CONSTITUTION OF RIFAMYCINS

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INTRODUCTION

Rifamycins (formerly known as rifomycins) are metabolic products which have been isolated in the Research Laboratories of Lepetit Ltd., Milan, from cultures of *Streptomyces mediterranei* n.sp.[1, 2]. Under certain growth conditions rifamycin B is the main product of this group of compounds. It is, however, unstable and is oxidized in buffered neutral solutions by mild oxidizing agents, or even in air, to rifamycin O with loss of two hydrogen atoms. Rifamycin S is obtained from rifamycin O by acid treatment in aqueous solution (and also from rifamycin B if air is present), one molecule of glycolic acid being removed hydrolytically. Rifamycin O is transformed by mild reducing agents, *e.g.* ascorbic acid, into rifamycin B; rifamycin S gives by the same reaction rifamycin SV, the sodium salt of which has found clinical application under the commercial name rifacin ®.
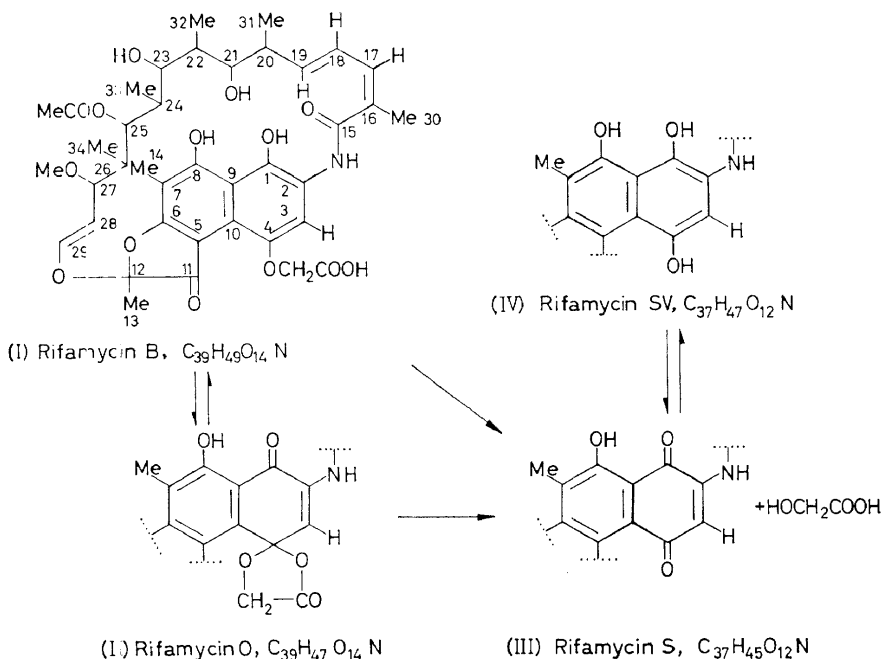

Figure 1. Constitutional formulae of rifamycins B, O, S, and SV

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From extensive investigations by W. v. Oppolzer in our laboratory, taking into account the numerous results obtained by Sensi and co-workers[3] of the Research Laboratories of Lepetit Ltd., Milan, constitutional formulae (I)–(IV) (*Figure 1*) have been derived for the four rifamycins mentioned above. These account for the relationships indicated by analytical data and chemical properties. The numbering system given in the complete formula of rifamycin B is the one which we shall use to identify the individual carbon atoms and their substituents.

ANALYTICAL DATA AND FUNCTIONAL GROUPS OF RIFAMYCINS

On the basis of analyses and of degradation results rifamycin B (I) has the empirical formula $C_{39}H_{49}O_{14}N$. It is a yellow, dibasic acid (pK^*_{MCS} 2·6 and 7·76) with typical ultraviolet, visible, and infrared absorption spectra that point to the presence of an aromatic chromophore and many functional groups. The oxidation of rifamycin B to rifamycin O (II), $C_{39}H_{47}O_{14}N$, is associated with strong changes in the absorption spectra; the appearance of a new infrared band at 1820 cm^{-1} is especially noteworthy. Rifamycin O is a yellow, monobasic acid (pK^*_{MCS} 7·61). It is remarkable that an acidic function is lost by oxidation. Although the reversible oxidation–reduction indicates some kind of hydroquinone–quinone relationship, transformation of rifamycin B into O must be accompanied by some further reactions linked with the above mentioned alterations. The existence of a spirane system at C–4 explains these facts and is further supported by model experiments with simple *p*-hydroxyphenyl ethers of glycolic acid. The quinone acetal ester group in rifamycin O reacts easily with aqueous acids with hydrolytic removal of glycolic acid and formation of rifamycin S (III), $C_{37}H_{45}O_{12}N$, which is a yellow, monobasic acid (pK^*_{MCS} 7·16). Reduction of rifamycin S to the yellow, monobasic acid rifamycin SV (IV), $C_{37}H_{47}O_{12}N$ (pK^*_{MCS} 2·96) is to all appearances simply the reduction of a quinone to a hydroquinone. Since rifamycin S does not react with lead tetra-acetate, it is probably not an *o*-quinoid compound.

Through these reactions the functions of four of the fourteen oxygen atoms in rifamycins B and O, and two of the twelve oxygen atoms in rifamycins S and SV are determined. Through group analysis, and the preparation of derivatives, the functions of a further six oxygen atoms, common to all four rifamycins are determinable. One of these oxygen atoms occurs in a strongly acidic phenolic hydroxyl group, which must obviously be in the peri-position to a hydroquinone or quinone oxygen respectively. This is indicated not only by the change in pK^*_{MCS}-values during the hydroquinone–quinone transformation, and by the colour reaction with pyroboric acid acetate, but also by the n.m.r. spectra (see p. 553). One methoxyl and one acetoxyl group can be determined microanalytically and are also easily recognizable in n.m.r. spectra. The existence of two hydroxyl groups is evident from the formation of an *O*, *O'*-diacetyl derivative on acetylation of 8-*O*-methyl ether of rifamycin S. The infrared absorption spectrum of this diacetyl derivative shows no bands corresponding to hydroxyl groups, so that no unacetylated hydroxyls can be present.

N.M.R. SPECTRA OF RIFAMYCINS O AND S†

Under routine conditions rifamycins B and SV do not yield good n.m.r. spectra because they are easily oxidized by air into paramagnetic semi-quinoid products. On the other hand the n.m.r. spectra of rifamycins O and S are well resolved (*Figures 2* and *3*). They confirm the analytical results and give further important information on the constitution of rifamycins.
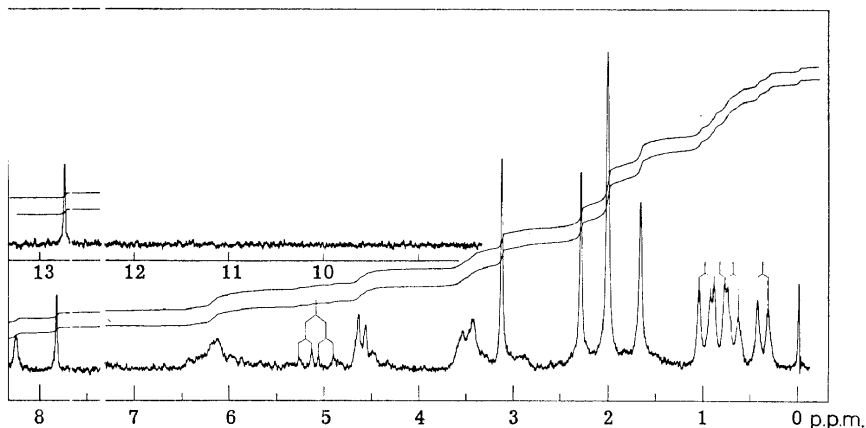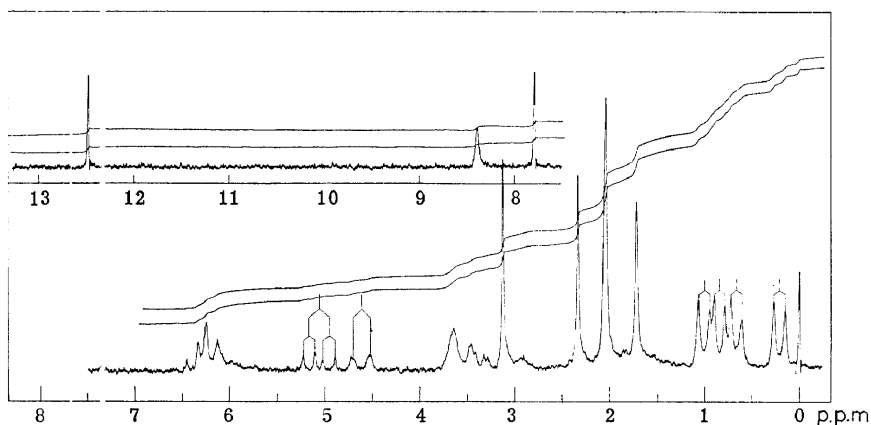

Figure 2. N.m.r. spectrum of rifamycin O

Figure 3. N.m.r. spectrum of rifamycin S

Apart from the signal due to the methylene of the glycolic acid residue in rifamycin O the two spectra are remarkably similar to one another, indicating that with the exception of hydrolytic removal of glycolic acid residue no further reactions take place during the transformation of rifamycin O to rifamycin S. Beginning at low magnetic fields the first feature in both

† All n.m.r. spectra in CCl_4 or $CDCl_3$, Varian A 60.

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spectra is a singlet at $\delta \sim 12{\cdot}5$ (one proton) corresponding to the strongly acidic proton of the hydroxyl group at C–8. Of two other singlets in the region of $\delta \sim 8$ (each one proton), one can be ascribed to an amide proton, the other to the "aromatic" proton at C–3. Next come signals that can be attributed to five protons at double bonds and the protons (six and four respectively) in the neighbourhood of oxygen functional groups. Especially important, however, are the singlets and doublets attributable to O- and C-methyl groups, of which nine are present in rifamycins. First comes the singlet of the methoxyl at C–27, then the singlet of the C–14 methyl on the aromatic nucleus, followed by unresolved singlets of acetoxyl at C–25 and of the C–30 methyl on the double bond. The position of the next singlet corresponding to the protons of the C–13 methyl group on C–12 is affected by adjacent oxygen atoms. The four last doublets ($J \sim 7$) correspond to four CH₃CH-groups, C–31, C–32, C–33 and C–34. It is thus apparent from the n.m.r. spectra that rifamycins contain 8 C-methyl groups; 7 such groups are indicated by the Kuhn-Roth procedure.

METHANOLYSIS OF THE IMINO METHYL ETHER FROM RIFAMYCIN S

Attempts to split rifamycins S and SV by hydrolysis or methanolysis were at first unsuccessful since side-reactions prevented the isolation of larger identifiable fragments. Eventually a way was found to overcome this diffi-culty. Rifamycin S on methylation with methyl iodide and silver oxide yields as the main product a neutral monomethyl derivative, that could be
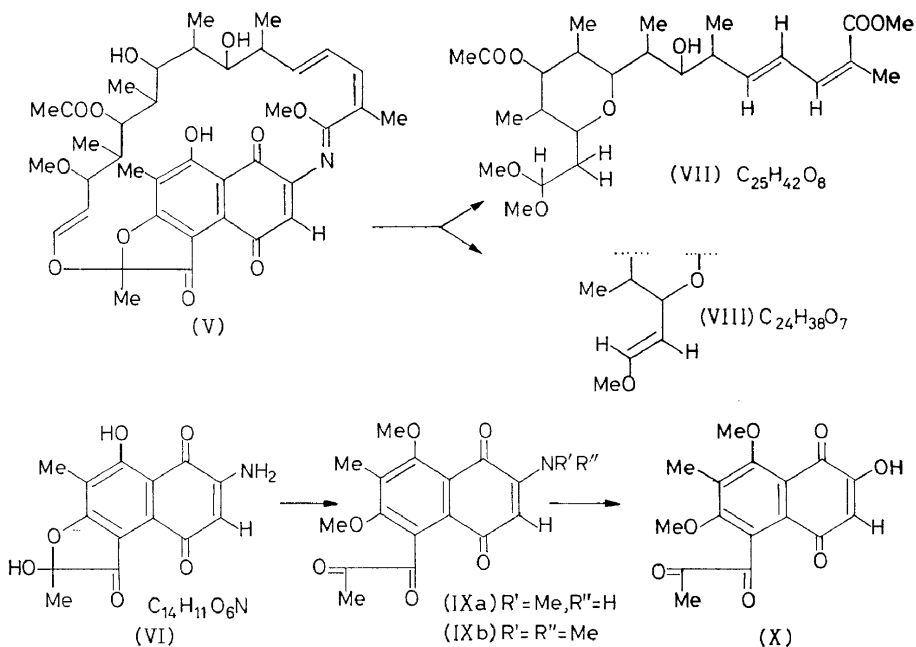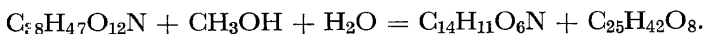

Figure 4. Methanolysis of the imino methyl ether from rifamycin S

identified as 8-O-methyl-rifamycin S. As a byproduct of this reaction there was obtained an acidic monomethyl derivative, whose chemical properties, i.r. and n.m.r. spectra indicate that it is the imino methyl ether (V) (*Figure 4*). It will be recalled that the formation of imino ethers by alkylation of secondary amides is a known reaction. Mild methanolysis of the imino methyl ether from rifamycin S gave as main products: an aromatic compound $C_{14}H_{11}O_6N$ (VI) and a compound $C_{25}H_{42}O_8$ (VII). The former contains no methoxyl, the second contains three. The over-all reaction can be represented by the equation

$$C_{38}H_{47}O_{12}N + CH_3OH + H_2O = C_{14}H_{11}O_6N + C_{25}H_{42}O_8.$$

As byproducts of the methanolysis the following were isolated: an isomeric (probably stereoisomeric) compound $C_{25}H_{42}O_8$ and a compound $C_{24}H_{38}O_7$ (VIII) containing two methoxyls.

Methanolysis of the imino methyl ether from rifamycin S played a key rôle in the determination of the constitution of rifamycins; it showed that they are constructed from two different large building blocks, which could be investigated separately.

CONSTITUTION OF THE COMPOUND $C_{14}H_{11}O_6N$ AND OF THE AROMATIC PART OF RIFAMYCINS

The compound $C_{14}H_{11}O_6N$ contains that part of the rifamycin molecule which is mainly responsible for the absorption in the visible and ultraviolet region. The constitution of the main part of its molecule was derived from the results of the degradation of rifamycin S with ozone (*Figure 5*). Energetic ozonolysis and subsequent treatment with performic acid yields two main
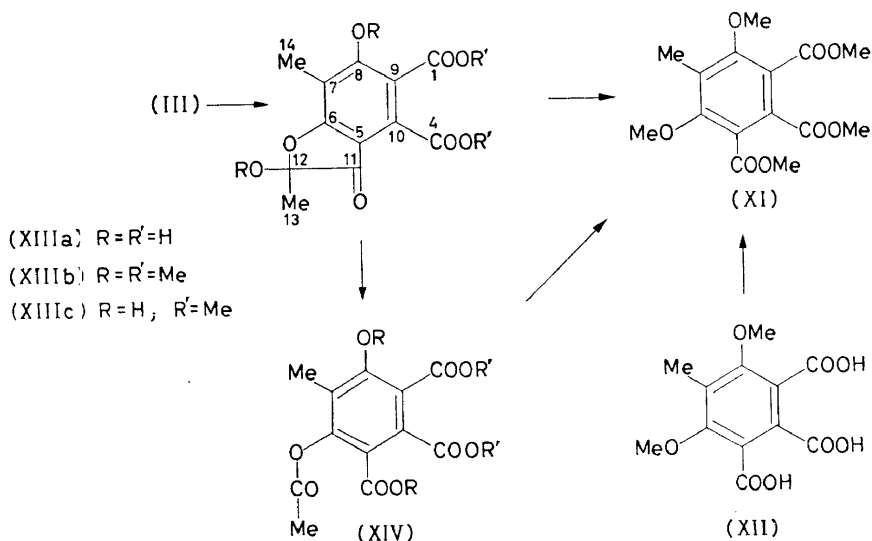

Figure 5. Degradation products of rifamycin S with ozone

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products: an aromatic tricarboxylic acid $C_{10}H_8O_8$ and an aromatic di-carboxylic acid $C_{12}H_{10}O_8$. The former could be identified as 1,2,3-tricarboxy-4,6-dihydroxy-5-methyl-benzene. Treatment of the acid with diazo-methane in methanol gives a dimethyl ether trimethyl ester (XI) identical with the material obtained by esterification with diazomethane of the known 1,2,3-tricarboxy-4,6-dimethoxy-5-methyl-benzene (XII) kindly sup-plied by Professor Birkinshaw. The constitution of the dicarboxylic acid $C_{12}H_{10}O_8$ (XIIIa) was derived as follows: treatment with methyl iodide and silver oxide yields, depending on the reaction conditions, either the dimethyl ester (XIIIc) or the dimethyl ether dimethyl ester (XIIIb). The former gives on oxidation with sodium periodate, and subsequent treatment of the oxidation product with diazomethane in methanol, 1,2,3-tricarboxy-4-methoxy-5-methyl-6-acetoxy-benzene trimethyl ester (XIVb). The acetoxyl group in this compound (detected from the i.r. and n.m.r. spectra) is re-moved by methanolysis, and the resulting phenol is etherified with diazo-methane in methanol to give the dimethyl ether trimethyl ester (XI) already mentioned.

These reactions, and especially the n.m.r. spectra of the ozonolysis pro-ducts, determine the constitution of a part of the compound $C_{14}H_{11}O_6N$ and account for twelve carbon and four oxygen atoms. The remainder of two carbon and two oxygen atoms must be arranged so as to give a p-quinone system. The only remaining task is to find out whether the amino group is at C–2 or C–3.

This group is found to be at C–2. Rifamycin O reacts with toluene-p-sulphonyl-hydrazine with elimination of glycolic acid and toluene-p-sulphinate ion to form a so-called quinone diazide, a reaction that has often
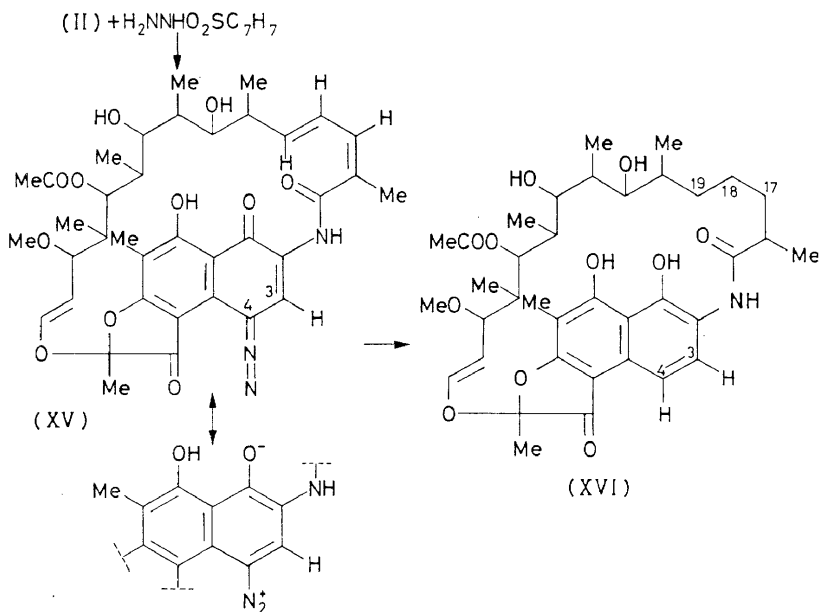

Figure 6. Quinone diazide and its hydrogenation product

been observed with simple quinones. The constitution (XV) (*Figure 6*) of this quinone diazide follows from its method of preparation, from analytical data, and especially from the presence in the infrared spectrum of the absorption band at 2120 cm^{-1} characteristic of quinone diazides, which are mesomeric with the anions of the corresponding p-hydroxy-phenyl-diazonium ions. The quinone diazide (XV) can be converted by catalytic hydrogenation into a compound (XVI) in which the diazonium group is replaced by hydrogen and the conjugated double bond system is saturated *i.e.* to a desoxy-tetrahydrorifamycin SV. This compound is still a strong monobasic acid (pK^*_{MCS} 4·44) and must therefore contain two hydroxyl groups in peri-position. The n.m.r. spectrum shows signals that correspond to two aromatic protons in o-positions. Thus, the newly introduced hydrogen must be placed at C–4 and the original hydrogen at C–3. C–2 therefore remains as the only possible site for nitrogen and the constitutional formula for the compound $C_{14}H_{11}O_6N$ is (VI). In accordance with that formula two products (IXa and b) are formed on methylation with methyl iodide and potassium carbonate. The compound (IXa) gave on acid hydrolysis the nitrogen-free o-hydroxy-quinone (X).

CONSTITUTION OF THE ALIPHATIC BRIDGE IN RIFAMYCINS AND OF THE COMPOUNDS $C_{25}H_{42}O_8$ AND $C_{24}H_{38}O_7$

The first information about the constitution of the aliphatic bridge in rifamycins was obtained by nitric acid oxidation of tetrahydro-rifamycin S (*Figure 7*). Rifamycin S rapidly absorbs 3 molecules of hydrogen to give
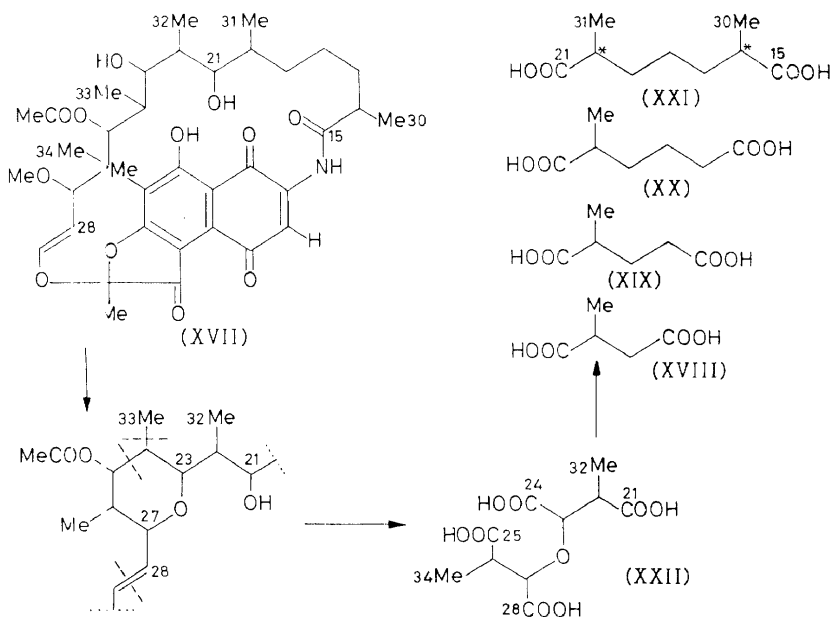

Figure 7. Nitric acid oxidation of tetrahydrorifamycin S

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tetrahydrorifamycin SV (XVII) which can easily be oxidized back to tetra-
hydrorifamycin S. Energetic hydrogenation saturated the remaining enol
ether double bond. On treatment with nitric acid tetrahydrorifamycin S
yields a mixture of acids, of which methylsuccinic acid (XVIII), α-methyl-
glutaric acid (XIX), α-methyladipic acid (XX), meso- and $(+)$-α,α'-
dimethylpimelic acid (XXI), as well as a tetracarboxylic acid $C_{10}H_{14}O_9$
(XXII), could be identified.

The isolation of two diastereomeric α,α'-dimethylpimelic acids indicates
that tetrahydrorifamycin is actually a mixture of two diastereoisomers that
can arise by hydrogenation of the conjugated double bond system between
C–16 and C–19. The isolation of these acids determine unambiguously the
constitution of the carbon chain between C–15 and C–21. The homologous
monomethyl dicarboxylic acids (XVIII)–(XX) are produced from the same
part of the chain.

The constitution of the tetracarboxylic acid $C_{10}H_{14}O_9$ (XXII) follows
from pK^*_{MCS}-values, n.m.r. spectrum of its tetramethyl ester, as well as from
its transformation into methylsuccinic acid (XVIII) on heating with hydro-
bromic acid and catalytic hydrogenation of the resulting reaction mixture.
The constitution of the tetracarboxylic acid (XXII) is important in the
derivation of the constitution of that part of the bridge lying between C–21
and C–28, and contains all carbon atoms of this part except the C–33 methyl
group. In interpreting these results one must of course assume that a tetra-
hydropyran ring is formed by the influence of acid prior to the oxidation.
The plausibility of this assumption is supported by the thorough investiga-
tion of the compounds $C_{25}H_{42}O_8$ (VII) and $C_{24}H_{38}O_7$ (VIII) (*cf. Figure 4*)
arising in the methanolysis of the imino methyl ether (V) from rifamycin S.
These two compounds can be transformed into one another: the compound
(VII) splits off methanol on pyrolysis giving compound (VIII) which in
turn can be converted by treatment with methanolic hydrochloric acid back
into the compound (VII). From the n.m.r. spectra it could be concluded
that the compound (VII) is the dimethyl acetal methyl ester of an aldehydo-
carboxylic acid, which gives the corresponding enol methyl ether (VIII)
with loss of methanol. In the n.m.r. of the latter compound (*Figure 8*) the
signals of the two protons on the enol methyl ether double bond can be
easily recognized; these signals are absent in the spectrum of the dimethyl
acetal (*Figure 9*). In both spectra the signals of the olefinic protons at C–17,
C–18 and C–19 are not only well recognizable but the multiplicity of the
signals and the coupling constants allow one even to derive the configuration
at the double bonds as given in the formulae. From the further well-recog-
nizable signals in both spectra the singlets of the dimethyl acetal, methyl
ester and acetoxy groups, the singlet of the methyl on double bond, and a
group of four doublets corresponding to the four CH_3CH-groups are worthy
of mention.

Other details of the constitution could be fixed by degradation of the
compound $C_{25}H_{42}O_8$. Ozonolysis, followed by oxidation of the product with
performic acid, and esterification with diazomethane, yields a saturated
hydroxy acetoxy dimethyl ester $C_{19}H_{32}O_8$ (XXIIIa) (*Figure 10*) in which
one carboxyl was produced by ozonolysis of the double bond between C–18
and C–19 and the other by oxidation of the aldehyde dimethyl acetal group

at C–29. The position of the conjugated double bond system, derived earlier from the ultraviolet light absorption spectra of rifomycins and their transformation products, is thereby confirmed. In the n.m.r. spectrum of the compound (XXIIIa) the singlets of two methyl ester groups and one acetoxyl group, as well as the unresolved signals from three CH_3CH-groups, can be recognized. A free hydroxyl group is indicated by formation of the
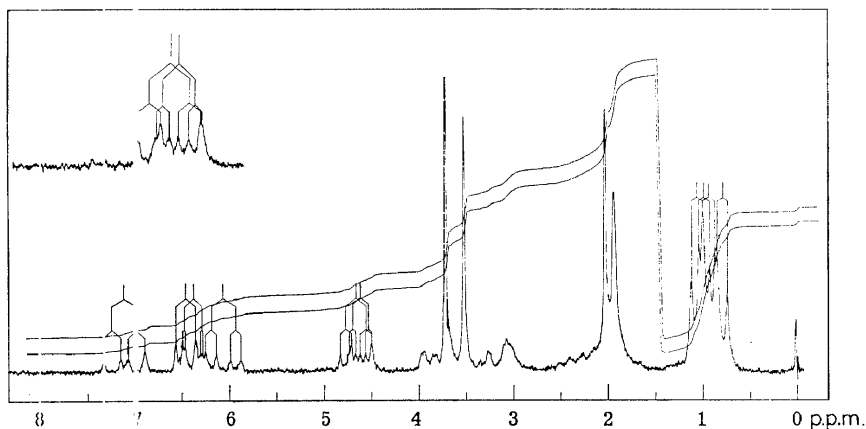

Figure 8. N.m.r. spectrum of the enol methyl ether $C_{24}H_{38}O_7$ (VIII)

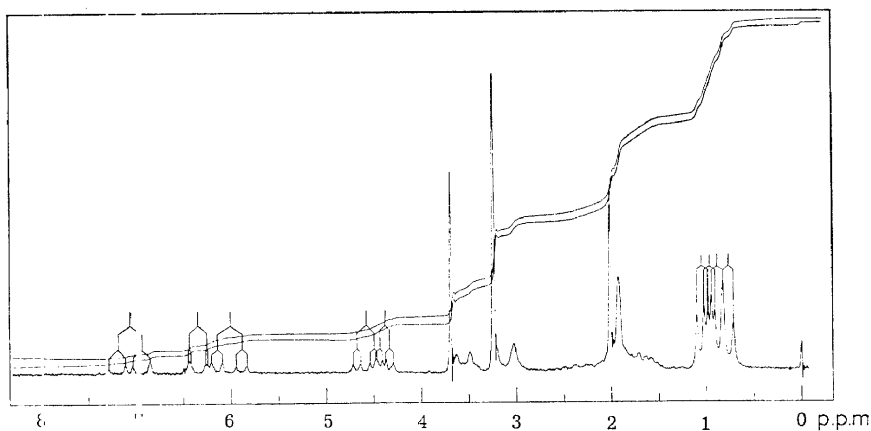*Figure 9.* N.m.r. spectrum of the dimethyl acetal $C_{25}H_{42}O_8$ (VII)

acetyl derivative (XXIIIb). Pyrolysis of this acetyl derivative produces an α,β-unsaturated acetoxy dimethyl ester $C_{19}H_{30}O_7$ (XXIV). This compound was again degraded by ozonolysis, followed by performic acid oxidation, and the resulting acids were esterified with diazomethane to give an acetoxy-dimethylester $C_{16}H_{26}O_7$ (XXVa). The presence of one acetoxyl group and only three CH_3CH-groups is indicated by the n.m.r. spectrum (*Figure 11*). In this degradation series one may ask whether the original acetoxyl group or the one introduced by acetylation was split off. This question is important

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(XXIIIa) R = H
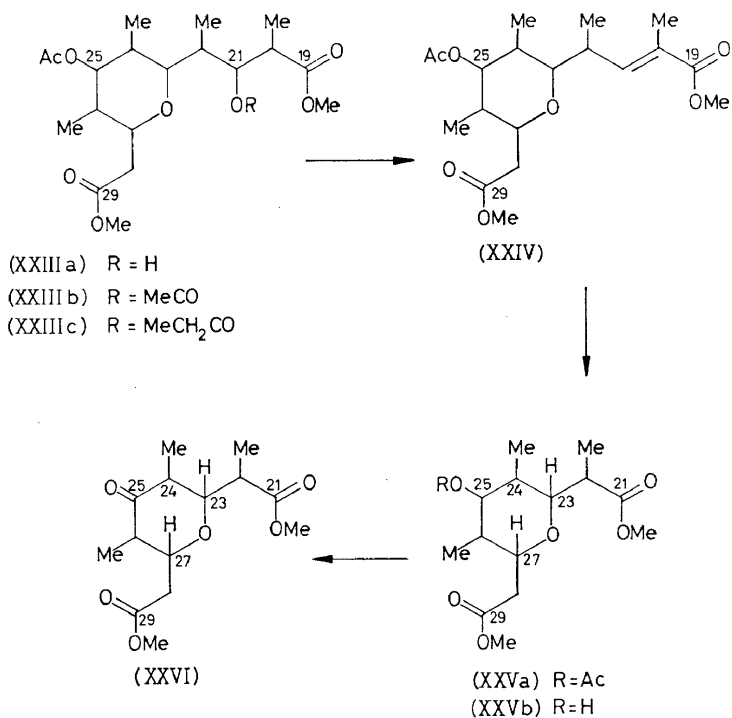(XXIIIb) R = MeCO
(XXIIIc) R = MeCH₂CO

Figure 10. Degradation of the dimethyl acetal $C_{25}H_{42}O_8$ (VII) with ozone

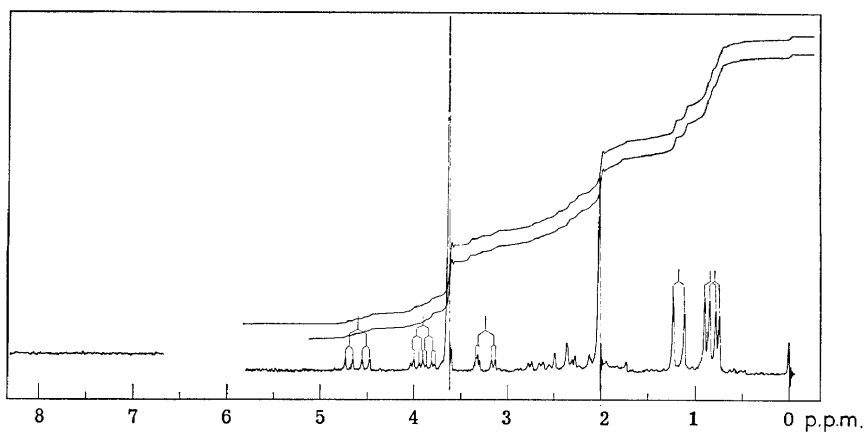*Figure 11.* N.m.r. spectrum of the acetoxy dimethyl ester $C_{16}H_{26}O_7$ (XXVa)

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for the placing of the acetyl group in rifamycins. To answer it the free hydroxyl group in the hydroxy-acetoxy-dimethyl ester, $C_{19}H_{32}O_8$, was esterified with propionic anhydride and the resulting propionyl derivative (XXIIIc) was pyrolized. This experiment yielded only propionic acid and the same α,β-unsaturated acetoxy-dimethyl ester (XXIV) as had been obtained from the acetyl derivative (XXIIIb). The original acetoxyl group can not, therefore, be placed at C–21. Further important information on the constitution of the aliphatic bridge in rifamycins was obtained by methanolysis of the acetoxyl group in the acetoxy-dimethylester $C_{16}H_{26}O_7$ (XXVa) and oxidation of the resulting hydroxy-dimethylester, $C_{14}H_{24}O_6$, (XXVb) to the oxo-dimethylester, $C_{14}H_{22}O_6$ (XXVI). Comparison of the n.m.r. spectra (*Figures 11* and *12*) of the degradation products (XXVa) and
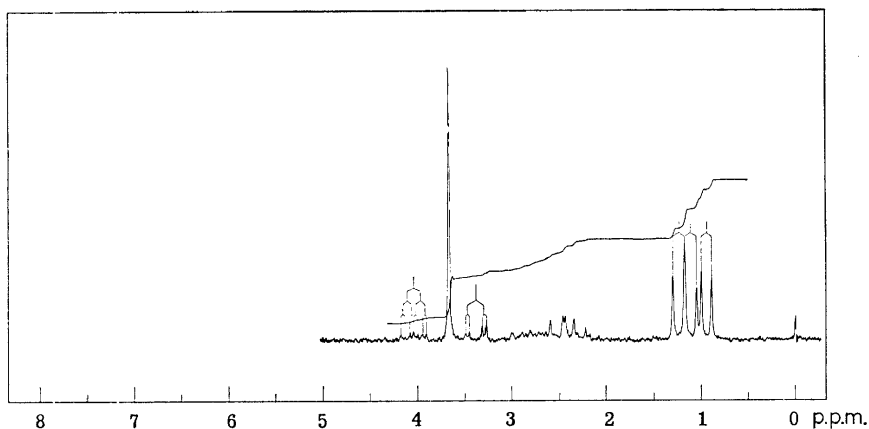

Figure 12. N.m.r. spectrum of the oxo dimethyl ester $C_{14}H_{22}O_6$ (XXVI)

(XXVI) leads to an important argument for the exact location of the acetoxyl group and of the C–33 methyl group. In the spectra of the acetoxy- and of the oxo-dimethylester the signals corresponding to the two protons at C–23 and C–27 adjacent to oxygen of the tetrahydropyran can clearly be recognized. These signals occur at practically the same place in both spectra, which could hardly be the case if the acetoxyl or carbonyl–oxygen were at C–24 and the methyl at C–25. In this connection it should be recalled that the constitution of this part of the bridge was initially derived from the constitution of the tetracarboxylic acid $C_{10}H_{14}O_9$ (XXII) (*cf. Figure 7*), and that the position of the C–33 methyl group was thereby left undetermined. It should be mentioned that the tetracarboxylic acid (XXII) can also be obtained by nitric acid oxidation of the enol methyl ether dimethyl ester $C_{24}H_{38}O_7$ (VIII).

ATTACHMENT OF THE ALIPHATIC BRIDGE TO THE AROMATIC PART

There remains the task of deriving the constitution of rifamycins from the constitution of the fragments obtained by methanolysis, taking into account any changes that might occur in this reaction.

There can be little doubt about the point of attachment to the aromatic part: only the nitrogen atom at C–2 and the oxygen atom at C–12 come into consideration. It is also fairly certain that the C–15 carboxyl group is attached to the nitrogen at C–2. The second point of attachment of the bridge is less obvious because during the methanolysis, apart from the breaking of carbon–oxygen bond, a ring closure takes place to give a tetrahydropyran ring.

From inspection of models it was apparent that on steric grounds only C–27 and C–29 could be candidates for this second point of attachment. Our preference is for C–29 since otherwise this carbon atom would be the site of the methoxyl group. In this event hydrogenation of the double bond between C–28 and C–29 in hexahydro-rifamycin S should lead to a shift of the methoxyl signal in the n.m.r. spectrum, but this has not been observed. For the placing of the methoxyl group in rifamycins there are thus two remaining possibilities, C–27 and C–23, which are linked in the compounds $C_{25}H_{42}O_8$ (VII) and $C_{24}H_{38}O_7$ (VIII) to the oxygen atom of the tetrahydropyran. On account of the smooth elimination of methoxyl in the formation of these products the allylic position at C–27 seems more probable. The first step of the acid methanolysis would then be elimination of the methoxyl group followed by ring closure, the enol-ether being split off subsequently.

THE ANSA-CONSTITUTION OF RIFAMYCINS

According to our formulae (I)–(IV) (*cf. Figure 1*) for rifamycins these compounds represent naturally occurring ansa-compounds in which the aliphatic bridge spans an aromatic system. One consequence of this ansa-constitution is that methyl groups of the aliphatic bridge are placed in the magnetic field of the aromatic π-electron current, and are therefore more-or-less shielded depending on their exact positions. An analogous situation arises for the middle methylene groups in certain cyclophanes[4]. The doublets attributed to two of the four CH_3CH-groups of rifamycins O and S indeed lie at unusually high magnetic fields δ = 0·22 and 0·67. This fact was interpreted by us right at the beginning of our investigation as an indication that rifamycins might be ansa-compounds.
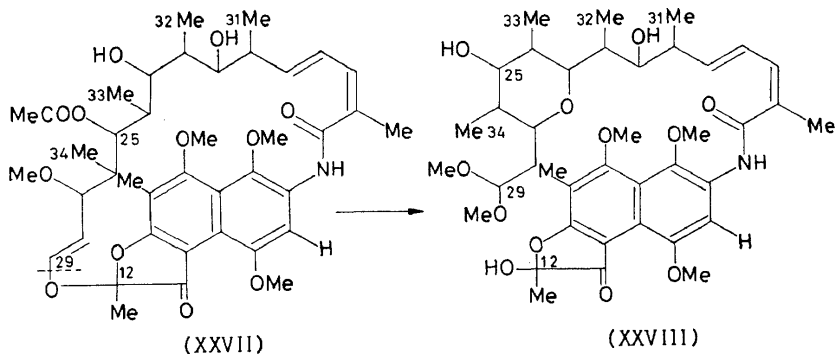

Figure 13. Rifamycin SV trimethyl ether (XXVII) and its methanolysis product

The shielding by the π-electron systems in rifamycin derivatives of the hydroquinone type, *e.g.* in the compound (XXVII), (*Figure 13*) is even stronger than in compounds of the quinone type. The n.m.r. spectrum (*Figure 14*) of (XXVII) shows CH_3CH-doublets at $\delta = -0.59$ and $+0.54$. The compound mentioned can be split between C–12 and C–29 by methanolysis to a compound (XXVIII) whose n.m.r. spectrum is reproduced in *Figure 15*. The doublets of the C–31, C–32, C–33 and C–34 methyl groups
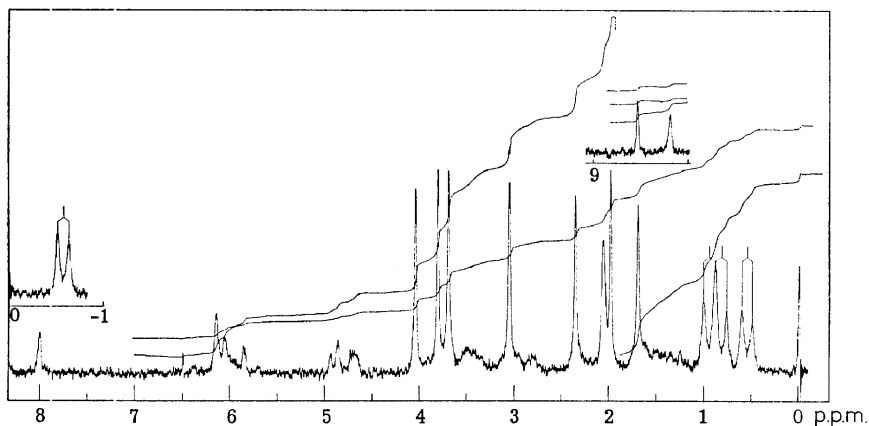

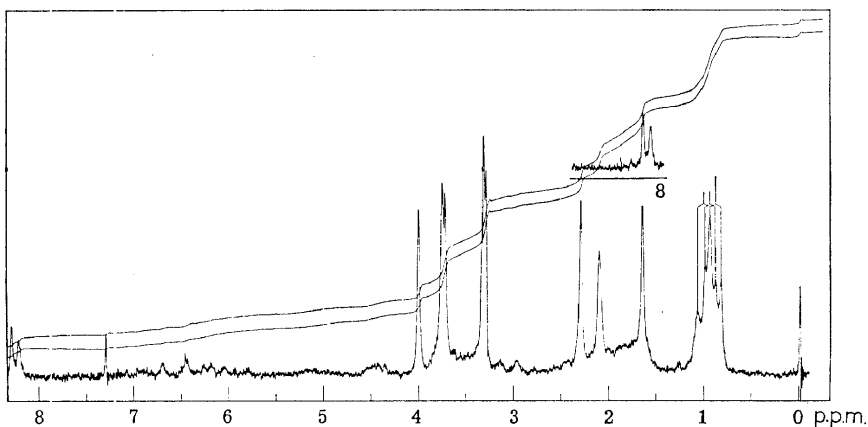*Figure 14.* N.m.r. spectrum of the rifamycin SV trimethyl ether (XXVII)

Figure 15. N.m.r. spectrum of the compound (XXVIII)

appear together in one composite peak whose centre lies at $\delta \sim 1$. The experimental facts that support the proposed formulae of rifamycins have been neither completely enumerated nor discussed because of lack of time.

An X-ray analysis of the *p*-iodoanilide of rifamycin B is under way in the laboratory of Professor Vaciago, Rome, and we await with interest (and confidence!) his verdict on the constitution derived chemically. The X-ray

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analysis should also answer many questions on the configuration and the conformation of the rifamycin molecule.

In conclusion I should like to say a few words about biogenesis. Their constitution suggests that rifamycins are built largely from acetate and propionate ions, or from their biogenetical equivalents. The distribution of the C-methyl groups and of the oxygen functions in the aliphatic bridge is very reminiscent of the constitutions of certain macrolides. In spite of this biogenetic relationship rifamycins represent a new type of metabolites of actinomyces and give further evidence of ability of micro-organisms to produce novel and unusual molecules.

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