A Comparative NMR Study between the Macrolide Antibiotic Roxithromycin and Erythromycin A with Different Biological Properties

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^{1}H nuclear Overhauser enhancement studies and ^{1}H NMR ^3J analysis establish the similarity between the major solution-state conformation of roxithromycin (1) and the erythromycin (2). A major difference between the structure of antibiotics 1 and 2 is the replacement of the 9-keto group in 2 by a 9-[*O*-(2,5-dioxahexyl)oxime] group. The NOE studies show that this oxime chain is oriented above the macrocyclic lactone ring and that the oxygen atoms of this chain are engaged in tight hydrogen bonding with a water molecule and with the 6- and 11-hydroxyl groups of the macrocycle. It results in a globular form of the whole roxithromycin molecule. These data explain also a relative hydrophobicity of this antibiotic. Erythromycin A (2), which presents a less rigid macrocycle with two free hydroxyl groups (6-OH and 11-OH), forms a dimer detected by FAB mass spectroscopy. ^{1}H and ^{13}C NMR relaxation measurements (T_1) for both antibiotics show that interresidue hydrogen bonds in roxithromycin reduce the rotational freedom of the macrocyclic lactone ring and consequently the motions of desosamine and cladinose sugars. In another way, an ionization of the amino function occurs in the various media according to the nature of the antibiotic. This would allow the reactivity modification of the desosamine unit. In the biological study, the modifications of the 455-nm metabolite-cytochrome P-450 complex formation are observed.

Introduction

Roxithromycin is a new, medically important macrolide antibiotic composed of an erythronolide ring (polyfunctionalized 14-membered lactone ring) substituted with desosamine and cladinose sugar units. The specificity of this molecule lies in its 9-[*O*-(2,5-dioxahexyl)oxime] chain.[1]

Because of its medical importance, its conformation in solution has been studied by several NMR spectroscopy techniques. The first NMR report presented was a complete and correct assignment and a good determination of vicinal proton–proton coupling constants used to elucidate the structure of roxithromycin and to analyze its conformation in solution.

A preliminary communication on roxithromycin has recently been published[2] and the solution conformation of erythromycin A and its motional properties have also been studied by NMR spectroscopy.[3] An extension of this work is to compare these two derivatives that show different biological effects.

Unlike erythromycin A (and troleandomycin), roxithromycin does not form an inhibitory P-450–metabolite complex in vivo, even at high doses (1 g/kg) and it does not induce hepatic cytochrome P-450. These properties are in good agreement with the absence of metabolic drug interaction observed after roxithromycin treatments.[4-7] In vitro, roxithromycin has little affinity for the cytochrome P-450 binding site, and it is unable to form significant quantities of P-450–metabolite complex.[7] In another way, recent reviews considered new macrolides as a possible alternative therapy in human toxoplasmic encephalitis.[8] Only small amounts of erythromycin A are found in human brain tissues after oral administration,[9] but a high brain tissue antibiotic concentration is found when roxithromycin is used.[10] Consequently the efforts to determine the factors playing a role in the penetration rate of this new macrolide into human brain tissue are fully warranted.

This paper reports on a comparative study of roxithromycin (1) and erythromycin A (2) (Figure 1): (i) comparison of their conformation in solution, including bending motions of some dihedral angles with respect to the crystalline-state structure; (ii) determination in solution of the motional properties of the sugar rings and of the methyl groups in the macrocycle, for 1 and 2, from ^{1}H and ^{13}C T_1 (relaxation) values; (iii) assignment of hydrogen bonds from NMR data and effect of concentration and temperature; (iv) ionization of the amino function and the pH dependence as observed by NMR and in biological studies; (v) possible correlations between the biological activities of 1 and 2 and their solution conformation.

Results and Discussion

I. Conformation in Solution. The X-ray crystal structures of various macrolide antibiotics have been studies[3,11] with the aim of obtaining structure–activity relationships.

Everett and Tyler proposed two diamond lattice conformation models for the aglycons of the 14-membered macrolides.[3] The crystal structure of erythromycin A (2) is characterized predominantly by a "folded-out" conformation whereas a "folded-in" model is found with the crystal structure of (9*S*)-9-*N*,11-*O*-[2-(2-methoxyeth-

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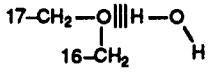
0022-2623/91/1834-1117$02.50/0

Table I. Torsion Angles for the Crystal Structures of Roxithromycin (1)[12] and Erythromycin (2)[3]

torsion angle ϕ, deg	roxithromycin (1)[a]	erythromycin (2)[b]	torsion angle ϕ, deg	roxithromycin (1)[a]	erythromycin (2)[b]
O14-C1-C2-C3	121.2	115.9	C7-C8-C9-C10	-63.3	-60.8
C1-C2-C3-C4	-69.6	-61.2	C8-C9-C10-C11	123.7	122.0
C2-C3-C4-C5	163.4	164.8	C9-C10-C11-C12	*-166.2*	*-173.3*
C3-C4-C5-C6	*-106.9*	*-116.1*	C10-C11-C12-C13	164.2	167.8
C4-C5-C6-C7	-72.0	-68.5	C11-C12-C13-O14	160.1	165.1
C5-C6-C7-C8	174.7	175.0	C12-C13-O14-C1	114.4	107.3
C6-C7-C8-C9	-75.5	-77.0	C13-O14-C1-C2	172.9	171.3

[a] The underlined dihedral angles increase for roxithromycin (1) while the dihedral angles around C4-C5 and C10-C11 (ital) decrease to about the same value (in absolute terms).

Table II. Intra- and Intermolecular Interactions (···) and Hydrogen Bonding (///) for the Crystal Structure[12] of Roxithromycin (1)

intramolecular interactions	d, Å	intramolecular interactions	d, Å	intermolecular interactions	d, Å
chain-macrocycle		intrachain		chain-cladinose	
19-Me--O///H-O6	2.128	9-C=N···H-CH16	2.346	9-C=N-O···H4″	2.698
9-C=N///H-O11	2.805	9-C=N-O···H_A-CH16	2.006	9-C=N-O···H-$CH_2$3″	2.761
6-H-O···H_A-CH17	2.696	9-C=N-O···H_B-CH16	2.102	chain-macrocycle	
6-H-O···H_B-CH17	2.968	9-C=N-O···H-CH17	2.813	12-H-O···H-CH18	2.471
9-C=N···H8	2.592	16-CH_2-O···H_A-CH17	2.009	inter-sugars	
9-C=N···H11	2.94	16-CH_2-O···H_B-CH17	2.057	4″-H-O///H-O2′	2.202
17-CH-H···H-$CH_2$6	2.394	16-CH_2-O···H-CH18	2.471	3′-$(Me)_2$-N///H-O4″	2.037
19-O-CH_2-H···H3	2.373	19-CH_3-O···H_A-CH18	1.91	2′-H-O///H-O4″	2.733
chain-water molecule		19-CH_3-O···H_B-CH18	1.95		
	2.391	19-CH_3-O···H-CH17	2.389		
		intramacrocycle			
		11-H-O///H-O12	2.567		
		macrocycle-water molecule			
		H-O///H-O11	1.989		
		intra-sugars			
		3′-$(Me)_2$-N///H-O2′	2.609		
		3″-Me-O///H-O4″	2.734		

oxy)ethylidene]erythromycylamine A (**3**), a derivative of erythromycylamine A with an ether side chain. In the crystal structure of **3** the C3 to C5 portion of the macrocyclic ring folds "inward" such that 3-H gets closer to 11-H. However, the similarity in the $\phi_{C3\text{-}C4}$ and $\phi_{C4\text{-}C5}$ of the two crystal structures of **3** and **2** implies that the orientation of the sugar rings with respect to one another is almost the same for both substances. It is therefore of interest to investigate the differences in the conformation of the aglycon in **2** and **1**, a derivative with an oxime chain. Table I compares the lactone ring torsion angles for the crystal structure of roxithromycin[12] and erythromycin A.[3] Their three-dimensional structures are quite similar. The torsion angles (Table I) are similar for the C6-C10 and C11-C13 portions of the aglycons, but they are slightly different for the C1-C3, C4-C6, C10-C11, and C13-O14 regions of the molecule.

Rotation about the C2-C3 and C4-C5 bonds leads to an increase of ca. 9° in $\phi_{C2\text{-}C3}$ and to a decrease of ca. 9° in $\phi_{C4\text{-}C5}$ of the **1** crystal structure. This indicates that the orientation of the sugar rings with respect to one another and with respect to the lactone ring in **1** are slightly different from that found in **2**. These data suggest that a conformational instability exists in the C3-C5 region of the native substance. Furthermore, a reorganization of another portion of the ring (C5-C6, C10-C11, C13-O14) has caused $\phi_{C10\text{-}C11}$ to decrease by ca. 7° while $\phi_{C5\text{-}C6}$ and $\phi_{C13\text{-}O14}$ increased by ca. 7°. This indicates that the orientation of the hydroxyl groups (6-OH and 11-OH) and the lactone group are slightly different from that found in **2**.

For **1**, in the solid state, some intramolecular hydrogen bonds occur between the different units. Intermolecular hydrogen bonds exists between roxithromycin and water molecules and also between roxithromycin molecules themselves (Table II).

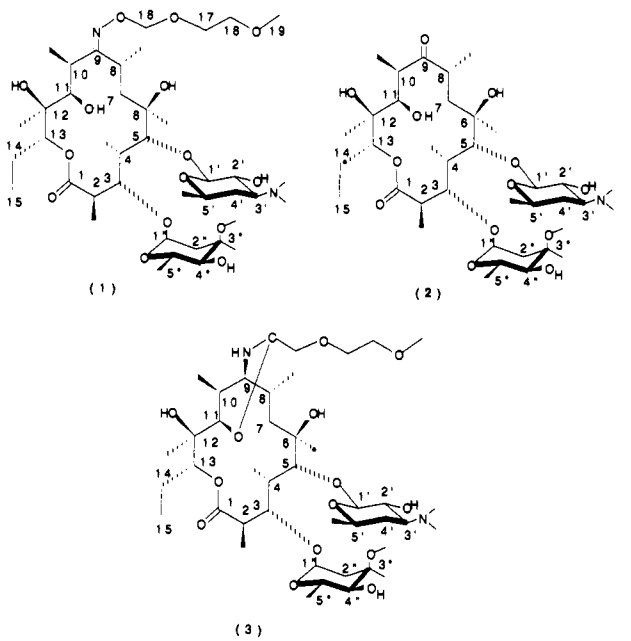


Figure 1. Representation of the structures of roxithromycin (**1**), erythromycin A (**2**), and (9*S*)-9-*N*,11-*O*-[2-(2-methoxyethoxy)-ethylidene]erythromycylamine A (**3**).

Vicinal proton-proton coupling constants (Table III) have been measured in various solvents ($CDCl_3$, CD_3OD, D_2O) and at different temperatures (273, 293, 323 K). For roxithromycin, angular distortions were deduced from correlations between observed and calculated coupling constants taking into consideration the orientation and the electronegativities of the substituents.[2] The observed $^3J_{HH}$ values for C2-C3, C7-C8, and particularly C4-C5 regions

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Table III. Dihedral Angles (ϕ) and Corresponding Coupling Constants for Vicinal Proton Pairs for Roxithromycin (1)[2] and Erythromycin (2)[3] in $CDCl_3$ and CD_3OD

vicinal proton pair	ϕ, deg		^3J, Hz: $CDCl_3$ 292 K		$CDCl_3$ 323 K		CD_3OD: 292 K	
	1	2	1	2	1	2	1	2
2-H, 3-H	170.5	174	9.9	9.4	9.9	9.0	9.2	9.1
3-H, 4-H	−71.5	−70	1.5	1.5	1.4	1.7	1.3	1.2
4-H, 5-H	129.6	125	7.4	7.7	7.3	7.4	7.8	7.8
7_{ax}-H, 8-H	164.0	164	10.5	11.7	10.2	11.0	9.6	8.4
10-H, 11-H	70.8	70	2.10	1.3	2.0	1.5	2.0	1.3
13-H, 14_{ax}-H	171.8	175	11.2	11.0	10.9	10.6	11.0	10.9
$1'_{ax}$-H, $2'_{ax}$-H	175.0		7.5	7.2	7.2		7.3	
$2'_{ax}$-H, $3'_{ax}$-H	176.3	172	10.5	10.3	10.2		10.4	
$3'_{ax}$-H, $4'_{ax}$-H	177.9		11.2	12.3	11.0		12.2	
$4'_{ax}$-H, $5'_{ax}$-H	176.7		10.8	10.8	10.6		10.7	
$1''_{eq}$-H, $2''_{eq}$-H	72.6		1.7	0.8	1.5		1.7	
$4''_{ax}$-H, $5''_{ax}$-H	178.6	177	9.5	9.7	9.2		9.5	

are slightly different for solution from those calculated for a crystal structure. The small differences between theoretical and empirical values are attributed to a slight variation in the dihedral angles. The difference in $J_{4,5}$, with respect to the previously calculated values for conformer **a** (*the conformer in the solid state*)[2] is more important. It can be accounted for either by a deformation in the torsion angle C4–C5 (by ca. 5°) for the conformation **a**, in solution, or by a participation of two different coupling constants for the protons (H4, H5) gauche or trans, respectively. This would result from the presence in solution of another minor conformation(s) that affect conformer **a** in this region as it is the case of the "folded-in" conformation model.

It is concluded that this compound exists in solution as one major conformer very similar to that found in the solid state; it is also similar to that of erythromycin A. The results of Table III show that the other minor conformation(s) participates a little more in solution, for **2**.

Nuclear Overhauser Enhancement (NOE) Experiments. 2D phase-sensitive ^1H NOESY experiments in $CDCl_3$ and CD_3OD solution were performed by using a time-proportional phase-increment method[13] and different mixing time (τ_m = 200 and 300 ms). Extensive 250-MHz ^1H NOE difference experiments in $CDCl_3$ and in CD_3OD solution were used to confirm 2D results.

Inspection of the matrix of ^1H NOEs for roxithromycin (Table IV) shows that the pattern of NOEs involving the lactone protons is similar to that found in erythromycin A[3] (if we neglect interactions involving the oxime chain). Differences are observed for all the NOEs from the hydroxyl groups and some other NOEs, particularly those from the methyl groups like [2-Me]2″-eq, [12-Me]4, [6-Me]5″-Me, and protons [4]7-ax. These HH interactions were found in the crystal structure of **1** and **2** but are not present in solution for **2**. The very small NOE [11]3 could not be predicted on the basis of the crystal structure and is only observed in CD_3OD solution. Everett and Tyler have already concluded, for erythromycin A, that these unexpected NOEs (NOE [3]11 and NOE [11]3) could be due to a minor conformer in equilibrium with a major one.[3]

For **1**, the phase-sensitive 2D ^1H NOESY in $CDCl_3$ solution at 300 ms (Table IV) demonstrates the spatial proximity of 11-OH to 16-H, 6-OH and 6-Me to 17-H and 18-H, and 3-H and 6-OH to 19-Me. The oxime chain is directed between 8-C and 11-OH, toward 6-Me and 6-OH;

(13) Marion, D.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 976.

Table IV. Qualitative Nuclear Overhauser Enhancement Data for Roxithromycin (1): Comparison of Distances from Erythromycin (2) NOEs[3] and from Roxithromycin (1) X-ray[12] Data[a]

protons observed	protons connected in $CDCl_3$ and CD_3OD solutions[b]
	Contacts Interunits
oxime chain–macrocycle	
19-OMe	3 (2.37), 6-OH (2.97), 5″Me*, 1″
18	3*, 6-OH*, 6-Me*
17	6-OH (2.69), 6-Me (2.79), 11-OH*
16	11-OH*, 11
sugar rings–macrocycle	
1′	5 (2.36), 4-Me (2.68)
1″	3 (2.46), 2-Me (2.16)
2″e	2-Me (2.88)
3″-OMe	4-Me (2.89)
5″	5 (2.32)
5″-Me	6-Me (2.889), 5*, 19-OMe*
desosamine–cladinose	
1′	5″ (2.14), 3″-OMe* (2.85)
3′	3″-OMe (2.76)
5′	5″ (2.16), 5″-Me
3′-N $(Me)_2$	3″-OMe*
	Contacts Intraunits
oxime chain	
19	18 (2.50), 17*
18	17 (2.31), 16*
17	16 (2.37), 19*
16	17 (2.37)
macrocycle	
3	2 (2.9), 4 (2.39), 5 (2.38), 6-OH (2.79), 11*
4	2 (2.63), 3, 6-OH (2.89), 7a (2.20), 11 (2.71), 4-Me, 12-Me* (2.93)
6-OH	3, 4, 5 (2.82), 6-Me (2.49), 11*
11	4, 7, 10 (2.36), 13 (2.44), 12-Me (2.78), 11-OH (2.07), 6-OH*
11-OH	11, 13 (2.48), 12-Me*
12-OH	10-Me, 12-Me* (2.45), 13*, 14e (1.837)
13	11, 12-OH, 11-OH, 15-Me, 14e*
desosamine	
2′-OH	2′ (2.33), 3′ (2.79), 3′-N$(Me)_2$ (2.98)
3′-N$(Me)_2$	2′ (2.27), 2′-OH, 3′, 4′a (2.26), 4′e (2.32)
cladinose	
2″e	2″a, 3″-OMe (2.20), 3″-Me, 1″*, 4″*
4″	3″-Me, 2″a, 2″e, 4″-OH (2.146), 5″-Me
4″-OH	3″-OMe, 3″-Me (2.82)

[a] The NOEs observed only for roxithromycin (1) are underlined. Some evident contacts intraunits found for the two molecules are not specified. [b] Distances in Å between two protons (less than 3 Å) obtained from crystal structure for roxithromycin (1) are in parentheses. An asterisk is used to denote NOEs observed with a 2D NOESY experiment (τ_m = 300 ms) corresponding approximately to interactions between protons of more than 3 Å.

finally, 19-OMe and 3-H are spatially proximate (even in a polar solvent like CD_3OD). The far NOE (only at τ_m = 300 ms) observed at 5″-Me from 19-OMe shows the presence of the oxime chain close to the cladinose.

The intra-sugar NOEs in $CDCl_3$ and CD_3OD (Table IV) suggest that the chair conformations of the cladinose and of the desosamine are identical with those found in the crystal structure and both are similar with those found in **2**. The NOE [3″-OMe]4″-OH (in $CDCl_3$ solution), involves 4″-OH as the donor group in an intramolecular hydrogen bond [3″-CH_3O///H-O4″], the existence of which is supported by X-ray crystal data (Table II). Similarly, the NOE [3′-N$(Me)_2$]2′-OH is also indicative of a hydrogen bond [3′-$(Me)_2$-N///H-O2′] that is confirmed by X-ray (Table II) data. Three inter-sugars' NOEs are observed for **1**, but not for **2**; the NOE from 3″-OMe to 3′-H is in agreement with a previous X-ray analysis ($r_{3''\text{-OMe},3'}$ = 0.23 nm), and the NOE [5″]5′ was as expected from the crystal structure ($r_{5'',5'}$ = 0.22 nm). These inter-sugars' NOEs

Table V. Experimental ^1H and ^{13}C NMR Relaxation Times in $CDCl_3$ and CD_3OD Solution for the Methyl Groups of Roxithromycin (1) and Erythromycin (2)[3a]

	roxithromycin (1)				erythromycin A (2)	
	$CDCl_3$		CD_3OD		$CDCl_3$	
methyl	^1H T_1, s	^{13}C NT_1, s	^1H T_1, s	^{13}C NT_1, s	^1H T_1, s	^{13}C NT_1, s
			Macrocycle			
2	0.28	1.02	0.28	0.60	0.20	0.78
4	0.27	1.95	0.29	1.59	0.33	1.80
6	0.26	1.20	0.28	1.08	0.30	1.90
8	0.24	1.20	0.28	1.44[b]	0.29	1.56
10	0.29	1.53	0.27	1.83	0.38	2.10
12	0.24	1.02	0.27	1.20	0.28	1.48
15	0.51	2.28	0.49	2.58	0.52	3.03
			Desosamine			
3'-$N(Me)_2$	0.36	1.50	0.31	1.32	0.35	1.80
5'	0.40	2.16		1.77	0.42	2.19
			Cladinose			
3''	0.39	1.98	0.35	1.62	0.38	1.99
3''-OMe	0.59	3.03	0.50	2.76	0.59	3.40
5''	0.32	1.68	0.34	1.44[b]	0.38	2.00
			Oxime Chain			
19-OMe	1.28	7.08	1.70	3.18		

[a] Experimental results in $CDCl_3$ and CD_3OD solutions at ambient temperature; T_1 = spin-lattice relaxation time; N = 3, the number of protons on each carbon. The average standard deviation of the ^1H T_1 and ^{13}C NT_1 determinations are respectively 0.02 ± 0.01 s and 0.03 ± 0.02 s. [b] 8-Me and 5''-Me have the same ^{13}C chemical shifts in CD_3OD.

confirm that the two α-faces of the cladinose and the desosamine rings are opposite to each other in 1, and the NOE from 3''-OMe to 3'-$N(Me)_2$ (Table IV) is indicative of inter-sugar interactions.

Two other cladinose–lactone NOEs are observed; the NOE [2''-eq]2-Me found in 1 only and [1'']2-Me that corresponds to the same interaction in the two molecules. Finally the NOE [5''-Me]6-Me is indicative of the weak distance between these two methyl groups. It can be noted that the NOE observed for [3''-OMe]4-Me will be further supported by the ^{13}C NMR relaxation time values measured for each methyl group.

In conclusion, the solution conformation of roxithromycin is not exactly the same as that of erythromycin A. The main differences are the orientations of the sugar rings with respect to one another and with respect to the lactone ring. A comparison of the two crystal structures shows interactions between protons (less than 0.3 nm) in 1 for which no corresponding solution structure exists for 2. Three missing NOEs, namely [2-Me]2''-eq-H, [12-Me]4-H, and [4-H]7-ax-H, are due to a puckered erythronolide ring in 1 whereas the equivalent portion of 2 is more planar.

Carbon-13 Spin–Lattice Relaxation Times (T_1). T_1's are used to probe the mobility of the protonated carbons in roxithromycin. The ^{13}C NT_1 values (N = number of hydrogen atoms on carbon) and ^1H T_1 values of the methyl groups are given in Table V. The T_1 data (^{13}C and ^1H) of 1 are available as supplementary material (Table S1). These results show that (i) the desosamine sugar has the same mobility as the cladinose sugar (The desosamine sugar of 2 was shown to be more mobile than the cladinose sugar; however, in absolute terms, the mobility of both sugars is very restricted.[3]) and (ii) the lactone ring of 1 is more sterically hindered than the macrocycle of 2.

The small NT_1 values for 2-Me, 6-Me, 8-Me, and 12-Me (1.02–1.2 s) are indicative of sterically hindered rotation. However the relatively long NT_1 values of 3''-OMe and 19-OMe (3.03–7.08 s) are indicative of near-free rotation. The remaining methyl groups have intermediate ^{13}C NT_1 values ranging between 1.95 and 2.28 s.

The 2-Me of 2 has a remarkably short NT_1 value, 0.78 s (compared to 1.02 s in 1); this phenomenon was accounted for by a very high rotation energy barrier (>20 kcal·mol^{-1}) due to an extremely close approach of its protons to 1''-H.[3] The greater mobility observed for 1 is in good agreement with a longer distance measured for the crystal structure and with the NOE [2''-eq]2-Me observed only for this molecule.

The position of 6-Me is in exact agreement with that of the oxime chain in 1; it has a shorter NT_1 value (1.20 s in 1 and 1.90 s in 2), which is indicative of a large steric hindrance to rotation for this molecule. Similar hindrance phenomena are observed for 10-Me, 12-Me, 8-Me, and 15-Me as evidence from ^{13}C NT_1 values measured from a $CDCl_3$ solution (Table V).

As for the macrocycle, the ^{13}C NT_1 values for the N,N-dimethylamino group (3'-Me) of the desosamine and for 3''-OMe and 5''-Me of the cladinose are smaller in 1 than in 2 (Table V). This result is indicative of the contribution of sugar ring conformations that restrict the rotation of these methyl groups, either by inter-sugar interactions or by rigid orientation of the sugar rings.

It can also be noted that the T_1 values in the flexible side chain are not significantly longer than those in the rigid ring system. The first methylene 16-CH_2 (0.19 s) is as rigid as the 7-CH_2 (0.20 s) of the lactone ring. The trend for T_1 values to increase slightly as we move down the side chain (17-CH_2, 0.44 s; 18-CH_2, 0.48 s; 19-OMe, 2.36 s) can be noted while a free side chain would show larger variations in the T_1 values.[14] This is in agreement with the existence of a hydrogen bond between the oxygen atom of the oxime chain and the macrolactone.

The ^{13}C relaxation rates for roxithromycin are solvent dependent: $T_1(CD_3OD) < T_1(CDCl_3)$ (Table V). The variation in the value of T_1 for 19-OMe is spectacular: $NT_1(CDCl_3)$ = 7.08 s and $NT_1(CD_3OD)$ = 3.18 s. Relaxation rates are sensitive to solvent viscosity and they reflect the degree of molecular mobility. From Table V, only 8-Me, 10-Me, 12-Me, and 15-Me in 1 seem to recover the same degree of mobility as in 2.

The preceding results are in agreement with the variations in chemical shifts observed in CD_3OD ($\Delta\delta$, in Hz): 8-Me, 17.5; 10-Me, 10; 12-Me, 13; 15-Me, 13.3. However,

(14) Abraham, R. J.; Fisher, J.; Loftus, P. *Introduction To NMR Spectroscopy*; John Wiley & Sons, New York, 1988.

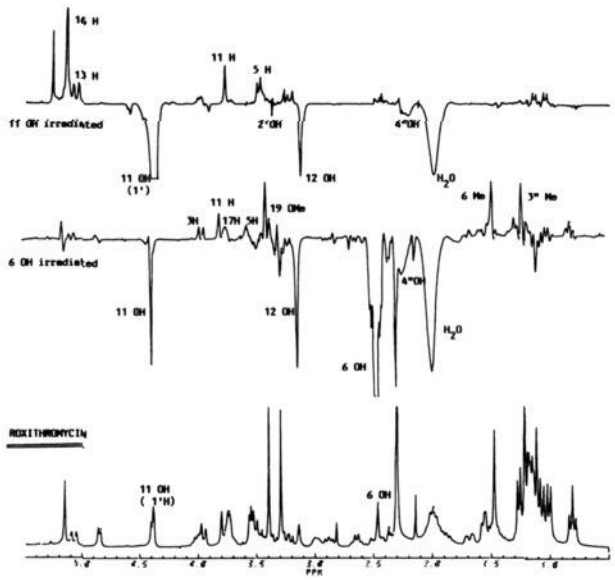

Figure 2. Two 250-MHz ^1H NOE difference spectra due to the irradiation of 6-OH and 11-OH in roxithromycin (**1**).

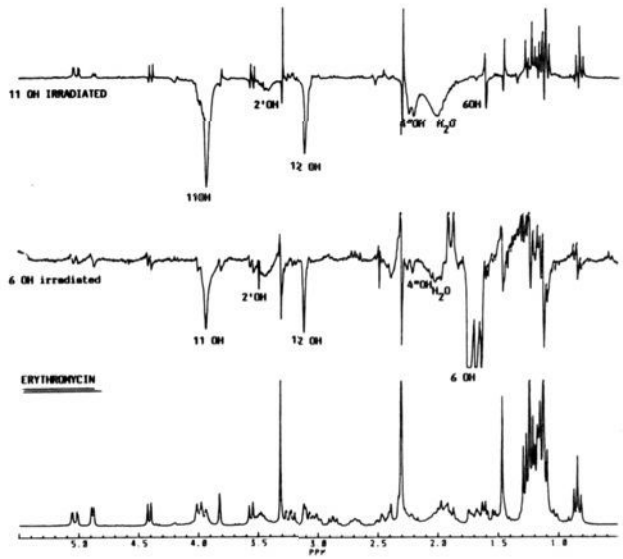

Figure 3. Two 250-MHz ^1H NOE difference spectra due to the irradiation of 6-OH and 11-OH in erythromycin A (**2**).

neither the desosamine nor the cladinose ring seems to be more mobile in this solvent. In the oxime chain, the carbon atoms 16–18 appear to be less restricted in CD_3OD than in $CDCl_3$ solution without recovering much mobility; this may be indicative of a greater mobility for this part of the oxime chain that had some interaction in $CDCl_3$.

The ^1H T_1 values reported in Tables V exhibit a similar trend to those of the ^{13}C NT_1 values. The ^1H T_1 data also indicate an enhanced mobility for some specific protons of the macrocycle in CD_3OD solution: 10-H, $\Delta T_1 = 2.5$ s ($\Delta\delta = 32.5$ Hz); 5-H, $\Delta T_1 = 0.25$ s ($\Delta\delta = 12.5$ Hz); 13-H, $\Delta T_1 = 0.16$ ($\Delta\delta = 35$ Hz).

^1H and ^{13}C NMR relaxation measurements (T_1 data) lead to the same conclusions as the NOEs data with respect to the conformation in solution. When compared to erythromycin A, the presence of the oxime moiety in roxithromycin reduces the mobility of the macrocyclic lactone ring, and consequently the motion of the desosamine and cladinose sugars, by adding more steric hindrance for 6-Me, 8-Me, 10-Me, 12-Me, and 15-Me while lessening the restriction to the rotation of 2-Me and 4-Me. The "folded-out" conformational model for the aglycon in erythromycin A is accentuated in roxithromycin. Consequently, the hydrophobic face of the latter presents more steric hindrance to all the methyl groups. Moreover, this provides for a better access to hydroxyl protons on the other face, to bind to the oxime chain by hydrogen bonding.

II. Hydrogen Bonding. The signal position of hydroxyl protons in **1** and **2** was confirmed from addition of D_2O in $CDCl_3$ and by a saturation transfer difference experiment performed in $CDCl_3$ under the same conditions as for the NOE difference experiments.

Hydroxyl protons are excellent starting points for NOE experiments because when the exchange processes that occur are sufficiently slowed down by intramolecular hydrogen bonding their residence time, at any one point, is longer, and as a result, their saturation will have local effects.

In roxithromycin, the NOE difference ^1H NMR spectrum upon irradiation of 6-OH (Figure 2) exhibits the expected NOEs at 3-H, 5-H, 6-Me; they are indicative of the spatial proximity of 6-OH and of these protons in solution as is the case in the crystal structure. Moreover, the NOEs [6-OH]19-H and [6-OH]17-H result from an

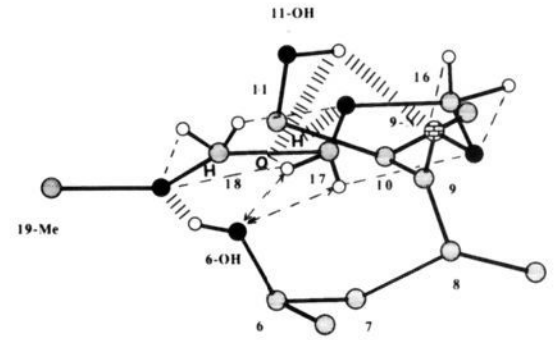

Figure 4. *Intra (···) and interresidue (<- - ->) interactions and* hydrogen bondings (///) for the 9-[*O*-(2,5-dioxahexyl)oxime] group in roxithromycin (**1**).

interresidue hydrogen bond 19-Me–O///HO-6 between the oxime chain and the macrocycle, again as for the crystal structure ($d = 0.21$ nm, Table II).

Similarly (Figure 2), 11-OH is found to be close in space to 11-H, 13-H, and 16-H. This last NOE [11-OH]16, and reciprocally [16]11-OH, are not predicted on the basis of the crystal-structure data (Table IV) although the X-ray diffraction results show an intramolecular hydrogen bond [9-N///HO-11 ($d = 0.28$ nm)] between the oxime chain and the macrocycle and also some intrachain interaction like 9-N///H-16 ($d = 0.235$ nm, Table II).

The use of difference spectroscopy for erythromycin A, principally allows detection of saturation transfer from 6-OH (or 11-OH) to all the exchanging hydroxyl protons (Figure 3). Hydrogen bonding in the macrocycle residue of **2** is weak, as evidenced by the small values of NOEs. Hydrogen bonds are not discernable, because the small amount of NOE, built up by 6-OH (or 11-OH) will rapidly disappear after exchange processes have occurred in the solution.

The oxygen (19-OMe, 17-OCH_2) and nitrogen (9-C=N) atoms are engaged in hydrogen bonding with the protons of two hydroxyl groups from the macrocycle (11-OH and 6-OH) and with water molecules. The presence of one (or several) water molecule and the presence in the chain of O and N atoms with a free electron doublet induce short interactions within this unit, e.g. the observation of NOE from 17-CH_2 to 16-CH_2 corresponds to interactions be-

Table VI. Chemical Shifts of Hydroxyl Groups in $CDCl_3$ Solution[a] at Different Concentrations[b] for Roxithromycin (1) and Erythromycin (2)

	C = 5.10^{-2} M		C = 10^{-2} M		C = 10^{-3} M	
	1	2	1	2	1	2
6-OH	2.43	1.51	2.36	1.50	2.34	1.50
11-OH	4.36	3.97	4.31	3.89	4.30	3.87
12-OH	3.13	3.15	3.13	3.09	3.12	3.08
2'-OH	3.41	3.45	c	c	c	c
4''-OH	2.23	2.23	2.20	2.21	2.19	2.20

[a] On molecular sieves. [b] At room temperature. [c] The proton signal was overlapped.

tween protons of less than 0.3 nm apart (Figure 4).

These observations may be rationalized by the presence of an intramolecular hydrogen bond network in both the macrocycle and the oxime chain residues. These weak hydrogen bonds (intrachain and intramacrocycle) are stabilized by the relatively strong interresidue (chain–macrocycle) hydrogen bond. The role of the nonpolar solvents ($CDCl_3$ solution) is important in the stabilization of the cooperative hydrogen bonding.[15] Nevertheless, even in CD_3OD, as evidenced from the constraint of the different NOEs observed in this solvent [[19-OMe]3-H, [18-CH_2]6-Me, [17-CH_2]6-Me], the relative rigidity of the oxime seems to be maintained. A good agreement was also found between the motions determined in $CDCl_3$ and in CD_3OD solutions (^1H T_1 values and ^{13}C T_1 values). Additional studies on the presence of hydrogen bonds have been performed (see below).

Concentration and Temperature Effects. If we examine the chemical shift values for protons of OH groups (Table VI), we notice that they span a range. Hydrogen bonding is the principal factor for this phenomenon. The high-frequency shift for the 6-OH proton in 1 (2.43 ppm) by comparison with that of 2 (1.6 ppm) may, however, be used as a criterion for the formation of a hydrogen bond. Similarly, the 11-OH hydroxyl groups resonate at 4.36 and at 3.97 ppm for 1 and 2, respectively. Therefore, 6-OH and 11-OH are more implicated in interesidue hydrogen bonding in roxithromycin than in erythromycin A.

The importance of hydrogen bonding can be studied by dilution effect as shown in Graph S1 of the supplementary material. When these molecules are dissolved at high dilution (10^{-3} M) in a non-hydrogen-bonding solvent such as $CDCl_3$, we observe a shift ($\Delta\delta = 10^{-2}$ ppm) of the OH signals to lower frequency, albeit small in absolute terms, for 1 [*6-OH (9)* > 11-OH (6) > 4''-OH (4) > 12-OH (1)] and 2 [11-OH (10) > 12-OH (7) > 4''-OH (3) > *6-OH (1)*].

Raising the temperature of the solution (25 to 55 °C) also reduces the hydrogen bonding to a predictable extent (Figure 5) for 1 ($\Delta\delta = 10^{-2}$ ppm to lower frequency) [*6-OH (16.5)* > 11-OH (13.2) > 2'-OH (12.9) > 12-OH (6.3) > 4''-OH (5.2)] and for 2 [11-OH (12.2) > 2'-OH (11.1) > 12-OH (8.1) > *6-OH (5.8)* > 4''-OH (4.5)].

Decreasing the temperature of the solution (25 to −10 °C) (Figure 5) strengthens the interresidue association by hydrogen bonding for 6-OH and 11-OH in 1. Consequently, a shift is observed for the higher frequency, in almost the same order for 1 ($\Delta\delta = 10^{-2}$ ppm to higher frequency) [*6-OH (22.4)* > 11-OH (21.3) > 2'-OH (9.3) > 4''-OH (7.6) > 12-OH (6.2)] but not for 2 [*6-OH (27.1)* > 2'-OH (13.1) > 11-OH (12.1) > 12-OH (7) 4''-OH (5.5)].

Possible conformational changes with temperature are the main source of ambiguities in interpreting the results (Figure 5) obtained by this method. Such changes are

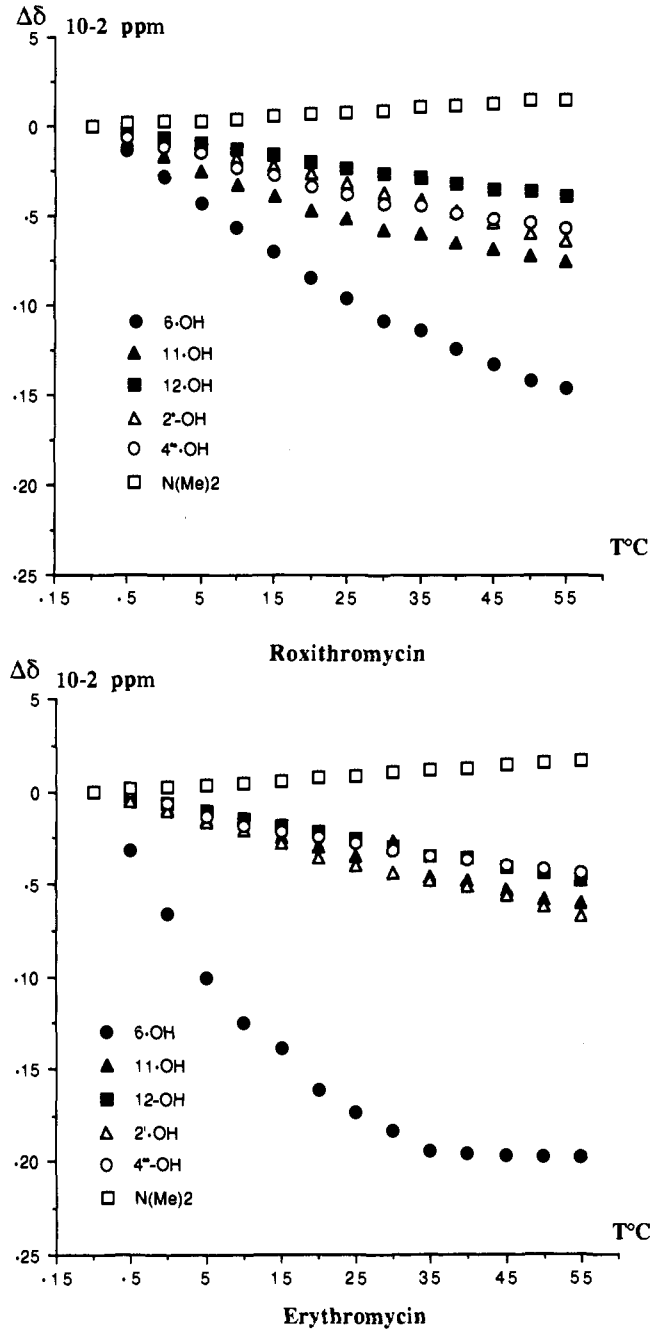

Figure 5. Temperature dependence for roxithromycin (1) and erythromycin (2).

characterized by a nonlinearity of the chemical shifts difference–temperature plot.[16]

The highest frequency chemical shift recorded particularly at low temperature for 6-OH, for erythromycin A (2), may be attributed to a weakly hydrogen-bonded proton in an intermolecular system. At low temperature (25 to −10 °C), (i) the dipolar attraction between 6-OH and a neighbor water molecule leads to intermolecular association, macrocycle–water molecule, or (ii) erythromycin A exists as a hydrogen-bonded dimer: the strength of these hydrogen bonds is only sufficient to maintain the dimeric association at low temperature.

A stable dimeric structure for erythromycin A and a less important dimeric structure for roxithromycin (10:1 ratio)

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(16) Patel, D. J. *Biochemistry* **1973**, *12*, 496. Kopple, K. D.; Ohnishi, M.; Go, A. *J. Am. Chem. Soc.* **1969**, *91*, 4264. Brewster, A. I.; Bovey, F. A. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, 68, 1199.

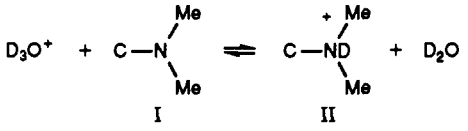
Table VII. Evolution of the Chemical Shifts of the *N,N*-Dimethylamino Signal with Solvent and Concentration According to the Ionization State of That Group for Roxithromycin (1) and Erythromycin (2)[a]

	D_2O		$CDCl_3$		$CDCl_3$ "100% D"		CD_3OD	
	1	2	1	2	1	2	1	2
$C = 10^{-2}$ M	2.83 (s), pH = 8.1	2.53 (bs), pH = 9.2	2.29 (s)	2.29 (s)	2.30 (s)	2.29 (s)	2.34 (s)	2.34 (s)
$C = 10^{-3}$ M	2.79 (s), pH = 8.4	2.40 (s), pH = 9.6	2.94 + 2.81 (2 d)	2.86 + 2.37 (2 bs)	2.30 (s)	2.29 (s)	2.35 (s)	2.34 (s)

[a] (s) singlet, (bs) broad signal, (d) doublet.

are observed by FAB mass spectrometry, corresponding to the detection of ions at *m/z 1468* and *1630*, respectively (shown in Figure S1 of the supplementary material). No mixed **2-1** (or **1-2**) aggregate was observed. Low energy collision experiments performed on the dimers lead to the corresponding monomers only. These results account for the fact that only the **2-2** dimer was observed in the NMR study.

III. Ionization of the Amino Function; pH Dependence. Working at constant temperature and varying the pH, the chemical shifts of the *N,N*-dimethyl protons will depend on the state of ionization of that group. The pH dependence of the *N,N*-dimethylamino group was studied with a 10^{-3} M D_2O solution, at ambient temperature (20 °C), for **1** and **2** and shown in Graph S2 of the supplementary material. When internal rotation occurs about the C3′–N bond, the two methyls are equivalent and give rise to an average single line.



I II

For I a single peak is observed as the methyl protons undergo a fast conformational exchange process:

1: δ = 2.28 ppm, solvent D_2O, 10^{-3} M, 293 K, pH 13

2: δ = 2.30 ppm, solvent D_2O, 10^{-3} M, 293 K, pH 12

By contrast, system II undergoes a slow conformational exchange process at room temperature, therefore in proton NMR is shows two distinct methyl signals of equal intensity separated by 0.11 ppm.

1: δ = 2.80 and 2.91 ppm, solvent D_2O, 10^{-3} M, 293 K, pH 2

2: δ = 2.81 and 2.92 ppm, solvent D_2O, 10^{-3} M, 293 K, pH 2

The fast conformational exchange process may be achieved for II above 343 K. A single proton peak is then observed, δ = 2.86 ppm (**1**), at midway between the frequencies for the two methyl protons at this temperature. This phenomenon could be caused by the presence of an intramolecular (or of some intermolecular) hydrogen bond with the 2′-OH equatorial and the 3′-$NH^+(Me)_2$ equatorial of the desosamine sugar. The strength of the hydrogen bond is associated with the nature of the two non-hydrogen atoms, their colinearity with and their symmetry about the central hydrogen atom, and an overall positive or negative charge.[17] Hydrogen bonds involving heteronuclear pairs such as nitrogen and oxygen tend to be asymmetric, the great majority involving NH···O interactions, e.g. as in peptides.[18] Fewer examples of strong OH···N bondings are known.[19]

The position of the methyl protons of the *N,N*-dimethylamino group in the two forms (protonated and deprotonated) being established, it was interesting to monitor different evolutions of the *N,N*-dimethylamino signal (with solvent and concentration variation), between **1** and **2**.

We report the result of a study in "commercial" $CDCl_3$, in $CDCl_3$ (100% D), in CD_3OD, and in D_2O solution (Table VII).

In $CDCl_3$ "100%" and in CD_3OD, when $C = 10^{-2}$ M or 10^{-3} M, the signal of the *N,N*-dimethylamino group was similar in **1** and **2**: δ = 2.30 ppm, like the singlet obtained at pH 12 or 13 (in D_2O) corresponding to the deprotonated form.

One observes the following in D_2O solution (10^{-2} or 10^{-3} M), for the amino group signal: (i) For **1** (pH 8.1, $C = 10^{-2}$ M), the singletlike signal, at 2.83 ppm, is similar to the resonance of the methyl in fast exchange in the protonated form, when pH = 3.5, $C = 10^{-2}$ M, and T = 343 K. (ii) For **1** (pH 8.4, $C = 10^{-3}$ M), the broad signal at 2.79 ppm corresponds to about 88% of the protonated form. (iii) For **2** (pH 9.2, $C = 10^{-2}$ M), the signal at 2.53 ppm is intermediate between the deprotonated form (60%, δ = 2.30 ppm) and the protonated form (40%, δ = 2.86 ppm). (iv) For **2** (pH = 9.6, $C = 10^{-3}$ M), the amino group signal is slightly shifted at δ = 2.40 ppm, corresponding to about 82% of the deprotonated form.

In "commercial" $CDCl_3$ (which is often acidic and wet), when the concentration of roxithromycin decreases, we observe an important shift of the initial *N,N*-dimethylamino signal: a singlet at δ = 2.29 ppm ($C = 10^{-2}$ M) toward its final position and two doublets of methyl nonequivalent in the protonated form at δ = 2.81 and 2.94 ppm ($C = 10^{-3}$ M).

Decreasing the concentration of erythromycin A in this solvent induces a progressive broadening and a shift of the initial *N,N*-dimethylamino signal at δ = 2.29 ppm ($C = 10^{-2}$ M) toward two broad signals at δ = 2.37 and 2.86 ppm ($C = 10^{-3}$ M), due to a dynamic process consistent with the exchange of population with protonated or deprotonated *N,N*-dimethylamino group. At low concentration, $C = 10^{-3}$ or 5.10^{-4} M, the spectrum shows a broadening of all the signals, presumably as a result of some process that averages the magnetic field of the protons. The hypothesis of the existence of a dimeric structure for compound **2** in solution brings in the possibility of dissociation to monomers, perhaps followed by recombination and exchange, two processes that must occur intermolecularly.

This study has shown that the reactivity of the nitrogen atom of the *N,N*-dimethylamino group causes shifts to be

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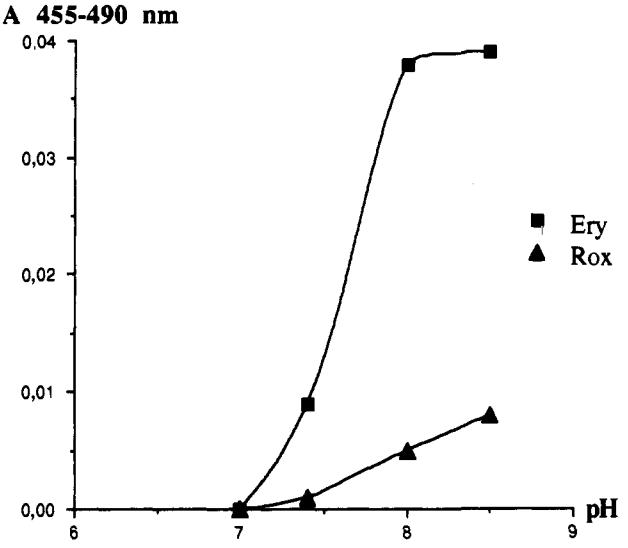

Figure 6. pH dependence of metabolite–cytochrome P450 complex formation for roxithromycin (1) and erythromycin (2): results are expressed as absorbance difference (455–490 nm) using 2 μM cytochrome P450 from dexametasone-treated rats, 10 μM substrate, and 0.5 mm NADPH (ref 7).

produced if the amine is protonated or by changing solvent or by changing the concentration in the same solvent. The magnitude of the shift depends on several factors, including pH, the nature of the counterion, and the degree of hydrogen bonding.

The presence of protonated erythromycin A or roxithromycin at physiological pH has consequences on their relative access to the hydrophobic active site of cytochrome P-450 and the formation of a 455-nm absorbing inhibitory cytochrome P-450–metabolite complex. For 1 and 2, a pH dependence of 455-nm metabolite–cytochrome P-450 complex formation is observed with a 4 times increase for erythromycin A between pH 7.4 and 8. Roxithromycin is only metabolized into 455-nm absorbing complex at pH 8 or higher (Figure 6). This phenomenon may occur in vivo since some tissues have alkaline pH and, thus, can lead to intense metabolization of 1 or 2 into cytochrome P-450–metabolite complex.

Conclusion

For roxithromycin, the hydroxyl protons 6-OH and 11-OH interact in a hydrogen-bond interunit, macrocycle–oxime chain, while erythromycin A is forming a weak association via 6-OH, with a water molecule or with another erythromycin molecule; the latter occurs particularly at low temperature. From these data, one can say that the macrocycle or roxithromycin has no free hydroxyl groups, so it results in greater hydrophobicity for this antibiotic. On the hand, 6-OH and 11-OH being implicated in hydrogen bonding with the oxime chain, it results in a globular form for the whole molecule. Erythromycin A presents a less rigid face with its hydroxyl groups 6-OH and 11-OH, which can interact with a polar solvent or associate with another unit.

In summary, the NMR data indicated that in $CDCl_3$ solution, the oxime chain is anchored to the erythronolide by the 6-OH hydroxyl group, with the participation of the 11-OH and a water molecule. The observed NOEs confirmed the close approach of the 19-OMe and 3-H. Thus the cladinose sugar bonded in C-3 pushes back on the desosamine, which consequently loses some of its mobility. The position of the oxime chain above the macrocycle and the different associations by hydrogen bonding reduce the freedom of the macrocycle lactone ring and consequently the motions of the desosamine and cladinose sugars.

Table VIII. Physicochemical Characteristics of Roxithromycin (1) and Erythromycin (2)

	1	2
M_R	837	734
pK_a	9.2	8.8
ionization (at pH = 7.4)	>96%	>96%
serum protein binding	95%	80%
α-1-glycoprotein binding	71.4%	73%
octanol/water partition (37 °C, pH = 7.4)	408	50

These results demonstrate the importance of the nature and orientation of the ether chain and can explain some of the differences observed between roxithromycin and erythromycin.

Similarly, the ionization of the amino group may occur in various media according to the nature of the antibiotic. This variation could allow for changes in the reactivity and in the motion of the desosamine unit. Biologically, differences are observed in the formation of a 455-nm metabolite–cytochrome P-450 complex.

Table VIII shows the physicochemical characteristics of these two compounds. Recently, it was considered that the octanol/water partition coefficient of 408 for 1 and 50 for 2, determined at pH 7.4 and 37 °C, can hardly account for the higher lipid solubility and hence the better permeability of roxithromycin through some tissues.[8] However, the resulting greater hydrophobicity and globular structure of roxithromycin with respect to erythromycin A and the effect of the pH could be related to its biological properties. Consequently, this study highlights other factors that play a role and that can explain some of the significant differences between these two antibiotics.

Experimental Section

The compound studied has an experimental formula $C_{41}H_{76}N_2O_{15}\cdot H_2O$. It is an antibiotic CID named roxithromycin, proprietary name Rulid, M_R = 854.99 (RU 28965-11).[1] ^1H NMR experiments were conducted essentially on WM250 (or AM400 or AM500) NMR spectrometers from Bruker, using a sample concentration of 5×10^{-2} M. The samples were at ambient temperature (20 °C) unless otherwise noted and were degassed, in $CDCl_3$ and CD_3OD solution. The ^1H NOE difference spectra were acquired automatically with one frequency list to define a series of irradiation points (on resonance) and one control (off resonance). The individual FIDS are stored. Typically eight to 10 irradiations would be performed in one experiment with automated cycling through the frequency list, using four dummy scans and 32 scans at each frequency. The pulse sequence utilized a relaxation delay (1.5 s) followed by a saturation time (2 s) using power S3 = 40 L and then acquired data with the decoupler gated off. Difference spectra were obtained by the subtraction of the control (off-resonance saturation) from every other spectrum.

The 2D phase-sensitive ^1H NOESY experiment was performed with variation of the mixing time (D8)(D9 P2 A0).[20] FIDS were acquired (256 scans, two dummy scans) over 1275 Hz into a 1K data block for 512 increment values of the evolution time, t_1. The raw data were zero filled to a 2K × 2K matrix and the first FID was divided by two. Different experiments in $CDCl_3$ and in CD_3OD solutions were performed with τ_m = 0.2, 0.3 s and the relaxation delay D_1 was 1.5 s.

The ^1H T_1 experiment was conducted by using a standard inversion-recovery pulse sequence (D_1-180°-VD-90°-FID) with the relaxation delay D_1 = 5 s and averaging 64 scans into an 8K data block. The experiment was repeated for 10 values of the variable delay VD ranging from 0.1 to 3.5 s. The T_1 values were calculated by using the Bruker DISNMR program. The 180° pulse calibrated in $CDCl_3$ and CD_3OD solution was respectively 12.0 and 11.4 μs.

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The ^{13}C T_1 experiment used an inversion-recovery pulse sequence with a relaxation delay D_1 = 4 s and averaging 2800 scans into an 8K data block. The experiment was repeated for 15 values of VD ranging from 0.01 to 3 s. The T_1 values were calculated by using the Bruker DISNMR program. The 180° pulse had been calibrated and the value was 19 μs in $CDCl_3$ and in CD_3OD solution.

FAB-PI mass spectra were obtained on a Nermag 1030 instrument fitted with an Ion Tech fast atom gun. Xenon and argon were used as the bombarding species. Glycerol was used as the matrix to dissolve the samples.

Acknowledgment. We would like to thank Dr. B. Bachet and J. P. Mornon for the crystal structure coordinates of roxithromycin.[12] We thank Laboratoire Roussel (Paris) for their scientific and financial support during this research.

Registry No. 1, 80214-83-1; 2, 114-07-8.

Supplementary Material Available: Table S1 listing ^1H and ^{13}C NMR relaxation times in $CDCl_3$ and CD_3OD solution for roxithromycin (1); concentration effect on the chemical shifts of hydroxyl groups for roxithromycin (1) and erythromycin (2) in $CDCl_3$ solution (Graph S1); chemical shifts variation with pH of the *N,N*-dimethylamino group for roxithromycin (1) and erythromycin (2) in D_2O solution (10^{-3} M) (Graph S2); Figure S1 is an FAB mass spectrum of a roxithromycin (1) and erythromycin (2) mixture (5 pages). Ordering information is given on any current masthead page.

Carboxylic Acids and Tetrazoles as Isosteric Replacements for Sulfate in Cholecystokinin Analogues

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A series of analogues of the satiety-inducing peptide cholecystokinin (CCK-8) was prepared in which the sulfated tyrosine required for activation of peripheral receptors was replaced with a carboxy(alkyl)- or tetrazolyl(alkyl)-phenylalanine to investigate whether an organic acid could serve the role of the sulfate group at the receptor. The necessary intermediates were prepared by previously reported procedures or by alkylation of carboxy(alkyl)- or tetrazolyl(alkyl)phenylmethyl bromides with a glycine-derived anion followed by protecting-group manipulations, and these were incorporated into derivatives of acetyl-CCK-7 using solid-phase synthesis. Peptide analogues were evaluated in a CCK-binding assay for affinity for either peripheral (CCK-A) receptors using homogenated rat pancreatic membranes as the receptor source or for central (CCK-B) receptors using bovine striatum as the receptor source. They were further evaluated for effects on food intake in rats after intraperitoneal (ip) injection. A number of the compounds reported are active in the CCK-A receptor binding assay although less potent than acetyl-CCK-7 and decrease food intake with comparable potency to acetyl-CCK-7. In a meal feeding model designed to assess appetite suppressant activity, acetyl-CCK-7 has an ED_{50} of 7 nmol/kg ip, while the ED_{50}s of Ac-Phe(4-CH_2CO_2H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (**28**) and Ac-Phe[4-(tetrazol-5-yl)]-Met-Gly-Trp-Met-Asp-Phe-NH_2 (**34**) were 9 and 11 nmol/kg ip, respectively. An analogue of **28** lacking the N-terminal acetamido group, 3-[4-(carboxymethyl)-phenyl]propanoyl-Met-Gly-Trp-Met-Asp-Phe-NH_2 (**50**), was also active in the meal feeding assay with an ED_{50} of 3 nmol/kg ip. Its anorexic effect was blocked by simultaneous administration of the CCK-A receptor antagonist MK 329, indicating that the observed anorexic activity is mediated by CCK-A receptors. We conclude from this work that the requirement for a negative charge at the CCK-A receptor provided in the natural substrate by a sulfate group can be satisfied by organic acids.

A number of neurohormones and secretory peptides such as cholecystokinin,[1] fibronectin,[2] hirudin,[3] and sulfakinin[4] contain a sulfated tyrosine, which is necessary for full expression of their biological activity. Because of the lability of tyrosine sulfates,[5] other investigators have developed methodology for their replacement in peptide analogues by phosphono-,[6] (phosphonomethyl)-,[7] and (sulfomethyl)phenylalanines.[7,8] We have been concerned for some time with the design of analogues of the putative satiety-signalling peptide cholecystokinin (CCK) as appetite suppressants, and in particular with the discovery of suitable stable and less polar replacements for the tyrosine sulfate which might ultimately be compatible with an orally active CCK mimetic. In this paper we report that analogues of CCK in which the tyrosine sulfate is substituted by either an appropriate (carboxymethyl)-phenylalanine or a tetrazolylphenylalanine retain binding affinity to the peripheral (CCK-A) receptor subtype[9] and, moreover, possess equivalent potency to CCK-7 as appetite suppressants in rats.

CCK represents a family of gut and neurohormones of which the 33 amino acid peptide CCK-33 and the equipotent eight amino acid peptide CCK-8 (1) are the most abundant circulating bioactive forms. Peripheral activities of CCK include stimulation of enzyme secretion from pancreatic acinar cells, inhibition of gastric emptying,

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0022-2623/91/1834-1125$02.50/0