3-AMINO-5-HYDROXYBENZOIC ACID IN ANTIBIOTIC BIOSYNTHESIS

VI.* DIRECTED BIOSYNTHESIS STUDIES WITH ANSAMYCIN ANTIBIOTICS

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(Received for publication July 8, 1983)

Biosynthesis of the ansamycin antibiotic actamycin (**2**) was markedly increased by the addition of the precursor 3-amino-5-hydroxybenzoic acid (**1**) to the producing Streptomyces fermentation. Similar addition of the 4-chloro, 6-chloro, *N*-methyl and *O*-methyl analogues **4**, **6**, **5** and **7** of the amino acid **1** reduced actamycin production and did not yield structurally modified ansamycins. These results with the analogues **4**, **5** and **7** indicate that the corresponding chlorine, *N*-methyl and *O*-methyl substituents present in the nuclei of various ansamycins are introduced at biosynthetic stages beyond the level of the amino acid **1**.

3-Amino-5-hydroxybenzoic acid (**1**) was identified in these laboratories as the key natural amino acid[1] which initiates formation of the carbon skeleton during biosynthesis[2] of the ansamycin antibiotic actamycin (**2**).[3,4] This result has subsequently been confirmed for other naphthalenoid and benzenoid antibiotics of the ansamycin group[5~7] by GHISALBA *et al.*,[8,9] who found that the same amino acid restored the production of rifamycin B in a transketolase-deficient mutant of *Nocardia mediterranei*, and by RINEHART *et al.*[10]) who demonstrated its efficient incorporation into geldanamycin by *Streptomyces hygroscopicus*. We have also demonstrated that 3-amino-5-hydroxybenzoic acid (**1**) is involved in the biosynthesis of the related maytansinoid antibiotic[11~14] ansamitocin P-3 (**3**) in *Nocardia* sp. C-15003,[15] and in the biosyn thesis of the structurally unrelated mitomycin antibiotics in *S. verticillatus*.[16]

The normal production of an antibiotic by a fermenting microorganism can potentially be altered by supplementation of the medium with either a natural biogenetic precursor of the antibiotic or an analogue of that precursor. In the former case, if the endogenous level of true precursor is limited either genetically or nutritionally, then increased antibiotic production may result. In the latter case, provided the precursor analogue can be actively transported into the organism and can compete with the true precursor for the synthetase enzymes, then an analogue of the original antibiotic may result. Such directed biosynthesis experiments assist in defining the specificity of the enzymes involved in biosynthesis, and may yield useful variants of the natural antibiotic which would be inaccessible by chemical modification.[17]

In this paper we examine the effects on ansamycin biosynthesis resulting from the addition of 3-amino-5-hydroxybenzoic acid (**1**) and its analogues **4**~**7** to an actamycin-producing fermentation of *Streptomyces* sp. E/784.

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* Part V, see reference 18.

Experimental and Results

General

NMR spectra were determined on Varian HA-100, Jeol JNM-MH-100 and Jeol JNM-FX-200 spectrometers, and mass spectra were run on a VG-Micromass 7070F instrument. UV spectra were obtained on a Varian DMS 90 spectrometer. Melting points were taken on a Kofler hot-stage apparatus and are uncorrected.

Supplementation Experiments

Streptomyces sp. E/784 was grown in 250 ml baffled Erlenmeyer flasks each containing a medium (100 ml) prepared from glucose (50.0 g), meat extract (4.0 g), peptone (4.0 g), soybean meal (10.0 g), yeast extract (1.0 g), calcium carbonate (5.0 g), sodium chloride (2.5 g), and inorganic salts in distilled water (1 liter). The various amino acids were added at the levels shown in Table 1 to separate sets of flasks as sterile aqueous solutions of their hydrochloride salts at intervals of 8 hours. After a total incubation time of 72 hours on a rotary shaker (220 rpm) at 28°C, the flasks in each set were combined, the mycelium was separated by centrifugation and filtration, and the various filtrates were extracted in parallel.

The culture filtrate (from 9 flasks) was extracted with ethyl acetate (3×400 ml) at pH 7, and the extracts washed with brine, dried (Na_2SO_4) and evaporated. The various neutral extracts were compared with the extract of the control set by NMR spectroscopy (in $CDCl_3$) and by TLC on silica in methanol - dichloromethane (1: 9) in order to detect metabolites of the added amino acids. From fermentations involving 3-hydroxy-5-methylaminobenzoic acid (**5**), a new metabolite was isolated by PLC and identified as 3-hydroxy-5-methylaminobenzamide by direct comparison with a sample prepared from methyl 3-hydroxy-5-methylaminobenzoate[18] by treatment with aqueous ammonia at room temperature: mp 145~146°C, NMR (MeOH-d_4) δ 6.58 and 6.54 (each 1H, t, H-2 and H-6), 6.22 (1H, t, H-4), and 2.76 (3H, s, NCH_3); MS *m/z* 166 (M^+, 100%), 165 (M−H, 25), 150 (M−NH_2, 21), 122 (M−$CONH_2$, 25); Found M^+ *m/z* 166.0739, $C_8H_{10}N_2O_2$ requires 166.0742.

The residual culture medium was adjusted to pH 2 with concentrated hydrochloric acid, extracted with ethyl acetate (3×400 ml), and the extracts dried (Na_2SO_4) and evaporated. The residue was extracted with dichloromethane (3×20 ml), the resulting brown insoluble material being removed by centrifugation and discarded. Comparison of these various acidic extracts by NMR (in $CDCl_3$) and TLC on silica in methanol - dichloromethane (1: 4) did not indicate the presence of any new ansamycin-type metabolites. In particular, actamycin analogues were absent as judged from their expected purple fluorescence under 366 nm light on TLC (detection limit for actamycin itself is ~3 μg).

The relative amounts of actamycin in these crude acidic extracts, as estimated by TLC and NMR spectroscopy, were in broad agreement with the values in Table 1 obtained after further purification as follows. The evaporated dichloromethane extract was first chromatographed in methanol - ethyl acetate (1: 99) on Sephadex LH-20 pre-equilibrated in these solvents (1: 4). The actamycin-containing fraction was then subjected to flash chromatography on acid-washed silica gel (Merck, particle size 0.040~0.063 mm, washed with 0.5 N aqueous hydrochloric acid, then with water, and activated at 130~140°C for 4 hours) with methanol - dichloromethane (2: 98 then 5: 95) as eluent. The resulting actamycin was estimated from its absorption at 565 nm in basic ethanol solution.

Amino Acids

3-Amino-5-hydroxybenzoic acid (**1**) and its *N*-methyl derivative **5** were prepared by the method of BECKER *et al.*,[18] whilst the chloro analogues **4** and **6** were obtained by chlorination of the methyl ester of the amino acid **1** (A.M. BECKER and R. W. RICKARDS, unpublished work).

The hydrochloride of 3-amino-5-methoxybenzoic acid (**7**) was prepared by hydrogenation of 3-methoxy-5-nitrobenzoic acid[19] (1.6 g) at atmospheric pressure for 3 hours in methanol (25 ml) containing concentrated hydrochloric acid (2 ml) over palladium-charcoal (10%, 0.25 g). After filtration through Celite to remove the catalyst, the solvent was evaporated and the residue recrystallized from ethanol - ether to give the hydrochloride (1.32 g, 80%): NMR (D_2O, CH_3CN internal reference) δ 3.73 (3H, s, OCH_3), 7.03, 7.31 and 7.38 (each 1H, m, 3×ArH).

Discussion

Two major effects resulting from supplementation of fermentations of *Streptomyces* sp. E/784 with the amino acid **1** or its analogues **4**~**7** are clearly demonstrated by the data in Table 1.

First, supplementation with the synthetic amino acid **1**, an established natural precursor[2] of actamycin (**2**), leads to a marked increase in the production of actamycin. Thus under the fermentation conditions employed here with this Streptomyces strain, actamycin production is severely limited by the availability of the endogenous amino acid. The same amino acid **1** has also been observed to promote the production of ansamitocin P-3 (**3**) by a *Nocardia* species[15] and of porfiromycin by *S. verticillatus* (A. M. BECKER and R. W. RICKARDS, unpublished work), although to a lesser extent than in the present case where the increase is more than four-fold.

Table 1. Supplementation experiments with *Streptomyces* sp. E/784.

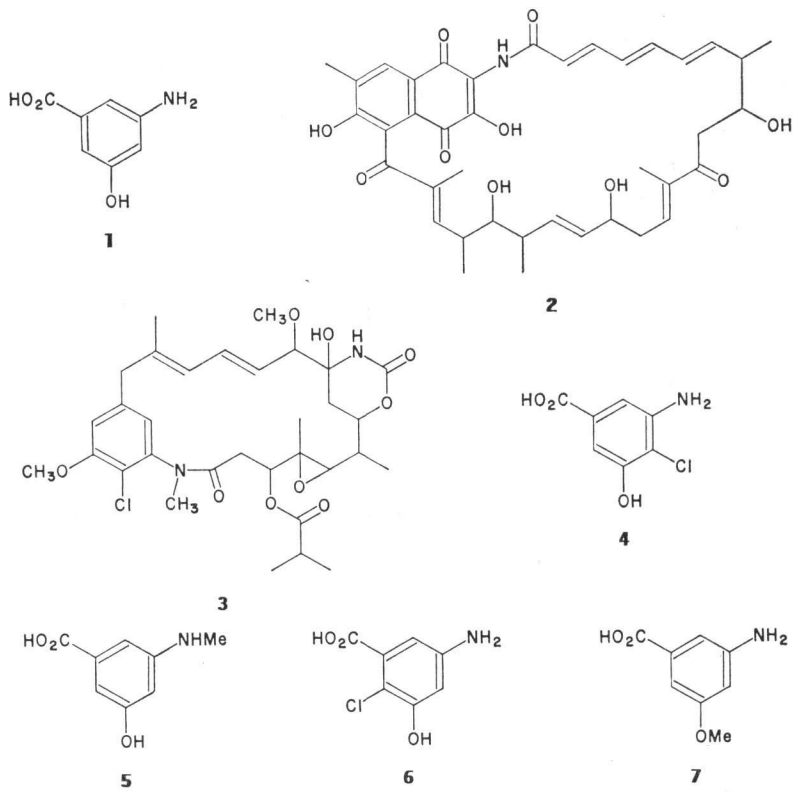
Amino acid hydrochloride	Amount fed per flask[a]		Actamycin produced (% of control)[b]
	Portions (mg)	Total (mg)	
None	—	—	100
1	5	30	460
4	1	6	20
	0.5	3	90
5	5	30	40
	0.5	3	40
6	2.5	15	30
	0.5	3	100
7	20	120	<10
	10	60	10
	5	30	20

[a] Amino acids **1**, **4**, **5**, and **6** were added at intervals of 8 hours to sets of 9 flasks after an initial growth period of 10 hours, the first two additions being half the quantity of the subsequent five. Amino acid **7** was added to sets of 5 flasks after an initial growth period of 18 hours, in six equal portions.

[b] For experiments with amino acids **1**, **4**, **5**, and **6**, the percentage is calculated from the absorption at 565 nm in basic ethanol solution of the total purified actamycin from the 9-flask set, relative to that from 9 control flasks. For experiments with amino acid **7**, the percentage is based upon NMR absorption of the total crude actamycin extract from the 5-flask set, relative to that from 5 control flasks.

Secondly, supplementation of the fermentation with the analogues **4**~**7** of the amino acid **1** does not lead to the production of structurally modified actamycins. Instead, the production of actamycin (**2**) itself is diminished to an extent which depends upon the particular analogue and the amount fed. The analogues are probably acting as competitive inhibitors of the enzyme systems responsible for the synthesis or the utilization of the natural substrate **1**. HATANO *et al.*[15] observed similar strong inhibition of ansamitocin P-3 (**3**) biosynthesis in a *Nocardia* species by 3-amino-, 3-hydroxy-, 3,5-diamino- and 3,5-dihydroxybenzoic acids.

Of the analogues **4**~**7** employed in these supplementation studies, the 4-chloro and *N*-methyl derivatives **4** and **5** were considered most likely to be converted into modified actamycins. Acceptance of the chloro acid **4** would lead to a 3-deoxy-3-chloro analogue of actamycin (**2**), which, configurational considerations aside, would be closely related to naphthomycin,[20~22] differing only in the absence of a *C*-methyl group



adjacent to the amide carbonyl. The acceptance of the *N*-methyl acid **5** would lead to *N*-methylactamycin, although in this connection it is notable that of the ansamycins[5~7] obtained to date from microorganisms only the ansamitocins[13,14] carry an *N*-methylated amide group. The 6-chloro and 5-*O*-methyl substituents of the remaining analogues **6** and **7** were designed to interfere with formation of the naphthoquinonoid nucleus of actamycin (**2**), thus leading to the production of benzoquinonoid or benzenoid ansamycins, respectively. The latter case would be particularly interesting, since the resulting *O*-methylated product would resemble the ansamitocins[13,14], *e.g.* ansamitocin P-3 (**3**), and the maytansinoids.[11,12] However, the non-utilization of these analogues **4**~**7** indicates the high specificity of the actamycin synthetase system for its natural substrate **1**. Furthermore, the results with the analogues **4**, **5** and **7** strongly suggest that the corresponding chlorine, *N*-methyl, and *O*-methyl substituents where present in naphthomycin,[20~22] the ansamitocins[13,14] and the maytansinoids[11,12] are introduced into these ansamycins at biosynthetic stages beyond the level of 3-amino-5-hydroxybenzoic acid (**1**), *i.e.* these analogues would not be precursors of such ansamycins.

TRAXLER and GHISALBA[23] recently demonstrated that the 4-hydroxy, 4-methyl, and 4-hydroxy-5-*O*-methyl analogues of 3-amino-5-hydroxybenzoic acid (**1**) were not converted into the corresponding 3-substituted rifamycins by a transketolase-deficient mutant of *N. mediterranei*. Since the addition of the amino acid **1** itself restored the production of rifamycin B in this mutant,[8,9] they also concluded that the 3-substituents in various rifamycin and other ansamycin chromophores, in particular the 3-hydroxyl and 3-methyl groups where present, were introduced in late biosynthetic steps.

The mechanisms which regulate the course of any antibiotic fermentation are complex and sensitive to many factors. The supplementation procedure with 3-amino-5-hydroxybenzoic acid which increased actamycin production in the present laboratory cultures was not optimised, and it may not be directly applicable to other fermentations. However, the potential of 3-amino-5-hydroxybenzoic acid to increase biosynthesis of antibiotics for which it is a precursor is clear, and the process may have applica-

tion in commercial antibiotic fermentations.

Acknowledgments

We are indebted to Dr. L. CORONELLI, Gruppo Lepetit S.p.A., Milan, for a culture of *Streptomyces* sp. E/784, fermentation of which was carried out by Mrs. M. G. ANDERSON and Mrs. J. M. ROTHSCHILD.

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