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THE BIOSYNTHESIS OF AUREOTHIN

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Abstract—Feeding experiments with specifically labelled precursors show that the nitroaromatic, C-6-C-1 unit of the antibiotic aureothin (9) biologically derives by degradation of the C-6-C-3, phenylpropanoid precursor D,L-*p*-aminophenylalanine (1) through hydroxylation β to the nitrogen to *erythro* and *threo* *p*-aminophenylserine (3 and 4). During the biosynthesis there is the loss of the hydrogen originally present in benzylic position in the phenylpropanoid precursor, and, further, the oxidation of the *p*-amino group to *p*-nitro takes place very late in the sequence.

INTRODUCTION

The mixed biosynthetic origin of the antibiotic aureothin (9) has been recently reported in preliminary accounts.[1,2] We refer now on the results of feeding experiments which rigorously establish the derivation of the nitroaromatic, C-6-C-1 unit of aureothin (9) from *p*-aminophenylalanine (1) and, further, define some of the operations of the biosynthetic process.

A *para*-amino substituted C-6-C-3 phenylpropanoid compound was tested as precursor of the C-6-C-1, nitroaromatic unit because of observation[3] that this type of intermediate is effectively incorporated into chloramphenicol through hydroxylation β to the nitrogen, and in the expectation that it could undergo degradation to the required C-6-C-1 unit either *via* the suitable isomer of *p*-aminophenylserine through the action of an aldolase (path *a*) or *via* substituted cinnamic acid (5), as does occur in the incorporation of phenylalanine into such a unit in higher plants,[4] (path *b*). Furthermore, owing to the observed[5] ability of *Streptomyces thiolutheus* to oxidise aromatic amino groups to nitro, experiments have been performed in order to establish the timing of the oxidation on the biosynthetic pathway leading from *p*-aminophenylalanine (1) to aureothin (9).

Synthesis of the radioactive precursors

The tritium labelling had been generally performed by acid-catalysed exchange of the aromatic compound chosen as precursor with tritiated water in evacuated sealed tube at 120° for 1–6 days with 2N HCl. However, either because of the instability of some compounds under the acidic conditions required for the labelling or because of their resistance to the electrophilic attack some syntheses were made starting from intermediates which were suitable for labelling.

D,L-[3,3',5'-^{3}H;3-^{14}C]p-*aminophenylalanine* (1). The multiply labelled compound was obtained by mixing the singly labelled isomers, which were obtained as follows. Tritium labels were inserted *ortho* to the aromatic amino group of D,L-*p*-aminophenylalanine (1) by acid-catalysed exchange with tritiated water. The labelling pattern of the radioactive (1) was determined by iodination[6] with ICl in diluted HCl to 3',5'-diiodo-4'-aminophenylalanine which retained less than 15% of the starting activity. D,L-[3-^{3}H]*p*-aminophenylalanine (1) was obtained through direct synthesis. Thus, *p*-nitrobenzaldehyde (7) was reduced (NaB^3H_4) to the ^{3}H-labelled alcohol, which was converted into the bromo derivative on HBr treatment in quantitative yield. Bromide displacement with an excess of sodio-acetamidomalonate in dimethylformamide at 50° led to a product which, after hydrolysis-decarboxylation and hydrogenation, yielded the required D,L-[3-^{3}H]*p*-aminophenylalanine without significant tritium loss in 65% overall yield. Our approach to the synthesis of D,L-[3-^{14}C]*p*-aminophenylalanine (1) started from the commercially available [*carbinol*-^{14}C] benzylalcohol, because the synthesis from phenylalanine through nitration and reduction resulted as unsatisfactory for the scaled-down radioactive work. Benzyl alcohol was converted into the *p*-nitrobenzoyl ester upon treatment with $p\text{-}NO_2C_6H_4COCl$ in pyridine. The ester was nitrated to *p*-nitrobenzyl-*p*-nitrobenzoate by means of HNO_3 in Ac_2O, and reduced with $NaBH_4$ and $AlCl_3$ in diglyma[7] to [*carbinol*-^{14}C]*p*-nitrobenzylalcohol. This was eventually converted into *p*-aminophenylalanine (1) as reported before, in 28% overall yield. The labelling pattern of D,L-[3,3',5'-^{3}H;3-^{14}C]*p*-aminophenylalanine (1) was determined by iodination. The ratio of the tritium labelling between positions 3 and those *ortho* to the aromatic amino group resulted as being 2·4:1.

D,L-erythro *and* D,L-threo[3,ring-^{3}H]p-*amino*-

phenylserine (3 *and* 4). They have been prepared through known procedures[3,8] from doubly labelled [*ring,formyl*-^{3}H]*p*-nitrobenzaldehyde (7). [*ring*-^{3}H]*p*-Nitrobenzaldehyde was prepared from [*ring*-^{3}H]toluene, obtained in two ways. The first one involved the acid-catalysed exchange with tritiated water of *p*-aminotoluene, followed by replacement of the activating amino group by means of diazotisation and treatment with $Na_3Co(NO_2)_6$ in the presence of Cu_2O and $CuSO_4$.[9] The second approach started from 2-amino-4-nitrotoluene which was labelled by acid-catalysed exchange. The radioactive compound was deaminated upon treatment with $NaNO_2$ and Cu powder. The *ring*-labelled nitrotoluene was converted into the required aldehyde by CrO_3 oxidation, according to well established procedures. [*formyl*-^{3}H]*p*-Nitrobenzaldehyde (7) was obtained by $Pb(AcO)_4$ oxidation of the previously prepared [*carbinol*-^{3}H]*p*-nitrobenzyl alcohol. The two samples were mixed to give [*ring,formyl*-^{3}H]*p*-nitrobenzaldehyde (7). The ratio of the tritium labels between positions in the aromatic ring and in the aldehydic group was determined by oxidation to *p*-nitrobenzoic acid (8) which retained *ca* 10% of the starting activity. The conversion of the aldehyde into the two abovementioned aminoacids proceeded without significant tritium loss.

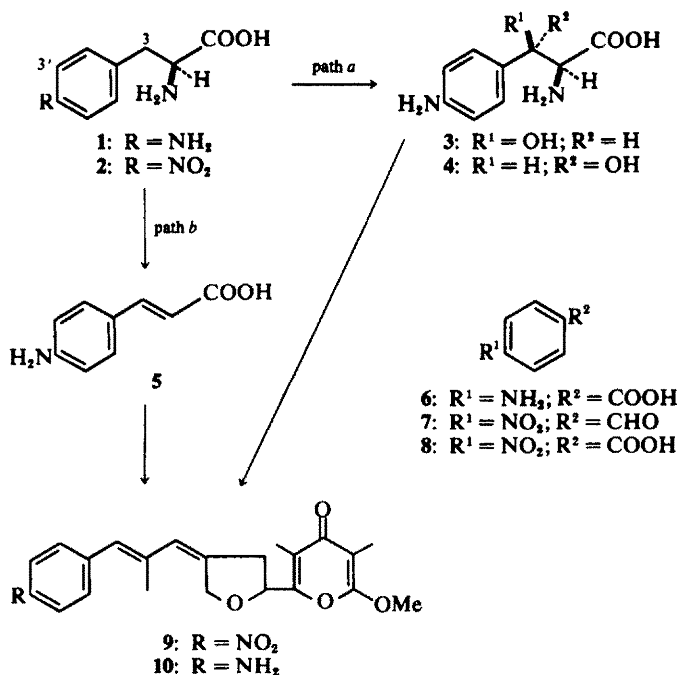
[ring-^{3}H]p-*Aminocinnamic acid* (5) *and* [ring-^{3}H]p-*aminobenzoic acid* (6). They have been prepared by direct exchange with tritiated water in 2N HCl.

[ring-^{3}H]*Aureothamin* (10). It has been prepared by reduction of biosynthetic [*ring*-^{3}H] aureothin.[10]

Results of the feeding experiments

The incorporation values into aureothin (9) of the abovementioned products and of D,L-[3-^{14}C]*p*-nitrophenylalanine (2) are reported in the Table. The labelling pattern of the radioactive aureothin (9) has been determined by degradation[10] to *p*-nitrobenzaldehyde (7) and to *p*-nitrobenzoic acid (8), which causes the removal of the H atom originally at benzylic position in 9. From the relative molar tritium activities of aureothin (9) and of the degradation products 7 and 8 (Table) it turns out that D,L-*p*-aminophenylalanine (1), D,L-*erythro* and D,L-*threo*-*p*-aminophenylserine (3 and 4), and aureothamin (10) are specifically incorporated into aureothin (9) with the loss of the H atom originally present at benzylic position in the C-6-C-3 precursor. The last result is coincident with that obtained in the conversion of phenylalanine into the C-6-C-1 unit of the *Amaryllidaceae* alkaloids.[11]

Furthermore, *p*-aminocinnamic acid (5), *p*-aminobenzoic acid (6) and D,L-*p*-nitrophenylalanine (2) seem to be outside the metabolic process to aureothin (9). The evidence therefore suggests that the degradation of the phenylpropanoid precursor to the nitroaromatic C-6-C-1



SCHEME*

*The L-forms of the aminoacids are depicted.

Table. Relative ^{3}H molar activities and (incorporations %)

Precursor	Aureothin (9)	p-$NO_2C_6H_4CHO$ (7)	P-$NO_2C_6H_4COOH$ (8)
D,L[3,3′,5′-^{3}H;3-^{14}C]*p*-aminophenylalanine (1)	100 (12)	98	96
D,L[3-^{14}C]*p*-nitrophenylalanine (2)	(<0·2)		
D,L-*erythro*[*ring*,3-^{3}H]*p*-aminophenylserine (3)	100 (26)	97	102
D,L *threo*[*ring*,3-^{3}H]*p*-aminophenylserine (4)	100 (11)	102	99
[*ring*-^{3}H]*p*-aminocinnamic acid (5)	(no incorp.)		
[*ring*-^{3}H]*p*-aminobenzoic acid (6)	(<0·01)		
[*ring*-^{3}H]aureothamin (10)	100 (4·4)	97	98

unit proceeds through hydroxylation β to the nitrogen, being the *erythro* and the *threo* forms of *p*-aminophenylserine incorporated with comparable efficiency, with the loss of the benzylic hydrogen at some stage of the biosynthesis (path *a*). Besides this, the lack of incorporation of the *p*-nitrosubstituted C-6-C-3 compound **2** and the specific incorporation of the sparingly soluble aureothamin (**10**) indicate that the oxidation of the *p*-amino function to *p*-nitro takes place late on the biosynthetic sequence to aureothin (**9**).

EXPERIMENTAL

Radioactive assays. The samples were counted at least in duplicate with a 3320 Packard liquid scintillation counter, using added ^{3}H-hexadecane and ^{14}C-hexadecane as internal standard.

Feeding experiments and isolation of aureothin (**9**). Generally, the precursors were fed as hydrochlorides in aqueous soln, except **10** which was fed in DMSO (50–100 mg for 1 litre of broth, producing 300–400 mg of **9**) after 48–72 h of grow. The cultures were harvested after 72 h. The mycelium was separated and suspended in MeOH. The surnatant is removed and the operation is repeated until all the coloured matter is removed. The liquid is evaporated and the residue partitioned between water and EtOAc. The organic layer is evaporated and the residue is chromatographed on silica with benzene-EtOAc 80–20. The crude **9** was diluted (1:2) with inactive material and crystallised from EtOAc and benzene-hexane to constant activity.

Degradation of aureothin by ozonolysis. Aureothin **9** (300 mg) in 15 ml $CHCl_3$ was ozonised at − 15° for 10 min. The solvent was evaporated in the cold and the residue treated with water (5 ml) at 100° for 2 h. The resulting suspension was extracted with ether. p-$NO_2C_6H_4COOH$ was separated from p-$NO_2C_6H_4CHO$ by alkaline extraction from ether. The aldehyde was purified by crystallisation from aqueous EtOH, whereas the acid was purified by crystallisation from benzene and sublimation at 0·01 mm/Hg.

Synthesis of D,L [3-^{3}H] *and* [3-^{14}]p-*aminophenylalanine* (1). To a soln made up with 500 mg diethyl acetamidomalonate and 50 mg of NaH in 2·5 ml dry DMF under N_2 after 2 h 275 mg of labelled *p*-nitrobenzyl bromide in 2 ml of DMF were added under stirring. The mixture was warmed at 50° for 2 h and then kept at room temp overnight. Upon dilution with water 2-carbethoxy-3-*p*-nitrophenyl ethylpropionate precipitated. The latter was hydrolysed upon treatment with AcOH (1·5 ml) and HBr (1 ml) at 100° for 5 h. The solvent was evaporated and the residue taken up with the minimum amount of boiling water and filtered. The soln was treated with 2N NH_3 to give *ca* 175 mg of 2 which was hydrogenated (H_2, Pd/C) to the required labelled amino compound.

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