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Structure-Activity Relationships in the Ansamycins

Molecular Structure and Activity of 3-Carbomethoxy Rifamycin S

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SUMMARY

The X-ray and NMR structural study of 3-carbomethoxy rifamycin S[5] was undertaken in order to determine whether its low antimicrobial activity was related to a conformation of the molecule which was unfavorable for interaction with bacterial DNA-dependent RNA polymerase. However, the molecule assumes a conformation similar to that of the active rifamycins. Indeed the compound was found to be active on the isolated enzyme, so that its low activity on whole bacteria has to be attributed to factors affecting its penetration through the bacterial cell wall.

INTRODUCTION

The rifamycins are a series of naphthalenic ansamycins with a 17-membered *ansa* chain. These compounds display a remarkable antimicrobial activity due to the inhibition of bacterial DDRP[6] (1, 2). The chemical and structural features of these antibiotics that allow the formation of a specific and stable complex with the bacterial enzyme have been defined by several structure-activity studies (3–6). In summary, the minimal requisites of the antibiotic for inhibition of the bacterial enzyme are (a) two oxygen atoms, on C(1) and C(8) in the naphthalenic nucleus, either as unsubstituted hydroxyls or as quinonic oxygens; (b) two unsubstituted hydroxyls on C(21) and C(23) in the *ansa* chain; and (c) a stable conformation of the molecule with a well-defined geometrical disposition of the hydroxyls on C(21) and C(23) (Structure 1).

Studies of the antimicrobial activity *in vitro* of several 3-substituted derivatives of both rifamycin S and of the closely related tolypomicinone have also been reported (4, 5, 7, 8). It was concluded that activity might be influenced by the steric features of the 3-substituent, the effect being related to a change in the conformation of the *ansa* chain.

Electronic effects of 3-substitution were investigated, and the inhibiting activities of a series of 3-substituted rifamycins on isolated DDRP from *Escherichia coli* were measured (9). It was found that the inhibiting power was increased by electron-withdrawing substituents and decreased by electron-donating substituents. This concurs with the hypothesis that the chromophore rings also take part in the formation of the complex through a π-π interaction.

The introduction of a bulky electron-withdrawing group, in position 3 of the chromophore rings, should thus make the aromatic nucleus a better electron acceptor and, in conjunction with the steric effect, increase the inhibiting power of the derivative.

To confirm this hypothesis two series of esters, amides and hydrazides of 3-carboxy rifamycin S and SV, were prepared. Their antimicrobial activities *in vitro* were tested, but all of the results were somewhat lower than expected (10). In order to verify that the low activity was related to a molecular conformation unfavorable for interaction with the bacterial enzyme, the X-ray crystal structure of CMRS was determined and compared with the known structures of the active rifamycins, RIFB (3) and RIFMP (11). At the same time the molecular con-

This work is dedicated, on the occasion of her 70th birthday, to Professor Dorothy M. C. Hodgkin, O.M., F.R.S., who has for many years inspired our crystallography.

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[5] It must be pointed out that, both at the 3rd Meeting of the Italian and Yugoslavian Crystallographic Associations (1979), held in Parma, Italy, and at the 5th European Crystallographic Meeting (1979), held in Copenhagen, Denmark, a paper was presented by us on the supposed structure of 3-carbomethoxy rifamycin SV, such as would have been expected on a chemical preparative basis. A further refinement of the data revealed that the structure of 3-carbomethoxy rifamycin S had been determined instead, an oxidation of the naphthohydroquinonic nucleus having occurred during crystallization.

[6] The abbreviations used are: DDRP, DNA-dependent RNA polymerase; CMRS, 3-carbomethoxy rifamycin S; RIFB, rifamycin B *p*-iodoanilide; RIFMP, rifampicin.

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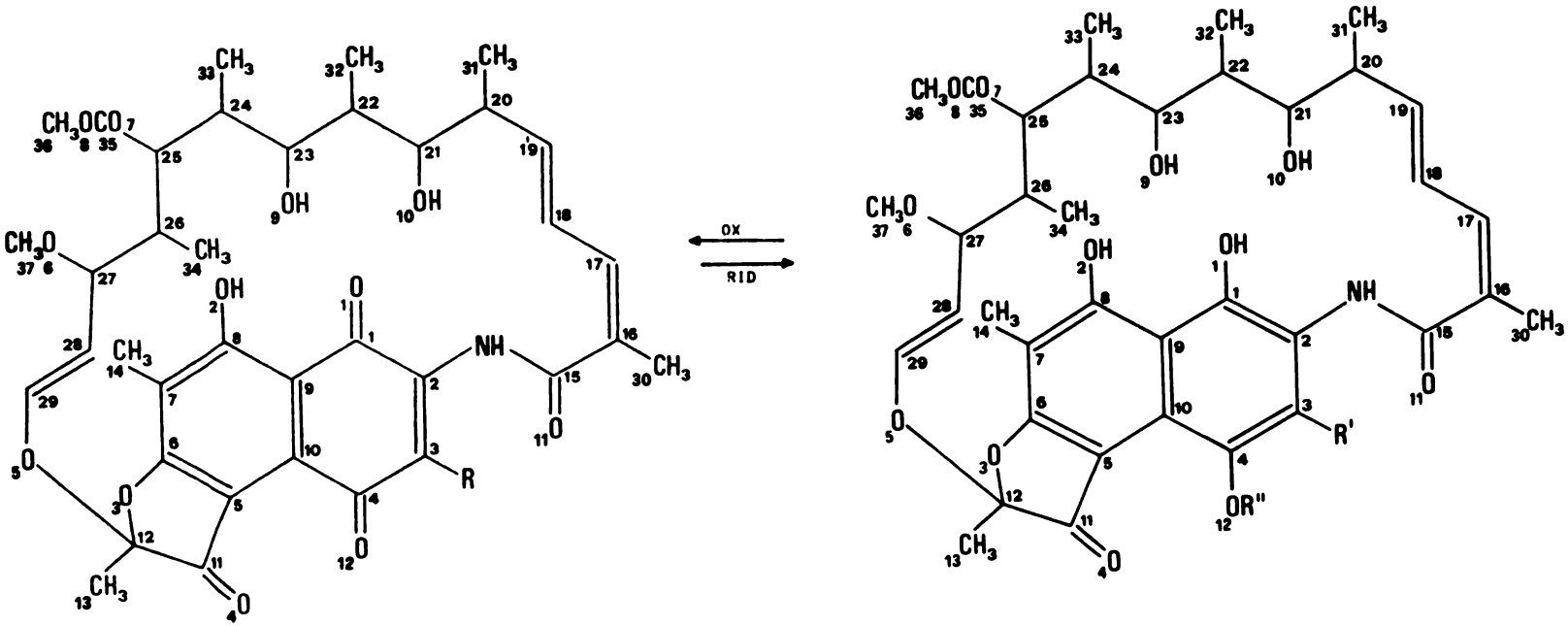

RS : R = H

CMRS : R = $COOCH_3$

RIFB : R' = H , R'' = CH_2COOH

RIFMP : R' = CH = N-N⟨ ⟩N-CH_3 , R'' = H

STRUCTURE 1

formation of CMRS was also studied in solution by ^{1}H-NMR. The inhibiting activity of CMRS on isolated DDRP from *E. coli* was also determined in order to discriminate between factors affecting inhibiting activity on isolated enzyme and those inhibiting antimicrobial activity *in vitro*. The results of this multiapproach investigation on the structure-activity relationships in CMRS are reported here.

MATERIALS AND METHODS

Molecular structure. The crystalline and molecular structure of CMRS, summarized here and shown in Fig. 1, is reported in detail in a separate paper (12). The molecule displays a conformation mostly resembling the other active rifamycins of known crystalline structure, RIFB and RIFMP. The O(9) and O(10) lie on the same side with O(1) and O(2) with respect to the *ansa* chain best plane. The C(21)—O(10) and C(23)—O(9) bonds are nearly parallel to the aromatic nucleus.

A comparison of the interatomic distances between O(1), O(2), O(9), and O(10) is reported in Table 1, and a comparison of the torsion angles along the *ansa* chain is reported in Table 2. It is noteworthy that, in CMRS, the torsion angles around the C(2)—N and C(15)—C(16) bonds are −141° and 63°, respectively, whereas the corresponding values are −32° and −43° for RIFB and −55° and −31° for RIFMP. The two rotations result in the reversal of the amidic group in CMRS with respect to its position in the other two rifamycins, but leave the orientation of the chain unchanged. The carbomethoxy group is strictly planar and makes an angle of 62° with the plane of the chromophore rings.

The ^{1}H-NMR spectrum of CMRS at 200 MHz (Fig. 2) is also discussed in more detail within two separate papers (12, 13). The spectrum is fully interpreted. It was

TABLE 1

Distances between O(1), O(2), O(9), and O(10) in RIFB, RIFMP, and CMRS as determined by X-rays

	CMRS	RIFB	RIFMP
	Å	Å	Å
O(1)-O(2)	2.54	2.6	2.48
O(1)-O(9)	7.00	6.7	6.16
O(1)-O(10)	5.76	5.7	5.41
O(2)-O(9)	7.42	7.8	6.82
O(2)-O(10)	7.03	7.5	6.93
O(9)-O(10)	2.73	2.7	2.72

TABLE 2

Torsion angles along the skeleton of the ansa chain in RIFB, RIFMP, and CMRS as determined by X-rays

	CMRS	RIFB	RIFMP
C(1)—C(2)—N—C(15)	−141°	−32°	−55°
C(2)—N—C(15)—C(16)	177	180	179
N—C(15)—C(16)—C(17)	63	−43	−31
C(15)—C(16)—C(17)—C(18)	2	5	4
C(16)—C(17)—C(18)—C(19)	169	168	155
C(17)—C(18)—C(19)—C(20)	−179	−175	−165
C(18)—C(19)—C(20)—C(21)	−30	−11	−19
C(19)—C(20)—C(21)—C(22)	180	170	169
C(20)—C(21)—C(22)—C(23)	−178	−179	−176
C(21)—C(22)—C(23)—C(24)	57	53	62
C(22)—C(23)—C(24)—C(25)	−174	174	165
C(23)—C(24)—C(25)—C(26)	169	155	159
C(24)—C(25)—C(26)—C(27)	180	174	153
C(25)—C(26)—C(27)—C(28)	180	−170	−171
C(26)—C(27)—C(28)—C(29)	−110	117	118
C(27)—C(28)—C(29)—O(5)	−176	−168	−175
C(28)—C(29)—O(5)—C(12)	−127	49	65
C(29)—O(5)—C(12)—O(3)	−52	−79	−78

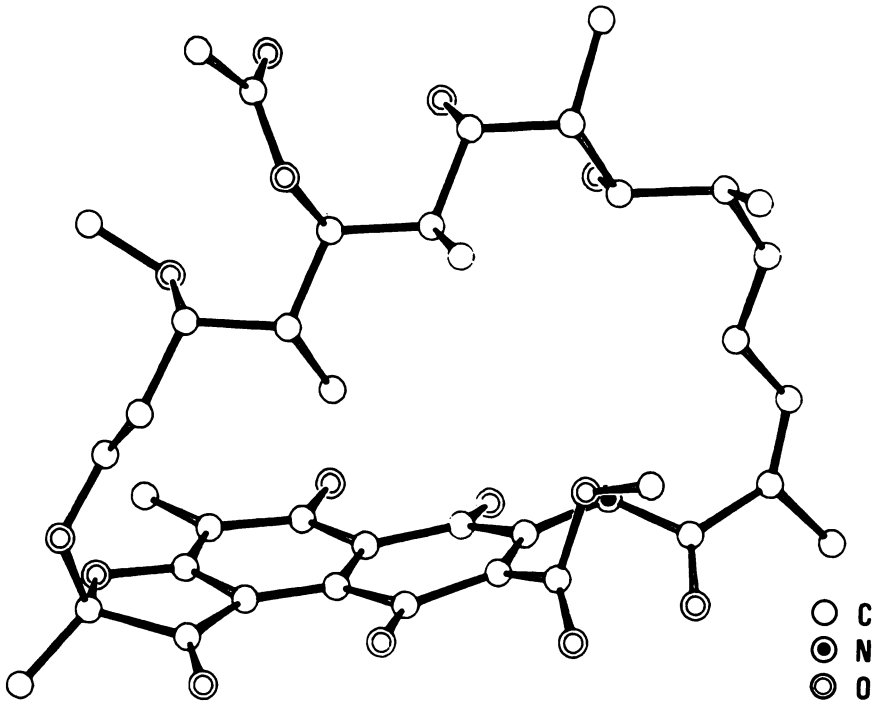

FIG. 1. *Molecular structure of CMRS (non-hydrogen atoms only)*

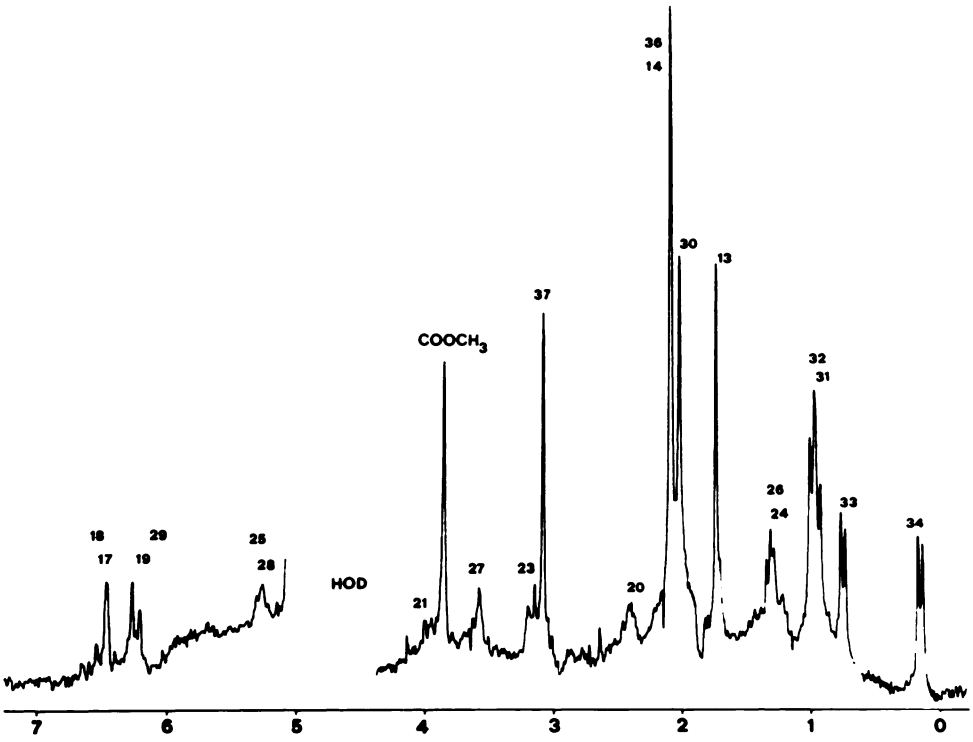

FIG. 2. *^{1}H-NMR spectrum of CMRS in ^2H_2O-phosphate buffer, pH 7.5*

possible to assign the chemical shifts unambiguously for most of the protons. In the case of too-close signals or overlapped multiplets, protons were assigned in groups. The values of the chemical shifts and of the observed coupling constants are reported in Table 3 together with the approximate dihedral angles of the C(21)—C(25) part of the *ansa* chain, estimated by an optimized Karplus relationship (14).

The choice of dihedral angles followed that of Gallo *et al.* (15). The dihedral angles in solution reported in Table 3 are comparable with those observed in the solid state (Table 2).

Activity Measurements. The activity tests of CMRS and rifamycin S were performed on isolated DDRP from *E. coli* B purified as described by Berg *et al.* (16) up to Step V. The product was concentrated by $(NH_4)_2SO_4$ precipitation, and stored[7] at −20°.

In order to facilitate the dissolution of the antibiotics, small quantities of dimethyl sulfoxide were added to a concentration of 0.4%. The antibiotic at different concentrations and 10 nM holoenzyme were preincubated[8] for 10 min at 4°. Then 10 μg of native calf thymus DNA were added and the mixture was kept for 10 min at 37°. The reaction was stopped by the addition of 10% cold trichloroacetic acid, and the precipitate was filtered on GF-A Whatman filters, washed with cold 5% trichloroacetic acid and counted.

Each test was performed in parallel for CMRS and rifamycin S and repeated twice for each concentration value. The complete series of tests was then repeated. The values obtained for each point are within 5% for inhibition higher than 20%. They have been averaged and are represented in Fig. 3, which shows a significant difference in the inhibiting power of the two compounds.

DISCUSSION AND CONCLUSIONS

The comparison of the structure of CMRS with those observed in the solid state for RIFB and RIFMP shows that CMRS has large conformational differences occurring near the junctions of the *ansa* chain with the naphthoquinonic ring, but that the conformation of the central part of the *ansa* chain is similar. Consequently the spatial arrangement and separation distances of the four oxygen atoms O(1), O(2), O(9), and O(10) are similar in RIFB, RIFMP, and CMRS.

The structural requirements for the activity mentioned in the introduction therefore seem to be fulfilled for CMRS, both in the solid state and in solution, despite the results of the antimicrobial tests *in vitro*. Indeed, cell-free tests have shown that CMRS is in fact active, and that the carbomethoxy substituent makes it even more active than the naturally occurring unsubstituted rifamycin S (Fig. 3).

A similar situation had been observed in RIFB, which carries a glycolic acid residue (OCH_2COOH) as C(4) substituent, and which has very low activity on whole bacteria (17), while being active on isolated DDRP from the same microrganism (18). The interpretation of this behavior was that the acidic character of the free carboxylic group made the antibiotic almost inactive on whole bacteria by preventing its penetration of the bacterial cell wall, while being of no relevance in cell-free tests.[9]

An analogous conclusion can be drawn for CMRS,

[7] DDRP storage buffer: 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, and 50% glycerol.

[8] Assay buffer (for each test 180 μl): 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 0.15 mM ATP, 0.15 mM GTP, and 0.3 μM I^3H I-UTP (10.1 Ci/mmole).

[9] This conclusion holds in view of the lack of any evidence for the existence of bacterial rifamycin-deactivating enzymes (19).

TABLE 3

Chemical shifts (parts per million from tetramethylsilane) and coupling constants for the ansa chain protons of CMRS in ^2H_2O-phosphate buffer, pH 7.5; approximate dihedral angles H—H (Φ) and corresponding C—C (τ) along C_{20}—C_{28}

	NH	
	C=O	
	C—CH_3	2.01
6.45	H—C=	
6.45	H—C	
6.2	H—C=	
2.4	H—C—CH_3	0.98
3.98	H—C—OH	
	H—C—CH_3	0.92
3.17	H—C—OH	
1.3	H—C—CH_3	0.75
5.2–5.3	H—C—$OCOCH_3$	2.09
1.3	H—C—CH_3	0.15
3.57	H—C—OCH_3	3.08
5.2–5.3	H—C	
6.23	H—C=	
	$C_{13}H_3$	1.72
	$C_{14}H_3$	2.08
	$COOCH_3$	3.86

	Hz	Φ	τ
JH_{20}–H_{21}	11	−175°	175°
JH_{21}–H_{22}	2.5–3	50	170
JH_{22}–H_{23}	2	55	55
JH_{23}–H_{24}	10–11	−175	−175
JH_{26}–H_{27}	1–2	70	−170
JH_{27}–H_{28}	5–6	−45	−105

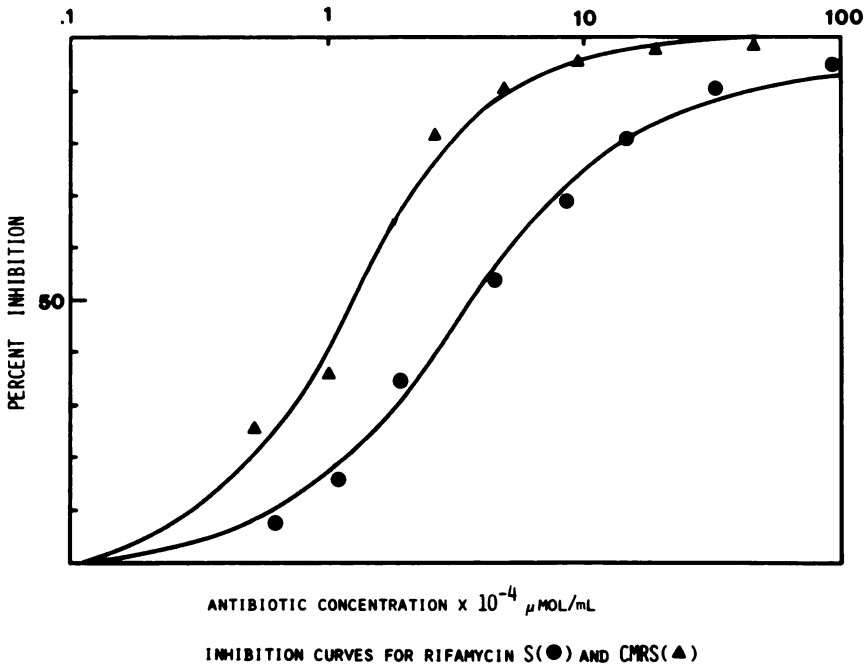

FIG. 3. *Inhibiting activity of CMRS (▲) and RS (●) on DDRP from Escherichia coli*

which, with respect to the unsubstituted rifamycin S, displays both a higher acidity of the phenolic hydroxyl on C(8) [pK_a 4.69 versus 5.03 (10)] and a reduced lipophilicity (ΔR_M −0.143) as measured by us according to the literature (20).

By analyzing the possible effects of the carbomethoxy group on the chromophore rings, one may exclude, on the basis of the X-ray and NMR studies, any significant conjugation as the planes of the two systems make an angle of 62°; but at least a σ-electronic effect is present, justifying an increase in the acidity of the phenolic hydroxyl, which is to a certain extent critical for the cell wall penetration.

On the basis of our data, the only possible comments on the interpretation of the higher inhibiting activity on isolated DDRP from *E. coli* found in CMRS, as compared with rifamycin S, are as follows:

1. The substituent involves the *ansa* chain in a steric interaction, as hypothized. In fact, the carbomethoxy group and the amidic carbonyl hinder each other; nonetheless, this strain effect is balanced by a rotation around the bond C(15)—C(16), thus leaving the essential part of the *ansa* chain unaffected.

2. The substituent exerts electronic effects (mainly σ-effects) on the chromophore rings, but no conclusion can be drawn on the way in which the effect is transferred into an increase of inhibiting activity on the isolated enzyme.

In conclusion, the introduction of a carbomethoxy group at C(3) of rifamycin S has no significant effect on the conformation of the active portion of the *ansa* chain. Instead it exerts electronic effects on the chromophore rings that lead both to an increase in the inhibiting activity on isolated bacterial DDRP and to a loss in the ability to penetrate the bacterial cell wall.

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