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A Study of Structure-Activity Relationships in 4-Deoxypyrido[1′,2′-1,2]imidazo[5,4-c]rifamycin SV Derivatives by Electron Spectroscopy for Chemical Analysis and ^{1}H NMR

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SUMMARY

A new class of rifamycins, 4-deoxypyrido[1′,2′-1,2]imidazo[5,4-c]rifamycin SV derivatives, has been synthesized. They are potent antibacterial agents and are not absorbed at the gastrointestinal level and can therefore probably be used as antibacterial intestinal disinfectants. From the present X-ray, electron spectroscopy for chemical analysis, and ^{1}H NMR study, it appears that this peculiar pharmacokinetic behavior is mainly to be attributed to the fact that the pyridoimidazo system exists in these compounds in a mesomeric betaine form, bearing one positively and one negatively charged nitrogen. If it is assumed that rifamycins are generally absorbed by passive diffusion, the presence of the two oppositely charged nitrogens, together with the presence of the phenolic hydroxyls, means that these molecules are ionized at all pH values encountered along the gastrointestinal tract, which thus prevents their absorption. These molecules also display a strong tendency to self-associate both in solution and in the solid state, and the increase in molecular size may also play a role in preventing their absorption.

INTRODUCTION

4-Deoxypyrido[1′,2′-1,2]imidazo[5,4-c]rifamycin SV derivatives (1) (Fig. 1) are a new series of semisynthetic rifamycins that display strong antibacterial activity *in vitro* and are very poorly absorbed when given *per os in vivo*. They therefore appear to be promising agents in the therapy of intestinal infections.

In order to explain this peculiar behavior, their structural features have been investigated using different spectroscopic techniques, i.e., X-rays[5] and ESCA in the solid state, and NMR in solution.

The N 1s ESCA and ^{1}H NMR analyses reported in this paper confirm the X-ray data (2). They have been carried out on one compound in particular, 4-deoxy-4′-methylpyrido[1′,2′-1,2]imidazo[5,4-c]rifamycin SV (**1**-SV),[6] i.e., on the one in the series actually under clinical evaluation.

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[5] The abbreviations used are: X-rays, single crystal X-ray diffraction; ESCA, electron spectroscopy for chemical analysis; N 1s b.e., binding energy of electrons in the core 1s orbital of nitrogen.

[6] This compound has been also reported under the names rifamycin L 105 and Rifaximin.

EXPERIMENTAL PROCEDURES

Synthesis. All derivatives were synthesized according to the systems described in the literature. They were isolated and purified by column chromatography on Silica Gel 60 (40–63 μm) eluting with mixtures of chloroform/methanol 95:5, 90:10. In particular the 8-methoxy derivative of **1**-SV was synthesized by reacting **1**-S with methyliodide, as described for the preparation of 8-methoxyrifamycin S (3), and reduction to the SV form with ascorbic acid (4).

ESCA measurements. Photoelectron spectra were run on a VG ESCA III MK2 spectrometer using Al K_α radiation, according to the usual technique (5). Samples were dusted as thin films onto gold sample plates. The C 1s line (b.e. = 285.0 eV) from residual pump-line oil contamination was used as internal standard for spectral calibration.

The binding energies reported were measured at half the width of the half-maximum of the bands. Possible changes in the products under the action of the X-ray radiation were monitored by repeated scans, the spectra being recorded at liquid nitrogen temperature. No significant changes in the spectra were observed, with the exception of 8-methoxy-**1**-SV. The average of three different measurements on different deposited samples was considered accurate to ±0.2 eV. Peak deconvolution was performed using a DuPont 310 curve resolver using a Gaussian shape fit.

^{1}H NMR measurements. The spectra were run at 200 MHz on a WP 200 Bruker spectrometer. Chemical shift values are given in parts per million relative to tetramethylsilane as 0.00.

RESULTS AND DISCUSSION

X-ray analysis. The molecular structure of 4-deoxy-3′-bromopyrido[1′,2′-1,2]imidazo[5,4-c]rifamycin S, **2**, de-

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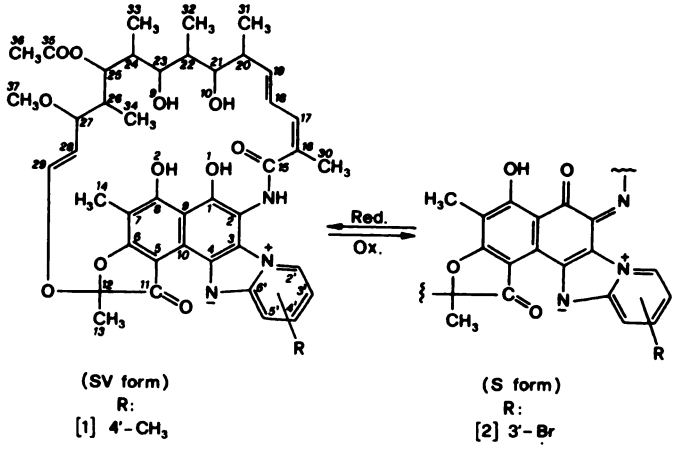

FIG. 1. *Structural formulas of some 4-deoxypyrido[1',2'-1,2]imidazo[5,4-c]rifamycin SV derivatives*

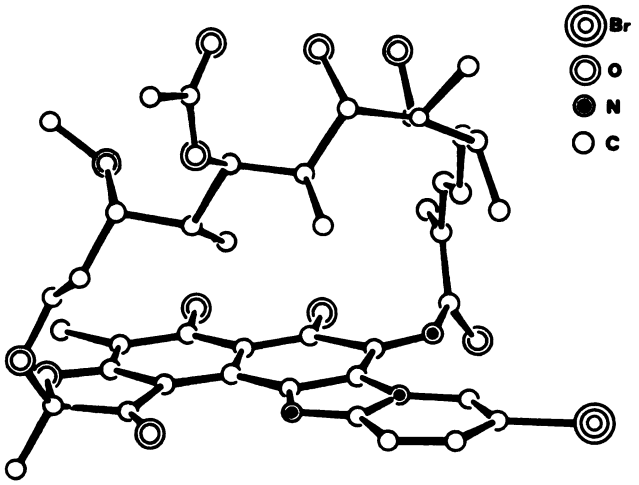

FIG. 2. *X-ray molecular structure of 4-deoxy-3'-bromopyrido[1',2'-1,2]imidazo[5,4-c]rifamycin S*

termined by single crystal X-ray diffraction (2) (Fig. 2) has shown that the newly formed pyridoimidazo system and the naphthoquinone system are completely co-planar within 0.05 Å. This fact implies that charged forms make a large contribution to the resonance structure, the pyrido nitrogen being positively charged, and the imidazo nitrogen being negatively charged, as shown in Fig. 1.

ESCA analysis. On the basis of the X-ray structural study, a further ESCA solid state study was performed in order to confirm that **1**-SV in particular, and all these new pyridoimidazo rifamycins in general, carry two oppositely charged nitrogens. For this purpose the N 1s b.e (6) in **1** both in the oxidized S and in the reduced SV forms were measured. Three b.e. values were found for **1**-S (Fig. 3a), corresponding respectively to one negatively charged nitrogen (N^-), one neutral nitrogen, and one positively charged nitrogen (N^+).

The analysis of **2** (S form), the derivative whose structure was determined by single crystal X-ray diffraction, gave a result very similar to that of **1**-S, with three peaks corresponding to one N^-, one neutral nitrogen, and one N^+ (Fig. 3c).

Although **1**-SV (Fig. 3b) also gave three signals, no signals could be attributed to N^-, while there was an

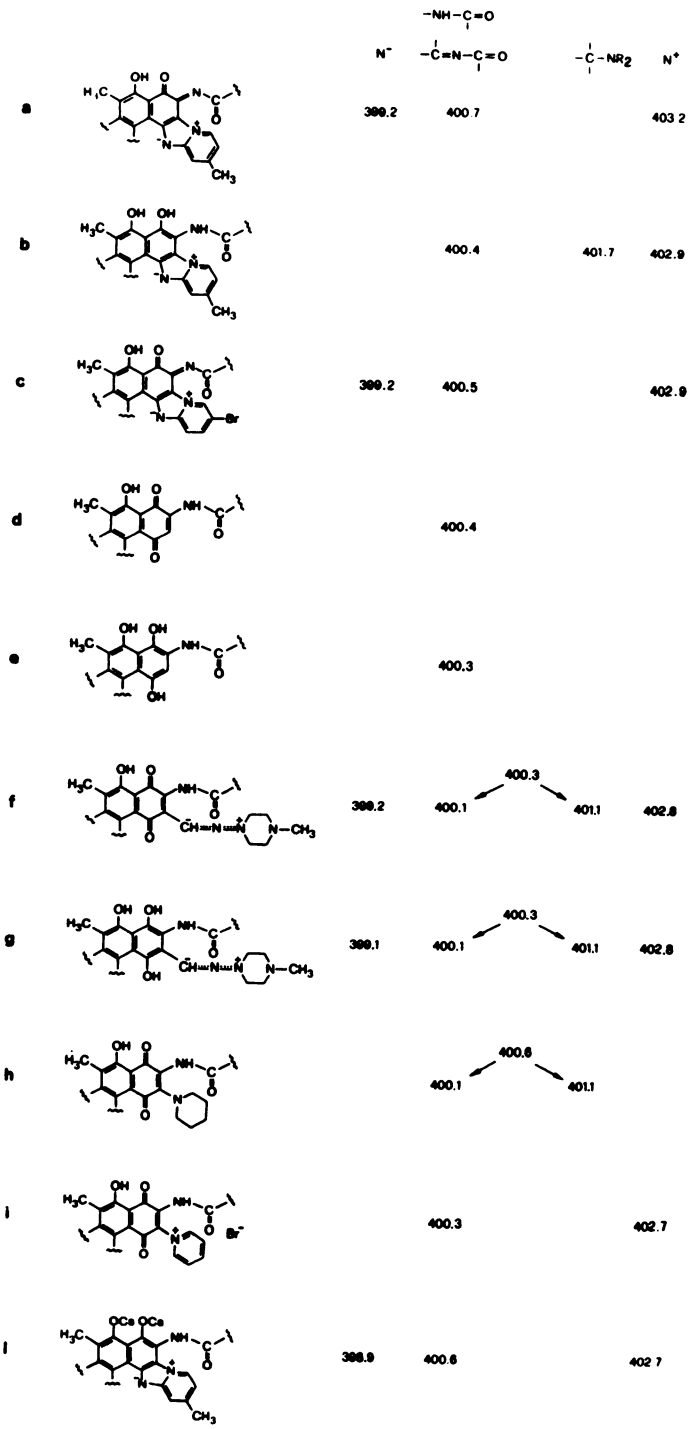

FIG. 3. *ESCA N 1s binding energy measurements (in electron volts)* Only the part bearing nitrogen is represented for each examined molecule. For complete formulas, see Fig. 1.

additional neutral N-type signal. In order to investigate the different behavior, analogous measurements were performed on other known rifamycins (Fig. 3, d–l).

Rifamycin S and rifamycin SV (4) (Fig. 3, d and e) were each found to give one b.e. value, owing to the presence of the amidic nitrogen; the two values are close together, 400.4 and 400.3 eV, respectively, and fall in the range of literature values for amidic nitrogens (6). The N 1s b.e. values in rifamycins thus do not seem to be

significantly influenced by the oxidation state of the naphthoquinone system. This is confirmed by the spectra displayed by the S and SV forms of rifampicin (7). Both these molecules display three types of signals, the corresponding values coinciding almost exactly. The values have been assigned in the following order: one to the nitrogen bound to the negatively charged carbon atom, one to the sum of the neutral amidic and *N*-methyl nitrogens, and one to the positively charged nitrogen, as shown in Fig. 3, f and g, in which rifampicin is represented in a mesomeric betaine resonance structure.

The X-ray molecular structure of rifampicin (8) displays bond distances of 1.292 Å for the $C_{1'}=N_{2'}$ group, and 1.398 Å for the following $N_{2'}-N_{3'}$, group, while the literature (9) reports 1.24–1.28 Å as the average value for the former bond type and 1.43–1.44 Å for the latter. The experimental X-ray values, which fall significantly outside the literature range, indicate that, in agreement with the ESCA measurements, the proposed mesomeric betaine structure is more representative of rifampicin than the corresponding neutral formula $C_{1'}=N_{2'}-N_{3'}$, at least in the solid state. Furthermore, the central signal corresponds to two superimposed signals, as found also in the analysis of 3-piperidinorifamycin S (10). In both cases, by deconvolution of the single broad band, two peaks were obtained with an intensity ratio 1:1, one of which has been attributed to the amidic nitrogen and the other to the aminic heterocyclic nitrogen, respectively, in order of increasing b.e. value (Fig. 3h).

A further example of positively charged nitrogen is provided by analysis of 3-pyridinium bromide rifamycin S (1), for which two b.e. values have been assigned, the lower to the neutral amidic nitrogen and the higher to the positively charged quaternary nitrogen (Fig. 3i).

On the basis of the comparison between **1**-SV and the other compounds studied, the lack of the N^- peak in the former was explained by the hypothesis that the negatively charged nitrogen 7′ is neutralized by one of the phenolic protons, either in an intra- or in an intermolecular exchange ($N^- \rightarrow NH$), and this would justify the presence of the peak at 401.3 eV.

To confirm this hypothesis, the 8-methoxy derivative of **1**-SV (Fig. 4) was first prepared. This derivative should be less likely to give such a proton exchange, since the acid proton on O_2 is substituted by a methyl group. However, this compound proved to be unstable under X-rays.

A final measurement was then carried out on the cesium salt of **1**-SV, which lacks both the acid protons, and should not be able to neutralize the charge of the negative $N_{7'}$ (Fig. 3l).

Indeed, three signals were found, one corresponding to an N^-, one to an amidic N, and one to an N^+.

These results clearly indicate that the spectrum of **1**-SV represents the compound in an associated form.

^1H NMR analysis. A detailed NMR study was performed in order to determine the structure of **1**-S and **1**-SV, and of the other related new pyridoimidazo rifamycins.

While chemically characterizing **1**-SV, it was observed that, although all values of chemical shifts and coupling

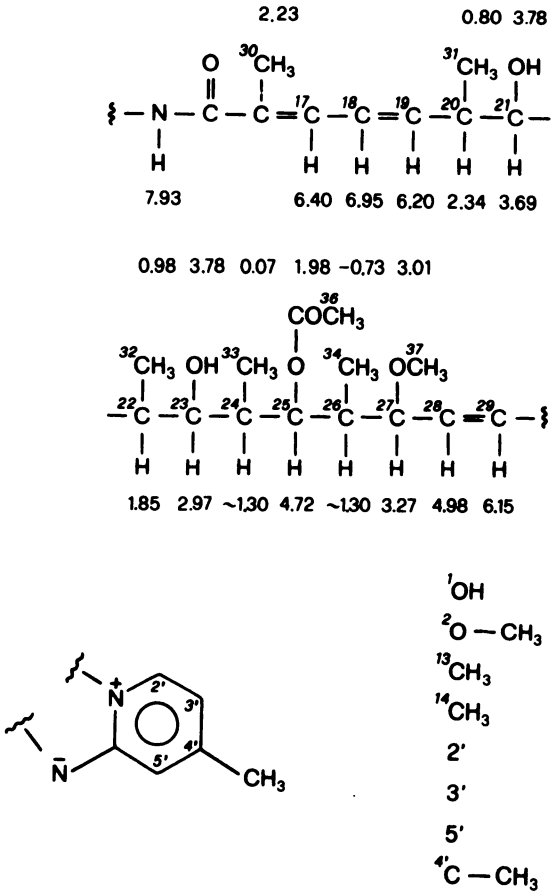

FIG. 4. *^1H NMR 200 MH$_z$ structure determination of 8-methoxy 1-SV in $CDCl_3$*

Chemical shift values in parts per million from tetramethylsilane; coupling constants in hertz.

constants with the exception of O_1H and O_2H resonances lie quite close to the corresponding values of other rifamycin derivatives (11), there were other significant differences. In detail, all the 3-substituted rifamycin SV derivatives studied give sharp, well resolved spectra in D_2O; most of them gave broad unresolved spectra in organic solvents, regardless of solvent polarity. One exception was rifampicin in $CDCl_3$, which was stabilized in a particular conformation by an intramolecular H bond between the amidic proton and the substituent in position 3 (12).

In $CDCl_3$, at low temperatures, these broad signals split into two sets, revealing the presence of two conformers. In none of the solvents used did any of the rifamycins examined show any noticeable chemical shift variation as a function of concentration. In $CDCl_3$, all phenolic protons for all SV derivatives gave rise to resonances at about 14 ppm.

On the contrary, **1**-SV gave a broad unresolved spectrum in D_2O and sharp, well resolved spectra in low polarity solvents. Broadening of peaks was observed in polar solvents. Splitting into two sets of signals was observed at low temperatues in CD_3OD, but not in $CDCl_3$.

In **1**-SV, O_1H and O_2H gave resonances at ~15 and ~17 ppm in $CDCl_3$. Furthermore, **1**-SV (but not **1**-S) showed a marked dependence of the chemical shift values

for all protons as function of its concentration in $CDCl_3$ solution. Chemical shift value variations as a function of concentration are shown in Fig. 5.

The marked dependence of chemical shift values in **1**-SV upon concentration (0.01–10 mM) in $CDCl_3$ can be explained by postulating that an intermolecular exchange takes place in solution, e.g., a monomer-dimer or a monomer-polymer fast equilibrium. It is noteworthy that such an equilibrium occurs even at rather low concentrations and mostly affects the phenolic protons, whose chemical shifts undergo the largest variations.

A B $\rightleftharpoons$ **2A** monomer-dimer equilibrium with dissociation constant K_d

$$K_d = \frac{[A]^2}{[B]} \qquad (1)$$

can be studied by NMR (13) and

$$\frac{1}{\delta_{obs} - \delta_\infty} = \frac{K_d}{8C_A\,\Delta\delta} - \frac{1}{2\Delta\delta} \qquad (2)$$

where δ_{obs} = actual value of chemical shift as a function of concentration C_A, δ_{dimer} = chemical shift of the dimeric species, $\delta_{monomer}$ = chemical shift of the monomeric species, and $\Delta\delta = \delta_{dimer} - \delta_{monomer}$.

Equation 2 is valid within the following approximations: 1) the structure of the monomer does not depend on concentration: 2) only one association site, forming a single H bond, is involved in the equilibrium; 3) $\delta_\infty = \delta_{monomer} = \delta$ at maximal dilution; and 4) resonances due to different protons are independent of one another at each concentration.

It must be pointed out that definite evidence exists

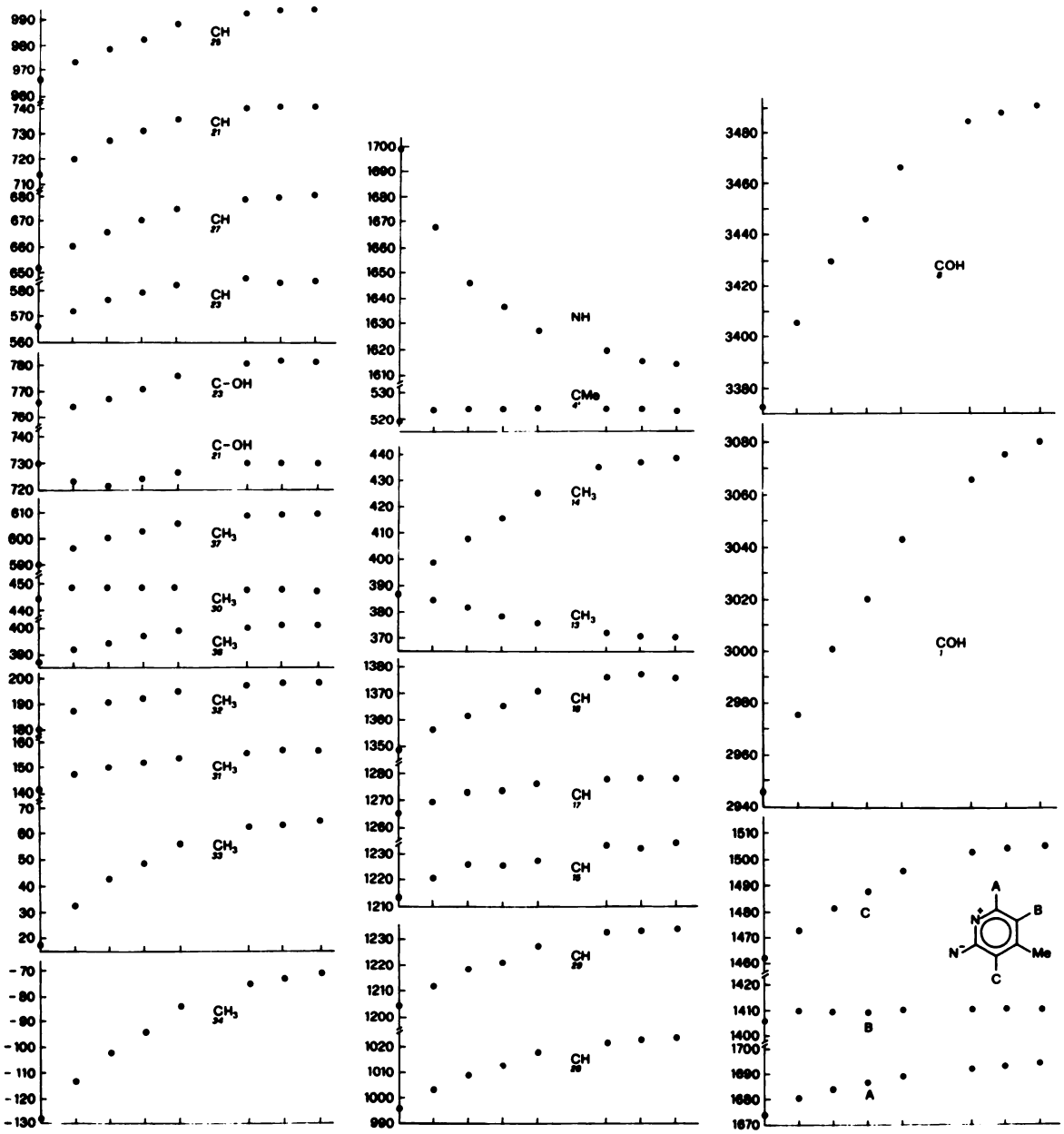

FIG. 5. *Chemical shift variations for some of the* ^1H *NMR signals of 1-SV in* $CDCl_3$ *as a function of concentration*

that approximation 2 is not correct, since chemical shift variations are very close together for signals due to O_1H and O_2H. Applying all these approximations, a linear dependence of $1/\Delta\delta$ with respect to $1/C_A$ was observed, which was true for all signals.

Rather arbitrarily, in order to minimize errors, only those protons were considered whose chemical shift variation, as a function of concentration, was larger than 25 Hz. The chosen signals are due to O_1H, O_2H, NH, $C_{33}H_3$, $C_{34}H_3$, $C_{14}H_3$, $C_{4'}$-CH_3, $C_{29}H$, $C_{28}H$, $C_{27}H$, $C_{25}H$, $C_{21}H$, $C_{18}H$. For all these signals, the linear dependence of $1/\Delta\delta$ versus $1/C_A$ holds with a correlation coefficient better than 0.997. Moreover, the straight lines $1/\Delta\delta \div 1/C_A$ obtained for these signals all give the same value of $K_d = 23 \pm 4$ mol/liter (Eq. 1), with the exception of the signal of the amidic proton giving a $K_d \approx 36$ mol/liter. Therefore, a model accounting for the observed behavior of 1-SV in $CDCl_3$ solution could be as shown in Fig. 6.

This dependence of chemical shift on 1-SV concentration was not studied in D_2O because of the low solubility limit (0.1 mM) and of the too narrow accessible temperature range. However, the 1-SV spectrum appears as broad in phosphate buffer, 40 mM, pD 7.0 as in D_2O. In acid solution (D_2O + DCl, pD 1) all lines became comparatively sharper, but the spectral resolution was still too poor for the coupling constants to be measured.

In a basic solution (D_2O + NaOD, pD 9), the broad lines split into two sets of signals of comparable intensity, although remaining fairly broad. This behavior could be explained in terms of the conformational equilibria taking place in rifamycins in solution and resulting in similar broadening and splitting phenomena (11), although these have never been observed in D_2O. Further studies were then carried out in other polar organic solvents, at concentrations of 0.1 mM.

In D_6-dimethyl sulfoxide, for instance, the spectrum displayed even broader lines than in D_2O. In CD_3OD the lines were still broad, and, at 190° K, a clear splitting of the signals due to $C_{33}H_3$ and $C_{34}H_3$ into two sets of lines occurred.

It may be concluded that 1-SV gives rise to monomer-dimer (or polymer) equilibria in all solvents. This would explain the observed chemical shift dependence on concentration in $CDCl_3$. Since no line splitting occurs in this solvent, even at 220° K, the observed phenomena are unlikely to be related to conformational variations.

In more polar organic solvents, and in D_2O, the existence of the monomer-dimer (or polymer) equilibria gives rise to a broadening of all lines, and is accompanied in some cases also by the contemporary existence of conformational equilibria, as revealed by the splitting of some of the lines.

Comparison of chemical shift values for corresponding protons on the pyrido ring in 1-SV and 1-S clearly shows that, despite any possible slight differences, both structures are rather similar. That is, both in its oxidized and reduced form, in 1 the pyridoimidazo rings are co-planar with the chromophore and display mesomeric betaine forms.

In $CDCl_3$, at concentrations higher than 10 mM, a small chemical shift variation can be observed also for the protons on the chromophore rings of 1-S and it has been attributed to the occurrence of intermolecular stacking. The measured variations are too small to allow any detailed interpretation, as they never exceed 8 Hz.

No other dependence of chemical shift values on concentration was observed in 1-S. This behavior confirms the proposed model since in any S form only the phenolic proton is strongly involved in an intramolecular H bond with the neighboring O_1. For a further confirmation of the model, the behavior of the 8-methoxy derivative of 1-SV was also examined in solution.

If the proposed model is correct, 8-methoxy 1-SV should not show any chemical shift dependence on concentration in chloroform solution, since an intermolecular H bond should be strongly impeded by the absence of one phenolic proton, and the presence of the hindering methyl group. In fact, no chemical shift dependence on concentration for 8-methoxy 1-SV was found in the range 0.01–10 mM in$CDCl_3$ solution. All these results indicate that a monomer-dimer equilibrium exists in solutions of 1-SV, as shown in Fig. 6.

CONCLUSIONS

The X-ray structural study has indicated the existence of pyridoimidazo rifamycins in the solid state in a mesomeric betaine form, with one positively and one negatively charged nitrogen. The N 1s b.e. values obtained by ESCA, also in the solid state, has confirmed the X-ray data. Furthermore, 1-SV has been found to give rise to association forms through the participation of the negatively charged nitrogen.

^1H NMR has shown the existence, in particular in $CDCl_3$ solution, of a monomer-dimer equilibrium in which the negatively charged nitrogen of one molecule most probably binds the phenolic hydroxyls of a second molecule. The existence of such an equilibrium in aqueous solution has not been directly demonstrated, owing to the rather low solubility of these molecules in this solvent, although it can be deduced from studies in other polar solvents.

In conclusion, the considerable contribution made by

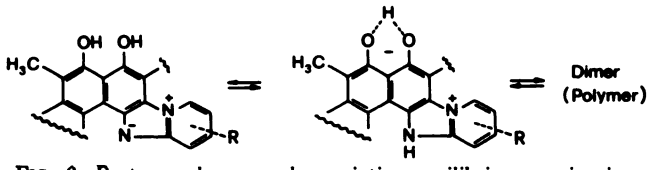

FIG. 6. *Proton exchange and association equilibria occurring in solutions of 1-SV*

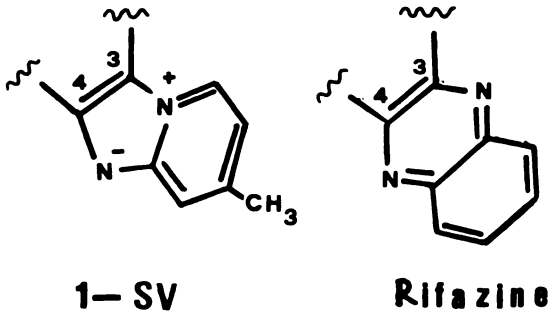

FIG. 7. *Structural formula of Rifazine (14)*

charged forms to the resonance structure of **1**-SV, and the tendency of **1**-SV to associate must contribute to the fact that this drug is poorly absorbed through the intestinal membrane. In fact, one can observe that benzophenazino rifamycins as Rifazine (Fig. 7) (14), given *per os*, are well absorbed drugs. They differ from pyridoimidazo rifamycins only by the presence of one additional carbon atom in the newly condensed aromatic system, for which, in this case, only neutral resonance structures can be written.

Therefore, assuming that the rifamycin absorption mechanism is generally a passive one, the presence of the charged nitrogens in **1**-SV must be the main factor responsible for its different pharmacokinetic behavior. That is, the presence of charged nitrogens in the pyridoimidazo system and the presence of the phenolic hydroxyls mean that these molecules are ionized at all pH values encountered along the gastroenteric tract, thus preventing their absorption through the lipoid membranes.

Furthermore, the hypothesis may be advanced that the association forms of **1**-SV, which have been found to exist particularly in chloroform may also exist in the lipoid layer of the intestinal membranes. This increase in molecular size could also be one of the factors determining the reduced absorption of this new drug.

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