SYNTHESIS AND BIOSYNTHESIS OF CANDIPOLIN, A METABOLITE OF PENICILLIUM CANADENSE.

T H E S I S

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by

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To Paul.

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SUMMARY

Candipolin, a mycelial metabolite of the fungus Penicillium canadense, is N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol. Its structure was deduced from spectroscopic and degradative studies and confirmed by synthesis. Esterification of N-benzoyl-L-phenylalaninol with the imidazolide of N-benzoyl-L-phenylalanine yielded a mixture of candipolin and its epimer, the latter arising by racemisation at the phenylalanine centre. Epi-candipolin crystallised from the mixture more easily than candipolin, making isolation of the desired product rather difficult. Candipolin was obtained free from its epimer by esterification of N-benzoyl-L-phenylalaninol with the imidazolide of N-CBZ-L-phenylalanine, followed by selective removal of the CBZ-group and benzoylation.

The presence of an ester link between an amino acid and an amino alcohol unit is unusual in nature and worthy of investigation. Biosynthetic studies using ^{14}C-labelled compounds showed that all four units of the candipolin structure (phenylalanine, phenylalaninol, and the two benzoyl moieties) arise from L-phenylalanine (incorporation 73%), this precursor showing equal incorporation into each. L-phenylalaninol (incorporation 2%) was not directly incorporated, but appeared to undergo oxidation to phenylalanine, which was itself incorporated. Benzoic acid (incorporation 28%) gave rise to the two benzoyl units with equal incorporation into each. N-benzoyl-L-phenylalaninol (incorporation 71%) was very efficiently and very specifically incorporated into its own unit. N-benzoyl-L-phenylalanine (incorporation 23%) was not directly incorporated, but appeared to undergo hydrolysis to benzoic acid and phenylalanine, which were each subsequently incorporated as expected. From these results, it was deduced that a possible

sequence of events in the biosynthesis of candipolin would be : N-benzoylation of enzyme-bound phenylalanine with reduction to the alcohol before release from the enzyme, followed by reaction of the N-benzoyl-L-phenylalaninol with phenylalanine to give N-benzoyl-O-(L-phenylalanyl)-L-phenylalaninol, and finally, benzoylation of the free amine.

Feeding experiments using $^{13}CD_3CO_2Na$ were performed on P. canadense and Trichothecium roseum, and the fatty esters and rosenonolactone isolated from the respective mycelia were studied using ^{13}C NMR spectroscopy. It was deduced that the deuterium atom at C-10 in the oleate from P. canadense, and hence, those in the even-numbered carbon atoms of the stearate precursor, was in the pro-R configuration. This suggests that in the reduction of crotonate to butyrate, delivery of hydrogen to C-2, and to C-3 if the reduction goes trans, is to the si face, giving hydrogen in the pro-S configuration. Studies on rosenonolactone from T. roseum and on the corresponding alcohol, aided by the use of ^{13}C shift reagents, indicated that published peak assignments were incorrect. Although the spectra require further investigation, using deuterium decoupling, they showed that C-18 carried d_1 and d_2, but not d_3 species (as expected from its derivation from C-2 of mevalonate), and provided convincing evidence for the accepted pathway of incorporation of acetate into rosenonolactone.

CONTENTS

I N T R O D U C I O N

INTRODUCTION

FUNGI

Fungi are classed as members of the plant kingdom, although they differ from higher plants in that they have no roots, stems, leaves, or chlorophyll[1]. Since they lack the pigment necessary for photosynthesis, they cannot utilise carbon dioxide, and, therefore, must obtain their carbon from sugars made by other organisms. Saprophytic fungi obtain their sugars from dead organic matter, such as leaves and trees, by secreting enzymes which break down the structure of the wood, releasing sugars in the appropriate form. By causing decay of dead vegetation, these fungi fertilise the soil, and by their ability to decompose structural timber under damp conditions, saprpohytic fungi cause dry and wet rot. Parasitic fungi obtain their sugars from living organisms, to which they can cause damage or even death, as in the rusts, smuts, and blights of crops. The lichens are a class of organisms consisting of a symbiotic association of fungi with algae, the algae photosynthetically converting carbon dioxide into sugars, which the fungi in turn metabolise.

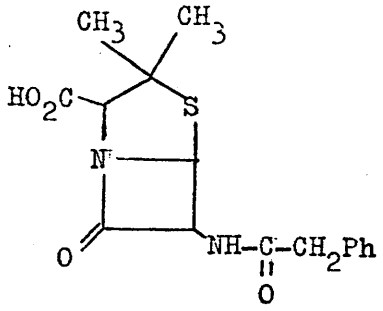
Mycelium, the material of which fungi are composed, consists of a closely interwoven network of microscopic tubes, called hyphae, through which nutrients are absorbed, and metabolites (and destructive enzymes) are excreted. The hyphae contain cytoplasm in which nuclei are randomly dispersed, without having any discrete cell structure. In some classes of fungi, the hyphae are divided into compartments by transverse walls (septa), which have central pores through which cytoplasm, and sometimes nuclei, can pass. Thus, even septate fungi are not organised onto mononucleate cells. There are two types of mycelium: vegetative and aerial. The former

serves to anchor the fungus to its food source, and acts as a permeable membrane through which nutrients and some metabolites can pass, while the latter produces spores, the "seeds" of the fungus, which are borne by wind and water to propagate the species. The spores, which do not grow actively, also serve to preserve the species through unfavourable conditions.

Fungi are classed according to the type of spores they produce and to the nature of the mycelium. Phycomycetes, the lowest class of fungi, are distinguished by the aseptate nature of the mycelium, where it is present. The lower members, which do not form mycelium, are unicellular and often aquatic organisms. Examples of this class are Pythium, a plant parasite, Mucor, which parasitises man, and Rhizopus, a common grey bread mould. (The term "mould" is a colloquialism, used loosely to refer to Fungi Imperfecti, q.v., and fungi which do not produce any macroscopic fruiting body.)

Ascomycetes bear sexual spores in a sac (ascus), and asexual spores (conidia) on aerial hyphae (conidiophores). This class includes unicellular yeasts, and fungi which produce large fruiting bodies, such as truffles. Many yeasts form loose aggregates of cells, and some are capable of forming mycelium. They reproduce asexually by budding, a process whereby an outgrowth which forms on the cell grows, accompanied by nuclear division, until it equals the size of the parent cell, after which a cell wall is formed to separate off the daughter cell. Many Ascomycetes, such as Claviceps purpurea, are parasites of plants, while some yeasts cause human disease.

Basidiomycetes, the highest class of fungi, bear their spores on basidia, which are often associated in large numbers to form fruiting bodies, as in mushrooms, bracket fungi, and puff balls.



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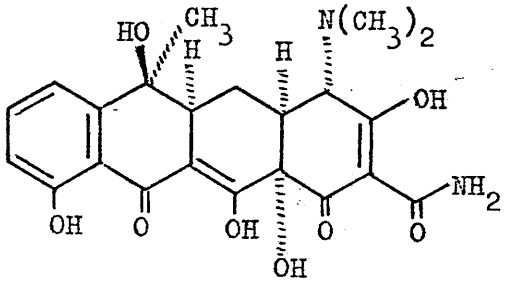

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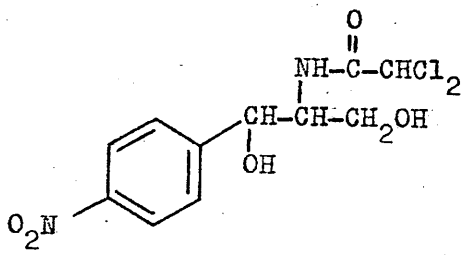

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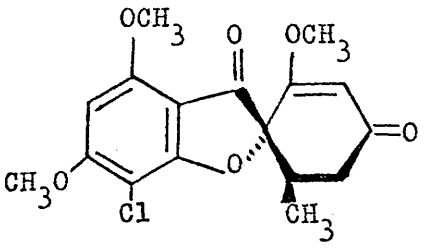
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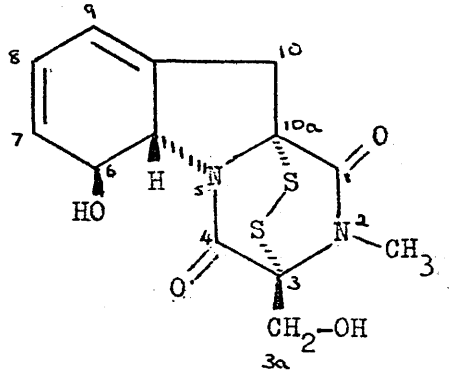
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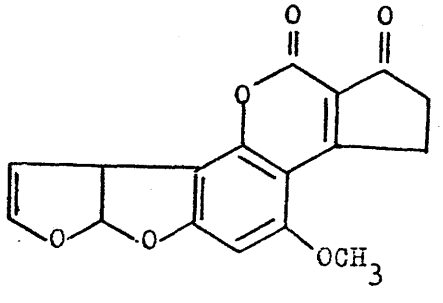
5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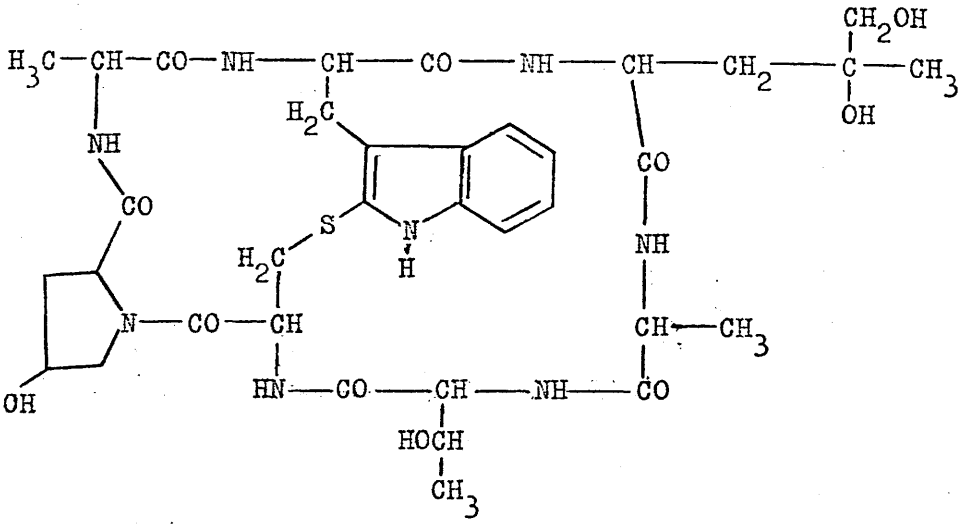

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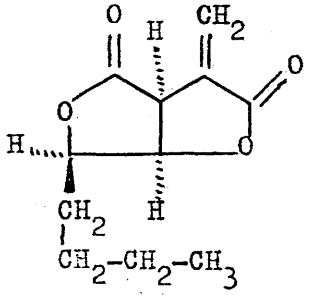

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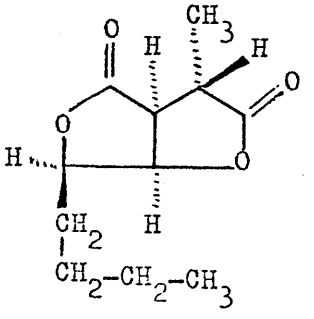

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Members of this class cause considerable crop damage in the form of rusts and smuts of cereals, and mycelium of Basidiomycetes is often perennial in soil or wood.

Fungi Imperfecti, which have no sexual ("perfect") stage of growth, form asexual spores on conidiophores which usually occur on the surface of the mycelium. Common examples of this class are Penicillia and Aspergilli, which cause the familiar grey-green growth on mouldy food. Some species classed as "Fungi Imperfecti" are simply the conidial stages of perfect fungi, for example, Fusarium moniliforme is the conidial stage of Gibberella fujikuroi.

Fungi play an important part in our everyday lives. Yeasts, by virtue of their ability to produce carbon dioxide from flour, are extremely important in the manufacture of bread. Yeasts are also very efficient in the conversion of fruit sugars to alcohol. Fungi are responsible for the maturing of cheeses such as Gorgonzola, Camembert, Roquefort, and, of course, some fungi, such as mushrooms and truffles, are edible. Fungi provide an important source of antibiotics, such as penicillin (1) (Penicillium notatum), tetracyclines (2) (Streptomyces species), chloramphenicol (3) (Streptomyces venezuelae), griseofulvin (4) (Penicillium griseofulvum), and many fungi produce potent toxins, such as gliotoxin (5) (Trichoderma viride), aflatoxins (6) (Aspergillus flavus), and phalloidine (7) (Amanita phalloides). The fungus Penicillium canadense, used in the present work, produces the antibiotic canadensolide (8) as well as dihydrocanadensolide (9), a compound which has found some use as an antiulcer agent[3].

Fungi and higher plants exhibit both primary and secondary metabolism. Primary metabolism is the process by which those compounds are produced which are necessary for the growth and

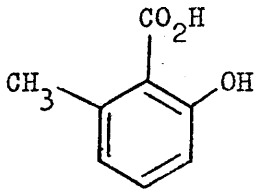
maintenance of the organism, and for providing the energy necessary for these processes. Primary metabolites are relatively few in number and are common to all classes of organisms. Secondary metabolites, on the other hand, are not essential for maintenance of the organism, and exist in enormous variety, many being produced only by one species of a particular genus. In order to study secondary metabolism, it is necessary to be able to grow cultures under controlled conditions in the laboratory.

The most convenient nutrient supply is an aqueous solution containing sugar, nitrogen, and various minerals, into which spores of the fungus may be inoculated. If this is left undisturbed, the mycelium grows as a mat covering the surface (surface culture), or, if it is shaken, the mycelium forms small submerged pellets (shake culture). In a surface culture, part of the mycelium is in contact with air, part is in contact with the nutrient medium, and part lies between these two extremes, making this type of culture relatively inhomogeneous. A shake culture is mich more homogeneous because all of the mycelium is submerged, thus presenting a much larger surface area to the nutrient supply, which is aerated as a result of the agitation. Nevertheless inhomogeneities exist between mycelium at the surface and that at the centre of the pellets. In a surface culture, metabolites are removed from the proximity of the mycelium, and fresh nutrients brought to it by the process of diffusion, which is relatively slow compared to the agitation of broth in shake cultures. These differences in the two methods of culturing often cause differences in the relative proportions of metabolites present, and, indeed, can lead to production of metabolites peculiar to one form of culture.

Borrow and Bu'lock, who independently studied the relationship

between nutrient supply and growth in cultures, discovered that fermentation occurs in several distinct phases. Borrow and co-workers[4,5], studying gibberellin production by shake cultures of Gibberella fujikuroi, described three metabolic phases in cultures where the nitrogen source was limited. Between inoculation and exhaustion of nitrogen was a period of rapid growth, the "balanced phase", during which the weight of mycelium increased while glucose, nitrogen, phosphorus, magnesium, and potassium were taken up. This was followed by the "storage phase", during which the weight of mycelium increased due to accumulation of fats and carbohydrates; the remaining nutrients were assimilated, and secondary metabolism commenced. At the "maintenance phase", no further increase in mycelial weight occurred, but glucose uptake and secondary metabolism continued. When glucose and mycelial fat reserves had been exhausted, the "terminal phase" of breakdown of the mycelium began, with release of mycelial components into the medium. In fermentations in which magnesium and phosphorus became exhausted, a "transition phase" was observed between the "balanced" and "storage" phases, during which cell proliferation proceeded at a reduced rate. Taber and Vining[6] discovered that during the "transition phase", cultures of Claviceps purpurea accumulated nitrogenous but not carbohydrate storage material. Whereas normally ergot alkaloids were accumulated during this period, cultures of Claviceps which did not produce alkaloids passed rapidly into a normal storage phase, accumulating fats and carbohydrates.

Bu'lock has suggested[7,8] that at some time during the initial period of rapid nutrient uptake, the "tropophase", some intermediate of primary metabolism, normally present in low concentrations, may suddenly accumulate and induce formation of the enzymes necessary



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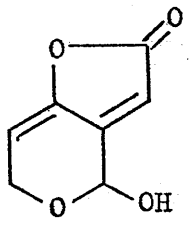

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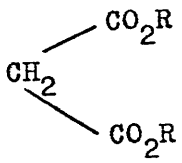
for secondary metabolism. The end of the tropophase, the period during which primary metabolism predominates, may be brought about by exhaustion of a nutrient, after which the "idiophase", the phase of secondary metabolite production, begins. It has been observed in Penicillium islandicum[9] and in Penicillium urticae[8] that the rate of oxidation of acetate fell during the tropophase, reaching a minimum when secondary metabolism commenced. This could be explained by an accumulation, towards the end of the tropophase, of primary metabolites, such as acetyl and malonyl Coenzyme A, and intermediates of the Krebs Cycle, with a concomitant decrease in rate of uptake of nitrogen and phosphorus, and in the rate of acetate metabolism. This accumulation of primary metabolites may have induced formation of new enzymes, or may have effected activation of enzymes already present, causing secondary metabolism to begin[10]. Formation of a secondary metabolite may induce enzymes for its modification, leading to production of a range of secondary metabolites. Bu'lock found evidence of this from studies of production and transformations of 6-methylsalicylic acid (10) by Penicillium urticae[11], in which this compound is converted to patulin (11) via gentisyl derivatives. 6-Methylsalicylic acid synthetase was shown to be a metabolically stable enzyme produced during the tropophase, and activated during the idiophase. Metabolically labile enzymes were formed during the idiophase which converted the acid to its gentisyl derivatives then to patulin.

Since accumulation of a compound in a culture may induce the formation of enzymes for its mofification, care must be taken when introducing compounds to cultures as part of a biosynthetic study. Addition of large quantities of material can, by this process, give rise to artefacts[2]. The use of radioactive labelling makes possible



12 R = H

13 R = C_2H_5OH

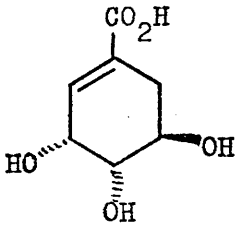
the administration of potential precursors in trace amounts, thus minimising the likelihood of artefacts (although if the compound is normally only present in minute quantities in the culture, even these trace amounts can behave as overdoses and cause misleading results). The potential intermediate must be introduced when the culture is actively producing the desired metabolite: adding it too late will result in poor uptake of label; adding it too early may result in its being metabolised to an undesired product before the desired metabolic process has begun. Because of their greater inhomogeneity, surface cultures are generally more easy to study than are shake cultures, since it is probable that at the time of addition of the "precursor", at least part of the mycelium will be actively producing the desired metabolite[1].

Care also has to be exercised in the interpretation of the results of a biosynthetic study. If a compound is not incorporated, it may be because it is not a precursor, or because it simply cannot penetrate the cell wall. For example, in some Penicillia, free malonic acid (12) cannot penetrate the cell walls, whereas diethyl malonate (13) does, and can function as a precursor of polyketides via malonyl-Coenzyme A[2]. If a compound does appear to be a biosynthetic intermediate, then it may either lie on the main pathway, or on a minor pathway, or may possibly exist in equilibrium with the true precursor, while not actually being one itself. The efficiency of a compound as a precursor is measured by its incorporation into the metabolite, given by the ratio:

$$\text{Incorporation} = \frac{\text{specific activity x weight of metabolite}}{\text{specific activity x weight of precursor}} \times 100\%$$

As mentioned earlier, secondary metabolites are produced in enormous variety by fungi, and are, in the words of Bu'lock[10],

"an expression of the individuality of the species in molecular terms". Many suggestions have been advanced as to the purpose of these apparently redundant compounds. The suggestion that they may be reserve foodstuffs does not account for the variety of these compounds, and seems somewhat inadequate, since fats and carbohydrates are used by fungi as food reserves. The production of an antibiotic obviously confers upon the organism the advantage of being able to kill off competition, thus making it more fit for survival[12]. Most secondary metabolites, however, possess no antibiotic activity, and it is possible that the activity of the relatively few compounds which do may be simply fortuitous. It is also possible that secondary metabolites may provide a means of waste disposal or detoxification of the environment in which a culture finds itself[13]. For example, if some product of primary metabolism may accumulate to such an extent that it becomes toxic to the organism, then conversion to a secondary metabolite may remove it from the pool of active metabolites. Bu'lock[14] has suggested that secondary metabolites are themselves of little significance, but that it is the process of secondary metabolism which is important because by removing intermediates which would otherwise accumulate, it allows the processes of primary metabolism to continue during times of stress. This explanation seems reasonable, since, despite their enormous variety and species-specificity, secondary metabolites arise by a limited number of pathways.



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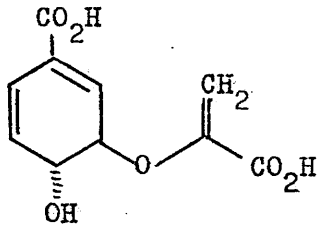

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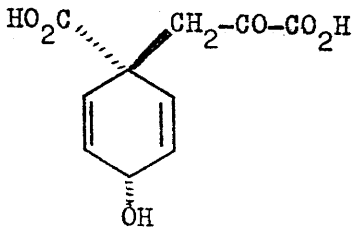

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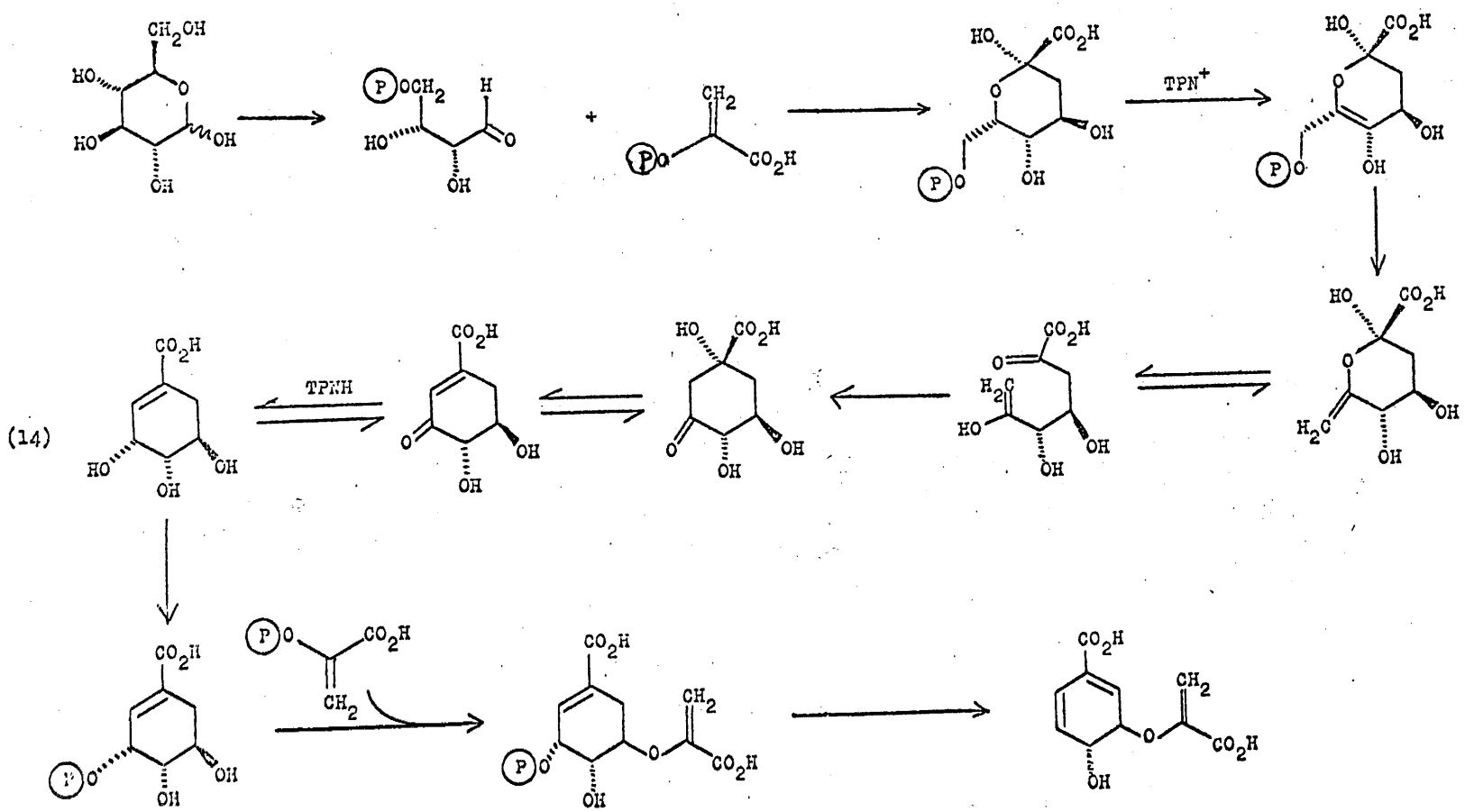
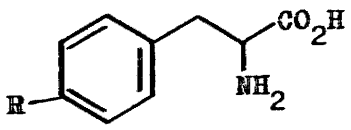
Scheme 1





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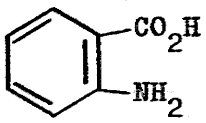
18 R = OH



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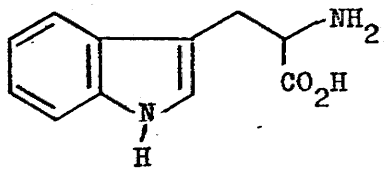

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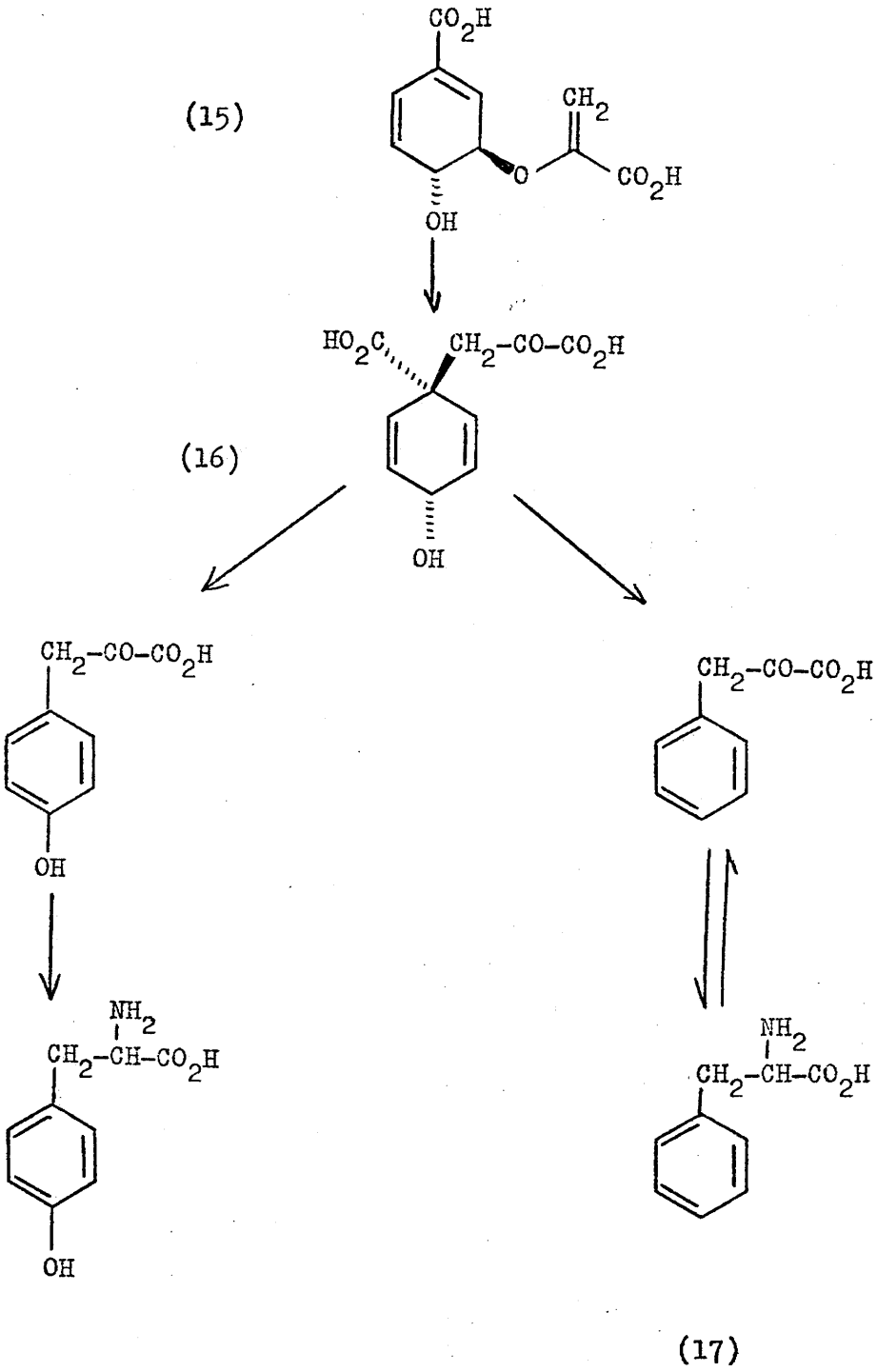
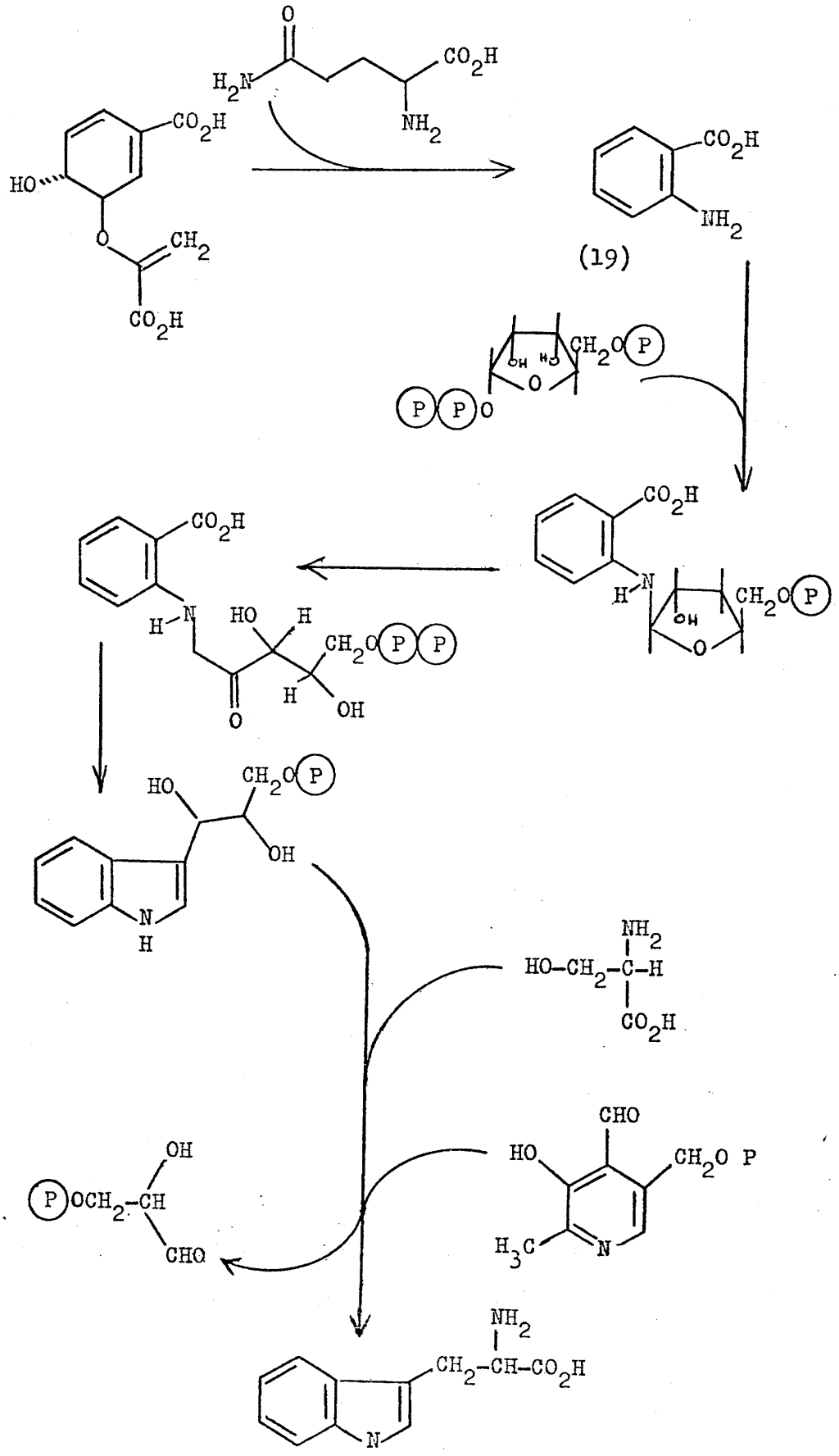
Scheme 2


(15)
CO_2H
CH_2
O
CO_2H
OH
HO_2C
$CH_2-CO-CO_2H$
(16)
OH
$CH_2-CO-CO_2H$
OH
NH_2
$CH_2-CH-CO_2H$
OH
(18)
$CH_2-CO-CO_2H$
NH_2
$CH_2-CH-CO_2H$
(17)

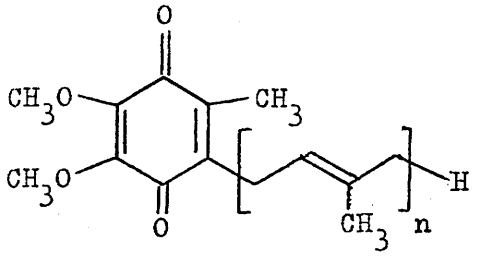
Scheme 3



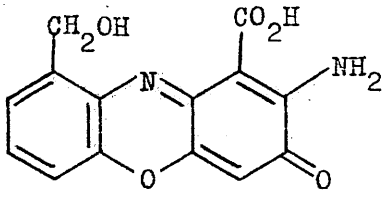
FUNGAL SECONDARY METABOLISM - THE SHIKIMIC ACID PATHWAY

Three major pathways of fungal secondary metabolism lead to polyketides, isoprenoids, and aromatic amino acids respectively. Whereas the first two pathways involve acetate as the fundamental starting material, the third involves glucose without intervention of acetate, and takes its name from one of its intermediates: shikimic acid (14). This compound was first discovered in 1885[15,16] in the seeds of the plant Illicium religiosum, but its importance was not realised until the 1950's, when it was identified as an intermediate in the biosynthesis of aromatic amino acids[17]. The pathway, elucidated using bacterial mutants[18], comprises an unbranching part, common to all shikimate-derived metabolites, and several branches, leading to various classes of metabolites[19]. The common part of the pathway consists of the sequence of transformations from glucose to chorismic acid[15] (Scheme 1)[2]. Transformations of chorismic acid give rise to the aromatic amino acids and isoprenoid quinones. For example, rearrangement gives prephenic acid (16), the precursor of phenylalanine (17) and tyrosine (18) (Scheme 2). A different sequence of reactions gives anthranilic acid (19), which leads to tryptophan (20) (Scheme 3) and to many alkaloids. This biosynthetic pathway could quite properly be called the chorismic acid pathway, but for the fact that shikimic acid was discovered first. It is of interest to note that chorismic acid was only discovered within the past fifteen years. The widespread importance of the shikimic acid pathway is illustrated by its occurrence in higher plants as well as in bacteria and fungi; in plants, it proceeds via the same intermediates as in microorganisms[20].

The shikimic acid pathway gives rise to many interesting



21 n = 1 - 12



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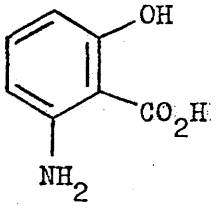

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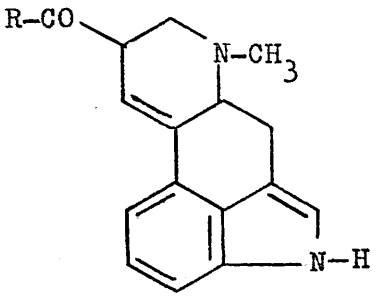
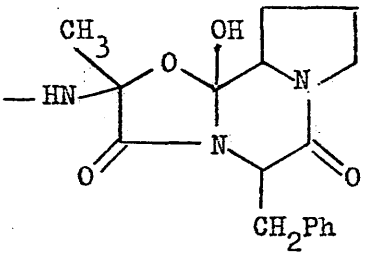
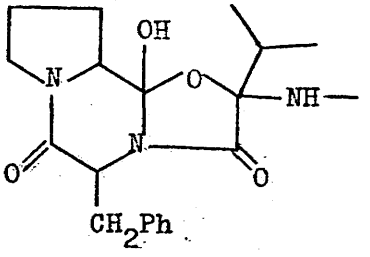


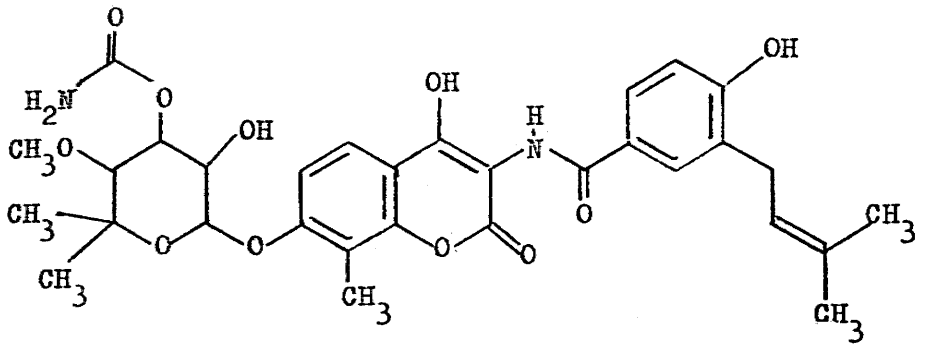
24 R = OH

25 R =



26 R =





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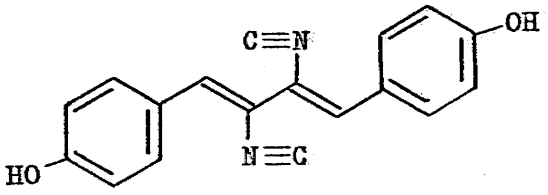

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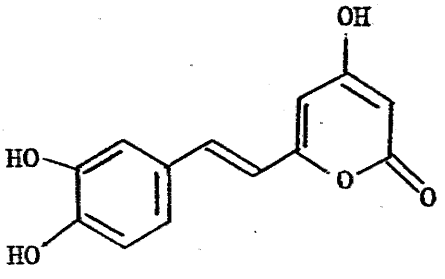

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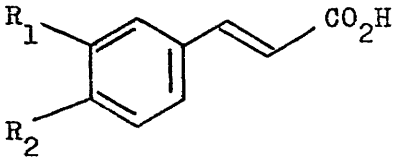
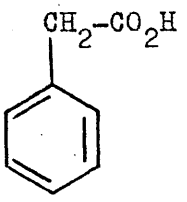


30 $R_1 = R_2 = H$

31 $R_1 = H$; $R_2 = OH$

32 $R_1 = R_2 = OH$

metabolites having a wide variety of structures and biological functions, including antibiotics, alkaloids, pigments, and toxins. For example, the ubiquinones or "Coenzyme Q" (21) occur widely in the mitochondria of animals and plants[21] and in the cell membrane of non-photosynthetic microorganisms, being involved in electron transport processes[22,23]. The quinone part of these is derived from shikimic acid, and the prenyl side chain from mevalonate[24]. Cinnibarin (22) is a red pigment found in the fruiting bodies of Polyporus cinnibarinus[25], which might arise[1] by oxidative coupling of 3-hydroxyanthranilic acid (23) or related compounds. The hallucinogenic ergot alkaloids ergotamine (25) and ergocristine (26), responsible for the toxic effects of rye infected with the fungus Claviceps purpurea[1], are amides of lysergic acid (24), a tryptophan-derived metabolite. The antibiotic novobiocin[26] (27), found in Streptomyces niveus, is a particularly interesting metabolite, having a mixed biosynthesis. The 3-aminocoumarin and p-hydroxy-benzoyl units are shikimate-derived, phenylalanine and tyrosine acting as equally effective precursors to these, while the sugar arises from glucose, the isopentenyl unit from mevalonate, and the methoxyl and gem-dimethyl groups from methionine[27]. Xanthocillin (28), a yellow antibiotic pigment occurring in Penicillium notatum, has the novel feature of isonitrile groups. Biosynthetic studies suggest that the C_6-C_2 units are most probably derived from tyrosine[28]. A final example of a shikimate-derived metabolite is the curious orange pigment hispidin (29), isolated from freshly-harvested fruiting bodies of Polyporus hispidus[29,30]. This pigment appears to form a lignin-like polymer when the fruits are allowed to stand and harden. It is probably formed from phenylalanine or tyrosine via caffeic acid (32)[10].



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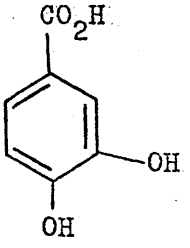

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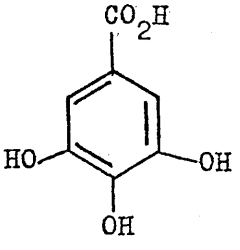
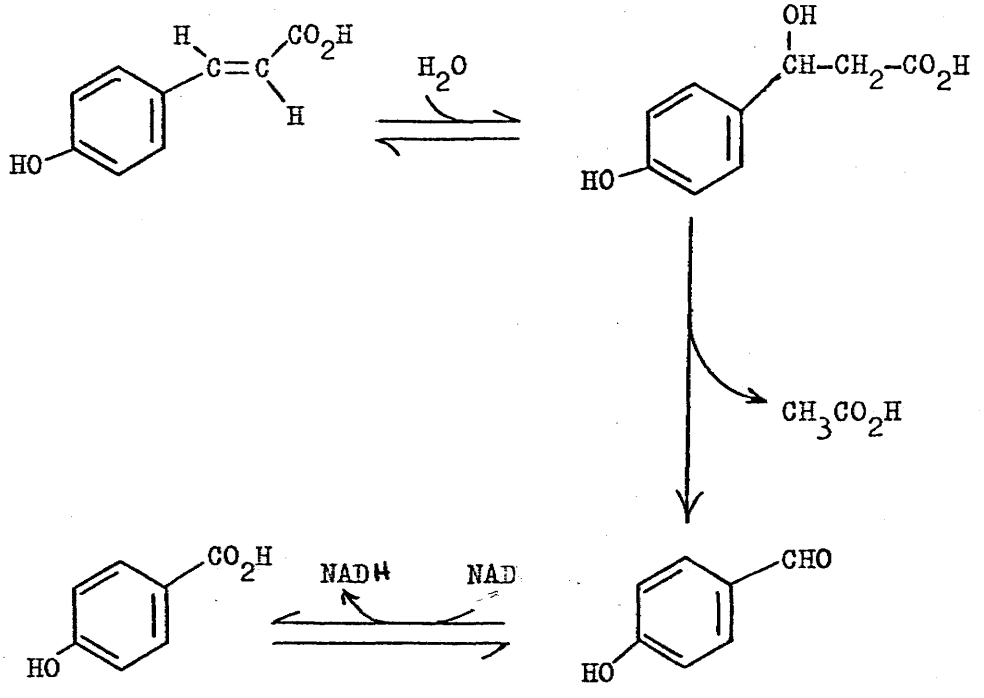


35

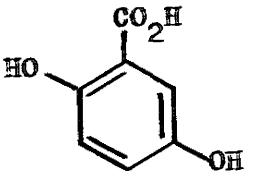
Scheme 4


H
C=C
CO2H
H
HO
H2O
OH
CH-CH2-CO2H
HO
CH3CO2H
CO2H
HO
NADH
NAD
CHO
HO

The shikimic acid pathway gives rise to a wide range of aromatic compounds, some of which can arise from more than one point on the pathway. C_6-C_3 acids, such as cinnamic acid (30), are frequently derived by deamination of the corresponding amino acids by the action of ammonia lyases. These enzymes have been shown to be present in Basidiomycetes and Actinomycetes[31,32]. p-Coumaric acid (31), in addition to being formed by deamination of tyrosine, can arise by enzymatic hydroxylation of cinnamic acid, as has been found to occur in a species of Polyporus[22].

Shortening of the side chain of cinnamic acids to give C_6-C_2 and C_6-C_1 compounds occurs in higher plants by important oxidation processes, and evidence that a similar pathway exists in fungi has been obtained from studies of Basidiomycetes. Schizophyllum commune has been found to convert cinnamic acid but not phenylacetic acid (33) into benzoic acid[33]. Washed cells of Sporobolomyces roseus convert cinnamic, p-coumaric, and caffeic acids into protocatechuic acid (34), probably via benzoic and hydroxybenzoic acids[32]. More recently, the conversion of p- coumaric acid to p-hydroxybenzoic acid by a non-oxidative mechanism has been shown to occur in Polyporus hispidus and in a cell-free extract of potato tubers[34]. When p- coumaric acid was supplied as substrate, the reaction was found to be fast and independent of NAD, suggesting that the chain-shortening step occurred via a non-oxidative mechanism[35] (Scheme 4). This is the first report of a C_6-C_3 compound giving a C_6-C_1 compound in a fungal cell-free system.

C_6-C_1 compounds can arise via C_6-C_3 compounds or from intermediates of the common part of the shikimic acid pathway. Gallic acid (35) has been found to come from 5-dehydroshikimic acid in Phycomyces blakesleeanus[36]. However, there is evidence that it



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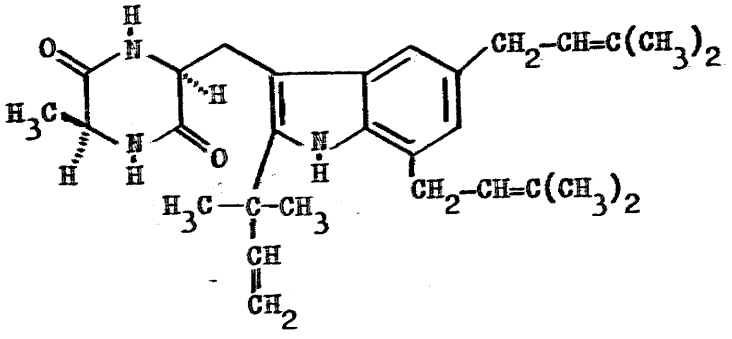

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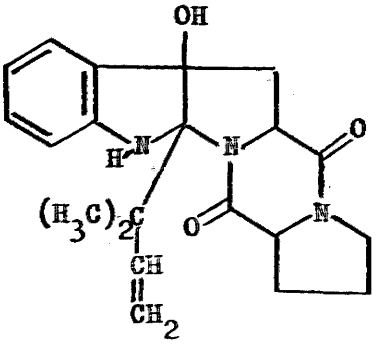

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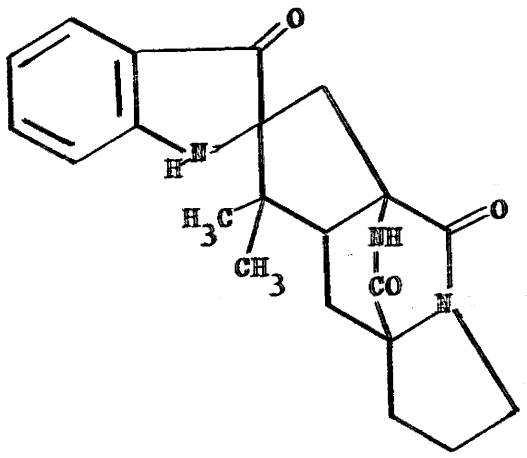

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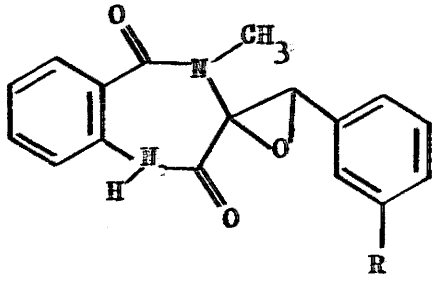


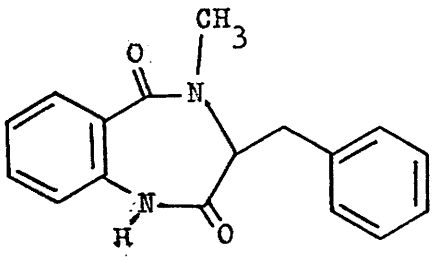
40 R = H

41 R = OH

may also be derived via a C_6-C_3 acid, since addition of L-tyrosine to cultures of the fungus stimulated gallic acid production[37]. Experiments on higher plants[38,39] suggest that this compound may arise by either route. Not only can C_6-C_1 compounds arise from different points on the shikimic acid pathway, they can arise by different pathways in different fungi. Gentisic acid (36) is a good example, coming from acetate in Penicillium urticae[40] (a member of the Fungi Imperfecti), but arising from the shikimic acid pathway in Polyporus tumulosus[41] (a Basidiomycete) and in higher plants[42]. Studies of this and other phenolic metabolites of P. tumulosus led Crowden[41] to conclude that C_6-C_1 compounds can be formed from C_6-C_3 precursors by a number of different routes operating simultaneously, the relative importance of an individual route depending on prevailing conditions.

The diketopiperazines are an important group of fungal metabolites, many of which are derived via the shikimic acid pathway. Echinulin (37) is found in various species of Aspergillus[43]. Biosynthetic studies using [2-^{14}C] mevalonate and [1-^{14}C] acetate have shown that the compound contains three isoprene units[44]. The specific incorporation of DL-[carboxy-^{14}C] alanine[44] and DL-[carboxy-^{14}C] tryptophan [45] with no randomisation of the label indicate that the diketopiperazine ring has the stereochemistry shown[46] (37). This is borne out by the observation that L-tryptophan is incorporated much more efficiently than the D isomer[45]. The substitution of tryptophan at the 2' position by an isoprene unit also occurs in brevianamide E (38), a metabolite of Penicillium brevicompactum[47]. Rearrangement of this isomer may lead to brevianamide A (39).

The benzodiazepine alkaloids cyclopenin (40) and cyclopenol (41), isolated from Penicillium cyclopium[48], were found to have a



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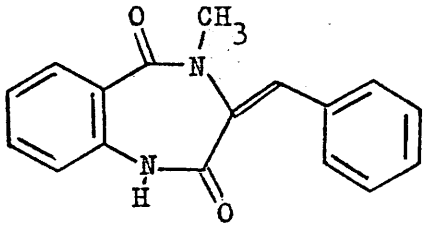

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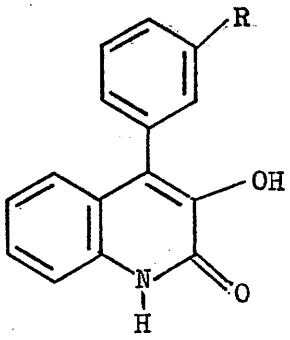
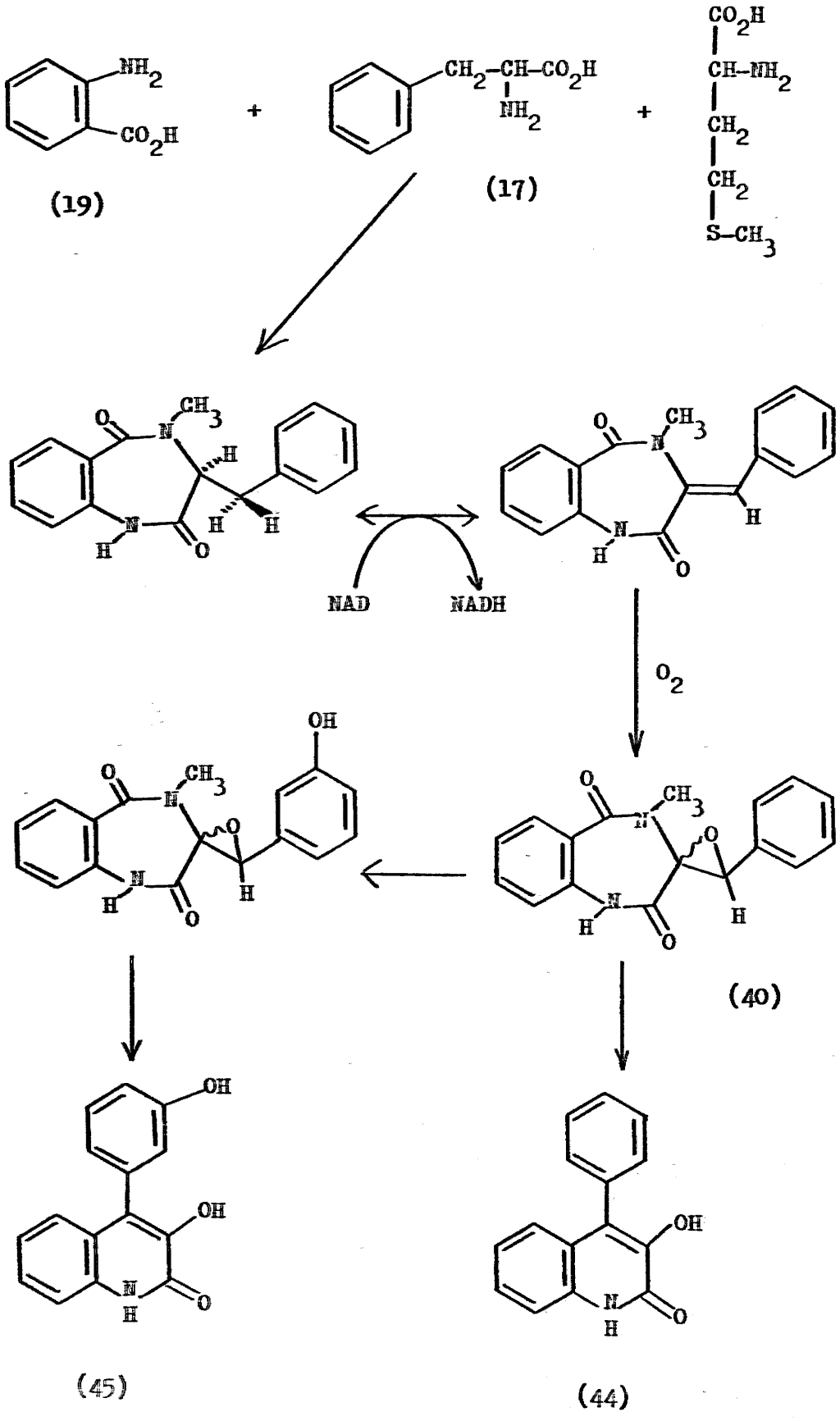


44 R = H

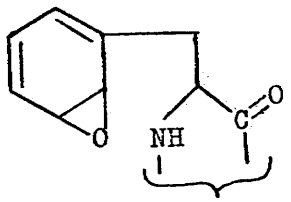
45 R = OH

Scheme 5



carbon skeleton derived from anthranilic acid, phenylalanine, and the methyl group of methionine[49]. The cyclic dipeptides cyclo-(anthranoyl-L-N-methylphenylalanyl) (42) and its 3,10- dehydro-derivative (43) were found to be specific precursors, while the corresponding acyclic compounds were not, suggesting that in the biosynthesis of cyclopenin, the cyclic peptide is formed in an enzyme-bound state, with cyclisation occurring as it leaves the enzyme[50]. These cyclic dipeptide intermediates were later isolated from cultures of P. cyclopium and named cyclopeptine (42) and 3,10-trans-dehydrocyclopeptine (43). m-Hydroxylated derivatives of these could not be found, leading to the conclusion that the biosynthetic sequence is: dehydrogenation of cyclopeptine to give the trans-3,10-dehydro derivative, followed by epoxidation of the double bond with molecular oxygen giving cyclopenin, and finally m-hydroxylation to give cyclopenol[50], catalysed by a mixed function oxygenase[49] (Scheme 5). The benzodiazepine alkaloids cyclopenin and cyclopenol are converted to the quinoline alkaloids viridicatin (44) and viridicatol (45) by the action of cyclopenase in the conidiospores of the fungus.

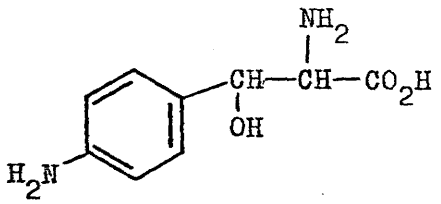
The epidithiodiketopiperazines are a group of extremely potent antiviral and antifungal agents which are produced by many of the Fungi Imperfecti[51]. Gliotoxin (5) the first member of the group to be discovered, was first isolated from a species of Trichoderma[52]. Biosynthetic studies have shown that serine is the source of carbon atoms 3,3a, and 4. DL-[1-^{14}C] and [2-^{14}C] phenylalanine give labelling at positions 1 and 10a, respectively, of the diketo-piperazine ring. Furthermore, DL-[^{3}H] m-tyrosine was found to be incorporated more efficiently than was phenylalanine[53], leading to the conclusion that phenylalanine is hydroxylated to m-tyrosine


O
NH
C
O

before cyclisation of the indole ring system. Care is always required in interpreting the results of a feeding experiment, and in this case, it seemed that more care was taken by other workers who obtained very poor incorporations of m-tyrosine compared to phenylalanine[54]. They proposed cyclisation of the indole system via an arene oxide-type intermediate (46), and suggested that the previous conclusion could have been the result of a phenylalanine impurity in the m-tyrosine giving good incorporation into gliotoxin. Their proposal was borne out by the finding that [G-^{3}H] phenylalanine was incorporated into gliotoxin without loss of ring tritium[54], which is inconsistent with intermediacy of m-tyrosine.

The origin of the nitrogen atoms has been the subject of much discussion. Feeding of L-[^{15}N] phenylalanine gave gliotoxin labelled only on N(5), whereas [^{15}N] glycine gave label in both N atoms[55]. Aspartic and glutamic acids have also been found to be capable of contributing nitrogen, but not carbon, to the diketopiperazine ring. This led to the conclusion that the nitrogen pool from serine gives rise to substantial quantities of phenylalanine. However, it has subsequently been shown that exchange of an amino group between L-serine and L-phenylalanine is limited[56].

Chloramphenicol (3), a metabolite of Streptomyces venezuelae[57] is a very potent wide spectrum antibiotic, causing inhibition of protein synthesis in bacteria[58]. The compound is biosynthesised via shikimic acid and L-p-aminophenylalanine[59], but neither L-phenylalanine nor L-tyrosine were incorporated into the C_6-C_3 skeleton, suggesting that it may arise at some point along the common part of the shikimic acid pathway between 3-enolpyruvylshikimic acid-5-phosphate and prephenate. Labelling studies indicate that the p-aminophenylalanine undergoes β-hydroxylation to



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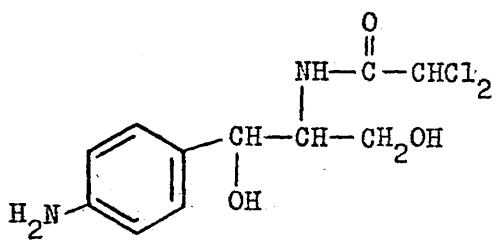
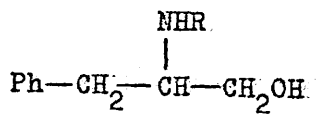


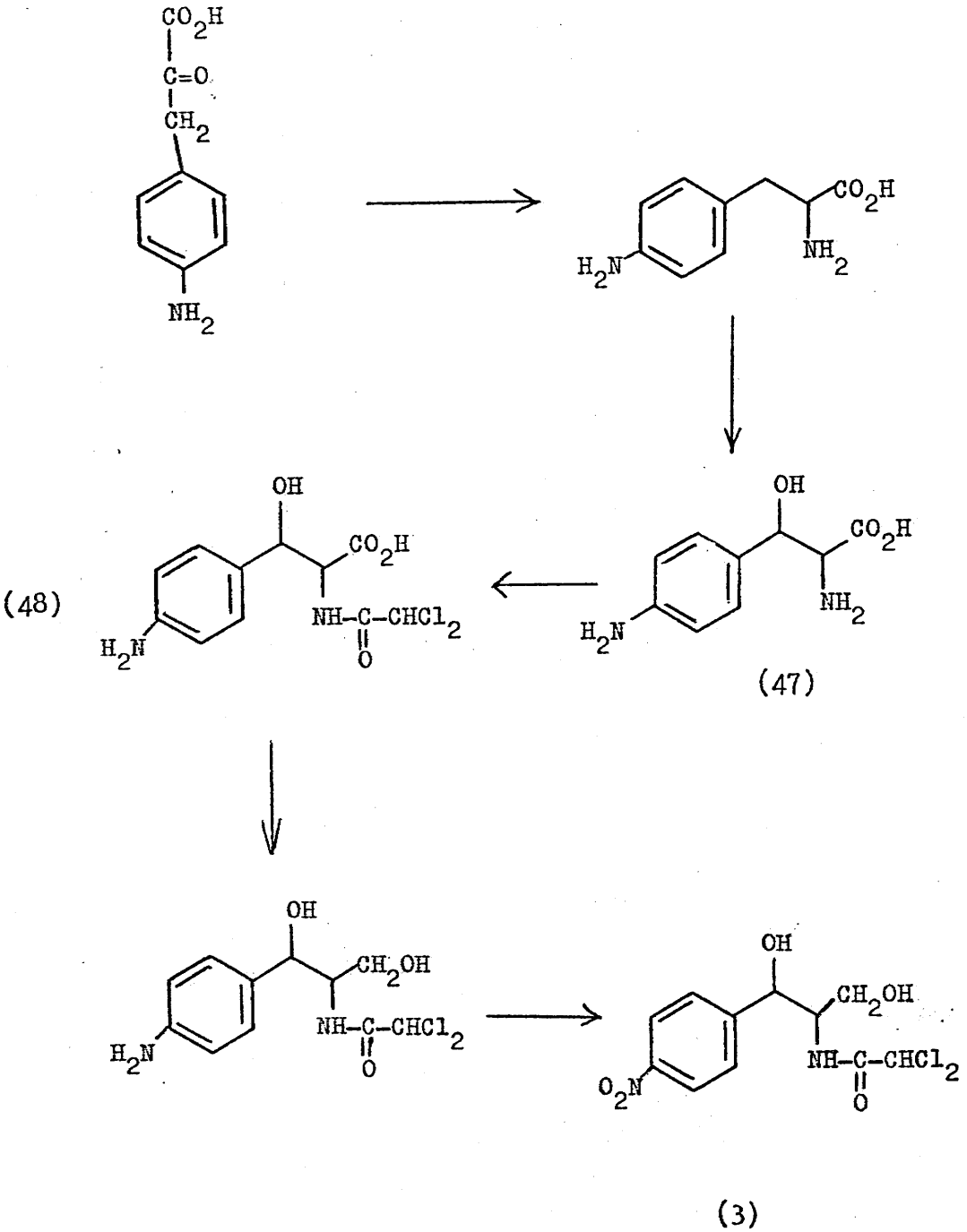
48

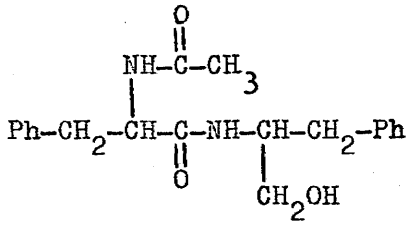


49 R = H

50 R = C=O
Ph

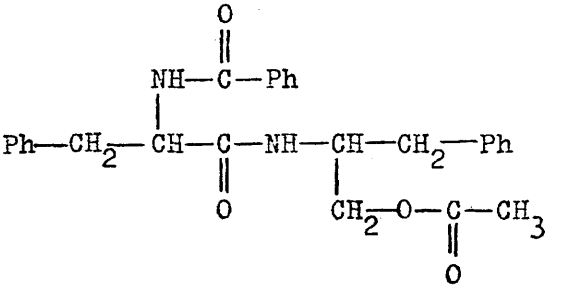
Scheme 6


CO2H
C=O
CH2
NH2
H2N
CO2H
NH2
OH
CO2H
NH2
H2N
(47)
OH
CO2H
NH-C-CHCl2
O
H2N
(48)
OH
CH2OH
NH-C-CHCl2
O
H2N
OH
CH2OH
NH-C-CHCl2
O
O2N
(3)



give threo-p-aminophenylserine (47), an efficient and specific precursor of chloramphenicol[60]. The sequence of steps following this transformation is rather uncertain, but the next precursor in line to be found was N-dichloroacetyl-D-threo-p-aminophenylserinol[60] (48). From the non-incorporation of ^{14}C-labelled dichloroacetic acid into chloramphenicol, it was suggested that the acylation may require several steps[61]. p-Aminophenylserinol and its nitro analogue do not act as precursors, indicating that N-acylation probably occurs before reduction of the carboxyl group. N-dichloroacetyl-threo-p-aminophenylserinol was incorporated with high efficiency and without randomisation of the label (hydroxymethyl-^{14}C), suggesting that it is close to the end of the biosynthesis[60]. This compound was found in trace quantities in chloramphenicol-producing cultures of S. venezuelae[62]. Feeding of unlabelled DL-serine with ^{15}N labelled potassium nitrate gave equal ^{15}N enrichment of both nitrogen atoms, irrespective of the serine: nitrate ratio in the medium[63]. This confirms that the nitro group of chloramphenicol does not come from a biological nitration involving nitrate. It appears to arise by direct oxidation of an amino group, as occurs in the biosynthesis of β-nitropropionic acid[1]. Although the intermediacy of some of the compounds has not yet been proved, evidence to date suggests that the biosynthesis of chloramphenicol is as in Scheme 6.

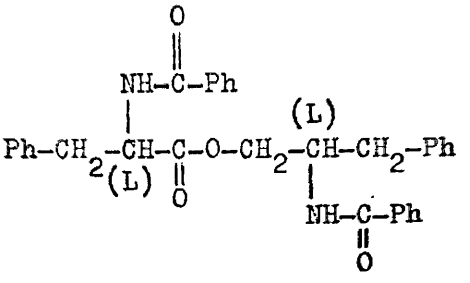
Chloramphenicol can be considered to be a substituted L-phenylalaninol derivative[64], and it is interesting to note that natural products found containing this amino alcohol are comparatively few in number. N-benzoyl-L-phenylalaninol (50) has been found in the leaves of the plants Catharanthus pusillus[64] and Alangium lamarkii[65]. N-acetyl-L-phenylalanyl-L-phenylalaninol (51)



has been isolated from culture filtrates of the fungus Emerecellopsis salmosynnemata[66]. N-(N-benzoyl-L-phenylalanyl)-O-acetyl-L-phenylalaninol (52) has been isolated from the fungus Aspergillus glaucus[67]. L-phenylalaninol (49) has also been identified as one of the hydrolysis products of the polypeptide antibiotic antiamoebin[68].

At the time of initiation of the present work, relatively few biosynthetic systems had been studied using late precursors. This thesis discusses the biosynthesis of candipolin, investigated using almost exclusively late precursors. Candipolin, a mycelial metabolite of Penicillium canadense, is a benzoylated phenylalaninol derivative, whose structure and synthesis are discussed herein.

D I S C U S S I O N



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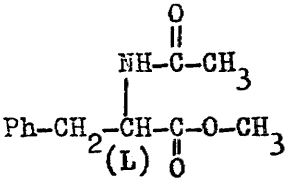

54

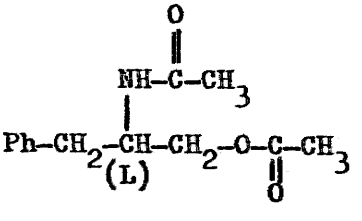

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Figure 1a

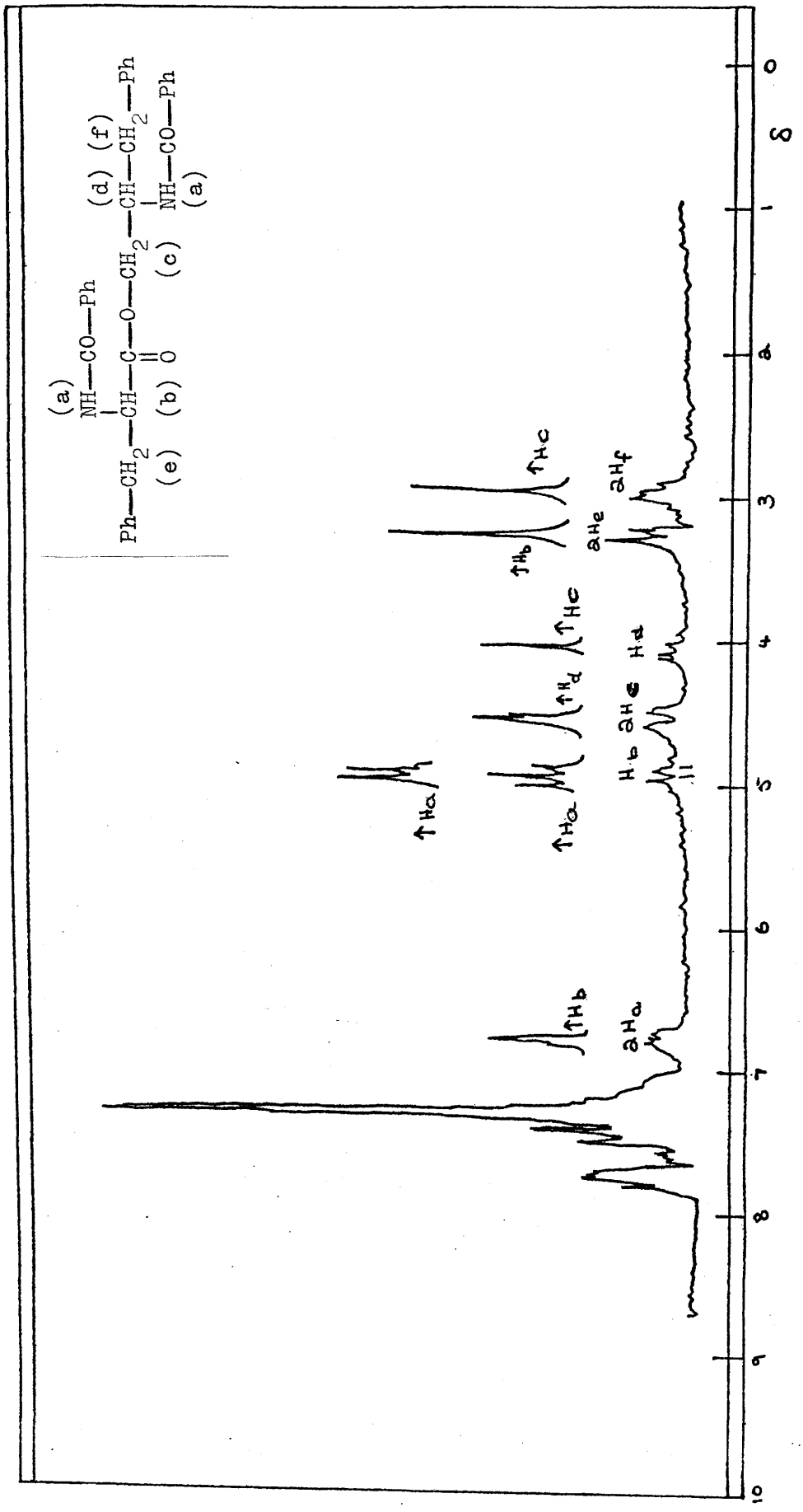
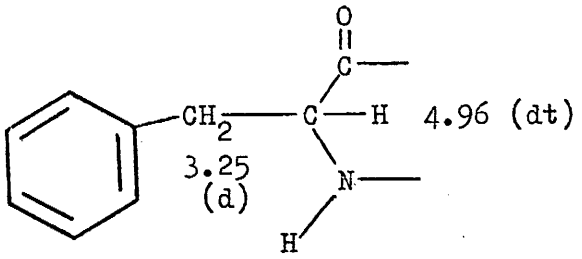
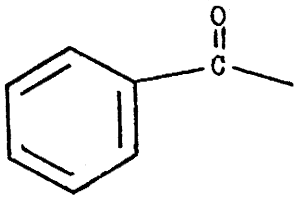

(a)
NH—CO—Ph
Ph—CH2—CH—C—O—CH2—CH—CH2—Ph
(e) (b) O (c) (d) (f)
NH—CO—Ph
(a)
↑Ha
↑Hb
↑Hd
↑Hc
↑Hb
↑Hc
aHa
Hb aHe Hd
aHe
aHf
0
1
2
3
4
5
6
7
8
9
10
δ

Figure 1b


O
C
CH2
C
H
4.96 (dt)
3.25
(d)
N
H
7.2 (s)
6.7 (m)

2 X


O
C
7.8 - 7.3 (m)

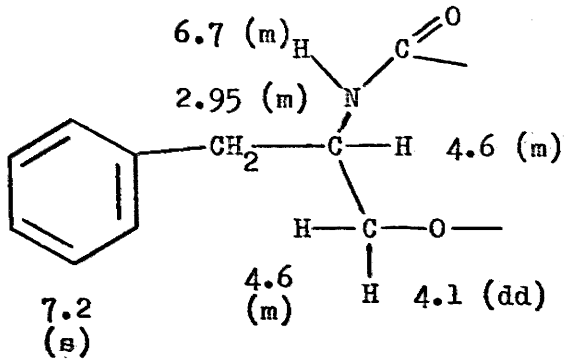
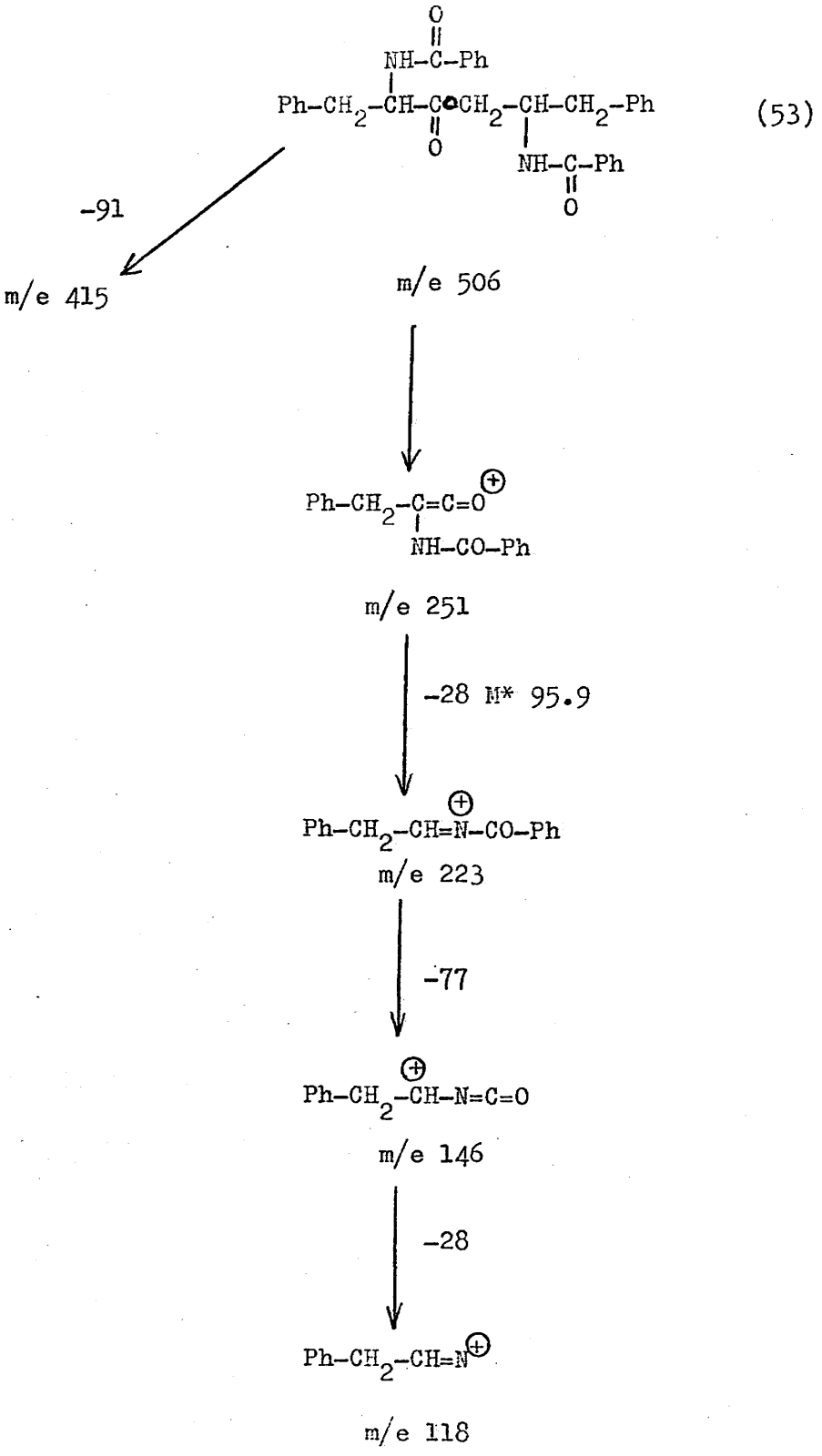
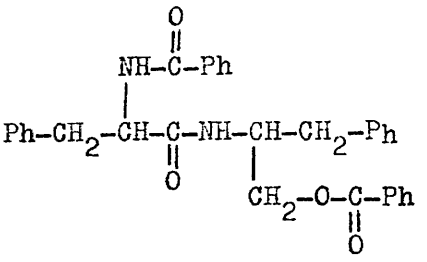
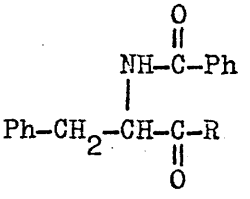

6.7 (m)
H
O
C
2.95 (m)
N
CH2
C
H
4.6 (m)
H
C
O
4.6
(m)
H
4.1 (dd)
7.2
(s)

Figure 2





56



58 R = OH

59 R = $N{=}\overset{\oplus}{N}{=}\overset{\ominus}{N}$

DISCUSSION

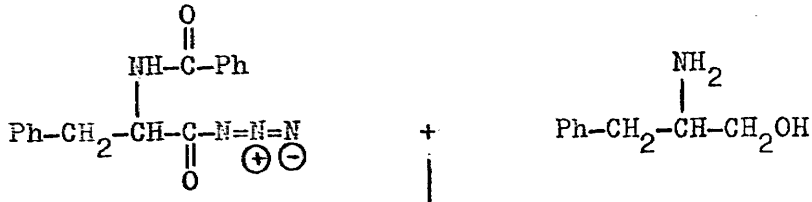
CHAPTER I

SYNTHESIS AND PROPERTIES OF CANDIPOLIN (53)

Candipolin (53) was first isolated from the chloroform extract of the mycelium of Penicillium canadense by silica column chromatography[69]. Elemental analysis and mass spectrometry[70] indicated that the compound had the general formula $C_{32}H_{30}N_2O_4$. Infrared spectrometry suggested the presence of ester (1750 cm^{-1}), amide (1635 and 1530 cm^{-1}), and aromatic (1600 and 1580 cm^{-1}) functions. The NMR spectrum (Fig. 1a) showed the structural features represented in Fig. 1b, which was consistent with the abundant ions at m/e 91 and 105 in the mass spectrum, corresponding to benzyl and benzoyl groups respectively. Hydrolysis of candipolin by heating at 130° with 5N hydrochloric acid in a sealed tube[71,72] gave benzoic acid plus two compounds which, although not actually isolated, showed T.L.C. behaviour (R_f and ninhydrin stain) identical to that of phenylalanine and phenylalaninol ; these were shown to be present as a 1:1 mixture by G.L.C. after trifluoroacetylation. The configuration of both hydrolysis products was shown to be L- by conversion to N-acetyl-L-phenylalanine methyl ester (54) ($[\alpha]_D$ + 25° in $CHCl_3$), and N,O-diacetyl-L-phenylalaninol (55) ($[\alpha]_D$ - 13° in $CHCl_3$) respectively. The mass spectral fragmentation pattern of candipolin (Fig. 2) showed some features apparently consistent with a peptide structure, namely, N-(N-benzoyl-L-phenylalanyl)-O-benzoyl-L-phenylalaninol (56). The peak at m/e 251 could be due to the ion (57), formed by cleavage of the peptide bond ; loss of CO from this could have given the ion at m/e 223.

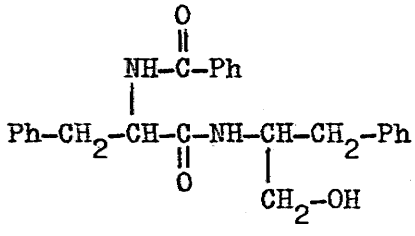
This compound was, however, synthesised by treatment of the azide of N-benzoyl-L-phenylalanine (59) with L-phenylalaninol (49),

Scheme 7



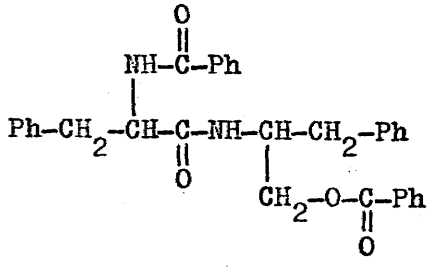
(59)

(49)



(81)

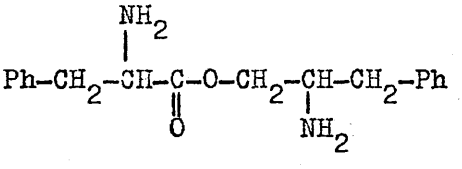
PhCOCl/pyridine



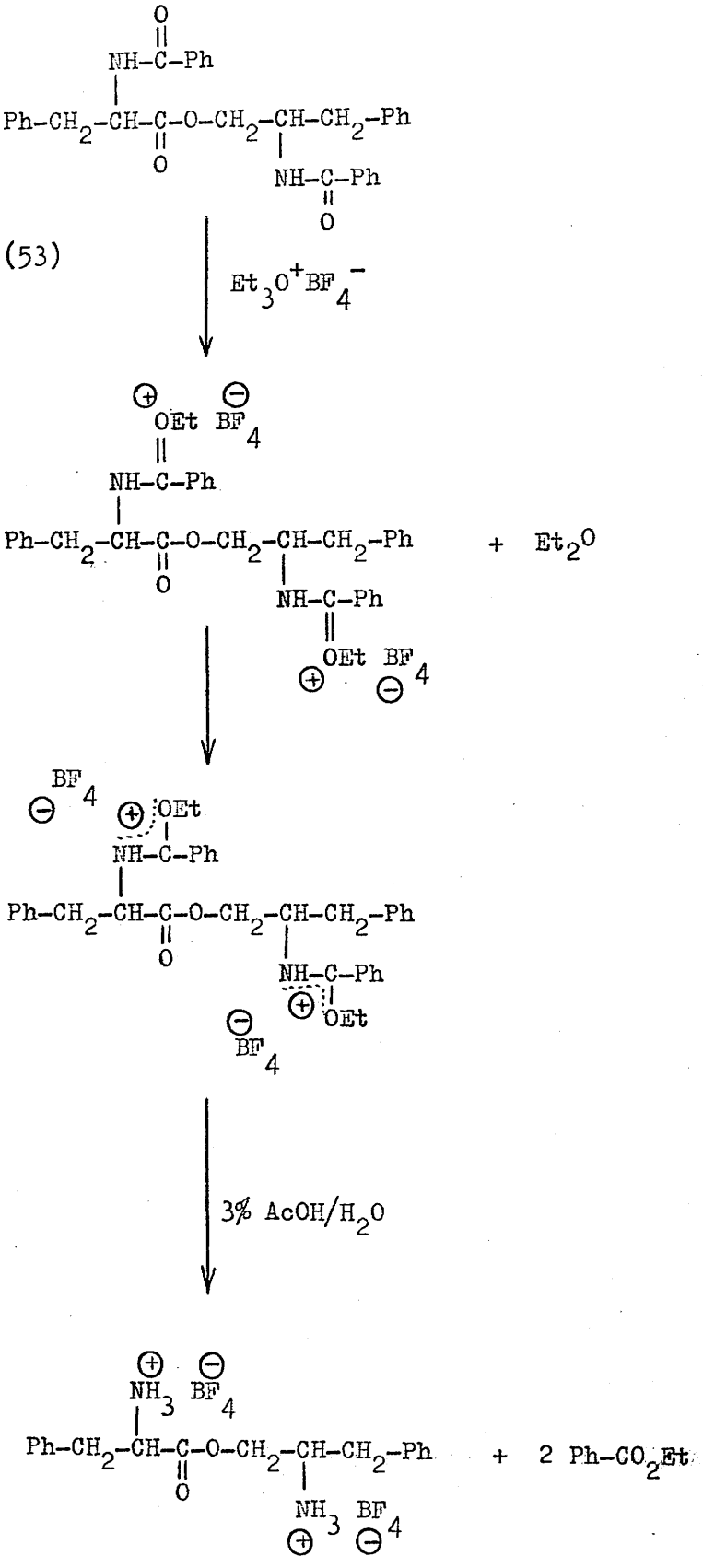
(56)

followed by benzoylation of the product (Scheme 7). Although it had the same R_f and staining properties as candipolin, and virtually the same melting point, mixture with candipolin caused depression of the melting point. Comparison of their I.R. and N.M.R. spectra showed that the two samples were distinct. While the ester carbonyl group in candipolin absorbs at 1750 cm^{-1}, the benzoate ester in the synthetic compound was found to absorb at 1720 and 1710 cm^{-1}. Whereas the synthetic peptide showed two amide I bands (1660 and 1630 cm^{-1}), assigned to the aliphatic and aromatic amides respectively, candipolin showed only one absorption (1635 cm^{-1}). Both compounds showed, in their N.M.R. spectra, a signal at 7.8 δ, assigned to the aryl protons ortho- to the carbonyl group of a benzamide function ; this corresponded to four protons in candipolin, and to two in the peptide. In addition, the peptide had a two-proton signal at 8.0 δ, due to the corresponding protons of the benzoate function. Candipolin showed a two-proton resonance at 6.8 δ, whereas the peptide showed two one-proton resonances at 6.04 and 6.79 δ, assigned to the NH protons of aliphatic and aromatic amides respectively.

A revised structure (53), consistent with all the previously observed data, was advanced for candipolin. Consideration of model esters and amides shows that a number of structural features of candipolin are in fact much better accommodated by the new structure. The ester C=O frequency 1750 cm^{-1} is more typical of aliphatic esters than of benzoates, which generally show lower carbonyl frequencies.[74] Aliphatic amides tend to have amide I absorptions at higher frequencies than the amide I band of candipolin (1635 cm^{-1}), which appears to be more typical of a benzamide. In their N.M.R. spectra, the NH protons of benzamides generally resonate at lower field than do those of aliphatic amides, and the aryl protons ortho- to the



Scheme 8

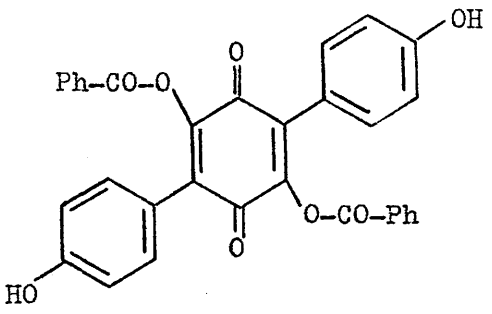


carbonyl group generally resonate at lower field in a benzoate than in a benzamide function.

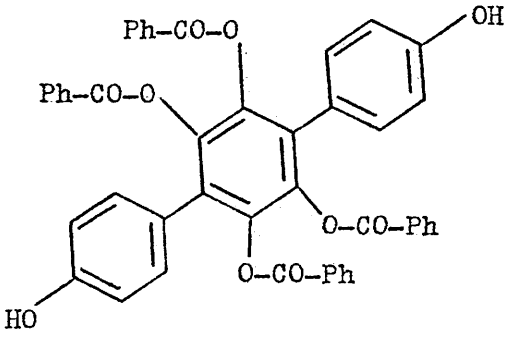
Subsequent studies of candipolin provided further evidence in favour of structure (53). Refluxing with ethanolic hydrazine hydrate yielded N-benzoyl-L-phenylalaninol (50) together with a very polar compound which showed the properties expected for N-benzoyl-phenylalanylhydrazide[73]. Some difficulty was experienced in the purification of this compound, and it reacted rapidly with traces of carbonyl compounds present in organic solvents. Treatment of candipolin with excess triethyloxonium fluoroborate yielded a crystalline compound, whose I.R., N.M.R., mass spectrum, and elemental analysis were consistent with those expected for the fluoroborate salt of the diamine (60)[73] (Scheme 8).

Although an attempt to synthesise N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (53) by esterification of N-benzoyl-L-phenylalanine (58) with N-benzoyl-L-phenylalaninol (50) (using dicyclohexylcarbodiimide) was unsuccessful[70], spectroscopic and degradative evidence described above establish unequivocally that candipolin has structure (53).

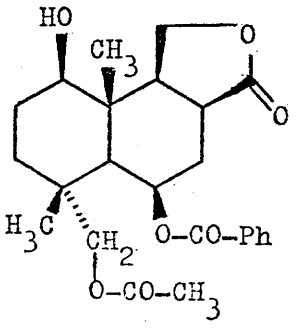
In commencing the present biosynthetic investigation, it was recognised that the candipolin structure involves at least three remarkable features. It incorporates an ester link between an α-amino acid and an α-amino alcohol (the first known natural product of this type). Until recently, only two natural products containing L-phenylalaninol were known, namely, antiamoebin, a biologically active polypeptide from the fungus Emerecellopsis poonensis[68,75], and N-benzoyl-L-phenylalaninol from the leaves of the plant Catharanthus pusillus[64]. In addition, chloramphenicol (3), a potent wide-spectrum antibiotic produced by Streptomyces


OH
O
Ph–CO–O
O–CO–Ph
O
HO

61

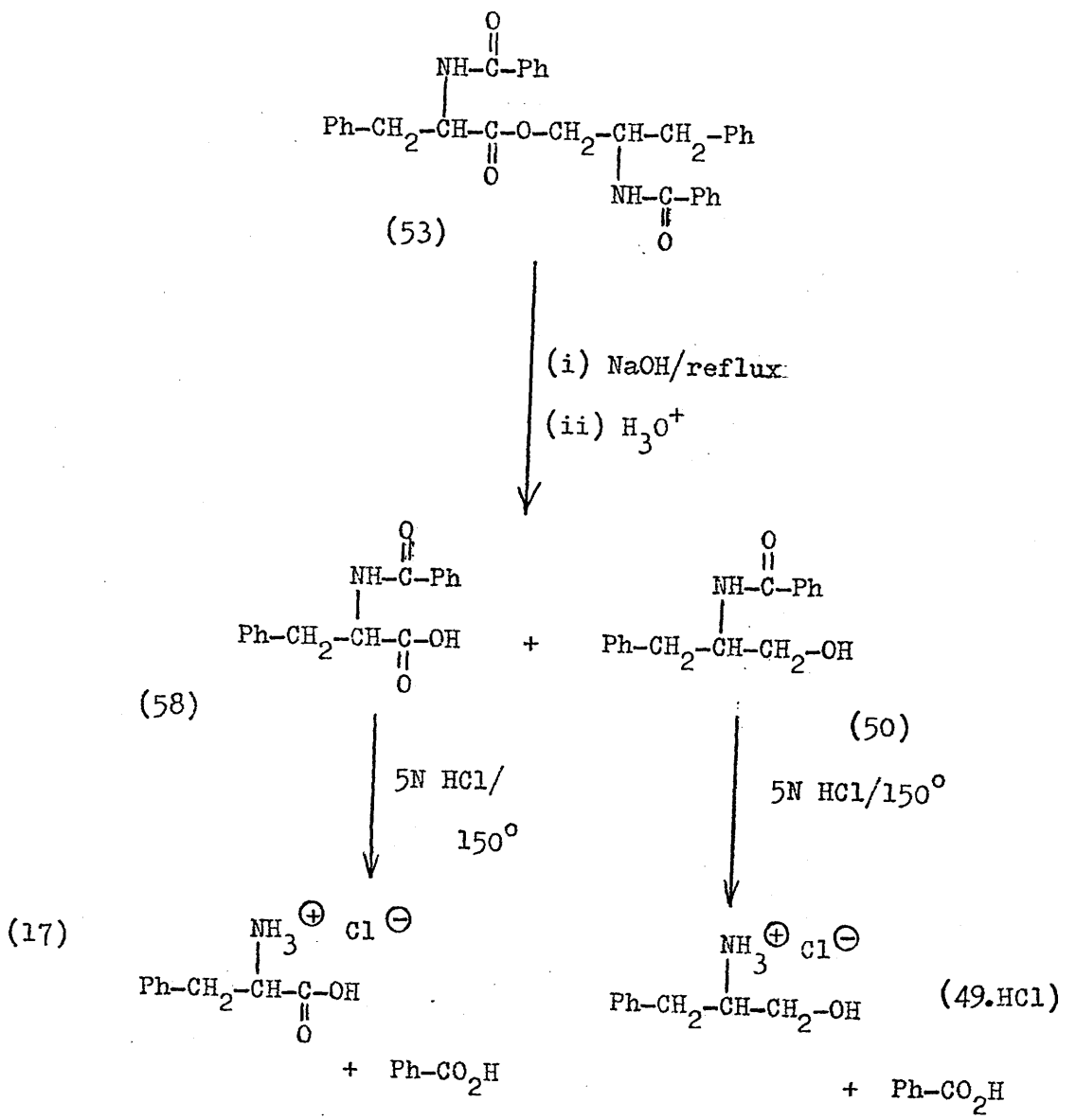

OH
Ph–CO–O
Ph–CO–O
O–CO–Ph
O–CO–Ph
HO

62


HO
CH3
O
O
H3C
CH2
O–CO–Ph
O–CO–CH3

63

Scheme 9


(53)
(i) NaOH/reflux
(ii) H_3O^+
(58)
(50)
5N HCl/ 150°
5N HCl/150°
(17)
(49.HCl)
+ $Ph-CO_2H$
+ $Ph-CO_2H$

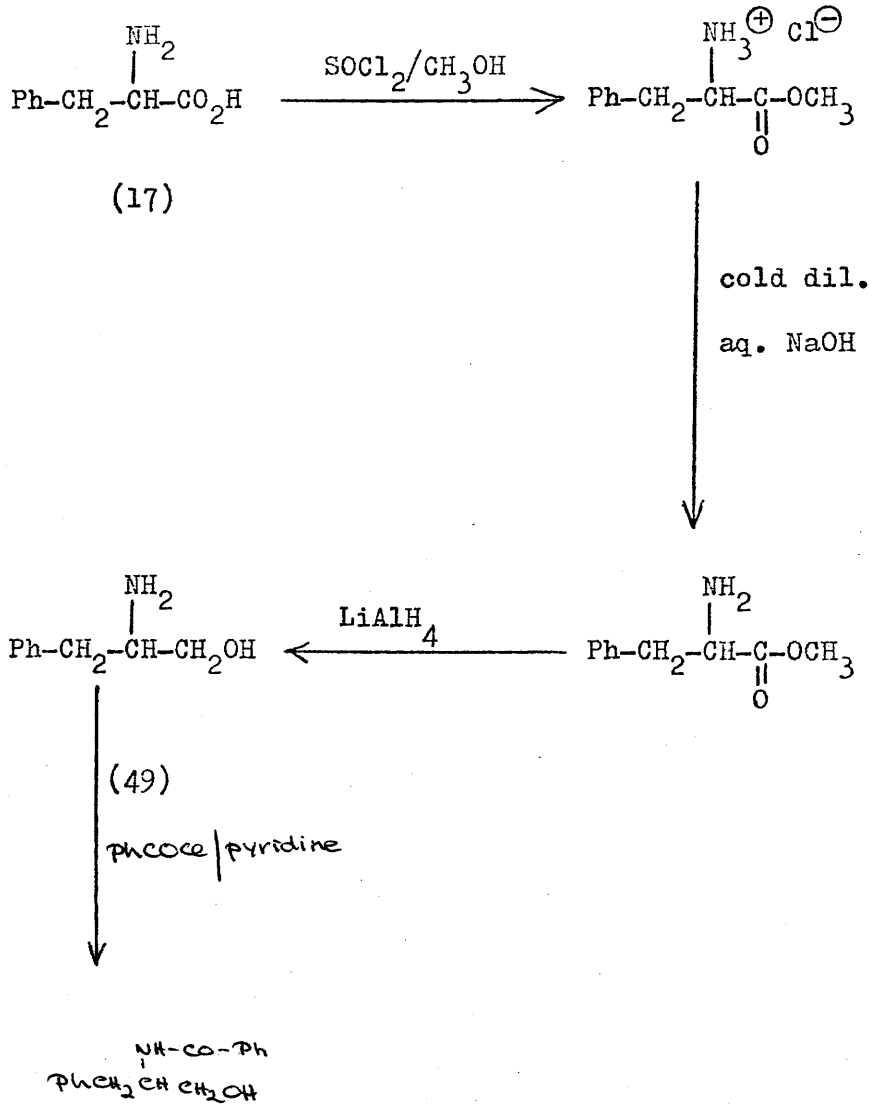
venezuelae[58,76], may be considered as a modified phenylalaninol derivative[64].

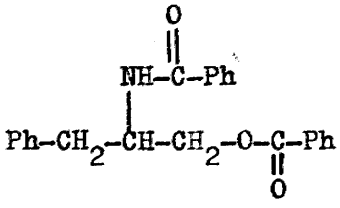
Finally, the benzoyl group, although found in many plant products, is an unusual feature to find in a fungal product. Previously-known metabolites are aurantiacin (61)[77] and debenzoyl leucoaurantiacin (62)[78], produced by Hydnum aurantiacum, and pebrolide (63) and related compounds from Penicillium brevi-compactum[79,80]. Hydroxybenzoic acids can be derived from three routes: by the acetate-malonate route; directly from shikimic acid; via phenylalanine and cinnamic acids[81]. In plants, benzoic acid itself seems to be formed by the last of these three routes, but no investigation of the pathway in fungi has been carried out. Candipolin is an attractive subject for such an investigation, since the presence of a benzoic acid and a phenylalanine unit in the same molecule permits direct comparison to be made of the relative efficiency of incorporation of a precursor into these units.

Before a biosynthetic study could be undertaken, a synthesis of candipolin was required in order both to confirm its structure, and to make available potential biosynthetic precursors. An efficient degradative scheme was also required, which would give access to each of the four units of which the structure (53) is composed.

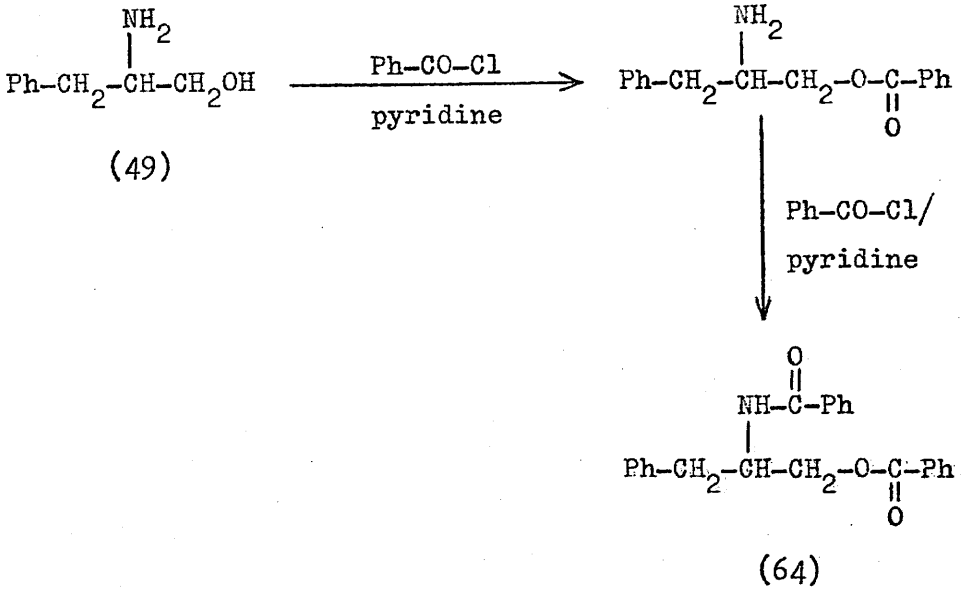
Selective hydrolysis of the ester link in candipolin was achieved by refluxing with 0.01 M sodium hydroxide. This gave N-benzoyl-L-phenylalanine (58) and N-benzoyl-L-phenylalaninol (50) in almost quantitative yield, and the products were optically pure. Acid hydrolysis of each component at 150° gave benzoic acid together with L-phenylalanine and L-phenylalaninol respectively (Scheme 9). The last compound was conveniently isolated as its hydrochloride.

Scheme 10


NH2
Ph-CH2-CH-CO2H
SOCl2/CH3OH
NH3⊕ Cl⊖
Ph-CH2-CH-C-OCH3
O
(17)
cold dil.
aq. NaOH
NH2
Ph-CH2-CH-C-OCH3
O
LiAlH4
NH2
Ph-CH2-CH-CH2OH
(49)
PhCOCl/pyridine
NH-CO-Ph
PhCH2CH CH2OH



Scheme 11



(49)

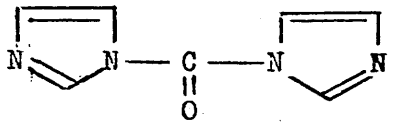
(64)

This degradative sequence, in addition to independently establishing the structure of candipolin as (53), makes available three optically pure compounds which may be intermediates in its biosynthesis, namely N-benzoyl-L-phenylalanine (58), N-benzoyl-L-phenylalaninol (50), and L-phenylalaninol (49).

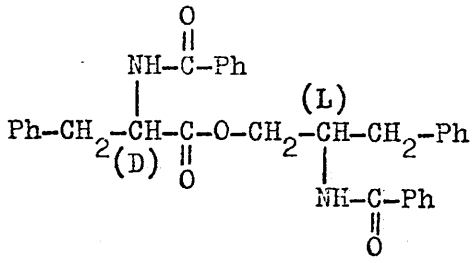
The most obvious way to synthesise candipolin was by esterification of N-benzoyl-L-phenylalanine with N-benzoyl-L-phenylalaninol. N-benzoyl-L-phenylalaninol was prepared in three stages from L-phenylalanine as outlined in Scheme 10. Yields in the step involving LAH reduction of L-phenylalanine methyl ester were at first found to be variable. Accordingly, an alternative method of reduction, using sodium bis-(2-methoxyethoxy) dihydro aluminate (SDA) was explored, but this gave poor results. Finally, the difficulties in the LAH reduction were resolved, the low yields previously obtained being attributed largely to inefficient extraction of the product, and unusually large losses upon drying extracts.

In benzoylation of L-phenylalaninol, the N,O-dibenzoyl derivative (64) occurred as a by-product, even when equimolar quantities of benzoyl chloride were used. Despite the apparently facile formation of this by-product, it was noted that O-benzoylation of N-benzoyl-L-phenylalaninol required a considerable excess of benzoyl chloride, and prolonged reaction time. It is possible that in the benzoylation of phenylalaninol, N- and O- benzoylation are competitive processes, and that the N,O-dibenzoyl derivative arises mainly via the O-benzoylphenylalaninol (Scheme 11).

The other reagent required for synthesis of candipolin, N-benzoyl-L-phenylalanine, was easily prepared by benzoylation of L-phenylalanine methyl ester, followed by selective hydrolysis of the ester function. Careful acidification of the sodium salt

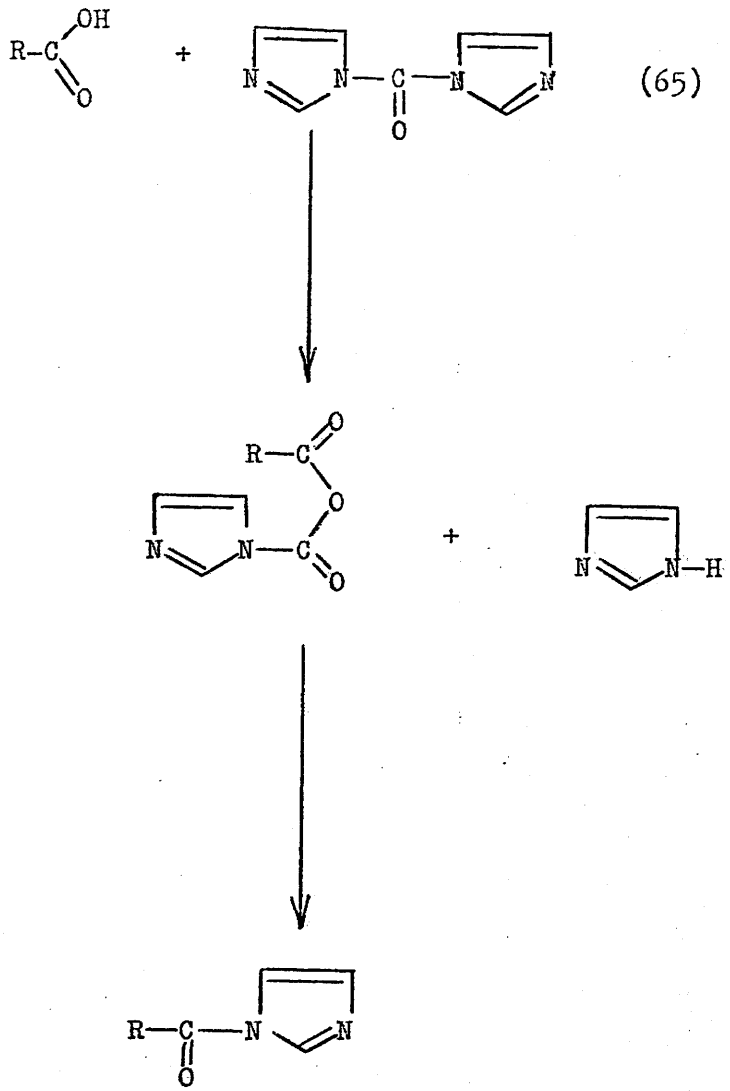


65



66

Scheme 12



(65)

Figure 3

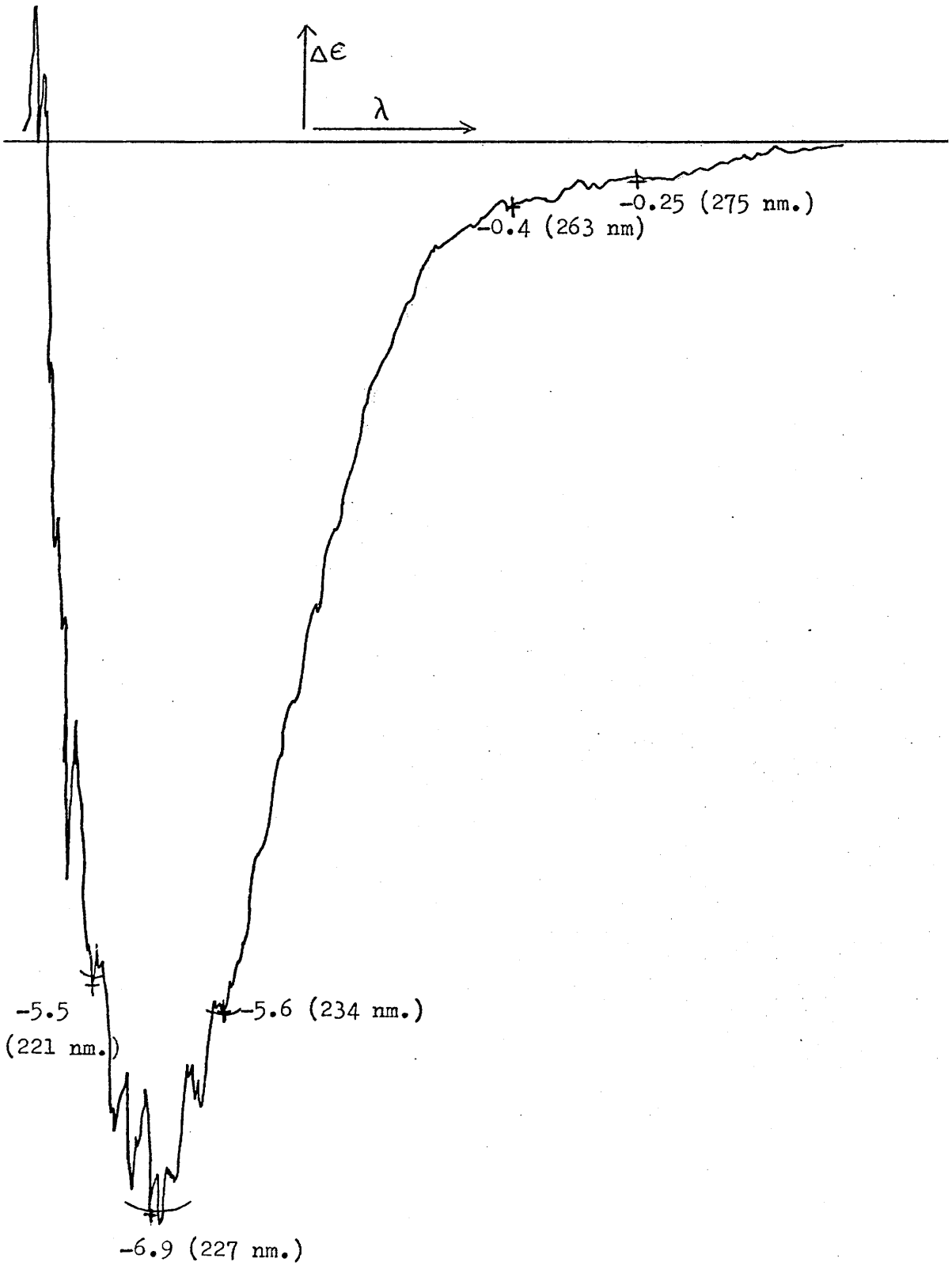
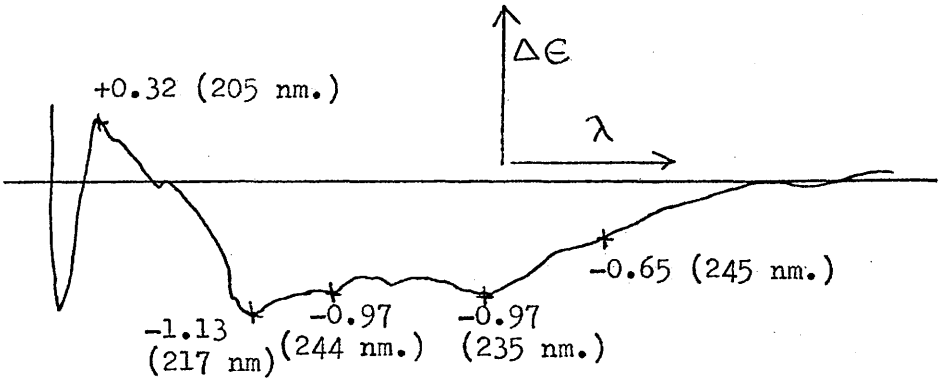


Figure 4

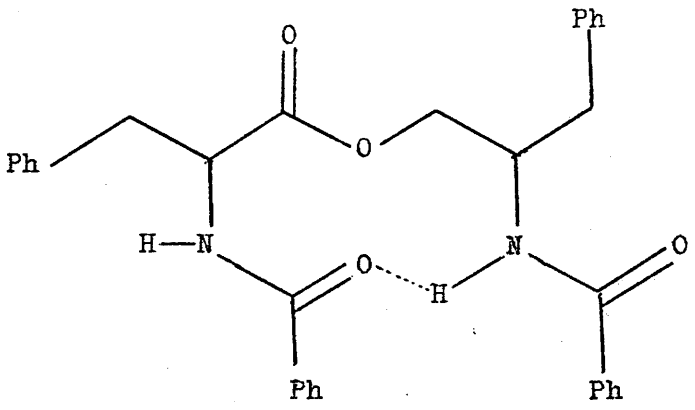


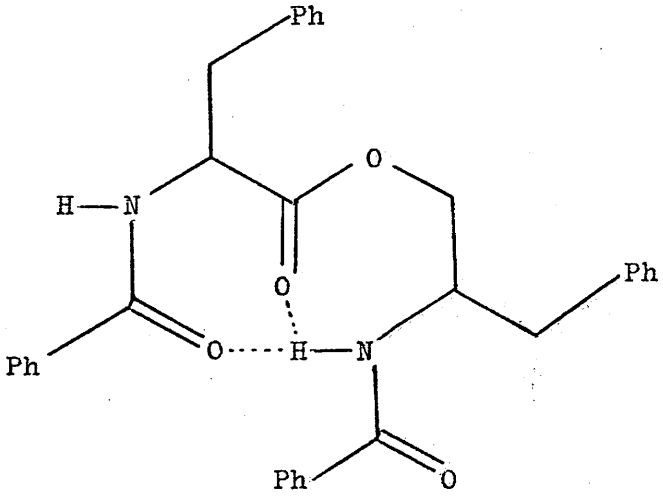
yielded optically pure acid.

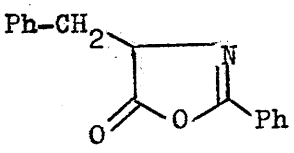
Since large supplies of commercial N-benzoyl-DL-phenylalanine were available, this was used in a preliminary investigation of the esterification with N-benzoyl-L-phenylalaninol. The somewhat sluggish behaviour of N-benzoyl-L-phenylalaninol towards benzoyl chloride noted above suggested that an efficient acylating system would be required to prepare candipolin and related esters. It has been shown that N,N'-carbonyldiimidazole (65) reacts with acids to give acylimidazolides which act as effective acylating agents not only towards amines[82,83], but also towards alcohols [84] (cf. Scheme 12).

Reaction of N-benzoyl-DL-phenylalanine with N-benzoyl-L-phenylalaninol using N,N'-carbonyldiimidazole yielded, in addition to some unreacted alcohol, a product, apparently homogeneous by T.L.C., having the same R_f and staining properties as candipolin. Repeated crystallisation of this material from ethanol afforded a compound, m.p. 218°, whose mass spectrum and elemental analysis were identical to those of candipolin (m.p. 210°). Its N.M.R. spectrum in $CDCl_3$ showed NH resonance as a 2H multiplet at 6.6 δ, whereas the corresponding resonance in candipolin came at 6.8 δ. No other N.M.R. differences were apparent. The compounds were, however, distinct, as shown by mixed melting point, circular dichroism, and infrared spectroscopy. The C.D. curves of the two compounds showed striking differences, that of candipolin (Fig. 3) having a very strong minimum of $\Delta\epsilon$ -6.9 (277 nm.), while the other curve (Fig. 4) showed several much weaker minima in the carbonyl region. The synthetic product thus appeared to be the (D,L) diastereoisomer of candipolin ("epicandipolin") (66). The most noticeable difference between the solid state I.R. spectrum of this compound and that of candipolin is the ester carbonyl frequency, which appears at 1733 cm^{-1} (cf.

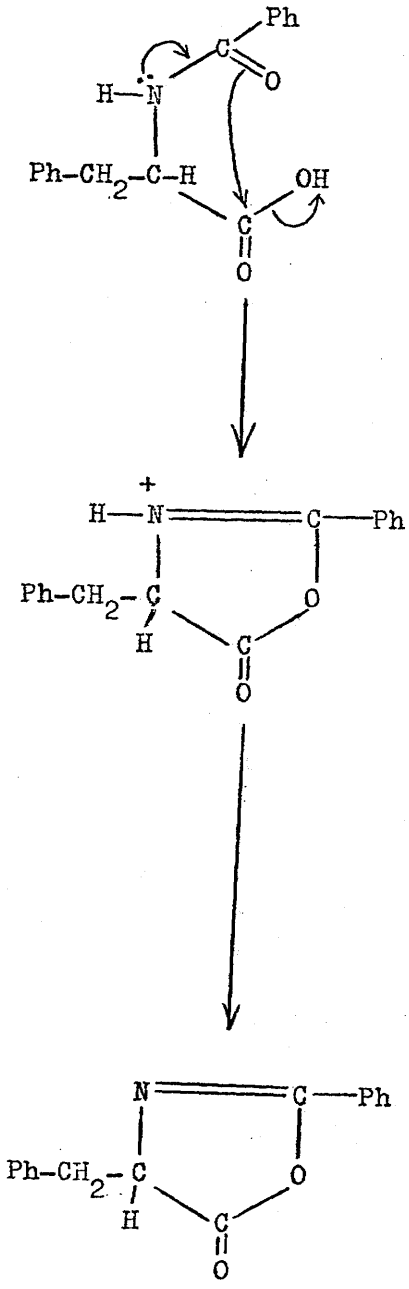
Figure 5





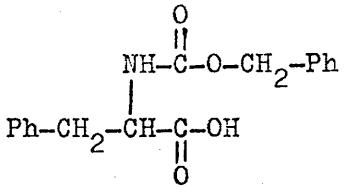

Ph–CH2
N
O
O
Ph

Scheme 13

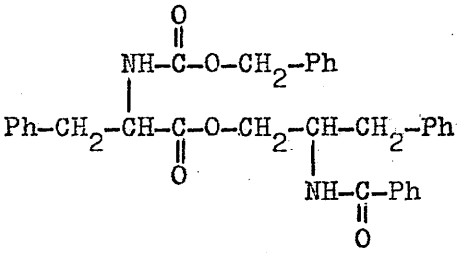


$\nu_{C=O}$ was 1750 cm.$^{-1}$ in candipolin). This difference may reflect the respective conformations adopted in the crystal states, since the carbonyl frequencies in solution are much closer together (1735 cm.$^{-1}$ for epicandipolin and 1740 cm.$^{-1}$ for candipolin). Two possible conformations are indicated in Fig. 5, in which the ester carbonyl group lies in different environments, particularly with regard to intramolecular hydrogen bonding. The mother liquors from the first crystallisation of epicandipolin contained a more soluble component, which was finally obtained in low yield after removal of eight successive crops of crystals. This proved to be identical with natural candipolin in T.L.C. behaviour, mixed melting point, and solid state I.R. spectrum.

Although the candipolin had proved difficult to isolate in this reaction, since an equal amount of epicandipolin was also obtained, it was hoped that similar condensation of N-benzoyl-L-phenylalaninol with N-benzoyl-L-phenylalanine might give candipolin accompanied by little or no epicandipolin. The required acid, obtained by benzoylation of L-phenylalanine methyl ester hydrochloride, followed by base hydrolysis, was esterified with N-benzoyl-L-phenylalaninol, using N,N'-carbonyldiimidazole. However, the solid state I.R. spectrum of the major product showed that it once more consisted of approximately equal amounts of candipolin ($\nu_{C=O}$ 1750 cm.$^{-1}$) and epicandipolin ($\nu_{C=O}$ 1733 cm.$^{-1}$). Also obtained in this reaction was a minor, less polar, product, which showed a double[85] carbonyl absorption at 1825 and 1810 cm.$^{-1}$. By comparison with an authentic sample[86], this was identified as 2-phenyl-4-benzyl-5-oxazolone (67), known to be the intermediate through which racemisation of N-benzoyl-L-phenylalanine occurs[87] (Scheme 13). In order to show that the oxazolone could in fact be

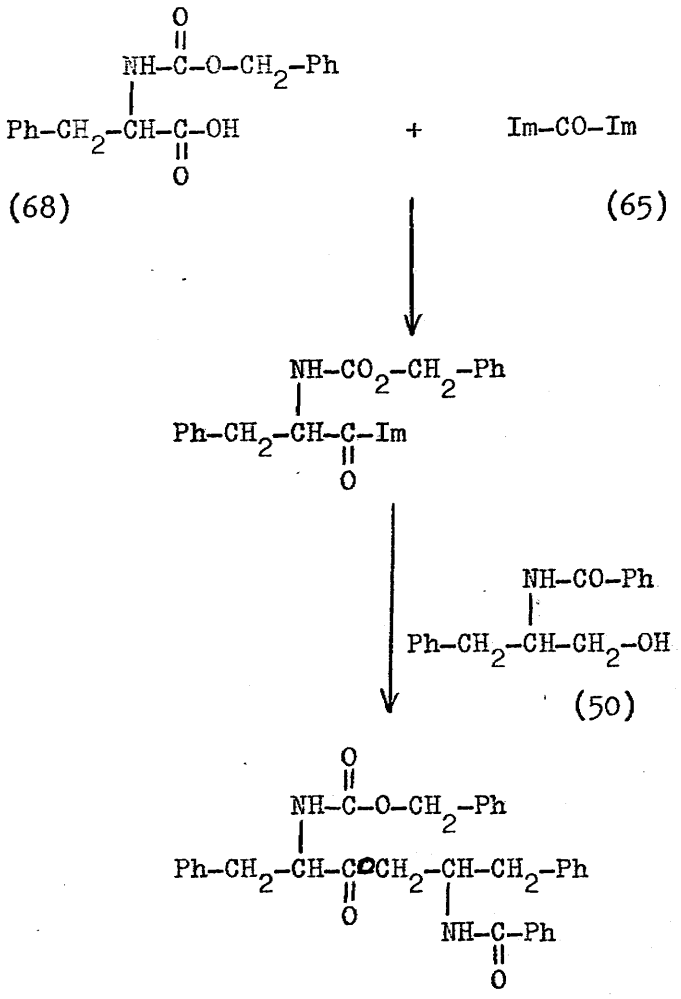


68



69

Scheme 14


NH-C-O-CH2-Ph
Ph-CH2-CH-C-OH
Im-CO-Im
(68)
(65)
NH-CO2-CH2-Ph
Ph-CH2-CH-C-Im
NH-CO-Ph
Ph-CH2-CH-CH2-OH
(50)
NH-C-O-CH2-Ph
Ph-CH2-CH-C-O-CH2-CH-CH2-Ph
NH-C-Ph
(69)

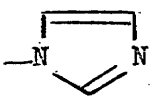
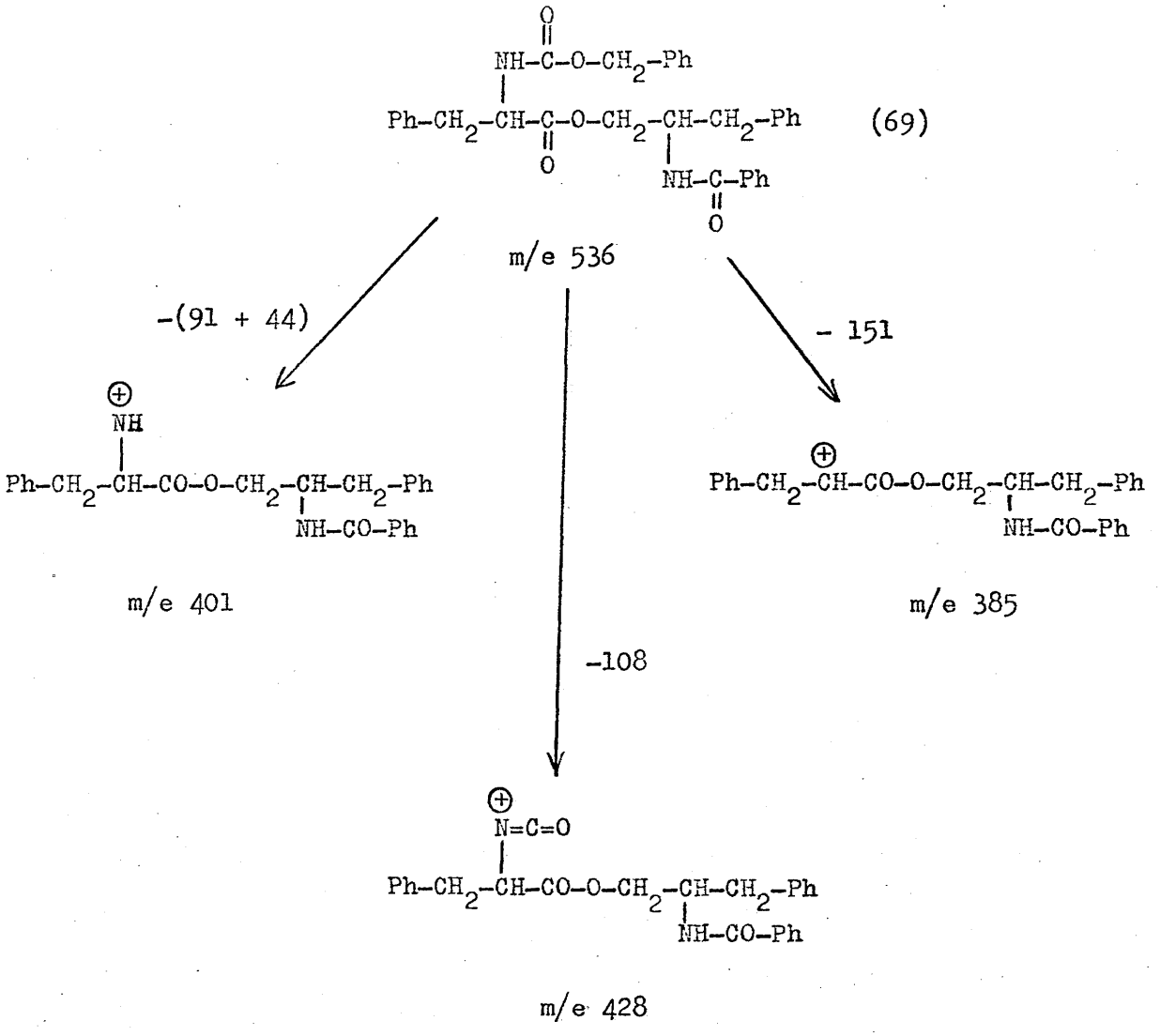
Im = 
N
N

Figure 6



Losses :

108 = $Ph-CH_2-OH$

91 + 44 = $Ph-CH_2$ + CO

151 = $Ph-CH_2-O-CO-NH_2$

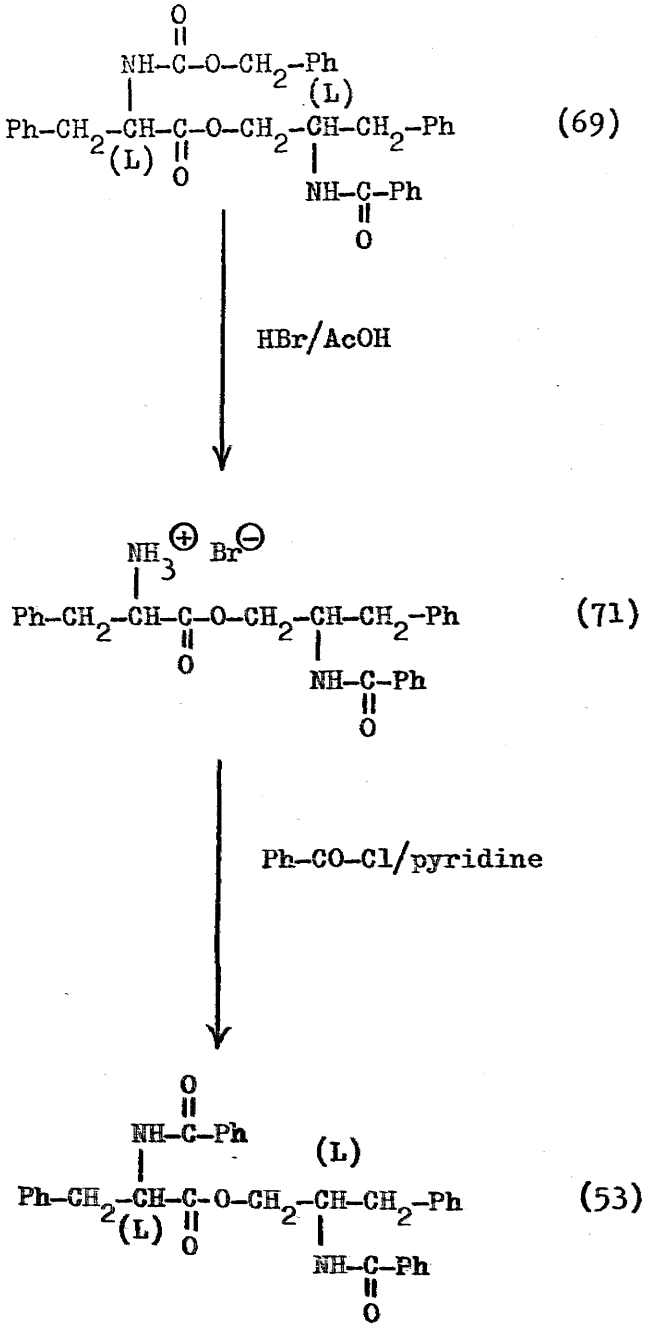
involved in the esterification reaction, an authentic sample (DL), was treated with N,N'-carbonyldiimidazole, followed by N-benzoyl-L-phenylalaninol. This gave a mixture of products, 40% of which was shown by T.L.C. to be the candipolin-epicandipolin mixture. Goodman and Levine[88] prepared the optically active oxazolone from N-benzoyl-L-phenylalanine and found that, on attempting to condense it with L-phenylalanine methyl ester, racemisation occurred much faster than did coupling. Activation of the acid carbonyl group is desirable in order to facilitate esterification. However, this renders it more susceptible not only to external attack by the alcohol, but also to intramolecular nucleophilic attack by the benzoyl group, which results ultimately in racemisation. Therefore, some alternative amino protecting group was required, which would permit esterification without loss of stereochemistry.

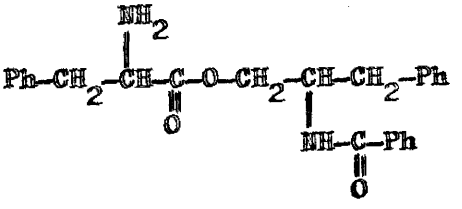
Many examples are known of N-carbobenzyloxy (CBZ) amino acids being used in peptide synthesis, with no racemisation occurring under coupling conditions. Treatment of commercial N-CBZ-L-phenylalanine (68) with N-benzoyl-L-phenylalaninol, using N,N'-carbonyldiimidazole (Scheme 14) gave two products along with much unreacted alcohol. The major product, a crystalline solid, apparently homogeneous by T.L.C. (R_f 0.54), showed three carbonyl frequencies in its I.R. spectrum, at 1740, 1690, and 1630 $cm.^{-1}$, which were assigned to ester, urethane, and amide groups respectively. The presence of the urethane function was confirmed by the appearance in the N.M.R. spectrum of a new NH resonance at 5.2 δ in addition to the amide signal at 6.5 δ, and also of a new 2H signal at 4.5 δ, assigned to the $-O-CH_2-$ protons. The mass spectral fragmentations shown in Fig. 6 provided further evidence that the compound was N-benzoyl-O-(N-CBZ-phenylalanyl)-L-phenylalaninol (69). With the

appearance of only one infrared absorption attributable to the ester carbonyl group, it was hoped that the compound had the desired (L,L) stereochemistry, however, caution was required, since it may have been simply fortuitous that the ester frequencies of candipolin and its epimer did not coincide. The melting point (185-7°) of the N-benzoyl-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol (69) remained unchanged with successive crystallisations from ethanol, suggesting that it consisted of a single compound, which, therefore, probably has the desired (L,L) stereochemistry.

A minor, less polar, product was again obtained, but this showed only one broad carbonyl absorption at 1720 $cm.^{-1}$, indicating that it was an acyclic ester rather than a cyclic compound comparable to an azlactone. The NMR spectrum showed that it contained a urethane grouping, but a new 3-proton singlet appearing at 3.8 δ suggested that it was a methyl ester. This was confirmed by the presence in its mass spectrum of peaks at m/e 282 and 254, corresponding to losses of $-OCH_3$ and $-CO_2CH_3$ respectively from the parent ion (m/e 313). The compound was identified as N-CBZ-L-phenylalanine methyl ester by comparison with an authentic sample. The origin of this was uncertain, since all the reagents had been found to be pure by m.p., T.L.C., I.R., and N.M.R. spectroscopy. However, since no corresponding ester had been found in analogous reactions using N-benzoyl-L-phenylalanine, the CBZ acid was investigated further. Although the N.M.R. spectrum of the commercial acid run under normal conditions showed no impurity, an almost saturated solution showed a very weak signal at 4.0 δ, which could be attributed to the methyl ester (cf. $-OCH_3$ at 3.8 δ in synthetic N-CBZ-phenylalanine methyl ester). However, the concentration of the impurity in the sample seemed insufficient to account for the amount isolated from the

Scheme 15





70

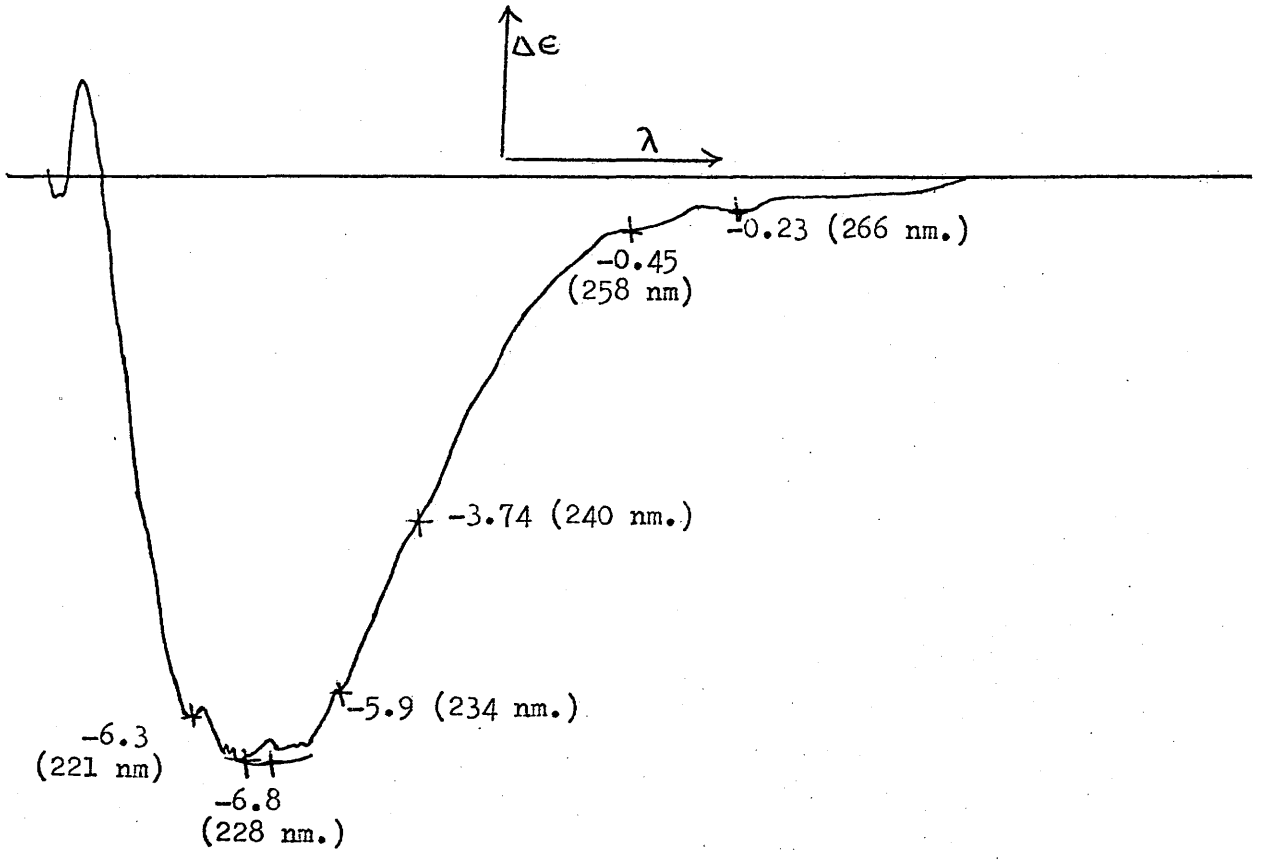
71 = 70.HBr

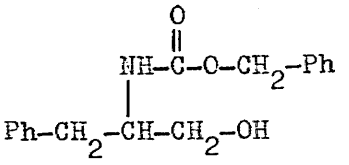
reaction, the origin of which remains to be explained.

In view of the amount of N-benzoyl-L-phenylalaninol recovered from these reactions, it seemed desirable to use a larger excess of N,N'-carbonyldiimidazole to ensure complete formation of the acyl imidazolide in the first stage. However, the fact that the coupling agent can itself react with alcohols[89] discouraged this idea. Improved yields in this type of esterification by the use of base (sodium ethoxide)[90] and by prolonged reaction times[91] have been claimed. (Heating apparently accelerates the reaction, but leads to undesirable side reactions[91]). A number of procedural variations were applied in the present case (e.g. using various solvents, adding sodium hydride, etc.), but no significant improvement in yield resulted.

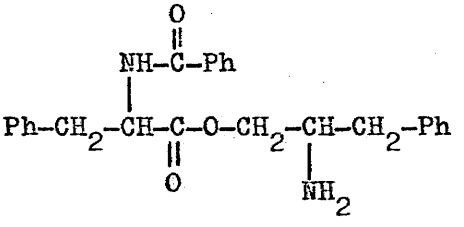
Candipolin was found not to suffer epimerisation or other transformation when treated with either hydrogen bromide in acetic acid (45%) or benzoyl chloride in pyridine. It, therefore, seemed likely that the steps of Scheme15 would proceed without undesirable side reactions. Treatment of N-benzoyl-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol (69) with 45% HBr in acetic acid gave quantitative yields of a crystalline hydrobromide, which showed a broad infrared absorption at 3420 $cm.^{-1}$, characteristic of the NH_3^+ grouping. The product showed only two carbonyl absorptions, at 1745 $cm.^{-1}$ (ester) and 1635 $cm.^{-1}$ (amide), whereas the urethane from which it was derived had shown an additional band at 1690 $cm.^{-1}$. Supporting the conclusion that the compound was the hydrobromide of N-benzoyl-O-(L-phenylalanyl)-L-phenylalaninol (71) (N'-debenzoylcandipolin hydrobromide), was the absence from its N.M.R. spectrum of the urethane NH resonance (5.2 δ), and a reduction in the intensity of the signal at 7.2 δ, corresponding to the loss of the benzyl group.

Figure 7

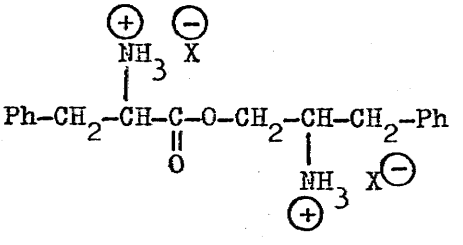




72



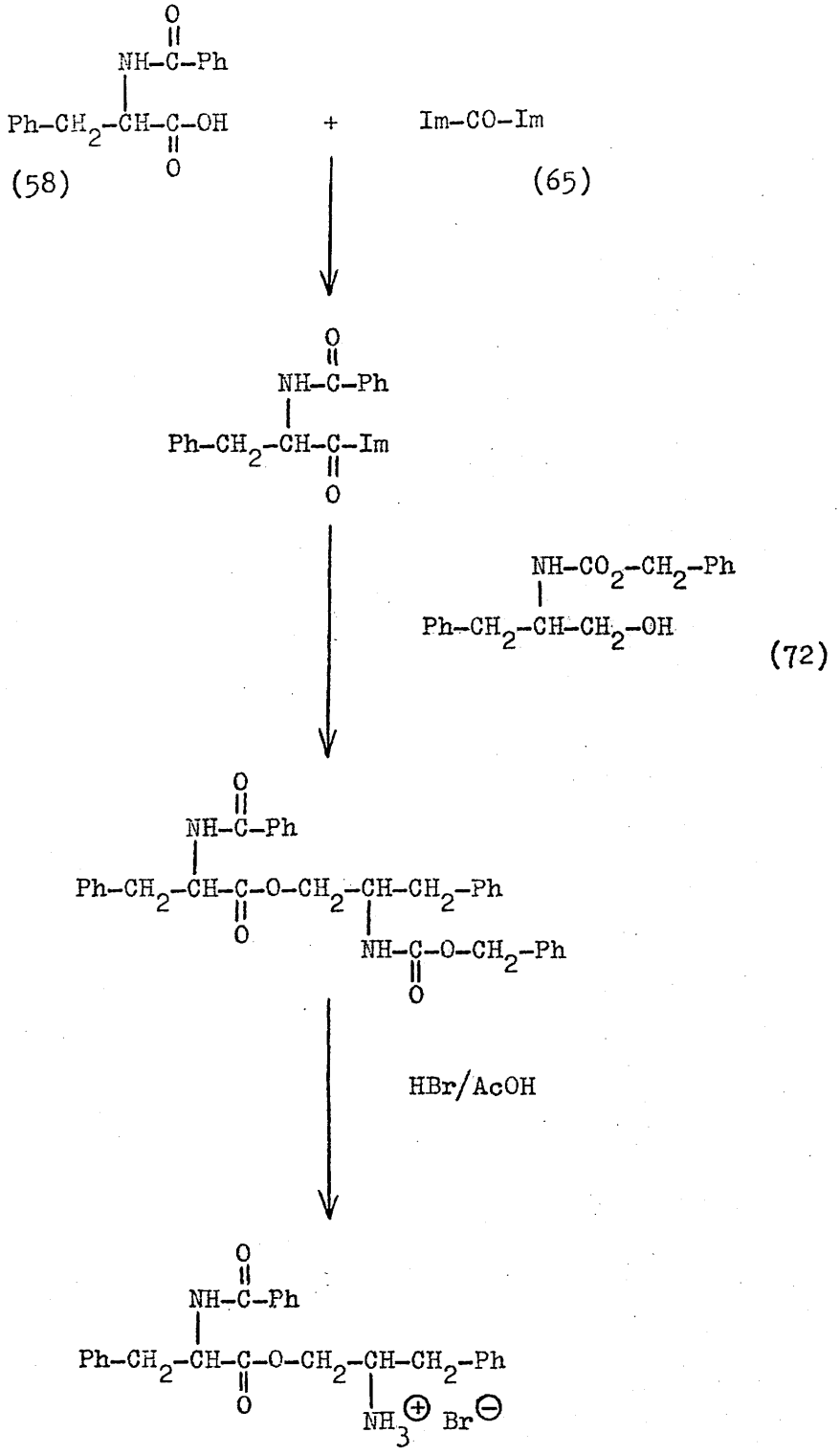
73



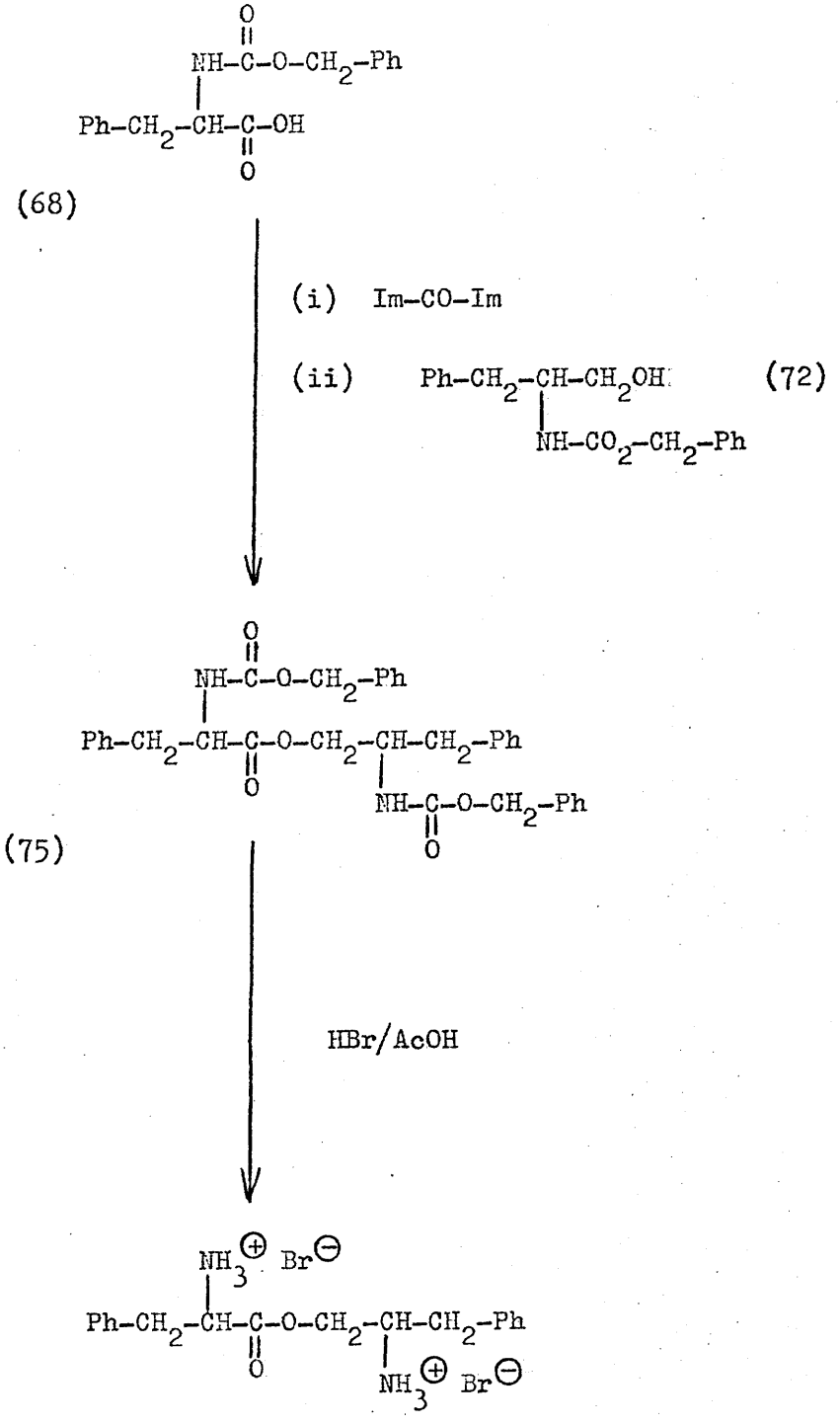
74 a X = $Br^{\ominus}$

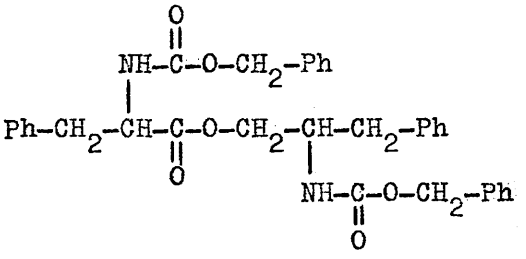
b X = $BF_3^{\ominus}$

Scheme 16



Scheme 17



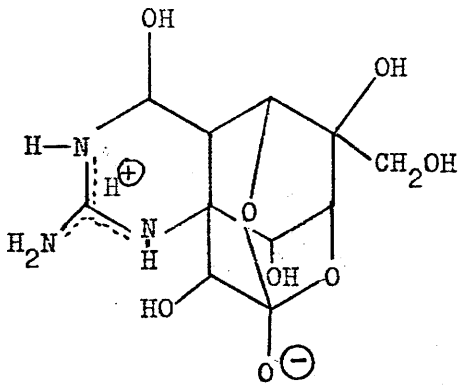


Benzoylation of this compound yielded a crystalline material, m.p. 210°, which was found not to suffer any change in melting point on repeated crystallisation from ethanol. The product showed only two infrared carbonyl frequencies, 1750 $cm.^{-1}$ (ester) and 1635 $cm.^{-1}$ (amide), the former indicating that it was in fact N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (53). Its identity with natural candipolin was demonstrated by mixed melting point, and by comparison of their circular dichroism curves, in which the natural material showed $\Delta\epsilon$-6.9 at 227 nm., while synthetic candipolin showed $\Delta\epsilon$-6.8 at 228 nm. (Fig. 7).

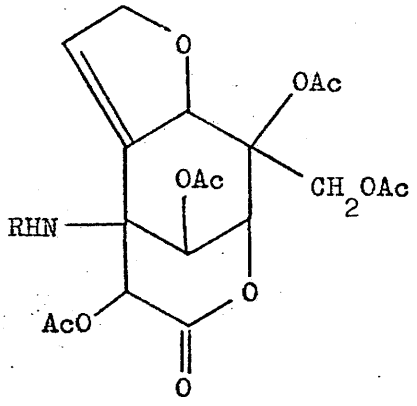
This synthesis has established the structure of candipolin as being N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (53), and has also made available the N'-debenzoyl compound (70), which could be a biosynthetic precursor of candipolin.

It seems probable that this synthesis of candipolin should be capable of adaptation to yield two further esters of this type which are potential intermediates in the biosynthesis of candipolin. Esterification of N-benzoyl-L-phenylalanine with N-CBZ-L-phenylalaninol (72), followed by cleavage of the CBZ-group should yield N-debenzoylcandipolin hydrobromide (73) (Scheme 16). The analogous reaction using N-CBZ-L-phenylalanine (Scheme 17) should give N,N'-didebenzoylcandipolin hydrobromide (74a). N-CBZ-L-phenylalaninol (72) required in both these schemes, was prepared by treatment of L-phenylalaninol with benzyl chloroformate in the presence of base[92,93]. Reaction of this alcohol with N-CBZ-L-phenylalanine/N,N'-carbonyldiimidazole gave a mixture in which the desired ester (75) was a relatively minor component. Due to a lack of time, these reactions were not investigated further.

An alternative method of obtaining the debenzoylcandipolins

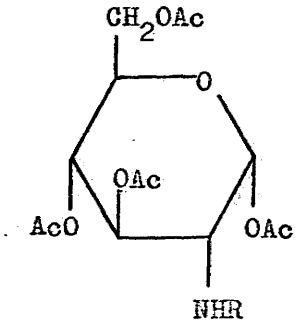


76



77 R = Ac

78 R = H



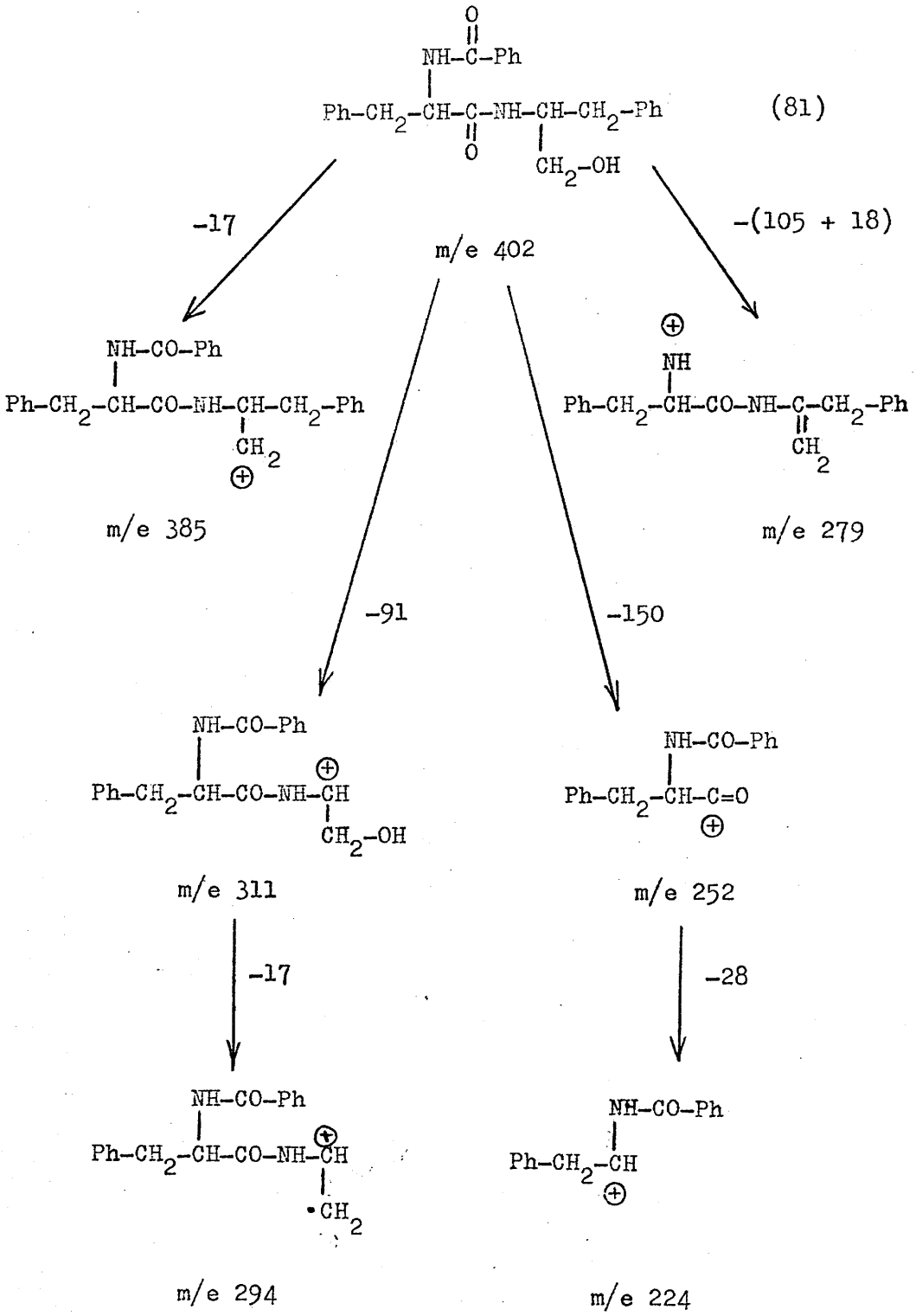
79 R = Ac

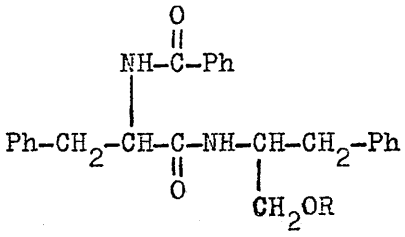
80 R = H

would be to selectively remove one or both of the N-benzoyl groups of candipolin while leaving the ester link intact. Triethyloxonium fluoroborate appears to have the required selectivity in its reactivity towards amides in the presence of ester functions. It was, for example, employed in the synthesis of DL-tetrodotoxin[94] (76) to selectively N-deacylate the peracetylated lactone (77) and give the corresponding diamine (78). In another example[95], treatment of the peracetylated amino sugar (79) with triethyloxonium fluoroborate, followed by a basic workup, yielded the diamine (80), in which all the acetoxy groups remained intact.

In these laboratories, a number of attempts to carry out this type of debenzoylation of candipolin were made[73]. Some indication that cleavage of the benzamide groupings was occurring was suggested by an unmistakable odour of ethyl benzoate in the product and its detection by T.L.C., although in most experiments, no other product could be isolated. On one occasion, however, using a very large excess of reagent, a crystalline salt was obtained which corresponded in properties to the difluoroborate of N,N'-didebenzoylcandipolin[73] (74b) (Scheme 8). Further studies of this reaction were undertaken. Reaction of candipolin with a slight excess of triethyloxonium fluoroborate, followed by treatment with dilute acetic acid gave a complex mixture containing ethyl benzoate and some unreacted starting material. Repeated preparative T.L.C. gave the major product as a white solid, ehich separated out of solution as a gel. The most striking feature of its infrared spectrum was the absence of an ester band, and the appearance of a broad hydroxyl absorption flanking the NH stretching band at 3300 cm.$^{-1}$. The absorption at 1640 cm.$^{-1}$ showed that the compound still contained a benzamide function, but the appearance of a new band at 1660 cm.$^{-1}$ indicated

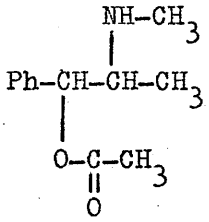
Figure 8


(81)
m/e 402
-17
-(105 + 18)
m/e 385
m/e 279
-91
-150
m/e 311
m/e 252
-17
-28
m/e 294
m/e 224



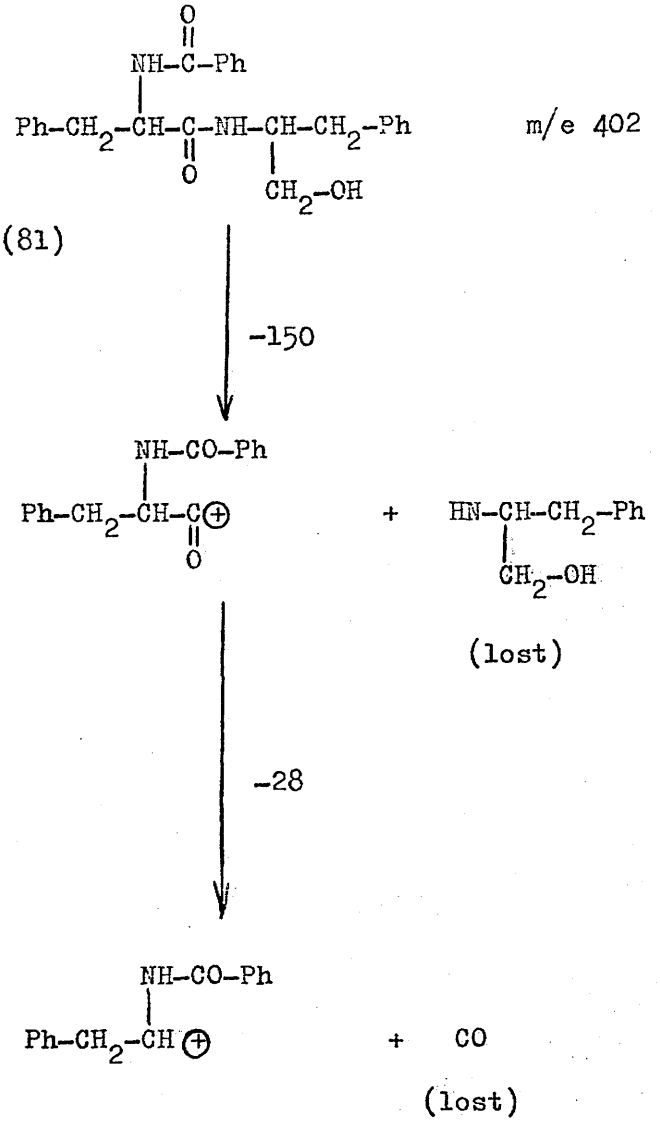
81 R = H

81a R = CO-CF_3



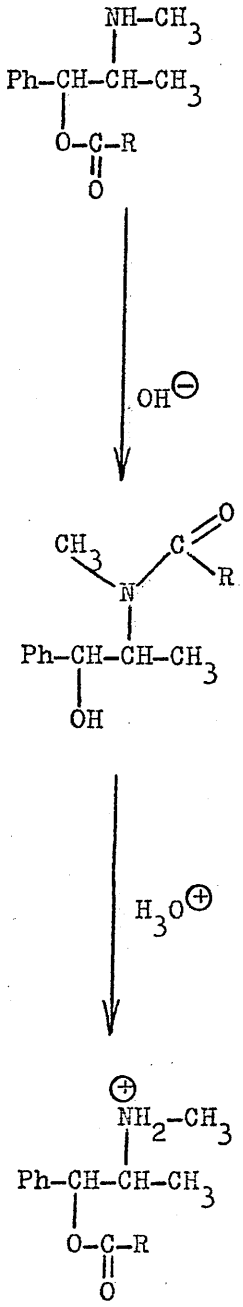
82

Scheme 18


NH–C–Ph
Ph–CH2–CH–C–NH–CH–CH2–Ph
CH2–OH
m/e 402
(81)
–150
NH–CO–Ph
Ph–CH2–CH–C⊕
+
HN–CH–CH2–Ph
CH2–OH
(lost)
–28
NH–CO–Ph
Ph–CH2–CH⊕
+
CO
(lost)

Scheme 19

(82)

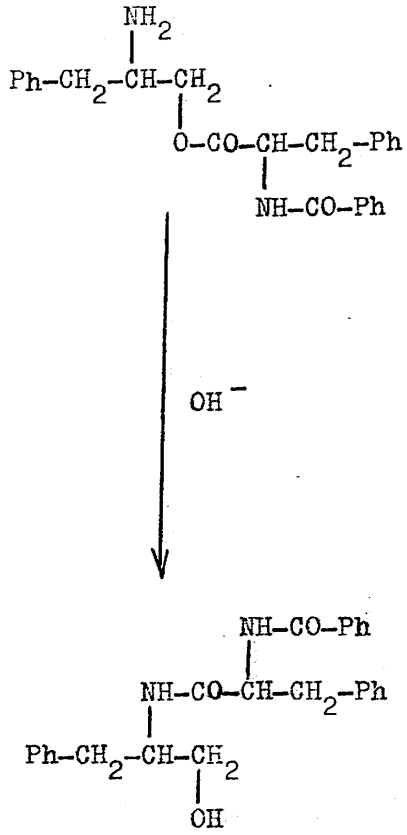

NH–CH3
Ph–CH–CH–CH3
O–C–R
O
OH⊖
CH3
C
O
R
N
Ph–CH–CH–CH3
OH
H3O⊕
⊕
NH2–CH3
Ph–CH–CH–CH3
O–C–R
O

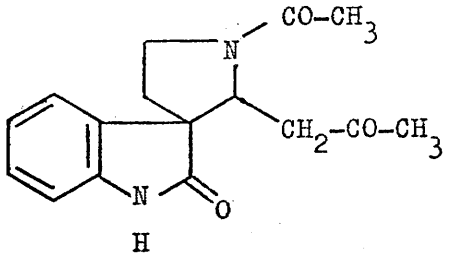
the presence of an aliphatic amide linkage. That this compound was not a debenzoylacndipolin was suggested by the number of fragment ions in the mass spectrum in which loss of water or an hydroxyl ion seemed to be involved (Fig. 8). The appearance of an abundant ion at m/e 252 suggested that the compound was N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (81). Cleavage of the central amide link, with loss of the fragment of mass 150, would give rise to this ion, which could easily lose CO to give the ion of m/e 224 (Scheme 18).

Confirmation of the structure of the alcohol (81) was obtained by benzoylation, which yielded a crystalline compound with melting point (208-10°) and T.L.C. (R_f 0.57) similar to those of candipolin. The I.R. and N.M.R. spectra of the product showed features identical to those of N-(N-benzoyl-L-phenylalanyl)-O-benzoyl-L-phenylalaninol (56), whose synthesis by a different route[70] was described earlier (see page 17). A characteristic of this compound was the double ester absorption (1720 and 1710 $cm.^{-1}$), caused by symmetric and asymmetric interactions with an amide group[74]. The positions of the two amide absorptions were the same as in the parent alcohol. The presence of the O-benzoyl group was demonstrated by the 2-proton signal at 8.0 δ in its N.M.R. spectrum, corresponding to the ortho-protons of the aromatic ring.

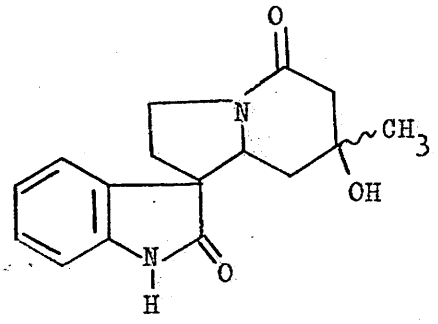
N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (81) could have arisen by debenzoylation of the phenylalaninol residue of candipolin to give N-debenzoylcandipolin (73), an analogue of N-acylephedrine (82). It has long been known [96,97] that O-acylephedrines, on treatment with base, readily undergo O→N migration of the acyl function (Scheme 19). Treatment of the product with acid reverses the process, yielding the ammoniom salt of the base. By analogy, N-debenzoylcandipolin (73) may have rearranged under the reaction conditions to give

Scheme 20

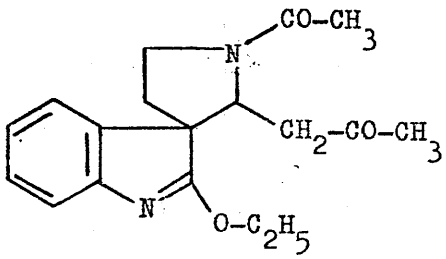

NH2
Ph–CH2–CH–CH2
O–CO–CH–CH2–Ph
NH–CO–Ph
(73)
OH⁻
NH–CO–Ph
NH–CO–CH–CH2–Ph
Ph–CH2–CH–CH2
OH
(81)



83

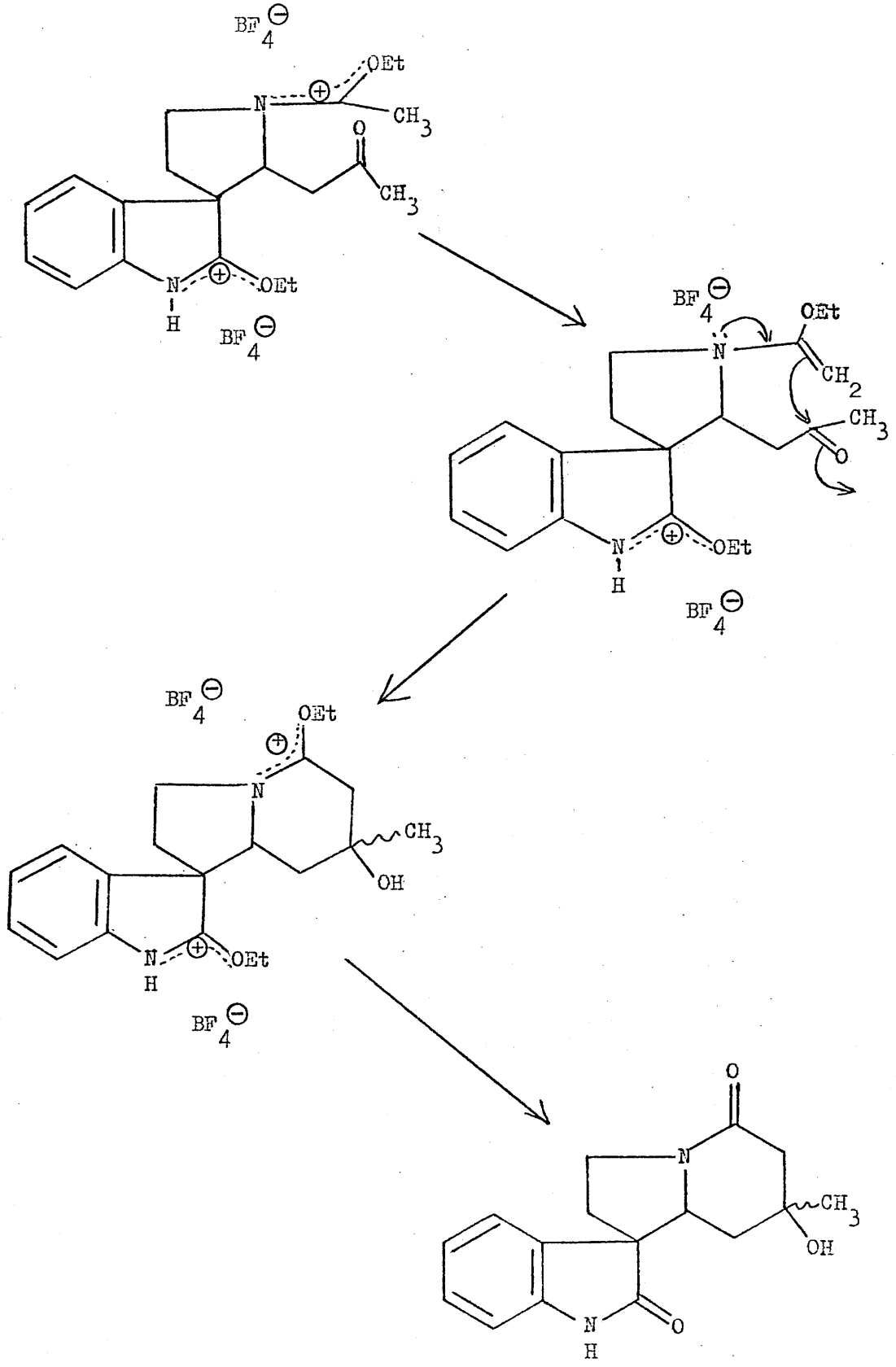


84



85

Scheme 21



N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (81), as shown in Scheme 20. The alcohol, on warming with trifluoroacetic acid, merely appeared to form the O-trifluoroacetate, as suggested by the appearance of a band at 1785 $cm.^{-1}$ in its I.R. spectrum. However, treatment of the alcohol with HBr/AcOH for several days at room temperature caused a new band to appear at 1730 $cm.^{-1}$ in the I.R. spectrum, suggesting that a degree of N→O migration of the acyl function may have been effected. The product, however, was a mixture and was not investigated further.

In connection with the fact that candipolin underwent only partial debenzoylation although treated with a slight excess of triethyloxonium fluoroborate, it has been reported[98] that even when a five-fold excess of this reagent was used, the amide (83) afforded not only the product (84), postulated to have arisen by attack at both amide functions (Scheme 21)[99], but also the monoamide (85). In the case of candipolin, further study is evidently required to establish the conditions whereby efficient debenzoylation can be achieved. Recently, a new method of selective N-debenzoylation in the presence of esters has been reported, which uses various phenols[100]. This method might also be applied to candipolin.

The alcohol (81) obtained from the debenzoylation has been found to occur naturally in *Emerecellopsis salmosynnemata*[66]. Recently, another new phenylalaninol derivative, isolated from a plant of the genus *Euphorbia*[67], was deduced to be N-(N-benzoyl-L-phenylalanyl)-O-acetyl-L-phenylalaninol (52), and its structure was confirmed by synthesis. Independently, the same compound was isolated from the fungus *Aspergillus glaucus*[101], the name asperglaucide being coined in this case. The alcohol (81) obtained in the present work is closely related to this, and , indeed,

Table 1 ^{13}C N.M.R. - δ_C in C_5D_5N

Compound	53	49	50	70	81
C-1	65.7	66.7	63.4	67.6	63.1
C-2	51.0	55.4	54.3	51.4	53.7
C-3	37.4	41.3	37.7	37.1	37.6
C-1'	172.4			170.4	171.8
C-2'	55.8			55.3	55.6
C-3'	37.5			37.1	38.6
CO-NH	167.7 168.5		167.8	167.7	167.7 171.8
aryl C p to CH_2	128.6 127.1	126.3	126.5	126.7 127.7	126.4 126.8
aryl C p to CO-NHR	131.3 131.8		131.2	131.4	131.4
quaternary aryl C	135.0 135.7 138.1 138.6	140.4	136.2 139.8	135.6 135.6 139.1	135.5 138.5 139.5
other aryl C (signals ⩾ 2C)	128.1 128.6 128.7 128.8 129.7	128.7 129.8	127.9 128.6 128.7 129.9	128.5 128.7 129.1 129.8 130.1	128.0 128.6 129.9

Notation :

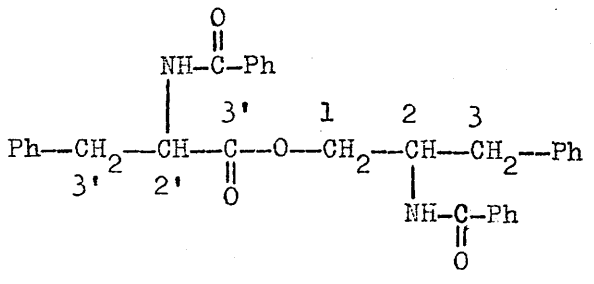
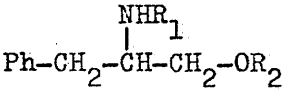


Table 2 Shielding effects in some acyl derivatives of phenylalaninol (49).

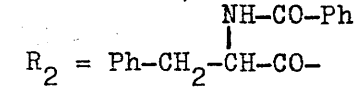
		C-1	C-2	C-3
-O-CO-R	$(\delta_C^{70} - \delta_C^{50})$	+4.2	-4.0	-0.6
	$(\delta_C^{53} - \delta_C^{50})$	+2.3	-3.3	-0.3
-NH-CO-Ph	$(\delta_C^{50} - \delta_C^{49})$	-3.3	-1.1	-3.6
-NH-CO-R	$(\delta_C^{81} - \delta_C^{49})$	-3.6	-1.7	-3.7

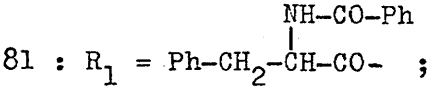


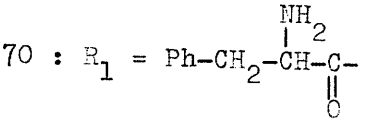
49 : $R_1 = R_2 = H$

50 : R_1 = CO-Ph ; $R_2 = H$

53 : R_1 = Ph-CO- ;







$R_2 = H$

acetylation gave a product which was indistinguishable from an authentic sample of asperglaucide[102] in melting point and I.R. spectrum. The similarities in the structures of asperglaucide (52) and candipolin (53) may reflect similarities in their biosyntheses.

The ^{13}C N.M.R. spectra of various derivatives of phenylalaninol obtained during the course of this work showed a consistent pattern (Table 1). The observed shielding effects resulting from N- and O-acylation are contrasted in Table 2. These give some indication as to the conformations adopted by the compounds. In the N-acyl derivatives, it is possible that the H atom of the amide might hydrogen bond to the H atom of the OH, in which case, the amide grouping would lie closer to C-1 than to C-3. It is also possible that the O atom of the amide may be repelled by the O atom of the OH group, in which case, the substituent would lie closer to C-3 than to C-1. The fact that equal shielding of both atoms occurs indicates that no preference is shown for either conformation of the molecules. It is noteworthy that C-2 is shielded by N-acylation. By the inductive effect, a deshielding would be expected. This result indicates that the conformation of the molecule allows shielding from the N-acyl group, particularly in the case of desacetylasperglaucide, where an additional benzamide function causes additional shielding at C-2. In the case of O-acylation, the expected deshielding effect at C-1 is observed. The strong shielding effect at C-2 indicates that there may be hydrogen bonding between the N-benzoylphenylalaninol unit and either the ester or the NH of the phenylalanine unit. The latter seems more likely, since the shielding is larger in N'-debenzoylcandipolin than in candipolin itself.

Table 3 Incorporation of label from L-[U-^{14}C], [G-^{3}H] phenylalanine into candipolin.

	Activity fed (d.p.m.)	Weight of candipolin (mg.)	Activity of candipolin (d.p.m./mg.)	Incorp. (%)
^{14}C	1.11×10^{8}	61.0*	7358	0.41
^{3}H	1.88×10^{9}	61.0*	102131	0.33

* Based on analytical T.L.C., the 13.8 mg. of candipolin originally obtained was estimated to contain at least 10 mg. candipolin.

Table 4 Distribution of label from L-[U-^{14}C], [G-^{3}H]phenylalanine in the two halves of the candipolin ester.

	Candipolin				N-benzoyl-L-phenylalanine				N-benzoyl-L-phenylalaninol			
	d.p.m./mg.	d.p.m./m.mol.	%	$^{3}H/^{14}C$	d.p.m./mg.	d.p.m./m.mol.	%	$^{3}H/^{14}C$	d.p.m./mg.	d.p.m./m.mol.	%	$^{3}H/^{14}C$
^{14}C	*	*	*	*	1015	2.73×10^5	-	14.4	947	2.42×10^5	-	13.9
^{3}H	*	*	*		14628	3.94×10^6	-		13140	3.35×10^6	-	
^{14}C	56	2.81×10^4	100	14.2	62	1.68×10^4	59.8	14.3	64	1.64×10^4	58.4	14.0
^{3}H	795	4.02×10^5	100		890	2.40×10^5	59.7		893	2.28×10^5	56.7	

* Activity not measured

\+ The number in the % column is $\frac{\text{d.p.m./m.mol of compound}}{\text{d.p.m./m.mol of candipolin}} \times 100\%$

Table 5 (a) Activity of benzoic acid from hydrolysis of N-benzoyl-L-phenylalanine.

	N-benzoyl-L-phenylalanine				Benzoic acid			
	d.p.m./mg.	d.p.m./m.mol.	%	$^3H/^{14}C$	d.p.m./mg.	d.p.m./m.mol.	%	$^3H/^{14}C$
^{14}C	1015	2.73×10^5	100	14.4	1167	1.43×10^5	52.4	14.5
^3H	14628	3.94×10^6	100		17248	2.11×10^6	53.5	

(b) Activity of benzoic acid from hydrolysis of N-benzoyl-L-phenylalaninol.

	N-benzoyl-L-phenylalaninol				Benzoic acid			
	d.p.m./mg.	d.p.m./m.mol.	%	$^3H/^{14}C$	d.p.m./mg.	d.p.m./m.mol.	%	$^3H/^{14}C$
^{14}C	947	2.42×10^5	100	13.9	998	1.22×10^5	50.4	15.4
^3H	13140	3.35×10^6	100		15426	1.56×10^6	46.6	

CHAPTER 2

THE BIOSYNTHESIS OF CANDIPOLIN

As indicated earlier, candipolin possesses a number of features of interest from a biogenetic point of view. In particular, the benzoyl groupings could conceivably arise from one of a number of different pathways as mentioned in the introduction. If phenylalanine happens to be the source of the benzoyl groups of candipolin, then its efficiency as their precursor can be gauged using the built-in phenylalanine unit as a reference. It was hoped to establish whether the biosynthesis merely involved a more or less random assembly of the subunits, i.e., whether several different pathways are followed, or whether there is a well-defined stepwise sequence of events leading to candipolin.

A preliminary investigation was carried out by adding L-[U-^{14}C], [G-^{3}H] phenylalanine to shake cultures of Penicillium canadense four days after inoculation. Incorporation of ^{14}C label into candipolin, which was isolated after six days' further incubation, was found to found to be 0.4% (Table 3). Only the ^{14}C results will be discussed at this point (the results based on ^{3}H will be discussed later). Base hydrolysis of candipolin to N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol showed that its ^{14}C activity was equally distributed between these two fragments (Table 4). Therefore, in this experiment, L-phenylalanine was incorporated just as efficiently into N-benzoyl-L-phenylalaninol as into N-benzoyl-L-phenylalanine. Hydrolysis of these two compounds gave samples of benzoic acid which were found to have half the ^{14}C activity of the parent amides (Table 5). By difference, the ^{14}C activity of the L-phenylalaninol unit was presumed to be the same as that of the phenylalanine unit. If all four units of candipolin are derived

Table 6 (a) Distribution (%) of label in units of candipolin.

	Candipolin	N-benzoyl-L-phenylalanine	N-benzoyl-L-phenylalaninol	Benzoic acid (single unit)
^{14}C	100	50	50	22
^{3}H	100	50	50	19

(b) Distribution (%) of label within the main units.

	N-benzoyl-L-phenylalanine	Benzoic acid	N-benzoyl-L-phenylalaninol	Benzoic acid
^{14}C	100	44	100	44
^{3}H	100	38	100	38

Table 7 Time-study using DL-[2-^{14}C]phenylalanine.

Day * fed	Day * harvested	Weight of candipolin mg.	Activity of candipolin d.p.m./mg.	Incorporation %
4	10	160	24609	14.4
4	13	121	31427	13.8
7	10	117	8721	3.7
7	13	148	6107	3.3

* Day = number of days after inoculation.

from L-phenylalanine with equal efficiency, the distribution of label among these would be as in Table 6, which is in reasonable agreement with the experimental results. This shows that not only was L-phenylalanine incorporated equally into both benzoyl groups, but that this process was as efficient as incorporation into the phenylalanine unit itself. This important result indicates that the benzoyl units of candipolin do not arise directly from any intermediate before chorismic acid on the shikimic acid pathway.

Since incorporations in the preliminary experiments had been somewhat low, a time study was undertaken in order to determine the optimum time of production of candipolin before carrying out further feeding experiments. DL-[2-^{14}C] phenylalanine was added to surface cultures four or seven days after inoculation, and candipolin isolated after various periods of incubation (Table 7). It became evident that, using surface cultures, incorporations much better than had been obtained using shake cultures were obtained by adding the radiotracer four days after inoculation. Although the day of harvesting did not significantly affect incorporation, isolation of candipolin from the crude mycelial extract was easiest if cultures were harvested ten days after inoculation. In all subsequent biosynthetic experiments, the radiotracer was accordingly fed to surface cultures of P.canadense four days after inoculation, these being harvested six days later.

Since remarkably high levels of incorporation of phenylalanine into candipolin were now possible, an efficient means of obtaining isotopically-labelled N-benzoyl-L-phenylalanine, N-benzoyl-L-phenylalaninol, and L-phenylalaninol also became available via hydrolysis of candipolin derived from suitably labelled L-phenylalanine. For this purpose, L-[U-^{14}C] phenylalanine was fed to

Table 8 Incorporation of label from L-[U-^{14}C]phenylalanine into candipolin.

Activity of phenylalanine fed d.p.m.	Weight of candipolin mg.	Activity of candipolin d.p.m./mg.	Incorporation %
6.66×10^8	168.2	2.88×10^6	72.7

Table 9 (a) ^{14}C Activity of N-benzoyl-L-phenylalanine and its proportion of total candipolin label.

	Candipolin activity	%*	Diluted N-benzoyl-L-phenylalanine activity	Calculated undiluted N-benzoyl-L-phenylalanine activity	%*
dpm/mg	2.88×10^6		44395	3.97×10^6	
dpm/mmol	1.46×10^9	100	1.20×10^7	1.06×10^9	72.6

(b) ^{14}C Activity of N-benzoyl-L-phenylalaninol and its proportion of total candipolin activity.

	Candipolin activity	%*	Diluted N-benzoyl-L-phenylalaninol activity	Calculated undiluted N-benzoyl-L-phenylalaninol activity	%*
dpm/mg	2.88×10^6		34965	2.68×10^6	
dpm/mmol	1.46×10^9	100	8.92×10^6	6.84×10^8	47.0

* $\% = \frac{\text{d.p.m./m.mol of compound}}{\text{d.p.m./m.mol of candipolin}}$

Table 10 (a) Hydrolysis of diluted [^{14}C] N-benzoyl-L-phenylalanine.

	N-benzoyl-L-phenylalanine activity	%	Benzoic acid activity	%*
dpm/mg	22416		28478	
dpm/mmol	6.04×10^6	100	3.48×10^6	57.6

(b) Hydrolysis of diluted [^{14}C] N-benzoyl-L-phenylalaninol.

	N-benzoyl-L-phenylalaninol activity	%	Benzoic acid activity	%*
dpm/mg	16216		19425	
dpm/mmol	4.14×10^6	100	2.37×10^6	57.2

$$* \% = \frac{\text{dpm/mmol of product}}{\text{dpm/mmol of parent amide}}$$

Table 11 Incorporation of label from L-[U-^{14}C]phenylalanine into candipolin.

Activity of phenylalanine fed dpm	Activity of diluted candipolin dpm/mg	Calculated activity of undiluted candipolin dpm/mg	Weight of undiluted candipolin dpm	Incorporation %
3.33×10^8	17990	1.90×10^6	89.9*	51.1*

* Weight = total weight actually recovered, not including a little candipolin remaining in the crude extract. Therefore, this is minimum incorporation.

Table 12 Hydrolysis of diluted [^{14}C] candipolin.

	Candipolin activity	%	N-benzoyl-L-phenylalanine activity	%*	N-benzoyl-L-phenylalaninol activity	%*
dpm/mg	17990		17399		17175	
dpm/mmol	9.10×10^6	100	4.69×10^6	51.5	4.38×10^6	48.1

* % = $\frac{\text{dpm/mmol of compound}}{\text{dpm/mmol of candipolin}}$

Table 13 Incorporation of N-benzoyl-L-phenylalaninol into candipolin.

Activity of N-benzoyl-L-phenylalaninol fed (^{14}C) dpm	Activity of diluted candipolin (^{14}C) dpm/mg	Calculated original candipolin activity dpm/mg	Weight of candipolin mg	Incorporation %
6.60×10^7	4734	509191	91.90	70.9

Table 14 Distribution of label in candipolin from [^{14}C] N-benzoyl-L-phenylalaninol feed.

	Candipolin activity	N-benzoyl-L-phenylalanine activity	%*	N-benzoyl-L-phenylalaninol activity	%*
dpm/mg	2186	184		4268	
dpm/mmol	1.11×10^6	4.96×10^4	5	1.09×10^6	98

* % = $\frac{\text{dpm/mmol of compound}}{\text{dpm/mmol of parent amide}}$

cultures of P.canadense, incorporation of label into candipolin being 72.7% (Table 8) on this occasion. Hydrolysis gave N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol, a diluted sample of each being used for radioactivity assay. In agreement with the preliminary results obtained using shake cultures, N-benzoyl-L-phenylalaninol was found to have half the ^{14}C activity of candipolin (Table 9). The ^{14}C activity calculated for N-benzoyl-L-phenylalanine based on the ^{14}C activity of a diluted sample was anomalously high and, in view of subsequent results, is considered to be in error owing to the sample having been weighed on a conventional laboratory balance instead of a microbalance). After further dilution, these amides were hydrolysed to give samples of benzoic acid. Although the ^{14}C activities of these seemed a little high (Table 10), each accounted for the same percentage (57%) of the ^{14}C activity of its parent monoamide, again in agreement with the experiment conducted on shake cultures. On another occasion, L-[U-^{14}C] phenylalanine was fed to surface cultures, and gave an incorporation of 51% (Table 11). Hydrolysis of diluted candipolin from this experiment gave N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol, each having half the ^{14}C activity of the ester (Table 12), which was, once again, in agreement with the results obtained using shake cultures.

The L-[U-^{14}C] N-benzoylphenylalaninol (undiluted) obtained from the former feeding (73% incorporation) was fed to 4-day old cultures of P. canadense. The candipolin recovered after six days' further growth showed a total incorporation of 71% (Table 13). It may be noted at this point that this is comparable to the total incorporation of L-phenylalanine itself into candipolin. Hydrolysis of candipolin revealed that 98% of the ^{14}C activity resided in the N-benzoyl-L-phenylalaninol unit (Table 14), showing that not only is this

Table 15 Incorporation of label from benzoic acid into candipolin.

Activity of benzoic acid fed (^{14}C) dpm	Activity of diluted candipolin (^{14}C) dpm/mg	Calculated activity of undiluted candipolin dpm/mg	Weight of candipolin mg	Incorporation %
5.5×10^8	10183	1.86×10^6	83.0	27.8

Table 16 Distribution of label in candipolin from benzoic acid feed.

	Candipolin activity (^{14}C)	N-benzoyl-L-phenylalanine activity (^{14}C)	%*	N-benzoyl-L-phenylalaninol activity (^{14}C)	%*
dpm/mg	6773	6615		6839	
dpm/mmol	3.43×10^6	1.78×10^6	52	1.74×10^6	51

$$* \% = \frac{\text{dpm/mmol of compound}}{\text{dpm/mmol of candipolin}}$$

compound a very good precursor, but that it is a very specific precursor in candipolin biosynthesis. Thus the specific incorporation of N-benzoyl-L-phenylalaninol into the N-benzoyl-L-phenylalaninol unit of candipolin (69.5%) is about double that of L-phenylalanine into the same unit (34.2%).

Commercial [carboxy-^{14}C] benzoic acid fed to surface cultures of P. canadense four days after inoculation gave 27.8% incorporation of label into candipolin (Table 15). After hydrolysis of candipolin, it was found that the compound had been incorporated with equal efficiency into the N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol fragments (Table 16). As shown earlier, 20% of the ^{14}C activity of [U-^{14}C] phenylalanine was incorporated into each benzoyl unit of candipolin. The result of the [^{14}C] benzoic acid experiment indicated that ca. 14% of the ^{14}C activity is incorporated into each benzoyl unit of candipolin, so that free benzoic acid and phenylalanine are both efficient precursors of the benzoyl units.

It may be presumed that the benzoic acid is incorporated via an enzymic benzoylation process. It is conceivable that the hypothetical benzoyl amidase might accept only one substrate, e.g. phenylalanine, or that it might be relatively non-specific and accept a variety of substrates, including, for example, phenylalaninol or a debenzoyl candipolin, such as O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol. In the case of L-phenylalaninol, the N-benzoylation product has already been demonstrated to be an excellent precursor for candipolin so that it should be evident whether or not the phenylalaninol is accepted as a substrate by the benzoyl esterase.

In order to ontain L-[U-^{14}C] phenylalaninol, several different samples of N-benzoyl-L-phenylalaninol obtained during the course of the previously described experiments were collected, purified, and

Table 17 Incorporation of label from L-[U-^{14}C]phenylalaninol into candipolin.

Activity of phenylalaninol fed dpm	Activity of candipolin dpm/mg	Weight of candipolin mg	Incorporation %
6.80×10^6	1015	130	1.94

Table 18 Distribution of label in candipolin from L-phenylalaninol feed.

	Activity of candipolin (^{14}C)	Activity of N-benzoyl-L-phenylalanine	%*	Activity of N-benzoyl-L-phenylalaninol	%*
dpm/mg	1015	977		957	
dpm/mmol	5.13×10^5	2.63×10^5	51	2.44×10^5	48

$$* \% = \frac{\text{dpm/mmol of compound}}{\text{dpm/mmol of candipolin}}$$

Table 19 Distribution of label within amide units of candipolin from [^{14}C] L-phenylalaninol feed.

(a) N-benzoyl-L-phenylalanine

	Activity of N-benzoyl-L-phenylalanine	Activity of benzoic acid	%*
dpm/mg	977	1131	
dpm/mmol	2.63×10^5	1.38×10^5	53

(b) N-benzoyl-L-phenylalaninol

	Activity of N-benzoyl-L-phenylalaninol	Activity of benzoic acid	%*
dpm/mg	957	1050	
dpm/mmol	2.44×10^5	1.28×10^5	53

* % = $\frac{\text{dpm/mmol of benzoic acid}}{\text{dpm/mmol of parent amide}}$

Table 20 Incorporation of label from [U-^{14}C]-N-benzoyl-L-phenylalanine into candipolin.

Activity of N-benzoyl-L-phenylalanine fed dpm	Activity of diluted candipolin dpm/mg	Calculated activity of undiluted candipolin dpm/mg	Weight of candipolin mg	Incorp. %
8.89×10^7	2001	228076	89.6	23.0

hydrolysed with acid to give L-[U-^{14}C] phenylalaninol (3.06 μCi) (Expt. 7a). This was fed as its hydrochloride to 4-day old cultures of P. canadense and the candipolin isolated after a further six days' growth. The incorporation was, however, less than 2% (Table 17). Before accepting this "negative" result for L-phenylalaninol, it seemed desirable to feed the free base rather than the hydrochloride. The successful use of DMSO in the benzoylphenylalaninol feeding suggested that the problems of water-insolubility and/or permeability might be overcome in this way. It was necessary in this case to biosynthesise a further supply of [U-^{14}C] candipolin from L-[U-^{14}C] phenylalanine in order to obtain the required L-[U-^{14}C] phenylalaninol (Expt. 3b). The pure amino alcohol (3.2 μCi) was fed in DMSO (Expt. 7b) to 4-day old cultures of P. canadense but the resulting candipolin again showed relatively low ^{14}C activity (incorporation ca. 1%). The weakly radioactive candipolin derived from the phenylalaninol hydrochloride was found to have half its ^{14}C activity located in the benzoylphenylalanine unit, and half in the benzoylphenylalaninol unit (Table 18). This suggests that the benzoyl amidase does not accept L-phenylalaninol as a substrate. Hydrolysis of these two compounds gave samples of benzoic acid, each having half the ^{14}C activity of the parent amide (Table 19). The distribution of label over the four units of candipolin was approximately the same as that found when phenylalanine was fed, suggesting that L-phenylalaninol is not a true precursor of candipolin, but that it may be oxidised to the amino acid and incorporated as such.

L-[U-^{14}C] N-benzoylphenylalanine obtained as mentioned above, gave 23% incorporation of label into candipolin (Table 20). After further dilution, the candipolin was hydrolysed to give N-benzoyl-L-

Table 21 Distribution of label in candipolin from [^{14}C] N-benzoyl-L-phenylalanine feed.

	Activity of candipolin	%	Activity of N-benzoyl-L-phenylalanine	%*	Activity of N-benzoyl-L-phenylalaninol	%*
dpm/mg	1550		1517		1539	
dpm/mmol	7.84×10^5	100	4.08×10^5	52.0	3.93×10^5	50.1

$$* \% = \frac{\text{dpm/mmol of compound}}{\text{dpm/mmol of candipolin}}$$

Table 22 Distribution of label within the amide units of candidolin from the N-benzoyl-L-phenylalanine feed. (^{14}C)

(a) N-benzoyl-L-phenylalanine

	Activity of N-benzoyl-L-phenylalanine	%	Activity of benzoic acid	%*	Activity of phenylalanine hydrochloride	%*
dpm/mg	392		845		56	
dpm/mmol	1.06×10^5	100	1.03×10^5	97	1.13×10^4	11

(b) N-benzoyl-L-phenylalaninol

	Activity of N-benzoyl-L-phenylalaninol	%*	Activity of benzoic acid	%*
dpm/mg	1539		2355	
dpm/mmol	3.93×10^5	100	2.88×10^5	73

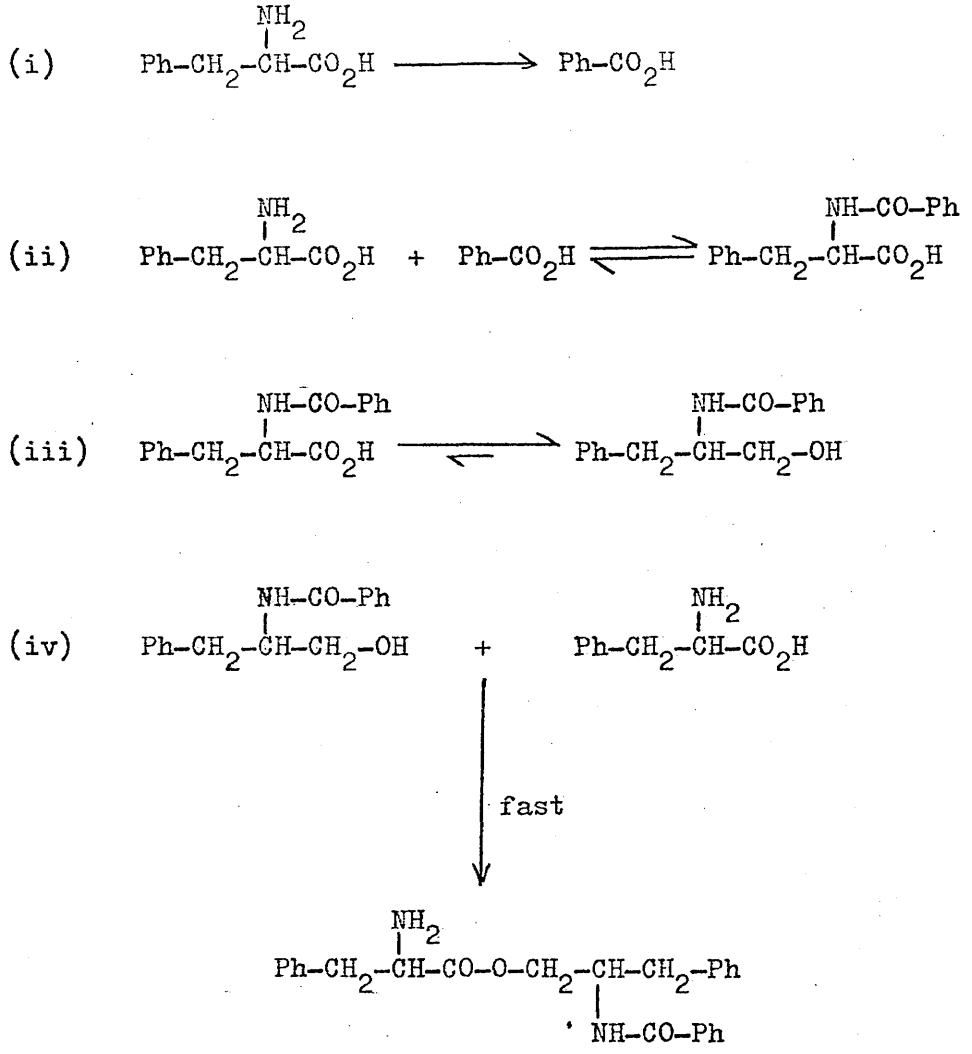
* % = $\frac{\text{dpm/mmol of compound}}{\text{dpm/mmol of parent amide}}$

phenylalanine and N-benzoyl-L-phenylalaninol, each with approximately half of the ^{14}C activity of the ester (Table 21). This seemed to suggest that the N-benzoyl-L-phenylalanine was incorporated into the N-benzoyl-L-phenylalaninol unit just as efficiently as into its own unit. Hydrolysis of the N-benzoyl-L-phenylalanine gave an interesting and unexpected result (Table 22a). Almost all of the ^{14}C activity was located in the benzoyl unit, with very little in the phenylalanine unit. Hydrolysis of the N-benzoyl-L-phenylalaninol showed that 73% of the label resided in the benzoyl unit (Table 22b).

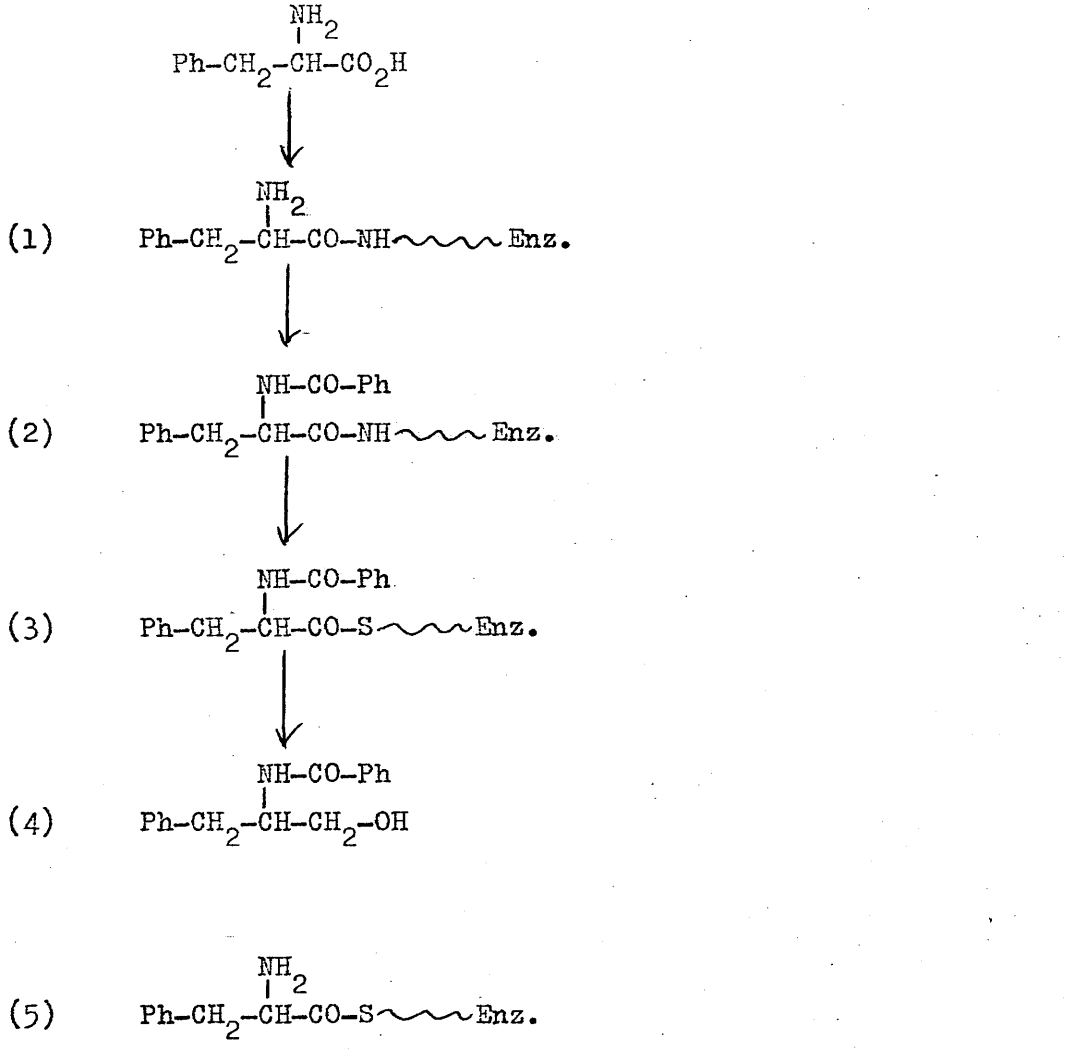
These results indicate that incorporation of intact N-benzoyl-L-phenylalanine was at least very low. It is possible that this compound is not a precursor of candipolin, or that it is a dynamic intermediate normally present only in small quantities so that on feeding an amount in excess of the natural pool, only a small percentage of the radiotracer would be incorporated intact. The results indicate that most of the N-benzoyl-L-phenylalanine was hydrolysed, before incorporation, to give L-phenylalanine and benzoic acid. The phenylalanine released could form the four units of candipolin, with concomitant reduction in ^{14}C activity of its own unit, while the benzoic acid would elevate the ^{14}C activity of the benzoyl residues. The low ^{14}C activity of the phenylalanine residue suggests that the phenylalanine released remains enzyme-bound, and does not become free to enrich the phenylalanine pool.

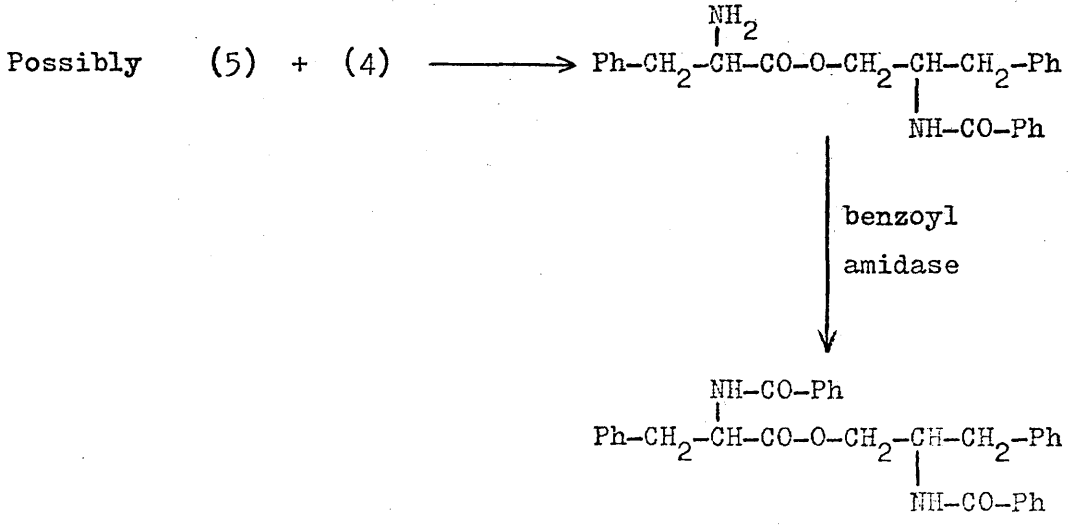
The fact that the proportion of label carried by the benzoyl group is less in the N-benzoyl-L-phenylalaninol fragment (75%) than in the N-benzoyl-L-phenylalanine fragment (90%) suggests that reduction of the fed N-benzoyl-L-phenylalanine (in which the benzoyl group carried 60% of the label) had occurred to some extent. The label distribution can be explained of production of N-benzoyl-L-

Scheme 22



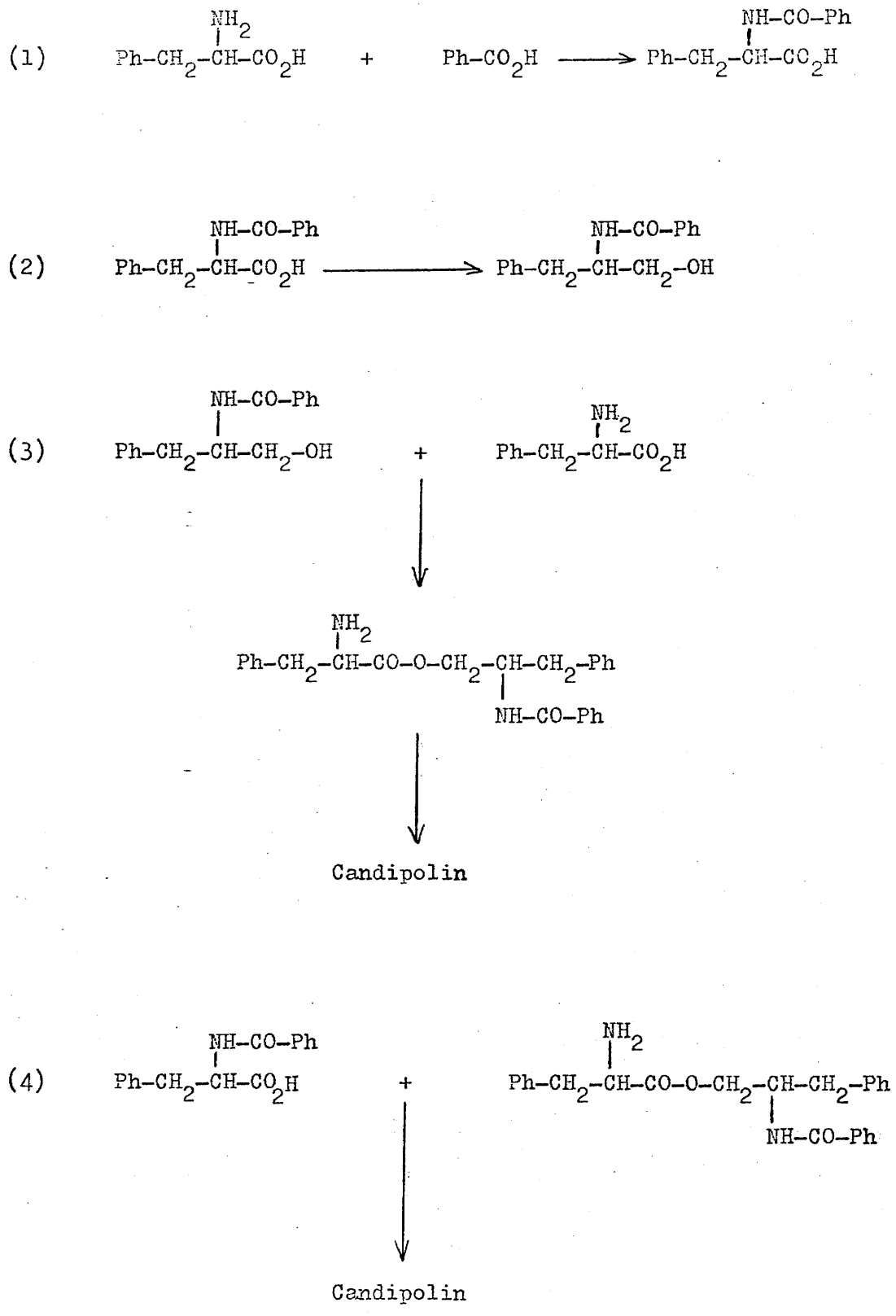
Scheme 23





Less frequently (2) + (4) ⟶ candipolin

Scheme 24


(1)
Ph-CH2-CH(NH2)-CO2H + Ph-CO2H
Ph-CH2-CH(NH-CO-Ph)-CO2H
(2)
Ph-CH2-CH(NH-CO-Ph)-CO2H
Ph-CH2-CH(NH-CO-Ph)-CH2-OH
(3)
Ph-CH2-CH(NH-CO-Ph)-CH2-OH + Ph-CH2-CH(NH2)-CO2H
Ph-CH2-CH(NH2)-CO-O-CH2-CH(NH-CO-Ph)-CH2-Ph
Candipolin
(4)
Ph-CH2-CH(NH-CO-Ph)-CO2H + Ph-CH2-CH(NH2)-CO-O-CH2-CH(NH-CO-Ph)-CH2-Ph
Candipolin

phenylalaninol by reduction is faster than its production from benzoic acid (labelled via the hydrolysis process) (Scheme 22). Indeed, it is difficult to see how N-benzoyl-L-phenylalaninol could be produced if not by these routes, since L-phenylalaninol does not appear to be a precursor of candipolin. Further investigation of this by feeding N-benzoyl-L-phenylalanine labelled only in the benzoyl carbonyl group is underway. Although time did not permit further study of the reduction and hydrolysis/recombination reactions, definite proof of intact incorporation of N-benzoyl-L-phenylalanine might be obtained by feeding N-benzoyl-L-phenylalanine synthesised from L-[^{15}N] phenylalanine and [carboxy-^{13}C] benzoic acid. Molecules of N-benzoyl-L-pehnylalanine (and N-benzoyl-L-phenylalaninol derived by reduction) would show ^{13}C-^{15}N coupling, whereas those derived via the hydrolysis step would show a ^{13}C singlet in their N.M.R. spectra. Comparison of the relative intensities of singlets and satellites should permit estimation of the contribution made by each pathway.

The results of these five feeding experiments suggest that a plausible pathway for biosynthesis of candipolin might be as outlined in Scheme 23, with N'-debenzoylcandipolin (71) being an intermediate. The feeding results do not rule out the possibility of other pathways existing.

It is possible that the concentrations of N-benzoyl-L-phenylalaninol may effect some degree of control over the enzymes of candipolin biosynthesis. In the hypothesis outlined in Scheme 24, steps 1 and 2 proceed faster than steps 3 and 4 until the concentration of N-benzoyl-L-phenylalaninol reaches a certain level. Then the rates of steps 3 and 4 increase until the alcohol is being esterified faster than it is being produced. When its concentration

Table 23 Predicted $^{3}H/^{14}C$ ratios.

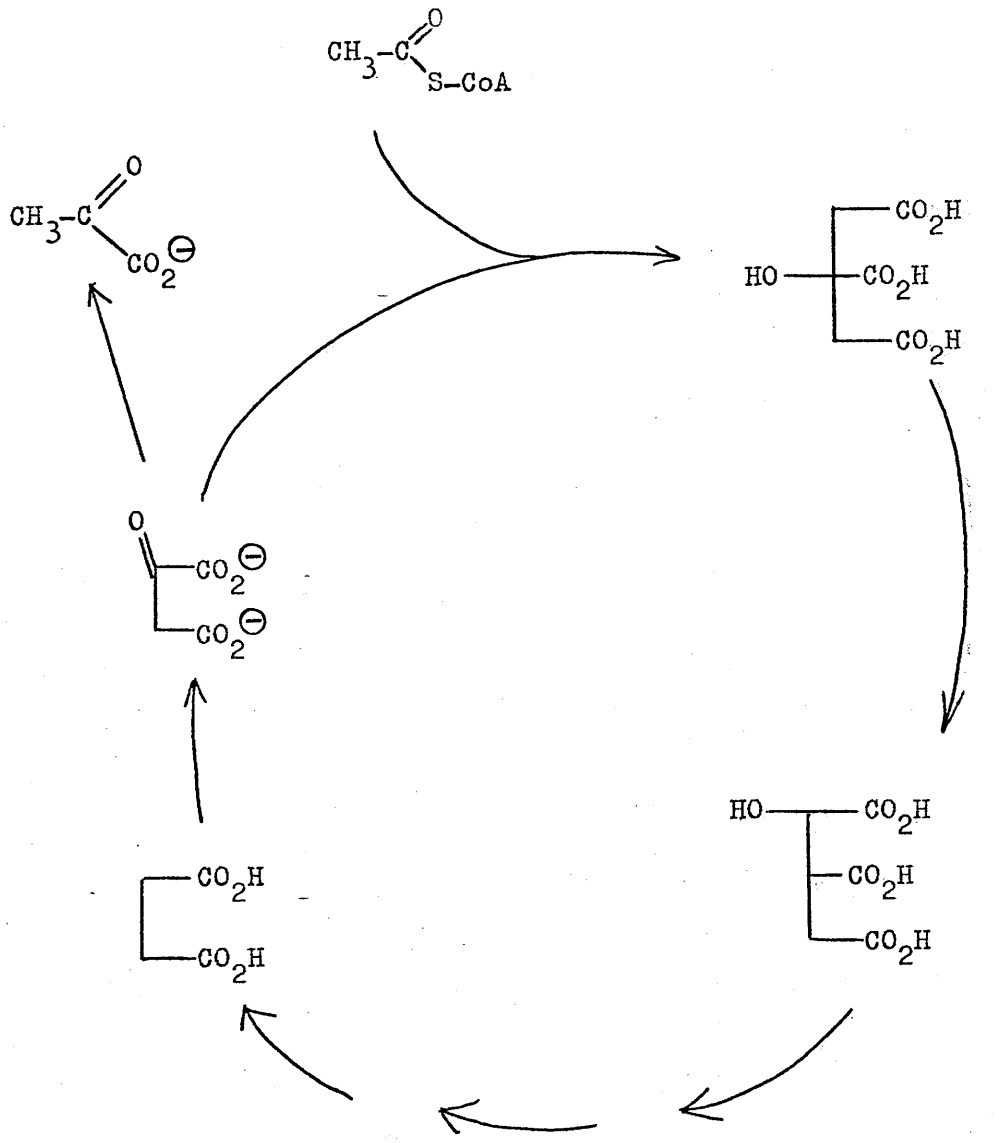
Phenyl-alanine fed	Candipolin	N-benzoyl-L-phenylalanine	N-benzoyl-L-phenylalaninol	Benzoic acid
16.9	15.4	15.5	15.5	13.6

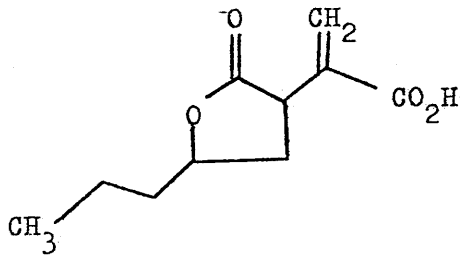
falls below the critical level, steps 3 and 4 become slower than the rate of production of the alcohol and the cycle begins again.

As was mentioned at the beginning of this discussion, a preliminary investigation of candipolin biosynthesis was carried out using L-[U-^{14}C],[G-^{3}H] phenylalanine. Hydrolysis showed that the label distribution over the four units of candipolin was the same as had been obtained for ^{14}C, i.e. N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol having the same ^{3}H activity and the benzoic acid samples from each having equal ^{3}H activities (Tables 4,5). The unexpectedly high ^{3}H/^{14}C ratios of the benzoic acid samples (Table 5, cf. Table 23) suggested an inhomogeneity in the labelling of [G-^{3}H] phenylalanine. An analysis of [G-^{3}H] phenylalanine has been reported[103] which indicates that this is so, since the side chain was found to carry 27 $\pm$ 3% of the label and the ring to carry 73 $\pm$ 3% of the label.

Other workers studying various phenylalanine-derived metabolites have reported stereospecific loss of tritium from the 3' position of phenylalanine, which occurs faster than incorporation. A fuller discussion of these interesting experiments is presented in Appendix 1. With the high generality of tritiation of the phenylalanine used in the current work, it is not possible to deduce reliably whether or not this tritium loss occurred during the biosynthesis of candipolin.

Scheme 25


CH3-C(=O)-S-CoA
CH3-C(=O)-CO2⊖
HO-C(CO2H)(CH2CO2H)2
HO-C(CO2H)(CH2CO2H)2
CH2(CO2H)2
O=C(CO2⊖)-CH2CO2⊖



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Investigation of Acetate and Succinate as potential Precursors of Candipolin.

As has been suggested earlier, it is possible that a minor pathway leading to the benzoyl groups of candipolin may be from acetate, via the Krebs cycle and the shikimic acid pathway (See Scheme 25). Similarly, succinate, another Krebs cycle intermediate, might act as a precursor to these functions.

^{13}C-labelled acetate and succinate were fed to cultures of P. canadense[104] during the course of an investigation not connected with this work, but dealing with the biosynthesis of several polyketide-derived broth metabolites. The metabolites under investigation were canadensolide (8), dihydrocanadensolide (9), which, as mentioned before, has been been found to act as an anti-ulcer agent, and canadensic acid (86). The mycelium from these experiments was, however, available, and afforded candipolin in the usual way. In each case, the ^{13}C N.M.R. spectrum of the candipolin showed no significant signal enhancemants above the natural abundance level, indicating that this pathway to the benzoyl groups is of little or no importance.

Occurrence of N-benzoyl-L-phenylalaninol in the Mycelium of P. canadense.

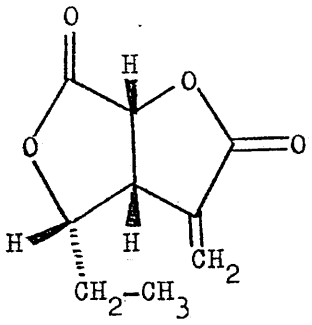
The mycelial extract of P. canadense which had been fed with L-[U-^{14}C] phenylalanine (Expt. 3b), was retained after removal of candipolin. Inactive N-benzoyl-L-phenylalaninol was added to the mixture of metabolites more polar than candipolin, and the corresponding band removed after preparative T.L.C. This was found to be radioactive, showing that N-benzoyl-L-phenylalaninol was present in the mycelium. After extensive chromatographic purification,

Table 24 Incorporation of label from L-[U-^{14}C]phenylalanine into N-benzoyl-L-phenylalaninol.

Activity of phenylalanine fed dpm	Activity of N-benzoyl-L-phenylalaninol dpm/mg	Minimum * weight of N-benzoyl-L-phenylalaninol mg	Minimum incorporation %
3.33×10^8	23912	45.4	0.33

* This is the weight of inactive N-benzoyl-L-phenylalaninol added as carrier.

0.33% of the fed ^{14}C activity was recovered in pure N-benzoyl-L-phenylalaninol (Table 24) . If the assumption is made that the incorporation into N-benzoyl-L-phenylalaninol is the same as into the same unit in candipolin, the recovered activity corresponds to 0.58 mg. of free N-benzoyl-L-phenylalaninol being present in the mycelium. The low level of this free alcohol is consistent with its being an intermediate in the biosynthesis of candipolin, as outlined in Scheme 23.

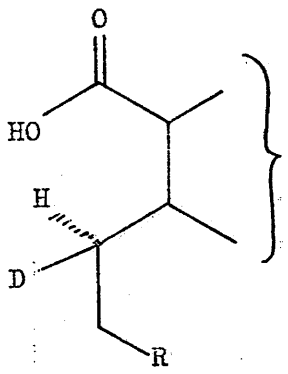

O
H
O
O
O
H
H
CH2
CH2–CH3

CHAPTER 3

FATTY ACID BIOSYNTHESIS IN P. CANADENSE : The Stereochemistry of Double Bond Formation.

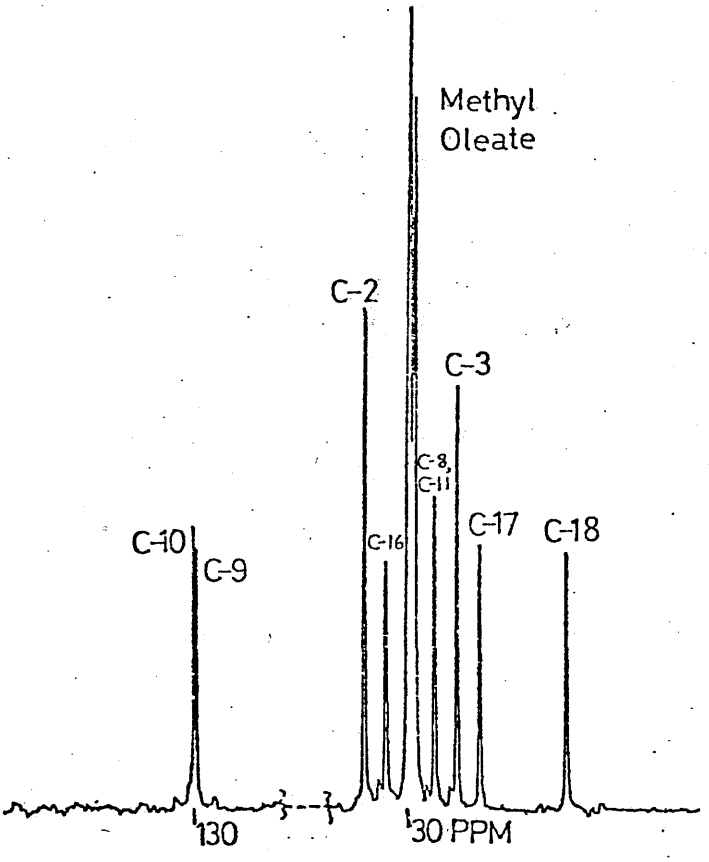
The accepted pathway of fatty acid biosynthesis from acetate involves a cycle of reactions including dehydration of a (3R)-hydroxy- fatty acyl derivative followed by reduction of the resulting trans-2-enoyl compound[120,121]. The initially formed compound is known to be the D-(-)-(3R)-hydroxybutyric acid derivative[120,121], and dehydration of this has recently been shown to be a syn process involving elimination of the pro-2S hydrogen (H^S) atom[122]. Hence, the pro-2R hydrogen atom (H^R) is retained in the crotonate. This predicts that only one deuterium atom will be retained at the even-numbered carbons in a fatty acid chain derived from deuterioacetate (CD_3CO_2Na). Assuming that the steps subsequent to butyrate formation and leading to stearate are repetitions of the first steps, all even-numbered methylene carbons in fatty acids such as stearic acid would retain one hydrogen atom derived from acetate in the same configuration (e.g. pro-R as shown in Scheme 27).

The antibiotics ethisolide (87) and dihydrocanadensolide (9) both contain polyacetate-derived moieties (ring A and the alkyl substituent) presumed to arise by standard fatty acid-type biosynthesis following studies using ^{14}C, ^{13}C, ^{3}H and ^{2}H singly- and multiply- labelled acetates[123]. Each is oxygenated at C-4 of the fatty acid chain. An interesting result was obtained using $^{13}CD_3CO_2Na$ whereby deuterium was retained at C-4 in ethisolide and lost from C-4 in dihydrocanadensolide[124]. The absolute configuration of these antibiotics was at that time uncertain, but the above



88

Figure 9



result was correctly deduced to reflect a difference in absolute configuration at C-4. These absolute configurations are now known to be as shown[125,126], so that the retained hydrogen atom derived from deuteroacetate is deduced to be in a pro-R configuration as in the structure 88. This assumes that hydroxylation at C-4 and lactonisation do not affect the configuration at C-4.

Since it has been established that in several systems (*C. diphtheriae*, *chlorella*, and chicken liver) the desaturation of stearic acid to give oleic acid involves loss of the 9-pro-R and 10-pro-R hydrogens[127,8], loss or retention of deuterium at C-10 in oleic acid derived from deuteroacetate will indicate whether the C-10 deuterium atom in the stearate precursor was in the pro-R or pro-S configuration respectively. The mycelial extract of *P. canadense* afforded, in addition to candipolin, a substantial quantity of lipid, consisting mainly of a mixture of triglycerides. The object of the present work was to derive evidence for the configuration of the acetate-derived hydrogen atoms in the fatty acid chains of the lipids.

A method was first developed for converting the triglyceride mixture, obtained by chromatography of the mycelial extract, into the corresponding mixture of fatty esters. This was achieved by successive treatments with 0.5M NaOH in methanol and with BF_3 in methanol[129]. Purification was effected by vacuum distillation. N.M.R. (^1H and ^{13}C) and G.L.C. showed that although the composition varied a little from batch to batch of fungus, the main components were methyl oleate (ca. 50%) and methyl palmitate (ca. 25%) with smaller amounts of other fatty esters. The ^{13}C spectrum of methyl oleate (Fig. 9) shows a feature advantageous to this study in that the signals corresponding to C-9 and C-10 are distinct, differing

Figure 10

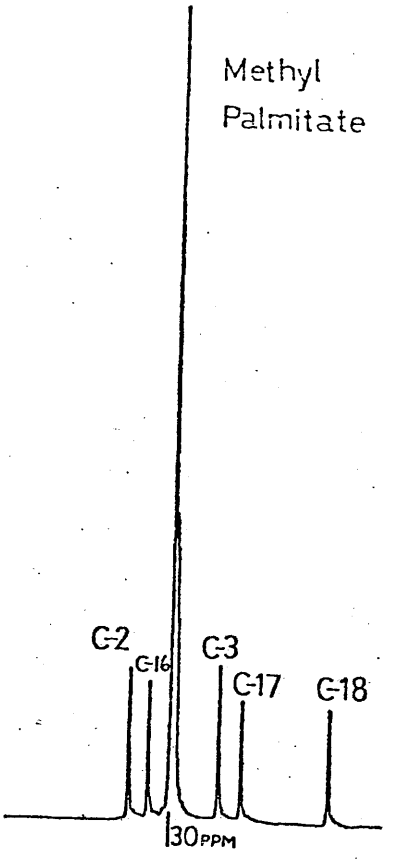


Figure 11

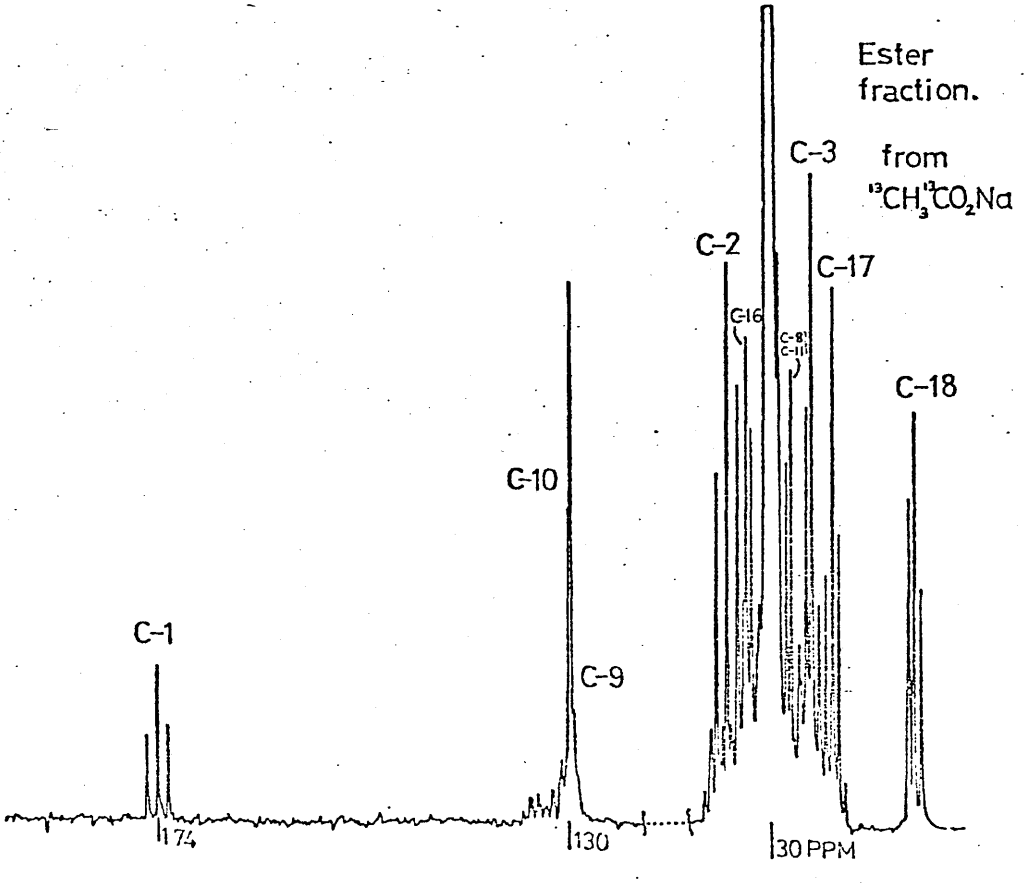


Table 25 ^{13}C N.M.R. Spectrum of methyl oleate (containing methyl palmitate and other esters) from $^{13}CH_3{}^{13}CO_2Na$.

Chemical shift δ	Carbon atom	$J_{^{13}C-^{13}C}$ Hz.
14.10	C-18	34.8
22.75	C-17	34.9
25.03	C-3	34.5
27.27	C-8, C-11	34.4
32.00	C-16	ca.36.4
34.09	C-2	57.6
174.07	C-1	57.5
130.02	C-10 } *	
129.52 inf.	C-9 }	

* Satellites not visible, but intermediate line appears at 129.85 p.p.m.

Notation :

$$\overset{18}{CH_3}-\overset{17}{CH_2}-\overset{16}{CH_2}-(CH_2)_4-\overset{11}{CH_2}-\overset{10}{CH}=\overset{9}{CH}-\overset{8}{CH_2}-(CH_2)_4-\overset{3}{CH_2}-\overset{2}{CH_2}-\overset{1}{CO_2}CH_3$$

by 0.25 p.p.m. It was considered that significant information might be obtained by studying the ^{13}C spectra of the total mixture of methyl esters since the saturated esters like methyl palmitate (Fig. 10) and methyl stearate have no signals in the olefinic region which would be the region of most interest.

At the time of this study, there was available mycelium of P. canadense which had been fed with [1,2-$^{13}C_2$] sodium acetate in connection with studies on canadensolide (a broth metabolite)[123,124]. It was decided to study the corresponding fatty ester fraction to check the method and the incorporation of acetate. The spectrum (Fig. 11) indicates that a good degree of ^{13}C enrichment has been achieved. In view of the accepted pattern of incorporation of acetate into fatty acids, $^{13}C-^{13}C$ coupling between the carbon atoms of each incorporated doubly enriched acetate unit will be expected to give rise to doublets associated with each carbon (other than the methoxyl group carbon). The assignments indicated in Fig. 11 are those established for these esters[130-3]. Although many of the methylene signals are overlapping, doublets associated with C-17 and C-18 and with C-1 and C-2 are both clearly seen (cf. Table 25). The $^{13}C-^{13}C$ coupling of C-9 and C-10 in doubly enriched molecules is represented, because of their small chemical shift difference, by a single line halfway between the natural abundance signals for C-9 and C-10.

It was, therefore, possible to follow the fate of acetate-derived hydrogen atoms following incorporation into methyl oleate by studying the ^{13}C spectrum of a sample enriched by culturing the P.canadense in the presence of $^{13}CD_3CO_2Na$ (95%). The presence of deuterium at a particular even-numbered carbon atom will result in a triplet (owing to $^{13}C-^{2}H$ coupling of characteristic magnitude)

Table 26 ^{13}C N.M.R. Spectrum of methyl oleate (plus methyl palmitate) from $^{13}CD_3CO_2Na$.

Chemical shift δ	Carbon atom	Enrichment factors (A)	(B)
14.14	C-18	0.36	0.56
22.75	C-17	-0.09	0.25
25.03	C-3	0.23	0.01
27.26	C-8, C-11	1.28	-0.12
29.21	$(CH_2)_n$	1.54	0.61
29.41		0.71	0.30
29.76		0.57	0.50
32.00	C-16	0.60	0.91
34.13	C-2	1.12	0.77
51.37	OCH_3	0	-
129.77	C-9	-0.08	-0.15
130.02	C-10	1.56	0.89
174.19	C-1	0.20	-

Figure 12

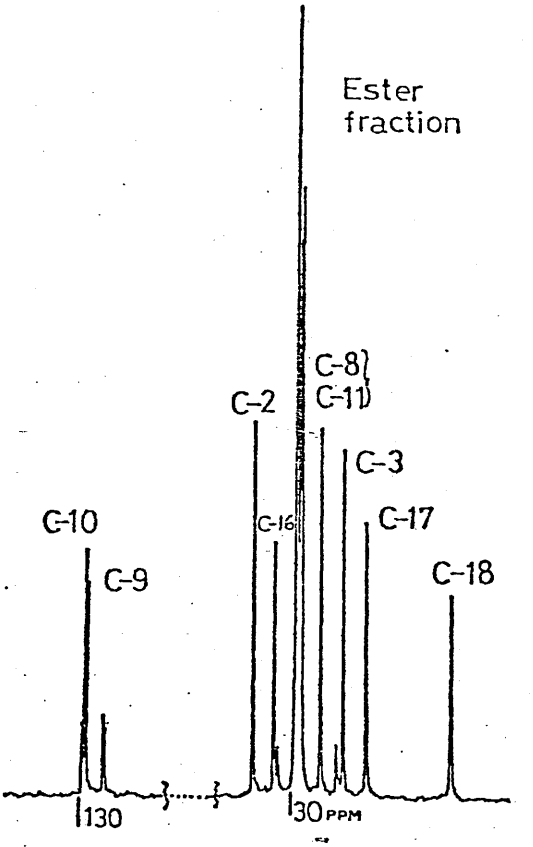
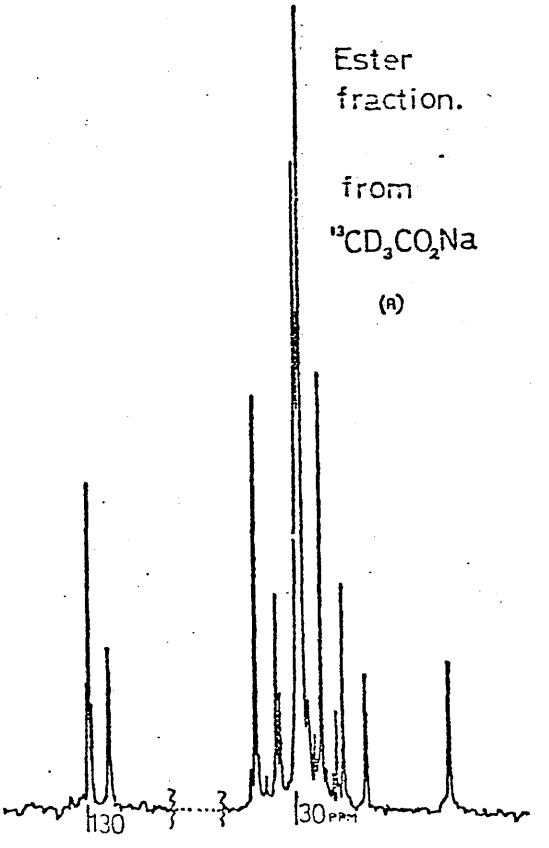


Figure 13



which is slightly upfield of the natural abundance signal (owing to the isotope effect) and which is relatively weak (owing to the greatly increased spin-lattice relaxation time and greatly reduced nuclear Overhauser effect associated with a $^{13}C-^{2}H$ signal as compared to $^{13}C-^{1}H$)[134]. The biosynthetic pathway to saturated fatty acids from $^{13}CD_3CO_2Na$ (fully deuterated) would predict that every even-numbered carbon atom would retain at least one deuterium atom. In practice, enhancement of the natural abundance signals are observed (cf. Table 26). This must correspond to the incorporation of ^{13}C atoms bearing hydrogen rather than deuterium atoms. This might arise because of some loss of deuterium by exchange during biosynthesis. Also, if a small amount (e.g. 1-2%) of the fed [2-^{13}C] acetate was not fully deuterated, any isotope effect would tend to this being preferentially carboxylated and incorporated into the fatty acid chain. In a recent study in which $^{13}CD_3CO_2Na$ was incorporated into a number of polyketides, extensive loss of deuterium was also noted[135].

The ^{13}C spectrum of the unlabelled ester fraction (Fig. 12) shows that the main olefinic ester is methyl oleate (cf. Fig. 9), the most significant signals being those for C-9 and C-10 (which are approximately the same in intensity) and for C-2 and the allylic carbon C-8. Other olefinic esters present in small amounts give rise to three weak signals in the 130 p.p.m. region. The sample derived by feeding $^{13}CD_3CO_2Na$ (experiment A, Fig. 13) shows triplets (J = ca. 19.3 Hz.*, isotope shift = ca. 0.35 p.p.m.) associated with

* Since $J_{HX}/J_{DX} = \gamma_H/\gamma_D$ = ca. 6.51[131] and a typical value of $J_{^{13}C-H}$ for a saturated CH_2 group is 128 Hz. and for an olefinic CH 158 Hz., values can be calculated for $J_{^{13}C-^{2}H}$ of 19.7 Hz. and 24.3 Hz. respectively. (N.B. Stothers p.317 for the 19.7 Hz. value). The calculated value of 24.3 Hz. is based on values of 157 Hz. for cyclohexene, 160 Hz. for cyclopentene, and 156 Hz. for ethylene.

Figure 14

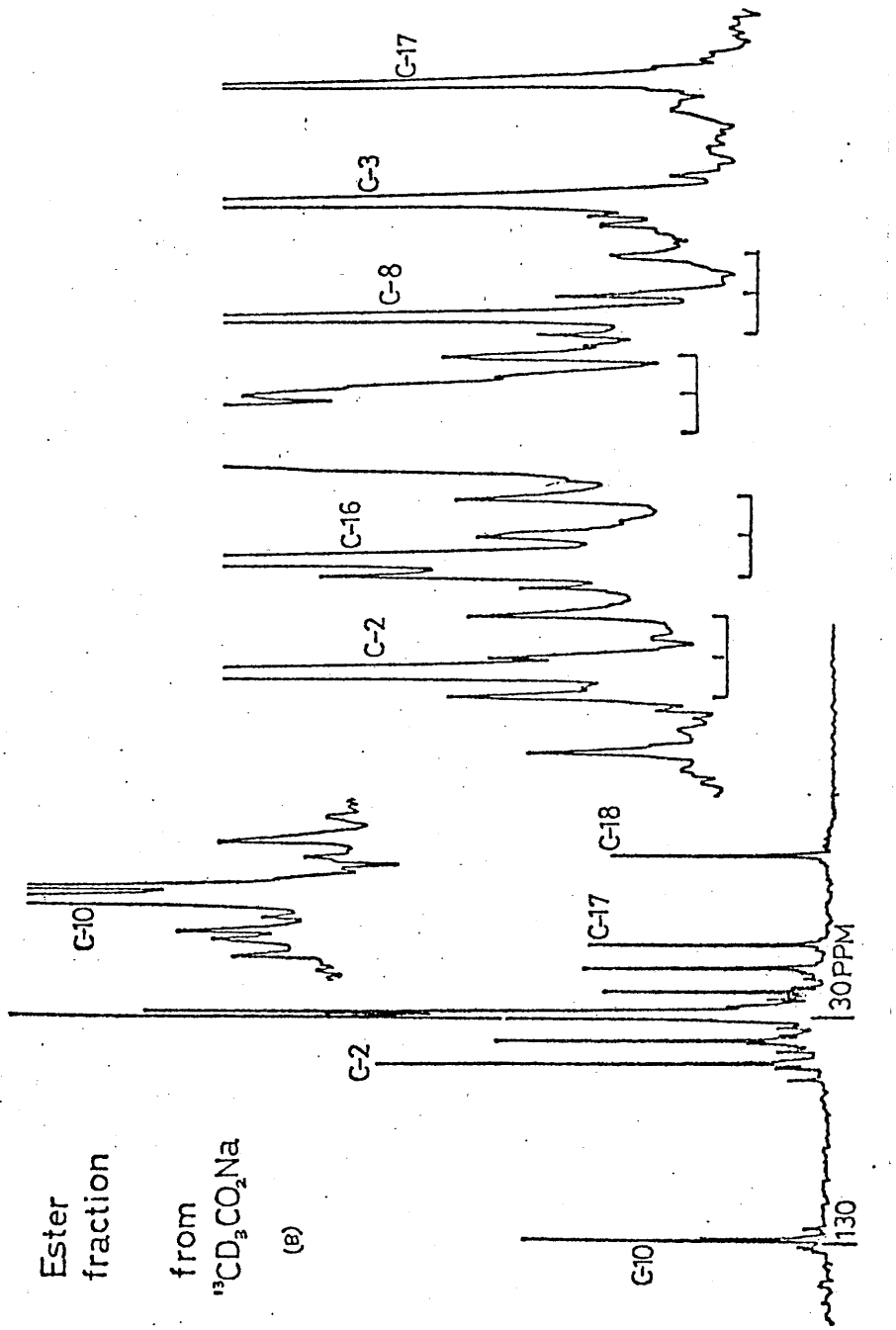

Ester fraction
from $^{13}CD_3CO_2Na$
(B)
C-10
C-2
C-17
C-18
C-16
C-8
C-3
C-17
C-2
C-10
30 PPM
130

Table 27 ^{13}C N.M.R. Spectrum of the ester mixture from $^{13}CD_3CO_2Na$ (Experiment B).

Chemical shift δ	Carbon atom	$J_{^{13}C-^{2}H}$ Hz.	Δ p.p.m.
27.20	C-8	19.1	0.37
31.95	C-16	19.1	0.46
34.12	C-2	19.6	0.28

C-2 and C-8 most clearly, indicating the presence of one deuterium atom at these centres[134]. The spectrum shows the same three "impurity" signals in the 130 p.p.m. region, but the signal for C-10 is clearly enhanced relative to C-9 (more than doubled in intensity) and enhanced considerably more than any of the other even-numbered carbon atoms. Also, there is no sign of a triplet (J = ca. 24.3 Hz.,* isotope shift = ca. 0.35 p.p.m.) corresponding to $-^{13}C^{2}H=CH-$.

The ester fraction obtained from a second $^{13}CD_3CO_2Na$ feeding experiment (B) was slightly less satisfactory. The spectrum of the ester sample (Fig. 14) showed the presence of slightly different impurity peaks in the 130 p.p.m. region. Again, the C-10 peak is strongly enhanced relative to the C-9 peak. It is, however, enhanced significantly more in this case than some of the other even-numbered carbon atoms, making invalid any conclusions about the absence of deuterium. Indeed, at first glance, the impurity peaks could suggest the presence of a deuterium triplet at C-10, but the signals do not satisfy the requirement of coupling constant and isotope effect indicated above. Also, an N.M.R. experiment involving reduction of Overhauser enhancements of C-H peaks by decoupler gating[136] confirmed that these were impurity C-H peaks rather than C-D peaks. The presence of these impurities was unfortunate, since, in this case, very well-defined triplets (J = ca. 19.3 Hz., Δ = ca. 0.35 p.p.m.) appear (cf. Table 27), associated with C-2, C-16, and C-8 as indicated in the inset.

The two experiments show almost certainly that there is no deuterium at C-10 in the methyl oleate, so that the deuterium atom at C-10 and other even-numbered methylene carbons in the precursor stearic acid can be deduced to be pro-R (assuming cis-dehydrogenation), in complete agreement with the results for ethisolide and dihydrocanadensolide.

* See footnote on p. 45.

Figure 15

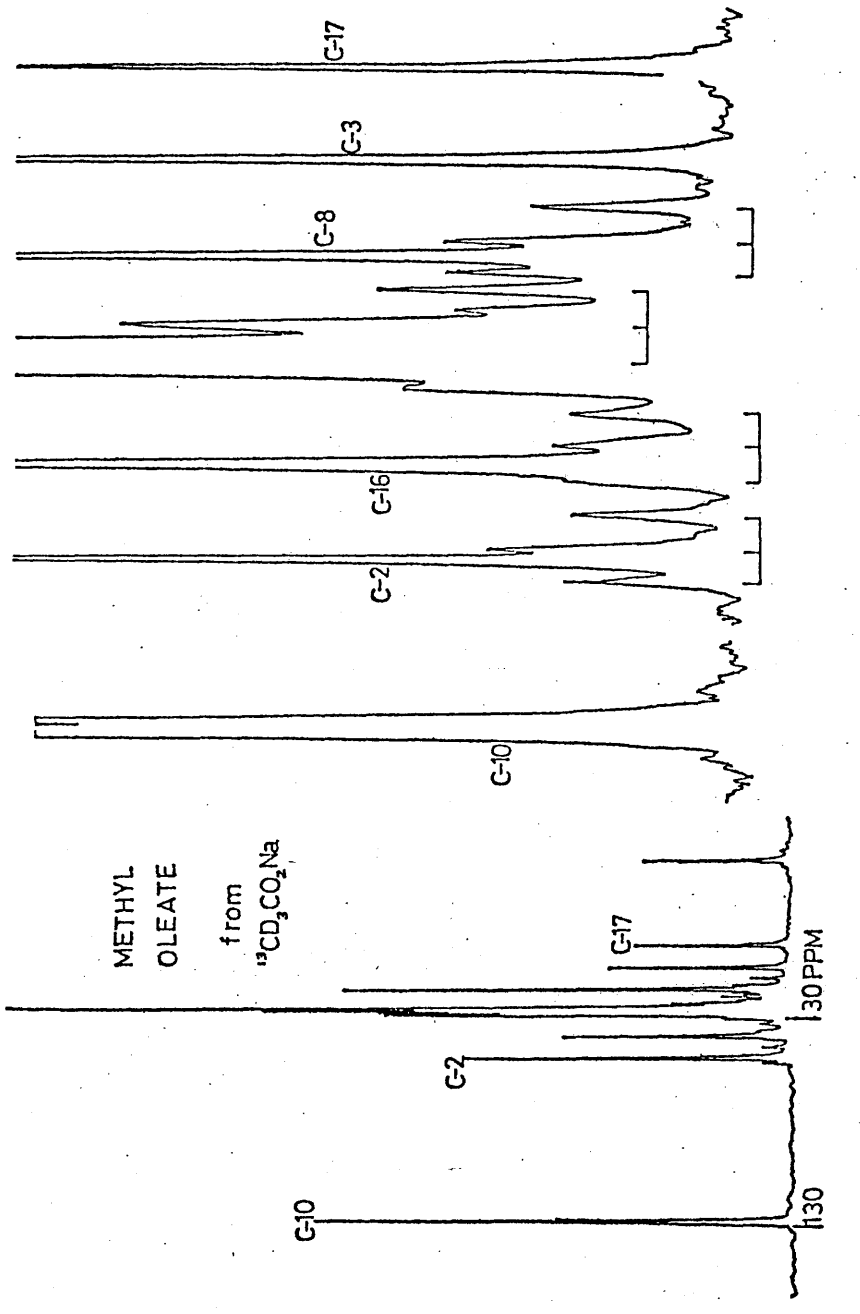
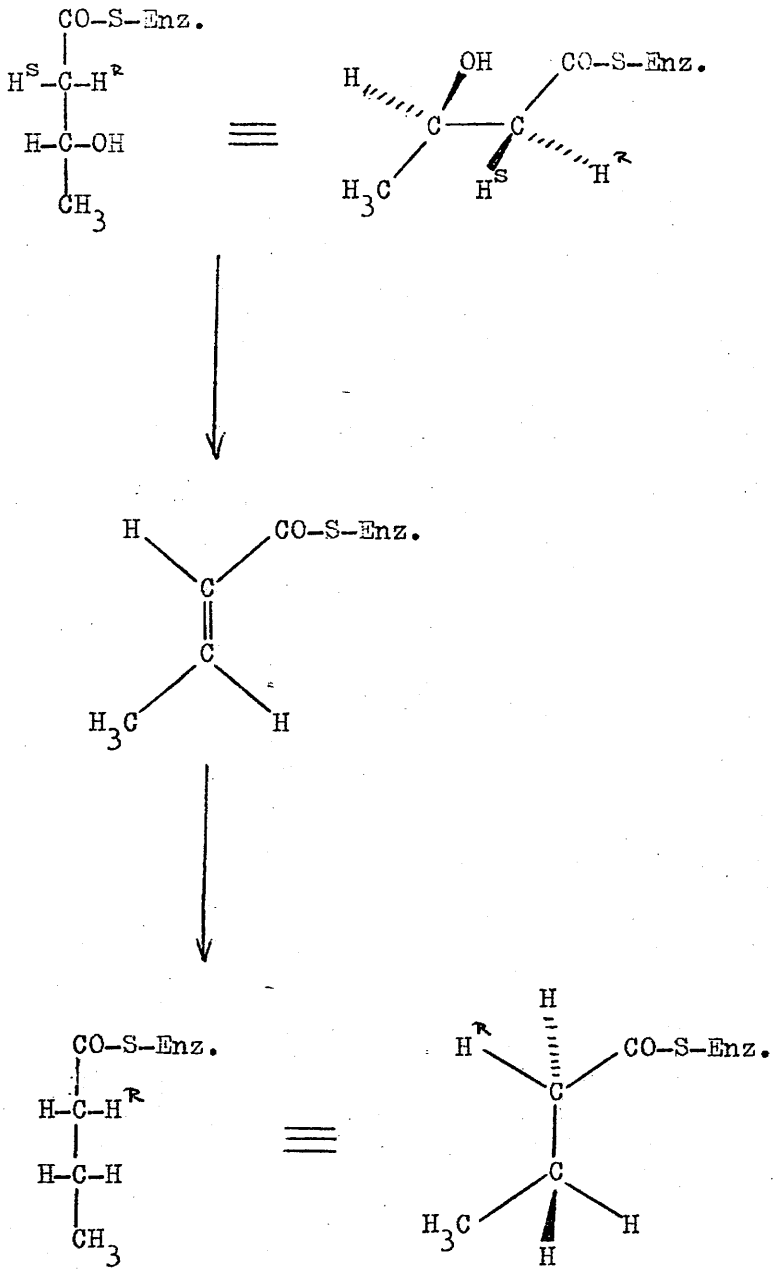

METHYL
OLEATE
from
$^{13}CD_3CO_2Na$
C-17
C-3
C-8
C-16
C-2
C-10
C-17
C-2
C-10
30 PPM
130

Table 28 ^{13}C N.M.R. Spectrum of methyl oleate from $^{13}CD_3CO_2Na$.

Chemical shift δ	Carbon atom	Enhancement factor
14.12	C-18	-0.14
22.71	C-17	-0.10
24.97	C-3	-0.36
27.20	C-8, C-11	1.22
29.14	$(CH_2)_n$	0.67
29.36		0.35
29.54		0.47
29.75		-0.24
31.93	C-16	0.44
34.11	C-2	0
51.41	OCH_3	-0.30
129.77	C-9	0.42
130.01	C-10	1.66
174.29	C-1	0.07

Scheme 27


CO–S–Enz.
H^S–C–H^R
H–C–OH
CH3
≡
H
OH
CO–S–Enz.
C
C
H3C
H^S
H^R
H
CO–S–Enz.
C
C
H3C
H
CO–S–Enz.
H–C–H^R
H–C–H
CH3
≡
H
H^R
CO–S–Enz.
C
C
H3C
H
H

Because of the unfortunate appearance of the olefinic region in the spectrum of the second sample, it seemed desirable that further purification of the methyl oleate should be carried out, although this was not possible owing to lack of time in the present work. A further feeding experiment with $^{13}CD_3CO_2Na$ has, however, since been carried out (by Mr. G. Johnstone and Dr. N.J. McCorkindale) and the transesterification carried out as before. The methyl oleate was, however, isolated free from both saturated and unsaturated impurities by T.L.C. on $AgNO_3$-silica gel. The resulting spectrum (Fig. 15, Table 28) is included for the sake of completeness (by courtesy of these workers). A very clean spectrum is obtained showing the $^{13}C-^{2}H$ triplets associated with C-2, C-16, and C-8 in particular. C-10 is again more strongly enhanced than any of the other atoms, and no triplet associated with C-10 can be detected, although prominent triplets associated with other carbons can be seen. This result confirms the above work.

It is now possible to deduce that the stereochemistry of reduction of the crotonate to butyrate (Scheme 27) involves delivery of hydrogen at C-2 from the *si* face, i.e. the hydrogen will adopt the pro-S configuration. Assuming *trans*- reduction as is observed in the biosynthesis of sterols[137], in the biosynthesis of griseofulvin[138], and in the enzymic reduction of cinnamyl alcohol using bakers' yeast[139], delivery of hydrogen to C-3 will also be from the *si* face with respect to C-3, i.e. hydrogen will adopt the pro-S configuration. This stereochemistry was recently verified[151] in the case of butyrate biosynthesis by use of the enoyl reductase to effect ^{3}H labelling of a crotonyl substrate at C-2 and C-3. On Treatment of the resulting butyryl substrate with pig liver dehydrogenase (which effects *anti*- elimination of the pro-2R and pro-3R hydrogen atoms) label was retained at both positions.

Scheme 28

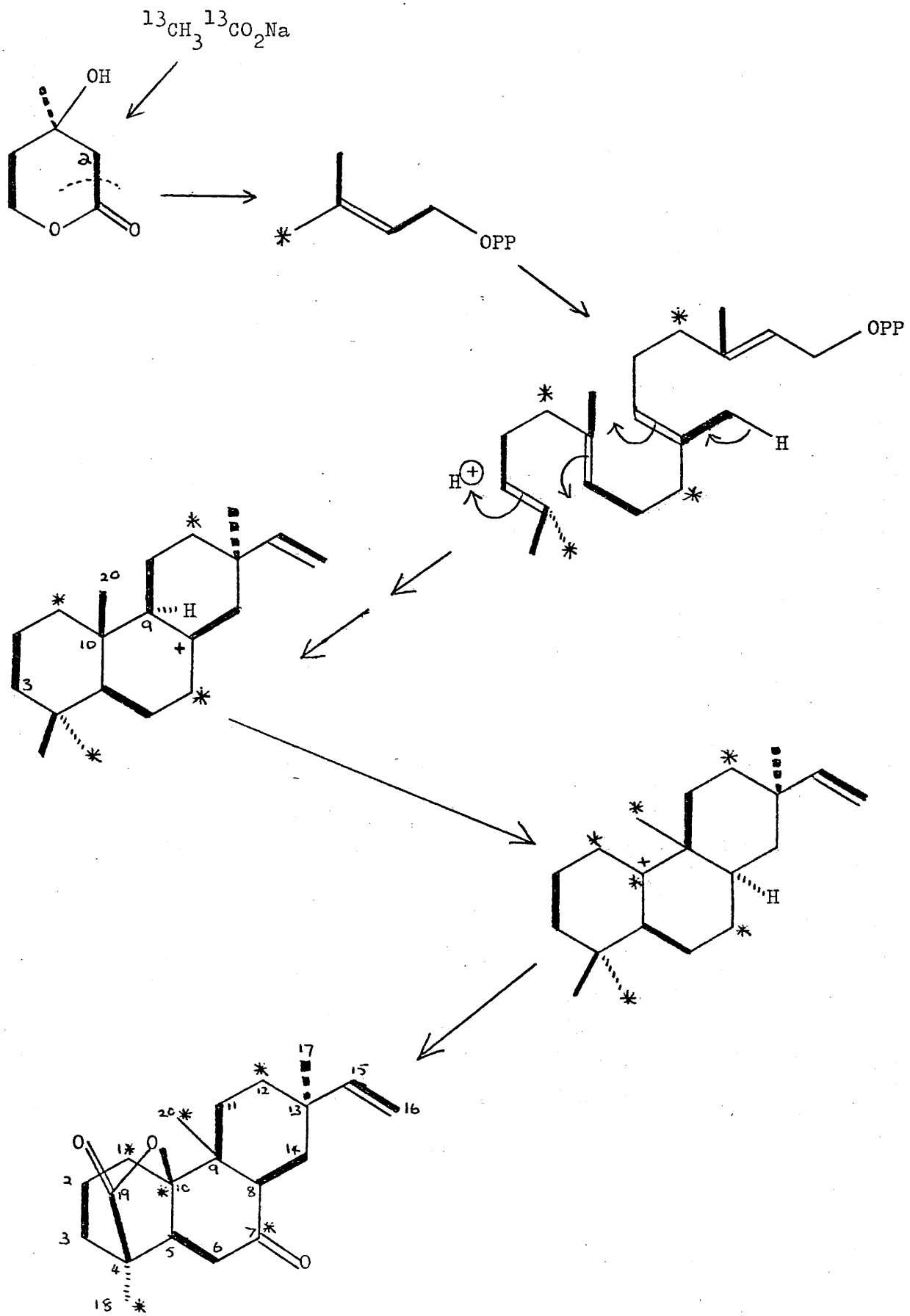

$^{13}CH_3\,^{13}CO_2Na$
OH
a
O
O
OPP
OPP
H
H
20
H
9
10
3
17
15
12
11
13
16
20
O
O
2
19
10
9
14
8
3
7
5
6
4
18
O

Table 29 ^{13}C N.M.R. data for rosenonolactone (89).

	Multiplicity in O.R.D. spectrum	Carbon atom	J_{C-C} in (89) from $^{13}CH_3{}^{13}CO_2Na$ † (Hz.)	Enhancements from [^{13}C] acetates			
				1-^{13}C	2-^{13}C	2-$^{13}CD_3$ ‡ (A)	2-$^{13}CD_3$ ‡ (B)
16.9	q	C-18		0	1.1*	0[a]	0.1*
16.9	q	C-20		0	1.1*	0[a]	0.1*
19.8	t	C-2	32.4	1.4	0.1	-0.2	-0.2
21.9	q	C-17	35.5	0	1.3	0[b]	-0.1
30.2	t	C-1		0	1.0	0.2[c]	0.2
30.8	t	C-6	33.9	1.1	0	0.1	0
31.4	t	C-12		0.1	1.1	0.2[d]	0.1
31.8	t	C-14	36.8	0	1.1	0.1[d]	0
35.1	s	C-13	35.5	1.4	-0.1	0	-0.1
35.4	t	C-3	31.9	0.1	1.2	0[d]	0
35.8	t	C-11	34.7	0.9	0.1	-0.1	-0.1
38.8	s	C-9	35.1	-0.1	0.4	0.4	0.2
47.3	s	C-4	47.6	1.1	-0.2	-0.1	-0.1
47.4	d	C-8	36.8	0.9	-0.1	-0.1	-0.1
50.8	d	C-5	33.4	-0.2	1.0	0.2[e]	0
87.0	s	C-10		1.0	-0.1	0.2	0.1
109.9	t	C-16	69.7	1.4	0.2	0	0
149.6	d	C-15	70.3	0	1.0	0.2[f]	0.3
179.2	s	C-19	48.0	0.1	0.6	3.1	2.2
210.0	s	C-7		0	1.0	1.5	1.4

Notes :

* Half the total enhancement of this signal.

† Summarised in (91).

‡ Deuterium and carbon-13 labelling pattern summarised in (93).

Notes on Table 29

a (C-18 and C-20) : 3^{et}, J = 19.5 Hz., $\Delta(d_1)$ = 0.27 ppm.;
5^{et}, $\Delta(d_2)$ = 0.45 ppm.

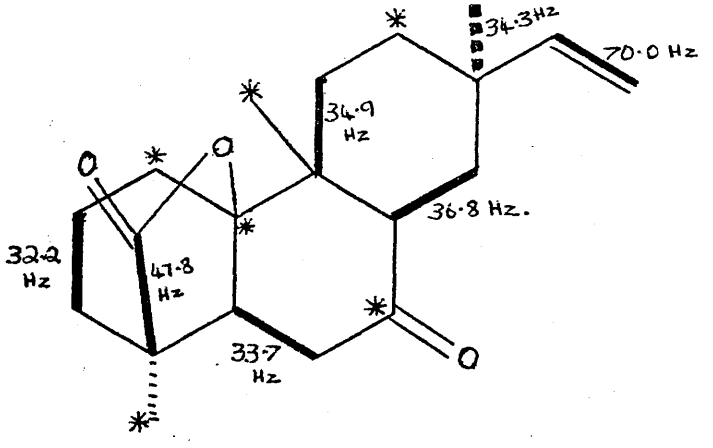
b (C-17) : 3^{et}, J = 19.3 Hz., $\Delta(d_1)$ = 0.29 ppm.; 5^{et}, $\Delta(d_2)$ = 0.63 ppm.;
7^{et}, $\Delta(d_3)$ = 0.88 ppm.

c (C-1) : Multiplets partially obscured by C-6, C-12 but centre lines of 3^{et}, $\Delta(d_1)$ = 0.32 ppm.; 5^{et}, $\Delta(d_2)$ =,0.65 ppm.

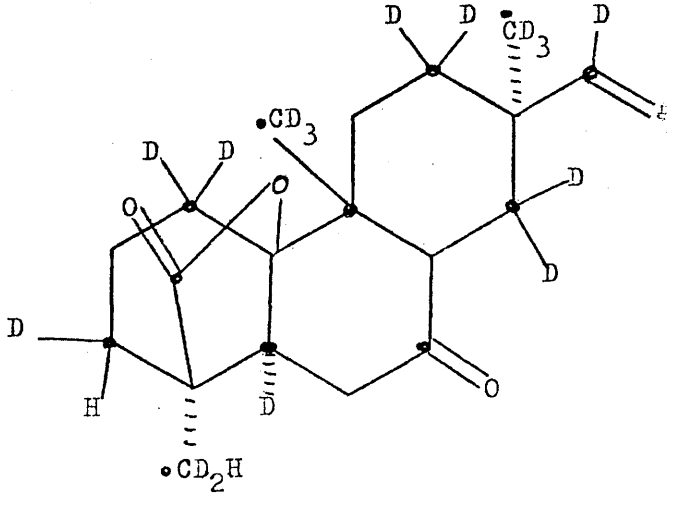
d Multiplets obscured by adjacent peaks.

e (C-5) : 3^{et}, J = 19.5 Hz., $\Delta(d_1)$ = 0.47 ppm.

f (C-15) : 3^{et}, J = 23.2 Hz., $\Delta(d_1)$ = 0.36 ppm.



91



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CHAPTER 4

Studies on the location of acetate-derived hydrogen atoms in rosenonolactone (89).

The way in which useful information concerning the location of acetate-derived hydrogen atoms in the antibiotics ethisolide and dihydrocanadensolide and in methyl oleate was obtained from $^{13}CD_3CO_2Na$ feedings has already been described. It was decided to explore this approach in the biosynthesis of rosenonolactone. In particular, it was hoped that the results of $^{13}CD_3CO_2Na$ feedings might demonstrate that the migration of the methyl group from C-10 to C-9, generally accepted to occur during the biosynthesis[140,1], occurs without loss of the original hydrogen, i.e. excluding the intermediacy of a cyclopropanoid intermediate (cf. Scheme 28).

The current work was initiated by Drs. J.Troke and N.J. McCorkindale, who obtained biosynthetically labelled samples of rosenonolactone by feeding Trichothecium roseum with singly- and doubly- labelled sodium acetates. The present analysis of the carbon-13 spectral data of these samples is indicated in Table 29. This includes an unambiguous assignment of most of the carbon resonances based on analogies, on off-resonance decoupled spectra, on enrichments of the appropriate carbons in the samples from either [1-^{13}C] or [2-^{13}C] acetate and on the patterns of carbon-carbon coupling in the sample derived from [1,2-^{13}C] acetate. One unfortunate feature of these spectra is that one of the signals of most interest, namely C-20, is overlapping with another (C-18). In the biosynthetic sequence leading from $^{13}CH_3{}^{13}CO_2Na$ to rosenonolactone (Scheme 28), the loss of a carbon atom in the transformation of mevalonate into dimethylallyl pyrophosphate will result in an enriched singlet in each of the four terpene units.

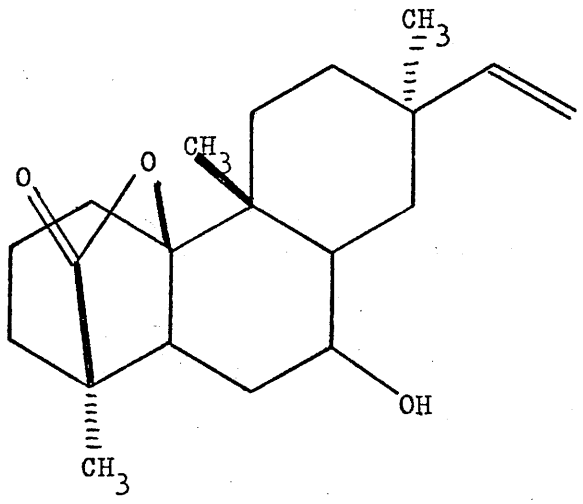
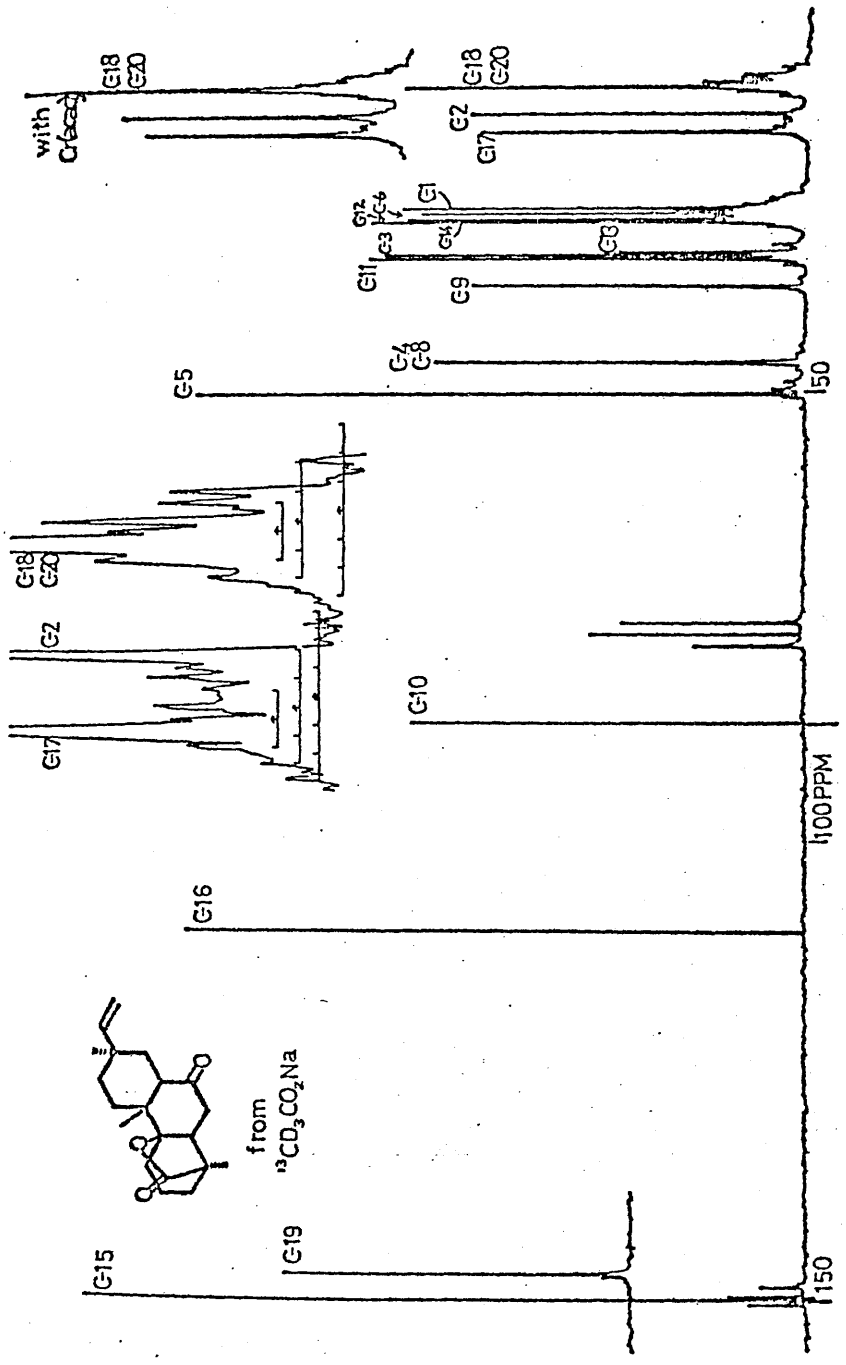

CH3
CH3
O
O
OH
CH3

Figure 16


with Cr-acac
C18
C20
C2
C17
C1
C12
C6
C3
C4
C13
C11
C9
C7
C8
C5
150
C18
C20
C2
C17
C10
100PPM
C16
from
$^{13}CD_3CO_2Na$
C19
C15
150

These appear at C-1, C-7, C-12, and C-18 in rosenonolactone. C-20 and C-10 also appear as singlets reflecting the breaking of the C-10/C-20 bond in the migration of C-20 from C-10 to C-9. It may be noted that the assignment of the signals for C-1 and C-12 could easily be interchanged on all the evidence from these spectra, but assignment is made as indicated by analogy with the values in rosenolactone (90), which were established from lanthanide shift data, as discussed later.

Comparison of the spectra of samples of rosenonolactone (89) derived from $^{13}CH_3CO_2Na$ and $^{13}CD_3CO_2Na$ shows that only three signals in the latter are enhanced, compared to twelve in the former (cf. Fig. 16). This indicates that substitution of one or more deuterium atoms at nine centres in the latter are causing suppression of the carbon-13 enhancements[134], as discussed earlier in the studies on [^{13}C, ^{2}H] methyl oleate. Since C-7, C-9, and C-19 are fully substituted carbons, however, the carbon-13 enrichment is apparent. Lower than average enhancements are observed for C-9 in both deuterated and undeuterated species.

The presence of deuterium is also indicated by various ^{13}C-D multiplets[134], e.g. C-5 shows a triplet (J = 19.5 Hz., Δ = 0.47 ppm) and the olefinic carbon atom C-15 another triplet (J = 23.2 Hz., Δ = 0.36 ppm). C-3 would also be expected to carry only one deuterium atom because of its origin as the olefinic carbon in geranylgeranyl pyrophosphate, whose protonation initiates the concerted cyclisation[130-3](cf. Scheme 28). However, it was not possible to observe a triplet because of the flanking signals for C-11 and C-13.

The signal for C-17 is associated with a triplet, quintet, and septet, corresponding to d_1, d_2, and d_3 species respectively

Table 30 Intensities of C-D multiplets relative to 16.9 ppm peak = 1.00 .

Species	δ ppm	Normal intensity	Intensity with $Cr(acac)_3$
CHD_2	15.61	0.138	0.132
CH_2D	15.90	0.157	0.151
CD_3	16.07	0.110	0.160
CHD_2	16.39	0.248	0.264
CH_3	16.9	1.00	1.00

Table 31 ^{13}C N.M.R. data for rosenololactone (90).

	Multiplicity in O.R.D. spectrum	Carbon atom	J_{C-C} in (90) from $^{13}CH_3{}^{13}CO_2Na$† (Hz.)	Enhancements from [^{13}C] acetates			
				1-^{13}C	2-^{13}C	2-$^{13}CD_3$‡ (A)	2-$^{13}CD_3$‡ (B)
17.2	q	C-18		-0.1	0.8	0.1[a]	-0.1
17.6	q	C-20		-0.1	0.7	0.1[b]	-0.1
19.9	t	C-2	32.3	1.1	-0.1	0	-0.1
22.0	q	C-17	35.9	-0.1	0.1	0[c]	-0.1
29.3	t	C-6	34.8	1.5	0.1	0.2	-0.1
30.0	t	C-1		0.1	1.0	0.2[d]	-0.1
31.9	t	C-11	34.8	1.0	0	-0.1	-0.1
32.0	t	C-12		0	0.8	0.1[e]	-0.1
34.1	t	C-14	35.1	-0.2	0.7	-0.1[f]	-0.1
35.7	t	C-3	32.2	0	0.9	0.2[g]	-0.2
35.9	s	C-13	35.8	0.9	0	0.1	0.3
37.4	d	C-8	35.1	1.1	-0.2	0	-0.2
38.4	s	C-9	34.9	0	0.8	0.4	0.7
47.2	s	C-4	48.7	0.9	-0.1	0	0.1
50.6	d	C-5	33.7	-0.2	0.8	0.2[h]	0.5
69.3	d	C-7		-0.3	0.9	1.7	0.5
87.9	s	C-10		1.0	-0.2	0	0
109.5	t	C-16	69.7	0.9	-0.1	0	-0.3
150.5	d	C-15	69.9	-0.3	0.5	0.4[i]	-0.2
180.3	s	C-19	48.7	-0.3	0.5	2.1	0.9

† Summarised in (92).

‡ Deuterium and carbon-13 labelling pattern summarised in (94).

Notes on Table 31.

a 5^{et}, J = 19.8 Hz., $\Delta(d_2)$ = 0.56 ppm.; 3^{et} obscured by C-20 5^{et}.

b 5^{et}, J = 19.8 Hz., $\Delta(d_2)$ = 0.66 ppm.; 3^{et} obscured by C-18; centre of 7^{et} would lie under 5^{et} of C-18.

c (3^{et}, weak), 5^{et}, J = 20.1 Hz., $\Delta(d_2)$ = 0.6 ppm; 7^{et}, $\Delta(d_3)$ = 0.94 ppm.

d 3^{et}, J = 19.3 Hz., $\Delta(d_1)$ = 0.34 ppm.; 5^{et} obscured.

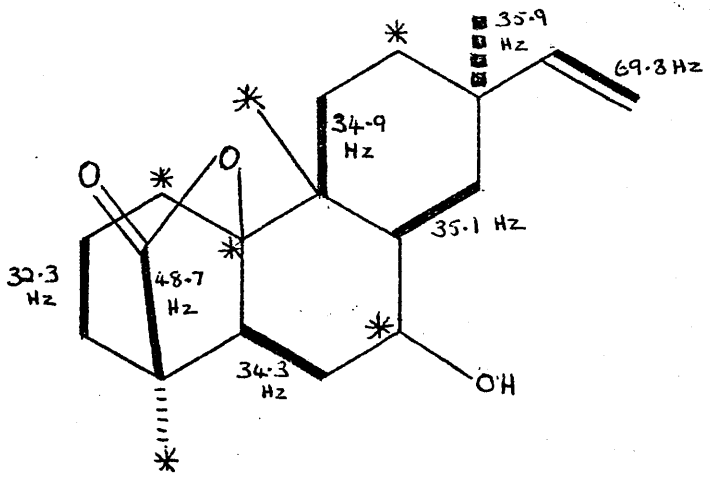
e 3^{et}, J = 19.5 Hz., $\Delta(d_1)$ = 0.36 ppm.; 5^{et}, $\Delta(d_2)$ = 0.79 ppm.

f 3^{et}, J = 19.5 Hz., $\Delta(d_1)$ = 0.39 ppm.; 5^{et}, $\Delta(d_2)$ = 0.83 ppm.

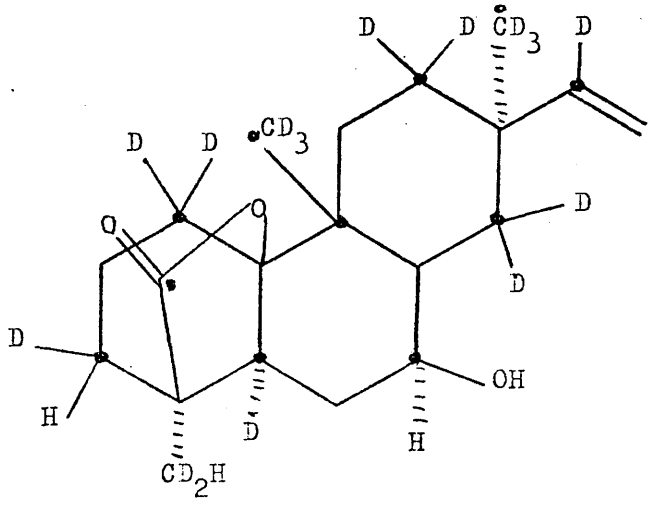
g 3^{et}, J = 20.0 Hz., $\Delta(d_1)$ = 0.40 ppm.

h 3^{et}, J = 19.6 Hz., $\Delta(d_1)$ = 0.49 ppm.

i 3^{et}, J = 23.1 Hz., $\Delta(d_1)$ = 0.36 ppm.



92



94

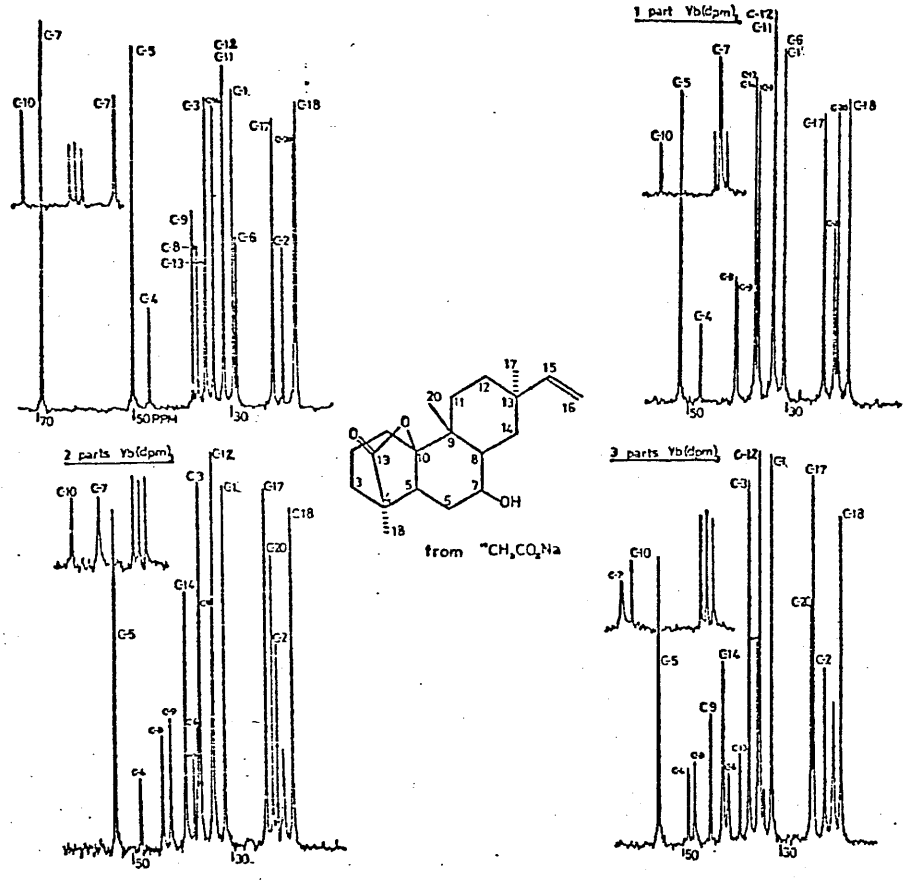
and the single line corresponding to both C-18 and C-20 is also accompanied by a triplet, quintet, and septet. The most significant line of these multiplets is the centre of the d_3 septet and this undergoes apparent enhancement relative to the lines of the other multiplets on addition of relaxation agent, $Cr(acac)_3$, which removes the nuclear Overhauser effect of the protonated species CD_2H and CDH_2 (cf. Table 30). Since C-18 is derived from the 2-position of mevalonate as indicated by the result with [1,2-$^{13}C_2$] sodium acetate and could only be CD_2H, this result indicates that C-20 is present at least partly as the d_3 species (to the same extent as C-17) and that methyl migration involves the intact grouping.

The disadvantage in these spectra was the overlapping of two sets of signals, namely, C-18/C-20 and C-4/C-8, and it was hoped that this could be circumvented by reducing the rosenonolactone samples to the corresponding enriched rosenololactones and applying lanthanide shift reagents [142-5].

Assignment of the chemical shift values to the various carbon atoms in rosenololactone (90) was facilitated again by off-resonance decoupled spectra, by enrichment data, and by the pattern of C-C coupling in the sample derived from $^{13}CH_3{}^{13}CO_2Na$ (Table 31). However, none of the carbon signals are overlapping and those for C-18 and C-20 in particular, differ by 0.4 p.p.m. In the sample derived from [2-^{13}C] acetate, C-9 shows a satisfactory degree of enhancement relative to the other enriched carbons.

In order to clarify the carbon assignments, and, if possible, obtain a spectrum in which the signals for C-18 and C-20 might be observed free from overlap by other signals (a requisite for studying ^{13}C-D multiplets associated with particular carbon atoms),

Figure 17


C-7
C-5
C-12
C-11
C-1
C-10
C-3
C-18
C-17
C-9
C-8
C-13
C-6
C-2
C-4
150PPM
70
30
1 part Yb(dpm)
2 parts Yb(dpm)
3 parts Yb(dpm)
OH
from $^{14}CH_3CO_2Na$

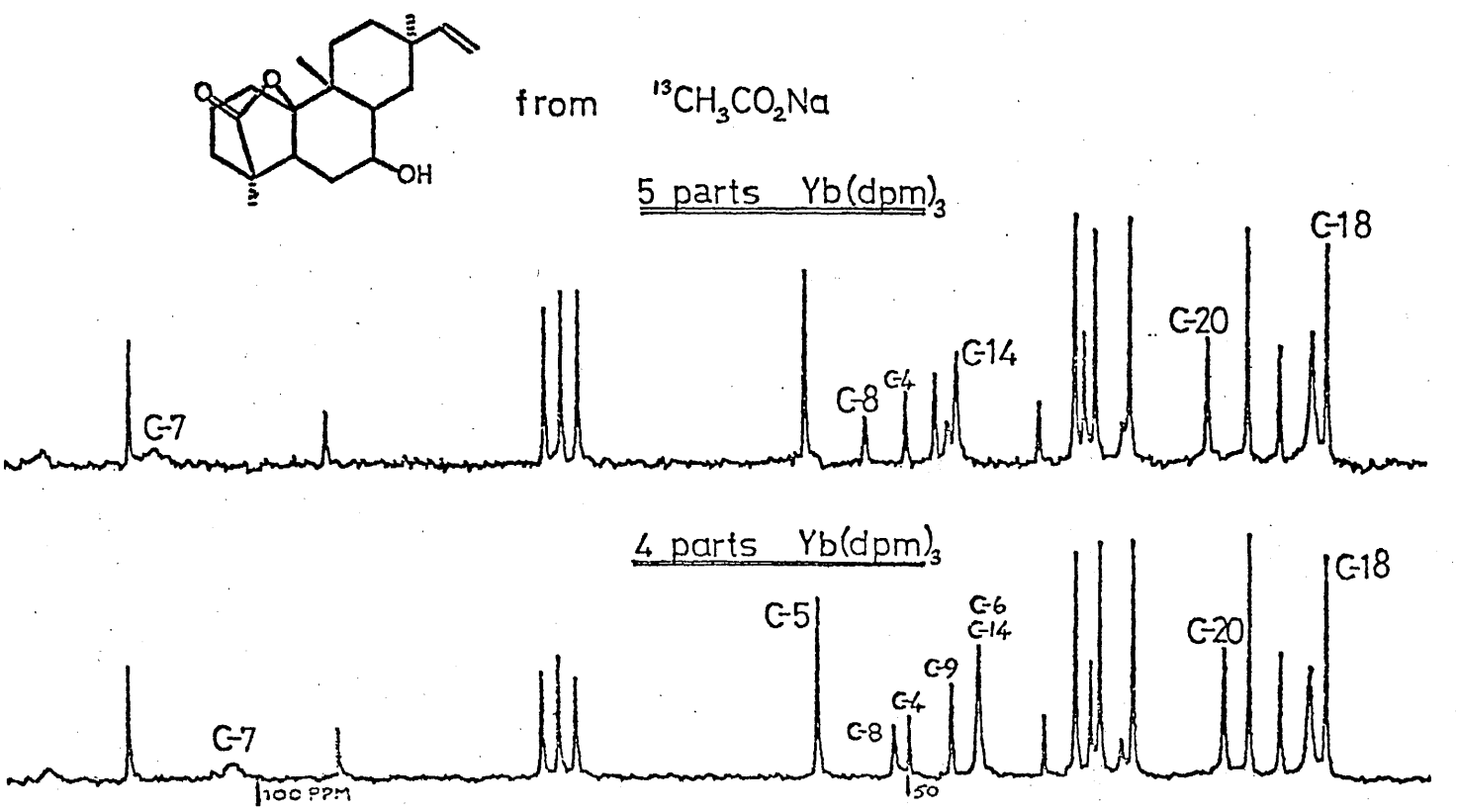

from $^{13}CH_3CO_2Na$
OH
5 parts Yb(dpm)$_3$
C-18
C-20
C-14
C-4
C-8
C-7
4 parts Yb(dpm)$_3$
C-18
C-5
C-6
C-14
C-20
C-9
C-4
C-8
C-7
100 PPM
50

Figure 18

Table 32 Lanthanide-induced shifts for rosenololactone (90).

Carbon atom	δ p.p.m.	r (Å)	θ°	Δ ppm calc.	Δ ppm * $Yb(dpm)_3$	$-\Delta$ ppm $Dy(dpm)_3$
C-1	30.0	7.96	15	3.9	3.9	3.1
C-2	19.9	9.04	15	2.7	2.6	2.3
C-3	35.7	8.56	25	2.5	2.3	2.1
C-4	47.2	7.36	25	4.0	3.7	3.4
C-5	50.6	6.08	20	8.0	7.9	6.8
C-6	29.3	4.84	20	15.8	13.0	15.9
C-7	69.3	3.76	20	33.8	38.5	30.6
C-8	37.4	4.64	25	15.9	16.6	16.1
C-9	38.4	5.76	15	10.3	10.3	8.8
C-10	87.9	6.64	10	7.1	7.1	6.2
C-11	31.9	6.96	20	5.3	5.4	4.9
C-12	32.0	6.68	30	4.6	4.5	4.1
C-13	35.9	5.92	38	4.5	4.8	4.6
C-14	34.1	4.00	35	17.2	18.5	20.5
C-15	150.5	6.24	50	1.1	1.8	2.2
C-16	109.5	7.32	54	0.1	0.4	0.8
C-17	22.0	6.68	47	1.5	3.0	2.8
C-18	17.2	7.44	35	2.6	1.7	1.6
C-19	180.3	7.54	22	4.0	3.7	3.5
C-20	17.6	5.52	15	11.7	10.4	9.0

* Summarised in (96).

Notes on Table 32 :

(1) The lanthanide-oxygen distance of 3Å gave the best fit. Shorter distances gave reasonable results, but not such a good fit. For instance, a distance of 2.75 Å and a C-O-Yb angle of 108° gave calculated for C-6, C-8, C-9, C-14 and C-20 shifts relative to C-7 = 38.5 ppm of 15.1, 16.3, 11.5, 16.7 and 13.0 (Found : 13.0, 16.6, 10.3, 18.5, 10.4).

(2) The C-O-Yb angle of 108° gave the best fit. An angle of 125°, for instance, gave a poor fit. For example (calculated with O-Yb of 3Å) for C-6, C-8, C-9, C-14, and C-20 was, relative to shift of 38.5 for C-7, 16.0, 17.4, 12.7, 14.1, and 15.0 respectively. (Found : 13.0, 16.6, 10.3, 18.5, and 10.4).

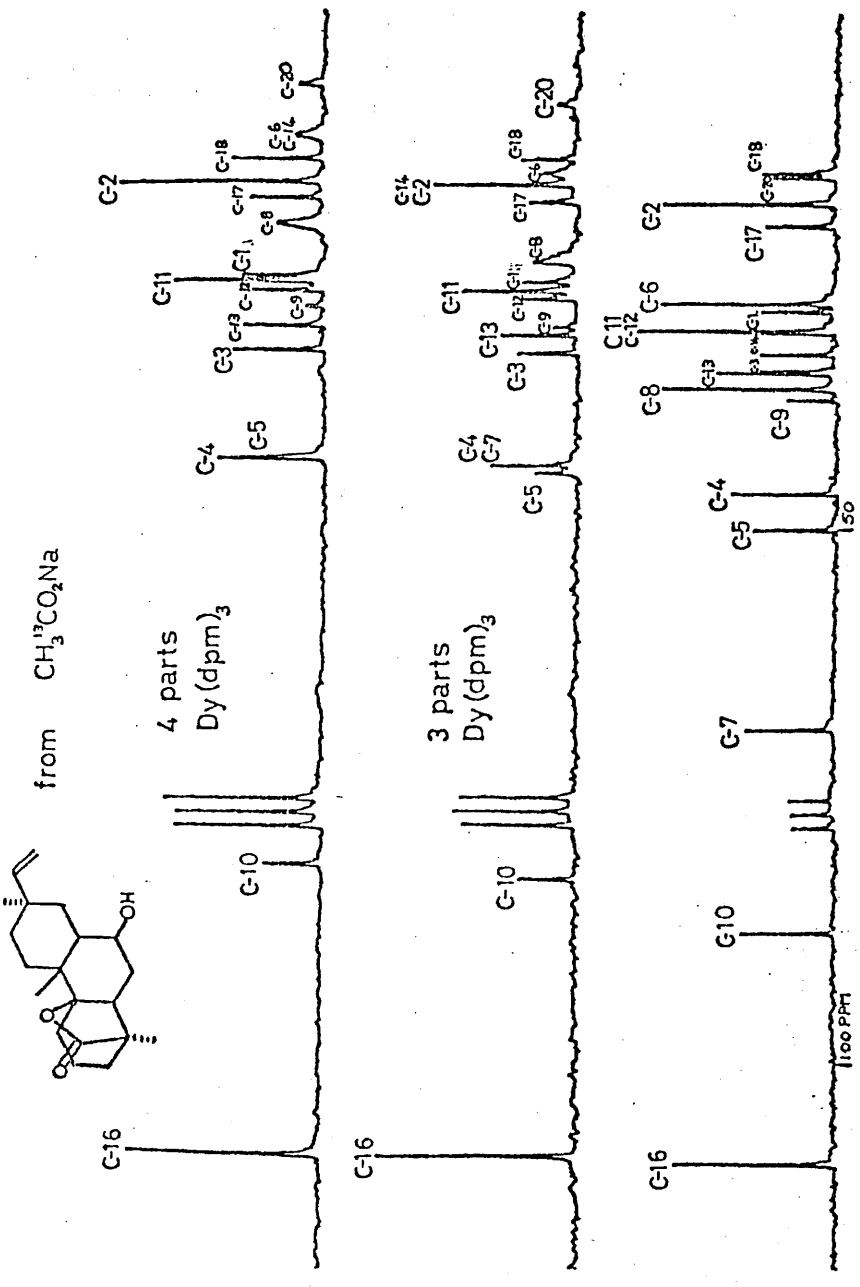
(3) A table of $(3\cos^2\theta - 1)$ is shown :

0°	2.00	30°	1.25
5°	1.9772	35°	1.013
10°	1.9095	40°	0.7604
15°	1.799	45°	0.50
20°	1.649	50°	0.2395
25°	1.464	55°	0

It may be deduced that an error of 1° in measurement of the angle will result in the following approximate error in the final calculated shift :

1° at ca.	45°	7%
	40°	6%
	35°	5%
	30°	4%
	25°	3%
	20°	2%
	15°	2%
	10°	1%
	5°	0.7%

Figure 19

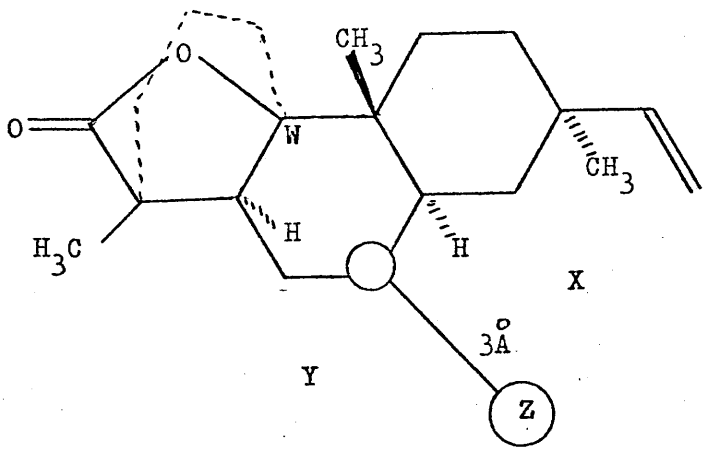

from $CH_3{}^{13}CO_2Na$
OH
4 parts
$Dy(dpm)_3$
3 parts
$Dy(dpm)_3$
C-16
C-10
C-4
C-5
C-3
C-11
C-2
C-18
C-20
C-7
C-13
C-9
C-8
C-6
C-12
C-17
C-14
100 PPM
50

spectra of rosenololactone derived from [2-^{13}C] acetate were obtained in which various amounts of shift reagent, Yb(dpm)$_3$, had been added (Fig. 17,18). The latter reagent is preferred to Eu(dpm)$_3$ in carbon-13 work, since it minimises the contact contribution to the lanthanide-induced shift[146,7]. Interpretation of these spectra was, to some extent, helped by the presence of a number of enhanced signals with this and the linear relationship shown by the observed shift vs. the amount of shift reagent added, the maximum downfield shift shown by each carbon atom was easily derived as shown in Table 32. In a complementary experiment, Dy(dpm)$_3$ was used to produce similar upfield shifts in the sample of rosenololactone derived from [1-^{13}C] acetate (Fig. 19 and Table 32).

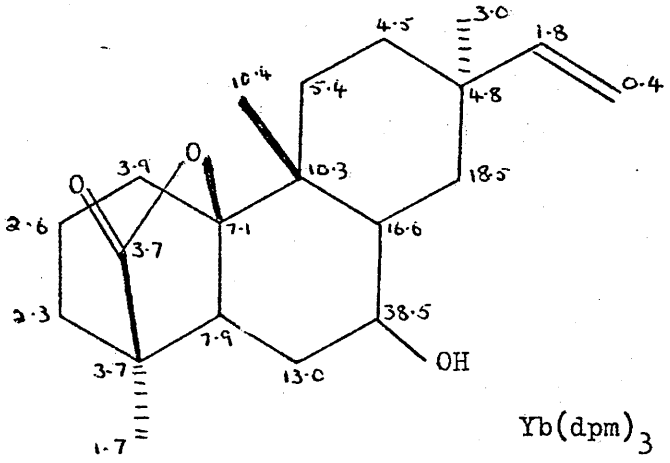
In order to use the magnitude of these shifts to obtain evidence for the carbon assignments, calculations were made of the pseudocontact shifts (Δ) using the McConnell-Robertson equation[148]:

$$\Delta = k\ (3\cos^2\theta - 1) / r^3$$

i.e. taking into consideration the probable location of the lanthanide atom, the length (r) of the vector joining the paramagnetic centre and the observed nucleus and the angle (θ) between that vector and the principal (z) magnetic axis of the paramagnetic atom[146,7]. Of the three possible staggered positions of the lanthanide atom about the C-O bond, W can be excluded on steric grounds and X can be excluded since the angle θ for nine carbon atoms would be in the range $55<\theta<125^\circ$. This would result in the angular term $(3\cos^2\theta - 1)$ being negative and predict upfield shifts, some of which, e.g. for C-13 and C-14, would be large because of proximity to the lanthanide atom (and hence a large $1/r^3$ term). This is clearly ruled out by the fact that all the atoms were observed to


O
CH3
O
W
CH3
H3C
H
H
X
3Å
Y
Z

95


4·5
3·0
1·8
10·4
5·4
4·8
0·4
3·9
O
O
10·3
18·5
2·6
7·1
16·6
3·7
2·3
38·5
7·9
OH
3·7
13·0
1·7
Yb(dpm)3

96

undergo downfield shifts with $Yb(dpm)_3$. Trial calculations of Δ with the lanthanide atom in the remaining staggered position Y gave a very poor fit with the observed lanthanide-induced shifts*. (The calculated shift for C-14, for example, was smaller than those for C-6 and C-8). A good fit with the observed data was, however, obtained assuming little or no contribution from the staggered conformers and the lanthanide atom being in the eclipsed conformation shown, i.e. with the site of coordination being essentially at Z in (95). (This looks very favourable sterically). Table 32 shows the estimated* values of r and θ and the calculated lanthanide-induced shifts, taking k arbitrarily as 2.656×10^5 to provide figures of the same order as the observed lanthanide-induced shifts. The 17.2 and 17.6 ppm signals can be assigned as C-18 and C-20 respectively in view of the very much larger lanthanide-induced shift shown by the 17.6 ppm signal. The signal at 32.0 ppm shows a larger lanthanide-induced shift than at 30.0 ppm and, on this basis, these signals can be assigned as C-12 and C-1 respectively to correspond with the calculated values of the lanthanide-induced shifts. It is reasonable to assume that similar assignments of C-1 and C-12 can be made for rosenonolactone.

In a recent publication[149], carbon-13 data were given for rosenonolactone and rosenololactone and assignments made. Some uncertainties in assignments, substantial discrepancies ($\geqslant$ 0.4 ppm) from the values we observed, and erroneous assignments are noted

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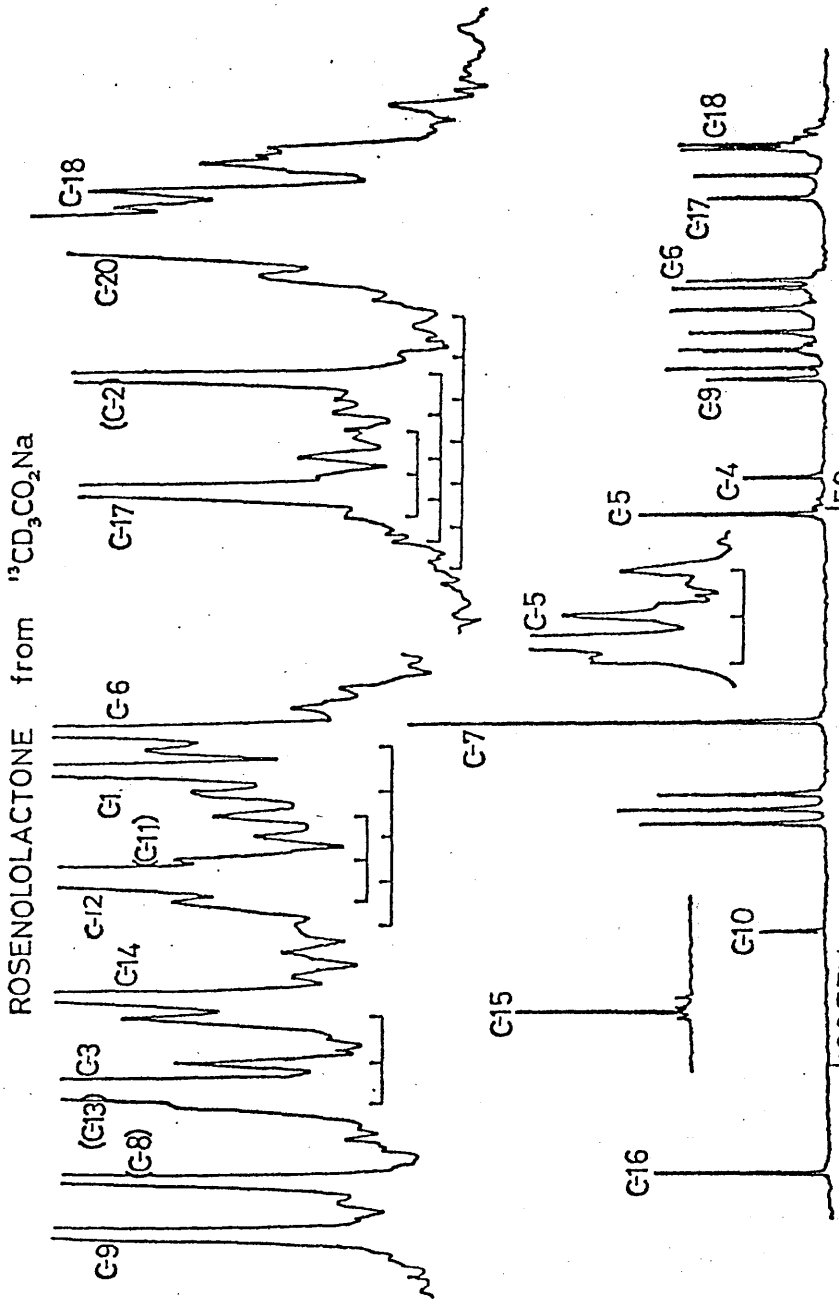
* The lanthanide-oxygen distance was taken as 3Å and r and θ were measured using Dreiding models with a piece of fine bore rigid plastic tubing to represent the oxygen-lanthanide bond.

Figure 20


ROSENOLOLACTONE from $^{13}CD_3CO_2Na$
C-9
(C-13)
(C-8)
C-3
C-14
C-12
(C-11)
C-1
C-6
C-7
C-15
C-16
C-10
C-5
C-17
(C-2)
C-20
C-18
C-5
C-4
C-9
C-6
C-17
C-18
150
100 PPM

as follows :

1) In the rosenololactone spectrum, signals at 31.0 and 31.9 ppm were assigned as C-1 and C-12 or vice-versa. Firm assignments are given in the present work for these carbons to the signals at 30.0 and 32.0 ppm as discussed above.

2) Signals at 34.0 and 31.8 ppm are assigned[149] to C-11 and C-14. This is clearly incorrect from the enrichment data and the correct assignment from our data is 31.9 and 34.1 ppm.

3) C-18 and C-20 are reported to appear as a single signal at 17.2 ppm. In our spectra, lines for these carbons appear at 17.2 and 17.6 ppm respectively as discussed above.

Attention was now turned to the sample of rosenololactone derived via $^{13}CD_3CO_2Na$ (Fig. 20). The spectrum shows the presence deuterium at several centres. The C-15 signal shows the triplet (J = 23.1 Hz., Δ = 0.36 ppm) typical of olefinic -CD- ; C-5 is associated with a triplet (J = 19.6 Hz., Δ = 0.49 ppm) ; C-13, being derived from C-1 of acetate, does not prevent the triplet associated with the -CHD- grouping at C-3 (J = 20.0 Hz., Δ = 0.40 ppm) being clearly seen and the absence of d_2 species to be evident ; similarly, C-11 is derived from C-1 of acetate and the triplet (CHD) and quintet (CD_2) associated with C-12 can be seen ; C-6 is superimposed on the multiplets associated with C-1 in this spectrum. In the methyl region, multiplets for d_1, d_2, and d_3 species associated with C-17 can all be detected. C-20 and C-18 are too close in this spectrum to permit unambiguous interpretation.

The spectrum of this [^{13}C, ^{2}H] sample was also run under special conditions, namely, the decoupling power was reduced to 103 dB so that protonated carbon signals were largely "smeared

Figure 21

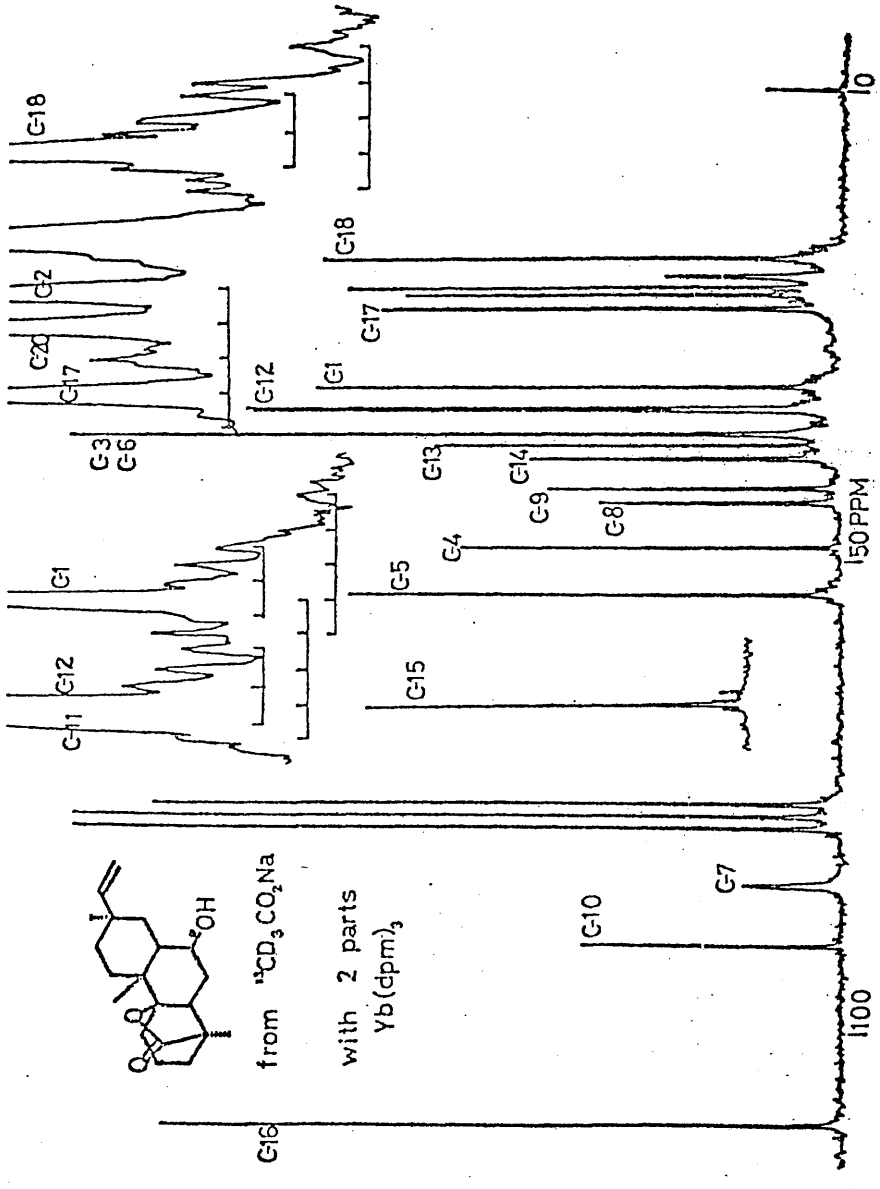
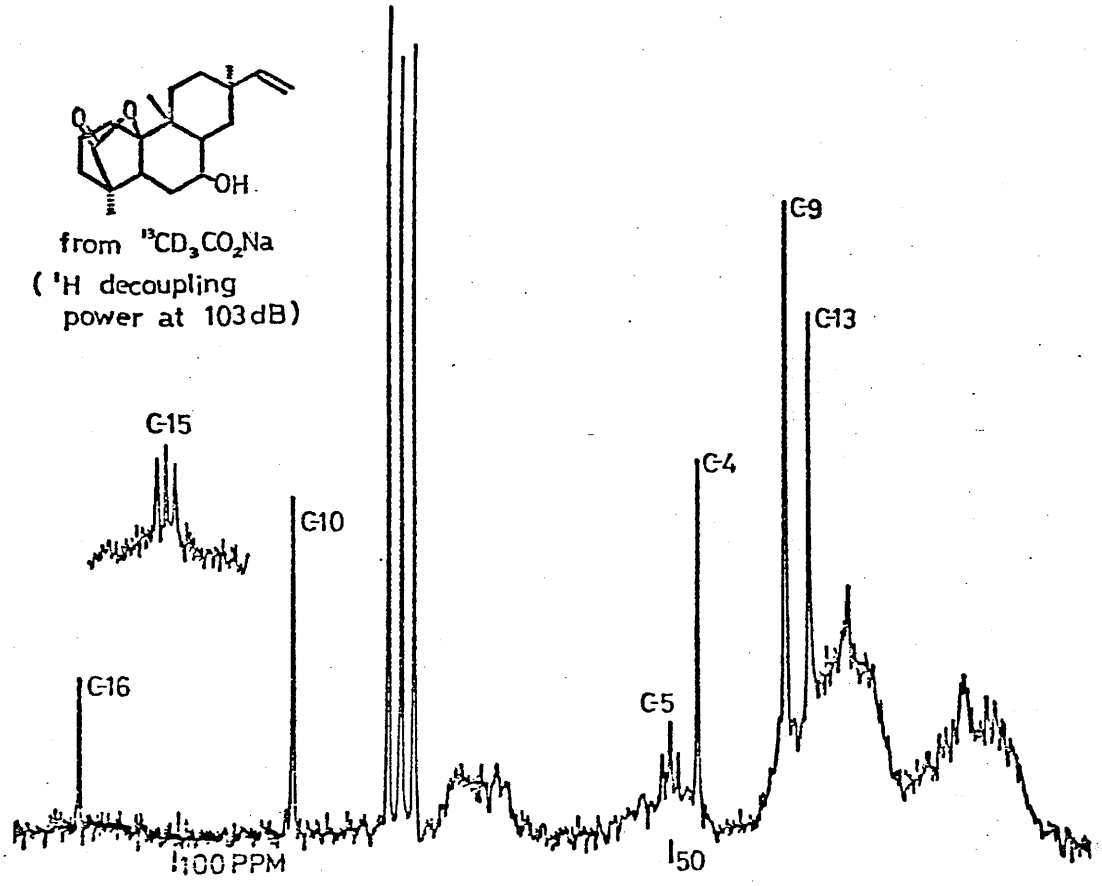

C-18
C-2
C-20
C-17
C-12
C-3
C-6
C-1
C-17
C-18
C-1
C-12
C-11
C-5
C-4
C-15
C-13
C-14
C-9
C-8
150 PPM
C-16
from $^{13}CD_3CO_2Na$
with 2 parts
Yb(dpm)$_3$
C-10
C-7
100
0

Figure 22


OH
from $^{13}CD_3CO_2Na$
(^1H decoupling
power at 103dB)
C-15
C-10
C-16
C-9
C-13
C-4
C-5
100 PPM
50

out"[150] (cf. Fig. 22). Quaternary carbons and carbons bearing only deuteriums now become the dominant peaks. C-9 is seen as the enriched quaternary carbon. The triplets associated with C-15 and C-5 are evident, but the multiplets associated with CD_2 and CD_3 species cannot, unfortunately, be made out.

Finally, spectra were obtained in the presence of shift reagents. It may be noted that $Dy(dpm)_3$ was considered unsuitable, since the shifted peaks suffered line broadening to an extent that satellites would be obscured (cf. C-20 in Fig. 19). When sufficient $Yb(dpm)_3$ was used to shift C-20 clear from the other methyl resonances (cf. Fig. 17, 18 ; spectrum not shown for the ^{13}C, ^{2}H sample), unfortunately, again, line broadening occurred and satellites could not be detected. However, a useful spectrum was obtained using a smaller amount of $Yb(dpm)_3$ (Fig. 21). The CD_2 quintets and CD triplets associated with C-12 and C-1 are now evident, and C-20 having been shifted clear of C-18, it was confirmed that the latter showed associated d_1 and d_2 species, but not d_3, as expected from its derivation from the 2-position of mevalonate. There is no deuterium at C-7 in this rosenololactone since it was obtained by chemical reduction of $[^{13}C, ^{2}H]$ rosenonolactone. It is hoped that deuterium-decoupled spectra will be obtained in the future, but these results provide convincing evidence for the deuterium substitution pattern indicated in (93) reflecting the accepted pathway of incorporation of acetate into the metabolite.

EXPERIMENTAL

EXPERIMENTAL

GENERAL

Instrumentation

Melting points were determined using a Reichert hot stage apparatus, and are uncorrected. Spectra were obtained using the following instruments :

^1H N.M.R. 60 MHz.	: Varian T 60
^1H N.M.R. 90 MHz.	: Perkin-Elmer R 32
^1H N.M.R. 100 MHz.	: Varian HA 100
^1H & ^{13}C N.M.R. (Fourier Transform)	: Varian XL 100
Mass spectrum	: A.E.I. MS 12
Mass measurement (high resolution)	: A.E.I. MS 9
I.R. (4000 - 625 cm^{-1})	: Perkin-Elmer PE 257
I.R. (4000 - 200 cm^{-1})	: Perkin-Elmer PE 225
U.V.	Pye-Unicam SP 800

Circular dichroism measurements were kindly performed by Dr. P.M. Scopes at Westfield College, London. Gas chromatograms were recorded on a Perkin-Elmer F 11 instrument with flame ionisation detector.

Presentation of N.M.R. data

N.M.R. data are presented in units of δ (parts per million), relative to tetramethylsilane as internal standard, using the following abbreviations : s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet ; the notation for coupling is as follows : δ 2.95 (2H, m ; irr. 4.6 $\rightarrow$ s ....) means that the two-proton multiplet at 2.95 p.p.m. collapses to a singlet when an

irradiating field is applied at 4.6 p.p.m.

Miscellaneous

Merck Kieselgel (Type 60) GF_{254}, of thickness 1 mm, was used for preparative thin layer chromatography ; 0.25 mm.-thick Kieselgel G was used for analytical T.L.C. All R_f values measured in 1 % $MeOH/CHCl_3$ eluant are standardised to that of p-aminoazobenzene, R_f 0.55 (candipolin R_f 0.54). The Ce^{4+} chromatographic spray used was a solution of 10 gm. ceric ammonium nitrate and 660 ml. 5N aqueous sulphuric acid in 330 ml. water. After spraying, plates were heated to 140° in an oven.

Unless otherwise stated, light petroleum refers to the fraction boiling between 40° and 60°. In solvent extractions, the volume given in brackets after the solvent name is the total volume accumulated over at least five extractions. All organic extracts were washed to neutrality with saturated aqueous sodium chloride and dried twice over AnalaR anhydrous sodium sulphate before evaporation of the solvent at reduced pressure on a Büchi Rotavapor rotary evaporator. Dilute aqueous HCl means 5N aqueous HCl.

Ethanol-free chloroform was obtained by slow passage of the solvent through a column (2 ft. x 1 in.) of silica gel, then passage through fresh silica gel. Methylene chloride was freed from acidic impurities and ethanol by successive passage through a column of basic alumina, then silica gel.

EXPERIMENTAL

CHAPTER I

Candipolin (N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol).

Candipolin (53) was obtained from the dried mycelium of Penicillium canadense (C.M.I. 95,493) by extraction with chloroform for 24 hours in a Soxhlet apparatus. The solvent was removed, and the brown residue taken up in boiling ethanol: candipolin crystallised out on cooling. A further quantity of this metabolite was obtained by preparative T.L.C., using 1% MeOH/$CHCl_3$ as eluant (R_f 0.54). Crystallisation from ethanol gave candipolin as small, colourless needles, m.p. 210° (yield ca. 150 mg./l.).

$[\alpha]_D$ -98.6° (c. 0.76, C_5H_5N); $[\alpha]_D$ -78.7° (c. 0.14, C_2H_5OH)

R_f (1% MeOH/$CHCl_3$) : 0.54 relative to p-aminoazobenzene (R_f 0.55); Ce^{4+} - beige .

ν_{max} (KBr) : 3300 (NH), 3020, 1750 (ester), 1635 (amide I), 1600, 1580, 1530 (amide II), 1485, 1450, 1390, 1350, 1320, 1305, 1270, 1210, 1185, 1150, 1095, 1070, 1025, 920, 910, 870, 850, 800, 740, 720, 690 cm^{-1}.

ν_{max} ($CHCl_3$) : 3400 (NH), 1740 (ester), 1660 (amide I), 1600, 1580 cm^{-1}.

λ_{max} (MeOH) : 215 (22,700), 227 (20,020) nm.

C.D. : $\Delta\epsilon(\lambda)$: -6.9 (227 nm.).

δ 100 MHz. ($CDCl_3$) : 2.95 (2H, m; irr. 4.6 → s : $-C\underline{H}_2-Ar$), 3.25 (2H, d, J = 6.5 Hz.; irr. 4.95 → s : $-C\underline{H}_2-Ar$), 4.1 (1H, dd, J = 5.0, 12.0 Hz.; irr. 4.6 → s : $-\underset{\underline{H}}{C}H-O-COR$), 4.6 (2H, m; irr. 4.1 → br. s : $-NH-C\underline{H}-\underset{\underline{H}}{C}H-O-COR$), 4.95 (1H, dt, $J_1 = J_2$ = 6.5 Hz.; irr. 6.7 → t (J = 6.5 Hz.); irr. 3.25 → d (J = 6.5 Hz.) : $NH-C\underline{H}-CO-$), 6.8 (2H, m; D_2O/CF_3CO_2H exchangeable ; irr. 4.95 → s :$-CO-N\underline{H}-$), 7.3 (16H, m : Ar-H), 7.8 (4H, m :

o-H of Ar-CO-N).

^{13}C N.M.R. ($CDCl_3$)* : C-3 and C-3' at 37.3 and 37.5, C-2' at 50.3, C-2 at 54.6, C-1 at 65.4, 6 aryl carbons m- or p- to CH_2R at 126.8 (1C), 127.1 (4C), and 127.3 (1C), 12 aryl carbons at 128.4 (2C), 128.6 (4C), 128.8 (2C), 129.2 (2C), and 129.3 (2C), 2 aryl carbons p- to CONR at 131.4 and 132.0, 4 quaternary aryl carbons at 133.4, 134.3, 135.9, and 137.2, CONR at 167.2 and 167.5, CO_2R at 171.9 p.p.m.

m/e (Rel. abundance) : M^+ 506 (0.05), 415 (0.1), 294 (0.2), 269 (1.5), 251 (3.3), 224 (3.3), 223 (2.2), 148 (33), 147 (17), 146 (91), 118 (34), 105 (80), 91 (100), 77 (72), 64 (17), 51 (27).

Analysis - Found : C : 76.11, H : 6.20, N : 5.33 % ; M^+ at m/e 506

$C_{32}H_{30}N_2O_4$ requires : C : 75.86, H : 5.79, N : 5.53 % ; M.Wt. 506.6 .

Base-catalysed hydrolysis of candipolin.

1 M. aqueous NaOH (1.5 ml.) was added to a solution of candipolin (75 mg.) in aqueous ethanol (30 ml. EtOH + 20 ml. H_2O), and the solution refluxed for 24 hours. On cooling, the mixture was neutralised (dil. HCl), and concentrated to low volume. This was basified by addition of saturated aqueous sodium carbonate (20 ml.) and extracted with chloroform (250 ml.), giving N-benzoyl-L-phenylalaninol (26.4 mg.; 70% yield), which crystallised from $CHCl_3$/light petroleum as long colourless needles, m.p. 171-3° (lit. 171-3°).[105]

$[\alpha]_D$ -74.5° (c. 0.87, C_5H_5N)

R_f (1% MeOH/$CHCl_3$) : 0.16 ; Ce^{4+} - beige.

* For ^{13}C N.M.R. (C_5D_5N), see Table 1, page 31.

The aqueous layer was acidified by careful addition of conc. aq. HCl until a white precipitate was formed. This was extracted with chloroform (250 ml.), to give N-benzoyl-L-phenylalanine (58) (19.9 mg.; 50% yield), which crystallises from glacial AcOH/H_2O as needles, m.p. 141-2° (lit. 142-3°).

$[\alpha]_D$ -42.2° (c.0.07, CH_3OH).

R_f (1% MeOH/$CHCl_3$) : 0.21 ; Ce^{4+} - dark beige.

Both acid and alcohol are identical to authentic samples.

For spectroscopic details, see pages

Attempted hydrolysis of N-benzoyl-phenylalanine (58).

When N-benzoyl phenylalanine was refluxed in acetic acid with either 5N. or concentrated aqueous HCl, or with 45% HBr in acetic acid + water, only partial hydrolysis occurred, even after prolonged reflux. At best, only small quantities of benzoic acid were detected by T.L.C. of crude reaction products.

Acid-catalysed hydrolysis of N-benzoyl phenylalanine.

A mixture of dilute aqueous HCl (3 ml.) and N-benzoyl phenylalanine (40 mg.) was sealed in a thick-walled borosilicate glass tube (capacity ca. 1 ml. per inch), and enclosed in a tube of copper gauze. This was heated at 150° (in stirred oil bath) for 24 hours. On cooling, benzoic acid crystallised out in the tube. After addition of chloroform (1 ml.), the solution was filtered. Extraction of the filtrate with chloroform (100 ml.) yielded benzoic acid (12.7 mg.; 70% yield), m.p. 121-2° (lit. 122°).[106]

Phenylalanine was obtained by preparative paper chromatography of the acidic aqueous extract, using Whatman P81 cellulose phosphate paper, with water : n-butanol : acetic acid (50:120:30)

as eluent. R_f 0.55 (purple stain on heating after ninhydrin spray).

Attempted hydrolysis of N-benzoyl-L-phenylalaninol (50).

A solution of N-benzoyl-L-phenylalaninol in glacial acetic acid was refluxed for 24 hours with 45% HBr in glacial acetic acid + water. T.L.C. of the product, after evaporation of solvent, showed that only partial hydrolysis had occurred.

Acid-catalysed hydrolysis of N-benzoyl-L-phenylalaninol.

Conditions used were identical to those given for hydrolysis of N-benzoyl phenylalanine. 35 mg. N-benzoyl-L-phenylalaninol yielded 11.2 mg. benzoic acid (67%), m.p. 121-2° (lit. 122°).

The aqueous layer was basified by addition of solid sodium bicarbonate, then extracted with chloroform (100 ml.) to give L-phenylalaninol, which crystallised from benzene/light petroleum as plates (10.4 mg.; 51%), m.p. 91-3° (lit. 91-2°).[105]
(For spectroscopic data, see page 63.

Preparation of L-phenylalanine methyl ester hydrochloride (54.HCl).

Thionyl chloride (12 ml.) was added dropwise (over a period of 2 hours) to a stirred solution of L-phenylalanine (20.2 gm.) in dry methanol (100 ml.) at 0°C, and stirring continued overnight, with heating to 50°C. After cooling, excess methanol and thionyl chloride were removed (in vacuo.) to give crude L-phenylalanine methyl ester hydrochloride as a white solid. Crystallisation from $CHCl_3$/light petroleum gave colourless needles (26.07 gm.; 99% yield), m.p.157-160° (lit. 158-162°).

R_f (1% MeOH/$CHCl_3$) : 0.22 ; Ce^{4+} - unstained.

ν_{max} (KBr) : 3500-3350 (broad, weak), 3100-2800 (broad, strong), 1745 (ester), 1580, 1495, 1445, 1290, 1240, 1145, 1080, 1060, 990, 935, 870, 810, 745, 705 cm^{-1}.

λ_{max} (MeOH) : 218 (1630), 243 (44.9), 248 (76.4), 252 (117) 258 (162), 264 (126), 267 (67.4) nm.

δ 100 MHz. ($CDCl_3$ + CD_3OD) : 3.35 (2H, d, J = 6.0 Hz.: $-C\underline{H}_2-Ar$), 3.8 (3H, s : $-CO_2-C\underline{H}_3$), 4.3 (1H, t, J = 7.0 Hz.: $-C\underline{H}-NH$), 7.3 (5H, br. s : $Ar-\underline{H}$) .

Preparation of L-phenylalaninol (49).

L-phenylalanine methyl ester hydrochloride (20.0 gm.) in chloroform (2 litres) was extracted with 5N. aqueous sodium hydroxide (total vol. 250 ml.) until the aqueous layer was basic. Evaporation of the chloroform gave L-phenylalanine methyl ester as a colourless oil (15.8 gm.; 95% yield).

A solution of this oil (15.78 gm.) in sodium-dried ether (200 ml.) was added dropwise (required 12 hours) to a suspension of lithium aluminium hydride (22.5 gm.) in sodium-dried ether (1 litre), stirred under nitrogen ; stirring was continued at room temperature for a further 18 hours. Excess L.A.H. was destroyed by very slow[107] dropwise addition of 10% aqueous ammonium chloride (200 ml.) over a period of several hours ; a reflux condenser was fitted to the flask to prevent loss of ether during this operation. The resulting pale grey precipitate was stirred at room temperature until white (36 hours). The ether layer was decanted, and the aqueous suspension basified with 5N. aqueous sodium hydroxide (200 ml.) and extracted with ether (2 litres) in portions. Since drying of the extract caused precipitation of phenylalaninol, the combined ether extracts were evaporated without drying, and the product taken up in benzene.

This solution was dried, and the drying agent extracted by bioling with benzene. Evaporation of the combined benzene solutions gave L-phenylalaninol, which crystallised from benzene as shiny colourless plates, m.p. 91-3° (lit. 91-5°)[105] (11.2 gm.; 80.2% yield w.r.t. phenylalanine methyl ester hydrochloride).

R_f (1% MeOH/$CHCl_3$) : 0.04 ; R_f (10% MeOH/$CHCl_3$) : 0.25 ; Ce^{4+} - unstained ; with ninhydrin, stains purple then red on heating.

ν_{max} (KBr) : 3450 (NH), 3390 (NH), 3100-2800 (strong, broad : OH), 3010, 2930, 2910, 2870, 1570, 1490, 1450, 1335, 1120, 1085, 1060, 990, 970, 960, 905, 860, 830, 755, 700, 695 cm^{-1}.

λ_{max} (MeOH) : 220 (1200), 245 (71.2), 248 (110), 253 (149), 259 (181), 261 (162), 265 (139), 268 (117) nm.

C.D. $\Delta\epsilon(\lambda)$: -1.11 (119 nm.), -1.29 (210), -0.010 (242), -0.016(248), -0.026 (255), -0.039 (261), -0.033 (267) .

δ 100 MHz.($CDCl_3$): ca. 2.7 (2H, ABX m : $-C\underline{H}_2-Ar$; δ_A = 2.82, δ_B = 2.55 J_{AB} = 14.1; J_{AX} = 5.4; J_{BX} = 9.9 Hz.), 3.1 (1H, br.m.: $-C\underline{H}-NH_2$) ca. 3.5 (2H, ABX m : $-C\underline{H}_2-OH$; δ_A = 3.63, δ_B = 3.4 ; J_{AB} = 10.41 J_{AX} = 2.7, J_{BX} = 7.9 Hz.), 7.2 (5H, m : Ar-H) .

^{13}C N.M.R. (C_5D_5N) : C-3 at 41.3, C-2 at 55.4, C-1 at 66.7, aryl carbon p- to CH_2 at 126.3, 4 aryl carbons at 128.7 (2C), and 129.8 (2C), quaternary aryl carbon at 140.4 p.p.m.

Attempted preparation of L-phenylalaninol using S.D.A.

L-phenylalanine methyl ester hydrochloride (6 gm.) in chloroform (500 ml.) was extracted with 5N. aqueous sodium hydroxide until the aqueous layer was basic. The chloroform extract yielded a colourless oil (phenylalanine methyl ester), which was taken up in sodium-dried

benzene (10 ml.). This solution was added dropwise (over 2½ hours) to a stirred solution of sodium bis-(2-methoxyethoxy)-dihydro aluminate (S.D.A.) (4.67 ml. of 70% solution in benzene) in sodium-dried benzene (20 ml.) at 0°. The reaction mixture was stirred at room temperature for 24 hours, after which excess S.D.A. was destroyed by slow dropwise addition of 5% aqueous ammonium chloride (10 ml.). After stirring for 3 hours and filtering, the benzene layer of the filtrate gave a mixture in which phenylalaninol was shown (T.L.C.) to be only a minor constituent.

Benzoylation of L-phenylalaninol.

A solution of benzoyl chloride (4.5 gm.) in dry pyridine (30 ml.) was added dropwise to a stirred solution of L-phenylalaninol (4.76 gm.) in dry pyridine (20 ml.) at 0°. The reaction mixture was stirred for 24 hours at room temperature. After dilution with chloroform (500 ml.) the solution was washed with dil. HCl, saturated aqueous Na_2CO_3, brine, then dried and evaporated to give crude product, which was a mixture of N-benzoyl-L-phenylalaninol with ca. 40% of the N,O-dibenzoyl derivative. Crystallisation from $CHCl_3$/light petroleum gave N-benzoyl-L-phenylalaninol (1.28 gm.; 15.8% yield) as long silky colourless needles, m.p. 171-3° (lit. 171-2°).[105]

Selective hydrolysis of the mother liquors by refluxing with aqueous base yielded a further 2.74 gm. of N-benzoyl-L-phenylalaninol, m.p. 171-3°, making the total yield up to 50.4% .

$[\alpha]_D$ -46.4° (c.0.49, $CHCl_3$) ; $[\alpha]_D$ -78.5° (c.1.11, C_5H_5N).

R_f (1% MeOH/$CHCl_3$) : 0.16 ; R_f (5% MeOH/$CHCl_3$) : 0.48 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3400-3200 (broad : OH), 3310 (NH), 1635 (amide I), 1600, 1580, 1540 (amide II), 1490, 1450, 1330, 1280, 1220,

1080, 1050, 1030, 1000, 915, 855, 800, 750, 700, 685, 675 cm^{-1}.

λ_{max} (MeOH) : 211 (13,000), 217 (12,000), 225 (11,000) nm.

C.D. $\Delta\epsilon(\lambda)$: -5.85 (226 nm.) .

δ 100 MHz. (d_5 pyridine) : 3.3 (2H, d, J = 7.0 Hz.: $-C\underline{H}_2-Ph$), 4.05 (2H, d, J = 6.0 Hz.: $-C\underline{H}_2-OH$), 4.9 (1H, m : $-C\underline{H}-NH$), 6.1 (1H, br. s ; exchangeable with D_2O : $-N\underline{H}-$) .

δ 100 MHz. ($CDCl_3$) : 7.3 (5H : Ar-H of benzyl group), 7.4, 7.7 (5H, m : Ar-H of benzoyl group) .

^{13}C N.M.R. ($CDCl_3$)* : C-3 at 37.1, C-2 at 53.4, C-1 at 64.5, 3 aryl carbons m- or p- to CH_2R at 126.9, 6 aryl carbons 128.6 (2C), 128.8 (2C), and 129.3 (2C), 1 aryl carbon p- to CONR at 131.7 p.p.m.

Selective hydrolysis of N,O-dibenzoyl-L-phenylalaninol.

A mixture of N-benzoyl and N,O-dibenzoyl-L-phenylalaninol (64) (3.39 gm.; estimated by T.L.C. to contain ca. 70% dibenzoyl derivative) in aqueous ethanol (20 ml. H_2O + 30 ml. EtOH) containing 3.3 M. aqueous sodium hydroxide (1.4 ml.) was refluxed for 1 hour. On cooling, needles of N-benzoyl-L-phenylalaninol crystallised out and were removed by filtration (m.p. 171-3° after washing and drying). Further material was obtained from the filtrate (pH 8) by concentration to low volume, dilution with water, and extraction with chloroform (500 ml.). Crystallisation from $CHCl_3$/light petroleum gave N-benzoyl-L-phenylalaninol as needles (2.41 gm.; 98%), m.p. 171-2° (lit. 171-3°).[105]

R_f (1% MeOH/$CHCl_3$) : 0.16 ; Ce^{4+} - pale beige.

For spectroscopic data, see page 63.

*For ^{13}C N.M.R. (C_5D_5N), see Table 1 (page 31).

Preparation of N,O-dibenzoyl-L-phenylalaninol. (64)

A solution of benzoyl chloride (0.511 gm.) in dry pyridine (30 ml.) was added to L-phenylalaninol (78 mg.) in dry pyridine (20 ml.) at 0°. After stirring at room temperature for 7 days, the reaction mixture was diluted with chloroform (100 ml.) and extracted with dil. HCl (until aqueous layer was acidic), then saturated aqueous Na_2CO_3. Evaporation of the chloroform gave crude N,O-dibenzoyl-L-phenylalaninol containing a non-polar impurity which was removed by trituration with light petroleum. Crystallisation from ethanol gave colourless needles of N,O-dibenzoyl-L-phenylalaninol (0.135 gm.; 73%), m.p. 173-4° (lit. 169°).[105]

R_f (1% MeOH/$CHCl_3$) : 0.62 ; Ce^{4+} - unstained.

ν_{max} (KBr) : 3300 (NH), 3060, 3020, 1720 & 1705 (ester), 1635 (amide I), 1600, 1575, 1535 (amide II), 1485, 1450, 1310, 1280, 1130, 1020, 800, 755, 710, 700 cm^{-1}.

λ_{max} (MeOH) : 221 (16,500), 228 (21,300) nm.

C.D. $\Delta\epsilon(\lambda)$: -6.12 (222 nm.), +1.50 (239), +0.72 (247).

δ 100 MHz. ($CDCl_3$) : 3.1 (2H, ABX multiplet, J_{AB} = 19 Hz, J_{AX} = 7 Hz. -C<u>H</u>$_2$-Ar), 4.4 (1H, m : -C<u>H</u>-), 4.75 (2H, m : -C<u>H</u>$_2$-O-COAr), 6.55 (1H, br. d, J = 8 Hz.: -N<u>H</u>-), 7.3 (5H, s : -CH_2-Ar-<u>H</u>), 7.4 (6H, m : <u>m</u>- & <u>p</u>- H of Ar-CO-NH and Ar-CO-O), 7.7 (2H, m : <u>o</u>- H of Ar-CO-NH), 8.05 (2H, m : <u>o</u>- H of Ar-CO-O).

Analysis - Found : C: 77.10, H: 6.13, N: 3.79 %.

$C_{23}H_{21}NO_3$ requires C: 76.86, H: 5.89, N: 3.90 %.

Preparation of N-benzoyl-L-phenylalanine methyl ester.

A solution of benzoyl chloride (1.555 gm.) in dry pyridine (20 ml.) was added to L-phenylalanine methyl ester hydrochloride

(2.294 gm.) in dry pyridine (50 ml.), and stirred for 3 hours at 0°. The reaction mixture was diluted with chloroform (30 ml.), and washed successively with dil. aqueous HCl, and saturated aqueous Na_2CO_3. Evaporation of the chloroform and crystallisation from ether/light petroleum gave needles of N-benzoyl-L-phenylalanine methyl ester (1.554 gm.; 60%), m.p. 79-81° (lit. 81°).[108]

R_f (1% MeOH/$CHCl_3$) : 0.61 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3300 (NH), 3010, 1750 & 1740 (ester), 1640 (amide I), 1600, 1580, 1525 (amide II), 1490, 1430, 1360, 1320, 1290, 1210, 1190, 1170, 1090, 1040, 980, 910, 800, 755, 700 cm^{-1}.

λ_{max} (MeOH) : 220 (9,930), 227 (10,300) nm.

C.D. $\Delta\epsilon(\lambda)$: +2.03 (211 nm.), -4.40 (225), -4.18 (230) .

δ 100 MHz. ($CDCl_3$) : 3.3 (2H, d, J = 7 Hz. : $-C\underline{H}_2-Ar$), 3.8 (3H, s : $-OC\underline{H}_3$), 5.1 (1H, m : $-C\underline{H}-$), 6.65 (1H, br. d, J = 7 Hz. : $-N\underline{H}$), 7.25 (5H, m : $-CH_2-Ar-\underline{H}$), 7.45 (3H, s : m- & p- H of Ar-CONH), 7.7 (2H, m : o- H of Ar-CONH) .

In another approach, L-phenylalanine methyl ester was treated in dioxan for 36 hours at room temperature with benzoylimidazole prepared from benzoic acid (25.3 mg.) and N,N'-carbonyldiimidazole (38 mg.) in dry dioxan. However, much of the benzoic acid was unreacted, and only a small amount of the benzoyl derivative was obtained.

With a view to possible small scale preparation of (^{14}C) labelled N-benzoyl-L-phenylalanine, this reaction was also carried out using commercial benzoyl chloride (23 mg.), giving yields of ca. 50%. When the benzoyl chloride was prepared from benzoic acid (23 mg.), essentially according to the method of Adams & Ulich,[109]

yields were unreliable. Preparation of (^{14}C) labelled N-benzoyl-L-phenylalanine by hydrolysis of (^{14}C) candipolin (see page 58) was preferred.

Preparation of N-benzoyl-L-phenylalanine (58).

N-benzoyl-L-phenylalanine methyl ester (0.999 gm.) in aqueous dioxan (47 ml. H_2O + 34 ml. 1,4-dioxan) containing 1M aqueous sodium hydroxide (3.7 ml.) was refluxed for 2 hours. After cooling, the reaction mixture was neutralised with dil. aq. HCl, and the solvent evaporated to give a white solid ; a suspension of this in dil.aq. HCl (20 ml.) was extracted with chloroform (700 ml.). Evaporation of the chloroform gave N-benzoyl-L-phenylalanine, which crystallised from glacial acetic acid/water as needles, (0.563 gm., 59%), m.p. 141-2° (lit. 142-3°).[106]

$[\alpha]_D$ -42.1° (c. 0.35, CH_3OH).

R_f (1% MeOH/$CHCl_3$) : 0.21 ; Ce^{4+} - dark beige.

ν_{max} (Nujol) : 3300 (NH), ~2800 (broad : OH), 1710 (acid C=O), 1630 (amide I), 1600, 1575, 1520 (amide II), 1485, 1290, 1260, 1230, 1180, 1090, 1020, 930, 795, 750, 695 cm^{-1}.

λ_{max} (MeOH) : 210 (13,200), 217 (12,000), 226 (10,500) nm.

δ 60 MHz. ($CDCl_3$) : 3.3 (2H, d, J = 5 Hz.:-C<u>H</u>$_2$-Ar), 5.1 (1H, m : -C<u>H</u>-NH), 6.8 (1H, d, J = 7 Hz.: -CO-N<u>H</u>), 7.3 (5H, s : -CH_2-Ar-<u>H</u>), 7.4 (3H, m : <u>m</u>- & <u>p</u>- H of Ar-CONH), 7.7 (2H, m : <u>o</u>- H of Ar-CONH), 8.5 (1H, s, D_2O-exchangeable : O<u>H</u>) .

Formation of candipolin and epicandipolin by esterification of N-benzoyl-L-phenylalaninol with :

(a) N-benzoyl-DL-phenylalanine

N-benzoyl-DL-phenylalanine (0.968 gm.) and N,N'-carbonyl-diimidazole (1.066 gm.) were stirred in ethanol-free chloroform (250 ml.) for 1½ hours. Solid N-benzoyl-L-phenylalaninol (0.591 gm.) was added in 5 portions over a period of 2 hours, and the reaction mixture stirred overnight at room temperature. After extraction with dil. aq. HCl, and saturated aq. Na_2CO_3, evaporation of the chloroform gave a white solid. T.L.C. showed that this contained only small quantities of unreacted starting material ; a non-polar impurity was present which could not be separated from the ester by trituration with light petroleum (b.p. 60-80°), but which was separated by preparative T.L.C., using $CHCl_3$ as eluant. Crystallisation from ethanol gave the mixture of candipolin + epicandipolin as needles (0.470 gm.; 40%), m.p. 184-190°. (See separation procedure below).

R_f (1% MeOH/$CHCl_3$) : 0.51 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3300 (NH), 3020, 1745 (candipolin ester), 1730 (epi-candipolin ester), 1635 (amide I), 1600, 1580, 1530 (amide II), 1490, 1450, 1390, 1305, 1290, 1275, 1180, 1160, 1095, 1075, 1025, 920, 870, 800, 750, 695 cm^{-1}.

λ_{max} (MeOH) : 215 (18,500), 227 (11,100) nm.

δ 100 MHz. ($CDCl_3$) : 2.9 (2H, m : $-C\underline{H}_2-Ar$), 3.25 (2H, d, J = 6 Hz. : $-C\underline{H}_2-Ar$), 4.1 (1H, m : $-C\underline{H}-$), 4.5 (2H, m : $-C\underline{H}_2-O-CO$), 4.9 (1H, t, J = 7 Hz.: $-C\underline{H}-CO-O$), 6.6 (2H, br. d, J = 6 Hz.: $-N\underline{H}-$), 7.2 (10H, s : $-CH_2-Ar-\underline{H}$), 7.4 (6H, m : m- & p- H of Ar-CO-NH), 7.7 (4H, m : o- H of Ar-CO-NH) .

m/e (%) : M^+ 506 (0.6), 415 (5), 293 (2), 252 (6), 224 (7), 148 (6),

147 (2), 146 (36), 118 (6), 106 (6), 105 (100), 104 (2), 91 (26), 77 (39).

Analysis - Found : C : 75.5, H : 6.13, N : 5.66 % .

$C_{32}H_{30}N_2O_4$ requires : C : 75.87, H : 5.79, N : 5.53 % .

(b) N-benzoyl-L-phenylalanine.

N-benzoyl-L-phenylalanine (0.140 gm.) and N,N'-carbonyl-diimidazole (0.154 gm.) in ethanol-free chloroform (250 ml.) were stirred at room temperature for 2 hours. Solid N-benzoyl-L-phenylalaninol (0.243 gm.) was added in 6 portions at ½-hour intervals, and a further 100 ml. ethanol-free chloroform was added to ensure dissolution of the material. After stirring overnight at room temperature, the reaction mixture was worked up as in the previous reaction (a). A white solid was obtained, which was found to contain three components which were separated by preparative T.L.C., using 1% MeOH/$CHCl_3$ as eluant :

(i) N-benzoyl-L-phenylalaninol (R_f 0.16), which crystallised as colourless needles (0.121 gm.) from $CHCl_3$/light petroleum , m.p. 171-3° : found to be identical (I.R., T.L.C., mixed m.p.) to an authentic sample.

(ii) A mixture of candipolin and epicandipolin (R_f 0.54), which crystallised as needles from ethanol (0.154 gm.; 63% yield w.r.t. unrecovered N-benzoyl-L-phenylalaninol), m.p. 192-7°. The I.R. spectrum was identical to that of the ester mixture obtained in the previous experiment (a), showing C=O bands at 1733 and 1745 cm^{-1}. Although apparently homogeneous by T.L.C., repeated crystallisation from ethanol raised the m.p. ultimately to 217-8°, and effected the

gradual removal of the component having $\nu_{C=O}$ at 1745 cm^{-1}. The m.p. (217-8°) of pure epicandipolin (N-benzoyl-O-(N-benzoyl-D-phenylalanyl)-L-phenylalaninol) was depressed by admixture with natural candipolin.

$[\alpha]_D$ -46.9 ± 20° (c. 0.006, C_5H_5N).

R_f (1% MeOH/$CHCl_3$) : 0.54 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3300 (NH), 3040, 3030, 1733 (ester), 1640 (amide I), 1600, 1580, 1535 (amide II), 1490, 1455, 1305, 1280, 1230, 1185, 1095, 1075, 1030, 1000, 870, 800, 750, 690 cm^{-1}.

ν_{max} ($CHCl_3$) : 3430 (NH), 1735 (ester), 1660 (amide I), 1600, 1580 cm^{-1}.

C.D. $\Delta\epsilon(\lambda)$: +0.32 (205 nm.), -1.13 (217), -0.97 (224), -0.97 (235), -0.65 (245) .

δ 100 MHz. ($CDCl_3$) : 2.9 (2H, m : $-C\underline{H}_2-Ar$), 3.25 (2H, d : $-C\underline{H}_2-Ar$), 4.15 (1H, m : $-C\underline{H}-$), 4.5 (2H, m :$-O-C\underline{H}_2-$), 5.0 (1H, m : $-C\underline{H}-CO$), 6.6 (2H, m : $N\underline{H}$), 7.3 (10H, m : $-CH_2-Ar-\underline{H}$), 7.5 (3H, m : m- & p- H of Ar-CONH), 7.8 (2H, m : o- H of Ar-CONH) .

m/e (%) : M^+ 506 (0.2), 415 (6), 385 (1), 294 (5), 270 (2), 268 (3), 264 (3), 252 (12), 251 (3), 238 (3), 226 (1), 225 (3), 224 (13), 148 (13), 147 (8), 146 (47), 131 (5), 122 (3), 118 (13), 117 (5), 106 (9), 105 (100), 104 (6), 103 (3), 91 (40), 77 (27) .

Analysis - Found : C : 75.60, H : 5.91, N : 5.36 % .

M^+ at m/e 506.2202 ± 0.001 .

$C_{32}H_{30}N_2O_4$ requires : C : 57.87, H : 5.97, N : 5.53 % .

M.Wt. : 506.2205 .

After exhaustive crystallisation of epicandipolin, the mother

liqours yielded a white solid (10 mg.), m.p. 204-7°, whose I.R. spectrum was identical to that of natural candipolin ; mixed m.p. with natural candipolin was not depressed.

R_f (1% MeOH/$CHCl_3$) : 0.54 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3300 (NH), 1745 (ester), 1630 (amide I), 1525 (amide II), 750, 690 cm^{-1}.

(iii) 2-phenyl-4-benzyl-5-oxazolone (R_f 0.74), which crystallised from light petroleum (b.p. 100-120°) as needles, m.p. 70-2°(lit. 69-71°);[110] its I.R. spectrum was identical to that of an authentic sample, prepared as described below.

A mixture of candipolin/epicandipolin (79.4 mg.) dissolved in a solution of 45% w/v HBr in glacial acetic acid (10 ml.); after standing for 1 hour at room temperature, excess HBr/AcOH was removed in vacuo, leaving a transparent solid, which crystallised from ethanol as needles. This was found (T.L.C.; I.R.) to be unchanged starting material.

The mixture (69 mg.) was also recovered unchanged after treatment with benzoyl chloride (38 mg.) and pyridine (20 ml.) at 0°.

Preparation of 2-phenyl-4-benzyl-5-oxazolone (67).

N-benzoyl-DL-phenylalanine (1.01 gm.) in acetic anhydride (10 ml.) was stirred at 90° for 40 min. Evaporation of excess acetic anhydride gave a yellow oil, which was taken up in boiling light petroleum (b.p. 100-120°) and filtered. On cooling, an oil separated out, which, on scratching, gave crystals of 2-phenyl-4-benzyl-5-oxazolone. Crystallisation from light petroleum (b.p.

100-120°) and washing with light petroleum (b.p. 40-60°) gave colourless needles (0.668 gm.; 71%), m.p. 70-2° (lit. 69-71°).[110]

R_f (1% MeOH/$CHCl_3$) : 0.74 ; Ce^{4+} - beige.

ν_{max} (KBr) : 1825 & 1810 (doublet : C=O), 1720, 1640, 1610, 1600, 1580, 1535, 1490, 1445, 1320, 1295, 1270, 1225, 1080, 1045, 1020, 990, 920, 905, 880, 780, 695 cm^{-1}.

λ_{max} (MeOH) : 211 (11,200), 242 (11,300) nm.

δ 100 MHz. ($CDCl_3$) : 3.25 (2H, m : $-C\underline{H}_2-Ar$), 4.65 (1H, m : $-C\underline{H}-$), 7.2 (5H, s : $-CH_2-Ar-\underline{H}$), 7.4 (3H, m : m- & p- H of Ar-C=N), 7.85 (2H, m : o- H of Ar-C=N) .

Attempted acylation of N-benzoyl-L-phenylalaninol with 2-phenyl-4-benzyl-5-oxazolone.

A solution of N-benzoyl-L-phenylalaninol (12.6 mg.), 2-phenyl-4-benzyl-5-oxazolone (20.4 mg.), and N,N'-carbonyldiimidazole (4.5 mg.) in ethanol-free chloroform (100 ml.) was stirred at room temperature for 7 days. After extraction with dil. aq. HCl, and saturated aqueous Na_2CO_3, evaporation of the chloroform gave a crude product which was shown (T.L.C.) to contain ca. 40% ester, the remainder being unreacted starting materials.

Preparation of N-benzoyl-O-(N-carbobenzyloxy-L-phenylalanyl)-L-phenylalaninol (69) by condensation of N-benzoyl-L-phenylalaninol with N-CBZ-L-phenylalanine.

A solution of N-CBZ-L-phenylalanine (0.318 gm.) and N,N'-carbonyldiimidazole (0.220 gm.) in ethanol-free chloroform (50 ml.) was stirred for 1 hour at room temperature. N-benzoyl-L-phenylalaninol (0.348 gm.) in ethanol-free chloroform (100 ml.) was then

added in 6 portions at half-hour intervals, and stirring continued for 4 days. After washing successively with dil. aq. HCl, and saturated aq. Na_2CO_3, evaporation of the chloroform gave a mixture. Preparative T.L.C., using 1% $MeOH/CHCl_3$ as eluant gave three compounds :

(i) The major component, R_f 0.54 (Ce^{4+} - beige), consisted of N-benzoyl-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol, (0.186 gm.), which crystallised from ethanol as needles, m.p. 185-7°.
Yield = 25% absolute, = 65% w.r.t. unrecovered N-benzoyl-L-phenylalaninol.

$[\alpha]_D$ -15.6° (c. 0.76, $CHCl_3$).

R_f (1%MeOH/$CHCl_3$) 0.54

ν_{max} (KBr) : 3300 (NH), 3020, 2920, 1740 (ester), 1690 (urethane C=O), 1630 (amide I), 1600, 1575, 1530 (amide II), 1490, 1450, 1380, 1340, 1310, 1295, 1260, 1210, 1190, 1045, 865, 795, 770, 745, 695 cm^{-1}.

λ_{max} (MeOH) : 215 (15,100), 227 (10,700) nm.

C.D. $\Delta\epsilon(\lambda)$: -3.90 (230 nm.) .

δ 100 MHz. ($CDCl_3$) : 2.9 (2H, m : $-C\underline{H}_2-Ar$), 3.1 (2H, d, J = 7 Hz. : $-C\underline{H}_2-Ar$), 4.05 (1H, m : $-C\underline{H}-NH$), 4.3 - 4.7 (complex m : $-O-C\underline{H}_2-CH$ & $-C\underline{H}-CO_2$), 5.0 (2H, s : $-NHCO_2-C\underline{H}_2-Ar$), 5.2 (1H, m ; D_2O/CF_3CO_2D - exchangeable : $-N\underline{H}-CO_2-CH_2-Ar$), 6.5 (1H, br. m : $-N\underline{H}-$), 7.2 (15H, m : $-CH_2-Ar-\underline{H}$), 7.4 (3H, m : m- & p- H of Ar-CONH), 7.7 (2H, m : o- H of Ar-CONH) .

m/e (%) : M^+ 536 (0.23), 447 (4), 445 (4), 428 (0.2), 415 (0.5), 401 (0.1), 385 (0.3), 337 (1), 334 (1), 323 (3), 294 (3), 239 (6), 238 (5), 148 (6), 147 (6), 146 (59), 118 (10),

105 (45), 91 (100), 77 (18) .

Analysis - Found : C : 74.00, H : 6.13, N : 5.20 % ;

M^+ at m/e 536.

$C_{33}H_{32}N_2O_5$ requires : C : 73.86, H : 6.01, N : 5.22 % ;

M.Wt. 536.2 .

Two minor components, R_f 0.16, and R_f 0.67, were obtained from the above separation. The first of these consisted of unreacted N-benzoyl-L-phenylalaninol (0.213 gm.), m.p. and mixed m.p. 171-2°. The second component was a colourless oil (32.7 mg.), found to be identical (I.R., N.M.R., T.L.C.) to an authentic sample of N-CBZ-L-phenylalanine methyl ester (see page 75).

This reaction was also carried out with a number of procedural variations : adding the alcohol in one portion ; varying stirring time from 12 hours to 7 days ; using different solvents - CH_2Cl_2, dioxan, CH_3CN ; adding a little NaH along with the alcohol. None of these had any significant effect on the yield of N-benzoyl-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol obtained.

Preparation of N-CBZ-L-phenylalanine methyl ester.

A solution of N-CBZ-L-phenylalanine (44.0 mg.) and N,N'-carbonyldiimidazole (22.9 mg.) in ethanol-free chloroform (50 ml.) was stirred at room temperature for 30 minutes. Dry methanol (10 ml.) was added, and stirring continued for 48 hours, after which the solvent was removed in vacuo, to leave an oil. This was taken up in chloroform (20 ml.), and washed successively with dil. aq. HCl, and saturated aq. Na_2CO_3; evaporation of the solvent gave a colourless oil, which was purified by preparative T.L.C., using 1% $MeOH/CHCl_3$ as eluant. This yielded N-CBZ-L-phenylalanine methyl

ester (20.2 mg.; 44%) as a colourless oil.

R_f (1% MeOH/$CHCl_3$) : 0.67 ; Ce^{4+} - olive green.

ν_{max} (KBr) : 3340 (NH), 3030, 2950, 1720 (broad : ester + urethane C=O), 1600, 1580, 1510 (broad : urethane), 1450, 1430, 1350, 1250, 1210, 1080, 1050, 1020, 910, 750, 700 cm^{-1}.

ν_{max} (CCl_4) : 3440 (NH), 3040, 2960, 1730 (broad : ester + urethane C=O), 1505 (urethane), 1460, 1440, 1350, 1210, 1060, 700 cm^{-1}.

λ_{max} (MeOH) : 219 (2,420), 248 (260), 253 (322), 258 (372), 265 (297), 268 (186) nm.

C.D. $\Delta\epsilon(\lambda)$: +2.83 (218 nm.), -0.056 (240), -0.026 (258), -0.026 (266) .

δ 60 MHz. ($CDCl_3$) : 3.2 (2H, d, J = 6 Hz.: $-C\underline{H}_2-Ar$), 3.8 (3H, s : $-OC\underline{H}_3$), 4.5 (1H, s : $-C\underline{H}-$). 5.2 (2H, s : $-O-C\underline{H}_2-Ar$), 7.4 (10H, m : $Ar-\underline{H}$) .

m/e (%) : 313 (2), 282 (0.1), 254 (1), 222 (0.7), 210 (4), 181 (4), 178 (3), 163 (6), 162 (33), 146 (4), 120 (2), 119 (2), 118 (4), 108 (4), 107 (2), 105 (2), 104 (2), 103 (2), 92 (11), 91 (100), 90 (3), 89 (4), 88 (3), 79 (3), 77 (5) .

Attempted preparation of this ester by an alternative route ($SOCl_2$/pyridine + methanol) gave a product which was difficult to purify, and, therefore, was not further investigated.

A solution of N-CBZ-L-phenylalanine (38.3 mg.) and N,N'-carbonyldiimidazole (133.5 mg.) in ethanol-free chloroform (150 ml.) was stirred at room temperature for 7 days. After extraction with dil. aq. HCl, and saturated aq. Na_2CO_3, the solvent was removed. T.L.C. showed that the crude product contained a moderate quantity of a compound having the same R_f (0.67) and staining properties

(olive green) as N-CBZ-L-phenylalanine methyl ester.

Preparation of N-benzoyl-O-(L-phenylalanyl)-L-phenylalaninol hydrobromide (70) (N'-debenzoyl candipolin hydrobromide).

N-benzoyl-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol (69) (0.172 gm.) in 45% w/v HBr/AcOH (25 ml.) was stirred at room temperature for 2 hours, after which excess HBr/AcOH was removed in vacuo. N'-debenzoyl candipolin hydrobromide (70) crystallised from ethanol/light petroleum as needles (0.138 gm.; 89%), m.p. 189°.

R_f (1% MeOH/$CHCl_3$) : 0.13, with baseline residue ; Ce^{4+} - unstained.

ν_{max} (KBr) : 3420 (broad), 3300 (NH), 3020, 2920 (broad), 1745 (ester), 1635 (amide I), 1600, 1575, 1530 (amide II), 1490, 1450, 1290, 1260, 1220, 1075, 1030, 800, 750, 700 cm^{-1}.

λ_{max} (MeOH) : 215 (10,300), 226 (11,700) nm.

C.D. $\Delta\epsilon(\lambda)$: +5.54 (214 nm.), -2.42 (238), +0.35 (250) .

δ 100 MHz. : ($CDCl_3$ + CD_3OD) : 3.0 (2H, d, J = 7.5 Hz.: $-CH_2-Ar$), 3.35 (2H, d, J = 7.5 Hz.: $-CH_2-Ar$ of phenylalanine part), 4.1 - 4.7 (complex m : 2 x $-CH-NH$ + 2 x $-OCH-H$), 6.95 (1H, D_2O/DCl exchangeable : $-NH$), 7.2 (10H, m : $-CH_2-Ar-H$), 7.45 (3H, m : m- & p- H of Ar-CONH), 7.8 (2H, m : o- H of Ar-CONH).

^{13}C N.M.R. (C_5D_5N) : C-3 and 3' at 37.1, C-2 at 51.4, C-2' at 55.3, C-1 at 67.6, 2 aryl carbons p- to CH_2 at 126.7 and 127.7, 12 aryl carbons o- and m- at 128.5, 128.7, 129.1, 129.8, and 130.1, aryl carbon p- to CONHR at 131.4, 3 quaternary aryl carbons at 135.6, (2C), and 139.1, CONH at 167.7, C-1' at 170.4 p.p.m.

m/e (%) : M^+ 403 (0.1), 402 (0.3), 312 (1), 311 (6), 294 (0.4), 267 (0.3), 239 (1), 238 (8), 224 (1), 207 (1), 190 (1), 147 (3), 146 (25), 122 (3), 121 (2), 120 (25), 119 (3), 118 (13), 117 (6), 106 (3), 105 (33), 104 (7), 103 (10), 102 (2), 92 (10), 91 (100), 89 (7), 78 (6), 77 (42) .

Analysis - Found : C : 61.99, H : 5.55, N : 6.08, Br : 16.8 %

$C_{25}H_{27}N_2O_3Br$ requires : C : 62.1, H : 5.58, N : 5.79, Br : 16.6 %

Synthesis of candipolin from N-benzoyl-O-(L-phenylalanyl)-L-phenylalaninol hydrobromide.

A solution of benzoyl chloride (9.4 mg.) in dry pyridine (5 ml.) was added dropwise to a stirred solution of N-benzoyl-O-(L-phenylalanyl)-L-phenylalaninol hydrobromide (33.9 mg.) in dry pyridine (10 ml.) at 0°. After stirring for 24 hours at room temperature, the reaction mixture was diluted with chloroform (50 ml.) and washed successively with dil. aq. HCl, and saturated aq. Na_2CO_3. Evaporation of the chloroform gave N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol, which crystallised from ethanol as needles (23.3 mg.; 75%), m.p. and mixed m.p. (with natural candipolin) 207-8°. The compound was identical to natural candipolin by T.L.C. (R_f 0.54 ; Ce^{4+} - beige), I.R. (ester: 1750 ; amide : 1635 & 1525 cm^{-1}), mass spectrum (same fragmentation pattern), mass measurement (M^+ at m/e 506.22055), C.D. ($\Delta\epsilon$ -6.8 at 228 nm.).

$[\alpha]_D$ -70.1 ± 20° (c. 0.01, C_5H_5N).

Preparation of N-CBZ-L-phenylalaninol.

L-phenylalaninol (0.157 gm.) in distilled water (6.4 ml.) containing sodium bicarbonate (0.2215 gm.) was treated with benzyl chloroformate (0.20 ml.) in 6 portions at 5-minute intervals :

white solid precipitated after each addition. The reaction mixture was stirred for 1 hour at room temperature, then acidified to pH 2 (dil. aq. HCl), and extracted with ethyl acetate (50 ml.). Evaporation of the organic solvent yielded a white solid, shown by T.L.C. to contain unreacted benzyl chloroformate, which was subsequently removed by trituration with light petroleum. N-CBZ-L-phenylalaninol crystallised from $CHCl_3$/light petroleum as needles (0.163 gm.; 55 %), m.p. 94-5°. (A mixture with L-phenylalaninol had m.p. 69-73°).

R_f (1% MeOH/$CHCl_3$) : 0.26 ; Ce^{4+} - grey .

ν_{max} (KBr) : 3350 (OH), 3300 (NH), 3020, 2960, 2930, 2880, 1690 (urethane C=O), 1600, 1585, 1540 (urethane), 1490, 1480, 1450, 1310, 1225, 1215, 1140, 1080, 1070, 1050, 1015, 975, 910, 780, 745, 730, 700 cm^{-1}.

λ_{max} (MeOH) : 220 (2,200), 243 (150), 249 (230), 253 (310), 259 (380), 265 (300), 268 (230) nm.

C.D. $\Delta\epsilon(\lambda)$: -4.0 (216 nm.), -0.30 (246), -0.50 (253), -0.065 (260), -0.054 (267) .

δ 90 MHz. ($CDCl_3$) : 2.0 (br. s., D_2O exchangeable : -OH), 2.8 (2H, d, J = 7 Hz.: $-CH_2-Ar$), 3.7 (2H, m : $-CH_2-OH$), 4.0 (1H, m : -CH-), 5.0 (1H, br. m : -NH-), 5.1 (2H, s : $-O-CH_2-Ar$), 7.25 (5H, s : Ar-H), 7.35 (5H, s : Ar-H) .

m/e (%) : M^+ 285 (2), 254 (1), 210 (4), 195 (3), 194 (24), 150 (12), 108 (4), 107 (4), 92 (14), 91 (100), 89 (2), 86 (4), 79 (6), 78 (1), 77 (5) .

Analysis - Found : C : 71.76, H : 6.65, N : 5.15 % .

$C_{17}H_{19}NO_3$ requires : C : 71.56, H : 6.71, N : 4.91 % .

Preparation of N-CBZ-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol (75) by esterification of N-CBZ-L-phenylalanine with N-CBZ-L-phenylalaninol.

A solution of N-CBZ-L-phenylalanine (0.108 gm.) and N,N'-carbonyldiimidazole (0.168 gm.) in dry dioxan (20 ml.) was stirred at room temperature for 1 hour. A solution of N-CBZ-L-phenylalaninol (41 mg.) in dry dioxan (10 ml.) was added, and stirring continued overnight. Evaporation of the solvent gave a colourless oil. Three main components were recovered by preparative T.L.C., using 1% $MeOH/CHCl_3$ as eluant :

(i) A white solid, probably N-CBZ-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol (75) (13.1 mg.), whose T.L.C. (R_f 0.54 ; Ce^{4+} - beige) is similar to that of N-benzoyl-O-(N-CBZ-L-phenylalanyl)-L-phenylalaninol (69) ; I.R. spectrum shows bands at 3320 (NH), 1710 (broad : ester + urethane C=O), 1530 (urethane) cm^{-1}.

(ii) N-CBZ-L-phenylalaninol (12.5 mg.) : R_f 0.26 ; Ce^{4+} - grey ; ν_{max} (Nujol) : 1690 (C=O), 1535 (urethane II) cm^{-1}.

(iii) White feathery crystals (96.0 mg.), having the same R_f (0.2 in 1% $MeOH/CHCl_3$) and staining properties (Ce^{4+} - beige) as N-CBZ-L-phenylalanine, but whose I.R. spectrum :

ν_{max} : 3500 - 2900 (broad, strong : OH), 1700 (broad : urethane C=O), 1600, 1530 (urethane), 1330, 1260, 1090, 1060, 830, 750, 700 cm^{-1}

differs from that of the starting material.

Due to the low yield of non-polar material, the products of this reaction were not further investigated.

Preparation of triethyloxonium fluoroborate.[111]

Apparatus used was dried at 50°, and assembled while hot ; drying tube containing silica gel protected assembly while cooling.

Epichlorohydrin (40 ml.) was added dropwise to a stirred solution of redistilled boron trifluoride-ether (80 ml.) in sodium-dried ether (160 ml.) at room temperature ; a gelatinous white precipitate formed during this process. The reaction mixture was agitated manually until addition of epichlorohydrin was complete (ca. 1 hour), then refluxed vigorously for 1 hour (oil bath temperature ca. 75°), and allowed to cool overnight. The dark brown fuming supernatant liquid was carefully decanted from the white crystalline mass. The crystals were washed with 3 x 300 ml. portions of sodium-dried ether, then transferred to a glass screw-top jar, and stored under ether in refrigerator.

Treatment of candipolin with triethyloxonium fluoroborate.

A solution of triethyloxonium fluoroborate (2.73 gm.) and candipolin (2.050 gm.) in dry methylene chloride (500 ml.) was stirred under N_2 for 24 hours at room temperature. Solvent was removed *in vacuo*, and the resulting dark oil taken up in dioxan (100 ml.); 3% aqueous acetic acid (100 ml.) was added, and the reaction mixture stirred at room temperature for 24 hours. The white solid which separated from solution was collected by filtration, and was added to further crops of solid obtained by concentration of the filtrate. After washing with ether, the solid was suspended in water and extracted with chloroform (500 ml.) to give a yellow solid (0.476 gm.), with an odour similar to that of ethyl benzoate. Preparative T.L.C., using 1% $MeOH/CHCl_3$ as eluant, gave two main components :

(i) Candipolin (0.265 gm.), R_f 0.54 (Ce^{4+} - beige). This was found to have m.p. and I.R. spectrum the same as the starting material.

(ii) N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (0.157 gm.), a white solid with R_f 0.21 (Ce^{4+} - beige). This was found to be identical (T.L.C., m.p., I.R., N.M.R.) to a sample prepared by reaction of N-benzoyl-L-phenylalanine p-nitrophenyl ester with L-phenylalaninol[73].

The aqueous layer was basified by addition of solid K_2CO_3, then extracted with chloroform (500 ml.) to give a white solid (0.727 gm.). Preparative T.L.C., using 10% MeOH/$CHCl_3$ as eluant, gave four major bands (R_f 0.95 : 33 mg.; R_f 0.85 : 45 mg.; R_f 0.7 : 296 mg.; R_f 0.5 : 153 mg.) plus a very minor band (R_f 0.2). The main band (296 mg.) was further separated into two major components (R_f 0.2 : 101 mg.; R_f 0.1 : 35 mg.) plus five minor components (each weighing less than 10 mg.) by preparative T.L.C., using 1% MeOH/$CHCl_3$ as eluant. The main component, R_f 0.2, consisting of N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (101 mg.), was combined with the sample of this compound obtained previously, and purified by preparative T.L.C., eluting twice with 100% $CHCl_3$. The alcohol, which separated from common organic solvents as a gel, was obtained as a very hard white solid (254 mg.), m.p. 189-191°, on trituration with ether.

Allowing for 75% average efficiency of recovery of material from preparative T.L.C. plates, the overall yield of N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol w.r.t. reacted candipolin is of the order of 25%.

$[\alpha]_D$ -47.7° (c. 0.67, C_5H_5N).

R_f (1% MeOH/$CHCl_3$) : 0.21 ; (5% MeOH/$CHCl_3$) : 0.6 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3300 (NH), 3300 (broad : OH), 3030, 2930, 1660 & 1640 (amide I), 1600, 1580, 1530 (amide II), 1490, 1450, 1380, 1330, 1300, 1080, 1050, 1035, 750, 700 cm^{-1}.

δ 90 MHz. ($CDCl_3$ + CD_3OD) : 2.8 (2H, m ; irr. 4.1 — br. s : $-C\underline{H}_2-Ar$), 3.15 (2H, m ; irr. 4.8 — br. s : $-C\underline{H}_2-Ar$ of phenylalanine part), 3.5 (2H, d, J = 5 Hz.: - $C\underline{H}_2-OH$), 4.1 (2H, m : $-C\underline{H}-$), 4.8 (1H, t, J = 7.5 Hz.: $-C\underline{H}-$), 6.8 (1H, m : $-N\underline{H}-$), 7.15 (5H, s : $-CH_2-Ar-\underline{H}$), 7.25 (5H, s : $-CH_2-Ar-H$), 7.5 (3H, s : m- & p- H of Ar-CONH), 7.75 (2H, m : o- H of Ar-CONH) .

^{13}C N.M.R. (C_5D_5N) : C-3 at 37.6, C-3' at 38.6, C-2 at 53.7, C-2' at 55.6, C-1 at 63.1, 2 aryl carbons p- to CH_2 at 126.4 and 126.8, 12 aryl carbons o- and m- at 128.0, 128.6, and 129.9, aryl carbon p- to CONHR at 131.4, 3 quaternary aryl carbons at 135.5, 138.5, and 139.5, CONH at 167.7 and 171.8, C-1' at 171.8 p.p.m.

m/e (%) : M^+ 402 (3), 385 (3), 384 (8), 311 (3), 294 (4), 293 (13), 279 (5), 269 (3), 264 (3), 253 (5), 252 (29), 225 (7), 224 (20), 190 (5), 172 (7), 146 (5), 131 (4), 120 (8), 118 (4), 117 (4), 106 (10), 105 (100), 104 (5), 103 (4), 95 (5), 91 (24), 78 (5), 77 (24).

Analysis - Found : C : 74.39, H : 6.77, N : 6.84 %

$C_{25}H_{26}N_2O_3$ requires : C : 74.60, H : 6.51, N : 6.96 %

The other components of the reaction product mixture (each shown by T.L.C. to contain impurities) were not further investigated.

Synthesis of asperglaucide from N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol.

A solution of N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (25.2 mg.) and acetic anhydride (1.5 ml.) in dry pyridine (10 ml.) was stirred overnight at room temperature. Evaporation of the solvent gave a white solid which was triturated with light petroleum to remove remaining traces of acetic anhydride. Crystallisation from ethyl acetate/light petroleum gave asperglaucide (20.6 mg.; 74%) as colourless needles, m.p. 185-7° (lit. 185-6°)[67]. Melting point was not depressed by admixture with natural material[102].

R_f (1% MeOH/$CHCl_3$) : 0.47 ; Ce^{4+} - beige.

ν_{max} (KBr) : 3310 (NH), 3020, 1725 (ester), 1660 & 1630 (amide I), 1600, 1580, 1530 (amide II), 1490, 1260, 1050, 750, 700 cm^{-1}.

δ 90 MHz. ($CDCl_3$) : 2.05 (3H, s : $C\underline{H}_3$-CO), 2.8 (2H, d, J = 7 Hz. : -$C\underline{H}_2$-Ar), 3.2 (2H, m : -$C\underline{H}_2$-Ar), 3.95 (2H. m : -O-$C\underline{H}_2$-), 4.4 (1H, m : -C$\underline{H}$-), 4.75 (1 H, m : -C$\underline{H}$-), 6.05 (1H, d, J = 8 Hz.: -N$\underline{H}$-), 6.75 (1H, d, J = 8 Hz.: -N$\underline{H}$-), 7.15 - 7.3 (10H, m : $-CH_2-Ar-\underline{H}$), 7.5 (3H, m : m- & p- H of Ar-CONH), 7.7 (2H, m : o- H of Ar-CONH) .

m/e M^+(%):444 (1), 384 (11), 293 (17), 252 (25), 224 (24), 172 (13), 105 (100), 91 (25).

M^+ at m/e 444.20467 ; $C_{27}H_{28}N_2O_4$ requires M^+ at m/e 444.20487 .

Benzoylation of O-debenzoyl iso-candipolin (81) to give N-(N-benzoyl-L-phenylalanyl)-O-benzoyl-L-phenylalaninol (56).

A solution of benzoyl chloride (55.7 mg.) and O-debenzoyl iso-candipolin (81) (23.0 mg.) in dry pyridine (10 ml.) was stirred

for several hours at 0°, then overnight at room temperature. The reaction mixture was diluted with chloroform (30 ml.), and extracted successively with dil. aqueous HCl and saturated aqueous Na_2CO_3. Evaporation of the solvent gave iso-candipolin (N-(N-benzoyl-L-phenylalanyl)-O-benzoyl-L-phenylalaninol), which crystallised from ethanol as colourless needles (17.7 mg.; 61%), m.p. 208-10°.

R_f (1% MeOH/$CHCl_3$) 0.57 ; Ce^{4+} - beige .

ν_{max} (KBr) : 3280 (NH), 3060, 3040, 1720 and 1710 (ester), 1660 and 1630 (amide I), 1600, 1580, 1540 (amide II), 1490, 1450, 1380, 1315, 1280, 1070, 1030, 750, 710 $cm.^{-1}$.

δ 100 MHz. (F.T.) ($CDCl_3$) : 2.85 (2H, d, J=7.1 Hz. : $-CH_2-Ar$), 3.15 (2H, ABX multiplet, J_{AB}= 13.6 Hz., J_{AX}= 5.97 Hz., J_{BX}= 8.44 Hz. ; δ_A= 3.23, δ_B= 3.03 : $-CH_2-Ar$), 4.1 (2H, ABX multiplet, J_{AB}= 11.6 Hz., J_{AX}= 4.6 Hz., J_{BX}= 4.6 Hz. ; δ_A= 4.17, δ_B= 4.06 : $-O-CH_2-$), 4.5 (1H, m : $-CH-NH$), 4.7 (1H, m : $-CH-NH$), 6.04 (1H, d, J= 8.4 Hz. : $-NH$), 6.79 (1H, d, J= 7.5 Hz. : $-NH$), 7.2 (10 H, br. s. : $-CH_2-Ar-H$), 7.5 (6H, m : m-, p- H of O-CO-Ar and NH-CO-Ar), 7.75 (2H, m : o- H of NH-CO-Ar), 8.0 (2H, m : o- H of O-CO-Ar) .

m/e (%) : M^+ 506 (0.05), 384 (90), 293 (100), 279 (23), 264 (10), 252 (11), 224 (11), 105 (56), 91 (61) .

Analysis : Found - C: 75.85, H: 6.03, N: 5.44 % .

$C_{32}H_{30}N_2O_4$ requires C: 75.87, H: 5.97, N: 5.53 % .

Treatment of N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (81) with trifluoroacetic acid.

A solution of N-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol (10 mg.) in trifluoroacetic acid (5 ml.) was allowed to stand at

room temperature for 2 hours, after which the solvent was removed by distillation. Trituration with iso-pentane gave a white solid mixture (m.p. 165-70°) which was shown (T.L.C.) to contain ca. 50% unchanged starting material plus a new compound, whose I.R. spectrum suggests that it is N-(N-benzoyl-L-phenylalanyl)-O-trifluoroacetyl-L-phenylalaninol (81a).

R_f (1% MeOH/$CHCl_3$) : 0.56 ; Ce^{4+} - beige .

ν_{max} (Nujol) : 3300 (NH), 1785 (ester C=O), 1660 and 1630 (amide I), 1520 (amide II), 1160, 960, 920, 780, 750, 700 $cm.^{-1}$.

EXPERIMENTAL

CHAPTER 2

Culture of Penicillium canadense in the presence of labelled compounds, and isolation of candipolin.

Penicillium canadense (Commonwealth Mycological Institute No. 95,493) was cultured on 2% malt agar as slants in test-tubes, then in seed bottles (18 x 7 cm.). A suspension of the spores from one seed bottle in sterile deionised water (100 ml.) was used to inoculate 10 Roux bottles (1 litre capacity), each containing culture medium A (200 ml.), which had been previously sterilised in an autoclave for half-an-hour with steam at a temperature of 120° and pressure of 15 p.s.i. The culture medium consisted of a solution of the following in 1 litre of deionised water : glucose (50 gm.), ammonium tartrate (2.8 gm.), potassium dihydrogen phosphate (5.0 gm.), magnesium sulphate (.7 H_2O) (1.0 gm.), sodium chloride (1.0 gm.), Oxoid yeast extract (0.5 gm), and 1 ml. of a solution of trace elements containing : $FeSO_4.7H_2O$ (0.1 gm.), $CuSO_4.5H_2O$ (0.015 gm.), $ZnSO_4.7H_2O$ (0.05 gm.), $MnSO_4.4H_2O$ (0.01 gm.), Na_2MoO_4 (0.01 gm.), 100 ml. deionised water. Cultures were allowed to grow undisturbed at 25°, and 50% relative humidity, with illumination from white fluorescent tubes for ca. 8 hours per day.

At the appropriate stage of growth, a sterile solution of radoitracer was injected, in equal portions, into the broth of two culture bottles. After the desired period had elapsed, the mycelium was separated from the broth, washed with chloroform, dried in an oven at 35° for 48 hours, then crushed, and extracted with chloroform for 24 hours in a Soxhlet apparatus. The solvent was removed in vacuo, and the resulting solid taken up in the minimum volume of boiling ethanol. Candipolin crystallised out on cooling.

A further quantity of candipolin (R_f 0.54) was obtained by preparative T.L.C. of the mother liquors, using 1% MeOH/$CHCl_3$ as eluant. Typically, two Roux bottles gave 5½ gm. mycelium, which gave ca. 800 mg. crude extract, yielding ca. 100 mg. candipolin.

Shake cultures of P. canadense were obtained by inoculating sterile culture medium (100 ml.) in conical flasks (250 ml. capacity) with a spore suspension and shaking at 150 r.p.m. The mycelium was collected by filtration.

Radioactive counting of samples.

All samples were weighed on a Mettler UM7 microbalance, then dissolved in a suitable solvent, and the volume made up to 15 ml. by addition of scintillator solution, which contains 4 gm. PPO (2,5-diphenyloxazole) and 0.1 gm. POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) per litre of sodium-dried toluene. The diluter solvents used were as follows : 6 drops dimethyl formamide for counting candipolin ; 0.5 ml. methanol for counting N-benzoyl-L-phenylalanine, N-benzoyl-L-phenylalaninol, benzoic acid, and L-phenylalaninol ; L-phenylalanine was counted as its hydrochloride dissolved in distilled water (3 drops) and methanol (3 ml.), then made up to 15 ml. with scintillator solution. The activity of these solutions was measured using a Philips Liquid Scintillation Analyser (PW 4510), giving detection efficiencies of the order of 85% for ^{14}C, and ca. 75% for dual labelling. Samples were counted for a time long enough to give a standard deviation of $\pm$ 2% on counts. Screening of T.L.C. plates for radioactivity was carried out using a Panax radiochemical T.L.C. plate scanner.

Incorporations were calculated as follows :

$$\text{Incorporation} = \frac{\text{Total activity recovered in candipolin}}{\text{Total activity fed}} \times 100\%$$

$$= \frac{\text{d.p.m./mg. X weight (mg.) of candipolin recovered}}{\text{d.p.m./mg. X weight (mg.) of "precursor"}} \times 100\%$$

In calculating incorporations, a pure sample of candipolin was crystallised and counted to constant activity. The remaining candipolin was crystallised until pure by T.L.C., and the quantity remaining in the mother liquors estimated by T.L.C. The combined weight of these three samples was used to calculate incorporation.

EXPERIMENTAL

CHAPTER 2

1. Biosynthesis of candipolin from L-[U-^{14}C], [G-^{3}H] phenylalanine by shake cultures of Penicillium canadense.

A sterile aqueous solution of L-[U-^{14}C] phenylalanine (50 μCi) and L-[G-^{3}H] phenylalanine (0.86 mCi) was added to two shake flasks of P. canadense on the 4th day after inoculation ; on the 7th day after inoculation, the mycelium was collected by filtration, washed, dried, and extracted with chloroform for 24 hours in a Soxhlet apparatus. Evaporation of the solvent gave crude extract (0.179 gm.), from which crude candipolin (13.8 mg.) was obtained by preparative T.L.C., using 1% MeOH/$CHCl_3$ as eluant. This was diluted by addition of unlabelled candipolin (50.5 mg.) and purified by preparative T.L.C., to give essentially pure candipolin (52.4 mg.), which crystallised from ethanol as needles, m.p. 210°. Crystallisation to constant activity gave the results shown in Table 3. A sample of this candipolin (31.6 mg.: 7358 d.p.m./mg. ^{14}C ; 102131 d.p.m./mg. ^{3}H) was further diluted to give candipolin (152.9 mg. : 1980 d.p.m./mg. ^{14}C ; 28280 d.p.m./mg. ^{3}H).

A sample of this candipolin (28.3 mg.) was hydrolysed by refluxing with base, giving pure samples of N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol, which were each in turn hydrolysed to give benzoic acid. On such a small scale, however, crystallisation of the products to constant activity was not possible.

Accordingly, candipolin (69.6 mg. : 1980 d.p.m./mg. ^{14}C ; 28280 d.p.m./mg. ^{3}H) was diluted still further by addition of inactive candipolin (131.7 mg.). This candipolin (201.3 mg.) was base hydrolysed to give N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol, which were crystallised to constant activity (from

AcOH/H_2O and $CHCl_3$/light petroleum respectively). (See Table 4).

By an oversight, the activity of the thrice-diluted candipolin used in this hydrolysis was not measured. A separate sample of candipolin was, therefore, obtained by preparative T.L.C. of residues of the crude mycelial extract after dilution with inactive candipolin. This was crystallised to constant activity, giving 56 d.p.m./mg. ^{14}C ; 795 d.p.m./mg. ^{3}H, and hydrolysed as before to give N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol. (See Table 4).

N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol from the former hydrolysis (201.3 mg candipolin) were separately hydrolysed (dil. aq. HCl at 150°) to give samples of benzoic acid, which were crystallised (from water) to constant activity. (See Table 5).

2. Time vs. incorporation study on surface cultures of Penicillium canadense using 2-[^{14}C] DL-phenylalanine.

A sterile aqueous solution of 2-[^{14}C] DL-phenylalanine (25 μCi) was added to surface cultures of P. canadense (4 bottles) 4 days after inoculation ; half of the cultures were harvested 6 days , and half harvested 9 days later. Similarly, 2-[^{14}C] DL-phenylalanine (25 μCi) was added to cultures (4 bottles) 7 days after inoculation, which were harvested 3 and 6 days later. Candipolin was isolated and crystallised to constant activity. The results are shown in Table 7 .

Investigation of the biosynthesis of candipolin by adding radiotracers to surface cultures of Penicillium canadense.

In all of these experiments, the radiotracer was added to 2 Roux bottles containing cultures of P. canadense 4 days after

inoculation ; cultures were harvested 10 days after inoculation. Candipolin was isolated and degraded as described previously.

3 (a). L-[U-^{14}C] phenylalanine.

300 μCi fed as sterile solution in distilled water. For incorporation, see Table 8 .

A sample of the candipolin isolated (92.9 mg. ; 2.88 x 10^6 d.p.m./mg.) was hydrolysed to give N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol, which were required for further feeding experiments, therefore, were crystallised once, but not counted directly. A sample of N-benzoyl-L-phenylalanine (0.9 mg.) was diluted by addition of unlabelled N-benzoyl-DL-phenylalanine (79.9 mg.). A sample of N-benzoyl-L-phenylalaninol (0.7 mg.) was diluted by addition of unlabelled material (53.0 mg.). Each of these diluted samples showed constant activity over three successive crystallisations (see Table 9). A sample (74.2 mg.) of once-diluted N-benzoylphenylalanine (44228 d.p.m./mg.) was further diluted by addition of inactive N-benzoyl-DL-phenylalanine to give material (147.4 mg.) of activity 28478 d.p.m./mg. , which was then hydrolysed to give benzoic acid (see Table 10a). A sample (46.2 mg.) of once-diluted N-benzoyl-L-phenylalaninol (34965 d.p.m./mg.) was further diluted by addition of the inactive compound (51.9 mg.) to give material (98.1 mg.) of activity 16216 d.p.m./mg. , which was then hydrolysed to give benzoic acid (see Table 10b).

3 (b). L-[U-^{14}C] phenylalanine (150 μCi) was fed as a sterile aqueous solution. Recrystallisation of the material which separated directly from an ethanolic solution of the crude mycelial extract gave candipolin (67.0 mg.), shown by T.L.C. to be free from impurity.

A sample (0.6 mg.) of it was diluted by addition of unlabelled candipolin (62.8 mg.), and the diluted sample was found to have constant activity over three successive crystallisations. Repeated preparative T.L.C. of the remaining crude mycelial extract, using 1% MeOH/$CHCl_3$ as eluant, gave a further sample of candipolin (22.8 mg) (see Table 11).

Diluted candipolin (58.3 mg.; 17990 d.p.m./mg.) was hydrolysed (base) to N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol, which were crystallised to constant activity (see Table 12). These compounds were not further hydrolysed.

3 (c). Detection of N-benzoyl-L-phenylalaninol in mycelial extract of P. canadense by radioisotopic dilution.

This investigation was carried out on the extract of the culture fed with L-[U-^{14}C] phenylalanine (150 μCi) in experiment 3 (b). The combined fractions of crude mycelial extract having lower R_f than candipolin were "diluted" by addition of unlabelled N-benzoyl-L-phenylalaninol (45.4 mg.) and separated by preparative T.L.C., using 1% MeOH/$CHCl_3$ as eluant. Elution of the major band and crystallisation to constant activity gave N-benzoyl-L-phenylalaninol, m.p. 169-172° (23912 d.p.m./mg.). Minimum incorporation is given in Table 24.

4. [U-^{14}C] N-benzoyl-L-phenylalanine.

[U-^{14}C] N-benzoyl-L-phenylalanine (8.894×10^7 d.p.m.), obtained by hydrolysis of candipolin derived from L-[U-^{14}C] phenylalanine (Expt. 3(a)), was converted to its sodium salt and fed as a sterilised aqueous solution. A sample of the isolated candipolin (1.1 mg.) was

diluted by addition of inactive candipolin (124.3 mg.) and crystallised to constant activity. Incorporation is given in Table 20.

A sample of the diluted candipolin (118.6 mg.; 2001 d.p.m./mg.) was further diluted to give candipolin (150.8 mg.; 1550 d.p.m./mg.), which was hydrolysed (base) to give N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol. Crystallisation to constant activity gave the results shown in Table 21.

N-benzoyl-L-phenylalanine (41.4 mg.; 1517 d.p.m./mg.) was diluted by addition of inactive N-benzoyl-DL-phenylalanine (79.4 mg.) to give material of activity 392 d.p.m./mg., then hydrolysed to give benzoic acid + phenylalanine (the latter was isolated as its hydrochloride by preparative paper chromatography : R_f 0.55 on Whatman P81 cellulose phosphate paper, using as eluant, a mixture of water (50 ml.), n-butanol (120 ml.), acetic acid (30 ml.)) (see Table 22a). N-benzoyl-L-phenylalaninol was hydrolysed (acid) to give benzoic acid (see Table 22b).

5. [U-^{14}C] N-benzoyl-L-phenylalaninol.

[U-^{14}C] N-benzoyl-L-phenylalaninol (6.598×10^7 d.p.m.), obtained by hydrolysis of candipolin derived from L-[U-^{14}C] phenylalanine (Expt. 3(a)), was fed in solution in dimethyl sulphoxide (2 ml.), which was not sterilised. A sample of the isolated candipolin (0.7 mg.) was diluted to give candipolin (75.3 mg.; 4734 d.p.m./mg.) (see Table 13). A sample of this (71.6 mg.) was further diluted to give candipolin (152.3 mg.; 2185 d.p.m./mg.), and then hydrolysed (base) to N-benzoyl-L-phenylalaninol. Crystallisation to constant activity gave the results shown in Table 14. These compounds were not further hydrolysed.

6. Carboxy-[^{14}C] benzoic acid.

Carboxy-[^{14}C] benzoic acid (250 μCi) was converted to its sodium salt by addition of aqueous sodium bicarbonate. This was fed as a sterile aqueous solution. A sample of the isolated candipolin (0.6 mg.) was diluted by addition of inactive carrier (108.9 mg.), and crystallised to constant activity (see Table 15). This diluted candipolin (106.3 mg.; 10183 d.p.m./mg.) was further diluted to give candipolin (136.4 mg.) of activity 6773 d.p.m./mg., which was hydrolysed to give N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol. Crystallisation to constant activity gave the results shown in Table 16. These compounds were not further hydrolysed.

7 (a). L-[U-^{14}C] phenylalaninol

L-[U-^{14}C] phenylalaninol (6.804 x 10^6 d.p.m.), obtained by degradation of candipolin derived from L-[U-^{14}C] phenylalanine (Expt. 3(a)), was fed as its hydrochloride in aqueous solution (sterilised). A sample of the isolated candipolin (ca. 100 mg.; 1015 d.p.m./mg.) (see Table 17) was hydrolysed (base) to give N-benzoyl-L-phenylalanine and N-benzoyl-L-phenylalaninol. Crystallisation to constant activity gave the results shown in Table 18. These compounds were each hydrolysed to give benzoic acid (see Table 19).

7 (b). The above experiment was repeated using L-[U-^{14}C] phenylalaninol (7.06 x 10^6 d.p.m.), fed in solution in dimethyl sulphoxide (2 ml.) (not sterilised). Candipolin isolated had activity of 519 d.p.m./mg., which is consistent with the previous result, therefore, no degradation was carried out on this material.

Feeding of ^{13}C- labelled Acetate and Succinate.

In these experiments, cultures of P. canadense (5 bottles, each containing 200 ml. of culture medium) were pulse fed on the 7^{th}, 8^{th} (10 a.m., 5 p.m.) and 9^{th} (3 p.m.) day after inoculation. Cultures were harvested 4 days later, and candipolin was isolated in the usual way.

In three separate experiments, $^{13}CH_3{}^{13}CO_2H$, $^{13}CD_3{}^{12}CO_2H$, and $(^{13}CH_2{}^{12}CO_2H)_2$ were added to cultures at the respective rates of 10, 12, and 8 mg. per bottle per feed. In each case, the ^{13}C N.M.R. spectrum of the isolated candipolin showed no ^{13}C enrichment.

CHAPTER 3

Preparation of $^{13}CD_3CO_2Na$*.

$^{13}CH_3CO_2Na$ (300 mg.) was dissolved in pure D_2O (6 ml.). NaOH (60 mg.) was added, and the solution was transferred to two identical thick-walled (10 mm. o.d.) tubes, which were then sealed off. The tubes were immersed in a magnetically stirred oil bath at 180° for 24 hours. After cooling, these were broken open and the contents washed into a 50 ml. flask. The solution was brought to pH 7 by dropwise addition of conc. HCl, and the water removed by evaporation in vacuo. The residue was dissolved in water (10 ml.), and the solution centrifuged to precipitate the suspended silicic acid. The supernatant solution was transferred into five 10 ml. flasks, and the sediment washed with water (2 x 2.5 ml.) to give a total of 3 ml. solution per flask (one flask per pulse was used in feeding).

Feeding of P. canadense.*

Labelled sodium acetate (250 mg.) was fed in 5 pulses 7, 8, and 9 days after inoculation, and the culture harvested 4 days later. The dried mycelium was extracted with chloroform for 24 hours.

Isolation of fatty esters.

The mycelial extracts were concentrated and candipolin removed. The fatty esters were isolated from the residual extracts by preparative T.L.C. (1% MeOH/$CHCl_3$). A yellow oil (0.1297 mg.) was obtained.

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Transesterification of the fatty ester mixture.

The fatty ester mixture (0.1221 gm.) was heated with 0.5N methanolic NaOH (4 ml.) at 95° for 5 minutes, after which, 14% w/w BF_3/MeOH solution (10 ml.) was added, and heating continued for 10 min. After cooling, the mixture was extracted with petroleum spirit (4 x 25 ml.; b.p. 40-60°). The extracts were washed (sat. NaCl), dried (Na_2SO_4), and the solvent evaporated to give a mixture of methyl esters (0.1218 gm.), which was shown by G.L.C. to consist of ca. 50% methyl oleate, ca. 25% methyl palmitate, and ca. 25% unidentified methyl esters. A crude separation of these was effected by vacuum distillation, but time did not permit further purification of the products.

CHAPTER 4

Feeding of Trichothecium roseum*.

Labelled sodium acetate (300 mg.) was fed in 6 pulses 7, 8, and 9 days after inoculation, and the culture harvested 3 days later. The mycelium was extracted with $CHCl_3$ for 24 hours in a Soxhlet apparatus.

Isolation of rosenonolactone*.

Crude rosenonolactone was obtained by washing the mycelial extract with ethanol. This solution was filtered through silica gel, and rosenonolactone recrystallised from $CHCl_3$/hexane to give colourless needles, m.p. 214° (yield 323 mg.).

Reduction of rosenonolactone.

KBH_4 (101.2 mg.; 1.88 m.mol) was dissolved in 12 drops distilled water + 1 drop 1M NaOH, and the solution added dropwise to rosenonolactone (139.1 mg.; 0.44 m.mol.) in methanol (25 ml.). The KBH_4 flask was washed into the reaction mixture with methanol (2 x 25ml) and the final solution stirred at 40° for 1 hr. 45 min. After cooling, a little distilled water (ca. 4 ml.) was added and the solution stirred at room temperature for 2 hours, during which time, a white solid precipitated. This was removed by filtration, and the methanol evaporated from the filtrate to leave an aqueous slurry. The filtered solid was dissolved in $CHCl_3$, and the slurry added. The resulting mixture was washed to neutrality (sat. NaCl), the organic layer was dried (Na_2SO_4), and the solvent evaporated to give crude rosenololactone. Recrystallisation from C_6H_6/$CHCl_3$/ petroleum spirit gave colourless needles of rosenololactone, m.p. 220-3° (110 mg.; 0.35 mmol.; yield 80%).

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REFERENCES

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1. W.B. Turner, "Fungal Metabolites", Academic Press, London and New York, 1971.
2. J.D. Bu'Lock, "The Biosynthesis of Natural Products", McGraw-Hill, London, 1965.
3. D.C. Aldridge, A. Borrow, and E.L. Gerring, Ger. Offen., 2,415,102 (1974), I.C.I.
4. A. Borrow, E.G. Jefferys, R.H.J. Kessell, E.C.Lloyd, P.B. Lloyd, and I.S. Nixon, Can. J. Microbiol., 1961, 7, 227.
5. A. Borrow, S. Brown, E.G. Jefferys, R.H.J. Kessell, E.C. Lloyd, P.B. Lloyd, A. Rothwell, B. Rothwell, and J.C. Swait, Can. J. Microbiol., 1964, 10, 407, 445.
6. W.A. Taber and L.C. Vining, Can. J. Microbiol., 1963, 9, 1.
7. J.D. Bu'Lock and A.J. Powell, Experientia, 1965, 21, 55.
8. J.D. Bu'Lock, D. Hamilton, M.A. Hulme, A.J. Powell, H.M. Smalley. D. Shepherd, and G.N. Smith, Can. J. Microbiol., 1965, 11, 765.
9. S. Gatenbeck and S. Sjoland, Biochim. Biophys. Acta., 1964, 93, 246.
10. J.D. Bu'Lock, "Essays in Biosynthesis and Microbial Development", Wiley, New York, London, Sydney, 1967.
11. J.D. Bu'Lock, D. Shepherd, and D.J. Winstanley, Can. J. Microbiol., 1967, 15, 279.
12. M.R. Pollock, Brit. Med. J., 1967, 4, 71.
13. M. Luckner, "Secondary Metabolism in Plants and Animals", Chapman and Hall, London, 1972.
14. J.D. Bu'Lock, "Advances in Applied Microbiology", 1961, 3, 293.
15. J.F. Eykmann, Rec. Trav. Chim., 1885, 4, 32.
16. J.F. Eykmann, Rec. Trav. Chim., 1886, 5, 299.

17. B.D. Davis, J. Biol. Chem., 1951, 191, 315.

18. D.B. Sprinson, Adv. Carbohyd. Chem., 1960, 15, 235.

19. E.Haslam "The Shikimate Pathway" Butterworths, London, 1974.

20. S.A. Brown, Ann. Rev. Plant Physiol.,1966, 17, 223.

21. F.L. Crane, "Biochemistry of Quinones", p. 183, Ed. R.B. Morton, Academic Press, London, 1965.

22. G.H.N. Towers in "The Filamentous Fungi, Vol. II - Biosynthesis and Metabolism", Eds. J.E. Smith and D.R. Berry, Edward Arnold, London, 1976.

23 D.R. Threlfall and G.R. Whitstance in "Aspects of Terpenoid Chemistry and Biochemistry", Ed. T.W. Goodwin, Academic Press, London, New York, 1971.

24. E.A. Doisy and J.T. Matscheiner, "Biochemistry of Quinones", p. 137, Ed. R.B. Morton, Academic Press, London, 1965.

25. J. Gripenberg, Acta Chem. Scand.,1958, 12, 603.

26. C.H. Stanmer, E. Walton, A.N. Wilson, R.W. Walker, N.R. Trenner, F.W. Holly, and K.W. Folkers, J. Amer. Chem. Soc., 1958, 80, 137.

27. A.J. Birch, D.W. Cameron, P.W. Holloway, and R.W. Rickards, Tetrahedron Letters, 1960, 25, 26.

28. H. Achenbach and H. Grisebach, Z. Naturforsch., 1965, 20B, 137.

29. R.L. Edwards, D.G. Lewis, and V.D. Wilson, J. Chem. Soc., 1961, 4995.

30. J.D. Bu'Lock, P.R. Leeming and H.G. Smith, J. Chem. Soc., 1962, 2085.

31. K. Moore, P.V. Subba Rao, and G.H.N. Towers, Can. J. Biochem., 1967, 45, 1863.

32. K. Moore, P.V. Subba Rao, and G.H.N. Towers, Biochem. J., 1968, 106, 507.

33. K. Moore and G.H.N. Towers, Can. J. Biochem., 1967, 45, 1659.

34. C.J. French, C.P. Vance and G.H.N. Towers, Phytochemistry, 1976, 15, 564.

35. M.H. Zenk, Rec. Adv. Phytochem., 1979, 12, 164.

36. E. Haslam, R.D. Howarth and P.F. Knowles, J. Chem. Soc., 1961, 1854.

37. M. Hashem and W. Brücker, Flora, 1962, 152, 57.

38. D. Cornthwaite and E. Haslam, J. Chem. Soc., 1965, 3008.

39. P.M. Dewick and E. Haslam, J.C.S. Chem. Commun., 1968, 673.

40. S. Gatenback and I. Lönnroth, Acta Chem. Scand., 1962, 16, 2298.

41. R.K. Crowden, Can. J. Microbiol., 1967, 13, 181.

42. G. Billek and F.P. Schmook, Monatsh. Chem., 1967, 98, 1651.

43. Z. Kitamura, U. Kurimoto, and M. Yokoyama, J. Pharm. Soc. Japan. 1956, 76, 972.

44. A.J. Birch, G.E. Blance, S. David, and H. Smith, J. Chem. Soc., 1961, 3128.

45. J.C. MacDonald and G.P. Slater, Can. J. Microbiol., 1966, 12, 455.

46. E. Houghton and J.E. Saxton, Tetrahedron Letters, 1968, 5475.

47. A.J. Birch and J.J. Wright, J.C.S. Chem. Commun., 1969, 644.

48. J.H. Birkinshaw, M. Luckner, Y.S. Mohamed, K. Mothes and C.E. Stickings, Biochem. J., 1963, 89, 196.

49. L. Nover and M. Luckner, Eur. J. Biochem., 1969, 10, 268.

50. J. Framm, L. Nover, A.E. Azzouny, H. Richter, K. Winter, S. Werner and M. Luckner, Eur. J. Biochem., 1973, 37, 78.

51. A.K. Bose, K.S. Khanchandani, R. Tavares and P.T. Funke, J. Amer. Chem. Soc., 1968, 90, 3593.

52. R. Weindling and O.H. Emerson, Phytopathology, 1936, 26, 1068.

53. J.A. Winstead and R.J. Suhadolnik, J. Amer. Chem. Soc., 1960, 82, 1644.

54. J.D. Bu'Lock and A.P. Ryles, J.C.S. Chem. Commun., 1970, 1404.

55. A.K. Bose, K.G. Das, P.T. Funke, I. Kugachovsky, O.P. Sukla, K.S. Kanchandani, and R.J. Suhadolnik, J. Amer. Chem. Soc., 1968, 90, 1038.

56. A.K. Bose, K.S. Kanchandani, R. Tavares, and P.T. Funke, J. Amer. Chem. Soc., 1968, 90, 3592.

57. D. Gottlieb, H.E. Carter, M. Legator and V. Gallicchio, J. Bact., 1954, 68, 243.

58. S. Pestka, Ann. Rev. Microbiol., 1971, 25, 487.

59. L.C. Vining and D.W.S. Westlake, Can. J. Microbiol., 1964, 10, 705.

60. R. McGrath, L.C. Vining, F. Sala, and D.W.S. Westlake, Can. J. Biochem., 1968, 46, 587.

61. D. Gottlieb, P.W. Robbins and H.E. Carter, J. Bact., 1956, 72, 153.

62. C.D. Stratton and M.C. Rebstock, Arch. Biochem. Biophys., 1963, 103, 159.

63. D.W.S. Westlake, F. Sala, R. McGrath and L.C. Vining, Can. J. Microbiol., 1968, 14, 587.

64. A.R. Battersby and R.S. Kapil, Tetrahedron Letters, 1965, 3529.

65. B. Achari, A. Pal, and S.C. Pakrashi, Ind. J. Chem., 1974, 12, 1218.

66. A.D. Argoudelis, S.A. Miszak, L. Baczynskyj, J. Antibiot., 1975, 28, 733.

67. D. Uemura, K. Sigura, and Y. Hirata, Chemistry Letters, 1975, 537.

68. P.V. Deshmukh and M.G. Vaidya, Nature, 1968, 217, 849.

69. R.L. Baxter, Ph.D. Thesis (Glasgow) 1968.

70. T.P. Roy, Ph.D. Thesis (Glasgow) 1970.

71. Dr. F. Cramer, "Paper Chromatography", Macmillan, London, 1954, p.46.

72. S.W. Fox and J.F. Foster, "Introduction to Protein Chemistry", Wiley, New York, 1957.

73. R.M. Stewart, B.Sc. Thesis (Glasgow) 1973.

74. L.J. Bellamy "Advances in Infrared Group Frequencies", Ch. 5, Methuen, London, 1968.

75. P.V. Deshmukh, Hindustan Antibiot. Bull., 1968, 10, 299. (Chem. Abs. 69 87459e)

76. D. Gottlieb, D.K. Bhattacharyya, H.W. Anderson and H.E. Carter, J. Bact., 1948, 55, 409.

77. J. Gripenberg, Acta Chem. Scand., 1956, 10, 111.

78. J. Gripenberg, Acta Chem. Scand., 1958, 12, 1411.

79. N.J. McCorkindale in "The Filamentous Fungi : Biosynthesis and Metabolism", Eds. D.R. Berry and J.E. Smith, Arnold, London, 1976, p. 406.

80. Unpublished work referred to by W.B. Turner in "Fungal Metabolites", Academic Press, London, 1971, p.230.

81. M.H. Zenk in "Biosynthesis of Aromatic Compounds", Ed. G. Billek, Pergamon, London, 1966, p. 45.

82. H.A. Staab, M. Lüking and F.H. Dürr, Chem. Ber., 1962, 95, 1275.

83. R. Paul and G.W. Anderson, J. Amer. Chem. Soc., 1958, 80, 4423.

84. H.A. Staab, Angew. Chem., 1959, 71, 194.

85. F. Michael and B. Schleppinghoff, Chem. Ber., 1955, 88, 763 (p.766 of paper).

86. H.E. Carter, Organic Reactions, Vol. III, 205, Ch.5, Eds. R. Adams, W.E. Bachman, L.F. Fieser, J.R. Johnson, and H.R. Snyder, Wiley, New York, 1946.

87. G.T. Young in "Peptides", Eds. H.C. Beyerman, A. van de Linde, W. Massen van den Brink, North-Holland, Amsterdam, 1967.

88. M. Goodman and L. Levine, J. Amer. Chem. Soc., 1964, 86, 2918.

89. H.A. Staab, Liebigs Ann. Chem., 1957, 609, 75.

90. H.A. Staab, W.Rohr, and A. Mannschreck, Angew. Chem., 1961, 73, 143.

91. R. Paul and G.W. Anderson, J. Amer. Chem. Soc., 1960, 82, 4596.

92. M. Bergmann and L. Zervas, Berichte, 1932, 65, 1192.

93. J.P. Greenstein and M. Winitz "Chemistry of the Amino Acids", Vol.2, Wiley, London and New York, 1961, p. 890ff.

94. Y. Kishi, F. Fukuyama, M. Aratani, F. Nakatsubo, T. Goto, S. Inoue, H. Tanino, S. Sugiura and H. Kakoi, J. Amer. Chem. Soc., 1972, 94, 9219.

95. S. Hanessian, Tetrahedron Letters, 1967, 1549.

96. L.H. Welsh, J. Amer. Chem. Soc., 1947. 69, 128.

97. L.H. Welsh, J. Amer. Chem. Soc., 1949, 71, 3500.

98. T. Oishi, M. Nagai and Y. Ban, Tetrahedron Letters, 1968, 491.

99. T. Oishi, M. Nagai and Y. Ban, Tetrahedron Letters, 1968, 498.

100. Y. Ishido, N. Nakazaki and S. Sakairi, J.C.S. Perkin I, 1977, 657.

101. R.E. Cox, K.K. Chexal and J.S.E. Holker, J.C.S. Perkin I, 1976, 578.

102. Natural asperglaucide was very kindly donated by Prof. R. Thomson, University of Aberdeen.

103. M.C. Clifford, E.A. Evans, A.E. Kilner, and D.C. Warrell, J. Labelled Compounds, 1975, 11, 435.

104. Feeding Experiment performed by Dr.J.A.Troke.

105. Dictionary of Organic Compounds, 5th and Cumulative Supplement, 4th Edition, Ed. R. Stevens, Eyre and Spottiswoode, London, 1969.

106. Dictionary of Organic Compounds, Eds. G. Harris, J.R.A. Pollock, and R. Stevens, Eyre and Spottiswoode, London, 1968.

107. V.M. Micović and M.J. Mihailović, J. Org. Chem., 1963, 18, 1190.

108. J.E. Snoke and H. Neurath, Arch. Biochem, 1949, 21, 351.

109. R. Adams and L.H. Ulich, J. Amer. Chem. Soc., 1920, 42, 599.

110. Organic Reactions, Vol. III, p.205, Ed. R. Adams, John Wiley and Sons Inc., New York, 1946.

111. H. Meerwein in Organic Synthesis, 1966, 43, 113, Ed. E.J. Corey, John Wiley & Sons Inc., New York.

112. J.D. Bu'Lock, A.P. Ryles, N.Johns, and G.W. Kirby, J.C.S. Chem. Commun., 1972, 100.

113. T. Oshima and N. Tamija, Biochem. J., 1961, 78, 116.

114. N. Johns, G.W. Kirby, J.D. Bu'Lock and A.P. Ryles, J.C.S. Perkin I, 1975, 383.

115. G.W. Kirby, and S. Narayanaswami, J.C.S. Perkin I, 1976, 1564.

116. R. Cardillo, C. Fuganti, D. Ghiringhelli, P. Grasselli and G. Gatti, J.C.S. Chem. Commun. 1975, 778.

117. A.M. Clark and C.D. Hufford, Lloydia, 1977, 40, 146.

118. N.J. McCorkindale, R.L. Baxter, T.P. Roy, H. Shields, R.M. Stewart, and S.A. Hutchinson, Tetrahedron, 1978, 34, 2791.

119. A.M. Clark and C.D. Hufford, Phytochemistry, 1978, 17, 552.

120. M.I. Gurr and A.T. James, Lipid Biochemistry, Chapter 2, Chapman and Hall, 2nd Edition, London, 1975.

121. P.R. Vagelos in "The Biochemistry of Lipids, M.T.P. International Review of Science, Biochemistry Series I,"Vol. 14, Chapter 3, Ed. T.W. Goodwin, Butterworths, London, 1974.

122. B. Sedgewick, C. Morris and S.J. French, J.C.S. Chem. Commun., 1978, 193.

123. N.J. McCorkindale, W.P. Blackstock, G.A. Johnstone, T.P. Roy, and J.A. Troke, IUPAC Symposium on Chemistry of Natural Products, Golden Sands, Bulgaria, 1, 151-154, September, 1978.

124. N.J. McCorkindale, W.P. Blackstock, G.A. Johnstone, and J.A. Troke, unpublished results.

125. R.C. Anderson and B. Fraser-Reid, Tetrahedron Letters, 1977, 2865.

126. M. Kato, R. Tanaka, K. Kuwahara, M. Kageyama and A. Yoshikoshi, Asai Garasu Hogyu Gijutsu Shoreikai Kenkyu Kokoku, 1974, 25, 169 (Chemical Abstracts, 84, 648c).

127. cf. W-H. Kunau, Angew. Internat., 1976, 15, 61 and references therein.

128. M.I. Gurr in The Biochemistry of Lipids, M.T.P. International Review of Science, Biochemistry Series 1, Vol. 4, Chapter 5, Ed. T.W. Goodwin, Butterworths, London, 1974.

129. cf. J.B. Harborne, Phytochemical Methods, Chapman and Hall, London, 1973, p.149.

130. F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour and H.S. Vedanayagam, Chemistry and Physics of Lipids. 1977, 18, 115.

131. J. Bus and D.J. Frost, Rec. Trav. Chim., 1974, 93, 213.

132. F.D. Gunstone, M.R. Pollard, C.M. Scrimgeour, N.W. Gilman, and B.C. Holland, Chemistry and Physics of Lipids, 1976, 17, 1.

133. E. Wenkert, B.L. Buckwalter, I.R. Burfitt, M.J. Gasic, H.E. Gottlieb, E.W. Hagaman, F.M. Schell and P.M. Wovkulich in Topics in Carbon-13 N.M.R. Spectroscopy, Vol.2, Ed. G.C. Levy, Wiley-Interscience, New York, 1976, p. 82.

134. J.B. Stothers in Topics in Carbon-13 N.M.R. Spectroscopy, Vol.1, Ed. G.C. Levy, Wiley-Interscience, New York, 1974, pp. 234-237.

135. U. Sankawa, H. Shimada and K. Yamasaki, Tetrahedron Letters, 1978, 3375.

136. J.R. Lyerla, Jr., and G.C. Levy in Topics in Carbon-13 N.M.R. Spectroscopy, Vol. 1, Ed. G.C. Levy, Wiley-Interscience, New York, 1974, p. 105.

137. cf. M. Akhtar and C. Jones, Tetrahedron, 1978, 34, 813-832, and references therein.

138. Y. Sato, T. Machida and T. Oda, Tetrahedron Letters, 1975, 4571.

139. C. Fuganti, D. Ghiringhelli, and P. Grasselli, J.C.S. Chem. Commun., 1975, 846.

140. B.A. Achilladelis and J.R. Hanson, J. Chem. Soc. (C), 1969, 2010.

141. D. Cane and P.P.N. Murthy, J. Amer. Chem. Soc., 1977, 99, 8327.

142. I.H. Sadler in Chem. Soc. Annual Reports, 1971, 68B, 18.

143. A.F. Cockerill, G.L.O. Davis, R.C. Harden and D.M. Rackam, Chem. Rev., 1973, 73, 553.

144. Nuclear Magnetic Resonance Shift Reagents, Ed. R.E. Sievers, Academic Press, New York, 1973.

145. J. Reuben, Prog. Nuclear Magnetic Resonance Spectroscopy, 1973, 9, 1.

146. J.W.M. de Boer, P.J.D. Sakkers, C.W. Hilbers and E. de Boer, J. Magn. Resonance, 1977, 25, 455.

147. R.E. Davis and M.R. Willcott III, Chem. Rev., 1973, 73, 150.

148. H.M. McConnell and R.E. Robertson, J. Chem. Phys., 1958, 29, 1361.

149. B. Dockerill, J.R. Hanson and M. Siverns, Phytochemistry, 1978, 17, 572.

150. I.H. Sadler, J.C.S. Chem. Commun., 1973, 809.

151. B. Sedgwick and C. Morris, J.C.S. Chem. Commun., 1980, 96.

APPENDICES

APPENDIX I

Selective loss of hydrogen from phenylalanine during the course of biosynthetic investigations.

During the course of biosynthetic investigations using phenylalanine as a precursor, it was discovered that the amino acid can undergo proton exchange at C-3 independently of its incorporation into secondary metabolites. In one study[112], DL-[3',3'-$^2H_2$] phenylalanine fed to Trichoderma viride gave rise to gliotoxin with a small enrichment of dideutero- species and a substantial enrichment of monodeutero- species. From the N.M.R. spectrum, it was deduced that during the course of the biosynthesis, the deuterium atom in the pro-S position had been lost, while that in the pro-R position was retained. Feeding of L-3'R-[3'-$^2H_1$]- and of DL-3'S-[3'-$^2H_1$]- phenylalanine to T.viride along with DL-[1'-^{14}C] phenylalanine (as an internal check) confirmed that the 3'- pro-R atom was retained while the pro-S atom was lost. Feeding of DL-[1'-^{14}C,3'-^3H] phenylalanine to the organism at a time when gliotoxin production was not yet underway yielded mycelial protein, the phenylalanine of which showed a 35% reduction in the $^3H/^{14}C$ ratio from the material fed. This indicates that stereoselective repalcement of the 3'-pro-S atom of phenylalanine by hydrogen occurs independently of the production of gliotoxin. It has been shown[113] that L-alanine underwent exchange of all hydrogen atoms when incubated in ^2H_2O with pyridoxal phosphate and alanine-glutamate transaminase. It is reasonable to assume that phenylalanine might undergo such an exchange, the exchanging species being, possibly, an enzyme-complexed pyridoxamine derivative of the α-keto acid[112].

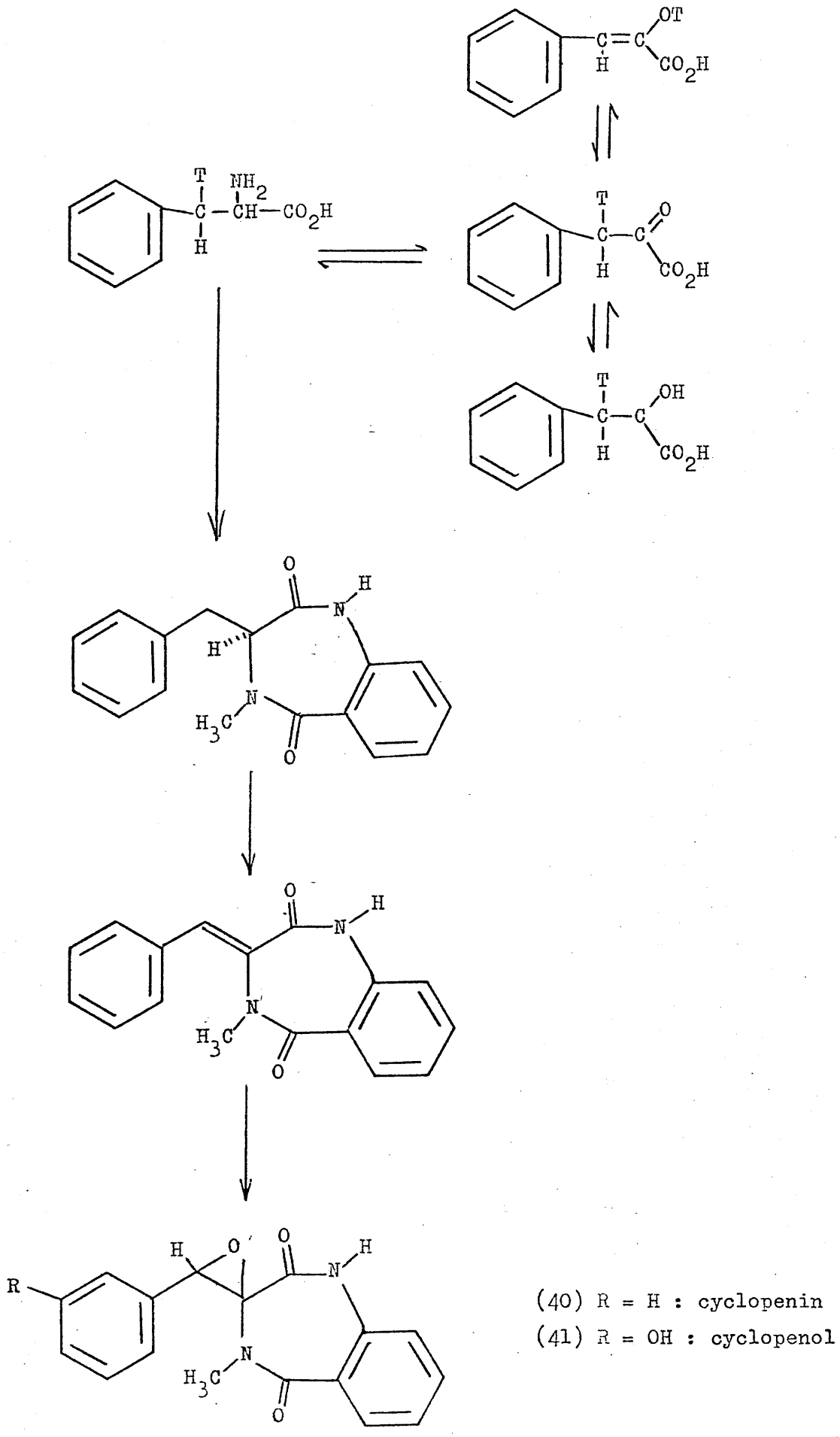
In another study[114], when L-(3'R)-[3'-^2H] and DL-(3'-S)-[3'-^2H]

phenylalanine were fed separately to T. viride along with DL-[1'-^{14}C] phenylalanine, it was found that the gliotoxin derived from the 3'R- labelled precursor carried only a little deuterium, while that from the 3'S- labelled phenylalanine was appreciably enriched with the isotope. This result was confirmed by feeding DL-(3'R)- and DL-(3'S)-[3'-^{3}H]phenylalanine with the same ^{14}C tracer as before. The mycelial protein from this latter experiment also showed loss of the 3'-pro-R label and retention of the 3'-pro-S tritium from the phenylalanine. In a time study, it was found that loss of label from the phenylalanine occurred much faster than incorporation of the precursor into gliotoxin. The proton introduced into the C-3' position of phenylalanine could have come from outside the molecule or from within by a 1,2- shift from C-2' to C-3'. The latter possibility was tested by feeding DL-[2'-^{3}H, 1-^{14}C] phenylalanine to T.viride, however, both the gliotoxin and mycelial protein phenylalanine isolated contained very little tritium. The loss of label could have resulted from rapid reversible transamination within the organism occurring before incorporation into protein, as has been previously shown to occur extensively[56].

In a further study[115], DL-(3'R)-, DL-(3'S)-, DL-(3'RS)-, and L-(3'R)-[3-^{3}H, U-^{14}C] phenylalanine were fed to Penicillium griseofulvum. The mycelianamide isolated after 55 days showed low retention of tritium from the 3S- labelled phenylalanine, but high retention from the 3R- labelled precursor. In the biosynthesis of cryptoechinulin A (144) in Aspergillus spp., the label from the [3'- ^{3}H]tryptophan precursor was found to be stereospecifically retained in the 3'R- position and lost from the 3'S- position.

In a study[115] of the biosynthesis of cyclopenin and cyclopenol in Penicillium cyclopium, D-,L-, and DL-(3'R), D-,L-, and DL-(3'S),

Scheme 26



(40) R = H : cyclopenin

(41) R = OH : cyclopenol

organism, with [1-^{14}C] phenylalanine as a reference label in each organism, with [1-^{14}C] phenylalanine as a reference label in each case. It was found that the tritium loss in each case was greater than 50% of that fed, and that the loss was independent of the configuration at C-3'. A possible explanation for these observations is that the phenylalanine might be reversibly converted into phenylpyruvic acid by a transaminase system. Reversible enolisation of the [3'-^{3}H] phenylpyruvic acid would remove part of the tritium with epimerisation of the remainder. Because of the kinetic isotope effect, hydrogen would be lost faster than tritium during enolisation, hence, enolisation would occur faster than tritium loss. If epimerisation were essentially complete, than half the remaining tritium would be lost in a stereospecific dehydration step[115] (Scheme 26).

The biosynthesis of candipolin in Penicillium canadense was studied using [G-^{3}H], [U-^{14}C] phenylalanine. With such a high generality of tritiation, it is not possible to deduce reliably from the ^{3}H/^{14}C ratios whether the 3'S tritium was lost or retained during the biosynthesis.

APPENDIX 2

Discovery of N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol in an Aspergillus species.

Since completion of the current work, and before its publication, some other workers found N-benzoyl-O-(N-benzoyl-L-phenylalanyl)-L-phenylalaninol in the mycelial extracts of Aspergillus flavipes, and named it asperphenamate[117]. The physical and spectroscopic properties quoted for asperphenamate are identical to those of candipolin (53)[118]. The only difference in the published data lies in the interpretation of the resonances at δ50.3 and 54.6 in the ^{13}C N.M.R. spectra. Clark and Hufford interpret these as being the methine carbon atoms of phenylalaninol and phenylalanine respectively, whereas, we feel that the reverse assignment is true.

This group of workers went on to synthesise the metabolite[119] by essentially the same route as has been used in this work. They used the anhydride of the protected phenylalanine, whereas we used the imidazolide for esterification with N-benzoyl-L-phenylalaninol.

In addition to this mycelial metabolite, N-benzoyl-L-phenylalaninol was isolated from broth extracts.