Biosynthesis of Aromatic Isoprenoids. Part 5.[1] The Preparation of 1-(3,3-Dimethylallyl)-L-tryptophan and *cyclo*-L-Alanyl-1-(3,3-dimethylallyl)-L-tryptophan and their Non-incorporation into Echinulin

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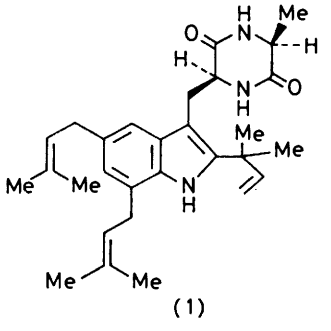
1-([1-^{3}H]-3,3-Dimethylallyl)-L-tryptophan and *cyclo*-L-alanyl-1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan have been synthesised. Neither compound served as a precursor of echinulin in surface cultures of *Aspergillus amstelodami*. In parallel feeding experiments, DL-[2-^{14}C]-mevalonic acid lactone and [*methylene*-^{14}C]-L-tryptophan were efficiently incorporated into echinulin.

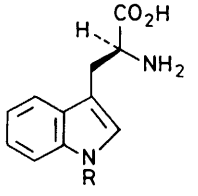
THE biosynthesis of echinulin (1), and of related fungal metabolites, has been extensively investigated.[2] The early work established that the basic framework of echinulin derived from the amino-acids L-tryptophan [3] (2a) and L-alanine.[4] Mevalonic acid [4] or L-leucine [5] served as precursors for the three prenyl substituents at C-2, C-5, and C-7 of the indole moiety, presumably *via* the formation of dimethylallyl pyrophosphate.

The sequence of events in the biosynthesis of echinulin is of crucial importance to the discussion below. *cyclo*-L-Alanyl-L-tryptophan (3a) was shown to be an efficient precursor of echinulin in *Aspergillus amstelodami*.[6] The same diketopiperazine formed a monoprenyl derivative, tentatively identified as compound (3b), when incubated with dimethylallyl pyrophosphate in the presence of a cell-free enzyme system from *A. amstelodami*;[7] in turn, the monoprenyl derivative was converted by the living fungus into echinulin in 14% yield.[8] From these data it was proposed that the substituent at C-2 of the indole moiety is introduced prior to those at C-5 and C-7 during the biosynthesis of echinulin. Isolation studies lend support to this suggestion; for example *cyclo*-L-alanyl-L-tryptophan [9] (3a) and the 2-prenyl derivative [10] (3b) co-occur in *A. chevalieri*. Furthermore both *A. ruber* [11] and *A. amstelodami* [12] contain diketopiperazine metabolites related structurally to (3b). Hence the biosynthesis of echinulin occurs, most probably, *via* the sequence: (2a)→(3a)→(3b)→(1).

Attention has also been given to the mechanisms by which the prenyl substituents of echinulin are introduced. It has been established that the methylene protons [13] and the C-4 and C-6 protons [14] are retained during *in vivo* conversion of tryptophan into echinulin. Furthermore the stereochemistry of the introduction from mevalonate of the C-5 and C-7 prenyl substituents into echinulin has been investigated.[15] Our primary interest

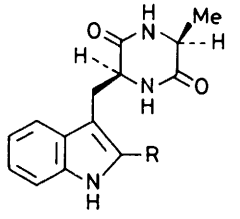


(1)



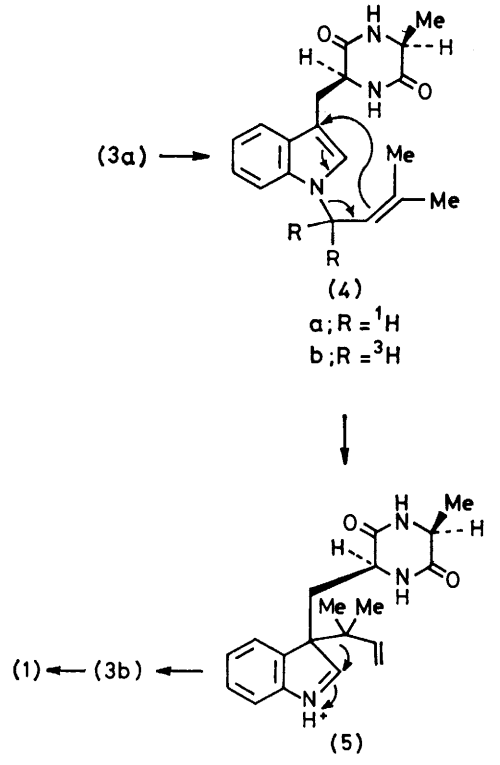
(2)

a; R = H
b; R = $CH_2CH{=}CMe_2$
c; R = $C^3H_2CH{=}CMe_2$



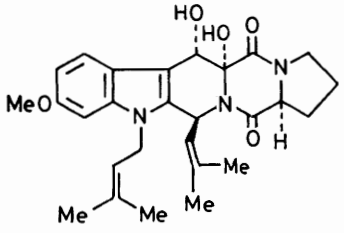
(3)

a; R = H
b; R = $CMe_2CH{=}CH_2$

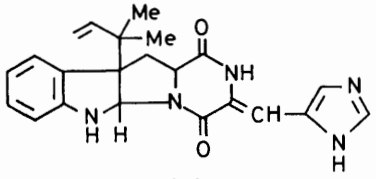


SCHEME

in the biosynthesis of echinulin concerns the mode of introduction of the ' reversed ' prenyl substituent at C-2 of the indole moiety. We felt that this substituent may be introduced biosynthetically *via* *N*-prenylation of the cyclic dipeptide (3a) (or of tryptophan), followed by sequential aza-Claisen and Plancher-like rearrangements as outlined in the Scheme. The occurrence of the *N*-prenylindole derivative fumitremorgin B (6) [16] and of related metabolites [17] in *Aspergillus* species supports this



(6)



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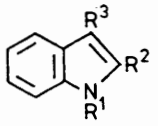
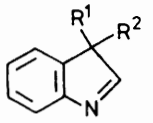


(8)

a; $R^1 = CH_2CH{=}CHMe$, $R^2 = R^3 = H$
b; $R^1 = R^2 = H$, $R^3 = CH(Me)CH{=}CH_2$
c; $R^1 = CH_2CH{=}CMe_2$, $R^2 = H$, $R^3 = Me$
d; $R^1 = H$, $R^2 = CMe_2CH{=}CH_2$, $R^3 = Me$
e; $R^1 = H$, $R^2 = CH_2CH{=}CMe_2$, $R^3 = Me$



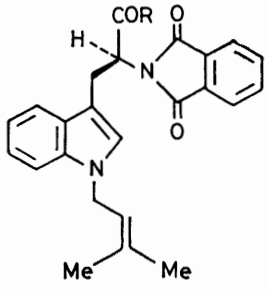
(9)

a; $R^1 = H$, $R^2 = CH(Me)CH{=}CH_2$
b; $R^1 = Me$, $R^2 = CMe_2CH{=}CH_2$

suggestion. Furthermore the existence of roquefortine (7) [18] and of other 3-(1,1-dimethylallyl)-2,3-dihydroindole derivatives [19] in related species may be rationalised by intramolecular cyclisation of 3,3-dialkyl-3*H*-indole biosynthetically related to (5). Useful *in vitro* evidence may be cited in support of the Scheme. In particular *N*-crotylindole (8a) formed the 3-(1-methylallyl) derivative (8b) at elevated temperatures, presumably *via* the product (9a) of aza-Claisen rearrangement.[20] However, other model *N*-allylindole derivatives were thermally stable [21] and the reverse rearrangement (9b)→(8c) has been observed indirectly.[22] On the other hand, Casnati and Pochini have reported the facile rearrangement of the (dimethylallyl)indole (8c) to the 2-substituted-indoles (8d) and (8e) in trifluoroacetic acid.[23] The ' reversed ' prenyl substituent in (8d) is believed to arise *via* acid-catalysed aza-Claisen rearrangement to (9b), followed by acid-catalysed 1,2-migration of the prenyl group.[24] On the basis of these considerations, we decided to synthesise 1-(dimethylallyl)-L-tryptophan (2b) and the derived diketo-piperazine (4a) and test their efficiencies as biosynthetic precursors of echinulin.

RESULTS AND DISCUSSION

1-(3,3-Dimethylallyl)-L-tryptophan (2b) was prepared in 38% yield by reaction of 3,3-dimethylallyl bromide with the disodium salt of L-tryptophan in liquid ammonia.[25] The constitution of the product was established by the further transformations described below and by the n.m.r. spectrum ([$^2H_6$]DMSO) which showed, *inter alia*, two three-proton singlets at τ 8.18 and 8.29 ($CH_2CH{=}CMe_2$), a one-proton triplet (*J* 6 Hz) at τ 4.68 ($CH_2CH{=}CMe_2$), and a two-proton doublet (*J* 6 Hz) at



(10)

a; R = OH
b; R = L-NHCHCO$_2$Et (Me)

τ 5.32, assigned to the methylene protons of an *N*-(3,3-dimethylallyl) substituent. 1-(Dimethylallyl)tryptophan (2b) was treated with *N*-ethoxycarbonylphthalimide in the presence of sodium hydride to give, in 54% yield, the *N*-phthaloyl derivative (10a). The latter compound reacted smoothly with L-alanine ethyl ester and dicyclohexylcarbodi-imide to furnish the protected dipeptide (10b) in 83% yield. Removal of the phthaloyl protecting group from (10b), by treatment with ethanolic hydrazine, was accompanied by cyclisation of the intermediate dipeptide ester to give the desired diketopiperazine (4a) in 72% yield. The constitution and stereochemical integrity of the latter was fully supported by the spectral data. In particular the i.r. spectrum showed bands at 1 680, 1 460, and 1 345 cm^{-1}, appropriate for a diketopiperazine [2a] and the ^1H n.m.r. spectrum ([$^2H_6$]DMSO) showed a doublet due to the alanyl methyl group at high field (τ 9.54) consistent only with

the *cis*-diketo-piperazine formulation indicated in (4a).[26] Furthermore the o.r.d. curve of (4a) (see Experimental section) showed satisfactory correspondence with that reported [27] for *cyclo*-L-alanyl-L-tryptophan (3a), in support of the stereochemistry assigned.

Tritium-labelled dimethylallyl-L-tryptophan (2c) was prepared by the condensation of labelled dimethylallyl *bromide* [28] *with* the disodium salt of tryptophan; the product was converted to the labelled diketopiperazine (4b) essentially as described above.

The labelled putative precursors (2c) and (4b) were dissolved in DMSO prior to feeding to surface cultures of *A. amstelodami*, owing to their low solubility in water; the use of DMSO in this manner has been shown to have no deleterious effect on the organism.[6,8] The observed incorporations into echinulin (see Table) were unexpectedly low. In contrast the known precursors DL-mevalonic acid lactone [4] and L-tryptophan [3] were efficiently incorporated into echinulin in our hands (see Table). Hence we are forced to conclude that 1-(3,3-dimethylallyl)tryptophan (2b) and the derived diketopiperazine (4a) are unlikely to be precursors of echinulin. Since it was argued above that the biosynthesis of echinulin follows the route (2a)→(3a)→(3b)→(1), the non-incorporation of the prenylated diketopiperazine (4a) in this study precludes the intermediacy of 1-(dimethylallyl)indole derivatives during the biosynthesis of echinulin.

Tracer experiments with *Aspergillus amstelodami*

Compound fed	Incorporation (%) into echinulin (1)
[2-^{14}C]-DL-Mevalonic acid lactone	1.3, 1.5, and 1.4
[*Methylene*-^{14}C]-L-tryptophan	2.1, 2.05, and 1.95
1-([1-^{3}H]-1,1-Dimethylallyl)-L-tryptophan (2c)	≤0.05
cyclo-L-Alanyl-1-([1-^{3}H]-1,1-dimethylallyl)-L-tryptophan (4b)	≤0.016, and ≤0.019

While this paper was in preparation Barrow *et al.* reported that the C-2 proton of tryptophan is lost during *in vivo* conversion to roquefortine (7).[29] This interesting observation is consistent with an alternative suggestion, by Bycroft and Landon,[30] for the biosynthesis of echinulin and related compounds.

EXPERIMENTAL

I.r. spectra were measured for KBr discs with Perkin-Elmer 157 and 457 spectrometers. N.m.r. spectra were recorded at 90 MHz with a Perkin-Elmer R32 spectrometer for $CDCl_3$ solutions (unless otherwise stated), using an internal tetramethylsilane lock.

Aspergillus amstelodami (Strain IMI 17455), was supplied by the Commonwealth Mycological Institute, Kew, Surrey, and was maintained on Czapek Dox agar slopes stored at 4 °C. All operations with the mould were conducted under sterile conditions.

[*Methylene*-^{14}C]-L-trytophan and DL-[2-^{14}C]mevalonic acid lactone were purchased from The Radiochemical Centre, Amersham.

Scintillation counting was performed as previously described [31] except that echinulin (*ca.* 0.3 mg) was dissolved in DMSO (50 μl) prior to addition of the POP–POPOP toluene-based liquid scintillation mixture (10 ml). Carbon-14 and ^{3}H were counted at efficiencies of *ca.* 80 and 30%, respectively.

Light petroleum refers to the fraction of b.p. 60—80 °C.

1-(3,3-*Dimethylallyl*)-L-*tryptophan* (2b).—Sodium metal (2.3 g) was added to a stirred solution of iron(III) nitrate (0.1 g) in dry liquid ammonia cooled in a solid carbon dioxide–acetone bath. After 30 min, when the initial blue colour had discharged, L-tryptophan (10.00 g) was added and stirring was continued for 1 h. 1-Bromo-3-methylbut-2-ene (8.14 g) was then added dropwise during 1 h with vigorous stirring. The cooling bath was subsequently removed and the ammonia allowed to evaporate overnight. The resultant gummy solid was dissolved in the minimum volume of water, which was immediately extracted with ethyl acetate. The aqueous phase was neutralised with 4M hydrochloric acid and stored at 0 °C overnight. The precipitate which formed was recrystallised from methanol to yield 1-(3,3-*dimethylallyl*)-L-*tryptophan* (5.17 g, 38%), m.p. 201—202 °C (decomp.); $\nu_{max.}$ 3 440, 3 300—2 300, 2 050w, 1 620(sh), 1 590, 1 520, 1 470, 1 420, 1 365, 1 315, and 740 cm^{-1} (Found: C, 70.2; H, 7.1; N, 10.0; $C_{16}H_{20}N_2O_2$ requires C, 70.6; H, 7.4; N, 10.3%).

N-*Phthaloyl*-1-(3,3-*dimethylallyl*)-L-*tryptophan* (10a).—*N*-Ethoxycarbonylphthalimide (4.2 g) was stirred overnight with a suspension of 1-(3,3-dimethylallyl)-L-tryptophan (5.42 g) and sodium hydride (50% dispersion in oil, 1.0 g) in dimethylformamide (50 ml). 0.5M Hydrochloric acid (200 ml) was added and the mixture extracted with chloroform. The extract was washed with water, dried over magnesium sulphate, and evaporated *in vacuo*. The resultant gummy solid was applied to a column of silica gel (200 g) which was eluted with chloroform–ethyl acetate (4 : 1, v/v) to yield the *phthaloyl derivative* (4.54 g), m.p. 131—132 °C (from benzene–light petroleum); $[\alpha]_D^{30}$ −174.5° ($CHCl_3$, *c* 1); $\nu_{max.}$ 3 300—2 200, 1 780, 1 720s, br, 1 620, 1 470, 1 440, 1 390, 1 275, 1 210br, 1 115, 1 018, 995, 950, 878, 748, 720, 655, and 630 cm^{-1}; τ 2.15—2.6 (5 H, m), 2.7—3.1 (3 H, m), 3.15 (1 H, s), 4.7—5.0 (2 H, m), 5.51 (2 H, d, *J* 7 Hz), 6.28 (2 H, d, *J* 8 Hz), 8.33 (3 H, s), and 8.36 (3 H, s) (Found: C, 71.3; H, 5.4; N, 7.2. $C_{24}H_{22}N_2O_4$ requires C, 71.6; H, 5.5; N, 7.0%).

N-*Phthaloyl*-1-(3,3-*dimethylallyl*)-L-*tryptophyl*-L-*alanine Ethyl Ester* (10b).—L-Alanine ethyl ester hydrochloride (2.50 g) was dissolved in 1M aqueous sodium hydroxide (20 ml) and extracted with dichloromethane (2 × 50 ml). The organic phase was dried over potassium carbonate and filtered. To the resulting solution was added the *N*-phthaloyl derivative (10a) (4.0 g) and *NN*-dicyclohexylcarbodi-imide (2.0 g). The solution was stirred for 6 h, and the precipitate of dicyclohexylurea was then removed by filtration. The dichloromethane phase was washed sequentially with 1M hydrochloric acid (2 × 100 ml), 1M sodium hydroxide solution (2 × 100 ml), and water (100 ml). The dried organic phase was evaporated *in vacuo* to yield a solid product which crystallised from ether–light petroleum to yield the *dipeptide derivative* (4.16 g, 83%), m.p. 93—94 °C; $[\alpha]_D^{30}$ −59.8° ($CHCl_3$, *c* 0.5); τ 2.30 (5 H, m), 2.85 (3 H, m), 3.05 (1 H, s), 3.25, (1 H, d, *J ca.* 8 Hz), 4.8 (2 H, m), 5.45 (3 H, m), 5.85 (2 H, q, *J* 7 Hz), 6.3 (2 H, m), 8.30 (3 H, s), 8.34 (3 H, s), 8.65 (3 H, d, *J* 7 Hz), and 8.75 (3 H, t, *J* 7 Hz) (Found: C, 69.2; H, 6.3; N, 8.0. $C_{29}H_{31}N_3O_5$ requires C, 69.4; H, 6.2; N, 8.4%).

cyclo-L-*Alanyl*-1-(3,3-*dimethylallyl*)-L-*tryptophan* (4a).—The protected dipeptide (10b) (3.00 g) and hydrazine hydrate (0.50 g) were added to ethanol (50 ml) and refluxed for 1 h. Solvent was removed *in vacuo* and the residual solid stirred with 1M aqueous sodium hydroxide (20 ml) and filtered. The crude product was recrystallised from ethanol to afford the *title compound* (1.40 g), m.p. 223—224 °C; $\nu_{max.}$ 3 170, 3 060(sh), 3 030, 2 950, 2 910, 2 860(sh), 1 680s, b, 1 460, 1 430, 1 322, and 728 cm^{-1}; o.r.d. (ethanol, *c* 0.01); $[\phi]_{205}$ +22 100, $[\phi]_{213}$ 0, $[\phi]_{225}$ −35 100 min., $[\phi]_{241}$ 0, $[\phi]_{246}$ +950 max., $[\phi]_{255}$ +260, $[\phi]_{270}$ 0, $[\phi]_{280}$ −390 tr., $[\phi]_{286}$ 0, $[\phi]_{293}$ +500 infl., $[\phi]_{300}$ +1 040 pk., $[\phi]_{305}$ +520, and $[\phi]_{320}$ +260; τ ([$^2H_6$]DMSO) 2.0—3.1 (5 H, m), 4.73 (1 H, t, *J* 7 Hz), 5.33 (2 H, d, *J* 7 Hz), 5.9 (1 H, m,) 6.4 (1 H, m,) 6.87 (2 H, m), 8.20 (3 H, s), 8.30 (3 H, s), and 9.52 (3 H, d, *J* 7.5 Hz) (Found: C, 70.2; H, 7.1; N, 13.2. $C_{19}H_{23}N_3O_2$ requires C, 70.2; H, 7.1; N, 12.9%).

Preparation of Tritium-labelled Precursors.—[1-^{3}H]-3,3-Dimethylallyl bromide [28] (*ca.* 4.2 × 10^6 disintegrations s^{-1} $mmol^{-1}$) was condensed with the disodium salt of L-tryptophan as described above to yield 1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan (2c). The latter was converted by the route described above to *cyclo*-L-alanyl-1-([1-^{3}H]-3,3-dimethylallyl)-L-tryptophan (4b) of activity 4.66 × 10^6 disintegrations s^{-1} $mmol^{-1}$.

Feeding of Labelled Compounds.—cyclo-L-*Alanyl*-1-([1-^{3}H]-3,3-*dimethylallyl*)-L-*tryptophan* (4b). *Method* 1. Aqueous Czapek Dox medium (1 500 ml) supplemented with sucrose (30%) was distributed equally between six 1-l culture flasks, which were loosely plugged with non-absorbent cotton wool. The flasks were autoclaved for 20 min at 120 °C (15 lb in^{-2}).

A small Petri dish containing Czapek Dox agar was innoculated with the fungus and incubated at 29 °C for 4 d. The agar was then macerated with distilled water (30 ml) in a Waring Blendor and the resultant suspension used immediately to innoculate the above liquid culture flasks. The labelled precursor (50 mg; activity 4.66 × 10^6 disintegrations s^{-1} $mmol^{-1}$) was dissolved in dimethyl sulphoxide (0.6 ml) and the resultant solution distributed equally among the six culture flasks which were vigorously swirled. The flasks were then allowed to stand undisturbed for 14 d at 29 °C.

The thick yellow mycelium which formed was filtered at the pump, dried *in vacuo*, and crushed to a yellow powder (20 g), which was extracted sequentially in a Soxhlet apparatus with light petroleum, ether, and chloroform. The ether extract was recrystallised once from ether to yield a buff-coloured solid which was combined with the solid obtained by the chloroform extraction. One recrystallisation from ethanol gave echinulin (typically 315 mg), m.p. 232—233 °C (lit.,[4] 230—242 °C) which showed one spot on t.l.c. [R_F 0.27 on silica gel, eluted with methanol–ethyl acetate (2 : 98 v/v)]. Further recrystallisation from ethanol yielded pure echinulin, m.p. 240—241 °C, activity ⩽1.57 × 10^2 disintegrations s^{-1} $mmol^{-1}$, equivalent to an incorporation of 0.019% based on the initial weight of echinulin.

Method 2. The procedure was identical to that described above except that the DMSO solution of the precursor was added to the aqueous culture medium 4 d after inoculation. The mycelium was permitted to grow for a further 10 d at 29 °C before harvesting. The incorporation of activity into echinulin was ⩽0.016%.

1-([1-^{3}H]-3,3-*Dimethylallyl*)-L-*tryptophan* (2c) was fed as described above (Method 1).

[Methylene-^{14}C]-L-*tryptophan* (3 μCi) diluted with unlabelled L-tryptophan (50 mg) was added as a sterile aqueous solution (5 ml) to the culture medium (1 500 ml) immediately after innoculation.

DL-[2-^{14}C]-*Mevalonic acid lactone* (16 μCi) in dilute aqueous potassium carbonate (1.5 ml) was added to the culture medium (1 500 ml) immediately following inoculation.

Incorporations observed into echinulin are recorded in the Table.

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