THIAZORIFAMYCINS. III

BIOSYNTHESIS OF RIFAMYCINS P, Q AND VERDE, NOVEL METABOLITES FROM A MUTANT OF *NOCARDIA MEDITERRANEA*

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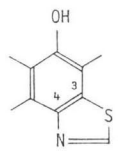
The thiazorifamycins are derived from rifamycin S and cysteine; rifamycins P and Verde are formed by chemical reactions, while rifamycin Q synthesis involves obligatory enzymatic assistance. All antibiotics come from a common six-member precursor lacking C-1 of cysteine; the thiazin-2-one ring of rifamycin Verde retains both C-2 and C-3 of cysteine, while the thiazole ring of rifamycins P and Q retains only C-2, C-3 becoming exocyclic in the ring-contraction C-3 is present in rifamycin Q but not in P.

In the first paper of this series[1] it has been shown that rifamycins P (**I**), Q (**II**) and Verde (**III**) obtained, together with rifamycin R[2], from the fermentation broths of a mutant of *Nocardia mediterranea* blocked in the conversion of rifamycin SV into rifamycin B, are characterized by the presence of a thiazole ring (rifamycins P and Q) and of a thiazin-2-one ring (rifamycin Verde) condensed on positions 3 and 4 of the chromophore of rifamycin S (**IV**) (Fig. 1).

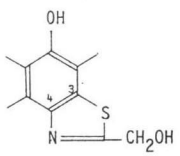
Two other examples of natural products containing a benzothiazole system are known: the degradation products of the pigments of reddish hair and feathers[3] and the "unique" molecule of firefly luciferin[4]. No example of natural products containing a benzothiazin-2-one system has been reported in the literature to date.

A probable biosynthesis of the luciferin benzothiazole nucleus by the condensation of *p*-benzoquinone with cysteine[5] and a possible mechanism of formation of the benzothiazole nucleus from benzothiazine in the biogenesis of the macromolecular pigments have been proposed[3].

Fig. 1.



Rifamycin P (**I**)



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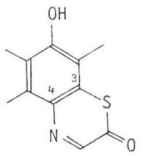
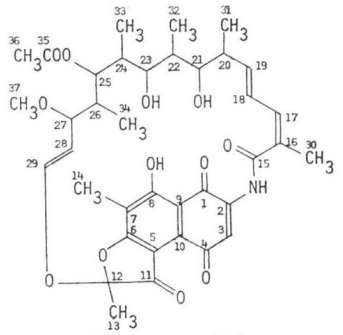


Rifamycin Verde (**III**)



Rifamycin S (**IV**)

We present in this paper evidence for the biosynthesis of three thiazorifamycins and we propose, moreover, a pathway which accounts for the formation from a common precursor of the thiazole-rifamycins P and Q and of the thiazin-2-one rifamycin Verde.

Materials and Methods

Organism

The *N. mediterranea* strain (mutant M/36), producer of the thiazorifamycins, was obtained by mutation of rifamycin B producing strain ATCC 21789[6] and was deposited with the ATCC number 31066.

Radiolabeled precursors

[^{35}S]-cysteine, [3-^{14}C]-cysteine and [U-^{14}C]-cysteine were obtained from the Radiochemical Centre (Amersham, England). [^{14}C]-labeled rifamycin S was produced as described before.[7]

Production and isolation of labeled rifamycins P, Q and Verde

Stock cultures of the producer organism were maintained as frozen vegetative mycelium at -20°C. After an intermediate growth (48 hours) in a soluble organic medium[8] the vegetative mycelium was used to inoculate a complex fermentation broth[8] which was then incubated at 28°C for 168 hours. Very little antibiotic was synthesized during the first 3 days, so radioactive precursors (both [^{14}C]-rifamycin S and labeled cysteine) were added at 72 hours. Fermentation broths were filtered, adjusted to pH 2 and extracted with ethyl acetate. Evaporation of the solvent gave a crude mixture of rifamycins.

Purification and identification of labeled rifamycins

Labeled rifamycins P, Q and Verde were purified by preparative TLC on silicagel HF plates (Merck), using chloroform - methanol (95: 5) as the solvent system, and were identified by comparison with authentic samples.

Assay of radioactivity

Labeled samples of rifamycins were dissolved in methanol and $0.1 \sim 0.5$ ml aliquots added to 10 ml of Instagel (Packard Instrument Company) scintillation cocktail. Radioactivity was measured with an Intertechnique liquid - scintillation spectrometer model SL 30, using an internal standard for the determination of the quenching factor.

Transformation of [^{14}C]-rifamycin S into rifamycins P, Q and Verde and incorporation of labeled precursors in thiazorifamycins

a) With "living" washed mycelium: Experiments with washed mycelium were carried out by growing the producer organism in a soluble organic medium[8] for 48 hours; then the mycelium was harvested, washed, starved for 5 hours in 0.7 M phosphate buffer (pH 6.5) and suspended in a volume equal to the original of 0.7 M phosphate buffer. [^{14}C]-Rifamycin S and the suitable precursors were added to the mycelial suspension and incubated again on a rotary shaker (260 rpm) at 28°C. After 24 hours the cultures of the washed mycelium suspensions were filtered, the cake washed and discarded, and the filtrate acidified to pH 2 was extracted with ethyl acetate. The purification of the rifamycins was carried out as described above.

b) With "boiled" washed mycelium: In order to assess if the synthesis of the thiazorifamycins is an enzymatic or a chemical reaction, a washed mycelium preparation obtained as before was heated at 120°C for 10 minutes in order to destroy all the enzymatic activity and then used in the experiments of incorporation described in section **a**.

c) Without washed mycelium: As a negative control the reactions described in section **a** were repeated without adding washed mycelium.

Results

Rifamycin S is a Precursor of the Thiazorifamycins

In Table 1 the results are presented for the transformation of [^{14}C]-rifamycin S into rifamycins P, Q and Verde with a washed mycelium preparation. The specific activity of rifamycin S and each of the thiazorifamycins produced at the end of the incubation period is the same and is slightly lower than that of added [^{14}C]-rifamycin S, owing to dilution from unlabeled endogenously generated molecules. These results show that rifamycin S can serve as a precursor of the thiazorifamycins.

Table 1. Transformation of [^{14}C]-rifamycin S into [^{14}C]-thiazorifamycins by washed mycelium of *N. mediterranea*.

[^{14}C]-Rifamycin	Specific activity μCi/mMole
S (added)	9.17
S (produced)	6.66
P	6.92
Q	6.96
Verde	6.92

The Thiazo Ring Comes from Cysteine

In Table 2 the results are presented for the incorporation of [^{35}S]-cysteine, [3-^{14}C] and [U-^{14}C]-cysteine into rifamycins S, P, Q and Verde, with a washed mycelium preparation.

The radioactivity of [^{35}S]-cysteine added to an actively synthesizing culture at hour 72 is incorporated into the thiazorifamycins, implicating cysteine as the source of the S atom of the thiazo ring. All three thiazorifamycins have the same specific activity suggesting both a common origin and close biosynthetic relation. Moreover similar experiments in which [U-^{14}C]-cysteine is used as precursor show that both rifamycin S and the thiazorifamycins are labeled; however the specific activity of rifamycin S, which is about 4~8-times lower than that of the thiazorifamycins is due to the incorporation into the rifamycin carbon skeleton of radioactive cysteine-derived precursors. Since the thiazorifamycins derive from the S form their specific radioactivity (Table 2, last column) is obtained by subtracting the observed value of the specific radioactivity found for rifamycin S. As is expected from the structural formulae, rifamycins Q and Verde possess identical specific activities which are double that of P. When [3-^{14}C]-cysteine is used as radioactive precursor, rifamycin P is not labeled, except for the background radioactivity of the carbon skeleton, while rifamycins Q and Verde are highly radioactive. This finding indicates the absence of the C-3 carbon atom of cysteine from the molecule of P.

Table 2. Incorporation of [^{35}S]-cysteine, [3-^{14}C]-cysteine and [U-^{14}C]-cysteine into thiazorifamycins by washed mycelium of *N. mediterranea*.

Rifamycin	Specific activity μCi/mMole				
	[^{35}S]-Cysteine added 530 μCi	[3-^{14}C]-Cysteine added 100 μCi		[U-^{14}C]-Cysteine added 100 μCi	
		observed value	calculated value	observed value	calculated value
S		8.76	0	11.12	0
P	871.0	8.85	0	51.66	40.54
Q	860.2	89.8	81.1	92.9	81.8
Verde	891.9	87.4	78.7	88.9	77.8

None of the Three Thiazorifamycins is the Precursor of the Other Two

Table 3 shows that a washed mycelium preparation obtained from a 48-hour culture is not able to transform rifamycin Verde into rifamycins P and Q, rifamycin P into Q and *vice versa*, thus indicating that none of the three thiazorifamycins is the precursor of the other two.

Rifamycins P and Verde are Formed by a Chemical Reaction between Rifamycin S and Cysteine, while Rifamycin Q Requires the Presence of Enzymatic Activity in the Microorganism

Table 3 shows that when rifamycin S and cysteine are added to a "washed mycelium preparation" obtained from a 48-hour culture all three thiazorifamycins are obtained, while by using a "boiled washed mycelium preparation" or without adding washed mycelium only rifamycins P and Verde are obtained.

These results indicate that only the synthesis of rifamycin Q requires obligatory enzymatic assistance,

Table 3. Transformation of rifamycins with
A) living washed mycelium preparation
B) boiled washed mycelium preparation
C) control: buffer solution pH 6.5

Substrate	Added precursor	Produced rifamycins			Experiment
		P	Q	Verde	
Rifamycin S	Cysteine	+	+	+	A
Rifamycin S	Cysteine	+	−	+	B
Rifamycin S	Cysteine	+	−	+	C
Rifamycin P		+	−		A
Rifamycin Q		−	+		A
Rifamycin Verde		−	−	+	A

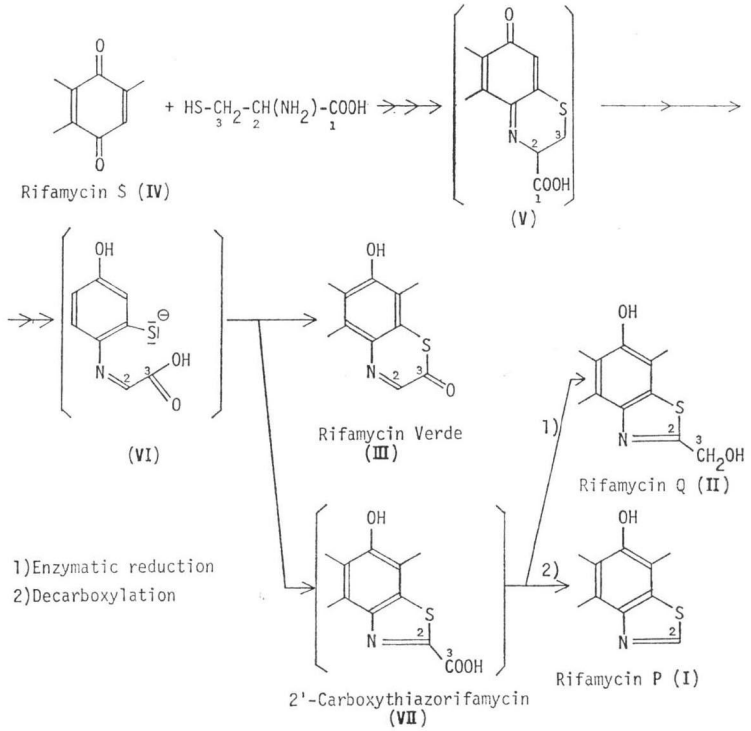
+ present, − absent

while rifamycins P and Verde are the product of a chemical interaction between rifamycin S and cysteine.

Discussion

The above reported results allow us to propose a biosynthetic pathway for the thiazorifamycins, which is illustrated in Scheme 1. Once rifamycin S has been produced in the fermentation broth a chemical reaction with cysteine takes place leading, after three steps, to the intermediate compound **V** which is transformed (as has been shown in the second paper of this series[9]) to the intermediate compound **VI** with a sequence of steps which includes the simultaneous cleavage of the S-C-3 bond and the elimination of C-1 (*i.e.* the carboxyl group of cysteine).

Scheme 1. Proposed biosynthetic pathway for the thiazorifamycins.



Compound **VI** is the key-intermediate in the biosynthesis of the thiazorifamycins: in fact it gives rise to both the thiazin-2-one rifamycin Verde by nucleophilic attack of S^- on C-3 and the thiazolorifamycin by nucleophilic attack of S^- on C-2. In the latter case a 2′-carboxythiazorifamycin (compound **VII**) is obtained through a ring-contraction which allows C-3 to become exocyclic.

Compound **VII** is the precursor of both rifamycin Q and rifamycin P: in fact rifamycin Q is obtained by enzymatic reduction of the carboxyl group, while rifamycin P is obtained by elimination of the carboxyl group (*i.e.* of the C-3 of cysteine). This is supported by the experiments of incorporation of [3-^{14}C]-cysteine in thiazorifamycins: rifamycins Q and Verde which retain the C-3 are labeled while rifamycin P which does not retain the C-3 is not labeled.

The biosynthesis of rifamycin P deserves a further consideration: we have shown[1] that compound **VII** is stable as sodium salt but not as the free acid; under acidic conditions, it decomposes on heating under vacuum giving rise to rifamycin P. Consequently the hypothesis can be made that rifamycin P is not directly produced in the fermentation broths but is obtained after the isolation procedure already described. In fact TLC of the crude material obtained from the fermentation broths without acidification showed the absence of rifamycin P and the presence of compound **VII**, while TLC of the crude material obtained after acidification showed the presence of rifamycin P and the disappearance of **VII**.

Our results support those reported in the literature; the benzothiazole is formed by condensation of *p*-benzoquinone with cysteine[5] and the ring-contraction benzothiazine-benzothiazole occurs in such a way that the C-3 of cysteine becomes exocyclic, while the sulfur atom becomes bonded to C-2 of the cysteine[3].

Acknowledgments

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