Stereochemical Studies on the Biosynthesis of the αβ-Didehydro-amino-acid Units of Mycelianamide, Cyclopenin, and Cyclopenol

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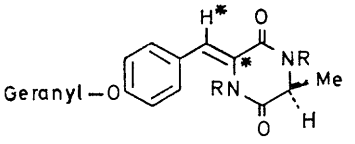
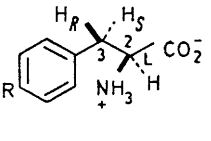
Feeding experiments with tyrosine, stereoselectively labelled with tritium at C-3, in *Penicillium griseofulvum* have shown that formation of the αβ-didehydro-amino-acid units of mycelianamide proceeds with high retention of the *pro-R*- and virtually complete loss of the *pro-S*-methylene hydrogen atom of the precursor. Analogous studies were conducted with phenylalanine in *Penicillium cyclopium*. Incorporation of [3-^{3}H]phenylalanine into the secondary metabolites, cyclopenin and cyclopenol, occurred with >50% (typically 65%) loss of tritium, irrespective of the tritium configuration at C-3. This non-stereospecific loss is attributed to an exchange process preceding entry of the precursors into the secondary metabolic pathway. In accord with this, incorporation of [^{3}H]phenylalanine into mycelial protein took place with substantial loss of tritium from C-3 and almost complete loss from C-2.

AROMATIC amino-acids labelled stereoselectively with tritium in the β-methylene groups are readily prepared by the oxazolinone route [1] and may be used to determine the steric course of a variety of biosynthetic reactions. We report here details of the first stereochemical studies [2] on the biological αβ-dehydrogenation † of aromatic amino-acids.

The mould metabolite mycelianamide [3] (1; R = OH) was chosen for initial study since the probable precursor,[4] tyrosine (2; R = OH), of the aromatic moiety was already to hand [1c] in suitably labelled form. Various specimens of [3-^{3}H]tyrosine ‡ (see Table 1) were



(1)



(2)

mixed with [^{14}C]tyrosine, to provide a reference label, and fed to *Penicillium griseofulvum* growing under standard conditions.[5] After 55 days, mycelianamide was isolated and crystallised to constant activity. Conversion [3a] into deoxymycelianamide (1; R = H) by reduction with zinc did not cause any significant change in molar activity or ^{3}H : ^{14}C ratio, thus confirming the radiochemical purity of the metabolite.

A simple procedure [6] was employed for the degradation of mycelianamide to establish unambiguously the location of ^{3}H and ^{14}C labels. Treatment of deoxymycelianamide (1; R = H) with hydriodic acid and red phosphorus under reflux caused, in one preparative step, removal of the geranyl group, reduction of the side-chain double bond, and cleavage of the amide linkages. Tyrosine (2; R = OH) was readily isolated in acceptable yield (typically 78%). Labelled deoxymycelianamide from experiment 3 (see Table 1) was degraded in this way to give tyrosine with high retention of ^{3}H (94%) and ^{14}C (98%). The tyrosine was methylated with dimethyl sulphate and alkali and the mixture was heated to effect Hofmann elimination. The total product was then oxidised directly with potassium permanganate to yield 4-methoxybenzoic acid lacking both ^{3}H and ^{14}C. Decarboxylation of the tyrosine in hot diphenylamine gave tyramine with retention of ^{3}H (95%) and ^{14}C (105%). This result confirmed the expected labelling pattern indicated by asterisks in structure (1). Similarly, degradation of deoxymycelianamide from experiment 4 gave tyrosine (96% ^{3}H and 99% ^{14}C retained) and thence 4-methoxybenzoic acid (79% ^{14}C and a negligible amount of ^{3}H retained). The L-tyrosine precursor used in this experiment was uniformly labelled with ^{14}C and the ^{14}C content of the

† 'Dehydrogenation' is used to indicate the overall structural change irrespective of the number and nature of the particular enzymic processes involved.

‡ MacDonald and Slater have shown [4] that both D- and L-[^{14}C]tyrosine are incorporated into mycelianamide with similar efficiency.

[1] (*a*) R. H. Wightman, J. Staunton, A. R. Battersby, and K. R. Hanson, *J.C.S. Perkin I*, 1972, 2355; (*b*) P. G. Strange, J. Staunton, H. R. Wiltshire, A. R. Battersby, K. R. Hanson, and E. A. Havir, *ibid.*, p. 2364; (*c*) G. W. Kirby and J. Michael, *ibid.*, 1973, 115; (*d*) G. W. Kirby, S. Narayanaswami, and P. S. Rao, *ibid.*, 1975, 645; (*e*) G. W. Kirby and M. J. Varley, *J.C.S. Chem. Comm.*, 1974, 833.

[2] G. W. Kirby and S. Narayanaswami, *J.C.S. Chem. Comm.*, 1973, 322.

[3] (*a*) A. J. Birch, R. A. Massey-Westropp, and R. W. Rickards, *J. Chem. Soc.*, 1956, 3717; (*b*) K. W. Blake and P. G. Sammes, *J. Chem. Soc.* (*C*), 1970, 980; (*c*) R. F. C. Brown and G. V. Meehan, *Austral. J. Chem.*, 1968, **21**, 1581.

[4] A. J. Birch, R. J. English, R. A. Massey-Westropp, and H. Smith, *J. Chem. Soc.*, 1958, 369; A. J. Birch, M. Kocor, N. Sheppard, and J. Winter, *ibid.*, 1962, 1502; J. C. MacDonald and G. P. Slater, *Canad. J. Biochem.*, 1975, **53**, 475.

[5] A. E. Oxford, H. Raistrick, and P. Simonart, *Biochem. J.*, 1939, **33**, 240.

[6] *Cf.* C. Gallina, A. Romeo, G. Tarzia, and V. Tortorella, *Gazzetta*, 1964, **94**, 1301.

4-methoxybenzoic acid agrees well with the calculated value (78%). Moreover, degradation of the precursor itself gave 4-methoxybenzoic acid with a similar retention (77%) of ^{14}C.

known [10] that phenylalanine (2; R = H) is incorporated intact into (4) *via* cyclopeptine (5) and didehydrocyclopeptine (6). Samples of (3*R*)- and (3*S*)-[3-^{3}H]phenylalanine were therefore prepared by the oxazolinone

TABLE 1

Incorporation of tyrosine (2; R = OH) into mycelianamide (1; R = OH) in *Penicillium griseofulvum*

Expt.	Labelling pattern in (2; R = OH)	Configurational purity (%) of 3-^{3}H in (2; R = OH)	Incorporation (%) of ^{14}C into (1; R = OH)	Tritium retention (%) in (1; R = OH)	Tritium retention (%) in (1; R = H)
1	DL-(3*R*)-[3-^{3}H, 2-^{14}C]	83	2.2	76.5	77.3
2	DL-(3*S*)-[3-^{3}H, 2-^{14}C]	87	1.7	15.5	15.5
3	DL-(3*RS*)-[3-^{3}H, 2-^{14}C]	50	3.3	44.2	44.2
4	L-(3*R*)-[3-^{3}H, *U*-^{14}C]	83	0.5	77.2	77.8

TABLE 2

Incorporation of [3-^{3}H, 1-^{14}C]phenylalanine (2; R = H) into cyclopenin (4; R = H), cyclopenol (4; R = OH), and mycelial protein in *Penicillium cyclopium*

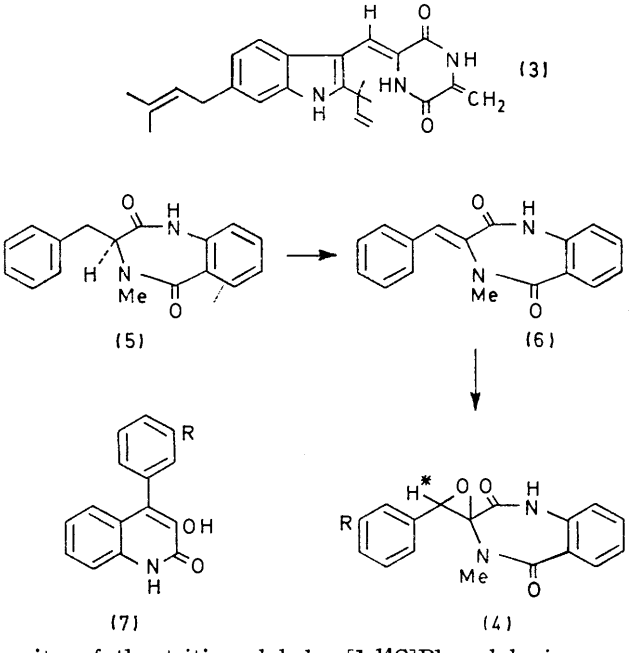
Expt.	Configuration [a] of (2; R = H); configurational purity (%) of 3-^{3}H	Incorporation (% ^{14}C) in (4; R = H)	Incorporation (% ^{14}C) in (4; R = OH)	Tritium retention (%) in (4; R = H)	Tritium retention (%) in (4; R = OH)	Tritium retention (%) in Protein phenylalanine
5	DL-(3*R*); 83	0.345	0.44	49	46	
6	DL-(3*R*); 79	0.82	1.01	38	32	76.5
7	L-(3*R*); 79	0.67	1.06	36	32.5	50.5
8	DL-(3*S*); 85	0.31	0.42	37	36	
9	DL-(3*S*); 87	0.71	1.13	37	32	89
10	L-(3*S*); [b] 83	0.64	0.77	18	16	36
11	DL-(3*RS*); 50	0.44	0.51	44	34	41
12	DL-[2-^{3}H] [c]	0.62	0.88	1.6	1.1	5.1

[a] The traditional symbols, D and L, are retained to denote configuration at C-2. [b] Labelled with ^3H equally at C-2 and C-3. [c] Labelled with ^3H only at C-2.

The stereoselectively tritiated (3*R*)-[3-^{3}H]tyrosine employed in this work (experiments 1 and 4) was known [1c] to contain both the (3*R*)- (83%) and the (3*S*)-form (17%). Conversely the (3*S*)-[3-^{3}H]tyrosine (experiment 2) contained (3*S*)- (87%) and (3*R*)-labelled (13%) species. Non-stereoselectively tritiated tyrosine (experiment 3) was fed to confirm the findings with the stereoselectively labelled materials. It is clear from the tabulated results that biosynthetic incorporation of [3-^{3}H]tyrosine into mycelianamide took place with high retention of (3*R*)-tritium and essentially complete loss of (3*S*)-tritium. Formation of the didehydro-amino-acid unit of the metabolite must therefore be subject to stereochemical and, presumably, enzymic control. Recently, Fuganti *et al.* have reported [7] the incorporation of stereospecifically labelled [3-^{3}H]tryptophan into cryptoechinulin A (3) in *Aspergillus* spp. They found that dehydrogenation proceeded stereochemically in the manner we have observed for mycelianamide, *i.e.* with stereospecific loss of (3*S*)-tritium and retention of (3*R*)-tritium. It remains to be seen whether the formation of didehydro-amino-acid units in nature [8] displays more generally this stereochemical consistency.[9]

The metabolites cyclopenin (4; R = H) and cyclopenol (4; R = OH) of *Penicillium cyclopium* Westling were chosen for our second study, with interesting, though stereochemically inconclusive, results. It is

method [1] and assayed with phenylalanine ammonia-lyase [11] to determine (see Table 2) the configurational



purity of the tritium label. [1-^{14}C]Phenylalanine was used throughout to provide a reference label. Feeding

[7] R. Cardillo, C. Fuganti, D. Ghiringhelli, P. Grasselli, and G. Gatti, *J.C.S. Chem. Comm.*, 1975, 778.

[8] B. W. Bycroft, *Nature*, 1969, **224**, 595; D. J. Aberhart and L. J. Lin, *J.C.S. Perkin I*, 1974, 2320; H. G. Floss, M. Tcheng-Lin, H. Kobel, and P. Stadler, *Experientia*, 1974, **30**, 1369.

[9] K. R. Hanson and I. A. Rose, *Accounts Chem. Res.*, 1975, **8**, 1.

[10] (*a*) L. Nover and M. Luckner, *European J. Biochem.*, 1969, **10**, 268; (*b*) J. Framm, L. Nover, A. El Azzouny, H. Richter, K. Winter, S. Werner, and M. Luckner, *ibid.*, 1973, **37**, 78.

[11] E. A. Havir and K. R. Hanson, *Biochemistry*, 1968, **7**, 1896.

experiments were conducted with cultures of *P. cyclopium* growing under standard conditions,[10a,12] the metabolites (4) being isolated 10 days after addition of the precursors. The cyclopenin (4; R = H) and cyclopenol (4; R = OH) from experiments 5 and 8 (Table 2) were separately degraded[13] with acid to give, respectively, viridicatin (7; R = H) and viridicatol (7; R = OH) with full retention (95—102%) of ^{14}C activity and complete loss of tritium; this accords with the expected location of tritium [asterisk in structure (4)]. Two broad generalisations emerge from the tabulated results: (i) incorporation of [3-^{3}H]phenylalanine into the metabolites (4) always proceeded with >50% loss of tritium, and (ii) the extent of tritium loss, though variable, did not depend in any regular way upon the configuration at C-3. Moreover, essentially the same results were obtained when either the DL- or the L-form of the precursor were used.* The (2*S*,3*S*)-[2,3-$^3H_2$]-phenylalanine (experiment 10) was labelled equally at both sites;[1d] loss of tritium from C-2 during incorporation into (4) was expected on structural grounds and was confirmed by feeding [2-^{3}H]phenylalanine (experiment 12). An allowance for this 50% loss must be made before comparing experiment 10 with experiments 8 and 9.

The observed lack of stereochemical control during the metabolism of phenylalanine cannot be merely a consequence of a non-stereoselective removal of tritium in the dehydrogenation step (5) ⟶ (6). The loss of tritium could then not exceed 50% and would probably be substantially less owing to the operation of a primary kinetic isotope effect. In contrast, the observed loss (Table 2) was typically 65% and always exceeded 50%. The results are most simply, but not uniquely, explained by assuming that phenylalanine is reversibly converted, by a transaminase system, into phenylpyruvic acid before entering the pathway (5) ⟶ (6) ⟶ (4). Reversible enolisation of [3-^{3}H]phenylpyruvic acid, without enzymic catalysis, would remove at least part of the tritium with epimerisation of the remainder. Owing to the kinetic isotope effect, hydrogen would be lost faster than tritium during enolisation and, consequently, epimerisation would be faster than tritium loss. If epimerisation were essentially complete then a stereospecific dehydrogenation step, (5) ⟶ (6), would remove half the remaining tritium. The overall loss would therefore exceed 50% and would not depend significantly on the configuration of tritium in the [3-^{3}H]-phenylalanine. It is not surprising if, in a dynamic system of this kind involving a growing organism, the extent of tritium loss should vary, as observed, from experiment to experiment.

The incorporation of [3-^{3}H]phenylalanine into mycelial protein was examined to test whether tritium loss could indeed occur by processes other than those leading directly to the secondary metabolites (4). The mycelia from various feeding experiments (Table 2) were thoroughly washed then digested with barium hydroxide to hydrolyse the protein. Phenylalanine was isolated from the resulting amino-acid mixture chromatographically and purified by crystallisation. In all cases the recovered phenylalanine contained less tritium than the precursor (Table 2). Tritium loss was variable but was generally less than that observed for the cyclopenin and cyclopenol produced in the same feeding experiment. However, critical comparison of the results for mycelial phenylalanine and the secondary metabolites is not possible without knowledge of, *inter alia*, the relative rates of competing processes at all times during growth of the organism, and the extent to which protein and secondary metabolite biosynthesis proceed from the same phenylalanine pool. Short-term feeding experiments were briefly investigated but the results were not encouraging: for example, 1 h after addition of [3-^{3}H]-phenylalanine to cultures the mycelial phenylalanine was found to have lost *ca.* 50% of the tritium. Determination of the steric course of dehydrogenation in cyclopenin biosynthesis must therefore await studies with a suitably labelled, later intermediate, for example cyclopentine (5), on the biosynthetic pathway.

* Both D- and L-phenylalanine are incorporated into (4), the former less efficiently than the latter.[10a] The incorporation of D-phenylalanine without randomisation was tentatively attributed[10a] to conversion into the L-isomer *via* phenylpyruvic acid.

EXPERIMENTAL

Counting Methods.—^{3}H and ^{14}C Activities were measured by liquid scintillation counting with use of a toluene-based scintillator solution. Phenylalanine and tyrosine were counted in dimethylformamide–toluene after conversion into the corresponding ethyl ester hydrochlorides.[14] Alternatively, phenylalanine was treated in dimethylformamide at room temperature with an excess of *NO*-bistrimethylsilylacetamide until a clear solution was obtained. This was diluted with toluene-base scintillator solution immediately before counting.

Labelled Tyrosine and Phenylalanine.—DL-(3*R*)-, DL-(3*S*)-, DL-(3*RS*)-, and L-(3*R*)-[3-^{3}H]Tyrosine of known stereochemical purity were available from an earlier study.[1c] DL-[2-^{14}C]Tyrosine and L-[U-^{14}C]tyrosine were obtained from the Radiochemical Centre, Amersham. DL-(3*R*)-, DL-(3*S*)-, DL-(3*RS*)-, and L-(3*R*)-[3-^{3}H]Phenylalanine were prepared by the oxazolinone route [1] and the configuration of the tritium assayed with phenylalanine ammonia-lyase.[11] L-(2*S*,3*S*)-[2,3-$^3H_2$]Phenylalanine, of defined configuration and labelling pattern,[1d] was obtained from the Radiochemical Centre, Amersham, as were L- and DL-[1-^{14}C]phenylalanine.

Mycelianamide (1; R = OH).—*Penicillium griseofulvum* Dierckx (Raistrick's strain; London School of Hygiene Catalogue No. *P.* 38) was grown on a defined glucose–mineral salts medium as before.[5] After 75 days the mycelia were collected, washed with cold water, dried *in vacuo*, and ground under liquid nitrogen. The resulting powder was extracted (Soxhlet) with light petroleum (b.p. 40—60°) to remove fats and with diethyl ether to remove

[12] A. Bracken, A. Pocker, and H. Raistrick, *Biochem. J.*, 1954, **57**, 587.
[13] J. H. Birkinshaw, M. Luckner, Y. S. Mohammed, K. Mothes, and C. E. Stickings, *Biochem. J.*, 1963, **89**, 196.
[14] W. R. Bowman, W. R. Gretton, and G. W. Kirby, *J.C.S. Perkin I*, 1973, 218.

mycelianamide. The mycelianamide crystallised from ethyl acetate as leaflets, m.p. 171° (lit.,[15] 170—172°).

Sterile solutions of labelled tyrosine (typically 5 mg) (Table 1) in water (*ca.* 30 ml), were added to 18-day-old cultures (3 × 350 ml) of *P. griseofulvum* with minimal disturbance of the mycelial mat. Incubation was continued for a further 55 days. Labelled mycelianamide (typically 40 mg) was isolated as described above and purified to constant activity by crystallisation from ethyl acetate; in general, the metabolite appeared radiochemically pure after the first crystallisation.

Degradation of Deoxymycelianamide (1; R = H).—Mycelianamide was reduced [3a] with zinc dust in acetic acid to yield deoxymycelianamide, m.p. 190—191° (from ethanol) (lit.,[6] 186—187°). Deoxymycelianamide (100 mg) in hydriodic acid (constant boiling mixture; 4 ml) containing red phosphorus (60 mg) was heated under reflux for 3 h. More red phosphorus (45 mg) was added and heating continued for 2 h. The mixture was diluted with water (10 ml) then filtered. The filtrate was evaporated and the residue treated with water which was then evaporated off. This procedure was repeated twice more. The residue was dissolved in water (5 ml) and the pH adjusted to *ca.* 7. The tyrosine (39 mg) which separated out was recrystallised from the hot water. The tyrosine was degraded (*a*) to 4-methoxybenzoic acid *via* methylation and permanganate oxidation,[1d] and (*b*) to tyramine by thermolysis under nitrogen at 280 °C in diphenylamine.[16]

Cyclopenin (4; R = H) *and Cyclopenol* (4; R = OH).—*Penicillium cyclopium* Westling (LSHTM No. 72) was grown on a Raulin–Thom medium as before.[12] The metabolites were isolated by preparative layer chromatography on silica gel.[10a]

Sterile solutions of labelled phenylalanine (typically 10 mg) (Table 2) in water (*ca.* 30 ml) were added to 7-day-old cultures (3 × 300 ml) of *P. cyclopium* with minimal disturbance of the mycelial mat. Incubations were continued for 10 days and cyclopenin (typically 28 mg) and cyclopenol (typically 45 mg) were isolated chromatographically. Cyclopenin, m.p. 182° (lit.,[10a] 183—184°), was crystallised from ether–light petroleum (b.p. 40—60°) and cyclopenol, m.p. 213° (lit.,[10a] 215°), from ethyl acetate–benzene to constant specific activity.

Cyclopenin and cyclopenol were converted [13] into viridicatin and viridicatol, respectively, in hot aqueous ethanolic hydrochloric acid.

Isolation of Phenylalanine from Mycelial Protein.—The mycelia were dried *in vacuo*, powdered, and extracted successively (Soxhlet) with light petroleum (b.p. 40—60°), chloroform, and methanol to remove secondary metabolites. The resulting powder (5 g) was washed well with water then digested with hot aqueous barium hydroxide (14% w/v) (150 ml) under reflux for 48 h. The mixture was filtered and the filtrate adjusted to pH 6—7 with aqueous sulphuric acid. Barium sulphate was filtered off and the filtrate evaporated. The residual amino-acid mixture was partially purified by ion-exchange chromatography (Dowex 50W-X8 resin; H^+ form) then separated by paper chromatography [Whatman No. 1 sheets developed with n-butanol–acetic acid–water (7 : 3 : 1)]. The phenylalanine-rich band was eluted with hot water and the crude phenylalanine further purified by paper chromatography and crystallisation from water.

We thank the S.R.C. for financial support.

[6/166 *Received*, 26*th January*, 1976]

[15] A. E. Oxford and H. Raistrick, *Biochem. J.*, 1948, **42**, 323.
[16] T. B. Johnson and P. G. Daschavsky, *J. Biol. Chem.*, 1925, **62**, 725.