activity in vitro (40, 24) and it is suggested

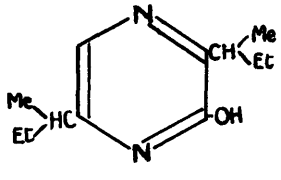
(40) that the mechanism is the formation of a ferric complex, thus depriving the organism of available iron, this mechanism being supported by the observation that the presence of ferric ions interferes with the activity of aspergillic acid. It has been shown (41) that bismuth in a concentration which in itself causes no growth inhibition decreases considerably the quantity of aspergillic acid necessary for inhibiting completely the growth of S. aureus. In vitro activity is shown by aspergillic acid against the anaerobic gas gangrene organisms (42) and anti-spirochaetal activity is shown against the organism Treponema pallidum (43).

The use of aspergillic acid as a chemotherapeutic agent in treatment of systemic infection is limited by its relatively high toxicity to animals (20). In mice, repeated intraperitoneal injection of one third of the lethal dose did not give protection against infection with haemolytic Streptococci or Pneumococci although some protection against gonococcal infection was observed (20). Aspergillic acid therapy has been applied without success to a virus infection of mice, encephalomyelitis (44).

Structure of Aspergillic Acid.

Initial investigation of the structure of aspergillic acid was made by Dutcher and Wintersteiner. The results of this investigation are summarised in a paper published by these authors in collaboration (45). Later a more detailed account of the chemistry of aspergillic acid was reported by Dutcher (46, 47). Aspergillic acid $C_{12}H_{20}O_2N_2$ is a weak monobasic acid (pKa 5·3) forming copper and silver salts and giving a crystalline salt with phenylhydrazine. The substance also shows weakly basic properties as was demonstrated by the formation of a hydrochloride in anhydrous medium and the formation of a crystalline salt with 3:5-dinitrobenzoic acid. Aspergillic acid gives an intense red colour on treatment of a methanolic solution with ferric chloride, and, on heating with copper chromite gives, with the loss of an atom of oxygen, deoxyaspergillic acid $C_{12}H_{20}ON_2$, an optically active neutral compound m.p. 102°. These last two reactions in conjunction with the formation of a green copper salt suggested the presence of the hydroxamic acid grouping $-\underset{\underset{OH}{|}}{N}-\overset{\overset{O}{\|}}{C}-$. Reduction of aspergillic acid with hydriodic acid or hydrazine also resulted in formation of deoxyaspergillic acid. The stability of aspergillic acid to acid and alkaline hydrolysis

indicated that the hydroxamic acid grouping could not be of simple aliphatic nature, since such groupings are readily hydrolysed. It was assumed, therefore, that the grouping formed part of a heterocyclic system. On the evidence of the similarity in ultra-violet light absorption spectra between deoxyaspergillic acid and hydroxypyrazine, the former was postulated as an alkyl-substituted hydroxypyrazine, the alkyl fragment having the asymmetry responsible for optical activity. Considering biogenetic possibilities in conjunction with the optical activity the hypothesis was adopted that the alkyl fragment consisted of two sec.-butyl groups symmetrically disposed in the pyrazine ring. Deoxy-aspergillic acid was therefore formulated as d-3-hydroxy-2:5-di-sec.-butylpyrazine (V) and aspergillic acid as d-1-hydroxy-2-keto-3:6-di-sec.-butyl-1:2-dihydropyrazine (VI). These formulations appeared to



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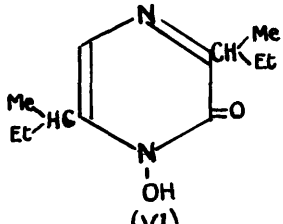
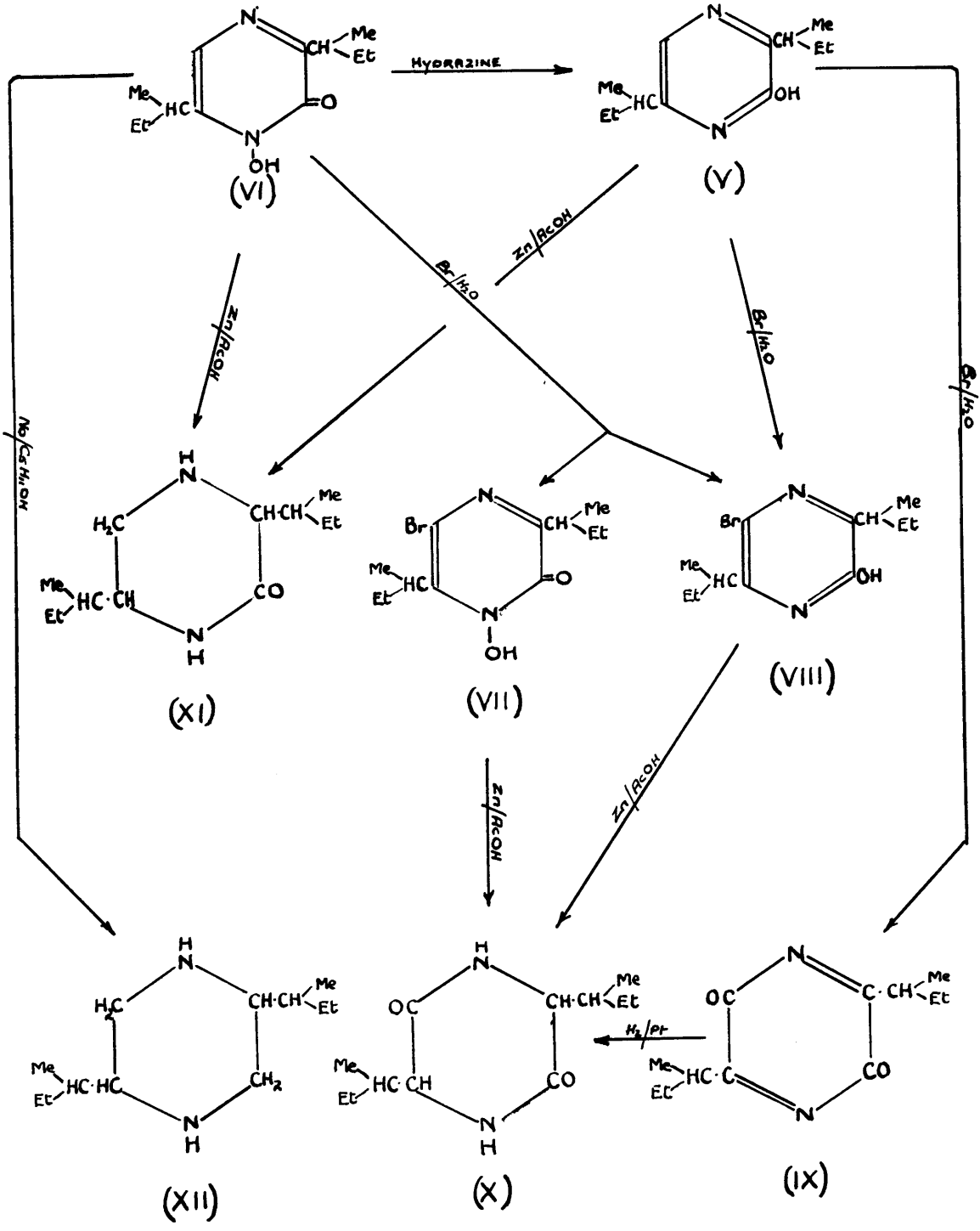


(VI)

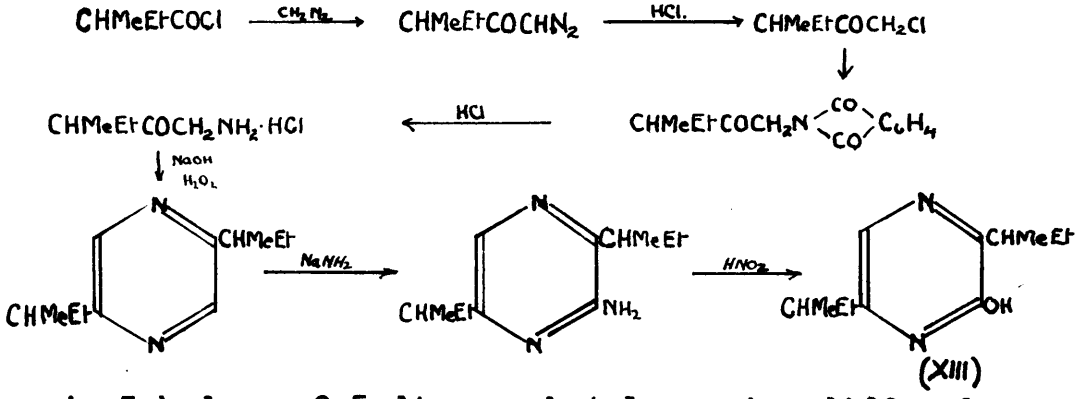
satisfy other properties of aspergillic acid and deoxyaspergillic acid. Bromination of aspergillic acid in aqueous solvents gave bromoaspergillic acid

$C_{12}H_{19}O_2N_2Br$ (VII) and bromodeoxyaspergillic acid $C_{12}H_{19}ON_2Br$ (VIII). The latter was also produced by direct bromination of deoxyaspergillic acid. That nuclear bromination in the unsubstituted position had taken place was demonstrated by the stability of bromoaspergillic acid and bromodeoxyaspergillic acid to alkaline hydrolysis. The direct bromination of deoxyaspergillic acid gave, in addition to bromodeoxyaspergillic acid, a quinone-like compound, a 2:5-diketo-2:5-dihydropyrazine $C_{12}H_{18}O_2N_2$(IX). Treatment of bromoaspergillic acid or bromodeoxyaspergillic acid with zinc in acetic acid solution gave in both cases the same compound $C_{12}H_{22}O_2N_2$(X) which had the properties of a 2:5-diketopiperazine and resembled DL-<u>iso</u>leucine anhydride. The same compound was also obtained by reduction of the diketodihydropyrazine (IX). Aspergillic acid and deoxyaspergillic acid were both reduced by zinc and acetic acid to the same compound, tetrahydrodeoxyaspergillic acid $C_{12}H_{24}ON_2$(XI) which was also produced by catalytic hydrogenation of deoxyaspergillic acid. Total reduction of aspergillic acid was brought about by sodium and amyl alcohol giving a piperazine base $C_{12}H_{26}N_2$(XII) which resembled the piperazine base obtained by sodium and amyl alcohol reduction of DL-<u>iso</u>leucine anhydride. These reactions are summarised schematically on p.14 .

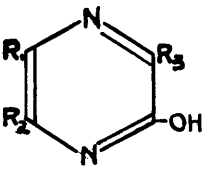

HYDRAZINE
Zn/AcOH
Br/H2O
Na/C5H11OH
(VI)
(V)
(XI)
(VII)
(VIII)
(XII)
(X)
(IX)
H2/Pt

Synthetic Compounds related to Aspergillic Acid and Deoxyaspergillic Acid.

Newbold and Spring (48) synthesised racemic 3-hydroxy-2:5-di-sec.-butylpyrazine (XIII) as a preliminary step in the synthesis of aspergillic acid assuming the formulation of aspergillic acid by Dutcher & Wintersteiner (45). The route followed was

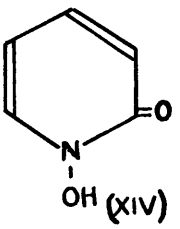


Racemic 3-hydroxy-2:5-di-sec.-butylpyrazine differed from deoxyaspergillic acid. Newbold and Spring examined the ultra-violet absorption spectra of 3-hydroxy-2:5-di-sec.-butylpyrazine, a number of other hydroxypyrazines, and deoxyaspergillic acid as supplied by Dr. O.Wintersteiner. The results they obtained are tabulated. Dutcher and Wintersteiner (45) in their examination of the ultra-violet light absorption of deoxyaspergillic acid observed only the band at 3250Å. Later, however, Dutcher (46) reported two-band absorption of similar location and intensity to that recorded by Newbold and Spring.



R_1	R_2	R_3	λm_1 Å	ε_1	λm_2 Å	ε_2
H	H	H	-	-	3160	4200
H	Me	Me	2270	7600	3230	3600
Me	Me	Me	2295	7900	3360	7400
H	CHMeEt	CHMeEt	2285	9600	3220	10,000
Deoxyaspergillic Acid			2295	6700	3250	8000

Towards the end of the investigation carried out by the author, Lott and Shaw (49,50) published the results of examination of analogs of aspergillic acid as antibacterial agents. These workers prepared pyridine cyclic hydroxamic acid (XIV), a number of nuclear substituted pyridine cyclic hydroxamic acids, quinoline cyclic hydroxamic acid (XV) and a cyclic pyrimidine hydroxamic acid, N-hydroxy-4:6-dimethyl-2-pyrimidone (XVI).



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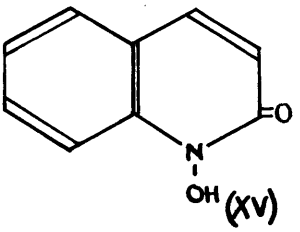

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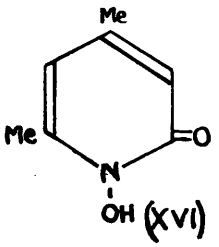
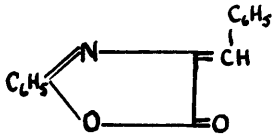


(XVI)

These synthetic cyclic hydroxamic acids were tested against Staphylococcus aureus, Klebsiella pneumoniae and Mycobacterium smegmatis and in general the activity was of the same order as aspergillic acid. Quinoline cyclic hydroxamic acid showed the greatest activity and the

pyrimidine cyclic hydroxamic acid showed the lowest antibacterial activity. Shaw and McDowell (51) have also prepared N-hydroxy-2-phenyl-4-benzylidene-5-imidazole (XIX) a five-membered ring analog of aspergillic acid. Treatment of 2-phenyl-4-benzylidene-5-oxazolone (XVII) with anhydrous methanolic hydroxylamine gave α-benzamidocinnamohydroxamic acid (XVIII) which on heating with dilute hydrochloric acid cyclised to the hydroxamic acid (XIX).



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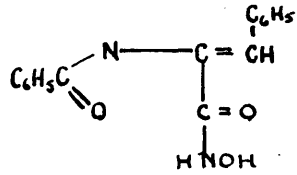

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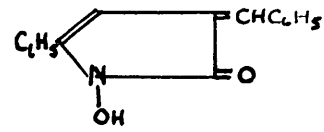


(XIX)

THEORETICAL.

This investigation was carried out as two main parts; firstly, degradative study with the object of reinvestigating the detailed structure of aspergillic acid, and secondly, model experiments on synthesis of compounds related to aspergillic acid and its primary degradation product deoxyaspergillic acid. Section I deals with investigations carried out on the natural product and Section II consists of model experiments, carried out in the light of knowledge gained by degradation, having as their ultimate aim a synthesis of aspergillic acid and deoxyaspergillic acid.

SECTION I.

In the historical introduction the evidence which lead Dutcher and Wintersteiner (45) to formulate aspergillic acid as 1-hydroxy-2-keto-3:6-di-sec.-butyl-1:2-dihydropyrazine and deoxyaspergillic acid as 3-hydroxy-2:5-di-sec.-butylpyrazine is summarised. The subsequent synthesis of this last compound by Newbold and Spring (48, p. 15.) did, in fact, confirm a very marked similarity between it and deoxyaspergillic acid although they differed in melting point. This difference could be considered to result from differences in optical properties between the optically active deoxy-

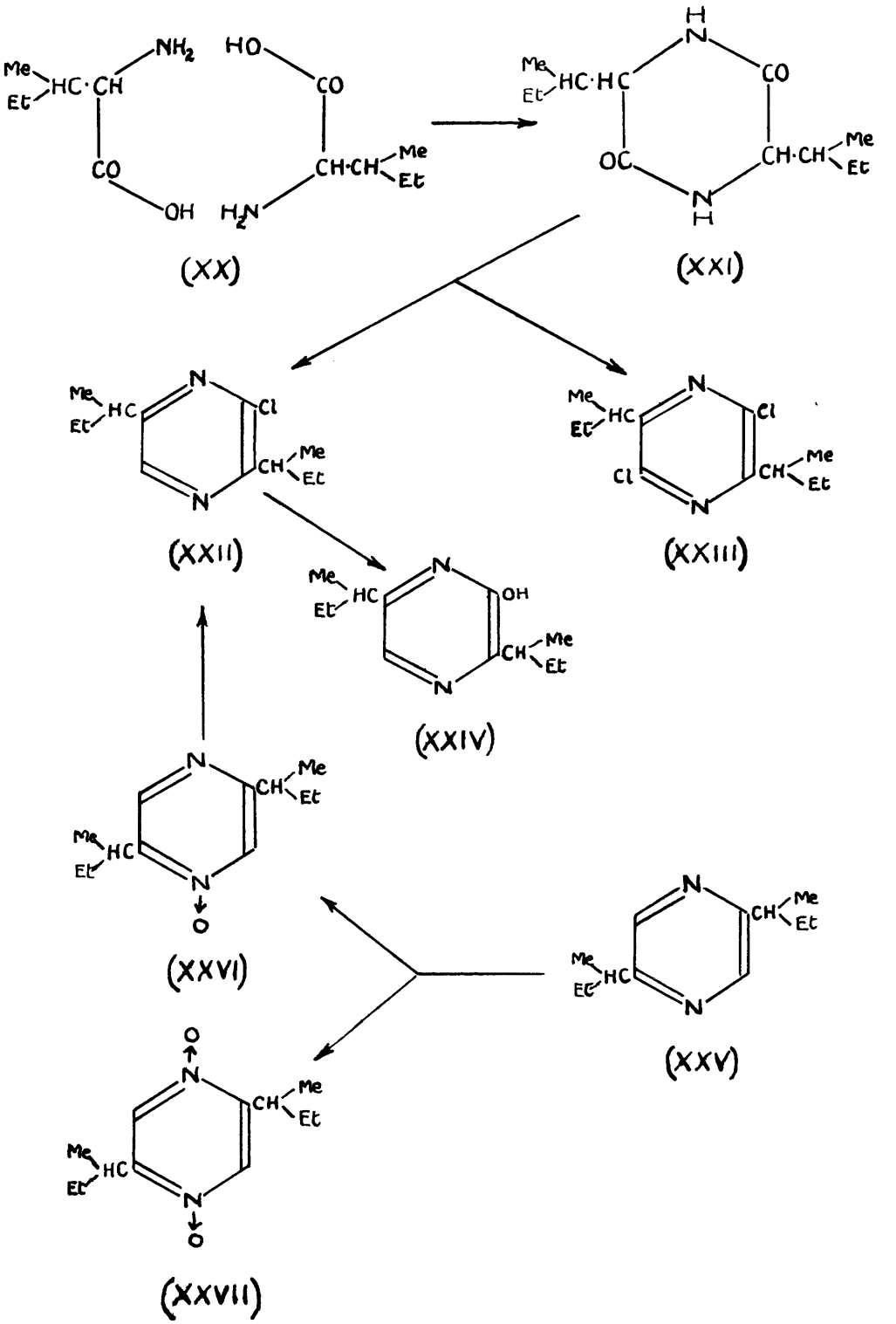
aspergillic acid and the racemic synthetic product. Newbold (52), however, drew attention to discrepancies in the ultra-violet absorption spectra of these two compounds, pointing out that, although the positions of the absorption maxima for deoxyaspergillic acid and 3-hydroxy-2:5-di-sec.-butylpyrazine are identical, there is a considerable difference in the values of the respective molecular extinction coefficients. This difference in ultra-violet absorption spectra indicated that the non-identity of deoxyaspergillic acid with 3-hydroxy-2:5-di-sec.-butylpyrazine was more fundamental than stereochemical, and stimulated this reinvestigation.

That the compound obtained by Newbold and Spring (48, p. 15) was in fact 3-hydroxy-2:5-di-sec.-butylpyrazine was more rigorously confirmed by alternative syntheses by Baxter and Spring (53) and Newbold and Spring (54). In the synthesis of Baxter and Spring DL-isoleucine anhydride (XXI), prepared by refluxing DL-isoleucine (XX) with ethylene glycol, was treated with phosphoryl chloride. This reaction gave a mixture of 3-chloro-2:5-di-sec.-butylpyrazine (XXII) and 3:6-dichloro-2:5-di-sec.-butylpyrazine (XXIII), separable by treatment with concentrated hydrochloric acid in which the former is soluble. Under intensive hydrolytic

conditions, with solid potassium hydroxide, 3-chloro-2:5-di-sec.-butylpyrazine was converted to 3-hydroxy-2:5-di-sec.-butylpyrazine (XXIV). The alternative synthesis by Newbold and Spring (54) started from 2:5-di-sec.-butylpyrazine (XXV). Treatment of this base, in acetic acid solution, with hydrogen peroxide gave a mixture of the corresponding pyrazine-mono and di-N-oxides (XXVI and XXVII). The mono-N-oxide reacted with phosphoryl chloride to give 3-chloro-2:5-di-sec.-butylpyrazine (XXII) which on alkaline hydrolysis gave 3-hydroxy-2:5-di-sec.-butylpyrazine (XXIV). These reactions are summarised schematically on p.21 . These dissimilar syntheses indicated unequivocally the nature of the synthetic product and showed that a re-examination of the evidence for the structure of aspergillic acid was desirable.

Isolation of Natural Products.

Aspergillic acid has been prepared by a modification of the method of White and Hill (20) by growth of Aspergillus flavus on a casein hydrolysate-sodium chloride medium. The strain of A. flavus employed was the active strain isolated by Jones, Rake and Hamre (21) from White's original mixed culture. As tryptone, an enzymic casein hydrolysate used by White and Hill (20)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(XXVI)
(XXVII)

was not readily available in quantity, another commercial enzymic digest of casein, "Pronutrin", was used. The cultural characteristics of A. flavus on a "Pronutrin" containing medium were the same as on tryptone medium and aspergillic acid was produced equally well on either. Aspergillic acid is not, however, produced when an acidic digest of casein is substituted for tryptone (55). The yield of aspergillic acid, under the conditions employed, 250-300mg./litre of culture filtrate was rather lower than that obtained by Jones, Rake and Hamre (21) who used a similar medium containing added brown sugar. In attempting to increase the yield to the value, 350-400mg./litre, reported by these workers, brown sugar was added to the basal medium. On this medium, however, the product could not be readily purified and after repeated crystallisation specimens were obtained melting as high as 137°. White and Hill (20) having observed similar variation in the melting point suggested that this was due to the existence of polymorphic forms of aspergillic acid. Accordingly the entity of high melting point was at first considered to be such a variant. Analysis, however, showed that the product was richer in oxygen than is required by the formula $C_{12}H_{20}O_2N_2$. Menzel, Wintersteiner and Rake (22) have shown that A. flavus on a simple

tryptone-sodium chloride medium produces aspergillic acid, whereas, on the same medium to which brown sugar was added they obtained hydroxyaspergillic acid $C_{12}H_{20}O_3N_2$ m.p. 149°. It is considered, therefore, that this high melting product, originally thought to be a polymorphic variant, is in fact a mixture of aspergillic acid and hydroxyaspergillic acid, although a satisfactory method for the isolation of this latter compound has not been evolved. After this preliminary work had been completed Dutcher (46) in an extensive report on aspergillic acid expressed the opinion that the high melting entities observed by White and Hill (20) and Bush et.al.(24) were, in fact, mixtures of aspergillic acid and hydroxyaspergillic acid.

The observed physical properties of aspergillic acid and the corresponding properties reported by Dutcher (46) are given in tabular form (p. 24).

Growth of A. flavus on a medium containing tryptone or "Pronutrin" and sodium chloride produced in addition to aspergillic acid another metabolite, which was named flavacol. In contradistinction to aspergillic acid which is soluble in sodium hydrogen carbonate solution, flavacol is insoluble but dissolves in aqueous sodium hydroxide, this difference being used as a basis for

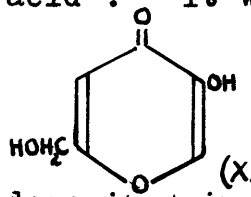
Physical Properties of Aspergillic Acid compared with those reported by Dutcher (46).

Properties	Observed	Dutcher (46)
Crystalline Form.	Radial Clusters of Yellow Needles.	Pale Yellow Rods.
Melting Point	97-99°	93°
Acidity (pka)	5·95	5·3
Optical Rotation (in ethanol)	$[\alpha]_D^{17} + 13{\cdot}3^\circ$	$[\alpha]_D^{24} + 12^\circ (\pm 3^\circ)$
Ultra-violet Light Absorption (in ethanol)	2340Å, ε=10,500 3280Å, ε= 8500	2350Å, ε =10,800 3300Å, ε = 9000

separation. The yield of flavacol was extremely low, amounting to 25 mg. of crude material from 33 litres of culture filtrate, being partly due to the repeated crystallisation required for purification. Analysis indicated the molecular formula $C_{12}H_{20}ON_2$, that is, it is isomeric with deoxyaspergillic acid. Like deoxyaspergillic acid, flavacol dissolves in dilute mineral acid and aqueous alkali and does not give a colouration with ferric chloride reagent. In particular it resembles deoxyaspergillic acid and the isomeric 3-hydroxy-2:5-di-sec.-butylpyrazine in its ultra-violet absorption spectrum, showing the characteristic two-band absorption associated with these compounds. The melting point 144-146° is, however, much higher than that of either of these isomers. Flavacol was identified by synthesis as 3-hydroxy-2:5-di-iso-butylpyrazine (p.56).

For the preparation of "high melting aspergillic acid" a number of batches were cultivated of A. flavus grown on a medium to which brown sugar was added. Although the cultural conditions were experimentally identical for each batch there was produced on one occasion, kojic acid (XXVIII) in addition to "high

melting aspergillic acid". It was isolated from the



(XXVIII)

culture filtrate as described in the experimental section and identified by analysis, titration equivalent and comparison with an authentic specimen provided by Dr. A. H. Cook.

The production of kojic acid by fungi has been reported frequently. It was first recognised in cultures of A. oryzae (56) and was investigated by Yabuta, who, besides elucidating the structure (57) observed that it possesses slight antibacterial and antifungal activity (58). Since then it has been shown that many other species of Aspergilli produce kojic acid (59,60,61). Production of kojic acid by A. flavus has been reported on several occasions (62,63,64) on a variety of media, a strain having been isolated (63) which, grown on a glucose medium, converts 67% of the sugar assimilated into kojic acid. Cultural conditions for the production of this acid, however, are critical as it is readily metabolised further by the organism (62). This last factor may account for its isolation, in association with "high melting aspergillic acid", on but one occasion although conditions of growth were apparently identical

for all batches.

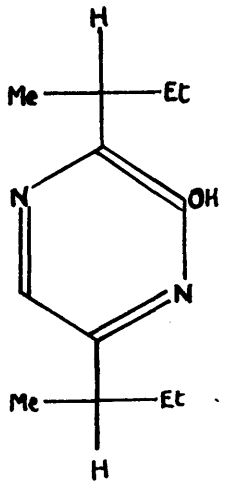
Investigations not concerning the Pyrazine Nucleus.

Dutcher and Wintersteiner (45) in their investigation of aspergillic acid stated that it could be converted to deoxyaspergillic acid by treatment with copper chromite or hydrazine, but did not give experimental details. Early attempts to reduce aspergillic acid by heating with copper chromite gave in good yield copper aspergillate and not the desired deoxyaspergillic acid. Reduction of aspergillic acid with 100% hydrazine hydrate under conditions more drastic than subsequently described by Dutcher (46) gave deoxyaspergillic acid in 60% yield. When, instead of 100% hydrazine hydrate the more readily available 90% aqueous solution was used, ammonia was produced and the resulting deoxyaspergillic acid was partially racemised. Deoxyaspergillic acid m.p. 98-100° shows light absorption in the ultra-violet consisting of two bands with maxima at 2280Å (ε =7500) and at 3250Å (ε =8000). These values are in close agreement with those obtained by Newbold and Spring (48) for a specimen of deoxyaspergillic acid supplied by Dr. O. Wintersteiner. The corresponding values (Newbold and Spring, loc.cit.) for 3-hydroxy-2:5-di-sec.-butylpyrazine, viz., 2285Å

(ε =9600) and 3220Å (ε =10,000) are outwith experimental error and confirmed the significant discrepancy previously observed by Newbold (52).

Treatment of deoxyaspergillic acid with aqueous alkali at elevated temperature resulted in total racemisation, the racemate having m.p. 102-104°. Aspergillic acid upon similar treatment with alkali was not totally racemised. When similarly treated 3-hydroxy-2:5-di-sec.-butylpyrazine m.p. 122-123° was recovered unchanged. A mixture of 3-hydroxy-2:5-di-sec.-butylpyrazine with racemic deoxyaspergillic acid showed a considerable depression in melting point. A consideration of the stereochemistry of 3-hydroxy-2:5-di-sec.-butylpyrazine indicates the significance of these results.

Since 3-hydroxy-2:5-di-sec.-butylpyrazine has two asymmetric centres, there are 2 pairs of optical antipodes which can be represented conventionally by (XXIX) to (XXXII).



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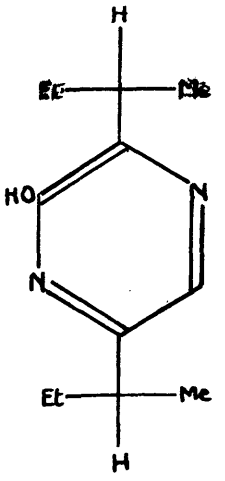

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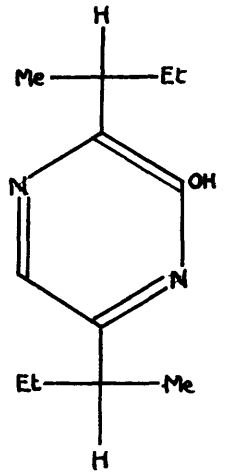

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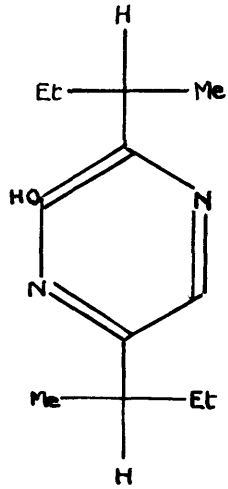


(XXXII)

Newbold (52) appreciating that two racemic forms of 3-hydroxy-2:5-di-sec.-butylpyrazine are possible examined the synthetic material m.p. 122-123° for evidence of heterogeneity. Fractional crystallisation of this hydroxypyrazine showed it to be homogeneous. 3-Amino-2:5-di-sec.-butylpyrazine, the precursor of 3-hydroxy-2:5-di-sec.-butylpyrazine in the Newbold and Spring synthesis (p. 15) was also examined for heterogeneity. Extensive fractional crystallisation of the picrate of this base showed that this amino-compound was also a single racemate. Newbold (52) therefore considered the possibility that the non-identity of 3-hydroxy-2:5-di-sec.-butylpyrazine with deoxyaspergillic acid was due to the latter belonging to the opposite optical series. Supposing this to be so, the fact that deoxyaspergillic acid was totally racemised indicates that the groups about both asymmetric centres are mobile, under the conditions of racemisation, and the racemate would therefore consist of a mixture of all four possible optical isomers. Likewise, treatment of 3-hydroxy-2:5-di-sec.-butylpyrazine with alkali at elevated temperature should give the same mixture of optical isomers. That this was not so, established conclusively that deoxyaspergillic acid is not a 3-hydroxy-2:5-di-sec.-butylpyrazine. The very definite evidence

for a hydroxypyrazine structure was recognised, however, and it was considered that Dutcher and Wintersteiner's formulation of aspergillic acid most probably erred in the nature of the alkyl residue. Experiments were therefore developed having as their object the elucidation of the nature of the alkyl side-chain or chains.

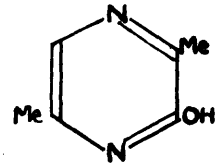
C-Methyl values for a number of compounds of possible relation to deoxyaspergillic acid were determined by Kuhn-Roth's method and are tabulated.

COMPOUND	C-Methyl Groups Theoretical	C-Methyl Groups Determined
DL-Leucine	1	0·82
DL-*iso*Leucine	2	1·42
r-Deoxyaspergillic Acid	-	2·65
3-Hydroxy-2(5)-*iso*-butyl-5(2)-*sec*.-butylpyrazine	3	2·71
3-Hydroxy-2:5-di-*sec*.-butylpyrazine	4	3·37

These results gave further confirmation of the non-identity of racemic deoxyaspergillic acid with 3-hydroxy-2:5-di-*sec*.-butylpyrazine and are in agreement with the values to be expected from the subsequently developed structure for deoxyaspergillic acid.

In alkaline solution deoxyaspergillic acid and racemic deoxyaspergillic acid both couple with benzene

diazonium chloride solution to give in good yield the corresponding phenylazo-derivatives both of which have m.p. 188-190°. This reaction confirmed the presence of an unsubstituted position in the pyrazine nucleus, a conclusion deduced by Dutcher and Wintersteiner (45) on the evidence of formation of bromodeoxyaspergillic acid. The coupling of hydroxypyrazine has not been extensively investigated and the directive influence of alkyl substituents is not known. Gastaldi and Princivalle(65) prepared the 6-phenylazo-derivative of 3-hydroxy-2:5-dimethylpyrazine (XXXIV) and also the corresponding o- and p-tolylazo-derivatives. The same 6-phenylazo-derivative (XXXIV) has also been prepared (66) from 3-hydroxy-2:5-dimethylpyrazine-6-carboxylic acid (XXXV).



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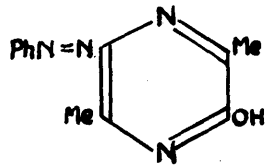

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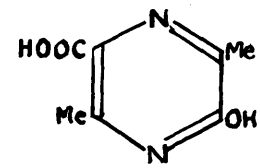
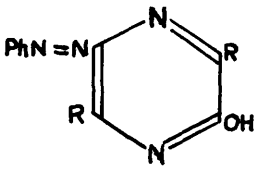


(XXXV)

by coupling with elimination of carbon dioxide.

Newbold (52) showed that the 6-phenylazo-derivative of 3-hydroxy-2:5-dimethylpyrazine (XXXVI,R=Me) is reduced in alkaline solution with sodium hydrosulphite to 3-amino-6-hydroxy-2:5-dimethylpyrazine (XXXVII,R=Me). He attempted to convert this last compound to 3:6-dihydroxy-2:5-dimethylpyrazine (XXXVIII,R=Me) by treatment with nitrous acid but was unable to isolate the desired



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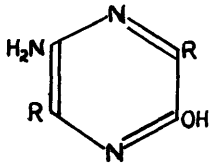

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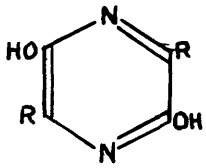
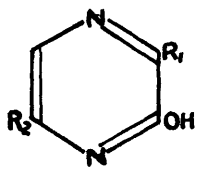


(XXXVIII)

product. It would be expected that mild reduction of a 2:5-dihydroxypyrazine would give the corresponding 2:5-diketopiperazine. As such a route suggested a method of converting the phenylazo-derivative of deoxyaspergillic acid to a 2:5-diketopiperazine this reaction series was investigated. The reduction of 3-hydroxy-6-phenylazo-2:5-di-sec.-butylpyrazine (XXXVI,R=sec.-butyl) with sodium hydrosulphite was also investigated by Newbold (52) who failed to isolate 3-amino-6-hydroxy-2:5-di-sec.-butyl-pyrazine (XXXVII,R=sec.-butyl). For this reason different conditions were used for the reduction of the phenylazo-derivative of deoxyaspergillic acid. The phenylazo-derivative of deoxyaspergillic acid was reduced catalytically with hydrogen at ordinary pressure. There was obtained a small yield of product which was difficult to purify but was eventually obtained crystalline m.p.138-140°. Analysis of the product, however, could not be interpreted. Although the nature of this catalytic reduction product was not confirmed it seems probable that the sensitivity of the nucleus to reduction by this method may account for the non-isolation of the primary

reduction product. As a satisfactory alternative method of converting deoxyaspergillic acid to a 2:5-diketo-piperazine was later adopted this line of investigation was not pursued.

Attempt was made to convert deoxyaspergillic acid to the corresponding pyrazine base. The object of this was to investigate the result of amination of this pyrazine base. Newbold (52) has shown that amination of 2:5-di-sec.-butylpyrazine gives exclusively 3-amino-2:5-di-sec.-butylpyrazine which is readily characterised as its picrate. Assuming Dutcher's structure of deoxyaspergillic acid (XXXIX), in which $R_1=R_2=$sec.-butyl, the corresponding pyrazine base (XXXX) would be symmetrical. Amination of such a pyrazine base would give a single amino-pyrazine, whereas, if $R_1 \neq R_2$ a mixture of two pyrazine bases (XXXXI and XXXXII) would result. As the structure proposed by Dutcher is the only one possible in which $R_1=R_2$ it was



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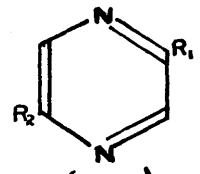

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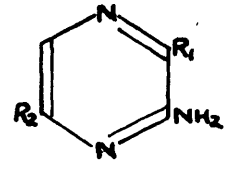

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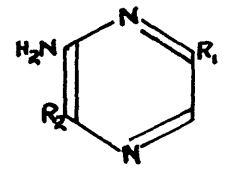


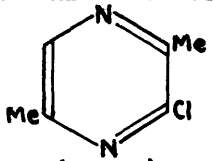
(XXXXII)

hoped by this method to obtain positive evidence of the non-identity of the alkyl groups. In attempting to convert deoxyaspergillic acid to the corresponding pyrazine base it was heated with zinc, and, in another experiment with zinc dust, sodium chloride and zinc

chloride mixture as recommended by Clar (67) who claims that enhanced yields are thus obtained in the conversion of hydroxy-aromatics to the corresponding aromatic systems. In neither case, however, was any pyrazine base isolated. 3-Hydroxy-2:5-dimethylpyrazine under similar conditions of reaction with Clar's reagent underwent deep-seated degradation, ammonia being the only product of the reaction identified.

The oxidation of alkylpyrazines to pyrazine-carboxylic acids has been used as a preparative method for the latter (68). The possibility of oxidising a suitable derivative of aspergillic acid to the corresponding pyrazine-carboxylic acid suggested itself as a means of confirming the location of the alkyl fragment. The isolation of a pyrazine-monocarboxylic acid would indicate that the alkyl fragment was disposed as a single alkyl side-chain, whereas, isolation of a pyrazine-dicarboxylic acid would prove that the alkyl fragment was disposed as two alkyl groups. As a model the oxidation of 3-chloro-2:5-dimethylpyrazine (XXXXIII) was examined. With alkaline potassium permanganate under essentially the conditions of Stoehr (69) for the preparation of pyrazine-2:5-dicarboxylic acid from 2:5-dimethylpyrazine, the chloropyrazine-dicarboxylic acid (XXXXIV) was not isolated.

Failure to isolate the acid was attributed to the low



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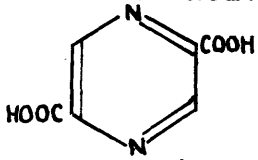

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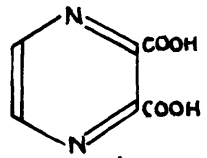

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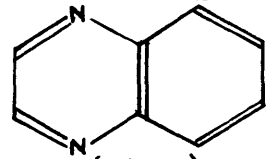


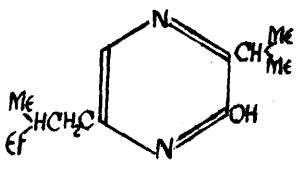
(XXXXVI)

yields (10-15%) general for this reaction (68). In contrast with the low yield of pyrazine-carboxylic acids obtained by oxidation of alkylpyrazines, quinoxaline (2:3-benzpyrazine, XXXXVI) has been converted to pyrazine-2:3-dicarboxylic acid (XXXXV) in 80% yield by oxidation with alkaline potassium permanganate (70). Concurrent with the model oxidation of 3-chloro-2:5-dimethylpyrazine the conversion of deoxyaspergillic acid to a chloropyrazine was examined. It was considered that deoxyaspergillic acid was unsuitable for oxidation studies as the presence of the hydroxyl group would increase the ease of oxidation of the nucleus. Conversion of deoxyaspergillic acid to a chloropyrazine appeared to be the most satisfactory method of enhancing the stability of the nucleus to oxidation. Prolonged treatment of deoxyaspergillic acid with phosphoryl chloride, however, failed to effect this conversion, the starting material being recovered unchanged.

The isolation of a pyrazine fragment by model oxidation of 3-chloro-2:5-dimethylpyrazine having failed, attention was next turned to the possibility of

identifying the alkyl fragment of aspergillic acid by oxidation. By treatment of aspergillic acid or deoxy-aspergillic acid with chromium trioxide in glacial acetic acid, under a variety of conditions, acetone was produced in very low yield and identified as its 2:4-dinitro-phenylhydrazone. This isolation of acetone showed that the group $= CMe_2$ formed part of the alkyl fragment of aspergillic acid.

Summarising the information concerning the alkyl fragment - it consists of a C_8 residue having an asymmetric centre. Three of the carbon atoms are accounted for by the group $Me_2C=$. The probability of the C_8 residue being a single C_8H_{17} side-chain carrying an asymmetric centre as well as a gem-dimethyl group appeared remote as did the possibility of the C_8 residue being disposed as two alkyl groups, one of which having both the asymmetric centre and the $= CMe_2$ group. These improbable structures being excluded the only alternative structures are those represented by (XXXXVII) and (IL), and the isomers (XXXXVIII) and (L) arising from the two possible positions of the hydroxyl group in the nucleus.



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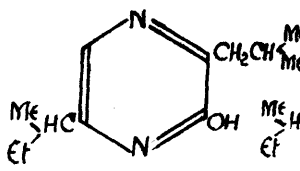

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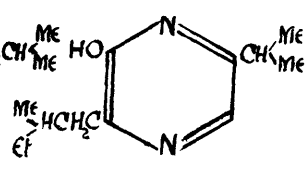
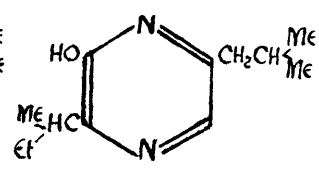


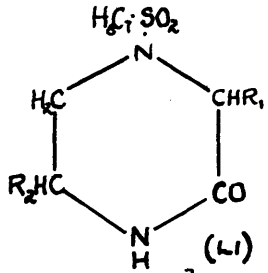
(IL)



(L)

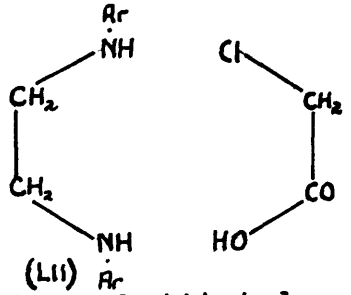
Reactions involving the Pyrazine Nucleus.

Deoxyaspergillic acid was rapidly reduced catalytically with hydrogen at ordinary pressure to tetrahydrodeoxyaspergillic acid and on treatment of the latter with toluene-p-sulphonyl chloride an almost quantitative yield of N-toluene-p-sulphonyl-tetrahydrodeoxyaspergillic acid m.p. 144-146° was obtained. The structure of this derivative is formulated as (LI).

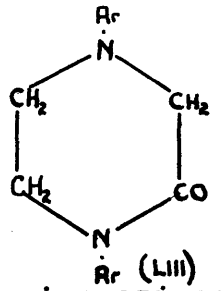


(LI)

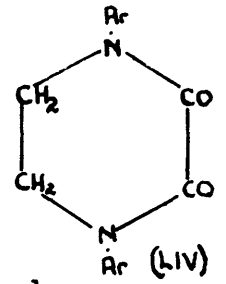
Monoketopiperazines as a class are but rarely mentioned in the literature. N-N'-Diaryl-monoketopiperazines (LIII) have been prepared by condensation of ethylene diarylamines (LII) with chloracetic acid (71) and by the reduction of N-N'-diaryl-2:3-diketopiperazines (LIV)(72).



(LII)

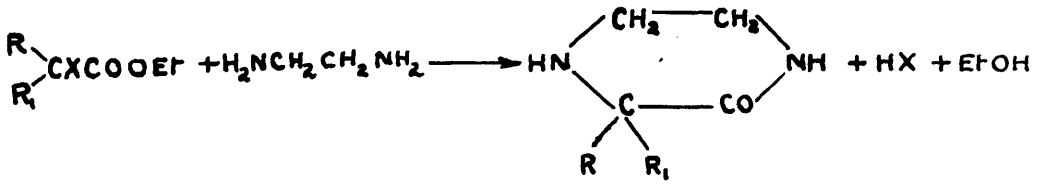


(LIII)

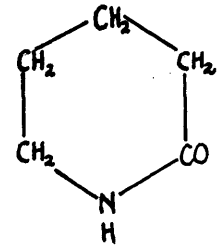


(LIV)

N-Unsubstituted-monoketopiperazines have been prepared analogously by Aspinall (73) according to the reaction



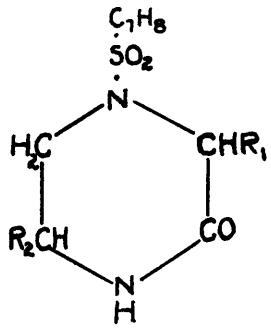
giving the monoketopiperazine (LV) in which R and R_1 are hydrogen or alkyl and X is halogen. These strongly basic compounds readily give crystalline toluene-p-sulphonyl-derivatives (73). It was hoped that on hydrolysis the toluene-p-sulphonyl-derivative of tetrahydrodeoxyaspergillic acid would be ruptured at the cyclic amide link to give the open chain acid (LVI) the conversion of which into an N-alkyl-N-toluene-p-sulphonylamino-acid (LVII) according to the reaction sequence shown (p.39) appeared feasible. Synthesis of compounds of this type can readily be effected (74,75), treatment of the amino-acid (LVIII) with aryl-sulphonyl chloride giving the intermediate (LIX) which is alkylated to the desired product (LX) by treatment with alkyl halide. Hydrolysis of N-toluene-p-sulphonyltetrahydrodeoxyaspergillic acid in either acid or alkaline solution was not achieved, the starting material being recovered under mild conditions. More drastic hydrolytic conditions resulted in total breakdown of the molecule. The analogous α-piperidone (LXI), however, is readily hydrolysed by mineral acid to δ-amino-n-valeric acid (LXII) (76).



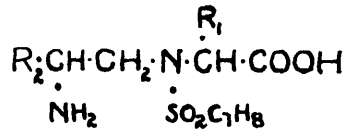
(LXI)

$H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2CH_2COOH$

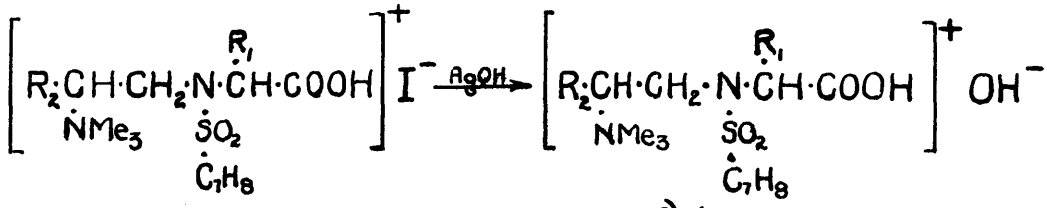
(LXII)

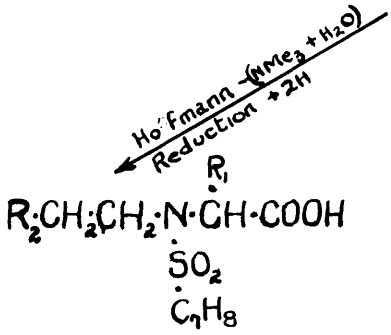


(LI)

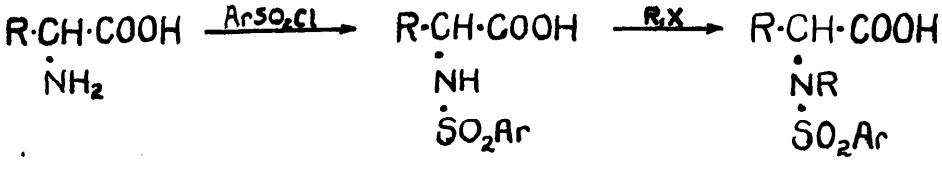


(LVI)





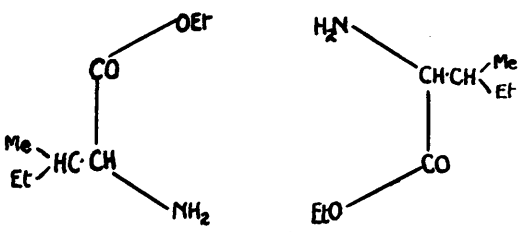
(LVII)



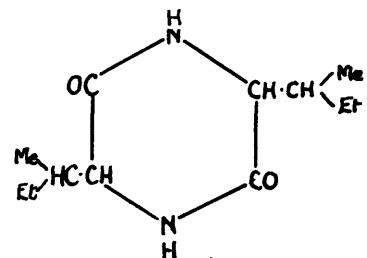
(LVIII) (LIX) (LX)

According to Dutcher (47) aspergillic acid can be converted into a compound which he considered to be identical with isoleucine anhydride. It must be emphasised that this compound was not analysed nor was satisfactory proof adduced that it was in fact a diketopiperazine apart from the fact that it showed physical similarity to isoleucine anhydride and was not depressed in melting point on admixture with it. Accordingly these experiments have been repeated. Aspergillic acid was converted to the compound $C_{12}H_{22}O_2N_2$ by the method of Dutcher (47) involving bromination of aspergillic acid in acid solution, followed by zinc and acetic acid reduction of the resulting bromoaspergillic acid. The compound $C_{12}H_{22}O_2N_2$ m.p.260-261° has $[\alpha]_D$+17° as compared with $[\alpha]_D$+13·8° for the compound $C_{12}H_{22}O_2N_2$ m.p.249-250° as described by Dutcher. This compound can, according to Dutcher, also be obtained by bromination of deoxyaspergillic acid followed by reduction of the resulting bromodeoxyaspergillic acid. The quinone produced along with bromodeoxyaspergillic acid also gives this compound $C_{12}H_{22}O_2N_2$ on reduction (p.13). This was considered as a method of obtaining the compound $C_{12}H_{22}O_2N_2$ in its racemic form from racemic deoxyaspergillic acid. The yield, however, from bromination of racemic deoxyaspergillic acid did not justify continuation of this project. For comparison purposes the

diketopiperazines, DL-isoleucine anhydride, DL-norleucyl-DL-isoleucine anhydride and DL-leucyl-DL-isoleucine anhydride have been synthesised. DL-isoLeucine anhydride (LXIV) m.p. 280-283° was prepared by two routes; by the method of Baxter and Spring (53, p.20) from DL-isoleucine by heating with ethylene glycol and by the method of Fischer (77, for the preparation of leucine anhydride) from DL-isoleucine ethyl ester (LXIII). This last method was that used by Dutcher to prepare DL-isoleucine anhydride although the melting point he quoted was 25° lower than that observed by the

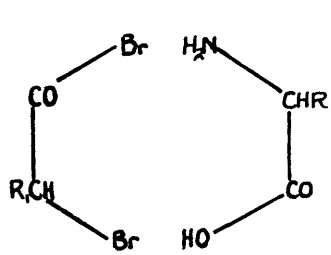


(LXIII)

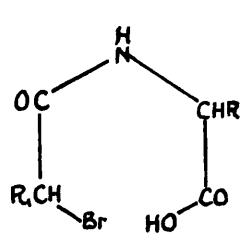


(LXIV)

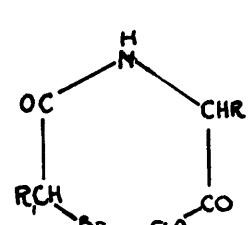
author, whose observed melting point is the same as that quoted by Ehrlich (78) for an inactive isoleucine anhydride obtained by thermal decomposition of isoleucine. DL-Norleucyl-DL-isoleucine anhydride and DL-leucyl-DL-isoleucine anhydride were prepared by Mr. J. J. Gallagher by the route



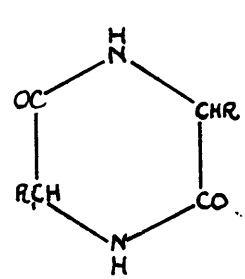
(LXVI) (LXV)



(LXVII)



(LXVIII)



(LXIX)

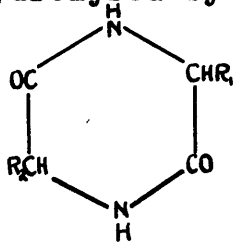
DL-α-Bromo-β-methylvaleryl bromide (LXVI, R_1=sec.-butyl) reacted with DL-norleucine (LXV, R=n-butyl) or DL-leucine (LXV, R=iso-butyl) to give the N-bromacylamino-acid (LXVII) which after esterification (LXVIII) was treated with ethanolic ammonia to give the appropriate diketopiperazine DL-norleucyl-DL-isoleucine anhydride (LXIX; R=n-butyl, R_1=sec.-butyl) or DL-leucyl-DL-isoleucine anhydride (LXIX; R=iso-butyl, R_1=sec.-butyl). They were also prepared by condensation of DL-α-bromo-n-hexanoyl bromide (LXVI; R_1=n-butyl) or DL-α-bromo-isohexanoyl bromide (LXVI; R_1=iso-butyl) with DL-isoleucine (LXV; R=sec.-butyl) and applying the above series of reactions to the resulting N-α-bromacylamino-acids.

It was confirmed that a mixture of the compound $C_{12}H_{22}O_2N_2$ and DL-isoleucine anhydride melted intermediate between the melting points of the components. This, however, was also true of a mixture of the compound with DL-leucyl-DL-isoleucine anhydride m.p.278°. A mixture of the compound with DL-norleucyl-DL-isoleucine anhydride m.p.258-260° showed a slight but significant depression of melting point. That a lack of melting point depression is inconclusive proof of identity of two diketopiperazines has been observed by Abderhalden and Rossner (79) who showed that a mixture of glycyl-

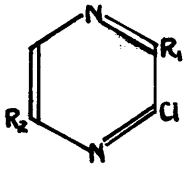
DL-norleucine anhydride m.p.219-220° and glycyl-DL-α-amino-heptanoic acid anhydride m.p.221-222° was not depressed in melting point. The literature did not disclose a satisfactory method for the characterisation of 2:5-diketopiperazines. The melting point was not a suitable criterion being somewhat variable, depending upon the rate of heating and complicated by a tendency to sublimation.

In attempting to confirm the structure of the compound $C_{12}H_{22}O_2N_2$ as a 2:5-diketopiperazine it was treated with phosphoryl chloride. This reagent is known to bring about the aromatisation of 2:5-diketopiperazines to pyrazine derivatives a reaction which is discussed on page 57 . By refluxing the compound $C_{12}H_{22}O_2N_2$ with phosphoryl chloride a mixture of products was obtained, separable by treatment with aqueous sodium hydroxide into an alkali soluble fraction and an alkali insoluble fraction. The former, after crystallisation from aqueous ethanol had m.p.85-91°, but by recrystallisation could not be purified to constant melting point. It was amphoteric, dissolving readily in dilute mineral acid and in dilute alkali; in this and other physical properties it resembled closely deoxyaspergillic acid, with which, on admixture, it was

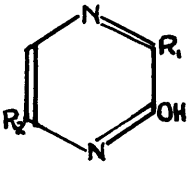
not depressed in melting point. These facts suggested that it was probably a mixture of hydroxypyrazines related to deoxyaspergillic acid. The alkali insoluble fraction, an oil b.p. 95-100°/1mm., contained halogen and on vigorous treatment with potassium hydroxide gave a halogen-free product indistinguishable in physical properties from the product m.p.85-91° isolated directly. These results are readily explained if the compound $C_{12}H_{22}O_2N_2$ is ascribed an unsymmetrical diketopiperazine structure (LXX), reaction with phosphoryl chloride giving a mixture of the two possible isomeric hydroxypyrazines (LXXII) and (LXXIII) in association with the corresponding chloropyrazines (LXXI) and (LXXIV), the mixture of chloropyrazines being hydrolysed by alkaline treatment



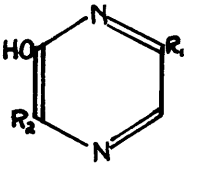
(LXX)



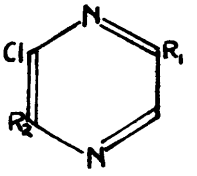
(LXXI)



(LXXII)



(LXXIII)

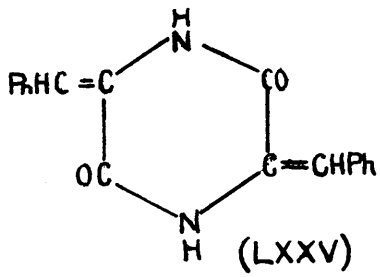


(LXXIV)

to give the same mixture of hydroxypyrazines (LXXII) and (LXXIII). Such a mixture of closely related hydroxypyrazines would be expected to prove extremely difficult to separate.

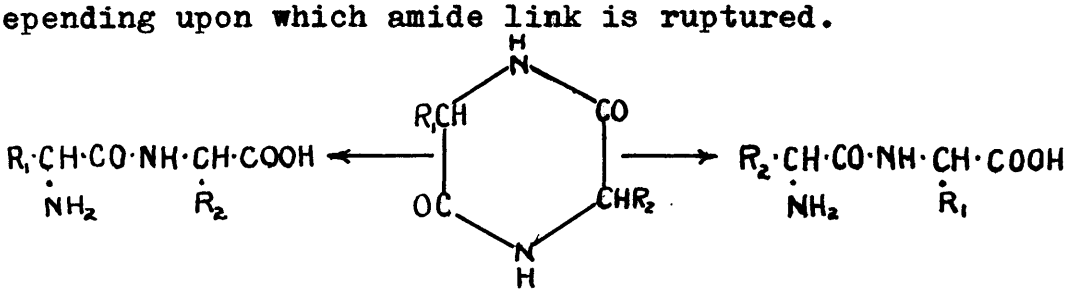
Attention was next turned to the hydrolysis of 2:5-diketopiperazines. Dutcher (47) in postulating the structure of the compound $C_{12}H_{22}O_2N_2$ as d-isoleucine anhydride considered this possibility as a means of more rigorous characterisation but rejected it on published evidence of the resistance to hydrolysis of isoleucine anhydride and leucine anhydride. In contrast to the considerable information on the hydrolysis of peptides, there was very little in the literature on the subject of hydrolysis of α-amino-acid anhydrides, especially those derived from the higher α-amino-acids. The only reference to hydrolysis of isoleucine anhydride (78) states that "by prolonged heating of isoleucine anhydride with hydrochloric acid a product is obtained as needles m.p.256-257°, probably a mixture if isoleucyl-isoleucine dipeptides." Fischer (80) found that by treatment of leucine anhydride in a sealed tube at 100° with concentrated hydrobromic acid for thirty minutes leucyl-leucine dipeptide was produced in 66% yield, and again (81), that leucine anhydride is stable to alkaline hydrolysis, more so than alanine anhydride which with dilute alkali at the boiling point is hydrolysed to the dipeptide alanyl-alanine. L-Alanyl-L-alanine has been hydrolysed by hydrochloric acid (82) to L-alanine. DL-Phenylalanine has been prepared (83) by condensation

of benzaldehyde with glycine anhydride followed by reduction of the 3:6-dibenzylidene-2:5-diketopiperazine (LXXV) with hydriodic acid, the cleavage to α-amino-acid



(LXXV)

occurring at the same time. The hydrolysis of 2:5-diketopiperazines involves intermediate formation of dipeptides and except in the case of symmetrical diketopiperazines this may give rise to two dipeptides depending upon which amide link is ruptured.



Levene and co-workers (84,85,86) have examined the hydrolysis of 2:5-diketopiperazines and shown that the ease of hydrolysis decreases rapidly with increasing alkyl substitution in the methylene groups of glycine anhydride.

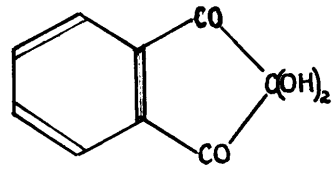
DL-isoLeucine anhydride was chosen for model experiments on the hydrolysis of 2:5-diketopiperazines. Dilute aqueous alkali at the boiling point did effect hydrolysis as evidenced by the ninhydrin reaction (p.48)

but the product could not be freed from hydrolytic agent. Likewise hydrolysis to the amino-acid was brought about, under intensive conditions by hydrochloric acid, hydrobromic acid and hydriodic acid, again as indicated by the ninhydrin reaction. Analysis of the product obtained by hydriodic acid hydrolysis, however, indicated that the hydrolysis was incomplete. The most satisfactory method was by refluxing DL-isoleucine anhydride with hydrobromic acid, DL-isoleucine being obtained in 50% yield and characterised as its N-formyl-derivative by heating with 98% formic acid (87,88).

At this stage the difficulty of identification of α-amino-acids is worthy of mention. In general the α-amino-acids have rather indefinite melting points, there is usually decomposition at the melting point, and the determined value is largely dependant on the rate of heating. Moreover, a lack of melting point depression with a mixture of two α-amino-acids is not conclusive proof of identity; a mixture of DL-leucine and DL-isoleucine, for example, has a melting point intermediate between the melting points of the components. Acylation has been found satisfactory for identification, in particular N-formylation is suitable, as the N-formyl-derivatives of many α-amino-acids have been described. Toluene-p-sulphonyl chloride (89) and 3:5-dinitrobenzoyl

chloride (90) have also been recommended for this purpose.

The ninhyrdin test was used to differentiate between 2:5-diketopiperazines, dipeptides and α-amino-acids. Ninhydrin (triketohydrindene hydrate LXXVI) was introduced by Ruhemann (91) as a test for α-amino-acids. That it

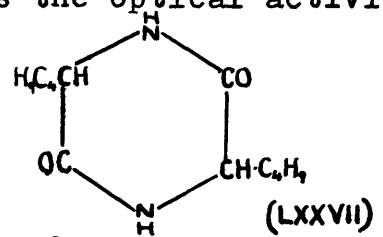


(LXXVI)

does not give a positive test with 2:5-diketopiperazines has been reported (92). This reagent has been claimed as giving a positive reaction with dipeptides (93) although other workers (94) have claimed that it is specific for α-amino-acids in which both of the functional groups are free. Model tests showed that dipeptides, as represented by DL-leucyl-DL-isoleucine, give a colour reaction with ninhydrin which is, however, readily distinguished from that given by α-amino-acids.

The compound $C_{12}H_{22}O_2N_2$ on hydrolysis gave in high yield a product having the properties of an α-amino-acid. The acid-free hydrolysate was subjected to partition chromatography on paper after the method of Consden, Gordon and Martin (93). On a unidimensional strip with n-butanol saturated with water as the mobile phase, the absence of glycine, alanine, valine and norvaline was readily shown. The behaviour of the

hydrolysate on the strip, however, was indistinguishable from that of DL-leucine, DL-<u>iso</u>leucine, DL-norleucine or a mixture of these α-amino-acids (p.50). This analytical method, therefore, although it did not establish the exact nature of the hydrolysate did confirm that it consisted of leucine isomers. The fact that the compound $C_{12}H_{22}O_2N_2$ gave leucine isomers on hydrolysis established conclusively that it is a 2:5-diketopiperazine. The diketopiperazine is therefore represented as (LXXVII), and, as the optical activity of aspergillic

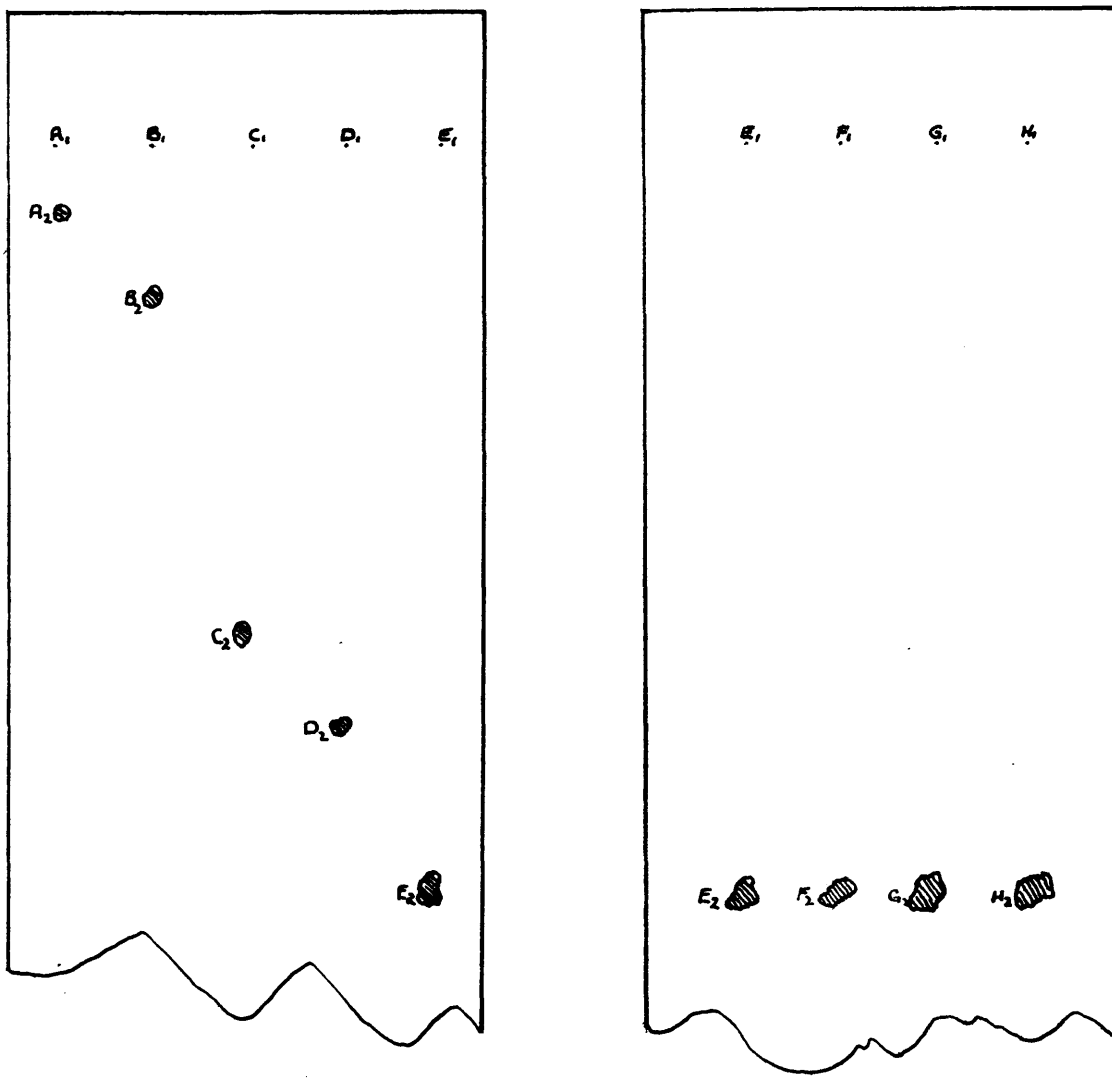


(LXXVII)

acid is to be ascribed to the asymmetry of the alkyl fragment, one at least, of the groups must be <u>sec</u>.-butyl. That both groups are not <u>sec</u>.-butyl, however, has previously been deduced. The isolation of acetone by oxidation of aspergillic acid indicates the presence of a=CMe_2 group which on the formulation (LXXVII) for the diketopiperazine must arise from the other alkyl group, which must therefore be <u>iso</u>-butyl. The diketopiperazine $C_{12}H_{22}O_2N_2$ is therefore 3-<u>iso</u>-butyl-6-<u>sec</u>.-butyl-2:5-diketopiperazine (LXXVIII) from which it follows that aspergillic acid is to be ascribed the structure (LXXIX) or (LXXX) and deoxyaspergillic acid

Partition Chromatography of Hydrolysate on Paper.

Appearance of paper strips.



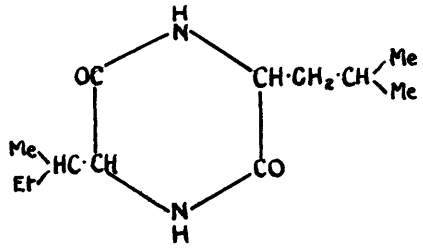
Solvent front advanced 26 cm. (same scale)

A = Glycine B = DL-Alanine C = L-Valine

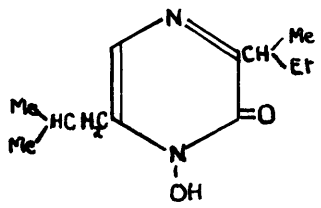
D = DL-Norvaline E = Hydrolysate F = DL-Leucine

G = DL-<u>iso</u>Leucine H = DL-Norleucine

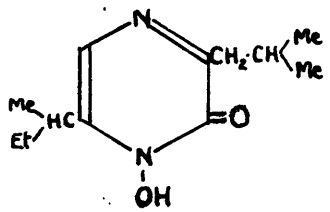
the structure (LXXXI) or (LXXXII).



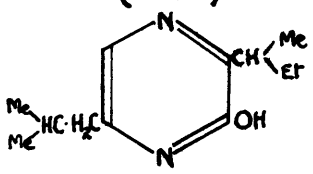
(LXXVIII)



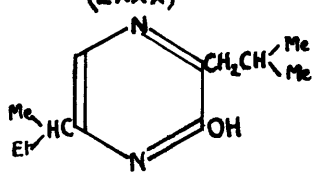
(LXXIX)



(LXXX)



(LXXXI)



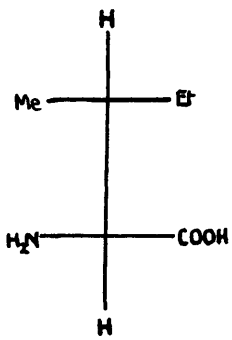
(LXXXII)

The fractionation of leucine isomers has been reported. Ehrlich (95) was able to separate L-leucine and L-isoleucine; a method based on the difference in solubility of their copper salts, and the method has been extended to the separation of the more complex mixture of α-amino-acids resulting from hydrolysis of the protein zein (96). An infra-red analysis of leucine and isoleucine mixtures has been evolved (97) using bands, given by the acetyl derivatives of these α-amino-acids in the 700 cm^{-1} region.

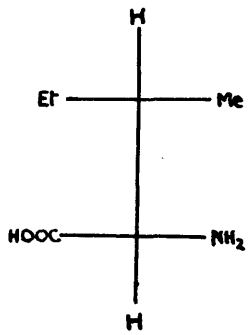
The hydrolysate was converted to its copper salt which was separated into a relatively water soluble fraction and a relatively water insoluble fraction.

These fractions were then individually reconverted to the parent α-amino-acid by treatment with hydrogen sulphide. Theoretical consideration of the nature of the hydrolysate will explain the subsequent procedure adopted for the confirmation of the nature of these two amino-acid fractions.

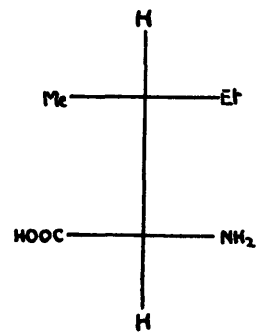
The diketopiperazine $C_{12}H_{22}O_2N_2$ has three asymmetric centres, two of which were introduced in its formation from aspergillic acid; it will therefore be racemic with respect to these two centres by virtue of their mode of formation. Hydrolysis of the diketopiperazine will therefore give rise to DL-leucine and α-amino-β-methylvaleric acid racemic with respect to the α-carbon. There are four possible isomers of α-amino-β-methylvaleric acid represented conventionally by (LXXXIII) to (LXXXVI), of which structures one optical pair represents



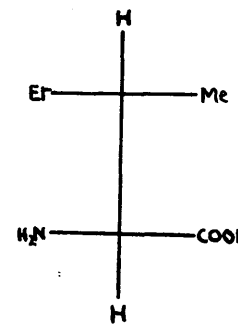
(LXXXIII)



(LXXXIV)



(LXXXV)



(LXXXVI)

DL-<u>iso</u>leucine and the other DL-<u>alloiso</u>leucine. The α-amino-β-methylvaleric acid component of the hydrolysate will consist of a non-optical pair (active <u>iso</u>leucine and

active alloisoleucine of the same β -configuration), or less likely, if β -racemisation has occurred, will consist of all four possible isomers (DL-isoleucine and DL-alloisoleucine). Theoretically the hydrolysate consists of 50% DL-leucine, 25% isoleucine and 25% alloisoleucine. If β -racemisation has not occured the $[\alpha]_D$ of such a mixture can be estimated using the physical constants of isoleucine and alloisoleucine reported by Abderhalden and Zeisset (98) and given in tabular form.

Amino-acid	$[\alpha]_D$	Amino-acid	$[\alpha]_D$
L(+)isoLeucine	$+10{\cdot}7^\circ$	D(-)alloisoLeucine	$-14{\cdot}2^\circ$
D(-)isoLeucine	$-10{\cdot}7^\circ$	L(+)alloisoLeucine	$+14{\cdot}2^\circ$
L-isoLeucine has the same β -configuration as D-alloiso-Leucine			

$[\alpha]_D$Hydrolysate$= {\cdot}25 \times [\alpha]_D$ isoleucine $+ {\cdot}25 \times [\alpha]_D$ alloisoleucine

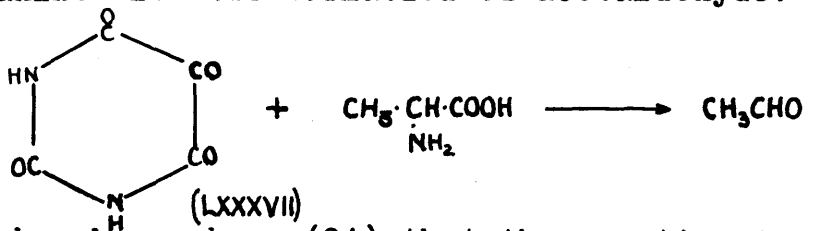
$$= \pm \frac{14{\cdot}2 - 10{\cdot}7}{4}$$

$$= \pm 0{\cdot}88^\circ$$

As this computed value is so low, the fact that the observed $[\alpha]_D$of the hydrolysate $= 0 \pm 1^\circ$ cannot be taken as evidence that β -racemisation of the α -amino-β -methyl-valeric acid has taken place during hydrolysis of the diketopiperazine $C_{12}H_{22}O_2N_2$.

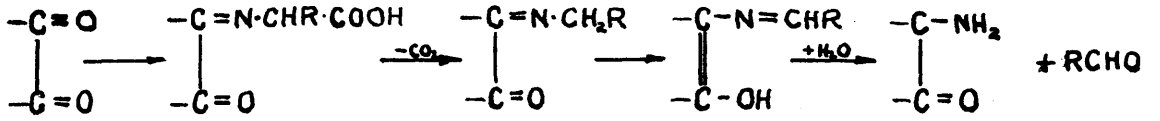
To avoid separating isoleucine and alloisoleucine

a method was sought of identifying the one in presence the other. This was most readily attainable by removal of one of the centres of asymmetry. The Strecker degradation presents a ready method of removing the asymmetry about this α-carbon atom. In this reaction α-amino-acids are degraded to the corresponding aldehydes or ketones containing one carbon atom less, by the action of certain carbonyl compounds. Strecker (99) observed that alloxan (LXXXVII) reacted with alanine with the formation of acetaldehyde.



(LXXXVII)

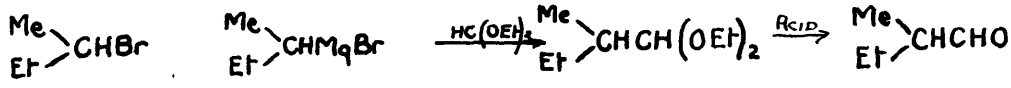
It has been shown (94) that the reaction is general for carbonyl compounds having the grouping $CO-(CH=CH)_n-CO$ where n=0 or any integer, and is specific for α-amino-acids in which the amino group is unsubstituted. The mechanism of the Strecker degradation has been postulated (94) as



In effecting Strecker degradation ninhydrin has been extensively used (94), it having been shown that both leucine (100,101) and isoleucine (101) undergo reaction with this reagent, although in neither case was the

resulting aldehyde characterised.

The amino-acid regenerated from the more water soluble copper salt (p.51) when reacted with ninhydrin in an inert atmosphere gave methyl ethyl acetaldehyde, characterised as its 2:4-dinitrophenylhydrazone, identical (melting point and mixed melting point) with that obtained by similar degradation of authentic DL-isoleucine. The observed melting point of methyl ethyl acetaldehyde 2:4-dinitrophenylhydrazone, 130°, was 9° higher than that recorded by Morgan and Hardy (102). Confirmatory evidence was deduced by a total synthesis of methyl ethyl acetaldehyde after Linstead and Mann (103) by the route



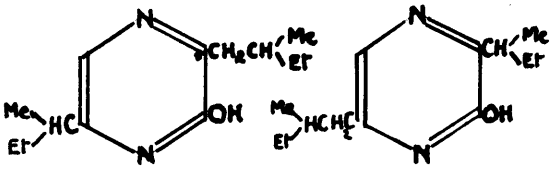
the 2:4-dinitrophenylhydrazone of this synthetic product being identical with that of the aldehyde obtained from DL-isoleucine.

The other component of the hydrolysate was confirmed as DL-leucine by two methods. It was converted to its N-3:5-dinitrobenzoyl-derivative (90) which was undepressed in melting point with N-3:5-dinitrobenzoyl-DL-leucine and depressed in melting point with either N-3:5-dinitrobenzoyl-DL-isoleucine or N-3:5-dinitrobenzoyl-DL-norleucine. Secondly it was subjected to Strecker degradation and the isovaleraldehyde produced

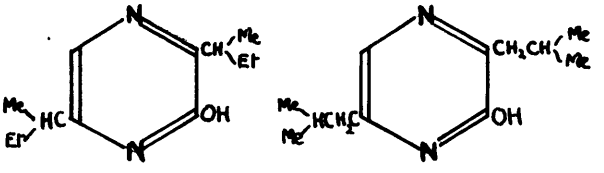
characterised as its 2:4-dinitrophenylhydrazone, which was identical with a specimen prepared from authentic DL-leucine.

Flavacol and 3-Hydroxy-2:5-di-iso-butylpyrazine.

As described on p.23 flavacol was separated from aspergillic acid, in association with which it is produced by growth of A. flavus on a tryptone or "Pronutrin" medium containing sodium chloride. In physical properties it resembles deoxyaspergillic acid with which it is isomeric. The similarity in ultra-violet absorption spectra between flavacol and deoxy-aspergillic acid was so marked as to suggest a very close relationship between the two. Deoxyaspergillic acid having been shown to have the structure (LXXXVIII) or (LXXXIX), the possibility of flavacol having the alternative structure (LXXXIX) or (LXXXVIII) was considered. Other structures which appeared worthy of consideration were the other isomeric 2:5-dialkylhydroxy-pyrazines in which the alkyl groups were iso-butyl or sec.-butyl. This latter consideration offered two further possibilities, 3-hydroxy-2:5-di-sec.-butylpyrazine (XC) and 3-hydroxy-2:5-di-iso-butylpyrazine (XCI).



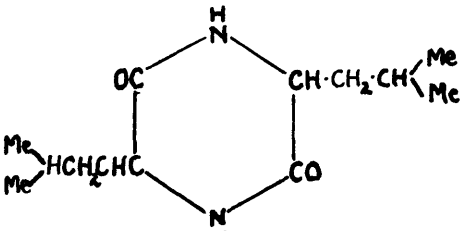
(LXXXVIII) (LXXXIX)



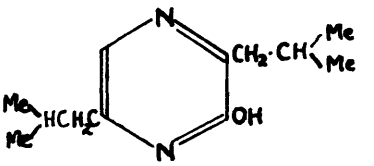
(XC) (XCI)

Flavacol differed from 3-hydroxy-2:5-di-sec.-butyl-pyrazine (XC). As synthetic routes to compounds (LXXXVIII) and (LXXXIX) are not known (see p.64 for consideration of these syntheses) the possibility of synthesis of (XCI) for comparison was examined.

The synthesis of 3-hydroxy-2:5-di-iso-butylpyrazine (XCIII) followed the general method of Baxter and Spring (53, p. 20) for the synthesis of 3-hydroxy-2:5-di-sec.-butylpyrazine. DL-Leucine anhydride (XCII) prepared after Sannié (104) by heating DL-leucine with ethylene glycol, on treatment with phosphoryl chloride gave



(XCII)

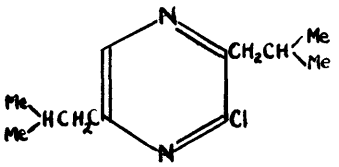


(XCIII)

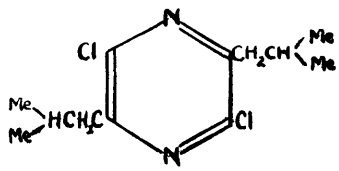
directly 3-hydroxy-2:5-di-iso-butylpyrazine (XCIII). Although Baxter and Spring (53) did not isolate directly the hydroxypyrazine, but obtained it by hydrolysis of the chloropyrazine produced, direct dehydration of 2:5-diketopiperazines has previously been observed. DL-Phenylglycine anhydride on treatment with phosphoryl chloride gives 3-hydroxy-2:5-diphenylpyrazine (105) and similar treatment of DL-leucyl-DL-isoleucine anhydride gives a hydroxy-2-iso-butyl-5-sec.-butylpyrazine.

Treatment of DL-leucine anhydride with phosphoryl

chloride gave in addition to 3-hydroxy-2:5-di-iso-butylpyrazine, a mixture of chloropyrazine derivatives which could not be separated directly. Analysis indicated that the mixture probably consisted of 3-chloro-2:5-di-iso-butylpyrazine (XCIV) and 3:6-dichloro-2:5-di-iso-butylpyrazine (XCV).



(XCIV)



(XCV)

The presence of the former was confirmed by treatment of the mixture with sodium ethoxide followed by acid hydrolysis of the product, 3-hydroxy-2:5-di-iso-butyl-pyrazine being isolated.

Flavacol (p.23) was identified as 3-hydroxy-2:5-di-iso-butylpyrazine. They have identical physical properties, show the same light absorption in the ultra-violet (Fig. I) and a mixture of the two was not depressed in melting point.

It has been shown that aspergillic acid can be degraded to a mixture of leucine isomers, and, based on the identification of these isomers, the alternative formulae of aspergillic acid were established. Such a mixture of leucine isomers would likewise be produced if aspergillic acid were a mixture of 1-hydroxy-2-keto-

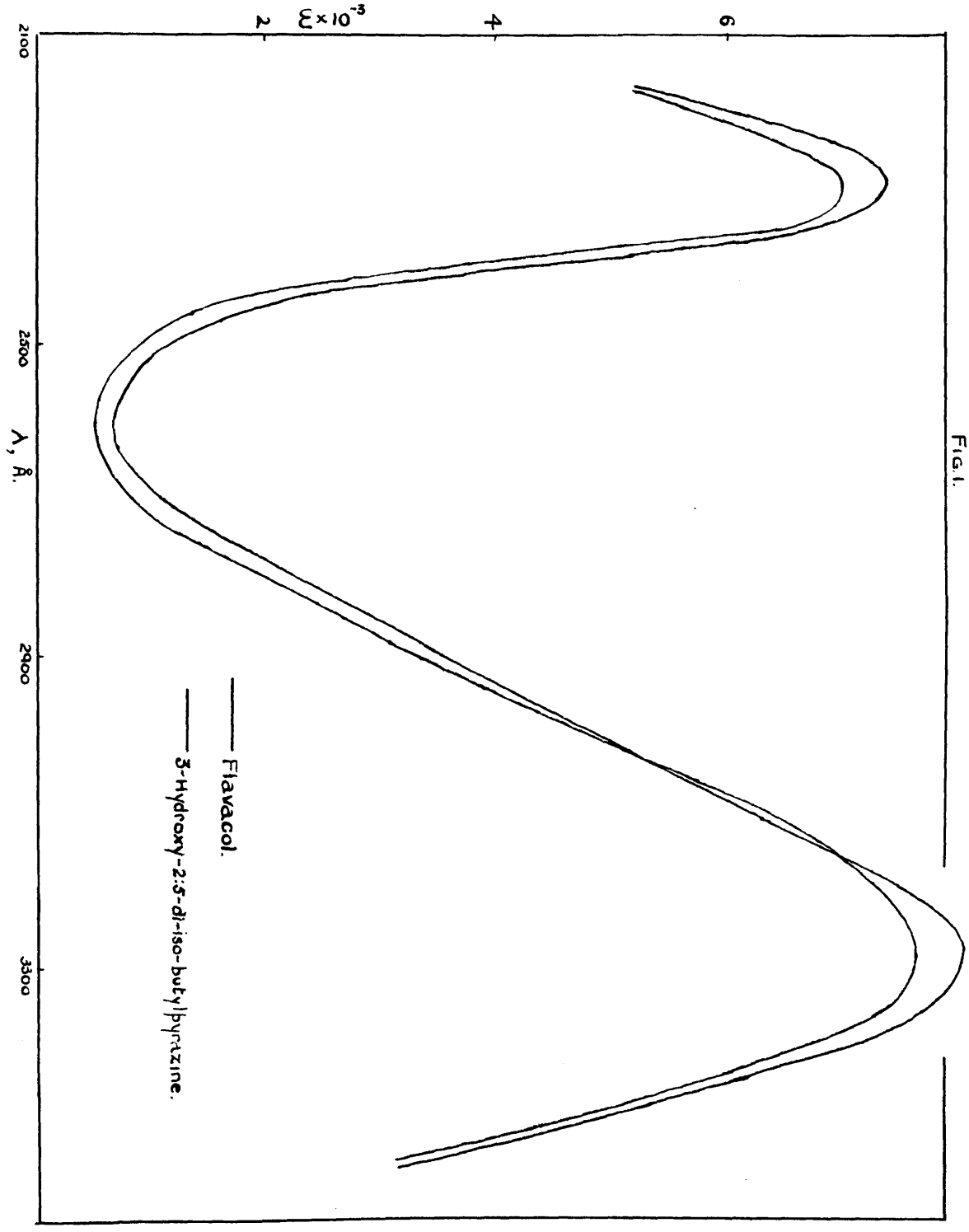
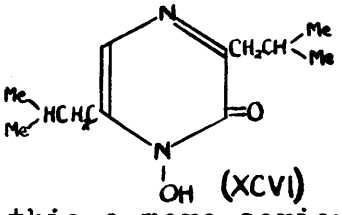
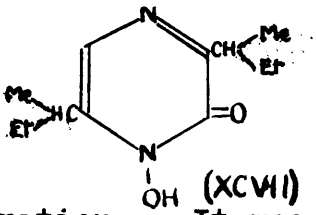

ε×10⁻³
2
4
6
2100
2500
2900
3300
λ, Å.
Flavacol.
3-Hydroxy-2:5-di-iso-butylpyrazine.

FIG. 1.

3:6-di-iso-butyl-1:2-dihydropyrazine (XCVI) and 1-hydroxy-2-keto-3-6-di-sec.-butyl-1:2-dihydropyrazine (XCVII). Establishment of the structure of flavacol showing as it does that A. flavus is able to synthesise 3-hydroxy-2:5-di-iso-butylpyrazine, which has the C-N skeleton of (XCVI),



(XCVI)



(XCVII)

made this a more serious consideration. It was, therefore, necessary to disprove the possibility of aspergillic acid being a mixed crystal formation of the two pyrazine cyclic hydroxamic acids (XCVI) and (XCVII) and deoxy-aspergillic acid a mixed crystal formation of the corresponding hydroxypyrazines. As 3-hydroxy-2:5-di-iso-butylpyrazine and 3-hydroxy-2:5-di-sec.-butylpyrazine were available the method adopted was to examine mixtures of these two hydroxypyrazines for evidence of formation of mixed crystals identical with deoxyaspergillic acid. A mixture of 50% 3-hydroxy-2:5-di-iso-butylpyrazine and 50% 3-hydroxy-2:5-di-sec.-butylpyrazine was examined. Fractional crystallisation of such a synthetic mixture failed to show any evidence of a sharp-melting entity, all crops melting over a wide range.

Biogenetic Considerations.

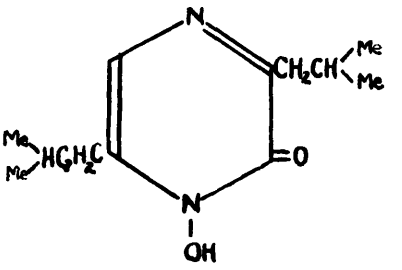
Elucidation of the structure of aspergillic acid stimulates interest in the biosynthesis of the antibiotic, the structure being novel amongst natural products. Produced as it is by growth of the organism on a protein hydrolysate medium, the utilisation of α-amino-acids immediately suggests itself. The amino-acid precursors of such a structure, leucine and isoleucine are known to be present in casein hydrolysate (106). Attempt was therefore made to produce aspergillic acid on a synthetic medium consisting of these amino-acids. It was known that aspergillic acid is produced on a medium of enzymic digest of casein but not on acid digested casein, a fact that was attributed to the destruction of tryptophane during acid hydrolysis of the protein. For this reason tryptophane was added to the synthetic medium of leucine and isoleucine. On this medium, however, not only was aspergillic acid not produced, but, in addition, there was negligible growth of the organism. Failure of A. flavus to grow on this synthetic medium is most probably to be attributed to vitamin defficiency rather than unsuitability of the primary components.

The conversion of leucine and isoleucine to aspergillic acid involves a dehydration and an oxidation

reaction expressed empirically.

$$2C_6H_{13}O_2N + O \longrightarrow C_{12}H_{20}O_2N_2 + 3H_2O$$

The isolation of flavacol may have some bearing on this reaction, showing as it does that A. flavus is able to synthesise a hydroxypyrazine. This suggests that deoxyaspergillic acid may be the precursor of aspergillic acid, that is, that the oxidation reaction occurs after cyclisation. That an isomer of aspergillic acid, 1-hydroxy-2-keto-3:6-di-iso-butyl-1:2-dihydropyrazine (XCVIII) is not also produced from flavacol must be attributed to the specificity of the oxidation reaction,

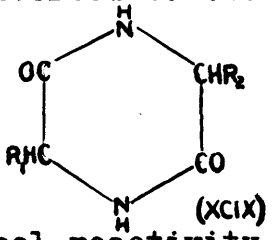


(XCVIII)

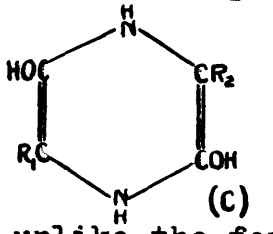
Although biogenetic formation of deoxyaspergillic acid seems probable, the prior mode of cyclisation is doubtful. The obvious mechanism, through intermediate formation of the diketopiperazine leucyl-isoleucine anhydride is improbable, as conclusive evidence of the biosynthesis of 2:5-diketopiperazines is rare. At one time considered to form part of many protein molecules, it has since been accepted that the isolation of

2:5-diketopiperazines from protein hydrolysates results from secondary condensation of α-amino-acid residues (107). It has, further, been shown that 2:5-diketopiperazines cannot be metabolised by animals (108) or micro-organisms (109).

Abderhalden has shown that α-amino-acid anhydrides can exist in two distinct isomeric forms, the true diketopiperazine (XCIX) and the enol-acid anhydride (C) (110). The 2:5-diketopiperazine can, by physical means (111), be converted to the enol form which shows greatly enhanced



(XCIX)

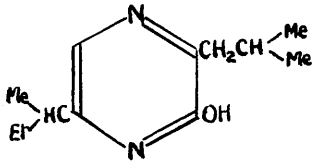


(C)

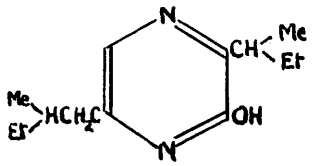
chemical reactivity (112) and, unlike the former is not resistant to enzymic attack. This enol-acid anhydride (C;R_1=<u>iso</u>-butyl,R_2-<u>sec</u>.-butyl) therefore may be the primary cyclic product in the biosynthesis of aspergillic acid, its structure as a dihydropyrazine suggesting that it would readily be converted, by loss of water, to the hydroxypyrazine deoxyaspergillic acid.

SECTION II.

This section is devoted to model experiments the object of which was to investigate synthetic routes to racemic deoxyaspergillic acid and racemic aspergillic acid. Considering firstly the synthesis of racemic deoxyaspergillic acid which has either the structure (CI) or (CII) the problem was one of developing a

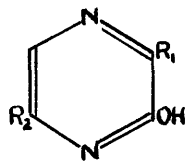


(CI)



(CII)

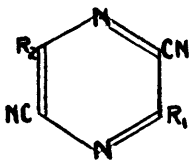
synthesis of 3-hydroxy-2:5-dialkylpyrazines in which the two alkyl groups are dissimilar. Having developed such a synthesis it would be expected that its application to appropriate starting materials would give either of the above structural forms. The methods available for the synthesis of 3-hydroxy-2:5-dialkyl-pyrazines will therefore be examined and their possible application to the synthesis of the general form (CIII) considered.



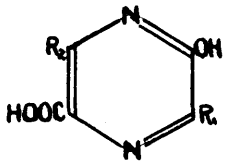
(CIII)

By treatment of the bisulphite compound of isonitroso-acetone with potassium cyanide followed by hydrochloric acid, Gastaldi (113) obtained 3:6-dicyano-2:5-dimethylpyrazine (CIV, R_1=R_2=Me) which on alkaline

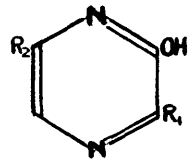
hydrolysis gave the hydroxyacid (CV) the latter on decarboxylation giving 3-hydroxy-2:5-dimethylpyrazine (CVI, R_1=R_2=Me). The same series of reactions applied to the bisulphite compound of isonitroso-acetophenone



(CIV)



(CV)

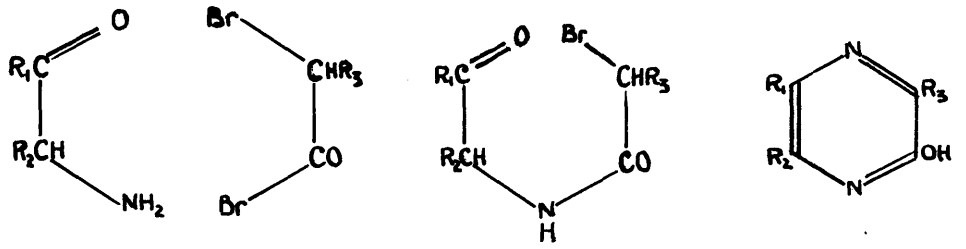


(CVI)

gave 3-hydroxy-2:5-diphenylpyrazine (CVI,R_1=R_2=Ph). The Gastaldi synthesis applied to the bisulphite compound of isonitroso methyl ethyl ketone (114) gave 3-hydroxy-2:5-diethylpyrazine (CVI,R_1=R_2=Et).. Golombok and Spring (115) attempted to extend the Gastaldi synthesis to the preparation of 3-hydroxy-2:5-dialkylpyrazines in which the alkyl groups were dissimilar. By reaction of potassium cyanide with a mixture of the bisulphite compounds of isonitroso-acetone and isonitroso-acetophenone with the bisulphite compound of a mixture of isonitroso-acetone and isonitroso-acetophenone they were unable to isolate any 3:6-dicyano-2-methyl-5-phenylpyrazine (CIV; R_1=Me, R_2=Ph) but obtained only 3:6-dicyano-2:5-dimethylpyrazine (CIV, R_1=R_2=Me) and 3:6-dicyano-2:5-diphenylpyrazine (CIV, R_1=R_2=Ph).

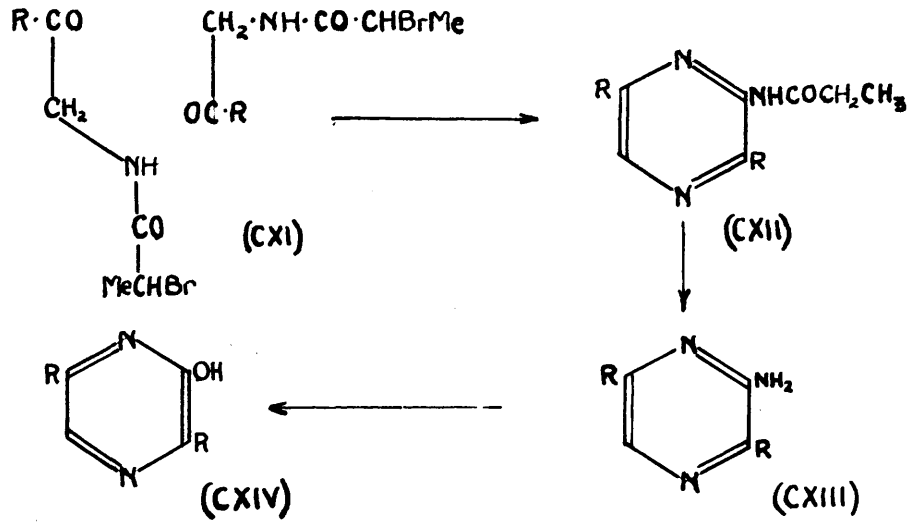
By treatment of the α-bromacyl-derivative of an α-amino-ketone (CIX) with ammonia Tota and Elderfield

(116) obtained hydroxypyrazines. By this method 6-hydroxy-2:3-dialkylpyrazines (CX, R_3=H) and 2-hydroxy-3:4:5-trialkylpyrazines (CX) were obtained.



(CVII) (CVIII) (CIX) (CX)

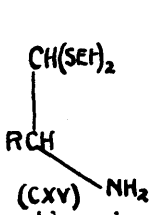
A modification of this method was adopted by Newbold, Spring and Sweeny (117) in which an α-amino-methyl ketone (CVII, R_2=H) was used in attempting to prepare 2-hydroxy-3:5-dialkylpyrazines (CX, R_2=H), but instead they obtained 3-acylamino-2:5-dialkylpyrazines which were readily converted to 3-hydroxy-2:5-dialkyl-pyrazines. α-Brompropionyl bromide (CVIII, R_3=Me) reacted with aminomethyl ethyl ketone (CVII; R_1=Et, R_2=H) to give (CXI, R=Et), two molecules of which reacted with one molecule of ammonia to give (CXII, R=Et). The last mentioned was readily hydrolysed to the amino-pyrazine (CXIII, R=Et) which on treatment with nitrous acid gave 3-hydroxy-2:5-diethylpyrazine (CXIV, R=Et). The same reaction was used to prepare 3-hydroxy-2:5-dimethylpyrazine (CXIV, R=Me) and 3-hydroxy-2:5-diphenyl-pyrazine (CXIV, R=Ph). Extension of this reaction to the preparation of racemic deoxyaspergillic acid would



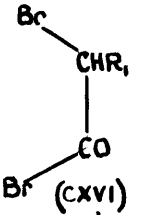
involve cyclisation of a mixture of two α-bromacylamino-ketones and would at best be expected to give the desired product in association with a mixture of the two symmetrically substituted 3-hydroxy-2:5-dialkyl-pyrazines.

Another modification of the method of Tota and Elderfield, in which an α-amino-aldehyde was substituted for an α-amino-ketone has been developed (118) as a synthetic route to 3-hydroxy-2:5-dialkylpyrazines, the carbonyl group being protected as a thioacetal group until the final stage of the reaction. The diethyl acetal of α-amino-propionaldehyde (CXV, R=Me) condensed with α-brompropionyl bromide (CXVI, R_1=Me) to give the intermediate (CXVII) treatment of which with mercuric chloride and cadmium carbonate resulted in the liberation of the free aldehyde which cyclised with ammonia to give 3-hydroxy-2:5-dimethylpyrazine (CXVIII, R=R_1=Me). This

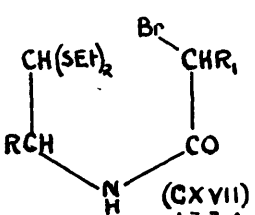
route could obviously be used for the unambiguous



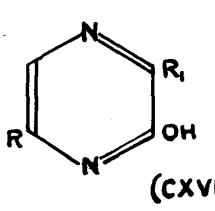
(CXV)



(CXVI)



(CXVII)



(CXVIII)

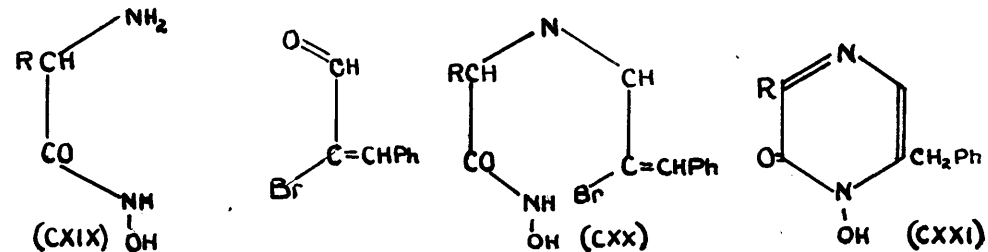
synthesis of racemic deoxyaspergillic acid and is at present being investigated by Mr. Sweeny.

Baxter and Spring (53,p. 19) prepared 3-hydroxy-2:5-dialkylpyrazines, in which both alkyl groups were the same, by the reaction of phosphoryl chloride with α-amino-acid anhydrides and hydrolysis of the resulting chloropyrazines. Mr. J. J. Gallagher has investigated the application of this reaction to mixed α-amino-acid anhydrides. Treatment of DL-leucyl-DL-isoleucine anhydride (p. 41) with phosphoryl chloride gave a hydroxy-2-iso-butyl-5-sec.-butylpyrazine of m.p.97°, which differed from racemic deoxyaspergillic acid. This is an ambiguous synthesis the location of the hydroxyl group at the 3-or-6-position being indeterminate.

The synthesis of racemic 3-hydroxy-2:5-di-sec.-butylpyrazine by Newbold and Spring (48) was outlined (p. 15). Extension of this synthesis to give mixed dialkyl-hydroxypyrazines would involve the condensation of different amino-ketones and would not be unidirectional.

Mr. Ramsay investigating pyrazine cyclic hydroxamic acids has developed a synthesis of 1-hydroxy-2-keto-

3:6-disubstituted-1:2-dihydropyrazines in which the substituents need not be similar. Condensation of α-bromcinnamaldehyde with glycine-hydroxamic acid (CXIX,R=H) gave the Schiff base (CXX,R=H) which cyclised and aromatised on treatment with sodium ethoxide to give

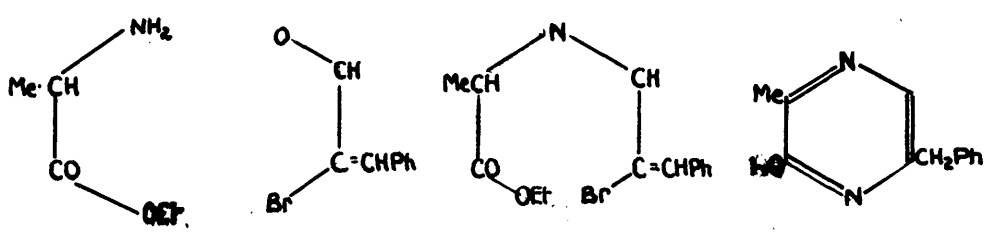


(CXIX) (CXX) (CXXI)

1-hydroxy-2-keto-6-benzyl-1:2-dihydropyrazine (CXXI,R=H). The same series of reactions starting from α-amino-n-butyro-hydroxamic acid (CXIX,R=Et) gave 1-hydroxy-2-keto-3-ethyl-6-benzyl-1:2-dihydropyrazine (CXXI, R=Et). This synthesis lending itself, as it does, to the synthesis of cyclic hydroxamic acids related to aspergillic acid, suggested analogous syntheses of compounds related to deoxyaspergillic acid by use of α-amino-acid amides in lieu of α-amino-hydroxamic acids.

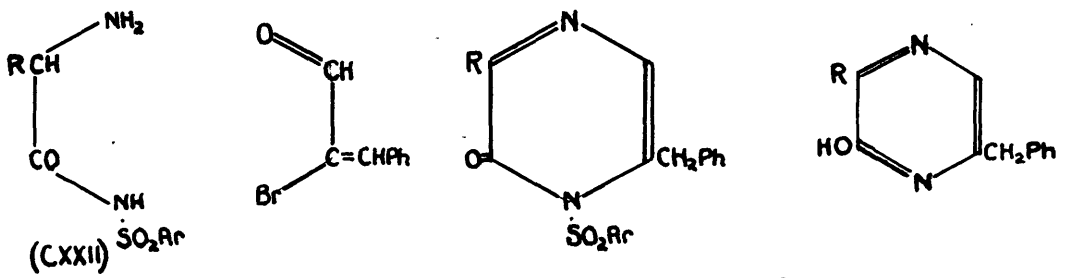
Initial investigation was carried out on the reaction between glycine amide and α-bromcinnamaldehyde. The former was prepared by the method of Yang and Rising (119) from glycine ester prepared by Fischer's method (77). α-Bromcinnamaldehyde was readily prepared from cinnamaldehyde by the method of Zincke and Hagen (120). Reaction of this aldehyde with glycine amide in boiling

ethanol was associated with considerable darkening in colour, in contradistinction to the clean interaction of this aldehyde with glycine-hydroxamic acid (121), and the Schiff base could not be isolated from the resulting tar. Allowing the reaction to proceed at lower temperature did not overcome this tar formation. As the Schiff base could not be isolated the reaction product was treated directly with sodium ethoxide and worked up for hydroxypyrazine but none was isolated, nor was any identifiable product obtained. Attention was next turned to the use of substituted glycine amide as it was realised that unsubstituted methylene groups, in the potential pyrazine ring, frequently cause anomalous reaction (p. 75). DL-Alanine amide prepared from DL-alanine ester (77) was therefore selected and subjected to the same series of reactions as glycine amide but with the same lack of success. In a similar manner DL-alanine ester and α-bromcinnamaldehyde gave an intractable reaction mixture which was heated with ammonia in view of the possibility of effecting the synthesis by the alternative route indicated. The reaction mixture, however, gave no hydroxy-pyrazine. As distinct from the



reaction with α-bromcinnamaldehyde, DL-alanine amide reacted smoothly with cinnamaldehyde to give cinnamylidene-DL-alanine amide m.p.154-155°. This Schiff base was readily hydrolysed by dilute mineral acid; on standing with Brady's reagent the 2:4-dinitrophenylhydrazone of cinnamaldehyde separated almost at once.

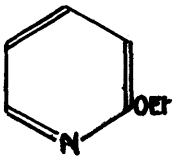
This disparity in results between condensation of α-bromcinnamaldehyde with α-amino-acid amides on the one hand and α-amino-hydroxamic acids on the other was attributed to the acidity of the latter. It was therefore considered desirable to investigate the reaction between α-bromcinnamaldehyde and α-aminoacyl-sulphonamides in the hope that the acidity due to the sulphonyl group would enforce the condensation, and at a later stage be removable by hydrolysis according to the scheme



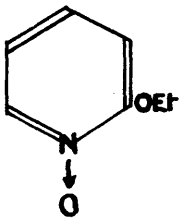
N-(α-Brompropionyl)-toluene-p-sulphonamide was prepared by a modification of the method of Openshaw and Spring (122) for the preparation of sulphonacetamides, α-brompropionyl bromide condensing with toluene-p-sulphonamide to give in 75% yield the bromacylsulphonamide,

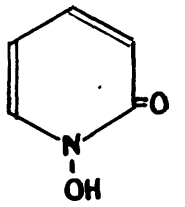
treatment of which with aqueous or ethanolic ammonia at room temperature gave N-(DL-alanyl)-toluene-p-sulphonamide (CXXII; Ar=p-tolyl,R=Me) m.p.234-235° (decomp.) in almost quantitative yield. With liquid ammonia in a sealed tube at slightly elevated temperature ammonolytic fission occurred and toluene-p-sulphonamide was regenerated. N-(DL-Alanyl)-toluene-p-sulphonamide after prolonged refluxing with α-bromcinnamaldehyde in ethanol was recovered unchanged. More drastic conditions caused the formation of an intractable tar.

Concurrent with these experiments, possible synthetic routes to compounds related to aspergillic acid were examined. There were two general approaches to this problem; firstly to obtain the cyclic hydroxamic acid by oxidation of preformed pyrazine derivatives and secondly to evolve a synthesis in which the final ring closure involves simultaneous formation of the pyrazine ring and the hydroxamic acid grouping. The former approach has been examined (123). 2-Ethoxypyridine (CXXIII) and 2-ethoxyquinoline being peroxidised to the corresponding N-oxides, acid hydrolysis of which gave the pyridine (CXXIV) and quinoline cyclic hydroxamic acids. Application of this method to2-ethoxypyrazine (124) and 2-ethoxy-quinoxaline (125) results however in oxidation of the



(CXXIII)





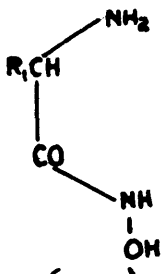
(CXXIV)

nitrogen atom remote from the ethoxy group. The peroxidation of 3-ethoxy-6-cyano-2:5-dimethylpyrazine has been examined (115) to find if the cyano group would enforce 4-oxidation, that is, oxidation of the nitrogen atom adjacent to the ethoxy group. The cyano-compound did give an N-oxide but subsequent hydrolysis caused total rupture of the molecule.

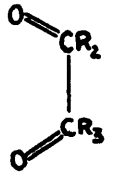
On the other approach to the synthesis of pyrazine cyclic hydroxamic acids, viz. by simultaneous formation of the pyrazine ring and the hydroxamic acid grouping there was no information in the literature. The synthesis of certain pyrazine cyclic hydroxamic acids by Mr. Ramsay has been outlined (p.68) and is being developed with the object of synthesis of aspergillic acid.

A further synthesis of pyrazine cyclic hydroxamic acids based on simultaneous formation of the pyrazine ring and the hydroxamic acid grouping has been developed in collaboration with Dr. Newbold and Mr. Sweeny. This synthesis consists of reacting α-amino-hydroxamic acids

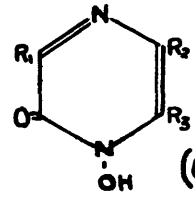
with α-dicarbonyl compounds, a development of the method of Jones (126) for the preparation of 2-hydroxy-3:5-disubstituted and 2-hydroxy-3:5:6-trisubstituted-pyrazines, from α-amino-acid amides and α-dicarbonyl compounds. Treatment of glycine-hydroxamic acid (CXXV, R_1=H) prepared after Jones and Sneed (127) with phenyl glyoxal (CXXVI; R_2=Ph, R_3=H) under essentially the conditions of Jones (loc.cit.) gave in very low yield 1-hydroxy-2-keto-5-phenyl-1:2-dihydropyrazine (CXXVII; R_1=R_3=H, R_2=Ph) m.p.194-196°(decomp.). With either methyl glyoxal



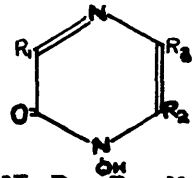
(CXXV)



(CXXVI)



(CXXVII)



(CXXVIII)

(CXXVI; R_2=Me, R_3=H) or diacetyl (CXXVI, R_2=R_3=Me) and glycine-hydroxamic acid (CXXV, R_1=H) there was indication of cyclic hydroxamic acid formation. In small yield in each case, a product was obtained which sublimed in high vacuum and gave the colour reaction of hydroxamic acids with ferric chloride reagent. In neither case however was sufficient material obtained for analysis.

Dr. Newbold and Mr. Sweeny have prepared a number of 3:5-disubstituted (CXXVII, R_3=H) and 3:5:6-trisubstituted-

1-hydroxy-2-keto-1:2-dihydropyrazines (CXXVII) using the appropriate starting materials. In all the cases they examined using α-keto-aldehydes (CXXVI, R_3=H) as the dicarbonyl compound they have shown that of the two possible products (CXXVII, R_3=H) and (CXXVIII, R_3=H) the unsymmetrical isomer is formed to the exclusion of the other. The structure of 1-hydroxy-2-keto-5-phenyl-1:2-dihydropyrazine could not be rigorously established but was implied from these results. In the condensations examined by the author between glycine-hydroxamic acid and α-dicarbonyl compounds, the yields were extremely low in comparison with the favourable yields observed by Dr. Newbold and Mr. Sweeny, using homologues of glycine-hydroxamic acid (CXXV, R_1=Me or Ph). Anomalous results have previously been observed where the first member of the series has been subjected to typical reactions. Thus glycine anhydride or pyrazine-N-oxide on treatment with phosphoryl chloride does not give the anticipated chloropyrazine derivatives (127).

EXPERIMENTAL

EXPERIMENTAL

All melting points are uncorrected.

SECTION I.

Aspergillic Acid.

(Adapted from White and Hill, J.Bact.,1943,45,433)

(a) The culture medium consisted of 2% "Pronutrin" (a commercial enzymic digest of casein prepared by Herts Pharmaceuticals Ltd.) and 2% sodium chloride dissolved in distilled water. Sterilisation was by steam autoclaving (15 lbs., 15 minutes), the resulting solution being pale yellow in colour and containing a small quantity of grey sediment. After autoclaving the solution was slightly acid (pH 5·4-5·6). The inoculum was prepared from beer wort agar slopes of Aspergillus flavus which had been incubated 10 days at 25°. Spores from two such slopes were added to sterile distilled water (100ml.) and shaken to give a uniform spore suspension. The medium was dispensed (350ml.) in standard penicillin flasks, giving a layer 1·5 cm. deep, and after sterilisation was inoculated with the standard inoculum (5ml.). The flasks were incubated at 23 ± 1° for 14 days (optimum time for maximal antibacterial titre; Gowenlock, private communication). Growth was scanty, isolated colonies appearing after 48 hours and after 7 days the surface was covered

by a thin white mycelial membrane. About the twelfth day there was slight sporulation. During incubation there developed a bright orange pigment, which settled to the bottom of the flask. In a typical batch of 100 flasks the mycelial matter was removed and the bulked culture filtrate (33l.; $\underline{p_H}$,7·8) was adjusted to $\underline{p_H}$ 4 by addition of dilute hydrochloric acid. Acidification precipitated much of the suspended matter as a reddish-brown sludge; this was filtered and the filtrate stirred with charcoal (660g.) for 3 hours. The charcoal was filtered, air-dried and ether extracted (Soxhlet) until the initially red percolate was colourless. The bright red ethereal solution was concentrated to about 250ml. and shaken with 2N-sodium hydroxide (150ml.). The brown amorphous solid which separated from the mixture was filtered and the layers separated. The ether layer was re-extracted with 2N-sodium hydroxide (150ml.) and the alkaline extracts bulked. The yellow alkaline solution was acidified with 33% aqueous acetic acid and chilled overnight. The solidified gum which had separated was filtered and shaken with 2% sodium hydrogen carbonate solution (400ml.) for 6 hours and filtered from gummy residue (solid A). The chilled sodium hydrogen carbonate solution was acidified to $\underline{p_H}$4 by dropwise addition of

3N-hydrochloric acid, with stirring, over several hours. The precipitated aspergillic acid was filtered and dried over phosphoric oxide to a free-running microcrystalline yellow powder m.p.80-85° (8g., 250mg./l of culture filtrate). Crude aspergillic acid was purified by re-dissolving in aqueous sodium hydrogen carbonate and precipitating with dilute hydrochloric acid at $\underline{p_H}4$, followed by sublimation at 80°/0·001mm. and repeated crystallisation from methanol. This gave aspergillic acid m.p.97-99° as radial clusters of yellow needles.

Found: C=64·2,64·5; H=8·90,8·91; N=12·3%

Calc. for $C_{12}H_{20}O_2N_2$: C=64·3, H=8·93, N=12·5%

$[\alpha]_D^{\eta}$ +13·3° (1,1,c,3·9, in ethanol)

Light absorption in ethanol: Maxima at 2340Å, ϵ =10,500;

3280Å, ϵ =8500.

pka =5·95.

(b) The conditions were the same as previously described except that the culture medium contained 2% "Difco" tryptone instead of 2% "Pronutrin". The cultural characteristics differed in that less pigment was produced. The culture filtrate on being worked up in the usual manner gave similar yields of aspergillic acid. The purified product m.p.96-98° was undepressed in melting point with a specimen described above.

Isolation of Flavacol.

The sodium hydrogen carbonate insoluble gum (solid A from preparation of aspergillic acid) was shaken with 2N-sodium hydroxide (20ml.) for 1 hour and filtered from gummy residue. The solution was neutralised (litmus) with dilute hydrochloric acid and extracted with ether (3 x 15 ml.). The combined ether extract was shaken with 3N-hydrochloric acid (3 x 10 ml.) and the acidic extract neutralised (litmus) with dilute sodium hydroxide. The precipitate which separated was filtered and sublimed in high vacuum (80°/0·005mm.). After two crystallisations from aqueous ethanol the product separated as needles m.p.120-130°(50mg.). On repeated crystallisation from aqueous ethanol pure flavacol was obtained as long needles mp.144-146°.

Analysis of specimens from different batches.

Found: C=68·9,68·9,69·0; H=9·35,9·55,9·54; N=13·7%

$C_{12}H_{20}ON_2$
requires: C=69·2, H=9·62, N=13·5%

Light absorption in ethanol: Maxima at 2280Å, ϵ =7300;
3260Å, ϵ =7900.

Flavacol was insoluble in cold water and aqueous sodium hydrogen carbonate. It dissolved readily in ether, methanol, ethanol, 3N-hydrochloric acid and 3N-sodium

hydroxide in the cold. It did not give a colouration with ferric chloride solution. The compound sublimed readily at 100°/0·001mm.

Growth of A. flavus on a Culture Medium containing Brown Sugar.

The culture medium consisted of 2% "Pronutrin", 2% brown sugar (Demerara type) and 0·5% sodium chloride dissolved in distilled water. Sterilisation, volume of medium per flask and inoculum were as in the production of aspergillic acid. The sterilised medium was slightly acid (p_H 6·2). The flasks were incubated at 23±1° for seven days (optimum time for maximal antibacterial titre; Gowenlock, private communication). There was prolific growth such that, after 48-72 hours the entire surface was covered by a thick white mycelial mat, and after five days the surface was covered with golden spores. The culture filtrate (p_H7·5) was worked up as described for aspergillic acid. The product could not be purified to constant melting point, continued crystallisation caused progressive rise in melting point.

Analysis of a sample of this material m.p.106-110°, $[\alpha]_D^{17}$ +13.7° (1,1,c,3·8, in ethanol)

Found: C=63·4, H=8·58%

Calc. for $C_{12}H_{20}O_2N_2$: C=64·3, H=8·93%

Calc. for $C_{12}H_{20}O_3N_2$: C=60·0, H=8·33%

After repeated crystallisation from methanol the product had m.p.130-132°.

Dried for analysis this specimen had m.p.135-137°.

Found: C=61·8, H=8·5%

Calc. for $C_{12}H_{20}O_2N_2$: C=64·3, H=8·9%

Calc. for $C_{12}H_{20}O_3N_2$: C=60·0, H=8·3%

Hydroxyaspergillic acid has m.p.149° (Menzel, Wintersteiner and Rake, J.Bact., 1943, 46, 109).

Kojic Acid.

Kojic acid was isolated from one batch of A. flavus grown on a medium of 2% "Pronutrin", 2% brown sugar and 0·5% sodium chloride. Cultural conditions were as previously described for growth of A. flavus on this medium. The culture filtrate (5 l.) was adjusted to p_H4 with dilute hydrochloric acid, stirred with charcoal (100g.) for several hours, and the charcoal air-dried. The charcoal was ether extracted (Soxhlet) and the ether extract concentrated to 150ml. The ether insoluble solid was filtered (2·1g., 400mg./l. of culture filtrate) and, crystallised from methanol, separated as brown coloured blades m.p.150-152° (880mg.), 2nd Crop m.p.148-151°(750mg.). After sublimation at 130°/0·0001mm. and crystallisation of the sublimate from ethanol, kojic acid separated as blades m.p.151-153°. The melting point was not raised

by further crystallisation.

Found: C=50·8, 50·9; H=4·30, 4·55%

Calc. for $C_6H_6O_4$: C=50·7, H=4·23%

Equivalent Weight by titration with barium hydroxide (phenolphthalein) 164,166. Theory 142.

Light absorption in ethanol: Maximum at 2710Å, ε =7000.

The melting point was undepressed on admixture with a specimen of authentic kojic acid supplied by Dr. A.H. Cook.

Attempted production of Aspergillic Acid on a Synthetic Medium.

The medium consisted of 1% DL-leucine, 1% DL-iso-leucine, 0·02% L-tryptophane and 0·5% sodium chloride. The medium after sterilisation was water clear and had p_H6·4. Inoculum and incubation were as described for production of aspergillic acid. After 14 days the only evidence of growth was a slight turbity of the culture solution. On working up as described for aspergillic acid there was no hydroxamic acid as evidenced by a negative ferric chloride test on the neutralised alkaline extract of the ether percolate.

Synthesis of 3-Hydroxy-2:5-di-iso-butylpyrazine.

DL-Leucine.

DL-Leucine was prepared from isohexanoic acid most conveniently by the method of Org. Syn., Vol.21, p.74.

isoHexanoic acid (250g.) gave DL-leucine (90g., 32% theory) m.p.275-278° (sealed tube).

DL-Leucine Anhydride.

DL-Leucine (30g.) was heated under reflux with ethylene glycol (180ml.) on an oil bath. After 20 minutes solution was complete and heating was continued for a further 90 minutes, the solution darkening slightly in colour. After standing 24 hours at 0° the solid was separated, washed with cold water (2 x 150ml.) and cold ethanol (2 x 150ml.) and dried over phosphoric oxide (Yield 14·6g., 58% theory) Crystallisation from ethanol gave DL-leucine anhydride as needles m.p.267-270°(sealed tube).

Found: C=63·5; H=9·70; N=12·7%

Calc. for $C_{12}H_{22}O_2N_2$: C=63·7; H=9·73; N=12·4%

Fischer (Ber.,1901,34,433) gives m.p.271°(corr.) for DL-leucine anhydride prepared from DL-leucine ethyl ester.

3-Hydroxy-2:5-di-iso-butylpyrazine.

(a) DL-Leucine anhydride (5g.) was refluxed for 1 hour with phosphoryl chloride (50ml.) in which it dissolved after a few minutes. The excess phosphoryl chloride was removed under reduced pressure from the red reaction solution. The semi-solid residue was treated with water (50ml.) and the mixture neutralised (litmus) with 2N-sodium hydroxide. The solution was extracted with ether (5x50ml.)

and the combined extract was dried (calcium chloride) and the ether evaporated. The residue partly crystallised, the solid was separated (filtrate A) and washed with small portions of chilled ether. Recrystallisation from ethanol yielded 3-hydroxy-2:5-di-iso-butylpyrazine as long needles m.p.144·5-147°. The yield was 700mg. at m.p.125-143°.

Found:	C=69·4;	H=9·70;	N=13·7%
$C_{12}H_{20}ON_2$ requires:	C=69·2;	H=9.62;	N 13·5%

Light absorption in ethanol: Maxima at 2290Å, ε =6900;
3260Å, ε =7600.

3-Hydroxy-2:5-di-iso-butylpyrazine was insoluble in water and sodium hydrogen carbonate solution but was soluble in 3N-hydrochloric acid and in 3N-sodium hydroxide. It did not give a colouration with ferric chloride reagent and sublimed at 120°/0·01mm.

A mixture of 3-hydroxy-2:5-di-iso-butylpyrazine m.p.144·5-147° with flavacol melted at 144-146·5°.

(b) Filtrate A.

The ether was removed and the residual oil distilled under reduced pressure. The distillate a colourless oil (2g.) was redistilled and the fraction b.p.117-119°/4mm. was collected.

Found: C=59·7; H=7·62; N=11·0; Cl=21·15%

$C_{12}H_{19}N_2Cl$ requires: C=63.6; H=8.4 ; N=12.4; Cl=15.7%

$C_{12}H_{18}N_2Cl_2$ requires: C=55.2; H=6.9; N=10.7; Cl=27.2%

The oil (1·5g.) was heated in an autoclave with an ethanolic solution of sodium ethoxide (from 0·6g. sodium and 25ml. dry ethanol) for 4 hours at 180°. To the reaction mixture water (50ml.) was added and the solution extracted with ether (3 x 20ml.). The ether extracts were combined, the solvent removed and the residual oil refluxed with 6N-hydrochloric acid (10ml.) for 24 hours. The solution was neutralised (litmus) with 3N-sodium hydroxide and extracted with ether (3 x 20ml.). The ether solution was dried (sodium sulphate), the solvent removed and the residue sublimed in high vacuum (100°/0·01mm.). The sublimate (250mg.) crystallised from aqueous ethanol to give 3-hydroxy-2:5-di-iso-butylpyrazine as needles undepressed with a specimen described above.

No other identifiable product was isolated from this reaction.

Attempted Mixed Crystal formation between 3-Hydroxy-2:5-di-iso-butylpyrazine and 3-Hydroxy-2:5-di-sec.-butylpyrazine.

3-Hydroxy-2:5-di-isobutylpyrazine (40mg.) and 3-hydroxy-2:5-di-sec.-butylpyrazine (40mg.) were dissolved together in ethanol. Sufficient water was added to cause precipitation

of ca.50% of the dissolved material, the mixture was heated until totally dissolved and allowed to cool. The crystalline fraction which separated melted over the range 100-130°. By addition of water to the mother liquor a second crop was obtained m.p.83-108°. Further crystallisation of either of these crops did not give material of sharp melting point.

Reaction of Copper Chromite with Aspergillic Acid.

(cf. Dutcher and Wintersteiner, J.Biol.Chem.,1944,135,359, Dutcher, J.Biol.Chem.,1947,171,321).

Aspergillic acid (1·61g.) and copper chromite (3·22g. Org. Syn., Vol.19, p.31) were intimately mixed, heated at 200° over 30 minutes and maintained at that temperature for 1 hour. A trace of sublimate formed on the cold surface of the vessel. The reaction mixture on cooling was extracted with ether (Soxhlet). The extract was grass-green in colour and from it separated on concentration to small bulk copper aspergillate (1·6g.) of m.p.200-202°. Dutcher, loc.cit. gives for aspergillic acid copper salt m.p.204-207°.

Deoxyaspergillic Acid.

(cf. Dutcher and Wintersteiner, J.Biol.Chem.,1944,155,359, Dutcher, J.Biol.Chem.,1947,171,321)

Aspergillic acid (2g.; m.p.85-92°) was dissolved in a

solution of 100% hydrazine hydrate (2ml.) and ethanol (60ml.) and the mixture heated in an autoclave for 9 hours (until the reaction mixture gave no colouration with ferric chloride reagent) at 170°. The solution was evaporated to dryness *in vacuo* on a boiling water bath and the brown gummy residue dissolved in ether (100ml.) and filtered from insoluble material. The ether solution was extracted with 3N-hydrochloric acid (5 x 10ml.) and the acidic extract neutralised (litmus) with 3N-sodium hydroxide. The precipitate was filtered, dissolved in ether (100ml.) and extracted with 3N-sodium hydroxide (5 x 10ml.). The solution was neutralised (litmus) with 3N-hydrochloric acid, the precipitate filtered, dried and sublimed at 100°/0.001 mm. The colourless sublimate (1.15g., 62% theory) m.p.91-96° crystallised from aqueous methanol to give deoxyaspergillic acid as irregular needles m.p.98-100°. It was not depressed in melting point on admixture with a specimen of deoxyaspergillic acid provided by Dr. O. Wintersteiner.

Found: C=69·3,69·35; H=9·27,9·61; N=13·4%

Calc. for $C_{12}H_{20}ON_2$: C=69·2; H=9·62; N=13·5%

$[\alpha]_D^{18}$ +21·3° (c,2·55; 1,1; in ethanol)

Light absorption in ethanol: Maxima at 2280Å, ϵ=7500;
3250Å, ϵ ≈8000.

Racemisation of Deoxyaspergillic Acid.

Deoxyaspergillic acid (260mg.; m.p.93-97°, $[\alpha]_D$ + 21·2°) and N-potassium hydroxide (12·5ml.) were heated together in an autoclave at 170° for 24 hours. The resulting solution was neutralised (litmus) with dilute hydrochloric acid and the precipitate which separated was filtered. Crystallisation from aqueous methanol gave racemic deoxyaspergillic acid as needles m.p.102-104°(180mg.). Recrystallisation from the same solvent did not cause further elevation of the melting point.

Found: C=69·0; H=9·57%

$C_{12}H_{20}O_4N_2$ requires: C=69·2; H=9·62%

Light absorption in ethanol: Maxima at 2280Å, ε =7000; 3250Å, ε =8400.

r-Deoxyaspergillic acid gave 2·65 mole C-Methyl per mole (Kuhn-Roth). A mixture of r-deoxyaspergillic acid m.p.102-104° and deoxyaspergillic acid m.p.98-100° melted at 100-102°.

A mixture of r-deoxyaspergillic acid and 3-hydroxy-2(5)-iso-butyl-5(2)-sec.-butylpyrazine m.p.95-96° melted at 90-93°.

3-Hydroxy-2:5-di-sec.-butylpyrazine (80mg.) was treated with N-potassium hydroxide (4ml.) as detailed above. On working up as for racemic deoxyaspergillic acid starting material was recovered m.p.121-123°.

A mixture of 3-hydroxy-2:5-di-sec.-butylpyrazine m.p.122-123° and r-deoxyaspergillic acid melted at 80-85°.

Attempted Racemisation of Aspergillic Acid.

Aspergillic acid (300mg., m.p.95-96°, $[\alpha]_D^{17}$ + 13·0°) was dissolved in N-potassium hydroxide (15ml.) and the solution autoclaved at 170° for 24 hours. The solution was filtered and adjusted to p_H4 with 3N-hydrochloric acid. The aspergillic acid which separated was filtered, dissolved in hot aqueous ethanol and allowed to crystallise. The recovered aspergillic acid had m.p.91-94°.

$[\alpha]_D^{19}$ + 10·5 ± 4° (c,1·15; 1,1, in ethanol)

Phenylazo-deoxyaspergillic acid.

A solution of aniline (1·2ml.) in hydrochloric acid (10·5ml., S.G.1·19 and water 10·5ml.) was treated with saturated sodium nitrite solution below -3° until a positive test was given with starch-potassium iodide indicator. The resulting benzene diazonium chloride solution was added, dropwise with shaking, to deoxy-aspergillic acid (700mg.) dissolved in ice-cold 3N-sodium hydroxide (35ml.). The yellow precipitate which formed was suspended in warm water (30ml.) and made just acid (congo red) with dilute hydrochloric acid with vigorous shaking. The product which separated was filtered,

dissolved in ethanol, warmed with charcoal and filtered. To the hot alcoholic solution water was added until opaque. On standing phenylazo-deoxyaspergillic acid separated (870mg.; 83% theory) m.p.183-187°. Crystallised from aqueous ethanol it separated as red needles m.p.188-190°(decomp.).

Found: C=69·4; H=7·37; N=18·1,17·8%

$C_{18}H_{24}ON_4$ requires: C=69·2; H=7·69; N=17·95%.

The phenylazo-derivative of r-deoxyaspergillic acid was prepared as above (yield 75%). Crystallised from aqueous ethanol it separated as red needles m.p.188-190°(decomp.).

Found: N=18·1%

$C_{18}H_{24}ON_4$ requires: N=17·95%

Reduction of Phenylazo-deoxyaspergillic Acid.

A solution of phenylazo-deoxyaspergillic acid (820mg.) in ethanol (75ml.) was hydrogenated at ordinary pressure using platinic oxide catalyst (Org.Syn.,Col.Vol.1,p.452; 40mg.). The absorption of hydrogen ceased after 10 hours (203ml.corr.; 1·73 mol.). The catalyst was filtered and the residual yellow solution evaporated under reduced pressure leaving an orange coloured oil. On addition of a little aqueous alcohol and standing there separated a solid which was filtered and washed with a few drops of

ethanol. The solid was dissolved in hot aqueous ethanol, treated with charcoal, and on standing there separated the product as orange needles m.p.138-140° (80mg.).

Found: C=63·9; H=9·01; N=18·8%

$C_{12}H_{25}ON_3$ requires: C=63·4; H=11·0; N=18·5%

The product sublimed unchanged at 100°/0·01 mm.

Attempted oxidation of 3-Chloro-2:5-dimethylpyrazine to 3-Chloro-pyrazine-2:5-dicarboxylic Acid.

To a stirred solution of 3-chloro-2:5-dimethyl-pyrazine (1·425g., 0·01 mole., prepared after Baxter and Spring, J.,1947,1179) in 3% potassium hydroxide (6ml.) at 70° was added a warm solution of potassium permanganate (6·32g., 0·04 mole.) in water (100ml.) by dropwise addition over 90 minutes. The mixture was maintained at 70° for 30 minutes with continued stirring. The residual permanganate was destroyed with sulphur dioxide. The manganese dioxide was filtered and the filtrate made acid (congo red) with dilute hydrochloric acid. The solution was concentrated in vacuo to about 20ml. and allowed to stand at 0°; there was no separation of solid. The solution was extracted with ether (5 x 20ml.) and the extract dried (sodium sulphate). On removal of the solvent there was no residue.

Chromium Trioxide oxidation of Aspergillic Acid and Deoxyaspergillic Acid.

The glacial acetic acid used in these experiments was freed from oxidisable impurity by distillation from chromium trioxide.

(a) To a standard solution of chromium trioxide in glacial acetic acid (46ml., 11·05m.mole. available oxygen by iodine titration) in a flask fitted with reflux condenser was added a solution of aspergillic acid (1g., 4·42m.mole.) in glacial acetic acid (20ml.). A heavy flocculent precipitate formed immediately. The flask was heated on a boiling water bath, the precipitate quickly dissolved and oxidation, as evidenced by the colour change, red to green, was rapid. After 20 minutes heating the solution was distilled under slight vacuum (ca.15mm.) the first 12ml. being collected. To the distillate was added a few drops of a saturated solution of 2:4-dinitrophenylhydrazine in 3N-hydrochloric acid. On dilution with water and standing there separated a yellow solid which was filtered and dried over phosphoric oxide (42mg.). Crystallised from ethanol-water the product separated as orange platelets m.p.108-112°. A mixture with acetone 2:4-dinitrophenylhydrazone m.p.123-125° melted at 110-120°. A mixture of the product with methyl ethyl ketone 2:4-dinitrophenylhydrazone m.p.110-113° melted at 100-110°.

(b) In another experiment using the same quantities as above, the oxidising agent was added over 20 minutes to the acetic acid solution of aspergillic acid at 100°, maintained under sufficient vacuum to give continuous distillation of 0·5ml./min. The distillate on treatment as before gave acetone 2:4-dinitrophenylhydrazone (5mg.). In a duplicate experiment the yield was 7mg. of acetone 2:4-dinitrophenylhydrazone from 1g. aspergillic acid.

(c) To a solution of deoxyaspergillic acid (500mg.; 2·4m.mole.) in 80% aqueous acetic acid (25ml.) was added a standard solution of chromium trioxide in glacial acetic acid (50ml.; 6m.mole..available oxygen by iodine titration). After standing overnight at room temperature there was no apparent change and the mixture was heated on a boiling water bath for 90 minutes. Under slight vacuum, distillate (3ml.) was collected. This distillate by treatment as described above gave a few mg. of yellow precipitate m.p.95-120°. Crystallised from aqueous ethanol the product separated as orange platelets m.p.103-118°. The product was undepressed on admixture with authentic acetone 2:4-dinitrophenylhydrazone.

Attempted conversion of Deoxyaspergillic Acid to a Chloropyrazine-derivative.

Deoxyaspergillic acid (1g.) was heated under reflux

for 4 hours with phosphoryl chloride (10ml.). Excess phosphoryl chloride was removed *in vacuo* and the residue dissolved in 3N-hydrochloric acid (20ml.). The solution was clarified by boiling with charcoal and on standing there separated a small quantity of crystalline material (*ca.* 5mg.). The solid was filtered (filtrate A) m.p. *ca.*200° with sintering above 150°. Sublimed at 100°/0·001mm. the product melted at 205° with sintering above 190°. There was not sufficient for analysis. Filtrate A was neutralised (litmus) with 3N-sodium hydroxide and the solid which separated was filtered and dried (640mg.) m.p.96-100°. The material gave a negative test (Lassaigne) for halogen and was undepressed in melting point on admixture with starting material.

Zinc Dust distillation of 3-Hydroxy-2:5-dimethylpyrazine.

3-Hydroxy-2:5-dimethylpyrazine (900mg.), zinc dust (1g.), zinc chloride (5g.) and sodium chloride (1g.) were finely ground and intimately mixed together. The mixture was heated in a distilling flask fitted with a receiver chilled in solid carbon dioxide. The flask was heated to 290° in 20 minutes, the mass sintering at 220°. No volatile product was collected. On cooling the reaction mass was ground up with water (20ml.), made strongly alkaline with 50% potassium hydroxide, and

steam distilled. Excess aqueous mercuric chloride was added to the distillate and the pale yellow flocculent precipitate which formed was filtered (200mg.). This solid was suspended in 50% potassium hydroxide (10ml.) and steam distilled. The distillate was treated with a few drops of methanolic picric acid and on standing there separated yellow rhombic crystals m.p.270-275°. The melting point was not depressed by admixture with ammonium picrate m.p.278-280°. Using zinc dust alone under similar conditions no identifiable product was isolated.

Deoxyaspergillic acid treated as detailed above either with zinc dust alone or with zinc dust, zinc chloride and sodium chloride mixture did not give any identifiable product.

N-Toluene-p-sulphonyltetrahydrodeoxyaspergillic Acid.

Adams platinic oxide catalyst (250mg., Org. Syn., Col.Vol.1, p.452) was suspended in glacial acetic acid (12·5ml.; distilled from chromium trioxide) and saturated with hydrogen at ordinary pressure at room temperature. To this suspension was added a solution of deoxyaspergillic acid (1·42g.) in glacial acetic acid (25ml.). The mixture was shaken in an atmosphere of hydrogen the theoretical uptake being complete in 1 hour, and thereafter absorbtion was negligible. The catalyst was filtered

and the acetic acid removed *in vacuo*. The residual oil was dissolved in pyridine (7ml.) and toluene-*p*-sulphonyl chloride (2g.) was added and the mixture refluxed for 2 hours. The reaction solution was poured into water and the oil which initially separated quickly solidified. The product crystallised from aqueous ethanol as needles m.p.144-146°(1·14g.). From the mother liquors a second crop m.p.128-132°(1·29g.; total yield 83% theory) was obtained by concentration. Recrystallised from aqueous ethanol N-*toluene*-p-*sulphonyltetrahydrodeoxyaspergillic acid* separated as needles m.p.146-148°. The compound gave a positive test for sulphur (Lassaigne).

Found: C=62·3; H=8·26; N=7·36%.

$C_{19}H_{30}O_3N_2S$ requires: C=62·3; H=8·20; N=7·57%.

Attempted hydrolysis of N-Toluene-p-sulphonyl-tetrahydrodeoxyaspergillic Acid.

(a) N-Toluene-*p*-sulphonyl-tetrahydrodeoxyaspergillic acid (100mg.) and N-sodium hydroxide (5ml.) were heated together under reflux for 4 hours. On cooling crystalline material (80mg.) separated and was identified as starting material.

(b) N-Toluene-*p*-sulphonyl-tetrahydrodeoxyaspergillic acid (200mg.) was heated in a sealed tube with 20% hydrochloric acid (8ml.) for 3 hours at 150°. On cooling a drop of oil separated from the clear acid solution and

solidified. The solid was dissolved in hot ethanol and water added until opalescent. On cooling an almost colourless solid separated m.p.125-138°. A mixture with starting material m.p.146-148° melted at 134-142°.

(c) N-Toluene-p-sulphonyl-tetrahydrodeoxyaspergillic acid (200mg.) was heated in a sealed tube with concentrated hydrochloric acid (8ml.) for 3 hours at 150°. On cooling an oily brown scum separated from the acid, it did not solidify and from it no identifiable product could be isolated. The aqueous phase was diluted to 20ml. and neutralised with 3N-sodium hydroxide but on standing at 0° there was no separation of solid.

Conversion of Aspergillic Acid to the Diketopiperazine $C_{12}H_{22}O_2N_2$.

(cf. Dutcher and Wintersteiner, J.Biol.Chem.,1944,155, 359, Dutcher, J.Biol.Chem.,1947,171,341).

To a solution of aspergillic acid (1g.) in 5N-hydrochloric acid (90ml.) was added at room temperature, over several hours, dropwise with shaking, saturated bromine water, until precipitation was complete. The separated solid was filtered and dried (750mg.; m.p.60° with initial softening). The solid was dissolved in glacial acetic acid (8ml.) and water (2ml.) added. To this solution under reflux, was added, over 4 hours,

zinc dust (10g.). Boiling water (100ml.) was added to the reaction mixture and filtered at the boiling point. The solid was extracted with boiling ethanol (100ml.) and the aqueous and alcoholic filtrates combined, concentrated to about 50ml. and allowed to stand at 0°. The diketopiperazine separated as a colourless solid (270mg.). Crystallised from aqueous methanol the diketopiperazine crystallised as needles m.p.261-262° (sealed tube).

$[\alpha]_D^{18}$ 10·7°(1,1; c,0·94,in methanol)

Dutcher, loc.cit. gives m.p.249-250° (with sublimation) $[\alpha]_D$+13·8°.

Found: C=63·8; H=9.38; N=12·5%.

Calc. for $C_{12}H_{22}O_2N_2$: C=63·7; H=9·73; N=12·4%.

The melting point was not depressed on admixture with DL-leucyl-DL-isoleucine anhydride m.p.273°. A mixture with DL-norleucyl-DL-isoleucine anhydride m.p.258-260° melted at 257-259°.

DL-Leucyl-DL-isoleucine anhydride and DL-norleucyl-DL-isoleucine anhydride were prepared by Mr. J.J. Gallagher.

Bromination of racemic Deoxyaspergillic Acid.

(cf. Dutcher, J.Biol.Chem.,1947,171,341).

Racemic deoxyaspergillic acid (1·1g.) was dissolved in glacial acetic acid (60ml.) and to this solution water

(40ml.) was added. A solution of bromine (847mg.) in glacial acetic acid (10ml.) was added to the solution of r-deoxyaspergillic acid, in portions with shaking, at room temperature. The colour was discharged almost at once and after addition was complete the pale yellow solution was allowed to stand overnight at 0°. The solution was slightly opalescent and water was added until no further opalescence developed. On standing at 0° for several hours crude r-bromodeoxyaspergillic acid separated as a pale yellow solid. Filtered and dried over phosphoric oxide it had m.p.85° (with initial sintering), yield 440mg. The aqueous filtrate was evaporated to dryness *in vacuo* leaving a brown solid residue. This residue was heated with water (100ml.) at the boiling point and filtered from insoluble solid. The filtrate was boiled with charcoal, filtered and re-evaporated to dryness under reduced pressure. Crystallised from aqueous ethanol the product separated as needles m.p.260-265° (130mg.). Recrystallised from the same solvent the m.p. was 266-268°(decomp.) not raised by further crystallisation. Lassaigne test for halogen on the product was negative.

Light absorption in ethanol: Maximum at 2860Å; ε =21,000.

Dutcher, *loc.cit.* gives m.p.268-270° for the quinone $C_{12}H_{18}O_2N_2$ and light absorption at 2850Å, ε =22,000.

Reaction of Phosphoryl Chloride with the Diketopiperazine $C_{12}H_{22}O_2N_2$.

Diketopiperazine $C_{12}H_{22}O_2N_2$(3g.) and phosphoryl chloride (25ml.) were heated under reflux for 1 hour, by which time evolution of hydrogen chloride had almost ceased. Excess phosphoryl chloride was removed in vacuo and the residue a red gum was treated with water (25ml.) and neutralised (litmus) with 3N-sodium hydroxide. The solution was extracted with ether (3 x 50ml.) and the combined extract shaken with 3N-sodium hydroxide (3 x 20ml.) The ether layer was retained (solution A). The alkaline extract was neutralised (litmus) with 3N-hydrochloric acid. The precipitate which separated was filtered, dried (700mg.) and sublimed at 80°/0·001mm. The sublimate crystallised from aqueous ethanol as irregular needles m.p.85-91°. The product gave a negative test for halogen (Lassaigne).

$[\alpha]_D^{17}$ +12·2° (c,2·94; 1,1, in ethanol)

The product could not be purified to constant melting point by crystallisation and after several crystallisations from the same solvent melted over the range 94-100°.

The product was insoluble in water, soluble in 3N-sodium hydroxide, 3N-hydrochloric acid, ether, methanol and ethanol in the cold. The product was not depressed

in melting point on admixture with deoxyaspergillic acid.

Solution A was washed with water and dried (sodium sulphate). The solvent was removed and the residual oil distilled under reduced pressure. The main fraction, a colourless oil (770mg.), was collected at 95-100°/1mm. and gave a positive test for halogen (Lassaigne). Optical rotation of the oil in ethanol,

$$[\alpha]_D^{16} + 18{\cdot}4^\circ (c, 7{\cdot}32;\ 1,1), +18{\cdot}2^\circ (c, 7{\cdot}29;\ 1,1)$$

The oil (0·75g.) and powdered potassium hydroxide (4g.) were heated together in an autoclave at 180° for 4 hours. The reaction mixture was dissolved in water (20ml.) and neutralised (litmus) with 3N-hydrochloric acid. The solid which separated (250mg.) was sublimed at 80°/0·01mm. The sublimate, crystallised from aqueous ethanol as irregular needles m.p.70-90°, gave a negative test for halogen (Lassaigne). After several crystallisations from aqueous ethanol the product melted over the range 90-100°. A mixture with the compound m.p.94-100°(above) melted over the range 90-100°.

DL-isoLeucine.

DL-isoLeucine was prepared by the malonic ester synthesis (Org. Syn., Vol.21, p.60) from sec.-butyl bromide. sec.-Butyl bromide (210g.; Org. Syn., Col.Vol.2, p.38) gave DL-isoleucine (45g., 23% theory) m.p.266-270°

(sealed tube). A mixture with DL-leucine m.p.275-278° melted at 270-273°.

DL-isoLeucine Anhydride.

(a) (cf. Fischer, Ber.,1901,34,433, Dutcher, J.Biol.Chem., 1947,171,341).

DL-isoLeucine (5·2g.) was suspended in refluxing ethanol (20ml.) and dry hydrogen chloride was passed through for 2 hours. The solvent was removed in vacuo and the syrupy residue dissolved in water (ca. 40ml.) and made alkaline (phenolphthalein) with 33% sodium hydroxide the temperature being kept below 10°. The alkaline solution was extracted with ether (3 x 50ml.) the extract dried (sodium sulphate) and the solvent removed. The residual oil was heated in a sealed tube at 180° for 2 hours. On cooling the crystalline mass was filtered, washed with a small portion of cold acetone and crystallised from aqueous methanol. DL-isoLeucine anhydride separated as needles m.p.280-283° (sealed tube, sintering above 270°).

Dutcher, loc.cit. gives m.p.255-257°

Found: C=63·4; H=9·62%.

Calc. for $C_{12}H_{22}O_2N_2$: C=63·7; H=9·73%.

(b) (Sannié, Bull.Soc.Chim.,1942,9,487, cf. Baxter and Spring, J.,1947,1179).

DL-isoLeucine anhydride was prepared in 47% yield from DL-isoleucine and crystallised from aqueous ethanol as needles m.p.280-282° (sealed tube, sintering above 270°). Baxter and Spring, loc.cit. give m.p.270-273° (sintering above 259°).

Found: C=63·6; H=10·05; N=12·1%.

Calc. for $C_{12}H_{22}O_2N_2$: C=63·7; H=9·73; N=12·4%.

A mixture of the diketopiperazine $C_{12}H_{22}O_2N_2$ (from aspergillic acid) m.p.261-262° and isoleucine anhydride melted intermediate between the melting points of the components as did a mixture with either DL-leucyl-DL-isoleucine anhydride or DL-norleucyl-DL-isoleucine anhydride. These last two compounds were prepared by Mr. J. J. Gallagher.

Ninhydrin Reaction with α-Amino-acids and derivatives of α-Amino-acids.

The substances tabulated were tested under identical conditions as follows:-

The substance (5mg.) was dissolved or suspended in water (0.5ml.) and ninhydrin reagent (3 drops; 0·1% in ethanol) added. In all cases there was no colour development after 2 minutes in the cold. The specimens were immersed in boiling water giving the results indicated.

- Indicates no colour, + indicates intensity of colour.

Compound	30sec	1min	1·5min	2min	2·5min	5min	10min	20min.
DL-Alanine	++	+++	++++	+++++	+++++	+++++	+++++	Fading
DL-Leucine	-	+	++	+++	++++	++++	++++	Fading
DL-isoLeucine	-	+	++	+++	++++	++++	++++	Fading
N-Formyl-DL-isoleucine	-	-	-	-	-	-	-	-
Dipeptide *	-	-	-	+	+	+	+	+
DL-isoLeucine Anhydride	-	-	-	-	-	-	-	-
Diketopiperazine $C_{12}H_{22}O_2N_2$	-	-	-	-	-	-	-	-
Blank **	-	-	-	-	-	-	-	-

* Dipeptide:- DL-Leucyl-DL-isoleucine anhydride

** Blank:- Water (0·5ml.) and standard ninhydrin solution (3 drops).

After 40 minutes the dipeptide colouration completely faded.

Hydrolysis of isoLeucine anhydride.

(a) Alkaline Hydrolysis.

DL-isoLeucine anhydride (250mg.) was heated in a sealed tube with N-potassium hydroxide (25ml.) at 200° for 20 hours. The solution was neutralised (litmus) with dilute hydrochloric acid and concentrated to 10ml.. To the hot solution an equal volume of ethanol was added and on standing there separated needles m.p.266°. The product gave a positive

ninhydrin reaction but could not be purified for analysis as after several recrystallisations from aqueous ethanol, it continued to give a positive test for ionic chlorine.

(b) Acid Hydrolysis.

(i) DL-*iso*Leucine anhydride (350mg.) was suspended in dry ethanol (25ml.) under reflux and dry hydrogen chloride was passed for 7 hours. The solvent was removed *in vacuo*, water (10ml.) added and re-evaporated. The residue was dissolved in water (50ml.) and the solution made just alkaline with ammonium hydroxide. The ammoniacal solution was warmed and clarified (charcoal), concentrated to 10ml. and an equal volume of ethanol added. On standing crystalline product separated as platelets sintering above 255°. The product gave a positive ninhydrin test but could not be freed from ammonium chloride by crystallisation.

(ii) DL-*iso*Leucine anhydride (500mg.) and hydriodic acid (5ml., S.G.1·5) were heated together under reflux for 8 hours. The residual solution was evaporated to dryness under reduced pressure, the residue dissolved in water (*ca*. 25ml.) and iodine removed by extraction with chloroform. The aqueous solution was treated with excess freshly precipitated silver hydroxide and filtered. The filtrate was desilvered by treatment with hydrogen sulphide and clarified (filteraid). The

aqueous filtrate was evaporated to dryness leaving a powdery residue which separated from aqueous ethanol as scintillating plates m.p.263-266° (sintering above 250°).

Found: C=54·4; H=9·70; N=11·4%.

Calc. for $C_{12}H_{24}O_3N_2$: C=59·0; H=9·84; N=11·5%.

Calc. for $C_6H_{13}O_2N_2$: C=55·0; H=9·92; N=10·7%.

The hydrolysis product gave a strong positive reaction with ninhydrin reagent.

(iii) DL-isoLeucine anhydride (2g.) was refluxed with 48% hydrobromic acid (20ml.) for 20 hours. The reaction mixture was worked up as for (ii) above and gave on evaporation of the silver-free solution a white powder (1·25g.). Crystallised from aqueous ethanol the product separated as plates m.p.260-263°.

Found: C=55·3; H=10·1; N=10·8%.

Calc. for $C_6H_{13}O_2N$: C=55·0; H=9·92; N=10·7%.

The product gave a positive ninhydrin test.

The hydrolysis product (500mg.) was refluxed for 90 minutes with 98% formic acid (3ml.), the acid removed in vacuo and the process repeated. The gummy residue was treated with cold 0·5N-hydrochloric acid (2ml.) and filtered. The product (300mg.) was crystallised from boiling water from which N-formyl-DL-isoleucine separated as platelets m.p.117-119°.

Found: C=53·4; H=7·87; N=9·29%.

Calc. for $C_7H_{13}O_3N$: C=52·8; H=8·18; N=8·81%.

The melting point was undepressed on admixture with an authentic specimen.

Hydrolysis of the Diketopiperazine from Aspergillic Acid.

The diketopiperazine (500mg.) and 48% hydrobromic acid (10ml.) were heated together under reflux for 12 hours, the diketopiperazine slowly dissolving. The solution was evaporated to dryness under reduced pressure, a few ml. water added and re-evaporated to remove excess acid. The residue was dissolved in water (30ml.) and treated with excess freshly precipitated silver hydroxide. The silver bromide and silver hydroxide was filtered and the filtrate desilvered by passing hydrogen sulphide and filtering. The solution was evaporated to dryness, dissolved in water (10ml.) and the solution clarified with charcoal (10mg.). The filtrate was evaporated to dryness leaving a white powder which was dried over phosphoric oxide (425mg.).

$[\alpha]_D^{18}$ 0 $\pm$ 1° (c,0·95; 1,1, in water)

The hydrolysate gave a positive ninhydrin reaction.

Partition Chromatography of Diketopiperazine $C_{12}H_{22}O_2N_2$ Hydrolysate on Paper.

(cf. Consden, Gordon and Martin, Biochem.J.,1944,38,224)

The apparatus consisted of an inverted glass cylinder 65cm. x 15cm. dia., with a close fitting ground glass base plate. The mobile phase reservoir was carried on a glass framework inside the cylinder and the paper strips were held in the reservoir by a microscope slide. The paper strips used were 8cm. broad, had an effective length of 60cm., and were cut from sheets of filter paper. The amino-acids were spotted along a horizontal line 1·5cm. apart. n-Butanol saturated with water was used as the mobile phase and tests were allowed to run 24 hours. The test-strips were then air-dried at 80°, wetted with a 0·1% solution of ninhydrin in n-butanol and redried at 80° for 5 minutes the new positions of the amino-acids being shown by purple stains. The amino-acids were used as 0·5% aqueous solution for spotting purposes. On strips on which the n-butanol front advanced 40cm. the respective distances advanced by amino acids were, glycine 1cm., DL-alanine 2·4cm., L-valine 7.9cm., DL-norvaline 9·6cm., DL-leucine, DL-isoleucine and DL-norleucine 12-13cm., $C_{12}H_{22}O_2N_2$ hydrolysate 12-13cm.. A mixture of DL-leucine, DL-isoleucine and DL-norleucine showed as a single stain 12-13cm. from

the origin under similar experimental conditions.

Preparation of N-3:5-dinitrobenzoyl-derivatives of DL-Leucine, DL-isoLeucine and DL-Norleucine.

(Saunders, Biochem.J.,1934,28,580)

The amino-acid (131mg., 1m.mole.) was dissolved in 0·5N-sodium hydroxide (4ml., 2m.mole.). To the solution was added powdered 3:5-dinitrobenzoyl chloride (231mg., 1m.mole.) and the mixture shaken vigorously for 2 minutes. The dinitrobenzoyl chloride dissolved giving an orange solution and after standing 1 hour was filtered from a small amount of insoluble matter, acidified with dilute hydrochloric acid and the precipitate filtered, water washed and crystallised from aqueous ethanol.

N-3:5-Dinitrobenzoyl-DL-leucine crystallised as pale yellow plates m.p.195-198° (sintering above 185°)

Found: C=48·1; H=4·57; N=12·8%.

$C_{13}H_{15}O_7N_3$ requires: C=48·0; H=4·62; N=12·9%.

Saunders, loc.cit., gives for N-3:5-dinitrobenzoyl-L-leucine m.p.186-187°.

N-3:5-Dintrobenzoyl-DL-isoleucine crystallised as small plates m.p.189-192° (sintering above 180°)

Found: C=48·2; H=4·59; N=13·2%.

$C_{13}H_{15}O_7N_3$ requires: C=48·0; H=4·62; N=12·9%.

A mixture of N-3:5-dinitrobenzoyl-DL-leucine and N-3:5-dinitrobenzoyl-DL-isoleucine melted at 182-186°.

N-3:5-Dinitrobenzoyl-DL-norleucine crystallised as aggregates of micro-plates m.p.202-204°

Found: C=47·6; H=4·87; N=12·9%.

$C_{13}H_{15}O_7N_3$ requires: C=48·0; H=4·62; N=12.9%.

A mixture of N-3:5-dinitrobenzoyl-DL-norleucine and N-3:5-dinitrobenzoyl-DL-isoleucine melted at 180-183° and with N-3:5-dinitrobenzoyl-DL-leucine melted at 180-186°.

Degradation of DL-isoLeucine to Methyl Ethyl Acetaldehyde by Ninhydrin.

(cf. Schönberg, Moubasher and Mostafa, J.,1948,176; Virtanen, Laine and Toivonen, Z.physiol.,Chem.,1940,266, 193).

To a solution of DL-isoleucine (100mg.) in water (25ml.) contained in a 50ml. flask fitted with splash-head and condenser, was added ninhydrin (75mg.). Carbon dioxide was passed through the mixture and the issuing gas passed through a solution of 2:4-dinitrophenyl-hydrazine in 3N-hydrochloric acid (3ml.). The flask was heated on an oil bath and the reaction mixture became intense blue in colour. Heating was such that over 30 minutes 10ml. of distillate was collected. From the

distillate there separated methyl ethyl acetaldehyde 2:4-dinitrophenylhydrazone as a yellow solid which was filtered and dried m.p.123-126°(58mg.). Crystallised from ethanol the product separated as orange plates m.p.129-130°. Morgan and Hardy, Chem. & Ind.,1933,52, 518, give for methyl ethyl acetaldehyde 2:4-dinitrophenylhydrazone m.p.120·5°.

Methyl ethyl acetaldehyde was prepared by the method of Linstead and Mann, J.,1930,2069, and converted to its 2:4-dinitrophenylhydrazone which crystallised from ethanol as orange plates m.p.127-129°.

Found: C=49·6; H=5·20; N=20·8%.

Calc. for $C_{11}H_{14}O_4N_4$: C=49·6; H=5·26; N=21·05%.

A mixture of this compound with the 2:4-dinitrophenylhydrazone of methyl ethyl acetaldehyde from DL-isoleucine melted without depression.

Degradation of DL-Leucine to isoValeraldehyde by Ninhydrin. (cf. Abderhalden, Z.physiol.Chem.,1938,252,87)

This was carried out as described for DL-isoleucine. isoValeraldehyde 2:4-dinitrophenylhydrazone crystallised from ethanol as orange plates m.p.123-125°.

Brady Analyst,1926,51,78 gives m.p.123°.

Separation of Diketopiperazine Hydrolysate into DL-Leucine and isoLeucine (and alloisoLeucine).

(cf. Ehrlich, Ber.,1904,37,1809,Brazier,Biochem.J.,1930, 24,1188).

The hydrolysate (425mg.) was dissolved in water (150ml.) and treated with a solution of cupric acetate (320mg.) in warm water (50ml.). After standing for several hours the precipitated copper salt was filtered (filtrate A), shaken with water (150ml.) and the suspension filtered. The solid was shaken with methanol (75ml.) and the mixture filtered. The solid was suspended in water and decomposed with hydrogen sulphide, the cupric sulphide filtered and the filtrate clarified (charcoal). The filtrate was evaporated to dryness and the residue crystallised from aqueous ethanol. DL-Leucine separated as plates (65mg.) m.p.270-273°.

Found: C=54·5,54·9; H=9·82,9·48%.

Calc. for $C_6H_{13}O_2N$: C=55·0; H=9·92%.

The product was converted to its N-3:5-dinitrobenzoyl derivative as previously described for DL-leucine. The derivative crystallised from ethanol as plates m.p.194-195.5°.

Found: C=48·2,48·0; H=4·80,4·80; N=12·9,13·2%

Calc. for $C_{13}H_{15}O_7N_3$: C=48·0; H=4·62; N=12·9%

The melting point was not depressed on admixture with N-3:5-dinitrobenzoyl-DL-leucine prepared from authentic DL-leucine and was depressed on admixture with either N-3:5-dinitrobenzoyl-DL-isoleucine or N-3:5-dinitrobenzoyl-DL-norleucine.

In a repeat experiment the DL-leucine obtained by hydrolysis of the diketopiperazine $C_{12}H_{22}O_2N_2$ was subjected to Strecker degradation as described for authentic DL-leucine and the product characterised as its 2:4-dinitrophenylhydrazone. The latter crystallised from ethanol as orange plates m.p.119-120°.

Found: C=49.6; H=5·05; N=21·3%.

Calc. for $C_{11}H_{14}O_4N_4$: C=49·6; H=5·26; N=21·05%.

This 2:4-dinitrophenylhydrazone was not depressed in melting point when admixed with isovaleraldehyde 2:4-dinitrophenylhydrazone. A mixture with methyl ethyl acetaldehyde 2:4-dinitrophenylhydrazone m.p.129-130° melted at 116-118°.

The filtrate A (p.112) was evaporated to dryness and the residue extracted with warm methanol (2 x 30ml.). The methanolic filtrates were combined and the solvent removed in vacuo. The residue was suspended in water and the copper removed as sulphide with hydrogen sulphide. The solution was clarified (filteraid) and on evaporation

to dryness left a powdery residue (92mg.) m.p.250-253°.

Found: N=10·1, 10·2%.

Calc. for $C_{12}H_{13}O_2N$: N=9·9%.

The product (90mg.) was degraded with ninhydrin as described for DL-isoleucine and the aldehyde characterised as its 2:4-dinitrophenylhydrazone. The latter (80mg.) m.p.121° was twice recrystallised from ethanol, methyl ethyl acetaldehyde 2:4-dinitrophenylhydrazone separating as orange plates m.p.127-129°.

Found: C=49·4,49·7; H=5·00,4·91; N=20·9%.

Calc. for $C_{11}H_{14}O_4N_4$: C=49·6; H=5·26; N=21·05%.

The product was not depressed in melting point on admixture with authentic methyl ethyl acetaldehyde 2:4-dinitrophenylhydrazone and was depressed in melting point on admixture with isovaleraldehyde 2:4-dinitrophenylhydrazone m.p.123-125° the mixture melting at 119-122°.

SECTION II.

Glycine Amide.

(Yang and Rising, J.Amer.Chem.Soc.,1931,53,3183)

To glycine ethyl ester (51g., Fischer, Ber.,1901,34, 433) was added a solution of ammonia in methanol (750ml. saturated at 0°). The mixed solution was held at 0° for 48 hours, the solution concentrated under reduced pressure to 50ml. and filtered from glycine anhydride. The filtrate was evaporated to dryness under reduced pressure the oily residue quickly solidifying on standing. Crystallised from chloroform-ether, glycine amide separated m.p.62-64° (14g.). Concentration of the mother liquors gave a second crop (3·5g.) Yield 48%.

Yang and Rising, (J.Amer.Chem.Soc.,1931,53,3183), give m.p.67-68°.

DL-Alanine Amide.

DL-Alanine amide was prepared by the method above. DL-Alanine ethyl ester (19·8g.) gave DL-alanine amide m.p.63-67°(12g.,80% theory).

Yang and Rising, (J.Amer.Chem.Soc.,1931,53,3183) give m.p.71-72° for L-alanine amide.

Attempted Condensation of α-Bromcinnamaldehyde with Glycine Amide and DL-Alanine Amide.

(a) Glycine amide (1·21g.) was added to a refluxing solution of α-bromcinnamaldehyde (3·46g., Zincke and Hagen, Ber.,1884,17,1817) in absolute ethanol (50ml.) and heated for 30 minutes by which time the solution was dark red in colour. The solution was concentrated to half bulk but there was no separation of solid on standing. Removal of solvent under reduced pressure left a dark viscous oil which could not be induced to crystallise. The oil was dissolved in ethanol (10ml.) and a solution of sodium ethoxide in alcohol (0·5g.sodium in 20ml. dry ethanol) was added. The mixture was refluxed for 30 minutes, sodium bromide separating. The alcohol was removed under reduced pressure, the residual tar dissolved in ether and the mixture acidified with 3N-hydrochloric acid. The acid layer was neutralised (litmus) with 3N-sodium hydroxide, ether extracted (3 x 20ml.) and the extract dried (sodium sulphate). Evaporation of the solvent left merely a trace of gum.

(b) The same quantities of glycine amide and α-bromcinnamaldehyde in ethanol (50ml.) were allowed to stand at room temperature for 2 days. The resulting solution was dark red in colour and on working up as

described under (a) no product was isolated.

Using the same molecular proportions of DL-alanine amide and α-bromcinnamaldehyde under the conditions described under (a) and (b) the reaction products were dark oils and from them no identifiable products were isolated on working up as above.

Attempted Condensation of DL-Alanine Ethyl Ester and α-Bromcinnamaldehyde.

To a solution of α-bromcinnamaldehyde (2·11g.) in dry ethanol (40ml.) under reflux was added DL-alanine ethyl ester (1ml.). Heating was continued for 30 minutes, the solution darkening in colour. The solvent was removed under reduced pressure leaving a dark oil which did not solidify. The oil was heated at 80-100° for 4 hours with liquid ammonia (5ml.) in an autoclave. To the reaction mixture water (50ml.) was added and the mixture ether extracted (3 x 15ml.). The ether solution was extracted with 3N-hydrochloric acid (2 x 10ml.) and the extract neutralised (litmus). Extraction of the aqueous layer with ether and removal of the solvent left no residue.

Cinnamylidene-DL-alanine Amide.

Cinnamaldehyde (1·32g., 1·2ml.) was dissolved in

dry ethanol (40ml.) and to this solution under reflux DL-alanine amide (880mg.) was added. The mixture was heated for 30 minutes, the solution concentrated to 15ml. and the resulting pale yellow liquid allowed to stand overnight at 0°. The separated solid was filtered and washed with a little ethanol m.p.150-153°. (Yield 920mg.). Recrystallised from aqueous ethanol cinnamylidene-DL-alanine amide separated as plates m.p.154-155°.

Found: C=71·1; H=6·87; N=14·4%.

$C_{12}H_{14}ON_2$ requires: C=71·3; H=6·93; N=13·9%.

Hydrolysis of Cinnamylidene-DL-alanine Amide.

To a saturated solution of cinnamylidene-DL-alanine amide in ethanol (2ml.) Brady's Reagent (4 drops) was added. In a few seconds a voluminous red precipitate separated. After standing several hours the precipitate was filtered. Crystallised from acetic acid cinnamaldehyde 2:4-dinitrophenylhydrazone separated as red micro-needles m.p.245° undepressed on admixture with an authentic specimen.

N-(α-Brompropionyl)-toluene-p-sulphonamide.

(cf. Openshaw and Spring, J.,1945,234)

Toluene-p-sulphonamide (20g.) and α-brompropionyl bromide (50g., 100% excess) were heated together at 100°

for 1 hour. The toluene-p-sulphonamide dissolved incompletely and on cooling the suspension was poured into cold water (500ml.). The mixture was shaken until the oil which separated solidified. The solid was water washed and ground up with excess of an aqueous suspension of sodium hydrogen carbonate. The unchanged toluene-p-sulphonamide and excess sodium hydrogen carbonate was filtered and the alkaline filtrate made just acid (litmus) with dilute hydrochloric acid. The solid which separated was filtered, and, crystallised from aqueous ethanol, N-(α-brompropionyl)-toluene-p-sulphonamide separated as needles m.p.125-130° (27·6g., 77% theory). The pure product obtained by recrystallisation from aqueous ethanol had m.p.130-132°.
Equivalent weight by titration with sodium hydroxide (phenolphthalien) 309, 310: Theory, 306.

Abderhalden and Riesz (German Patent 539049) give for N-(α-brompropionyl)-toluene-p-sulphonamide m.p.127°.

N-(DL-Alanyl)-toluene-p-sulphonamide.

(a) Ammonium hydroxide (100ml., S.G., 0·88) was added to N-(α-brompropionyl)-toluene-p-sulphonamide (10g.) and the solution held, in a stoppered bottle, for 5 days at room temperature. The solution was evaporated under reduced pressure and the residue dissolved in water (50ml.). The solution on standing soon

crystallised. The solid was filtered (7·5g., 95% theory) and, crystallised from boiling water N-(DL-alanyl)-toluene-p-sulphonamide separated as needles m.p.234-235° (decomp., heated from 230°).

Found: N=11·9%.

$C_{10}H_{14}O_3N_2S$ requires: N=11·6%.

(b) A solution of ethanolic ammonia was prepared by saturating ethanol with ammonia gas at -5°. This solution (20ml.) was added to N-(α-brompropionyl)-toluene-p-sulphonamide (2g.) and the mixture held in a pressure vessel for 4 days at room temperature. The alcoholic ammonia was removed under reduced pressure and the residue dissolved in water (10ml.), from which solution on standing N-DL-alanyl-toluene-p-sulphonamide crystallised. Recrystallised from boiling water the product separated as needles m.p.228°(decomp.) undepressed on admixture with a specimen prepared as above.

Ammonolysis of N-(α-Brompropionyl)-toluene-p-sulphonamide.

A mixture of N-(α-brompropionyl)-toluene-p-sulphonamide (10g.) and liquid ammonia (20ml.) was heated at 80° in an autoclave for 3 hours. The reaction mixture was diluted to 150ml. with water and the solid which separated was filtered m.p.137°(3g.). The melting point

was not depressed on admixture with toluene-p-sulphonamide (m.p.138°). Evaporation of the mother liquor to 50ml. gave a second crop (1g.) also identified as toluene-p-sulphonamide.

Attempted condensation of N-(DL-Alanyl)-toluene-p-sulphonamide and α-Bromcinnamaldehyde.

N-(DL-Alanyl)-toluene-p-sulphonamide (1·2g.) was added to a solution of α-bromcinnamaldehyde (1·05g.) in ethanol (40ml.). The suspension was autoclaved for 4 hours at 100°. On cooling the undissolved solid was identified as starting material. Heating for a further period of 4 hours at this temperature caused no change. After 4 hours at 140° complete solution resulted but it was dark in colour. Evaporation of the solvent left an intractable tar which could not be induced to crystallise.

Glycine-hydroxamic Acid.

(cf. Jones and Sneed J.Amer.Chem.Soc.,1917,39,673)

To glycine methyl ester (12·5g., Fischer, Ber., 1901,34,433) was added methanolic hydroxylamine (3mole., Org.Syn.,Vol.26,p.74) and the mixture allowed to stand overnight at 0°. The glycine-hydroxamic acid which separated was filtered and dried.

Condensation of Di-acetyl with Glycine-hydroxamic Acid.

Glycine-hydroxamic acid (1·9g.) was suspended in methanol (10ml.) and the suspension chilled in acetone-solid carbon dioxide. Di-acetyl (1·85g.) was dissolved in methanol (10ml.) and after similar pre-cooling was added to the suspension. To the resulting mixture 5N-sodium hydroxide (5·3ml.) was added, dropwise with shaking, the cooling being maintained. The reaction solution was then held at 0° for several hours the glycine-hydroxamic acid dissolving to give a brown solution. After adjusting to $p_H 4$ by addition of dilute hydrochloric acid the solution was evaporated under reduced pressure. The residue was extracted with boiling chloroform (50ml.) and the chloroform evaporated. The resulting tar could not be induced to crystallise but, under high vacuum (100°/0·001mm.), gave a small quantity of pale yellow glassy sublimate. The sublimate liberated carbon dioxide from aqueous sodium hydrogen carbonate and, in ethanol solution, gave a claret colouration with aqueous ferric chloride.

Condensation of Methyl-glyoxal and Glycine-hydroxamic Acid.

The reaction was carried out as above using glycine-hydroxamic acid (9g.), methyl-glyoxal (7·2g.) and 5N-sodium hydroxide (25ml.). On working up as described

above a pale yellow glassy sublimate was obtained. This sublimed product gave a positive ferric chloride reaction and liberated carbon dioxide from aqueous sodium hydrogen carbonate.

1-Hydroxy-2-keto-5-phenyl-1:2-dihydropyrazine.

Glycine-hydroxamic acid (3g.) was suspended in methanol (20ml.) and the suspension chilled with acetone and solid carbon dioxide. To this suspension was added a pre-cooled solution of phenyl-glyoxal hydrate (4·5g.) in aqueous methanol (15ml. methanol 5ml. water). To the resulting yellow suspension, 5N-sodium hydroxide (8·3ml.) was added at low temperature in portions with shaking. The mixture was allowed to warm to -5° over 2 hours and held at that temperature overnight. After standing a further 2 hours at room temperature the suspension was held at 40° for 15 minutes and water (ca. 20ml.) added. The solution was cooled and on adjusting to $p_H 4$ with dilute hydrochloric acid and standing, a semi-solid separated. This material, sublimed at $100^{\circ}/0{\cdot}001$mm., gave a yellow waxy solid (250mg.). Crystallised twice from methanol 1-hydroxy-2-keto-5-phenyl-1:2-dihydropyrazine separated as plates m.p.194-196°(decomp.).

Found: C=63·9; H=4·15; N=15·1%.

$C_{10}H_8O_2N_2$ requires: C=63·8; H=4·25; N=14·9%.

Light absorption in ethanol: Maxima at 2700Å, ϵ=13,600;
3620Å, ϵ=5800.

1-Hydroxy-2-keto-5-phenyl-1:2-dihydropyrazine was slightly soluble in water, methanol and ethanol in the cold. It sublimed readily at 100°/0·001mm., liberated carbon dioxide from aqueous sodium hydrogen carbonate and gave a claret colouration, in ethanol solution with aqueous ferric chloride.

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