and calculations based on the integral showed that XIa composed approximately one-third of the mixture. Most of the nmr signals for XIb could be assigned from the spectrum and are in Table I. Because the desulfurization product would be identical from both isomers XIa and XIb, no attempt was made to purify the product further.

Desulfurization of 7-Phenoxyacetamido-3-methylcepham-4-carboxylic Acid to Methyl Phenoxymethyldesthiopenicillinate (VII).—The same procedure used to desulfurize the penicillin was used for the dihydrodesacetoxycephalosporin. Crude 7-phenoxyacetamido-3-methyl-3-cepham-4-carboxylic acid (1 g, 2.86 mmoles) was dissolved in 80 ml of H_2O by adjusting the solution to pH 7 with 1 *N* NaOH. Then 6 g of Raney Ni was added; the mixture was heated for 15 min in an oil bath at 105° and then quickly chilled. After the catalyst was filtered and washed (H_2O), the cooled filtrates were acidified to pH 2.5 below a layer of EtOAc. The EtOAc solution was washed (H_2O), dried ($MgSO_4$), and evaporated to give an oil which in tlc (silica; Et_2O–AcOH–H_2O, 15:3:1) showed three spots.

The catalyst above was extracted with cold 0.05 *N* NaOH (500 ml) from which an additional small amount of oily product could be recovered by acidification and extraction as above. In tlc this fraction showed the same spots as the first fraction.

The two fractions were combined in 15 ml of EtOAc and cooled in an ice bath while excess CH_2N_2 in CH_2Cl_2 was added. After 15 min the yellow color of CH_2N_2 was discharged with a few drops of AcOH and the solvent was removed by evaporation *in vacuo*. The residue was redissolved in EtOAc, washed (5% $NaHCO_3$, H_2O), and dried ($MgSO_4$). After the solvent was evaporated, the oily residue crystallized after standing overnight in the cold. A tlc (SiO_2; EtOAc) of this product compared with the methyl ester of the penicillin desulfurization showed a major spot moving as did that product, and in addition two quite minor, less mobile spots. The product (400 mg) was chromatographed over a column of SiO_2 (16 g), eluting with C_6H_6 containing increasing amounts of EtOAc. The recovered product (180 mg) corresponding to the major tlc spot was recrystallized from C_6H_6–petroleum ether and showed only one spot in tlc: mp 112°. *Anal.* Calcd for $C_{17}H_{22}N_2O_5$: C, 61.06; H, 6.63; N, 8.38. Found: C, 61.06; H, 6.36; N, 8.28.

The above product melted at 112°, that from the penicillin at 109–110°, and the mixture of the two at 109–112°, all observed at the same time in a Mel-Temp melting point apparatus.

The two 6-phenoxymethyldesthiopenicillin methyl esters possessed identical ir and nmr spectra, and X-ray diffraction powder diagrams. The X-ray pattern of the ester from the cephalosporin appeared to be sharper, indicating a more highly crystalline material. This is also supported by the slightly higher melting point of the cephalosporin-derived ester.

Valine from Cephalosporin-Derived Methyl Phenoxymethyldesthiopenicillinate (VII).—An initial experiment[15] designed to produce valine methyl ester from the desthiopenicillin ester VII (500 mg) gave instead an α,β-diaminopropionic acid derivative (205 mg) due to simple β-lactam opening as its water-soluble hydrochloride; an nmr spectrum possessed the requisite signals. A portion (150 mg) of this acid was hydrolyzed in 25 ml of 2 *N* HCl at reflux for 12 hr. After being cooled and extracted with ether to remove neutral, amphoteric, acidic materials, the reaction mixture was evaporated to dryness *in vacuo*. The residue in 2.3 ml of 1 *N* HCl was placed on a Dowex 50W-X4 (acid form) column (0.9 × 30 cm) and the amino acids eluted with 1 *N* HCl. Fractions (1–2 ml) enriched in valine were combined and evaporated to give 78 mg of product. The product was purified by chromatography[16] over Sephadex G-25 (25 g in a 2.5 × 25 cm column) using *n*-BuOH–AcOH–H_2O (62:15:25). Valine was cleanly separated. No attempt was made to crystallize the product. Its specific rotation in 0.01 *N* HCl was compared with that of pure D-valine at 250 mμ. D-Valine standard showed a specific rotation, $[\alpha]$ −563°, whereas the isolated product had a specific rotation of −245°. Therefore, less than 30%, of the material racemized in the process if no account is taken of degree of solvation and experimental errors. The product is predominantly of the D configuration.

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Acetyl Migration in Rifampicin[1]

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Mild alkaline treatment of rifampicin affords 25-desacetylrifampicin and two other rifamycins, identified as 25-desacetyl-23-acetyl and 25-desacetyl-21-acetyl derivatives. From the antibacterial activity data of the two compounds, the presence of the C-21 and C-23 hydroxyls in rifamycins seems to be an essential prerequisite for their antibacterial activity.

Rifampicin (I) is a new semisynthetic rifamycin[2,3] selected from many derivatives of 3-formylrifamycin SV[4] for its high antibacterial activity *in vitro* and *in vivo*.[2,5,6] Clinical experiments on this antibiotic are presently underway and the data available indicate its effectiveness in the systemic diseases induced by gram-positive bacteria and in tubercular infections. Like other rifamycins, rifampicin undergoes desacetylation by alkaline treatment, affording the corresponding desacetyl derivative (II) without substantial loss of antibacterial activity.[7] Under milder alkaline conditions I yields two additional products whose identification and antibacterial activity evaluation was considered of interest for the knowledge of activity–structure relationships in rifamycins. To these two rifamycins structures III and IV were assigned on the basis of physical and chemical data.

By treating I in a pH 8.2 buffered aqueous solution at 90–95° for 5 hr, II–IV were detected by tlc as reaction products besides minor by-products and a fair amount of starting I. The reaction mixture was separated by

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(7) N. Maggi, V. Vigevani, and R. Pallanza, *Experientia*, **24**, 209 (1968).

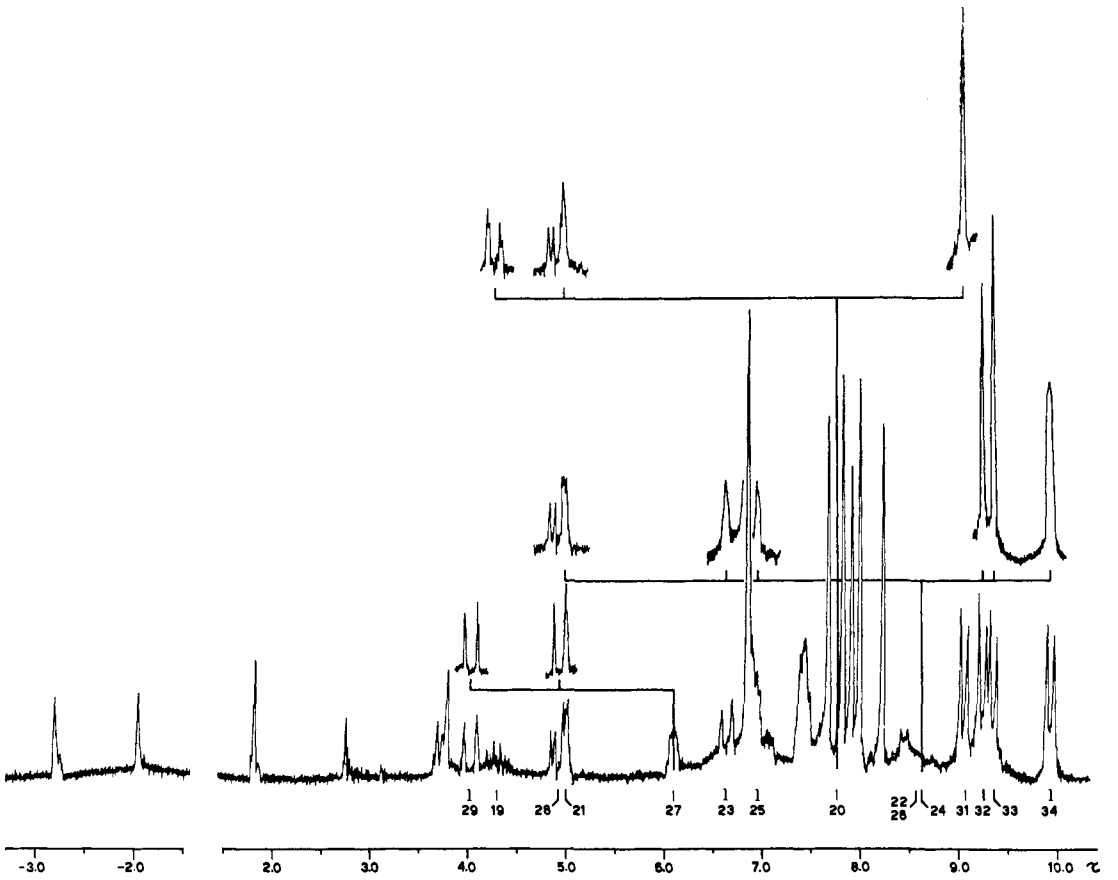
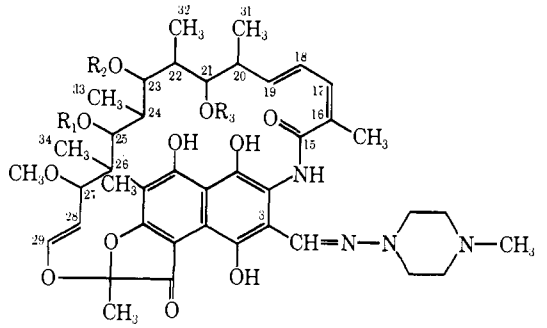


Figure 1.—Nmr spectrum of 25-desacetyl-21-acetylrifampicin (III).

combined extraction, crystallization, and column chromatography. One of the products (R_f 0.23) was identified as II by comparison with an authentic sample.[7] The other two products, III (R_f 0.35) and IV



I, $R_1 = COCH_3$; $R_2 = R_3 = H$
II, $R_1 = R_2 = R_3 = H$
III, $R_3 = COCH_3$; $R_1 = R_2 = H$
IV, $R_2 = COCH_3$; $R_1 = R_3 = H$

(R_f 0.62), showed most physical properties identical with those of I, *e.g.*, color, water solubility, ionization behavior, electronic spectrum, and polarographic oxidation wave. Elemental analyses (C, H, N) gave for both the same $C_{43}H_{58}N_4O_{12}$ formula, like I. No useful information could be derived from the ir spectra which, however, showed some differences from that of I. Nmr spectra ($CDCl_3$ solution) of III and IV (Figures 1 and 2) revealed the presence of all the proton signals previously reported for rifampicin[3] with changes in chemical shifts for some of them.

The chemical behavior of III and IV was therefore investigated by subjecting them to a mild acidic and basic treatment under controlled conditions. (a) After heating separately III and IV at 90° for 2 hr in a buffered aqueous solution (pH 6.0), two distinct products were found which on nmr analysis were found to be 3-formyl derivatives, different from the hydrolysis product of I, *i.e.*, 3-formylrifamycin SV,[4] thus indicating the persistence of the structural differences after the removal of the basic side chain at C-3. (b) When heating pH 8.5 buffered solutions of III and IV for a few hours at 90–95°, a mixture of I, III, and IV along with small amounts of II was detected by tlc in both cases. Examination of the reaction solutions at various time intervals indicated that when III was the starting substance, the transformation into IV was initially detected, whereas I was formed subsequently. Starting from IV, the formation of I and III was practically simultaneous. Since, starting from I, with the same treatment, an early formation of IV followed by III was observed, the equilibria I $\rightleftarrows$ IV and IV $\rightleftarrows$ III can be considered as effective. (c) Stronger basic conditions, *e.g.*, treatment in a water–ethanol (1:1)–1 *N* NaOH solution, induced a rapid transformation of both III and IV into desacetylrifampicin (II), formerly obtained from rifampicin[7] (I).

The chemical data referred to above are thus consistent with a reversible isomerism of III, IV, and rifampicin (I). Since the physicochemical data do not provide evidence for differences in the chromophoric moiety of III, IV, and I, the only possible interpretation is the migration of the acetyl group during the mild alkaline treatment. It is noteworthy that the

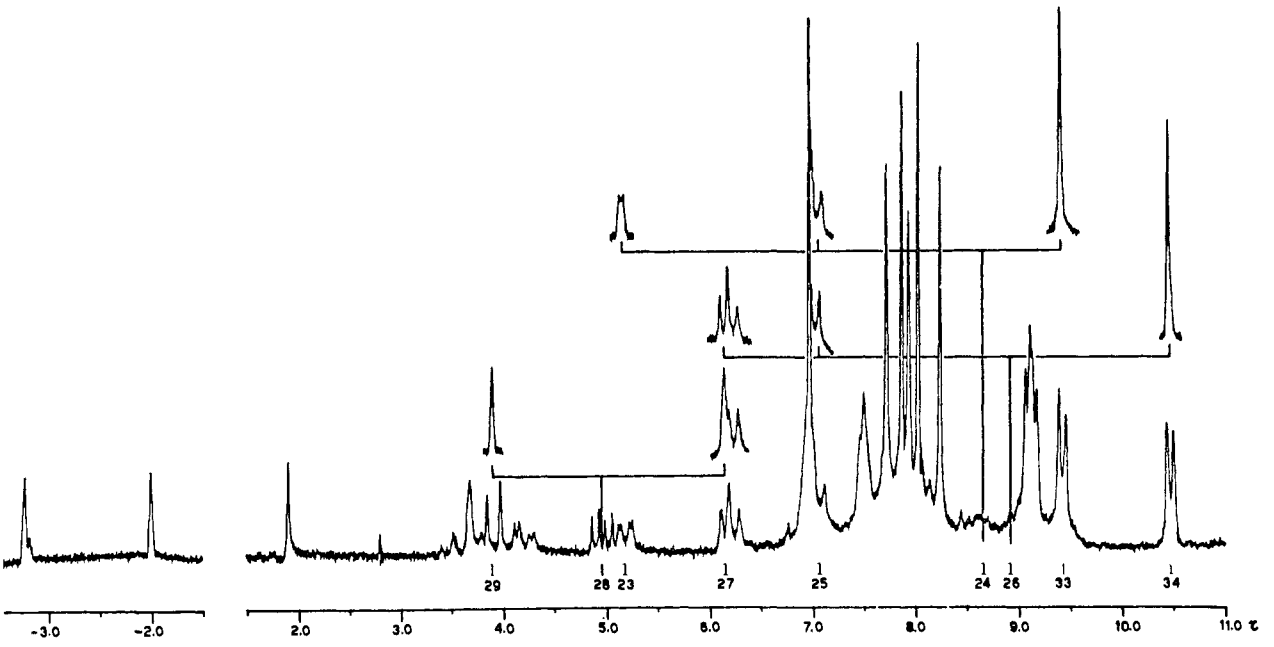


Figure 2.—Nmr spectrum of 25-desacetyl-23-acetylrifampicin (IV).

only *trans* acetylation reported for an inactive series of rifamycins is described to occur under acidic conditions.[8]

To ascertain whether the position of the acetyl group in III and IV was at the 21- and 23-OH, respectively, or *vice versa*, nmr spin decoupling experiments were performed, based on the fact that the hydrogen at the carbon atom bearing the acetoxy group is at lower field than the hydrogen at the carbon atom bearing the hydroxyl.[7] In Figures 1 and 2 the nmr spectra of III and IV are shown together with the principal spin decouplings, which allowed us to assign the reported structures. The signals of the C-26, C-24, and C-22 protons of III are almost at the same field, as shown by the excitation of the C-24 proton which causes, besides the complete decoupling of 33-CH_3, also the partial decoupling of 34- and 32-CH_3 (Figure 1). For this reason a partial decoupling of the signal of the C-21 proton is observed by exciting the C-24 proton. The excitation of the C-20 proton confirms the above attributions and permits the location of the acetoxy group at C-21. Concerning IV, the assignment of the signal of the proton at C-24 and its excitation permits the location of the acetoxy group at C-23 (Figure 2).

The *in vitro* antibacterial test, carried out by Dr. R. Pallanza of our laboratories, gave MIC values for III and IV, against *Staphylococcus aureus*, of 10 and 0.5 μg/ml, respectively, as compared to 0.002 μg/ml for I with the same test.[2] A similar decrease of activity was also observed on other gram-positive strains, whereas a complete loss of activity was found on gram-negative ones.

Discussion

Intramolecular *trans* acetylation from 25-OH to 23-OH and 21-OH of rifampicin (I) occurs in a stepwise way in aqueous solution under very mild basic conditions, the process being determined by the temperature. The isomeric 25-desacetyl-21-acetylrifampicin (III) and 25-desacetyl-23-acetylrifampicin (IV), despite their physical and chemical similarity with I, do not display useful antimicrobial behavior.

Since, as already mentioned, the replacement of the C-25 hydroxyl with the acetoxy group in rifamycins does not affect seriously the antibacterial activity,[7] the loss of the activity is of relevant significance when one of the residual hydroxyls at C-21 and C-23 is acetylated. The low but appreciable activity of IV might be explained as due to a slight isomerization to I during the incubation time of the test microorganism, while the slower isomerization of III into I might account for its virtual inactivity.

Due to the very close physical properties of III, IV, and I, it seems unlikely that the difference of the activity of the three isomers can be attributed to different ability to penetrate the bacterial cell wall. Hence, the most probable explanation is that both the C-21 and C-23 OH's are essential for allowing the rifamycin to act on the RNA-polymerase system of the bacterial cell.[9,10]

Experimental Section

Thin layer chromatography (tlc) was performed on silica gel HF (E. Merck, A.G. Darmstadt) plates, using $CHCl_3$–MeOH (9:1) as solvent; the running distance was 10 cm. Melting points (open capillary method) are uncorrected. Column chromatographies were carried out on silica gel 0.01–0.2 mm (E. Merck, A.G. Darmstadt) prewashed with $CHCl_3$. Acidic pK_a values were determined spectrophotometrically in aqueous solution. Basic pK_a values (pK_{MCS}) were obtained by potentiometric titration in MCS–H_2O (4:1) solution. Nmr spectra were recorded on a Varian HA-100 instrument in $CDCl_3$ solution (TMS as internal reference), by courtesy of Dr. A. Segre of the Politecnico, Milan.

Isolation of III and IV.—To a warm (60–70°) solution of 30 g of I in 3.0 l. of Sörensen phosphate buffer, pH 8.2, an excess (30 g) of 1-amino-4-methylpiperazine was added in order to prevent the hydrolysis to the aldehyde and the oxidation by atmospheric oxygen to I quinone. After adjusting the pH to the appropriate value (8.2) with 2 *N* NaOH, the solution was

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stirred on a steam bath for 5 hr. Tlc analysis of the solution showed the presence of four major orange-yellow spots: R_f 0.23 (II), R_f 0.35 (III), R_f 0.52 (I), and R_f 0.62 (IV) in the approximate ratio 2:1:3.5:1.5, besides other colored by-products at R_f <0.1. After cooling, the solution was worked up by extracting the rifamycins twice with $CHCl_3$. The organic layer was evaporated to dryness under vacuum. The crude residue taken up in CCl_4 gave, after evaporation to a small volume, a crystalline crop (19.6 g) consisting mainly of I–III. The mother liquor showed the presence of I and IV, besides other degradation products (R_f <0.1).

III was recovered by column chromatography using a $CHCl_3$ solution of the crystalline crop on a pH 6.0 buffered (McIlvaine) silica gel column, by stepwise elution with $CHCl_3$ containing 2 and 4% MeOH. The selected fractions were collected and evaporated to dryness. The residue, crystallized from $CHCl_3$ and recrystallized from acetone, gave 0.7 g of pure (tlc) III: mp 158–160° dec; pK_a = 2.3, pK_{MCS} = 6.7.

The CCl_4 mother liquor was evaporated to dryness. The residue (12 g) was taken up in $CHCl_3$ and passed through a silica gel column using mixtures of $CHCl_3$ with 1, 2, and 3% MeOH as eluent. The first colored eluate, containing IV, was collected and evaporated to dryness. After crystallization of the residue from EtOH–H_2O, 1.7 g of pure (tlc) IV was obtained: mp 166–170° dec; pK_a = 2.5, pK_{MCS} = 6.8.

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Structure of Sangivamycin[1]

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Sangivamycin is an antitumor substance and a metabolite of a streptomycete culture. It has the molecular formula $C_{12}H_{15}N_5O_5$ and shows spectral properties similar to those of nucleosides. The presence of a pentose residue was established indirectly by periodate oxidation and hydrolysis. The compound also has an aromatic amino group and a carboxamide group. Some of the similarities in properties to toyocamycin led to the hypothesis that sangivamycin may be closely related to this compound. This hypothesis is shown to be correct in that sangivamycin has a carboxamide group and toyocamycin has a nitrile group on the same carbon skeleton. This relationship is established by three different methods. A brief study of the biological activity of some of the derivatives of sangivamycin is described.

Sangivamycin is an antitumor substance produced by an unidentified species of *Streptomyces*.[2] It shows significant activity against leukemia L1210 in mice and is strongly cytotoxic toward HeLa cells grown in cell culture. It has very slight antibacterial or antifungal activity. The compound is currently under clinical trials. Only the method of isolation from the broth and some preliminary characterizations have appeared so far.[2] Degradation reactions which led to the elucidation of its structure are presented here.

Sangivamycin is a colorless crystalline solid of low solubility in water and common organic solvents. It is weakly basic and forms salts such as hydrochloride, sulfate, and picrate. Titration of the hydrochloride shows that it is a monoacidic base with a pk_a of 3.4 and an equivalent weight of 310 (Figure 1A). The molecular formula, $C_{12}H_{17}N_5O_6$, proposed earlier appears to be that of the monohydrate of the compound. The formula $C_{12}H_{15}N_5O_5$ is now considered correct for sangivamycin as is shown by the analysis of several derivatives.

The spectral properties of the compound have been briefly described earlier. The uv spectrum has maxima at 228 mμ (ε 9250) and at 278 mμ (ε 14,500). In acid solution the major maximum is shifted to 272 mμ. The ir spectrum has prominent peaks to suggest the presence of amino and/or hydroxyl groups (2.90 and 3.05 μ) and either a conjugated carbonyl, an amide, or a C=N system (6.10 μ).

The nmr spectrum of sangivamycin run in trifluoroacetic acid shows the following characteristics: two broad based singlets at τ 1.42 and 1.53 equal to two to four protons, a doublet at τ 3.59 and 3.68 equal to one proton, and the rest, several broad peaks in the region τ 4.87–5.75. The spectrum shows some resemblance to the spectra of purine nucleosides.

In harmony with this observed similarity, sangivamycin forms a tetraacetyl derivative. Its nmr spectrum indicates clearly that one of the acetyl groups (τ 7.57, 3 protons) is different from the other three (τ 7.93, 3 protons) which appear to be due to three O-acetyl groups.

Further support for the possible presence of a sugar is provided by the fact that sangivamycin reacts with 1 mole of periodate. No formaldehyde or formic acid is formed and the main product retains the original carbon skeleton. In spite of the foregoing suggestive evidence for the nucleosidic nature of the compound, direct acid hydrolysis of sangivamycin failed to yield a sugar component even under relatively drastic conditions. However, the periodate-reaction product underwent smooth hydrolysis to yield a crystalline aglycone of composition $C_7H_6N_4O_2$ together with NH_4Cl. The loss of a $C_5H_8O_4$ residue thus established the existence of a pentose residue in sangivamycin.

Sangivamycin is readily deaminated when treated with sodium nitrite in acetic acid. The oxydesamino compound thus formed is a much weaker base than sangivamycin. Its uv maximum at 268 mμ is unchanged when acidified. These properties indicate that the amino group is of aromatic type and that it is part of the chromophore. The broad signal in the nmr spectrum at τ 1.42 (or 1.53) is in agreement with this. Also, the acetyl signal (τ 7.57, 3 protons) in tetraacetyl-

(1) Research supported by Contract No. PH43-64-50, with the National Institutes of Health, Public Health Service, Department of Health, Education and Welfare. This paper was presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965. Sangivamycin is also known as BA-90912.

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