16,17-DIHYDRORIFAMYCIN S AND 16,17-DIHYDRO-17-HYDROXYRIFAMYCIN S, TWO NOVEL RIFAMYCINS FROM A RECOMBINANT STRAIN C 5/42 OF *NOCARDIA MEDITERRANEI*

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The structures of 16,17-dihydrorifamycin S and 16,17-dihydro-17-hydroxyrifamycin S, two novel ansamycins from the recombinant strain C 5/42 of *Nocardia mediterranei*, were elucidated by physico-chemical methods. In addition structure-activity relationships among 16,17-hydrogenated rifamycins are discussed.

In the course of our search for new ansamycins by mutation or recombination we found that a recombinant strain C 5/42 of *Nocardia mediterranei* produces the two hitherto unknown ansamycins 16,17-dihydrorifamycin S and 16,17-dihydro-17-hydroxyrifamycin S together with the already known 16,17-dehydrorifamycin G[2], rifamycin W[3] and rifamycin W-lactone[2] (Fig. 1)*.

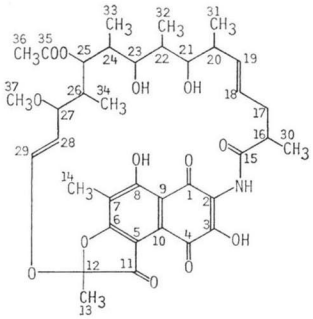
In the first part of this paper we report on the isolation and characterization of recombinant strain C 5/42 and on the fermentation, purification and structural elucidation of the two new rifamycins. In the second part we discuss some biological properties of the compounds from recombinant strain C 5/42 and of 16,17-dihydrorifampicin SV which is a derivative of 16,17-dihydrorifamycin S, together with the results of some transformation studies. The structures of the ansamycins from the C 5/42 strain provide useful information about structure-activity relationships among the rifamycins.

Isolation and Characterization of Recombinant Strain C 5/42

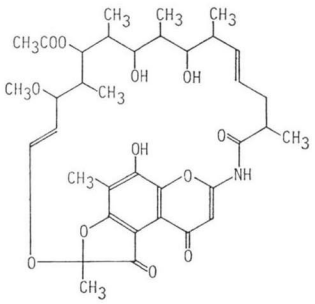
Strain C 5/42 was isolated as a recombinant strain from a cross between two *Nocardia mediterranei* mutants, T101 and F 1/24, which are blocked at different steps in rifamycin biosynthesis. Strain T 101 is blocked in the transformation of rifamycin SV to rifamycin B. It produces rifamycin SV and is auxotrophic for proline and resistant to streptomycin. Strain F 1/24 is blocked in the transformation of protorifamycin I to rifamycin W and produces protorifamycin I. The two strains were crossed on agar slants using a method described in an earlier paper[5] and recombinant colonies were isolated on minimal agar[6] containing 25 μg/ml streptomycin. By this means a selection was achieved which allowed only those recombinants to grow which had inherited the *pro*$^+$ allele from strain F 1/24 and the *str*^{R} allele from strain T 101. Out of 150 recombinant colonies isolated and checked for their stability by streaking out on the same selective medium, 105 with stable *pro*$^+$ *str*^{R} phenotype were selected. These were tested for rifamycin production by fermentation in liquid medium 148[7] in 200-ml Erlenmeyer flasks at 28°C. After 7 days incubation, 4 ml samples of culture broth from each strain were filtered, adjusted to pH 2 with HCl and extracted once with an equal volume of methylene chloride. The organic phases were concentrated 20 fold and analyzed by thin-layer chromatography (Merck silica gel 60 F_{254} plates).

* R. CRICCHIO *et al.* from the Gruppo Lepetit describe the same compound, isolated from a mutant of *Nocardia mediterranei*, in a later publication[4].

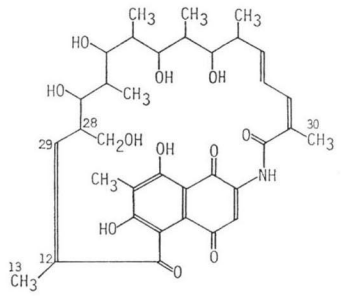
Fig. 1. Rifamycins from the recombinant strain C 5/42.



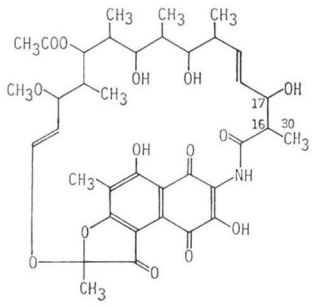
16,17-Dihydrorifamycin S



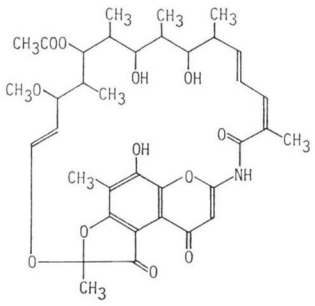
Rifamycin G



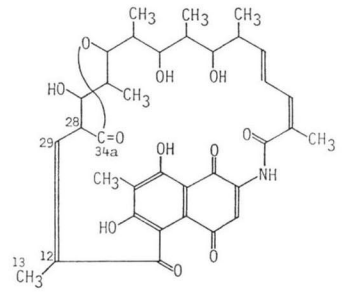
Rifamycin W



16,17-Dihydro-17-hydroxyrifamycin S



16,17-Dehydrorifamycin G



Rifamycin W - lactone

After developing with chloroform - methanol, 4: 1, new rifamycins could be distinguished from the rifamycins produced by the parental strains. Of the 105 recombinants tested, 67 produced rifamycin SV, 34 protorifamycin I and one strain with the number C 5/42 a mixture of new products and no rifamycin SV or protorifamycin I. The remaining 3 strains were found to produce a mixture of protorifamycin I, rifamycin SV and rifamycin B. It could be shown by further cultivation on selective plates that these 3 strains were not stable haploid recombinants but possessed unstable phenotypes which segregated into the two parental phenotypes.

Strain C 5/42 has inherited the *pro*$^+$ gene from parent strain F 1/24 and the *str*^{R} gene from parent strain T 101. The morphology of this recombinant strain is the same as that of the two parental strains. It does not sporulate under our conditions and the mycelium breaks up into rod like fragments of $5 \sim 20$ μm length.

To obtain material for a preliminary characterization of the fermentation products, strain C 5/42 was fermented in shake flasks with medium 148 and the culture filtrate was extracted as described above.

Thin-layer chromatography using different solvent systems for development showed that C 5/42 produces rifamycin G as its main product and two new products, but no rifamycin SV or B, which are the normal endproducts or rifamycin fermentation.

Fermentation, Isolation and Purification of the Ansamycins

To obtain large amounts of the new products, strain C 5/42 was grown in 30 liters of liquid complex medium 151 b[7]. After 120-hour fermentation the culture broth was filtered through Celite, the filtrate was acidified to pH 2.5 and then extracted five times with ethyl acetate. The combined ethyl acetate

extracts were washed with water, dried over Na_2SO_4 and evaporated to dryness, leaving 60.5 g of a reddish brown residue of ansamycins. Separation of this mixture into its components was achieved by repeated chromatography on silica gel columns or by preparative TLC on silica gel PF_{254} plates (Merck, 1.5 mm) with chloroform and increasing amounts of methanol as solvents. The following amounts of chromatographically pure ansamycins were obtained: Rifamycin G (15 g), 16,17-dihydrorifamycin S (4.5 g), 16,17-dihydro-17-hydroxyrifamycin S (0.1 g), 16,17-dehydrorifamycin G (0.35 g), rifamycin W (0.5 g) and rifamycin W-lactone (0.2 g).

Structures of 16,17-Dihydrorifamycin S and 16,17-Dihydro-17-hydroxyrifamycin S

The structures of 16,17-dihydrorifamycin S and 16,17-dihydro-17-hydroxyrifamycin S were elucidated by spectroscopic methods. The main evidence came from a direct comparison of their UV, IR, ^{13}C NMR, ^1H NMR and mass spectra with those of rifamycin S.

16,17-Dihydrorifamycin S

16,17-Dihydrorifamycin S forms yellow-reddish crystals from ether - hexane; $[\alpha]_D +337 \pm 1°$ ($CHCl_3$); UV (λ_{max}, ε in ethanol) 235 (22,800), 275 (18,700), 304 (13,600), 385 (4,100), 330 (sh), 530 nm (sh.).

In the FD-mass spectrum there is a difference of only two mass units in the localization of the molecular ion M^+ between 16,17-dihydrorifamycin S (M^+ at m/z 697, $C_{37}H_{47}NO_{12}$) and rifamycin S (M^+ at m/z 695, $C_{37}H_{45}NO_{12}$). Furthermore there are no substantial differences in the chemical shifts of the C-atoms in the ^{13}C NMR spectra of these two compounds, except for the signals from C-16 to C-19 and C-30 (Table 1). Due to the missing 16,17-double bond the signals of C-16 and C-17, present as olefinic carbons at 131.0 and 133.2 ppm in rifamycin S, shifted upfield to 44.6 and 38.8 ppm, respectively, whereas the vinylic C-30 methyl group of rifamycin S at 20.0 ppm is now present as an aliphatic secondary methyl group at 15.8 ppm. As a consequence of the hydrogenated 16,17-double bond in 16,17-dihydrorifamycin S, the signal for C-18 shifted downfield from 124.4 ppm in rifamycin S to 130.2 ppm whereas the C-19 signal shifted upfield from 142.4 ppm (rifamycin S) to 130.2 ppm.

The 360 MHz ^1H NMR spectrum of 16,17-dihydrorifamycin S (Fig. 2) is in a good accordance with the postulated structure. The signals of the olefinic H-17 proton as well as the vinylic C-30 methyl group, found at 6.2 and 2.0 ppm, respectively, in rifamycin S, have shifted upfield. The C-30 methyl protons could be localized as a doublet at 1.30 ppm within the group of doublets of the four secondary methyl groups C-31 (0.75 ppm), C-32 (1.01 ppm), C-33 (0.60 ppm) and C-34 (0.05 ppm). The CH_2-17-methyl-

Fig. 2. 360 MHz ^1H NMR spectrum of 16,17-dihydrorifamycin S in $CDCl_3$.

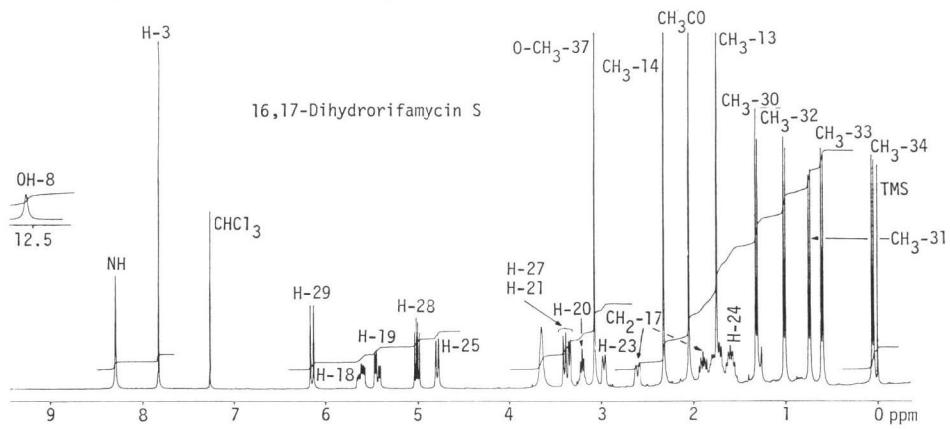


Table 1. ^{13}C NMR data of rifamycin S, 16,17-dihydrorifamycin S, 16,17-dihydro-17-hydroxyrifamycin S and 16,17-dihydrorifampicin SV in $CDCl_3$ (δ-values in ppm).

C	Rifamycin S	16,17-Dihydrorifamycin S	16,17-Dihydro-17-hydroxyrifamycin S	16,17-Dihydrorifampicin SV	C	Rifamycin S	16,17-Dihydrorifamycin S	16,17-Dihydro-17-hydroxyrifamycin S	16,17-Dihydrorifampicin SV
1	184.5	185.0	184.9	138.5	22	33.0	33.5	33.2	34.1
2	139.4	139.2	138.9	106.0	23	77.7	77.3	74.2	76.8
3	117.4	118.2	119.1	111.9	24	37.4	35.4*	37.3*	34.6*
4	181.6	182.3	182.4	147.9	25	73.6	74.1	71.3	74.4
5	111.2	111.4	111.3	113.0	26	37.4	37.4*	38.7*	37.0*
6	172.2	174.5	174.2	176.1	27	81.9	78.8	77.8*	77.3
7	115.7	115.3	115.3	119.0	28	115.7	118.2	117.8	120.1
8	166.5	167.0	167.0	174.2	29	145.3	143.3	142.5	143.5
9	111.0	110.6	110.5	104.7	30	20.0	15.8	9.3	15.8
10	131.3	131.1	131.1	117.8	31	16.8	17.0	16.8	18.4
11	191.1	191.9	192.1	194.4	32	11.4	11.4*	11.3	10.9
12	108.6	108.0	107.9	109.3	33	8.8	8.5	8.5	8.1
13	22.4	21.6	21.5	21.8	34	11.4	10.0*	9.8	8.3
14	7.4	7.6	7.7	7.6	35	172.6	172.7	172.9	172.4
15	169.0	172.9	172.9	172.3	36	20.8	20.8	20.8	20.7
16	131.0	44.6	43.9	45.0	37	56.8	57.1	57.4	56.8
17	133.2	35.8	77.3*	34.9	-CH=N-				153.3
18	124.4	130.2	129.2	127.2					{ 54.2
19	142.4	130.2	131.3	131.7					{ 50.4
20	39.2	38.5	43.1	40.4	>N-CH_3				45.8
21	73.6	72.4	70.4	71.1					

* Tentative assignment.

ene group forms an AB-system at 2.2 ppm. Double resonance experiments showed the coupling of the AB-system with the multiplet of the H-18 proton at 5.6 ppm. By further irradiation experiments the coupling of H-18 with H-19 (doublet at 5.45 ppm) and of H-19 with the H-20 proton (multiplet at 3.24 ppm) was demonstrated. With these findings the structure of 16,17-dihydrorifamycin is well established.

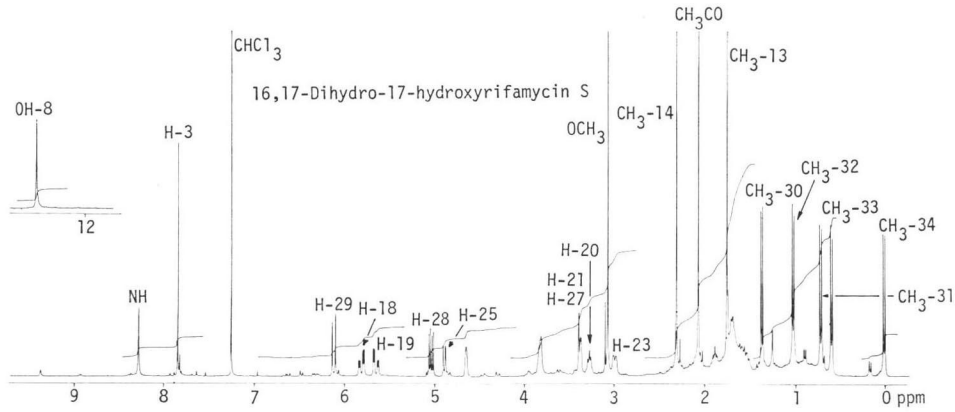
16,17-Dihydro-17-hydroxyrifamycin S

The postulated structure, especially with regard to the missing 16,17-double bond and the position of the hydroxyl group at C-17, is based on the following spectroscopic observations:

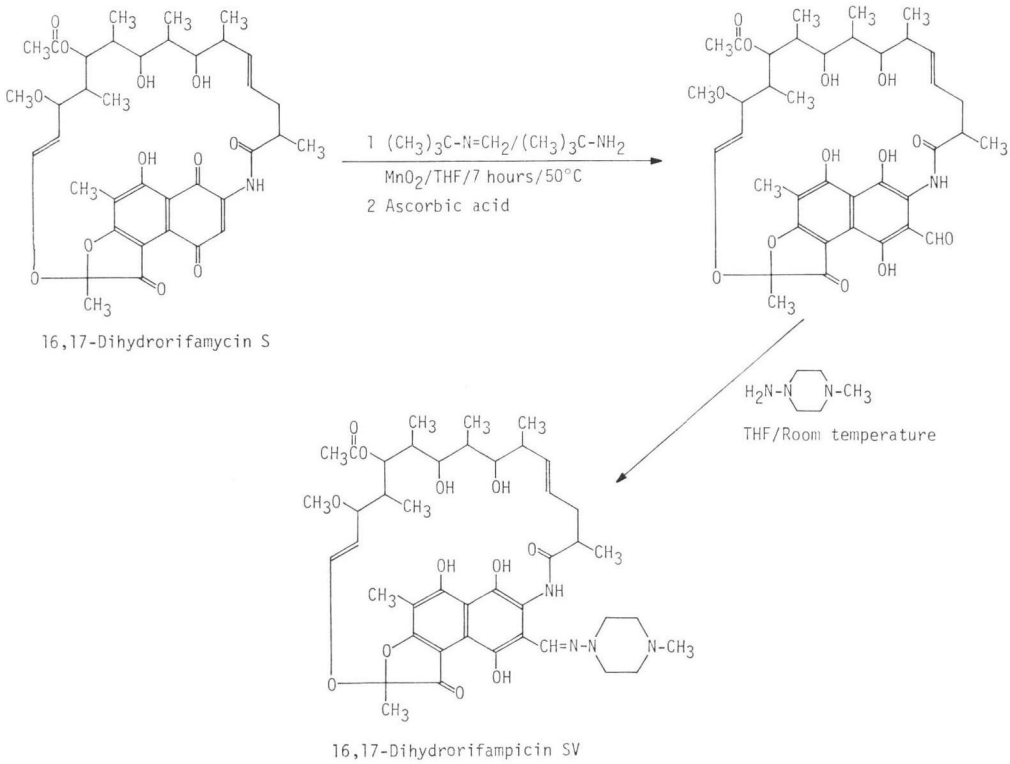
In the FD-mass spectrum the molecular ion M^+ was found at m/z 713 ($C_{37}H_{47}NO_{13}$) which is 16 mass units higher than found for 16,17-dihydrorifamycin S.

In the ^{13}C NMR spectrum (Table 1) the C-16 and C-17 signals are missing again in the olefinic part of the spectrum. But while the C-16 signal was localized at 43.9 ppm along with the four signals of C-22 (33.2 ppm), C-24 (37.3 ppm), C-26 (38.7 ppm) and C-20 (43.1 ppm), five signals of oxygen substituted carbons were found between 70.4 and 77.8 ppm. Because four of them have to be attributed to the carbons C-21, C-23, C-25 and C-27, the additional one must belong to C-17, thus indicating a hydroxyl group in this position. This assumption is in good agreement with the 360 MHz ^1H NMR spectrum of 16,17-dihydro-17-hydroxyrifamycin S. Due to the hydroxyl group at position 17, the former AB-system at 2.2 ppm of the two H-17 protons in the ^1H NMR spectrum of 16,17-dihydrorifamycin S has disappeared. The H-17 proton of 16,17-dihydro-17-hydroxyrifamycin S is now found at a lower field (3.7 ppm) as a

Fig. 3. 360 MHz ^1H NMR spectrum of 16,17-dihydro-17-hydroxyrifamycin S in $CDCl_3$.



Scheme 1. Preparation of 16,17-dihydrorifampicin SV.



double doublet. Its exact position was confirmed by a decoupling experiment with the olefinic H-18 proton at 5.6 ppm. Except for the signals of the C-30 methyl group which shifted from 1.30 ppm in 16,17-dihydrorifamycin S downfield to 1.35 ppm in 16,17-dihydro-17-hydroxyrifamycin S, no serious shift differences were observed for the rest of the protons in these two compounds.

16,17-Dihydrorifampicin

Rifampicin SV (Rimactan®) is a derivative of rifamycin S which is used worldwide as drug against tuberculosis. The derivative 16,17-dihydrorifampicin was prepared in order to compare its biological

Table 2. Antibacterial spectrum of 16,17-dihydrorifamycin S, 16,17-dihydro-17-hydroxyrifamycin S, 16,17-dehydrorifamycin G and 16,17-dihydrorifampicin SV compared with rifamycin S and rifampicin SV (MIC values in μg/ml)*.

Organism	16,17-Dihydrorifamycin S	16,17-Dihydro-17-hydroxyrifamycin S	16,17-Dehydrorifamycin G	16,17-Dihydrorifampicin SV	Rifamycin S	Rifampicin SV
Staphylococcus aureus 10 B	1	1	1	16	0.01	0.05
Staphylococcus aureus 2999	8	1	1	16	0.01	0.05
Streptococcus pyogenes Aronson	16	—	1	—	—	0.05
Streptococcus faecalis 1362/3	>128	>128	128	>128	16	8
Neisseria gonorrhoeae 1317/4	16	—	0.5	—	—	0.1
Haemophilus influenzae NCTC 4500	32	—	4	—	—	0.5
Escherichia coli 205	>128	>128	>128	>128	128	16
Escherichia coli 1074	>128	>128	>128	>128	>128	16
Klebsiella pneumoniae 327	>128	>128	>128	>128	>128	32
Enterobacter cloacae P99	>128	>128	>128	>128	>128	64
Proteus mirabilis 774	>128	>128	128	>128	64	16
Proteus rettgeri 856	>128	>128	128	>128	>128	16
Proteus morganii	>128	>128	128	>128	64	32
Pseudomonas aeruginosa ATCC 12055	>128	>128	32	>128	64	32

* Rifamycin G in all tests: >128.

properties with those of Rimactan. The compound was synthesized starting from 16,17-dihydrorifamycin S by the sequence of reactions (Scheme 1).

The ^{13}C NMR and ^{1}H NMR spectra of 16,17-dihydrorifampicin SV (Table 1) were in good agreement with the corresponding spectra of rifampicin SV.

Biological Activity

The various 16,17-dihydrorifamycins have been tested for their *in vitro* activity against a variety of bacteria (Table 2). Surprisingly the loss of the 16,17-double bond leads to a 100~300 fold lower activity against Gram-positive bacteria.

Rifamycin G[1] also belongs to the 16,17-dihydrorifamycins and contains a γ-pyrone ring instead of a quinone ring system. This compound has been reported to be totally inactive against bacteria.[1] In contrast the 16,17-dehydro derivative of rifamycin G[2] has some antibacterial activity. This demonstrates the importance of the 16,17-double bond of the rifamycins for biological activity.

This fact is borne out even more clearly by measuring the inhibitory activity of the various derivatives on RNA polymerase, the target enzyme for the rifamycins[8,9]. As shown in Table 3, hydrogenation of the 16,17-double bond leads to compounds with a 100~500 fold decrease in enzyme inhibition. This also holds for 16,17-dehydrorifamycin G and rifamycin G, showing clearly that the very low activity of the latter compound is not only due to the replacement of the $C_1=O$ by O, but also to the hydrogenation of the 16,17-double bond.

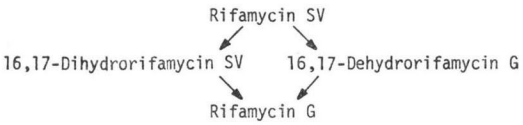
The importance of the 16,17-double bond for enzyme inhibition is quite unexpected, since 16,17,18,19-tetrahydrorifamycin S is only about 3 times less active on the enzyme than rifamycin S[10]. It seems that the partial hydrogenation in position 16,17, apparently leads to a conformation of the ansa-ring which is less favorable for the specific binding to RNA polymerase[11].

Table 3. Inhibition of RNA polymerase by various rifamycin derivatives.

Compounds	Inhibition of RNA-polymerase, $ED_{40\sim60}$, μg/ml
16,17-Dihydrorifamycin S	3
16,17-Dihydro-17-hydroxyrifamycin S	2
16,17-Dihydrorifampicin SV	6
Rifamycin G	25
16,17-Dehydrorifamycin G	0.3
Rifamycin S	0.01
Rifampicin SV	0.01

RNA polymerase from *E. coli* ETH 2018 was isolated and its activity assayed as previously described[8,11].

Fig. 4. Possible pathways for the biosynthesis of rifamycin G.



Transformation Studies

Transformation experiments were performed in order to study the biosynthetic relation between rifamycin S, 16,17-dihydrorifamycin S, 16,17-dehydrorifamycin G and rifamycin G. Washed mycelium of strain C 5/42 grown for 3 days in liquid medium 148 was used and the transformation was performed in 15 ml phosphate buffer (pH 7.0, 0.06 M) with the addition of the appropriate rifamycin precursor at a concentration of 250 μg/ml. After incubation at 28°C on a rotary shaker (250 rpm) for 24 and 48 hours, the culture broth was extracted with methylene chloride and analyzed by thin-layer chromatography.

The results obtained show that strain C 5/42 transforms added 16,17-dihydrorifamycin S, 16,17-dehydrorifamycin G and rifamycin S to rifamycin G. In the case of 16,17-dihydrorifamycin S and rifamycin S, 16,17-dihydrorifamycin SV and rifamycin SV, respectively, were formed as intermediate reduction products during the transformation reaction.

Discussion

The isolation of recombinant strain C 5/42 is a further example of the biosynthetic potential of intraspecific recombination between *N. mediterranei* strains blocked in rifamycin biosynthesis (see Reference 5). The recombinant strain produces mainly rifamycin G, which so far has only been detected as a side-product along with the rifamycin complex in a fermentation with a rifamycin B producing strain[1]. In addition C 5/42 produces 16,17-dihydrorifamycin S, and 16,17-dihydro-17-hydroxyrifamycin S, two hitherto unknown rifamycins, together with 16,17-dehydrorifamycin G which was first isolated from recombinant strain R-21[2].

In addition to rifamycin G[1], 16,17-dihydrorifamycin S and 16,17-dihydro-17-hydroxyrifamycin S are two further examples in the rifamycin series where the $\alpha,\beta,\gamma,\delta$-diene system of the ansa-chain is partially hydrogenated.

As is shown by the biological activity as well as the activity against RNA polymerase of these two compounds and of 16,17-dihydrorifampicin SV, the presence of the 16,17-double bond is essential for full biological activity of the rifamycins.

16,17-Dihydro-17-hydroxyrifamycin S is another one of the numerous examples[12~14] where hydroxylation in the ansa-chain has taken place, suggesting again that the hydroxylating enzymes do not act very specifically.

The transformation data together with an analysis of the structural differences of the new rifamycins allow two possible biogenetic pathways to be postulated for the synthesis of rifamycin G starting from rifamycin SV (Fig. 4).

Acknowledgment

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