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Studies in the Biochemistry of Micro-organisms

111. THE PRODUCTION OF L-PHENYLALANINE ANHYDRIDE (*cis*-L-3,6-DIBENZYL-2,5-DIOXOPIPERAZINE) BY *PENICILLIUM NIGRICANS* (BAINIER) THOM*

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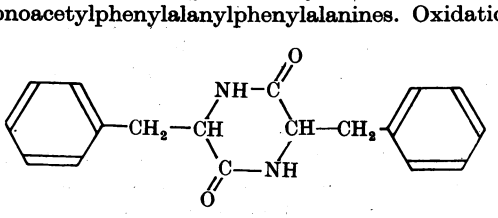
From the culture filtrates of certain strains of *Penicillium nigricans* (Bainier) Thom [= *P. janczewskii* Zaleski] the metabolites griseofulvin and dechlorogriseofulvin, of known structure, have been isolated (MacMillan, 1953). By substituting potassium bromide for potassium chloride in the Czapek–Dox medium the bromo analogue of griseofulvin was obtained (MacMillan, 1954). A further metabolite of the same mould, extractable from the culture fluid by chloroform, was isolated in the form of an antibiotic red pigment, $(C_{10}H_8O_4)_n$, of undetermined structure (Curtis & Grove, 1947).

In the present study of *P. nigricans*, directed more particularly to the mycelial constituents, we have obtained, in addition to griseofulvin and the frequently recorded mould metabolites mannitol and *meso*erythritol, a new optically active nitrogen-containing product. The yield of this product reached a maximum after 7 days' incubation. The yield had fallen considerably at 20 days when griseofulvin was beginning to accumulate. The new product, obtained by acetone extraction of the dried mycelium after removal of lipids by light petroleum, possessed the molecular formula $C_{18}H_{18}N_2O_2$. From a study of its derivatives and breakdown products, the structure of this substance is *cis*-L(−)-3,6-dibenzyl-2,5-dioxopiperazine (L-phenylalanine anhydride) (I). This structure was confirmed by synthesis from L-phenylalanine ethyl ester. Being a substituted dioxopiperazine, the molecule could exist as a *trans*-isomer (*meso* form) or as a *cis*-isomer; only the *cis*-isomer could exist in two optically active forms which together give a racemic modification. The 3,6-dibenzyl-2,5-dioxopiperazine, m.p. 296°, obtained by heating DL-phenylalanine or its ethyl ester, has long been known and may be either the *trans*- or the racemic *cis*-modification, or a mixture of these forms. Vejdělek (1951) has reported the synthesis of the *trans*-isomer from D-phenylalanyl-L-phenylalanine, and of the D-*cis*-isomer by heating D-phenylalanyl-D-phenylalanine for 3 hr. at 200–210°. He records m.p. 315–316°, $[\alpha]_D^{25} + 107°$. No synthesis of the L-form has been described. There is reason to believe that Vejdělek's product was partly racemized by the heat treatment, since our synthetic L-*cis*-isomer produced under much milder conditions had the rotation $[\alpha]_{5461}^{20} - 258 \pm 9°$, in agreement with the natural product, $[\alpha]_{5461}^{20} - 267 \pm 3°$. The optical rotations of the D- and L-forms should be opposite in direction but numerically equal.

The following reactions led to identification of the new fungal product as L-phenylalanine anhydride.

Under drastic conditions *cis*-L-3,6-dibenzyl-2,5-dioxopiperazine gave (+)-diacetyl-3,6-dibenzyl-2,5-dioxopiperazine, which on hydrolysis with methanolic potassium hydroxide yielded two acidic monoacetylphenylalanylphenylalanines. Oxidation



(I)

* Part 110: Birkinshaw & Gowlland (1962).

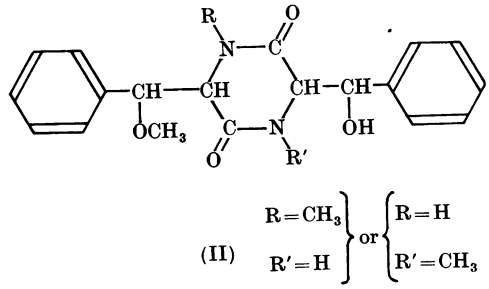
of the diacetyl compound with potassium permanganate gave benzoic acid as the main product, together with benzaldehyde and a compound of empirical formula $C_{11}H_{11}NO_2$ isolated in small amounts.

Methanolic hydrogen chloride opened the dioxopiperazine ring and methylated the liberated carboxyl group to give (+)-L-phenylalanyl-L-phenylalanine methyl ester hydrochloride. This derivative on acetylation gave what we suggest to be (+)-*N*-acetyl-L-phenylalanyl-L-phenylalanine methyl ester, which on hydrolysis of the ester group yielded one of the monoacetyl derivatives obtained by hydrolysis of the diacetyl compound. Acid hydrolysis of (+)-L-phenylalanyl-L-phenylalanine methyl ester hydrochloride gave L-phenylalanine hydrochloride, from which L-phenylalanine was isolated and characterized. Attempts to prepare the L-phenylananyl-L-phenylalanine from the hydrochloride ester were unsuccessful, since it is immediately recyclized to the dioxopiperazine.

When methylated with methyl iodide and silver oxide, the (+)-L-phenylalanyl-L-phenylalanine methyl ester hydrochloride gave a crystalline ester of phenylalanylphenylalanine ωN-trimethylammonium hydroxide in small yield, the major product being an amorphous material; both products on treatment with alkali gave trimethylamine and two isomeric optically inactive cinnamoylphenylalanines. One of these was known previously (Fischer, 1904) and supposed to be the *trans*-form; the other was not known and we suggest that it is the *cis*-form. The structure of the *cis*-cinnamoylphenylalanine was proved through acid hydrolysis which yielded cinnamic acid and DL-phenylalanine.

Methylation of the dipeptide ester hydrochloride with diazomethane or dimethyl sulphate gave anomalous results. By reaction with either the methyl or ethyl ester hydrochloride diazomethane gave the same crystalline compound analysing for $C_{21}H_{25}N_3O_4$, whereas dimethyl sulphate yielded a compound $C_{21}H_{20}N_2O_3$. Neither of these reactions can be interpreted as straightforward methylation and the reactions require further investigation.

The synthesis of the optically inactive phenylalanine anhydride from DL-phenylalanine was carried out by two different procedures (Ovakimian, Kuna & Levene, 1940; Vegotsky, Harada & Fox, 1958) for direct comparison with the optically-active natural form. The infrared spectrum in Nujol of the synthetic material differed slightly from that of the metabolite, and attempts to prepare a synthetic diacetyl compound or a phenylalanylphenylalanine ester hydrochloride from the inactive isomer were unsuccessful. However, a sample of DL-phenylalanine ethyl ester that had been left at room temperature for 15 days gave the



(II) $R=CH_3$, $R'=H$ or $R=H$, $R'=CH_3$

anhydride in small amount. By applying the same treatment to L-phenylalanine ethyl ester, *cis*-L-3,6-dibenzyl-2,5-dioxopiperazine was obtained. The yield could be improved by heating on the water bath for 20 hr. This synthetic product was, in all properties examined including infrared spectrum, identical with the L-phenylalanine anhydride derived from *P. nigricans*.

Other dioxopiperazines in addition to L-phenylalanine anhydride are obtained from fungi. Thus Chen (1960) isolated from the fungus *Rosellinia necatrix* Berlese L-prolyl-L-leucine anhydride, L-prolyl-L-valine anhydride and L-prolyl-L-phenylalanine anhydride. L-Prolyl-L-leucine anhydride has been obtained from *Aspergillus fumigatus* (Johnson, Jackson & Eble, 1951). Direct linkage of the two similar or dissimilar α-amino acids is the most likely mechanism of biosynthesis. Other closely related fungal metabolites, e.g. flavacol, aspergillic acid, hydroxyaspergillic acid and pulcherriminic acid, probably originate in a similar manner, the dioxopiperazine ring initially formed being then modified by oxidation or reduction. MacDonald (1961*a*, *b*) has indeed shown in tracer studies with ^{14}C-labelled leucine and isoleucine that aspergillic acid and hydroxyaspergillic acid are synthesized by direct incorporation of the amino acids.

The structure of picrorocellin (II) obtained from the lichen *Roccella fuciformis* (Forster & Saville, 1922) possesses the same carbon and nitrogen skeleton as L-phenylalanine anhydride. This could arise from phenylalanine anhydride with subsequent oxidation and methylation or from β-hydroxy-β-phenylalanine anhydride with methylation only. A third possibility is appropriate methylation of the hydroxyamino acids before anhydride formation as suggested by Forster & Saville (1922).

EXPERIMENTAL

The combustion analyses were carried out by Dr Ing. A. Schoeller. All melting points are uncorrected.

Organism. The culture employed was isolated by Mr G. Agosti from Irish roadside soil and was identified by Mr G. Smith as *Penicillium nigricans* (Bainier) Thom. It has the L.S.H.T.M. catalogue no. G.A. 658.

Cultural conditions. The culture medium employed was the usual Czapek–Dox medium containing: glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; water to 1·0 l. This was distributed in 350 ml. amounts in 1 l. conical flasks, sterilized by steaming on 3 successive days, sown with a suspension of spores from malt–agar slopes and incubated at 24° in the dark.

Optimum time of incubation for production of L-*phenylalanine anhydride.* In preliminary experiments five flasks were harvested after 7, 14 and 21 days and the mycelium after drying in a vacuum oven was ground up and extracted, first with light petroleum (b.p. 40–60°), then with ether then with acetone. Both the light petroleum and ether left small amounts of gummy residue on evaporation, whereas the acetone extract gave the new metabolite as colourless needles, m.p. 326°; the best yield was obtained at 7 days. To define more closely the optimum incubation period for the preparation of the L-phenylalanine anhydride 25 flasks of culture medium were inoculated and five flasks were harvested after incubation for 5, 7, 9, 14 and 17 days respectively. The filtrates were examined for pH and for residual glucose (by polarimeter), and the L-phenylalanine anhydride was extracted from the mycelium. The results are recorded in Table 1, which shows that the optimum yield of the anhydride is obtained at 7 days.

Identification of other metabolic products. Ten flasks harvested after 24 days' incubation, when the residual glucose had fallen to 0·3%, were examined for other metabolic products. The culture filtrate when extracted with ether gave a very small amount of L-phenylalanine anhydride, m.p. over 300°, and 0·8 g. of crude griseofulvin, m.p. 200°. Extraction of the dried mycelium with ether (after light petroleum) gave crude griseofulvin (1·6 g.), m.p. 200°, and the acetone extract gave L-phenylalanine anhydride (0·07 g.), m.p. over 300°, then mannitol (0·24 g.) and *meso*erythritol (20 mg.), m.p. 100°. After recrystallization from ethanol, the additional products had the following properties: griseofulvin, shining prisms, m.p. 219–220°, $[\alpha]_{5461}^{20}+326\pm3°$ (*c* 0·54 in acetone); mannitol, needles, m.p. 163°; *meso*erythritol, prisms, m.p. 120°. None of these products showed any depression in m.p. on admixture with authentic specimens.

Isolation and properties of L-cis-3,6-*dibenzyl*-2,5-*dioxopiperazine* (L-*phenylalanine anhydride*). The dried ground mycelium (360 g.) from a typical batch of 100 flasks after 7 days' incubation was defatted in a percolator with light petroleum, then extracted with ether. Both extracts on drying formed a gum at this stage of growth. The mycelium was then extracted with acetone to give the metabolite which separated from the acetone solution and was periodically collected. The combined extracted material was washed with water and crystallized from methanol, giving long colourless needles (3 g.), m.p. 326°.

The product, L-cis-3,6-*dibenzyl*-2,5-*dioxopiperazine* L-*phenylalanine anhydride*, was sparingly soluble in most organic solvents and could be crystallized from most of them in needles. It sublimes unchanged in high vacuum at 250°, $[\alpha]_{5461}^{20}-267\pm3°$ (*c* 0·132 in pyridine). It was insoluble in camphor and no colour reactions could be carried out owing to its insolubility (Found: C, 73·1; H, 6·2; N, 9·5. $C_{18}H_{18}N_2O_2$ requires C, 73·5; H, 6·2; N, 9·5%).

Table 1. *Production of* L-*phenylalanine anhydride by* Penicillium nigricans

Experimental details are given in the text.

Incubation period (days)	pH	Glucose in culture filtrate (%)	Yield of L-phenylalanine anhydride (5 flasks) (mg.)
5	4·6	3·4	125
7	4·0	2·2	152
9	4·0	1·85	120
13	4·0	1·29	97
17	4·0	0·81	51

Derivatives

L(+)-cis-*Diacetyl*-3,6-*dibenzyl*-2,5-*dioxopiperazine.* A suspension of L-phenylalanine anhydride (0·2 g.) in a mixture of acetic acid (15 ml.) and acetic anhydride (20 ml.) was refluxed for 10 hr. The reaction mixture was then poured on to crushed ice and left overnight at 0°. The solid (0·2 g.) was collected, dried and repeatedly extracted with hot ether. On concentration the ethereal extracts yielded aggregated needles, m.p. 178°, $[\alpha]_{5461}^{20}+201\pm2°$ (*c* 1·28 in $CHCl_3$), mol.wt. (cryoscopic in camphor) 364. L(+)-cis-*Diacetyl*-3,6-*dibenzyl*-2,5-*dioxopiperazine* is soluble in $CHCl_3$, hot benzene or hot ethanol, insoluble in water or light petroleum (Found: C, 69·6; H, 6·1; N, 7·3. $C_{22}H_{22}N_2O_4$ requires C, 69·8; H, 5·8; N, 7·4%).

DL-*Diacetyl*-3,6-*dibenzyl*-2,5-*dioxopiperazine.* A suspension of L(−)-3,6-dibenzyl-2,5-dioxopiperazine (0·1 g.) in acetic anhydride (20 ml.) was treated with anhydrous sodium acetate (2 g.) and the mixture was refluxed for 10 hr. The DL-*diacetyl*-3,6-*dibenzyl*-2,5-*dioxopiperazine*, when worked up in the usual manner, crystallized from ethanol in needles or plates, m.p. 149°. The product was optically inactive (Found: C, 70·0; H, 5·9; N, 7·5. $C_{22}H_{22}N_2O_4$ requires C, 69·8; H, 5·8; N, 7·4%).

Monoacetylphenylalanylphenylalanine. L(+)-Diacetyl-3,6-dibenzyl-2,5-dioxopiperazine (0·25 g.) was refluxed with methanolic 5% (w/v) KOH (20 ml.) for 3 hr. The reaction mixture was diluted with water and the methanol was evaporated. The solution was extracted with $CHCl_3$ (which removed some of the metabolite) and the aqueous layer was acidified with HCl. The crystalline precipitate which separated was collected (0·19 g.), m.p. 160–220°. This product was boiled with water (20 ml.) and immediately filtered from the insoluble fraction. Concentration of the filtrate gave a product crystallizing in needles, the *monoacetate A*, m.p. 177–178°. This product is soluble in the usual organic solvents, except light petroleum, and in boiling water (Found: C, 67·8; H, 6·3; N, 7·8. $C_{20}H_{22}N_2O_4$ requires C, 67·8; H, 6·2; N, 7·9%).

The water-insoluble product, which crystallized from aqueous ethanol in needles, was the *monoacetate B*, m.p. 247°. It is soluble in organic solvents except light petroleum, and insoluble in boiling water (Found: C, 67·9; H, 6·3; N, 8·0. $C_{20}H_{22}N_2O_4$ requires C, 67·8; H, 6·2; N, 7·9%).

L(+)-*Phenylalanylphenylalanine methyl ester hydrochloride.* A suspension of L-phenylalanine anhydride (0·25 g.) in methanolic HCl (150 ml.) was refluxed for 3 hr. The metabolite gradually dissolved. The reaction mixture was evaporated to dryness under reduced pressure and the

crystalline residue (0·25 g.) was recrystallized from methanol–ether (1:50, v/v), giving needles of L(+)-*phenylalanylphenylalanine methyl ester hydrochloride*, m.p. 195° (decomp.), $[\alpha]_{5461}^{20}+13\pm0·3°$ (*c* 2·2 in water). When this product is treated with aqueous $NaHCO_3$ or Na_2CO_3 it quickly reverts to the original metabolite (Found: C, 62·6; H, 6·0; N, 7·8; Cl, 10·0; OMe, 8·0. $C_{19}H_{22}N_2O_3$, HCl requires C, 63·0; H, 6·3; N, 7·7; Cl, 9·8; 1 OMe, 8·6%).

L(+)-*Phenylalanylphenylalanine ethyl ester hydrochloride.* This derivative was prepared as described for the corresponding methyl ester. From ethanol–ether (1:50, v/v) L(+)-*phenylalanylphenylalanine ethyl ester hydrochloride* forms needles, m.p. 167° (decomp.), $[\alpha]_{5461}^{20}+16\pm0·5°$ (*c* 2·28 in water) (Found: C, 63·4; H, 6·6; N, 7·8; Cl, 9·3. $C_{20}H_{24}N_2O_3$,HCl requires C, 63·7; H, 6·4; N, 7·4; Cl, 9·4%).

N-*Acetyl*-L-*phenylalanylphenylalanine esters.* A solution of L(+)-phenylalanylphenylalanine methyl ester hydrochloride (0·15 g.) in pyridine (5 ml.) was treated with acetic anhydride (6 ml.) and the reaction mixture was kept overnight at room temperature. It was then poured into cold water and the crystalline precipitate (0·15 g.) was collected. N-*Acetyl*-L-*phenylalanylphenylalanine methyl ester* crystallized from water in needles, m.p. 177°, $[\alpha]_{5461}^{20}+37\pm1°$ (*c* 0·89 in $CHCl_3$) (Found: C, 68·5; H, 6·8; N, 7·7; OMe, 83. $C_{21}H_{24}N_2O_4$ requires C, 68·5; H, 6·8; N, 7·6; 1 OMe, 8·4%).

By similar treatment the ethylated hydrochloride gave N-*acetyl*-L-*phenylalanylphenylalanine ethyl ester* as needles, m.p. 154°, $[\alpha]_{5461}^{20}-12·6\pm0·5°$ (*c* 2·0 in methanol) (Found: C, 69·2; H, 6·3; N, 7·8. $C_{22}H_{20}N_2O_4$ requires C, 69·4; H, 6·8; N, 7·3%).

N-*Acetyl*-DL-*phenylalanylphenylalanine.* A solution of *N*-acetyl-L(+)-phenylalanylphenylalanine methyl ester (0·1 g.) in 0·1 N-NaOH (15 ml.) and a few drops of methanol was heated on the water bath for 30 min. and allowed to cool. On acidification with HCl, a crystalline precipitate (0·10 g.) was obtained which, after recrystallization, had m.p. 248°. This proved to be identical with the monoacetate B obtained from the alkaline hydrolysis of L(+)-diacetyl-3,6-dibenzyl-2,5-dioxopiperazine, since the m.p. was unchanged on admixture.

Degradations

Oxidation of L(+)-*diacetyl*-3,6-*dibenzyl*-2,5-*diketopiperazine by potassium permanganate.* $KMnO_4$ (1·5 g.) was added to a solution of the diacetyl derivative (0·28 g.) in dry acetone (100 ml.) and the solution was refluxed for 4 hr. The MnO_2–organic salts mixture was collected and washed with anhydrous acetone. The organic salts were then extracted from the MnO_2 by warm water and the filtrate was extracted with ether in a continuous extractor. The residue from the ether smelt strongly of benzaldehyde and gave a crystalline precipitate with 2,4-dinitrophenylhydrazine in HCl. This product, m.p. 237°, after recrystallization, proved to be benzaldehyde 2,4-dinitrophenylhydrazone since it showed no depression in m.p. when mixed with an authentic specimen. The aqueous extract was acidified with HCl and again extracted. The ether residue gave benzoic acid (0·17 g.; 90% yield), m.p. 122°, unchanged on admixture with authentic material.

The acetone filtrate and washings from the oxidation process were evaporated to dryness and the residue was extracted with boiling ether. The crystalline residue from the ether, when recrystallized from ethanol, gave a *product*, m.p. 193°, as shining plates (30 mg.) (Found on product dried at 100° *in vacuo*: C, 70·0; H, 6·0; N, 7·6. $C_{11}H_{11}NO_2$ requires C, 69·8; H, 5·8; N, 7·4%).

Methyl ester of phenylalanylphenylalanine ωN-*trimethylammonium hydroxide.* A solution of L(+)-phenylalanylphenylalanine methyl ester hydrochloride (1 g.) in methanol (20 ml.) was treated with a large excess of methyl iodide (20 ml.) and silver oxide (5 g.). The mixture was kept overnight at 0°, and the precipitated silver salts were separated by filtration and washed with methanol. The combined filtrates were evaporated to dryness on the water bath and the gummy residue was dissolved in water (30 ml.). The crystalline product (70 mg.) obtained on keeping the solution was collected and recrystallized. The product was the *methyl ester of phenylalanylphenylalanine* ωN-*trimethylammonium hydroxide*, as needles, m.p. 241° (decomp.), soluble in ethanol or boiling water but not in ether (Found: C, 67·8; H, 7·7; N, 7·7. $C_{22}H_{30}N_2O_4$ requires C, 68·4; H, 7·8; N, 7·3%).

The filtrate, after the separation of the methyl ester, was concentrated under reduced pressure and dried, giving an amorphous solid (0·90 g.), which could be obtained in granular form from methanol–acetone.

Action of N-*sodium hydroxide on the methyl ester of phenylalanylphenylalanine* ωN-*trimethylammonium hydroxide or on the amorphous solid.* The amorphous solid (0·90 g.) was suspended in N-NaOH (70 ml.) and heated for 4 hr. on the water bath in a stream of nitrogen. The volatile base was carried off and was trapped in picric acid solution. The picrate (0·58 g.) was collected from the trap and recrystallized. It had m.p. 216° which was not depressed on admixture with trimethylamine picrate.

The alkaline reaction mixture was acidified with dilute H_2SO_4 and the precipitated product was extracted with ether. A crystalline residue (0·26 g.) was obtained from the ether, which on washing with ether and recrystallization yielded *trans*-cinnamoyl-DL-phenylalanine as needles, m.p. 202° (lit. 199°), optically inactive (Found: C, 73·2; H, 6·0; N, 4·9. Calc. for $C_{18}H_{17}NO_3$: C, 73·2; H, 5·8; N, 4·7%). The combined ether washings from *trans*-cinnamoyl-DL-phenylalanine, allowed to evaporate slowly, yielded another crystalline product (0·35 g.). By recrystallization from ethanol–water, fine needles of optically inactive *cis-cinnamoyl*-DL-*phenylalanine* were obtained, m.p. 164° (Found: C, 73·3; H, 5·8; N, 5·1. $C_{18}H_{17}NO_3$ requires C, 73·2; H, 5·8; N, 4·7%).

Acid hydrolysis of cis-*cinnamoyl*-DL-*phenylalanine. cis*-Cinnamoyl-DL-phenylalanine (0·32 g.) was suspended in 4N-HCl (150 ml.) and the reaction mixture was boiled under reflux for 6 hr. during which the solid completely dissolved. After cooling, the precipitated cinnamic acid (0·14 g.) was collected and crystallized from water. It was obtained as needles, m.p. 134°, unchanged on admixture with an authentic specimen (Found: C, 72·8; H, 5·2. Calc. for $C_9H_8O_2$: C, 73·0; H, 5·4%).

The acidic filtrate was evaporated to dryness under reduced pressure and the resulting DL-phenylalanine hydrochloride (0·16 g.) crystallized from methanol–acetone in needles, m.p. 214°. DL-Phenylalanine was obtained from the hydrochloride by dissolving in water and neutralizing with silver carbonate. The filtrate was dried off and the residue was sublimed, then crystallized from water–

ethanol. The product, DL-phenylalanine, was obtained as needles, m.p. 267° (decomp.), unchanged on admixture with an authentic specimen. The infrared spectra were also identical.

Hydrolysis of L(+)-*phenylalanylphenylalanine methyl ester hydrochloride.* A solution of L(+)-phenylalanylphenylalanine methyl ester hydrochloride (0·5 g.) in 4N-HCl (200 ml.) was refluxed for 10 hr. The reaction product after drying under reduced pressure was crystallized from methanol–acetone, giving L-phenylalanine hydrochloride as needles, m.p. 235°, $[\alpha]_{5461}^{20} - 9 \pm 0{\cdot}1°$ (*c* 10·8 in water). L-Phenylalanine was obtained from the hydrochloride by neutralization with silver carbonate and purification of the crude product by sublimation and crystallization from water–ethanol. It formed fine needles, m.p. 283° (decomp.) (alone or when mixed with authentic material), $[\alpha]_{5461}^{20} - 35 \pm 1°$ (*c* 2·95 in water).

Action of diazomethane on L(+)-*phenylalanylphenylalanine methyl or ethyl ester hydrochloride.* A solution of L-phenylalanylphenylalanine methyl ester hydrochloride (0·3 g.) in methanol (20 ml.) was treated with ethereal 1% (w/v) diazomethane solution (150 ml.) and the mixture was kept at room temperature for 24 hr. Slow evaporation of the solvent gave the product as needles (crystallized from ethanol–water), m.p. 202° (Found: C, 65·8; 65·8, H, 6·4, 6·5; N, 11·1, 11·0; OMe, 8·2. $C_{21}H_{25}N_3O_4$ requires C, 65·8; H, 6·5; N, 11·0; 1 OMe, 8·1%). The ethyl ester hydrochloride under similar treatment yielded the same product, m.p. and mixed m.p. 203°.

Action of methyl sulphate on L(+)-*phenylalanylphenylalanine methyl ester hydrochloride.* To a solution of the ester hydrochloride (0·42 g.) in aq. 10% (w/v) NaOH (50 ml.), dimethyl sulphate (5 ml.) was added dropwise during 1·5 hr., with stirring. The reaction mixture was then heated for 1 hr. on the water bath and extracted with $CHCl_3$. The extract left a gummy residue (0·27 g.) which failed to crystallize. Acidification of the aqueous layer and extraction with $CHCl_3$ gave a gummy residue which later crystallized. Recrystallization from $CHCl_3$–light petroleum or from ethanol–water gave the product as needles, m.p. 163° (Found: C, 72·6; H, 5·8; N, 8·3. $C_{21}H_{20}N_2O_3$ requires C, 72·4; H, 5·8; N, 8·0%).

Syntheses

Formation of DL-3,6-*dibenzyl*-2,5-*dioxopiperazine.* DL-Phenylalanine ethyl ester (1 g.) kept at room temperature for 15 days formed fine needles. The amount increased with time and, after 2 months, the solid was separated from DL-phenylalanine ethyl ester by washing with ethanol. The resulting solid (70 mg.) crystallized from ethanol in needles, m.p. 296°, unaltered on mixing with authentic samples of DL-3,6-dibenzyl-2,5-dioxopiperazine synthesized according to Ovakimian *et al.* (1940) and Vegotsky *et al.* (1958).

Synthesis of L(−)-cis-3,6-*dibenzyl*-2,5-*dioxopiperazine.* L(−)-Phenylalanine ethyl ester (0·5 g.) was kept at room temperature for 15 days, then heated on the water bath for 20 hr. The solid (60 mg.) was washed with methanol to remove unchanged ester and crystallized from methanol. The L(−)-*cis*-3,6-dibenzyl-2,5-dioxopiperazine had m.p. 326°, unchanged on mixing with the metabolite, $[\alpha]_{5461}^{20} - 258 \pm 9°$ (*c* 0·163 in pyridine). The infrared spectrum showed complete identity with that of the metabolite (Found: C, 73·7; H, 6·2; N, 9·5. Calc. for $C_{18}H_{18}N_2O_2$: C, 73·5; H, 6·2; N, 9·5%).

SUMMARY

1. From the dried mycelium of a strain of *Penicillium nigricans* (Bainier) Thom grown on Czapek–Dox glucose medium the following metabolites were extracted: mannitol, *meso*erythritol, griseofulvin and a new product, L-phenylalanine anhydride [*cis*-L(−)-3,6-dibenzyl-2,5-dioxopiperazine). The yield of the last named was maximal after 7 days' incubation.

2. The structure of the new metabolite was deduced from a study of its derivatives and breakdown products. It was also compared with a synthetic sample of L-phenylalanine anhydride prepared under mild conditions from L-phenylalanine ethyl ester. The synthetic and natural products were found to be identical.

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