A GENETIC APPROACH TO THE BIOSYNTHESIS OF THE RIFAMYCIN-CHROMOPHORE IN *NOCARDIA MEDITERRANEI*

V. STUDIES ON THE BIOGENETIC ORIGIN OF 3-SUBSTITUENTS

PETER TRAXLER and ORESTE GHISALBA

Pharmaceuticals Division and Central Research Laboratories of
CIBA-GEIGY Limited, Basel, Switzerland

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4-Substituted 3-amino-5-hydroxybenzoic acids were tested in mutasynthesis experiments as potential starter-units for the biosynthesis of 3-substituted rifamycins. From our results it can be concluded that 3-substituents in rifamycin and other ansamycin chromophores must be introduced in a late biosynthetic step.

3-Amino-5-hydroxybenzoic acid was identified as a direct precursor of the seven-carbon amino starter-unit for the biosynthesis of rifamycins by GHISALBA *et al.*[1,2] and independently by KIBBY *et al.*[3] for the biosynthesis of the ansamycin actamycin. A biogenetic model for the biosynthesis of all types of ansamycins (*e.g.* rifamycins, streptovaricins, geldanamycins, maytansins) starting from shikimate pathway intermediates *via* 3-amino-5-hydroxybenzoic acid was therefore proposed by both groups of authors[2,3].

A number of ansamycins bear different substituents in position 3 of the aromatic nucleus (rifamycin numbering) *e.g.* hydroxyl in 3-hydroxyrifamycin S[4,5], actamycin[6] and rubransarol[7]; thiomethyl in 3-thiomethylrifamycin S[8]; chlorine in naphthomycin[9], maytansinoids[10] and ansamitocins[11]; methyl in streptovaricins[12], protostreptovaricins[13] and damavaricins[14].

For the insertion of a 3-hydroxyl group during the biosynthesis of 3-hydroxyrifamycin S (or 8-deoxy-3-hydroxyrifamycin S[15]) three different biogenetic pathways have been proposed[5,15]:

1. 3-Hydroxylation of rifamycin B (or 8-deoxyrifamycin S).
2. 3-Hydroxylation of a precursor such as protorifamycin I[16] or proansamycin B[16,17].
3. Ansamycin biosynthesis starting with a seven-carbon amino starter-unit already hydroxylated in the position which later becomes position 3 of the final rifamycins (ansamycins).

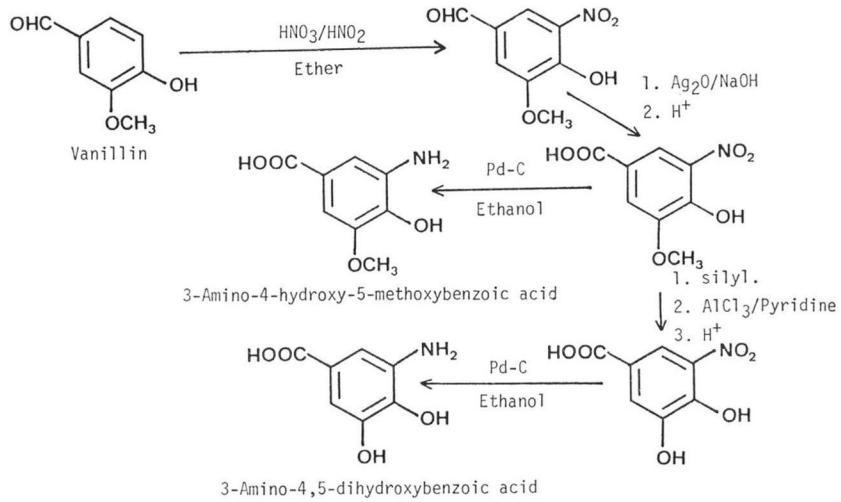
Quite recently transformation experiments showed that no 3-hydroxyrifamycin S is formed from rifamycin S with the recombinant strain *Nocardia mediterranei* R21 which is able to produce considerable amounts of 3-hydroxyrifamycin S in normal fermentations[4]. Pathway 1 can therefore be excluded.

In order to investigate the operation of pathway 3 the following 4-substituted 3-amino-5-hydroxybenzoic acids were synthesized and used as supplements in a mutasynthesis system with *Nocardia mediterranei* A8: 3-amino-4,5-dihydroxybenzoic acid, 3-amino-4-hydroxy-5-methoxybenzoic acid and 3-amino-4-methyl-5-hydroxybenzoic acid.

3-Amino-4,5-dihydroxybenzoic acid and 3-amino-4-hydroxy-5-methoxybenzoic acid were synthesized starting from vanillin, whereas for the synthesis of 3-amino-4-methyl-5-hydroxybenzoic acid *p*-toluic acid was used as starting material (Schemes 1 and 2). The acceptance of 4-substituted 3-amino-5-hydroxybenzoic acids as precursors for ansamycin biosynthesis would lead to 3-substituted rifamycins

(ansamycins) and could therefore be an interesting approach for the mutasynthesis of 3-substituted ansamycins (Fig. 1).

Scheme 1. Preparation of 3-amino-4,5-dihydroxybenzoic acid and 3-amino-4-hydroxy-5-methoxybenzoic acid.



Scheme 2. Preparation of 3-amino-4-methyl-5-hydroxybenzoic acid.

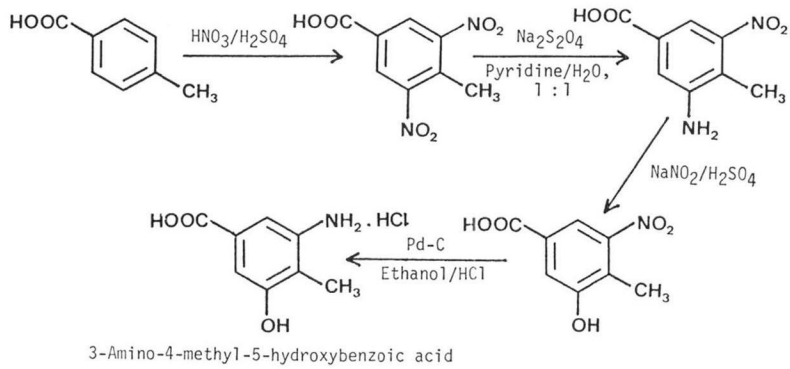
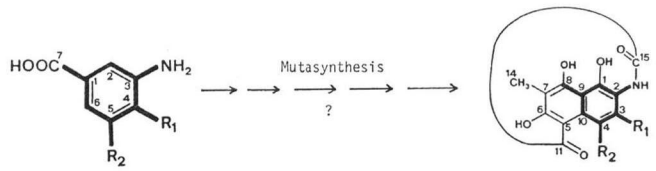


Fig. 1. Working hypothesis for the mutasynthesis of 3-substituted ansamycins.



R_1=OH, CH_3
R_2=OH, OCH_3

R_1=R_2=OH→3-Hydroxyrifamycin SV (S)

R_1=CH_3, R_2=OH → 3-Methylrifamycin SV (S)

R_1=OH, R_2=OCH_3 → 3-Hydroxy-4-methoxyrifamycin SV

Experiments and Results

Synthesis of 4-Substituted 3-Amino-5-hydroxybenzoic Acids and 3-Amino-5-methoxybenzoic Acid

3-Amino-4,5-dihydroxybenzoic Acid and 3-Amino-4-hydroxy-5-methoxybenzoic Acid

3-Nitro-4-hydroxy-5-methoxybenzaldehyde (3-nitrovanillin): 3-Nitrovanillin was prepared according to a method described in HOUBEN-WEYL[19]. From 10 g vanillin 9.6 g 3-nitrovanillin of mp 174°C (from acetic acid) were obtained.

3-Nitro-4-hydroxy-5-methoxybenzoic Acid: Oxidation of 25 g 3-nitrovanillin with silver oxide, analogous to a method described in Organic Synthesis[19] yielded 12.46 g yellow crystals of 3-nitro-4-hydroxy-5-methoxybenzoic acid of mp 217~218°C (from methanol - water).

3-Amino-4-hydroxy-5-methoxybenzoic Acid: 3-Nitro-4-hydroxy-5-methoxybenzoic acid (7.5 g) in 800 ml ethanol were hydrogenated with 2 g 10% Pd-C at room temperature for 45 minutes. The catalyst was filtered off and the filtrate evaporated to dryness leaving 7 g 3-amino-4-hydroxy-5-methoxybenzoic acid which was used for incorporation experiments without further purification.

3-Nitro-4,5-dihydroxybenzoic Acid: To a slurry of 1.4 g 3-nitro-4-hydroxy-5-methoxybenzoic acid in 150 ml of absolute CH_2Cl_2 2.5 ml of pyridine and an excess of hexamethyldisilazane were added. The mixture was stirred until a clear solution was obtained. Evaporation and high vacuum drying gave an oily residue which was dissolved in 100 ml of absolute CH_2Cl_2. To this solution 1.1 g $AlCl_3$ and 2.5 ml of pyridine were added with stirring under a stream of nitrogen. The mixture was heated to reflux over night and then 2 N HCl was added. The organic layer was separated and the aqueous phase extracted twice with CH_2Cl_2. Extraction of the acidic water phase with ether (3 times) and evaporation of the combined ether extracts to dryness yielded 870 mg of oily residue. By crystallization from $CHCl_3$ - cyclohexanone 825 mg of 3-nitro-4,5-dihydroxybenzoic acid of mp 147~150°C were obtained.

3-Amino-4,5-dihydroxybenzoic Acid: A solution of 1 g 3-nitro-4,5-dihydroxybenzoic acid in 50 ml ethanol was hydrogenated for 30 minutes with 400 mg 10% Pd-C catalyst at room temperature. The catalyst was then filtered off and the filtrate evaporated to dryness to give 970 mg of 3-amino-4,5-dihydroxybenzoic acid (M^+ at m/z 169) which was used for incorporation experiments without further purification.

3-Amino-4-methyl-5-hydroxybenzoic Acid

3,5-Dinitro-4-methylbenzoic Acid (3,5-Dinitro-*p*-toluic Acid): 3,5-Dinitro-*p*-toluic acid was prepared from 50 g *p*-toluic acid by a method analogous to the preparation of 3,5-dinitrobenzoic acid (in Organic Synthesis[20]). Yield: 77.5 g of 3,5-dinitro-4-methylbenzoic acid of mp 161°C (from ethyl acetate - petroleum ether)

3-Nitro-4-methyl-5-aminobenzoic Acid: 3,5-Dinitro-*p*-toluic acid (43 g) was partially reduced with $Na_2S_2O_4$ according to a described method[21]. Yield: 22.5 g of 3-nitro-4-methyl-5-aminobenzoic acid of mp 171°C (M^+ at m/z 196).

3-Nitro-4-methyl-5-hydroxybenzoic Acid: The preparation of this compound from 3-nitro-4-methyl-5-aminobenzoic acid is described in the Journal of Medicinal Chemistry[21]. From 27.5 g 3-nitro-4-methyl-5-aminobenzoic acid 7.23 g of 3-nitro-4-methyl-5-hydroxybenzoic acid of mp 218°C (lit. 215°C) were obtained (M^+ at m/z 197).

3-Amino-4-methyl-5-hydroxybenzoic Acid: 3-Nitro-4-methyl-5-hydroxybenzoic acid (5 g) in 150 ml ethanol and 2.35 ml concentrated HCl were hydrogenated with 1.2 g 10% Pd-C catalyst for 45

minutes at room temperature. The catalyst was then filtered off and the filtrate evaporated to dryness. Yield: 4.75 g of 3-amino-4-methyl-5-hydroxybenzoic acid hydrochloride (M^+ at m/z 167).

Supplementation Studies with 4-Substituted 3-Aminohydroxybenzoic Acids and 3-Amino-5-methoxybenzoic Acid

Supplementation experiments were carried out with *Nocardia mediterranei* A8 (transketolase$^-$-mutant with drastically reduced rifamycin production[23]) in industrial fermentation medium 151b as described in a preceding paper for 3-amino-5-hydroxybenzoic acid[2].

Five different concentrations (0, 0.5, 1, 2 and 4 g/liter) of the 4-substituted 3-amino-5-hydroxy- (or 5-methoxy) benzoic acids were tested and two parallel experiments were carried out for each concentration. After fermentation the culture filtrates and ethyl acetate extracts[16] were investigated for new rifamycins (ansamycins) on TLC silica gel plates (Merck) using $CHCl_3$ - CH_3OH (4: 1) as solvent system. Rifamycin B, 3-hydroxyrifamycin S and 3-hydroxyrifamycin SV were used as reference standards.

In all the mutasynthesis experiments only rifamycin B and not even traces of the expected 3-hydroxyrifamycin S or SV (when supplemented with 3-amino-4,5-dihydroxybenzoic acid), 3-hydroxy-4-methoxyrifamycin SV (when supplemented with 3-amino-4-hydroxy-5-methoxybenzoic acid) or 3-methylrifamycin S or SV (when supplemented with 3-amino-4-methyl-5-hydroxybenzoic acid) were detected. However, in all the experiments the added 3-amino-5-hydroxy (5-methoxy) benzoic acid derivative was partially metabolized. Using 3-amino-4-hydroxy-5-methoxybenzoic acid as a supplement, 3-acetamido-4-hydroxy-5-methoxybenzoic acid (M^+ at m/z 225) and a condensation product thereof with 3-amino-4-hydroxy-5-methoxybenzoic acid (M^+ at m/z 358, $C_{17}H_{14}N_2O_7$) could be isolated.

Discussion

The mutasynthesis experiments with *Nocardia mediterranei* A8 demonstrated that only 3-amino-5-hydroxybenzoic acid[2] but not 4-substituted 3-amino-5-hydroxybenzoic acids can substitute for the seven-carbon amino starter-unit in the biosynthesis of rifamycins. The failure to isolate even traces of 3-hydroxyrifamycin S or SV*, 3-hydroxy-4-methoxyrifamycin SV or 3-methylrifamycin S (SV) using 4-substituted 3-amino-5-hydroxy (5-methoxy) benzoic acids in our mutasynthesis system indicates that the activating enzyme system for the seven-carbon amino unit seems to be highly specific and does not accept 4-substituted 3-amino-5-hydroxybenzoic acids as substrates.

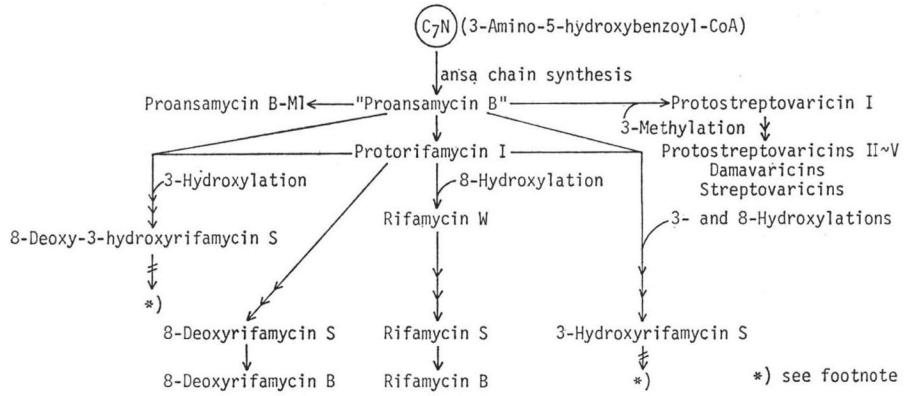
Pathway 3 (see introduction) for the insertion of the 3-hydroxyl group (and other substituents) into rifamycins (ansamycins) can therefore be excluded. The partial transformation of the added supplements at least indicates the uptake of the three tested 4-substituted 3-amino-5-hydroxy (5-methoxy)-benzoic acids into the cells.

If we assume comparable substrate specificities for the 3-amino-5-hydroxybenzoic acid-activating enzymes in all the other ansamycin-producing actinomycetes we can conclude from our negative results with the incorporation of 3-amino-4-methyl-5-hydroxybenzoic acid that the 3-methyl group in antibiotics of the streptovaricin type and most likely also the 3-chlorine in other ansamycins (*e.g.* maytansins) is not present in the seven-carbon amino starter-unit. Therefore, pathway 3 is also ruled out for these other ansamycin-types.

Since pathway 1 (3-hydroxylation of rifamycin S or 8-deoxyrifamycin S) was already excluded by earlier experiments[4] only pathway 2 (see introduction) is left, proposing 3-hydroxylation (methylation) of an early intermediate such as proansamycin B or protorifamycin I. This pathway is depicted in Fig. 2.

* It has recently been demonstrated that 3-hydroxyrifamycin S is not transformed into 3-hydroxyrifamycin B by the mycelium of *Nocardia mediterranei*[22].

Fig. 2. A biogenetic model for the introduction of C(3)-substituents in rifamycins and streptovaricins. (For the biosynthesis of proansamycin B see Fig. 2 in ref. 2)



Our results are in good agreement with the general pathway for the biosynthesis of ansamycins proposed in the preceding paper[2] (see Fig. 2 in part IV) and also support our proansamycin B hypothesis[15,16,17]. Similar pathways for the introduction of 3-substituents could be postulated for the maytansinoids, ansamitocins, rubransarols, naphthomycin and actamycin but in all these cases the branching points from the general ansamycin biosynthetic pathway are located before the level of proansamycin B (see Fig. 2 in part IV[2]).

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