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

III. ISOLATION AND IDENTIFICATION OF AN EARLY AROMATIC ANSAMYCIN-PRECURSOR CONTAINING THE SEVEN-CARBON AMINO STARTER-UNIT AND THREE INITIAL ACETATE/PROPIONATE-UNITS OF THE ANSA CHAIN

ORESTE GHISALBA, HERMANN FUHRER, WILHELM J. RICHTER and SERGE MOSS

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

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A number of rifamycin non-producing UV-mutants derived from *Nocardia mediterranei* strains N813 (rifamycin B producer) and A10[1] (*aro*$^-$-mutant excreting shikimate derived from strain N813) were found to accumulate an identical complex of aromatic components instead of rifamycin B. The main component of this aromatic complex, product P8/1-OG, was isolated from six of these P^--mutant strains and identified spectroscopically as a very early precursor in the biosynthesis of rifamycins.

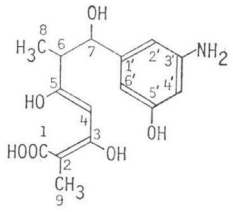
As described in parts I and II of this series of papers[1,2] we could demonstrate by chemical and biochemical analysis of *aro*$^-$-mutants of *Nocardia mediterranei*, that the seven-carbon amino unit of rifamycin is derived from an intermediate of the shikimate pathway between 3-deoxy-D-arabinoheptulosonic acid-7-phosphate and shikimate. These findings are also supported by the ^{13}C incorporation experiments published by KARLSSON *et al.*[3] and WHITE *et al.*[4].

In order to identify accurately the branch point intermediate between the shikimate pathway and rifamycin-chromophore biosynthesis, we tried to isolate *aro*$^-$-mutants of *Nocardia mediterranei* strains N813 or A10 blocked either in DAHP synthetase, 3-dehydroquinate synthetase, 3-dehydroquinate dehydratase or shikimate reductase. A number of rifamycin non-producing UV-mutants (P^--mutants) were isolated and tested for excretion of shikimate pathway intermediates differing from shikimate. This approach has not yet led to the isolation of mutants blocked in one of the four enzyme activities mentioned above. All the P^--mutants derived from strain N813 are *aro*$^+$, rifamycin$^-$, whereas the P^--mutants derived from strain A10 are *aro*$^-$, rifamycin$^-$.

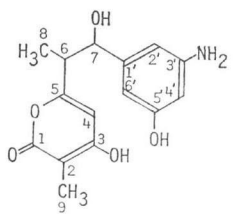
As all the mutants from A10 still excrete shikimate and no other shikimate pathway intermediates, the rifamycin$^-$-mutation is not due to a second block in the shikimate pathway before the branch point but is due to additional blocks in the rifamycin biosynthesis behind the branch point.

Nevertheless, the chemical analysis of these mutants led to some interesting results. Some of the strict P^--mutants were found to accumulate a chromatographically identical complex of aromatic compounds instead of rifamycin B (leaky mutants did not show this effect). The

Fig. 1. Structure of product P8/1-OG.



I: Product P8/1-OG



II: Product P8/1-OG-lactone

main component of this aromatic complex, product P8/1-OG, was isolated and identified by means of spectroscopic methods as 2,6-dimethyl-3,5,7-trihydroxy-7-(3′-amino-5′-hydroxyphenyl)-2,4-heptadienoic acid. Product P8/1-OG can be isolated either as the free acid or as its 1,5-lactone, using different isolation procedures.

The structure of this compound supplies us with considerable biosynthetic information (see discussion).

Experiments and Results

Mutant Selection and Characterization

Using the UV-irradiation technique for the induction of auxotrophic mutants of *Nocardia mediterranei* described by SCHUPP[5] and GHISALBA[6], P^--mutants of the strains N813 and A10 were isolated as pale colonies clearly different from the orange colonies of strains N813 or A10. The colonies were transferred into liquid complex medium 148, grown at 28°C and the mycelium was conserved as a frozen skim milk suspension at −30°C[5,6].

The culture filtrates of fermentations in liquid complex medium 148[2] or industrial fermentation medium 151b[7] were investigated by TLC-methods as described earlier[1,2]. On silica gel TLC plates using solvent system 1 the culture filtrates of some of the isolated strict P^--mutants showed (after air-drying) an identical distribution of one strong brown spot with Rf 0.81 ~ 0.82 and two weak brown spots with Rf 0.86 and 0.92, respectively. These three spots could not be detected in the corresponding tests with culture filtrates of the strains N813 and A10. Likewise for the P^--leaky mutants these spots did not occur. The special behaviour of these new spots (darkening during the air-drying) is often observed for aromatic acids such as gallic acid or dihydroxybenzoic acids.

After spraying the plates with periodic acid - benzidine reagent[1,2], shikimate (Rf 0.56) and sometimes also 3-dehydroshikimate (Rf 0.38), but no other intermediates of the shikimate pathway, could clearly be detected in all the tests with P^--mutants derived from *Nocardia mediterranei* A10. The tests with the P^--mutants derived from strain N813 showed no intermediates of the shikimate pathway after spraying with periodic acid - benzidine reagent.

From these results we can conclude that in all the investigated strains the rifamycin$^-$-mutation is due to a block in the rifamycin biosynthesis behind the branch point intermediate of the shikimate pathway and rifamycin-chromophore biosynthesis, respectively.

Therefore it seemed very likely that the three additional "aromatic" compounds detected in the culture filtrates are early precursors in the biosynthesis of rifamycin and the knowledge of their structures could provide us with interesting biosynthetic information.

The following P^--mutant strains show this accumulation of aromatic compounds:

strains derived from *Nocardia mediterranei* A10: P8, P14, P17, P18 and NS 17 (accumulation of aromatic compounds and shikimate)

strains derived from *Nocardia mediterranei* N813: F3/2, F3/15, F3/16 and F3/22 (only accumulation of aromatic compounds).

Isolation of Product P8/1-OG

Isolation of Product P8/1-OG as its Free Acid (I)

To obtain large enough amounts of the main component of the aromatic complex (TLC: Rf 0.81 ~ 0.82), the following fermentations were carried out. Two 200-ml shake flasks with 40 ml of liquid com-

plex medium 148[2] were inoculated with mycelium of P^--strain (60 mg total dry weight per flask) and fermented for 4 days (250 rpm, 28°C). Three ml of this culture were transferred into each of 10~20 500-ml shake flasks (one baffle) with 100 ml industrial fermentation medium 151b[7] and fermented for another 10 days (250 rpm, 28°C).

The mycelium was separated by centrifugation (20 minutes, 3,000×*g*). The supernatant (1,900 ml) of a fermentation with *Nocardia mediterranei* P8 was passed over a column with 1,100 g Dowex 2×8 (100~200 mesh, Cl^-) to adsorb acidic compounds and washed with 3 liters of deionized water to remove the neutral and alkaline compounds. The effluents were controlled by TLC (solvent system 1, periodic acid - benzidine reagent). The acidic compounds were then eluted (360 ml/hour) with 2 liters of 0.05 N HCl and 6 liters of 0.1 N HCl. Fractions of 300 ml were collected and tested by TLC. Shikimate and 3-dehydroshikimate were eluted first, together with some other compounds not identified, whereas product P8/1-OG is found in the later fractions well separated from shikimate. The fractions containing product P8/1-OG (but not shikimate) were pooled, passed over a porcelain suction filter with 200 g charcoal (activated GR, Merck Art. 2186) and washed with deionized water to remove the HCl. The neutral charcoal with the adsorbed product P8/1-OG was then eluted with water and increasing amounts of ethanol. Product P8/1-OG was found in the fractions with 60~100% ethanol as eluent. These fractions were evaporated to dryness under reduced pressure and 780 mg of chromatographically pure product P8/1-OG (free acid) were obtained as a light brown amorphous powder.

Isolation of Product P8/1-OG as its 1,5-Lactone (II)

Fermentation and fractionation of the fermentation broth by chromatography on Dowex 2×8 were carried out as described above. The fractions containing product P8/1-OG (but not shikimate) were pooled, neutralized (pH 7.0) with NaOH and evaporated to dryness under reduced pressure. The solid brown residue was then stirred for 60 minutes with 1 liter of absolute ethanol. The insoluble NaCl-fraction was separated by filtration and the operation was repeated twice with 500 ml of absolute ethanol. The yellow filtrates were pooled, treated with 500 mg of charcoal to remove high molecular impurities, dried with molecular sieve (3 Å, Merck Art. 5704) and, after filtration, evaporated to dryness under reduced pressure. Product P8/1-OG as its 1,5-lactone (light brown amorphous powder, TLC: minor impurities) was obtained by this procedure from the following strains of *Nocardia mediterranei*: P14 (2,035 mg from 1,600 ml fermentation broth), P17 (110 mg from 1,600 ml), P18 (440 mg from 1,600 ml), NS17 (310 mg from 1,300 ml) and F3/22 (195 mg from 1,500 ml).

Physico-Chemical Properties and Structure of Product P8/1-OG

The product has no defined melting point but decomposes at 120~145°C in the form of its free acid (**I**) or above 200°C in the lactone form (**II**).

From elemental analysis and from the data of ^{13}C-NMR and mass spectroscopy the molecular formula of product P8/1-OG was deduced to be $C_{15}H_{19}NO_6$ (**I**) or $C_{15}H_{17}NO_5$ (**II**) respectively. Elemental analyses: calculated for $C_{15}H_{19}NO_6$ (**I**): C 58.3, H 6.2, N 4.3, O 31.1% and found for P8/1-OG (**I**): C 56.0, H 6.5, N 4.9, O 32.0%.

The IR spectrum of **I** (KBr) shows bands at (wave numbers in cm^{-1}): 3400 (OH, NH), 3020 and 2950 (various CH), 2650 (bridged OH), 1675 (C=O, conjugated), 1590 (C=C, aromatic and vinylic), 1465, 1420, 1380 (CH_3), 1340, 1310, 1255, 1175 (various C–O), 1140, 1085, 1040, 1002, 985, 960, 935, 845 (aromatic H), 760, 727 and 700. The carbonyl band of **I** shows a strong shift from 1675 cm^{-1} (in KBr) to 1690 cm^{-1} or to 1715 cm^{-1} when the spectrum is recorded in DMSO-d_6 or in dioxane, respectively.

This indicates that product P8/1-OG (**I**) is present as its free acid.

The IR spectrum of **II** (KBr) shows bands at (cm^{-1}): 3400 (OH, NH), 2980 and 2940 (various CH), 1600 (C=O, conjugated), 1600 and 1500 (C=C, aromatic and vinylic), 1440, 1355, 1300, 1265, 1175 (various C–O), 1080, 1040, 995, 960, 930, 840 (aromatic H), 760, 725 and 700. The band at 2650 cm^{-1} (bridged acid OH) present in I is missing in **II**. The IR spectrum of **II** in DMSO-d_6 shows no shift of the carbonyl band compared to the KBr-spectrum, thus indicating that product P8/1-OG (**II**) is present in its lactone form.

The lactone (**II**) can easily be converted into the free acid (**I**) by the following treatment: 100 mg lactone are dissolved in 100 ml 0.1 N HCl and kept at 25°C for 3 days. The acidic solution is then passed over a porcelain suction filter with 25 g of charcoal (Merck) and washed to remove the HCl. The free acid (**I**) is eluted with increasing amounts of ethanol and isolated as described above. The IR spectrum of this conversion product is identical with the spectrum of the original free acid (**I**) and shows the typical carbonyl shift in DMSO-d_6 (compared to KBr). The IR spectra were recorded for the free acid (**I**) isolated from strain P8 and for all the lactone preparations (**II**) isolated from the strains P14, P17, P18, NS17 and F3/22 (all the lactone-spectra were identical).

The electron impact (EI) mass spectrum of the lactone **II**, obtained at 120°C, displays a molecular ion peak $M^{+\cdot}$ at *m/z* 291 of moderate intensity. It disappears, due to thermal decomposition of **II**, when raising the temperature. The chief fragment ions, *m/z* 154 (base peak; $C_8H_{10}O_3$, determined by accurate mass measurement at high instrumental resolution) and *m/z* 138, are both formed through benzylic cleavage of the C(6)~C(7) bond, with and without hydrogen transfer, respectively. The former, a product of a McLAFFERTY rearrangement (H-transfer probably from 7-OH to C(4)), embodies the α-pyrone moiety together with C(6) and C(8); the latter comprises the aromatic portion of the molecule as the complementary structural unit. On silylation, (treatment with *bis*(trimethylsilyl)acetamide - pyridine at 60°C, 2 hours), **II** forms a *tetrakis* (trimethylsilyl)-, *i.e.* TMS_4-derivative with $M^{+\cdot}$ at *m/z* 579.

Table 1. ^{13}C-NMR data of product P8/1-OG. (δ values in ppm)

Carbon	Product P8/1-OG free acid (**I**) (CD_3OD)	Product P8/1-OG 1,5-lactone (**II**) (CD_3OD)
C(1)	168.0	167.9
C(2)	99.3	~100*
C(3)	169.3	164.2
C(4)	102.3	96.5
C(5)	166.0	149.7
C(6)	47.0	30.7
C(7)	77.0	77.3
C(8)	15.3	16.0
C(9)	8.3	8.8
C(1′)	145.9	145.9
C(2′)	108.1	108.9
C(3′)	147.1	146.1
C(4′)	104.4	103.1
C(5′)	159.2	159.1
C(6′)	106.8	107.2

* Tentatively assigned.

Table 2. 360-MHz NMR data of product P8/1-OG. (δ values in ppm, coupling constants *J* (Hz) in brackets)

Protons	Product P8/1-OG free acid (**I**) (DMSO-d_6)	Product P8/1-OG 1,5-lactone (**II**) (DMSO-d_6)
H(4)	6.00s	5.30s
H(6)	2.62m (9) DR	2.38m (9)
H(7)	4.28d (9) DR	4.27d (9)
H(2′)	6.02s	6.00s
H(4′) H(6′)	} 5.93s	} 5.89s and 5.92s
CH_3(8)	0.87d (7)	0.76d (7)
CH_3(9)	1.77s	1.56s
OH, NH_2	5.17 and 8.78	4.76 and ~8.8

DR: Position confirmed by double resonance experiment.

This corresponds to a TMS substitution of the 3 hydroxyl functions in addition to the usual substitution of 1 of the 2 amino hydrogen atoms. Accordingly, *m/z* 138 is shifted to 354 (3 TMS groups), *m/z* 154 to 298 (1 TMS group *plus* another one by TMS transfer instead of H, as is frequently observed in related cases[8]). Obviously due to facile conversion in to **II**, the free acid **I** gave essentially identical results irrespective of whether analyzed as such or after TMS derivative formation. For that very reason, field desorption (FD) mass spectrometry also failed to furnish evidence of intact **I**. In all attempts made only *m/z* 291 ions (M (**II**)) were observed instead of the expected ones at *m/z* 309 (M (**I**)).

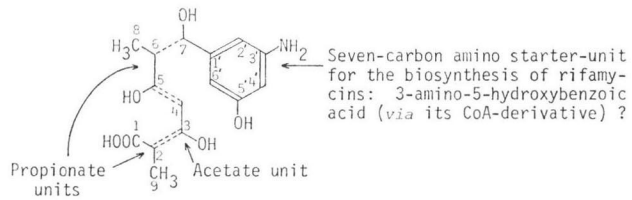
The ^{13}C-NMR spectra (data see Table 1) are in good agreement with the postulated structures. In contrast to the free acid (**I**), considerable shift differences (upfield drift) were found for C(3) to C(6) of the lactone (**II**) due to the formation of the lactone ring.

In the 360-MHz NMR spectra (data see Table 2) strong shift differences between structures **I** and **II** are found for H(4) and H(6) only. With all these findings the structures of product P8/1-OG as its free acid (**I**) and as its 1,5-lactone (**II**) seem to be well established. Product P8/1-OG (**I**) showed no antibiotic activity.

Discussion

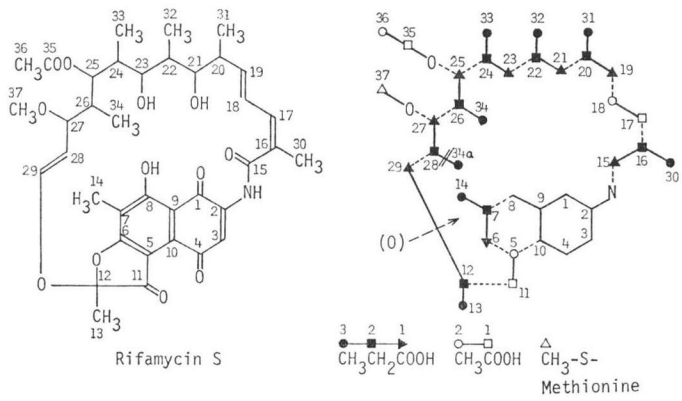
The structure of product P8/1-OG supplies us with considerable biosynthetic information. By structural comparison with rifamycin S or rifamycin W the isolated product P8/1-OG can easily be recognized as a very early ansamycin precursor containing the seven-carbon amino starter-unit and three initial acetate/propionate units of the ansa chain (see Fig. 2). 3-Amino-5-hydroxybenzoyl-coenzyme A might act as a starter-molecule for the biosynthesis of product P8/1-OG and of the rifamycins. To this seven-carbon amino unit a first propionate unit (*via* methylmalonyl-CoA), then an acetate unit

Fig. 2. Biosynthetic interrelationship between product P8/1-OG and rifamycin.



Product P8/1-OG

Incorporation of acetate, propionate and methyl from methionine into rifamycin.



(*via* malonyl-CoA) and finally another propionate unit are added by condensation and decarboxylation. The resulting aromatic triketide is then converted into product P8/1-OG by hydrogenation of the keto group C (7) and by enolization of the keto groups C (3) and C (5). The CoA is split off (possibly during the excretion of the product).

If we compare this biogenetic model for P8/1-OG with the well-known incorporation patterns for ^{13}C-acetate and ^{13}C-propionate into rifamycin S[3,9,10] or rifamycin W[11], we can deduce that the two structures must have the same biogenetic origin. The methyl groups C (14) and C (13) in the rifamycins correspond to the methyl groups C (8) and C (9) in P8/1-OG. Thus our product P8/1-OG, or its CoA-derivative, respectively, is the earliest precursor of the rifamycins to be described so far. The fact that nine independent UV-mutants all accumulating this precursor could be isolated might be explained in different ways: *e.g.* mutational hot-spot or multienzyme complex (matrix), where various mutational events might lead to an early interruption of the polyketide (ansa chain) synthesis. With the present knowledge we can not decide which of these explanations is correct.

We have observed similar frequent appearances of phenotypically identical mutations in some of the later steps in rifamycin biosynthesis. A considerable number of mutants accumulating rifamycin S, rifamycin W or protorifamycin I[7] have been isolated.

In conclusion the isolation of product P8/1-OG provides strong evidence that the seven-carbon amino starter-unit for the ansamycin biosynthesis is 3-amino-5-hydroxybenzoic acid or its coenzyme A derivative, respectively. Our experiments with 3-amino-5-hydroxybenzoic acid will be discussed in the following paper[12].

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