Mechanism of Alkylation of the Benzene Unit of Tryptophan in the Biosynthesis of Echinuline and Neoechinuline

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Feeding experiments with specifically labelled precursors show that in the biosynthesis of echinuline (8) and neoechinuline (9) from tryptophan (1) the isoprenylation of the benzene ring involves removal of the hydrogen atoms at the positions undergoing alkylation and retention of those at the vicinal positions.

TRACER work has indicated the mode of biosynthesis of echinuline (8) from tryptophan (1) and mevalonate, with the introduction of the isoprene units into the benzene ring occurring late in the sequence, after the dioxopiperazine has been built up.[1] The lack of precise information on the mechanism of alkylation of the benzene unit of tryptophan (1) in the biosynthesis of isoprenylated natural indole derivatives, and the observation [2] that *in vitro* the indole system undergoes alkylation at the benzene ring only when it is hydroxylated or when it is reversibly modified to give an indoline system, induced us to study the manner of incorporation of specifically ^{3}H-labelled tryptophan into the mould metabolites echinuline (8) and neoechinuline (9).[3] Since previous work has shown the migration to position 4 of *ca.* 85% of a tritium label at position 5 of tryptophan during its biological conversion into 5-hydroxytryptophan,[4] we considered that the occurrence of intermediate hydroxylation of the benzene ring of tryptophan (1) on the biosynthetic pathway to echinuline (8) and neoechinuline (9), with or without the above-mentioned hydrogen shifts to vicinal carbon atoms, could be detected by measuring tritium retentions in the two metabolites (8) and (9) biosynthesised from the same tryptophan sample carrying ^{3}H labels at positions 5 and 7 or 4 and 6, respectively.

The labelled precursors were synthesised from [2,4,6-$^3H_3$]aniline and from the [3,5-$^3H_2$]-compound by known procedures.[5] Thus, in the first case, aniline was labelled *ortho* and *para* to the amino-group by acid catalysed exchange with tritiated water. The distribution of tritium activities amongst the various positions was determined as follows. Conversion of the ^{3}H-labelled aniline into acetanilide caused no tritium loss, but bromination of the latter in deuterioacetic acid–sodium acetate to give 4-bromoacetanilide caused *ca.* 38% tritium loss, without incorporation of deuterium from the medium. 2,4,6-Tribromoaniline, obtained similarly, was devoid of activity. The relative activities for positions 2, 4, and 6 of the labelled aniline were thus *ca.* 30 : 40 : 30. Since in the conversion of this labelled aniline into tryptophan (1) a considerable (>40%) tritium loss took place during the Fischer cyclization step, the labelling pattern of the final radioactive precursor was determined. Ozonolysis [6] of the labelled *N*-acetyltryptophan (2) led to acetylkinurenine (3), which was methylated (CH_2N_2) to give compound (4). Bromination in deuterioacetic acid then gave *N*-acetyl-4,6-dibromokinurenine methyl ester (7) without incorporation of deuterium but with almost complete tritium loss. The 4-bromo-derivative (6), obtained by bromination of (5) under identical conditions, retained *ca.* 55% of the tritium activity, without incorporation of deuterium from the medium. Thus, the labelled tryptophan (1) possessed tritium labels at positions 5 and 7 in the ratio *ca.* 45 : 55.

The [4,6-$^3H_2$]tryptophan (1) was prepared similarly from [3,5-$^3H_2$]aniline. The latter was obtained by inserting ^{3}H labels *ortho* to the phenolic hydroxy-group of 4-acetamidophenol by base-catalysed exchange with tritiated water, followed by reductive removal of the activating hydroxy-group *via* hydrogenolysis of the *N*-phenyltetrazole derivative,[7] and hydrolysis of the acetyl group. Its labelling pattern was deduced from n.m.r. studies in the deuteriated series. The conversion

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[4] J. Renson, J. W. Daly, H. Weissbach, B. Witkop, and S. Udenfriend, *Biochem. Biophys. Res. Comm.*, 1966, **25**, 504; J. W. Daly, D. M. Jerina, and B. Witkop, *Experientia*, 1972, **28**, 1129.
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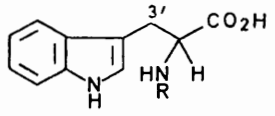
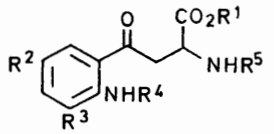
[6] A. Previero, E. Scoffone, P. Pajetta, and C. A. Benassi, *Gazzetta*, 1963, **93**, 841.
[7] W. J. Musliner and J. W. Gates, jun., *J. Amer. Chem. Soc.*, 1966, **88**, 4271.

of [3,5-^{3}H]aniline into [4,6-^{3}H]tryptophan (1) proceeded without tritium loss.

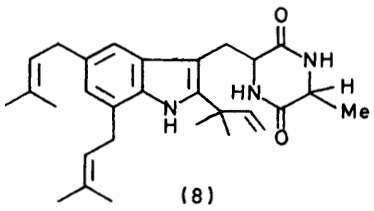
Aspergillus amstelodami incorporated doubly labelled [5,7-$^3H_2$;3′-^{14}C]tryptophan (1) into echinuline (8) and



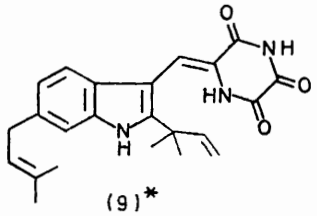
(1) R = H
(2) R = Ac



(3) R^1 = R^2 = R^3 = R^4 = H, R^5 = Ac
(4) R^1 = Me, R^2 = R^3 = R^4 = H, R^5 = Ac
(5) R^1 = Me, R^2 = R^3 = H, R^4 = R^5 = Ac
(6) R^1 = Me, R^2 = Br, R^3 = H, R^4 = R^5 = Ac
(7) R^1 = Me, R^2 = R^3 = Br, R^4 = H, R^5 = Ac



(8)



(9)*

SCHEME

* The stereochemistry of (9) has not been determined.

neoechinuline (9) with 2 and 103% retention of tritium activity, respectively. The isomeric [4,6-$^3H_2$;3′-^{14}C]-tryptophan was incorporated into echinuline (8) with 102% tritium retention and into neoechinuline (9) with 48% tritium loss. The two experiments give complementary results, suggesting that the isoprenylation of the benzene ring of the tryptophan system in (8) and (9) takes place with removal of the hydrogen atoms at positions undergoing alkylation, and retention of those at the vicinal carbon atoms. These results appear to exclude the intermediacy of hydroxylated tryptophan derivatives in the biosynthesis of echinuline (8), and of a 7-hydroxy-derivative of (1) in the biosynthesis of neoechinuline (9).

EXPERIMENTAL

Radioactivity Assay.—The measurements were taken at least in duplicate with a Beckman LS 100 liquid scintillation counter. [^{3}H]- and [^{14}C]-Hexadecane were added to each sample as internal standards.

Exchanges with Tritiated Water.—Aniline (100 mg) was heated in 2N-hydrochloric acid (1 ml) containing tritiated water (*ca.* 200 mCi) in an evacuated sealed tube at 120° for 4 days. The solvent was removed on a vacuum line, and the exchangeable tritium in the residue was removed by dissolution in methanol and evaporation. 4-Acetamidophenol (150 mg) was heated in a mixture of tritiated water (*ca.* 200 mCi; 0·5 ml), dioxan (0·5 ml), and triethylamine (0·1 ml) under the above-mentioned conditions for 6 days.

Labelling Patterns of the Precursors.—*N*-Acetyldibromokinurenine methyl ester (7) was obtained as follows. *N*-Acetylkinurenine methyl ester (200 mg) in deuterioacetic acid (4 ml) containing dry sodium acetate (300 mg) was treated with bromine (2·2 mol. equiv.) in acetic [^{2}H]acid at room temperature with stirring. After 2 h the mixture was poured into ice–water, and the precipitate was crystallized from aqueous methanol (m.p. 190°). The n.m.r. spectrum ($MeCO_2D$; 60 MHz; Me_4Si standard) showed, in the aromatic region, signals at δ 7·99 and 7·81 (d, 1H each, J_{AB} 1·5 Hz).

NN′-Diacetyl-4-bromokinurenine methyl ester (6), m.p. 178° (from ethanol), was obtained upon bromination of *NN′*-diacetylkinurenine methyl ester, prepared in turn from *N*-acetylkinurenine methyl ester by acetylation with acetic anhydride–pyridine. The n.m.r. spectrum of (6) ($MeCO_2D$; 60 MHz) showed δ 8·67, 8·21, and 7·79 (1H each, ABX, J_{AB} 9, J_{BX} 1·5, J_{AX} 0·5 Hz).

Feeding Experiments and Isolation of Metabolites.—The tryptophan samples (typically 100 mg) were dissolved in the culture medium (40% w/v of sugar beet molasses and 0·2% KH_2PO_4 in distilled water), which had been inoculated with *Aspergillus amstelodami* spores in Roux flasks (10 flasks, each containing 50 ml of culture medium). After 10—12 days the cultures were harvested, and the dried fungal mats were extracted (Soxhlet) with petroleum (b.p. 40—60°) (discarded), ether [for neoechinuline (9)], and chloroform [for echinuline (8)]. The two metabolites were purified by column chromatography [SiO_2 (Merck)] and crystallised to constant activity. Neoechinuline (9) was diluted 1 : 1 with inactive material. The incorporation values were *ca.* 7—15% for the two metabolites.

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