Articles

A Multifaceted Approach to the Study of the Side-Chain Conformation in β-Lactamase-Resistant Penicillins

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The resistance of some penicillins to β-lactamase enzymes was previously attributed to the nature of their C(6) side chain. In order to find explicitly the influence of the conformation of this side chain in the enzymatic mechanism, we have analyzed by experimental and theoretical methods (X-ray diffraction, NMR, IR, PCILO) the molecular structure of six resistant penicillins and derivatives: oxacillin, cloxacillin, dicloxacillin, flucloxacillin, methicillin, nafcillin, cloxacillin sulfoxide, and oxacillinpenicilloic acid. X-ray crystallography of flucloxacillin and nafcillin is fully described. We observe that the side chains of these penicillins have no influence on the electronic properties of the penam nucleus but are much more rigid than in the sensitive ones. The molecular conformations are mostly governed by the nonbonded Van der Waals interactions and, in the oxacillins, partly by the conjugation between exocyclic groups. The lack of flexibility could result in a distorting effect on the structure of the active site of the β-lactamase, leading to the deactivation of the enzyme.

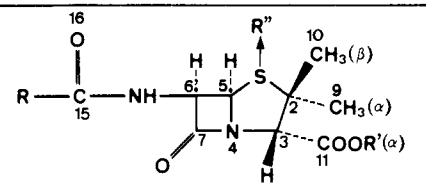
Penicillins and cephalosporins form one of the most important of the antibiotic families.[1] Their antibacterial activities are limited by the growing resistance of the pathogenic bacteria. This resistance proceeds from β-lactamase enzymes which transform these antibiotics into biologically inactive molecules.[2] Unfortunately, only partial results are available on this specific enzymatic action,[3] but a great deal of effort is being devoted to try to find resistant antibiotics or specific inhibitors of β-lactamases.[4] Although the resistance can also come from altered targets or lack of penetration besides from β-lactamases, it seems that the nature and, more particularly, the three-dimensional geometry of the side chain of the β-lactam ring are among the most important factors which induce conformational changes in the enzyme, resulting in the deactivation of the active site.[5-7]

In order to understand the role of the side chain in the enzymatic process, we have analyzed by experimental and theoretical methods (X-ray diffraction; ^{1}H NMR, ^{13}C NMR, and IR spectroscopy; and PCILO computation) the molecular structure of six resistant penicillins and derivatives: oxacillin, cloxacillin, dicloxacillin, flucloxacillin, methicillin,and nafcillin. By this approach, we try to put forward the influence of the C(6) side chain on the properties of the penam nucleus and to delimit the allowed conformational space of the side chain. Beyond the immediate pharmacological interest of the β-lactamase inhibitors, this multifaceted approach could also help to understand the antibacterial activity of penicillins and enzymatic mechanisms in general.

Experimental Section

Materials. The different penicillins and related compounds studied here are reported in Table I. Oxacillin, cloxacillin, dicloxacillin, methicillin, and cephalothin sodium salts are commercially available. Oxacillin, cloxacillin, dicloxacillin, and methicillin methyl esters, oxacillin and cloxacillin sulfoxides, as well as the methyl diester of the penicilloic acid corresponding to oxacillin were synthesized by SKF-RIT (Belgium) laboratories. Sodium flucloxacillin was obtained from Beecham Pharma (Belgium) and sodium nafcillin from Wyeth laboratories (USA). Flucloxacillin and nafcillin methyl esters were easily prepared by a reaction with diazomethane.[8]

Table I. List of Penicillins and Related Compounds Studied



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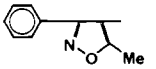
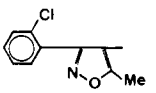
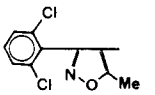
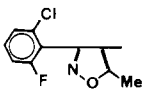
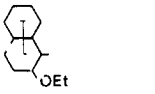
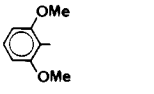
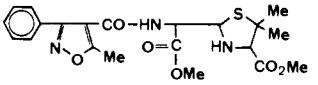
	Na	*a*	oxacillin
	CH_3		
	H		
	Na		cloxacillin
	CH_3	*a*	
	CH_3	0[a]	
	Na		dicloxacillin
	CH_3	*a*	
	Na	*a*	flucloxacillin
	Na		nafcillin
	CH_3	*a*	
	Na		methicillin
	CH_3	*a*	
		*a*	oxacillin penicilloic methyl diester

[a] Compound analyzed by X-ray diffraction.

(1) Flynn, E. H., Ed. "Cephalosporins and Penicillins, Chemistry and Biology"; Academic Press: New York, 1972.
(2) Abraham, E. P.; Chain, E. *Nature (London)* **1940**, *146*, 837.
(3) Perlman, D. Structure-Activity Relationships among the Semisynthetic Antibiotics; Academic Press: New York, 1977.
(4) Elks, J., Ed. "Recent Advances in the Chemistry of β-Lactam Antibiotics"; *Spec. Publ. Chem. Soc.* **1977**, *no. 28*.
(5) Samuni, A.; Citri, N. *Biochem. Biophys. Res. Commun.* **1975**, *62*, 7.
(6) Samuni, A.; Citri, N.; Zyk, N.; *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1048.
(7) Labia, R.; Fabre, C. *Biochim. Biophys. Acta* **1976**, *452*, 209.

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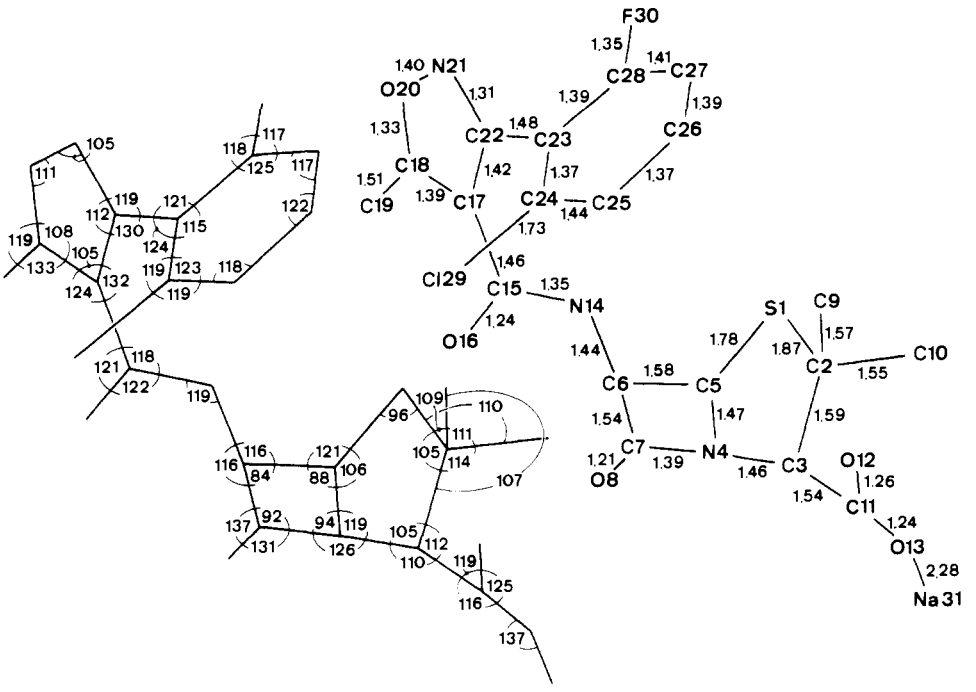


Figure 1. Flucloxacillin: atomic numbering and bond lengths (Å) and angles (deg). Standard deviations for distances and angles are smaller than 0.02 Å and 1°, respectively.

Table II. Relative Values of the Ratio v/v_i in the Inhibition of *Enterobacter aerogenes* β-Lactamases (Oxacillin Sulfoxide = 1)

compounds	rel inhibitory powers
oxacillin sulfoxide	1
methicillin methyl ester	2
methicillin	50
oxacillin methyl ester	120
nafcillin (sodium salt)	130
cloxacillin methyl ester	270
cloxacillin sulfoxide	370
dicloxacillin methyl ester	1500
oxacillin (sodium salt), flucloxacillin (sodium salt), cloxacillin (sodium salt), dicloxacillin (sodium salt)	±5000

Kinetic Study. In order to estimate the relative inhibitory powers of the various molecules listed in Table I, initial rates concerning the degradation of cephalothin by β-lactamases from *Enterobacter aerogenes* were measured at different concentrations (3.4×10^{-7} to 8.5×10^{-5} M) with (v_i) and without (v) inhibitors according to O'Callaghan's[9] method using a Pye-Unicam SP 1800 spectrophotometer. The enzyme was a partially purified preparation obtained from SKF-RIT, taken at constant concentration in a pH 8.5 phosphate buffer at 25 °C. The absorption variations of the substrate (2.6×10^{-4} M) were recorded at 260 nm (initial absorbance 1.8), and the initial rate of hydrolysis was graphically estimated. The relative values of the ratio v/v_i are compared in Table II.

Single Crystal X-Ray Diffraction. (a) Flucloxacillin Sodium Salt Monohydrate ($C_{19}H_{16}ClFN_3O_5SNa \cdot H_2O$). **Crystal Data.** Crystals suitable for X-ray analysis were obtained by the slow evaporation of an acetonitrile solution. The lattice constants and estimated standard deviations of the orthorhombic unit cell measured in Weissenberg photographs and from single crystal diffractometry are as follows: $a = 10.952$ (2), $b = 25.862$ (3), $c = 7.985$ (1) Å; $\alpha = \beta = \gamma = 90°$. The space group is $P2_12_12_1$.

Table III. Flucloxacillin Hydrogen and Ionic Bond Distances and Angles

bond	distance or angle
O(32)-O(8) [$^3/_2 + x, \bar{y}, ^1/_2 + z$]	2.87 Å
O(32)-O(12) [$x, y, 1 + z$]	2.72 Å
O(8)-O(32)-O(12)	107°
Na(31)-O(13) [x, y, z]	2.28 Å
Na(31)-O(16) [$^3/_2 + x, \bar{y} - 1, ^1/_2 + z$]	2.33 Å
Na(31)-O(32) [x, y, z]	2.34 Å
O(8)-O(32)-Na(31)	124°
O(12)-O(32)-Na(31)	115°

The observed density of 1.43 (1) g/cm^3 measured by flotation in mixed liquids can be compared to the density of 1.45 g/cm^3 calculated with four formula units per cell ($Z = 4$). Crystals obtained from a 1-butanol/methanol solution have been shown to have the same space group and the same unit cell dimensions.

Determination of the Structure. Following the $\omega/2\theta$ scan method, intensities were collected on a four-circle Nonius CAD-4 diffractometer with a graphite monochromated Cu Kα (1.54178 Å) radiation. Among the 1785 independent reflections with $2° \leq \theta \leq 72°$, 1223 had $I \geq 3\sigma(I)$ [$\sigma^2(I) = S + B + (0.03S)^2$, S being the scan and B the background count] and were used for the subsequent analysis. Data were corrected for Lorentz and polarization effects but not for absorption. The structure was solved by direct methods using the MULTAN program.[10] The best figure of merit gave an E map in which 24 of the 32 nonhydrogen atoms were recognized; the others were located by Fourier synthesis. The positional and anisotropic thermal parameters were refined according to the full-matrix least-squares method. It was impossible to locate the hydrogen atoms on difference Fourier maps. The final R index ($\Sigma[|F_o| - |F_c|]/\Sigma|F_o|$) was 0.056 for the 1223 observed reflections. All the computations were carried out on the SDP system of crystallographic programs written for the PDP-11 computers.[11]

Description of the Structure. Figure 1 shows the interatomic distances and angles, Figure 2 a stereoscopic view of the molecular conformation of flucloxacillin, and Table VIII lists the main torsion

(8) Clarke, H. T.; Johnson, J. R.; Robinson, R. "The Chemistry of Penicillins", Princeton University Press: Princeton, NJ, 1949.
(9) O'Callaghan, C. H.; Muggleton, P. W.; Ross, G. W., *Antimicrob. Agents Chemother.* **1968**, 57.
(10) Germain, G.; Declercq, J. P. "The MULTAN Program"; Version of 1976, University of Louvain, Belgium.
(11) Okaya, Y.; Frenz, B. "The S.D.P. System"; Molecular Structure Corp.: College Station, Texas, 1976.

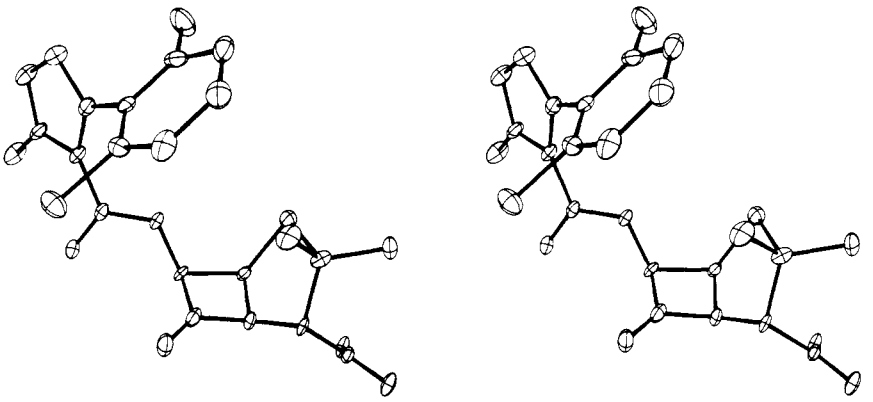


Figure 2. Flucloxacillin: stereoscopic view of the molecule with 50% probability thermal ellipsoids (ORTEP).[14]

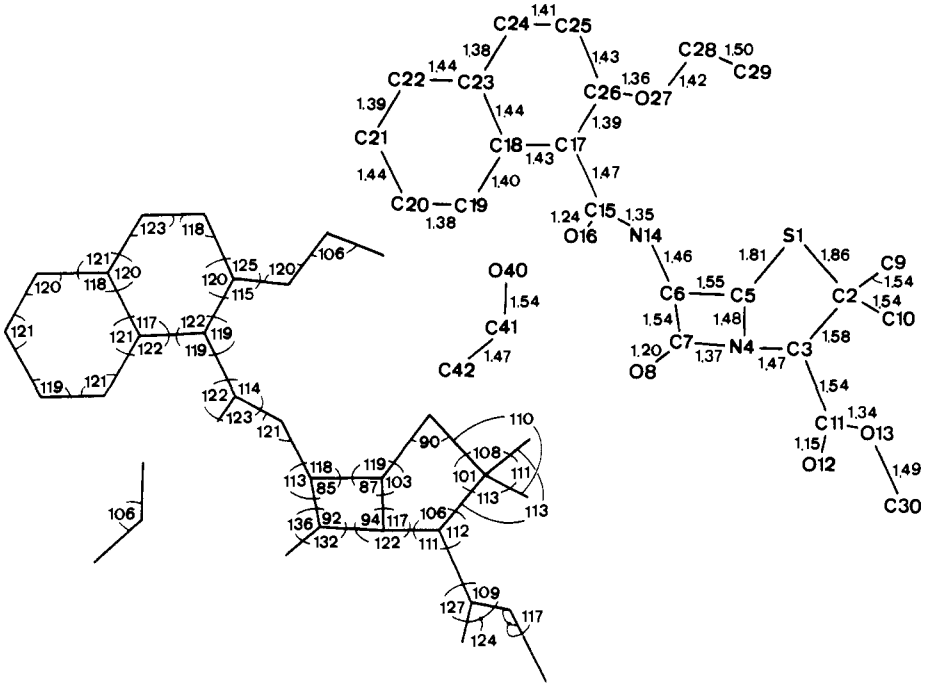


Figure 3. Nafcillin: atomic numbering and bond lengths (Å) and angles (deg). Standard deviations for distances and angles are smaller than 0.013 Å and 1°, respectively.

angles and deviations from the mean planes. The bond distances and angles of the nucleus, β-lactam and thiazolidine rings, agree with those of other penicillin derivatives.[12,13] The thiazolidine ring adopts the conformation α-CH_3 equatorial, β-CH_3 axial, and α-COO^- axial; four atoms are nearly coplanar, S(1), C(2), N(4), and C(5), while the fifth, C(3), is out of this plane (0.048 Å). N(4) in the β-lactam ring lies 0.38 Å from the plane of C(3), C(5), and C(7), and the sum of the bond angles around N(4) is 339°. Atoms C(6), N(14), C(15), O(16), and C(17) belonging to the exocyclic amide group are coplanar within experimental deviations; this plane is nearly perpendicular to the plane of the β-lactam group. The side chain is folded up to the nucleus with a close intramolecular contact, 3.4 Å, between the carbon atoms C(26) of the phenyl and the thiazolidine ring β-CH_3 substituent. The molecules are linked to each other through ionic Na^+-O^- and hydrogen bonds, the values of which are reported in Table III.

(b) Nafcillin Methyl Ester ($C_{22}H_{24}N_2O_5S \cdot C_2H_5OH$). Crystal Data. Suitable crystals were obtained by the slow evaporation of an ethanol solution. Unit cell parameters determined from Weissenberg photographs and single crystal diffractometry [a = 12.385 (2), b = 27.824 (5), c = 7.199 (3) Å; α = β = γ = 90°), and systematic absences, indicated the presence of space group $P2_12_12_1$. The measured density of 1.26 (1) g/cm^3 agrees with the calculated density of 1.26 g/cm^3, assuming that there are four nafcillin and four ethanol molecules per unit cell (Z = 4).

Determination of the Structure. The intensities of 2663 independent reflections were collected by the $\omega/2\theta$ scan method ($2° \leq \theta \leq 72°$); 2214 had measured intensities larger than $3\sigma(I)$ and were considered observed. They were corrected for Lorentz and polarization effects but not for absorption. The structure was solved by direct methods using the MULTAN program.[10] One of the eight computed phase sets permitted us to locate 13 of the 33 nonhydrogen atoms. The 20 remaining atoms were located by Fourier synthesis. Refinement was carried out by full-matrix least-squares calculations with anisotropic thermal factors; the final R index was 0.067 for the observed reflections. No hydrogen atoms were located on difference Fourier maps. All the calculations were carried out according to the SDP system.[11]

Description of the Structure. The enumerations of the atoms, bond distances, and angles are shown in Figure 3; the geometry of the nafcillin molecule can be seen from the stereoscopic drawing in Figure 4; and the principal torsion angles and deviations from mean planes are listed in Table VIII.

The thiazolidine ring adopts the conformation α-CH_3 axial, β-CH_3 equatorial and α-CO_2CH_3 equatorial; four atoms of the ring, C(2), C(3), N(4), and C(5), were approximately planar, but the fifth, S(1), is out of this plane by 0.88 Å. The nitrogen atom N(4) in the β-lactam ring lies 0.43 Å from the plane of its three sub-

(12) Sweet, R. M., "Cephalosporins and Penicillins, Chemistry and Biology"; Flynn, E. H., Ed.; Academic Press: New York, 1972; pp 280–309.
(13) Domiano, P.; Nardelli, M.; Balsamo, A.; Macchia, B.; Macchia, F. *Acta Crystallogr., Sect. B* **1979**, *35*, 1363.

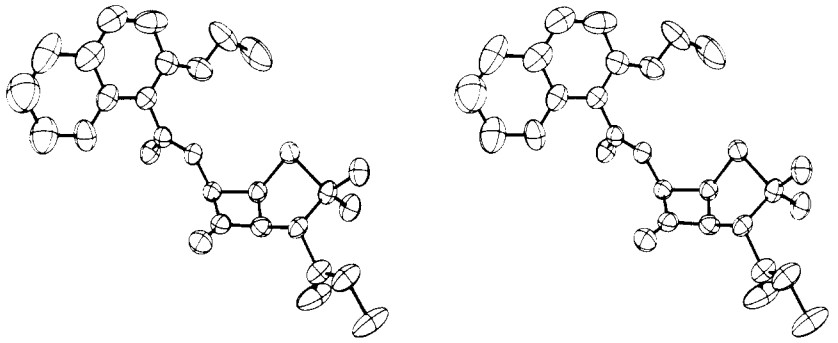


Figure 4. Nafcillin: stereoscopic view of the molecule (ORTEP).[14]

Table IV. Carbonyl Infrared Absorption Frequencies

	IR, cm^{-1}								
	C=O β-lactam			C=O exocyclic amide			C=O ester or acid		
compd	CCl_4	$CHCl_3$	D_2O[a]	CCl_4	$CHCl_3$	D_2O[a]	CCl_4	$CHCl_3$	D_2O[a]
(a) Methyl Esters in Solution									
oxacillin	1793	1788		1677	1672		1756	1751	
cloxacillin	1793	1788	1761	1679	1669	1638	1756	1750	1601
dicloxacillin	1794	1790	1760	1682	1675	1641	1757	1751	1600
flucloxacillin	1793	1788	1760	1674	1669	1638	1752	1746	1600
methicillin	1794	1788	1762	1692	1678	1625	1758	1751	1600
nafcillin	1792			1680			1757		
penicillin V		1790[b]	1760			1656			1599
penicillin G			1761			1639			1601
oxacillin sulfoxide		1802			1669			1740	
cloxacillin sulfoxide		1804			1675			1743	
(b) Na Salts in KBr Disks									
cloxacillin		1762			1659			1604	
nafcillin		1760			1655			1605	

[a] Reference 46. [b] Reference 44.

stituents C(3), C(5), and C(7), and the sum of the bond angles around N(4) is 334°.

The plane of the exocyclic amide group [C(6), N(14), C(15), O(16), C(17)] is almost perpendicular (82.3°) to the naphthyl ring of the side chain. The oxygen atom O(40) of the ethanol molecule participates in hydrogen bonding, 2.78 (1) Å, with the oxygen atom O(16) of the exocyclic amide group of nafcillin.

Energy Conformation by the PCILO Method. From bond lengths and bond and dihedral angles obtained for oxacillin[39] by X-ray diffraction, the conformational energy map corresponding to $\alpha(0\rightarrow180°)$ and $\beta(0\rightarrow360°)$ rotations was computed by the "perturbative configuration interaction using localized orbitals" (PCILO) method,[15] keeping γ at its crystallographic value. This semiempirical method for the calculation of electronic ground-state properties of a molecular system partially includes the correlation energy and avoids the iterative process. The rotation of the phenyl was carried from 0 to 360°, but only the mean energy values of α and $\alpha+180°$ were retained in order to take into account the delocalization of π electrons in phenyl. The calculations were performed on a Siemens 4004/151 computer. In Figure 5, energy contours are plotted above the global minimum energy of 0. The map obtained is not significantly different from the one published by Samuni and Meyer[16] for the side chain of oxacillin without the penam nucleus.

Infrared Spectroscopy. IR spectra were run from 1900 to 1450 cm^{-1} on a Perkin-Elmer 377 spectrometer with a ±1 cm^{-1} precision. For the $CHCl_3$ and CCl_4 solutions (0.01 M) of the tested penicillins, conventional liquid cells, about 0.5-mm thick, were used as well as a cell filled with the reference solvent. Spectra in the solid phase were recorded using KBr disks. Stretching vibration frequencies of the carbonyls (β-lactam, exocyclic amide, and ester) are compared in Table IV.

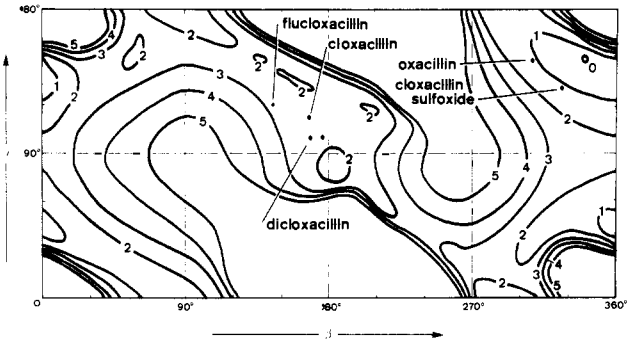


Figure 5. Conformational energy map (PCILO) for oxacillin at $\gamma = 174°$, $0° \leq \alpha \leq 180°$, and $0° \leq \beta \leq 360°$. Energy contours at 0.5, 1.0, 2.0, 3.0, 4.0, and 5 $kcal \cdot mol^{-1}$ above the deepest minimum. Conformations observed in the crystalline state are indicated for the oxacillin series.

Table V. ^1H NMR Chemical Shifts of the Thiazolidine Ring Substituents

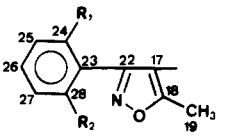
	^1H NMR (DSS in D_2O), ppm			conformation of the thiazolidine ring in solid state
compd	β-CH_3	α-CH_3	H-C(3)	
oxacillin	1.45	1.43	4.08	α-COOH ax
cloxacillin	1.41	1.38	4.03	
flucloxacillin	1.43	1.43	4.09	
dicloxacillin	1.43	1.43	4.08	
nafcillin	1.52	1.52	4.18	α-COOH eq
methicillin	1.62	1.51	4.17	
6-APA	1.57	1.48	4.11	α-COOH ax

Nuclear Magnetic Resonance. (a) ^1H NMR Measurements. ^1H NMR spectra were recorded at room temperature using a JEOL JNM-MH/100 spectrometer (sweep time = 250 s;

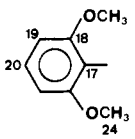
(14) Johnson, C. K. "The ORTEP Program"; Oak Ridge, Tenn., June 1965; ORNL-3794, UC-4 Chemistry.
(15) Pullman, B.; Maigret, B.; Perahia, D. *Theor. Chim. Acta*, **1970**, *18*, 44.
(16) Samuni, A.; Meyer, A. Y. *Mol. Pharmacol.* **1978**, *14*, 704.

Table VI. ^{13}C NMR Chemical Shifts

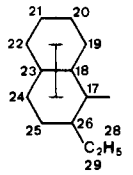
	^{13}C NMR (Me_4Si in D_2O), ppm							
							sulfoxides	
	oxacillin	cloxacillin	flucloxacillin	dicloxacillin	methicillin	nafcillin	oxacillin	cloxacillin
	(a) Penam Nucleus[a]							
C(2)	66.0	65.7	65.7	65.9	65.5	66.7	76.4	78.4
C(3)	74.5	74.2	74.1	74.3	74.4	74.5	67.5	68.5
C(5)	67.4	67.0	67.1	67.2	67.4	67.7	76.4	77.6
C(6)	59.2	58.6	58.7	58.6	58.7	59.0	56.6	57.7
β-CH_3	32.1	32.0	31.9	32.5	31.0	31.5	19.5	20.7
α-CH_3	27.8	27.6	27.5	27.6	27.5	27.8	18.9	19.8
C(7)	175.4	175.5	175.7	174.9[b]	175.5[b]	175.3[b]	175.2	176.5
C(11)	175.4	175.5	175.7	176.5[b]	175.9[b]	175.7[b]	171.6	172.7
C(15)	162.3	160.0	154.9	157.7	158.1	154.4	162.2	162.6



	oxacillin (R_1 = H; R_2 = H)	cloxacillin (R_1 = Cl; R_2 = H)	flucloxacillin (R_1 = Cl; R_2 = F)	dicloxacillin (R_1 = Cl; R_2 = Cl)	sulfoxides	
					oxacillin	cloxacillin
	(b) Side Chains (R)					
C(17)	111.8	112.5	112.9	112.1	111.7	114.0
C(18)	164.3	163.5	162.9	162.2	163.5	164.1
C(19)	13.3	13.4	13.3	13.7	12.8	13.9
C(22)	175.4	175.5	175.7	175.3	175.2	176.5
C(23)	132.2	133.6	126.6	134.4		134.4
C(24)	129.9	134.2		136.4	128.1	135.5
C(25)	130.7	133.1	127.4	130.2	128.6	132.6
C(26)	128.1	127.1	134.5	126.7		128.7
C(27)	130.7	129.1	117.1	130.2	129.8	130.1
C(28)	129.2	132.2	161.6	136.4		133.8



methicillin



nafcillin

C(17)	169.5	C(17)	119.5
C(18)		C(18)	132.0
C(19)	105.9	C(19)	128.9
C(20)	133.4	C(20)	124.9
C(24)	57.1	C(21)	
		C(22)	
		C(23)	129.3
		C(24)	133.0
		C(25)	115.6
		C(26)	169.9
		C(27)	66.0
		C(28)	15.5

[a] See atomic numbering in Table I. [b] Reverse assignment is also possible. [c] The ^{13}C-F coupling constants are $J_{C_o\text{-}F}$ = 252.1 Hz; $J_{C_m\text{-}F}$ = 23.9 Hz, and $J_{C_p\text{-}F}$ = 9.0 Hz, where subscript o, m, and p = ortho, meta and para, respectively.

sweep width = 1080 Hz). Because of the concentration dependence of the chemical shifts of most of the penicillin functional groups,[17] sample concentrations were 10% w/w in D_2O. 3-(Trimethylsilyl)propanesulfonic acid sodium salt (DSS) was used as the internal reference; chemical shifts are reported in Table V in parts per million downfield from the reference (δ values).

(b) ^{13}C NMR Measurements. ^{13}C NMR spectra have been obtained on a BRUKER WP-60 spectrometer operating in the pulsed Fourier transform mode with complete proton noise decoupling; 30° (3 μs) pulses and ±10000 scans were used. Chemical shifts are reported in Tables VI and VII in parts per million from tetramethylsilane (Me_4Si). Sample concentrations were 10% w/w. Spectra calibration was realized using the decoupling frequency of benzene with reference to the carrier frequency and with different ^2D locking modes of CD_3COCD_3, D_2O, or Me_2SO-d_6.

Discussion

Penicillins are formed by a β-lactam and a fused thiazolidine ring[18] (see Table I). Their physicochemical (lipophilicity, stability, etc.) and biological properties (spectrum of antibacterial activity, resistance to β-lactamases, toxicity, etc.) have been shown to depend on the side chain R.

The damages caused to growing bacteria by penicillins are related to the ability of these antibiotics to bind to several proteins and to inactivate several enzymes which, with few exceptions, are membrane bound.[19-21]

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Table VII. ^{13}C NMR Chemical Shifts for Methicillin at Different Temperatures[a]

	^{13}C NMR (Me_4Si), ppm			
	CD_3-$COCD_3$ (183 K)	CD_3-$COCD_3$ (300 K)	Me_2SO-d_6 (300 K)	Me_2SO-d_6 (400 K)
C(2)	64.6	64.4	65.1	65.2
C(3)	70.5	70.7	71.5	71.7
C(5)	68.3	68.3	68.8	69.0
C(6)	58.5	59.1	59.2	60.5
β-CH_3		30.6	31.0	31.9
α-CH_3		26.7	28.0	27.8
C(11)	169.0		169.5	
C(12)	52.9	52.0	53.6	53.0
C(7)	175.2		175.2	
C(15)	157.2	157.2	158.2	
C(24)	55.3	55.6	57.0	57.6
C(17)			116.7	116.8
C(18)	175.5		166.1	
C(19)	103.9	104.3	105.5	106.8
C(20)	131.6	131.0	131.7	131.9

[a] See atomic numbering in Tables I and VI.

The penicillins described in this paper are more or less resistant to most of the bacteria which produce β-lactamases. Some features are known about this type of monomeric enzyme which is characterized by a unique active site.[22] Sequences of four β-lactamases have been so far determined,[23-26] and X-ray crystallographic studies on three of them are in progress.[27,28] Conformational studies in solution seem to show a certain lability of such enzymes.[29] However in spite of the large amount of available information about the chemistry of these enzymes, little is known about their catalytic mechanism. The involvement of a tyrosine and a histidine residue has been proposed,[30] but little evidence has been accumulated in favor of this hypothesis; a tryptophan residue could be responsible for the adequate conformation leading to enzymatic action and a cationic site would interact with the carboxylate of the antibiotic.[31] Recently, a serine residue of β-lactamase I from *B. cereus* has been shown to react with β-bromopenicillanic acid.[32] The presence of this serine residue is conserved in the amino acid sequences of all known β-lactamases. Fisher and Knowles[33] suggest the formation of a transient acyl enzyme from a serine residue, in the catalytic pathway of β-lactamases.

Previously, the resistance of semisynthetic penicillins was attributed to a steric effect of the side chain R. The importance of the steric hindrance around the exocyclic amide bond is emphasized in the enzymatic hydrolysis, while the rate of the nonenzymatic reaction is not influenced very much by the presence of different side chains.[34] In general, the modifications in the side chain which enhance the resistance unfortunately depress the antibiotic activity at the same time. In our study we examine penicillins (Table I) in which the α carbon [C(17)] to the exocyclic carbonyl is built into a five- or six-membered ring. In order to observe the resistance, the substituents in the oxacillin series (five-membered ring) must, in general, be greater than in the methicillin series (six-membered ring) because the external angles are larger for the former than for the latter.[35]

According to the results reported in Table II, oxacillins are the most powerful inhibitors among the penicillins studied here. Nafcillin and methicillin are less active. Similar trends were also found for some of these inhibitors of β-lactamase from *Staphylococcus aureus*.[36] As mentioned previously, the effects of the corresponding sulfoxides are far less pronounced.[37,38] The methyl esters are also less active, thus showing the importance of the carboxylic function in the enzyme–inhibitor interaction; however, the relative inhibitory power is conserved in this series.

Crystallographic Data. Thiazolidine Ring. Two different conformations are possible in the solid state[12] one with an α-CH_3 equatorial, β-CH_3 axial, and α-COOH axial (see Figure 6), and the other with an α-CH_3 axial, β-CH_3 equatorial, and α-COOH equatorial. Like penicillins G and V, oxacillins adopt conformation "α-COOH axial", while methicillin, nafcillin, and cloxacillin sulfoxide adopt conformation "α-COOH equatorial", which is also found in ampicillin. The main structural features of the analyzed molecules are summarized in Table VIII. Depending upon the conformation, atoms S(1) and C(3) are more or less displaced above or below the plane formed by the other four atoms. In conformation α-COOH axial, C(3) is situated below the mean plane S(1), C(2), N(4), C(5); for flucloxacillin, C(3) is 0.45 Å apart from this plane, while the deviations for S(1), C(2), N(4), and C(5) are only, respectively, −0.01, 0.01, −0.01, and 0.01 Å. In conformation α-COOH equatorial, in nafcillin, for example, S(1) is above (0.88 Å) the mean plane C(2) (0.04 Å), C(3) (−0.07 Å), N(4) (0.07 Å), C(5) (−0.04 Å). Moreover the C-S-C angles are also different, rising from 96.1 ± 2° in type α-COOH axial to 89.3 ± 2° in type α-COOH equatorial. Other angles are equivalent within the standard deviations.

The conformations of cloxacillin, α-COOH axial, and its sulfoxide, α-COOH equatorial, are different. The same difference was found in penicillin V and its sulfoxide by Cooper et al.[44]

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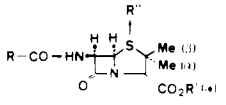
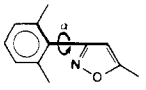
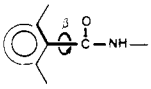
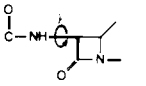
Table VIII. Comparison of the Main Structural Features from X-ray Diffraction

	oxacillin[a]	cloxacillin[b]	flucloxacillin	dicloxacillin[c]	methicillin[d]	nafcillin	cloxacillin sulfoxide[e]
	axial equatorial axial	axial equatorial axial	axial equatorial axial	axial equatorial axial	equatorial axial equatorial	equatorial axial equatorial	equatorial axial equatorial
C(5)-S(1)-C(2) angle, deg	97.0	95.1	95.6	96; 97	89.6	90.4	88
distance between N(4) and the plane of its 3 substituents, Å	0.38	0.39	0.38	0.41; 0.41	0.44	0.43	0.41
sum of the angles around N(4), deg	339	338	339	335	331	334	335
distance between C(5) and the mean plane N(4)-C(6)-C(7)-O(8), Å	0.31	0.34	0.22	0.35; 0.34	0.41	0.22	0.30
 , deg	31	67	58	81; 82			51
 , deg	124 (56)[f]	−15	−37	−15; −6	67	82	138 (42)[f]
 , deg[g]	174	125	102	118; 118	189	137	159
$\beta + \gamma$, deg	298	110	65	103; 112	256	219	297
intermolecular bonds from the exocyclic amide	N(14)···H_2O				O(16)···N(14)	O(16)···HOC_2H_5	N(14)···O(12)

[a] Reference 39. [b] Reference 40. [c] Reference 41. [d] Reference 42. [e] Reference 43. [f] In parentheses: $\beta' = 180° - \beta$. [g] Angle is positive for counterclockwise rotation looking from C(6) to N(4). The values 0 and 180° correspond, respectively, to the syn-planar and anti-planar conformations.

β-Lactam Ring. The main characteristic of the β-lactam ring in biologically active penicillins is its pyramidal geometry: this can be described either by the distance between N(4) and the plane formed by its three substituents or by the sum of the angles around N(4). Data are reported in Table VIII. In the penicillins with conformation α-COOH equatorial, the nitrogen atom N(4) is more remote from this plane than in conformation α-COOH axial. This corresponds to the diminution of the C-S-C angle. Another characteristic is the coplanarity of atoms N(4), C(6), C(7), and O(8). The distance of C(5) from this plane is ca. 0.3 Å toward S(1).

Side Chain. Side-chain conformations are described by the torsion angles α, β, and γ (Table VIII). Atoms C(17), C(15), O(16), N(14), and C(6) of the exocyclic amide group are coplanar with the carbonyl pointing below the β-lactam ring. According to the general orientation of their side chain described by the sum of dihedral angles β and γ, the penicillins can be divided into two classes: one "F" with $65° \leq \beta + \gamma \leq 112°$ (cloxacillin, flucloxacillin, and dicloxacillin) and the other "O" with $219° \leq \beta + \gamma \leq 298°$ (oxacillin, methicillin, nafcillin, and cloxacillin sulfoxide). Molecules from class F have a more compact structure with their side chain folded back onto the penam nucleus; the others (class O) adopt an open conformation. The α and β rotations are restricted due to the presence of bulky ortho substituents. However, the possible conjugation between the amide, isoxazole, and phenyl groups results in more coplanarity between these three groups. Around the β bond, the substituents are disposed at Van der Waals contacts. Table IX shows the experimental distances between atoms in contact compared to the sum of their Van der Waals radii. It is clear that in the solid state the differences are small. In the oxacillin series, the torsion angle α is a function of the number and dimension of the ortho substituents: oxacillin, 31°; penicilloic acid of oxacillin, 30°; cloxacillin sulfoxide, 51°; cloxacillin, 67°; flucloxacillin, 58°; dicloxacillin, 81-82°. In this series the general shape of the molecule is mainly governed by this α angle. For oxacillin [$\alpha = 31°$; $\beta' = 56°$ ($\beta' = 180 - \beta$)] the open form is preferred to the folded form because angle β would become greater due to the Van der Waals interactions between the phenyl and the exocyclic NH groups. On the contrary, in cloxacillin, flucloxacillin, and dicloxacillin, the increase of α (67, 58, and 81/82°, respectively) stabilized the folded form by avoiding this unfavorable interaction; this results in a smaller value of β: 15, 37, and

Table IX. Shortest Experimental Intramolecular Distances between Atoms of the Side Chain and Atoms of the Exocyclic Amide Group or Nucleus, Compared with the Sum of Their Van der Waals Radii[a]

compd		distance, Å	Σ Van der Waals radii, Å	Δ, Å
oxacillin	O(16)-C(24)	3.34	3.1	0.2
cloxacillin	O(16)-C(19)	3.02	3.4	−0.4
	C(9)-C(27)	3.75	3.7	0.05
flucloxacillin	O(16)-C(19)	3.10	3.4	−0.3
	C(9)-C(26)	3.40	3.7	−0.3
dicloxacillin	O(16)-C(19)	3.04	3.4	−0.4
	C(9)-C(27)	3.70	3.7	0.0
methicillin	O(16)-O(23)	2.97	2.8	0.2
nafcillin	O(16)-C(19)	3.30	3.1	0.2
cloxacillin sulfoxide	H(14)-C(19)	3.00	3.2	−0.2
oxacillinpenicilloic acid	O(16)-C(24)	3.40	3.1	0.3

[a] Reference 59. See atomic numbering in Tables I and VI.

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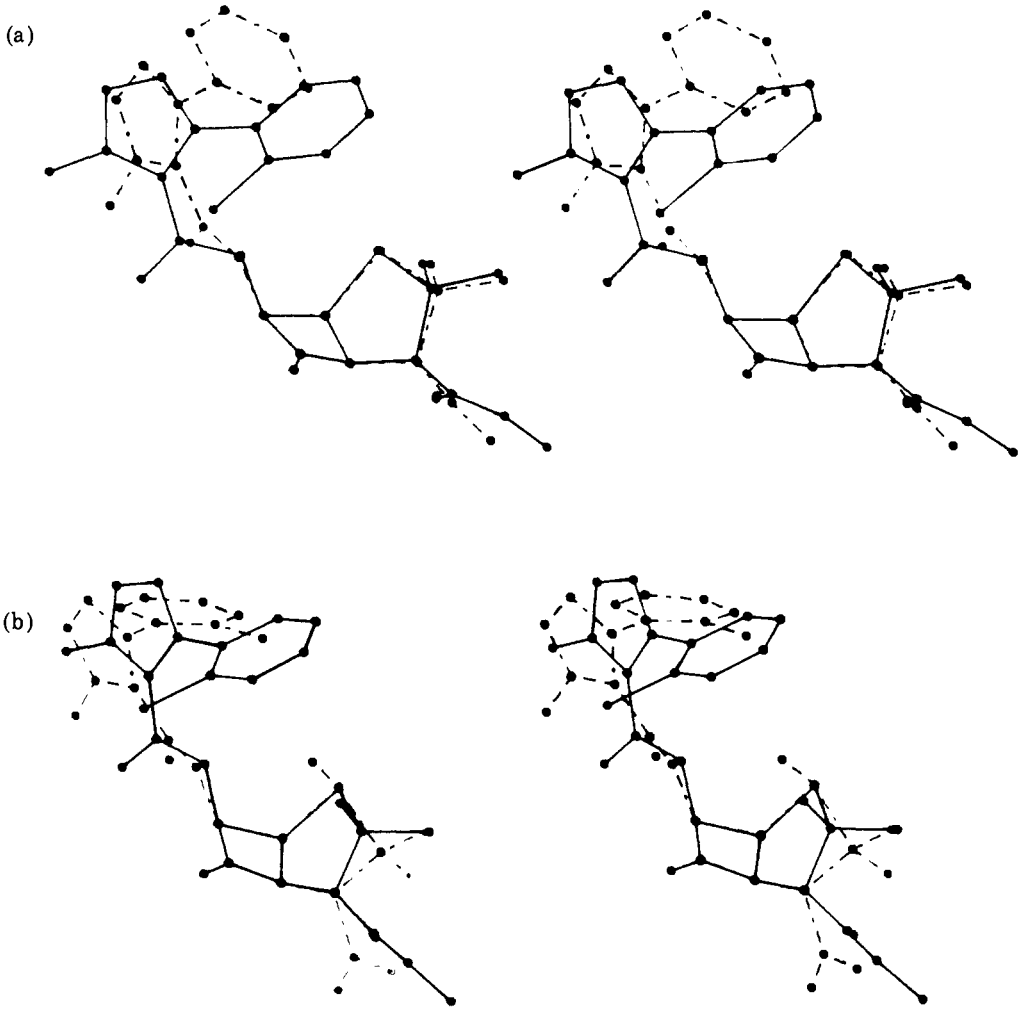

Figure 6. Stereoscopic view of pairs of penicillins after a least-squares fitting of their β-lactam ring (OSIRIS):[45] (a) cloxacillin ("F" conformation) and oxacillin ("O" conformation) (dotted lines); (b) cloxacillin sulfoxide (dotted lines) and cloxacillin; thiazolidine ring in conformation α-COOH equatorial and α-COOH axial, respectively.

15/6°, respectively. These molecules are very compact, as seen by the distances observed between the phenyl and β-CH_3 of the thiazolidine ring. In cloxacillin sulfoxide, angle β′ (42°) is larger compared to the cloxacillin angle because the repulsion which exists between the phenyl and sulfoxide groups prevents a folded conformation from being obtained; this value is still smaller than the corresponding one in oxacillin (β′ = 56°), since the α angles are in the reverse order, 51° for cloxacillin sulfoxide and 31° for oxacillin. We can conclude that, in the solid state, the side chains of penicillins try to adopt a conformation in which the largest π overlappings are realized not only between the phenyl and the isoxazolyl but also between the isoxazolyl and amide groups. Modifications in the penam nucleus do not significantly affect the side-chain conformation, as shown by the similarity of α and β angles in oxacillin (α = 31°; β′ = 56°) and in the corresponding penicilloic acid (α = 30°; β′ = 57°). In the open form the exocyclic amide group can participate in intermolecular hydrogen bonds, while in the folded conformation this group is well protected by the side chain. The molecular superpositions[45] given in Figure 6 illustrate the differences in the conformations obtained: α-COOH axial or α-COOH equatorial for the thiazolidine ring and F or O for the side chain.

IR Spectroscopy. In Table IV we notice that the carbonyl stretching frequency of the β-lactam ring is constant in the same solvent and not influenced by the nature of the side chain. On the contrary, the exocyclic amidic CO stretching frequency is very sensitive to the nature of the side chain; it is also a function of the solvent, where the better hydrogen donor ability is accompanied by the lowering of the vibration frequency. It does not appear possible to link this variation to the resistant character of penicillins.

As already observed with penicillin V sulfoxide,[46] oxacillin and cloxacillin sulfoxides have their β-lactam carbonyl frequencies at some 15 cm^{-1} higher. In the crystalline cloxacillin sulfoxide, the nitrogen N(4) atom is further away from the C(5)–C(6)–C(7) plane (0.41 Å), resulting in less conjugation with the carbonyl group; the sum of the angles around N(4) is equal to 335°, compared to 337.6° in cloxacillin where the above-mentioned distance is 0.39 Å.

It is impossible to differentiate between the two possible conformations (α-COOH axial or equatorial) of the thiazolidine ring on the basis of the β-lactam carbonyl frequencies. Indeed, in the solid state, cloxacillin and naficillin, although having opposite conformations, show the same absorption frequencies.

^{1}H and ^{13}C NMR Spectroscopy. Our ^{1}H NMR measurements have been assembled in Table V with regard

(45) Michel, A. "OSIRIS Molecular Graphic System", Department of Chemistry, Facultés Universitaires, B-5000 Namur, Belgium, 1978.

(46) Casu, B.; Ventura, P. *J. Pharm. Sci.* **1974**, *63*, 211.

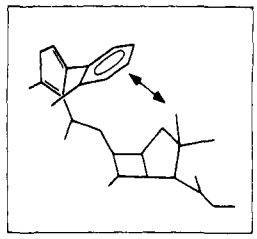

Figure 7. Illustration of the interaction between the phenyl and the thiazolidine β-CH_3 substitutent in cloxacillin.

to the thiazolidine ring conformations observed in the crystalline state. As far as the α-CH_3, β-CH_3, and H–C(3) resonances are concerned, the molecules can be separated into two distinct groups. In the first one, the oxacillin group has the following average chemical shifts: β-CH_3, 1.43; α-CH_3, 1.42; H–C(3), 4.07 ppm. Methicillin, nafcillin, and 6-APA constitute the second group: β-CH_3, 1.57; α-CH_3, 1.50; H–C(3), 4.15 ppm. Neither of these groups corresponds to the different thiazolidine conformations; indeed, 6-APA has the same conformation as the oxacillins (α-COOH axial) even though it belongs to the nafcillin, methicillin group according to its ^1H chemical shifts. Moreover, in solution both conformers[47] can probably coexist, but the NMR differences cannot be adequately explained on this ground.

One important difference exists between the two classes: in the first class, the oxacillin group, the side chain can fold back onto the thiazolidine ring, while the second class has a rigid open chain (see also ^{13}C NMR results below). Figure 7 illustrates the influence of the aromatic group on the α-CH_3, β-CH_3, and H–C(3) chemical shifts. The diamagnetic anisotropy of the phenyl ring causes a high-field shift of these three groups: α-CH_3, 1.50–1.42; β-CH_3, 1.57–1.43; and H–C(3), 4.13–4.07 ppm. On an average, the β-CH_3 experiences a higher field variation (it is closer to the phenyl group) than the corresponding α-CH_3 group.

The ^{13}C chemical shifts are reported in Table VI. Assignments could be carried out either by additivity relationships[48] or by reference to the works of Archer et al.[49] and Harrison et al.[50] for the thiazolidine and β-lactam rings and to Buchan et al.[51] for the isoxazolyl group. Our results correspond to those recently published by Chang et al.[52]

The chemical shifts of the β-lactam and thiazolidine rings are quite insensitive to the side-chain variations. On the contrary, the exocyclic amidic carbonyl group is really influenced by the nature and the electronegativity of the substituents; δ varies in a range of 154.4 to 162.3 ppm. These observations corroborate the IR results which have already shown the independence between the nucleus and the side chain. Contrary to ^1H NMR, the ^{13}C chemical shifts of α- and β-CH_3 groups cannot differentiate between open and folded conformations.

Another interesting problem is the configuration around the sulfur atom in the sulfoxides. Kinetic measurements quite clearly showed the very different behavior (much less efficient) of the sulfoxides from the corresponding sulfides.

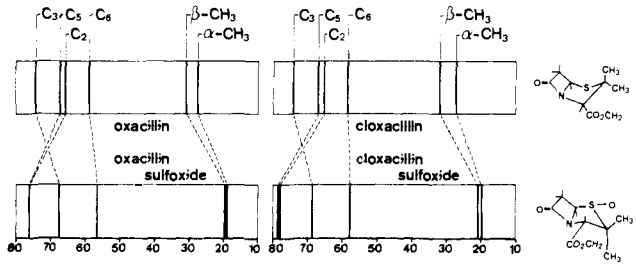

Figure 8. ^{13}C chemical shift (parts per million from Me_4Si) correlation for the process: penicillin → *S*-sulfoxide.

Two different isomers (*S* and *R*) can be obtained by the oxidation of the sulfur atom. X-ray analysis revealed that, in the solid state, cloxacillin sulfoxide is an *S* isomer. By analogy with the work of Archer et al.,[49] we could predict that, in solution, oxacillin sulfoxide is also *S* (see Figure 8). Compared to sulfides, the C(2) and C(5) of the sulfoxides experience a low-field shift of ±10.4 and ±9.0 ppm, respectively. In a first approximation, this shift variation can be interpreted by the diminution of the electronic charge due to the greater electronegativity of the oxygen atom. The high-field shifts of the γ atoms to sulfoxide oxygen are more difficult to explain: α-CH_3, −8.9; β-CH_3, −12.6; C(3), −7.0 ppm. Steric shifts can be involved in the case of β-CH_3 and also, but to a lesser extent, in the case of the other two carbon atoms. The chemical-shift anisotropy of the sulfoxides must also be taken into account, but at present no data exist to evaluate it correctly. Archer et al.[49] attribute the high-field shift of α-CH_3 to a conformational change of the thiazolidine cycle (from conformation α-COOH axial to conformation α-COOH equatorial), resulting in a 1,3-diaxial interaction with the C(5) hydrogen. This change was also observed for penicillin V sulfoxide in the solid state. Moreover, in conformation α-COOH equatorial a 1,3-diaxial interaction exists between the C(3) hydrogens and the sulfoxide oxygen atom[53] and then again a hydrogen bond could be made between the exocyclic amide and the sulfoxidic oxygen.[44] These effects could favor this conformation in the solid state. From NOE experiments, Cooper et al.[44] also propose conformation α-COOH equatorial for the sulfoxides in solution but conformation α-COOH axial for the corresponding sulfides. In opposition, Dobson et al.[47] showed that sulfides, as well as the sulfoxides, are in equilibrium in both conformations; conformation α-COOH equatorial would be preponderant in solution, while conformation α-COOH axial is more frequently observed in the solid state. One must ask whether the lanthanide complexes used in this experiment do not favor one of the conformations. Thus, the conformations in solution still remain an open question. Our NMR results do not favor either of the possibilities and equilibrium probably exists between both conformers.

Finally, a last important question can be raised concerning the rigidity of the methicillin side chain. In the crystalline state, the angle formed by the dimethoxyphenyl and the amide group is 67°. The distance between one of the methoxy oxygens and the carbonyl oxygen atom (2.97 Å) is somewhat larger than the sum of Van der Waals radii. Our ^{13}C NMR experiments on methicillin in deuterated acetone and Me_2SO are reported at three different temperatures, 183, 300, and 402 K (Table VII). At room temperature, only one resonance signal of the methoxy group is observed. This must be due either to the high rotation rate of the dimethoxyphenyl groups around the C(15)–C(17) bond or to an equivalence of the two methoxy groups in an environment where the phenyl is nearly perpendicular to the amide group. But free rotation can

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be excluded; indeed,Siddal and Garner[54] determined a barrier height of rotation >20 kcal·mol^{-1} in *O*-dimethoxybenzamide (coalescence is obtained at 438 K in ^{1}H NMR). Moreover, we observe no change in either the chemical shift or the line width at 183 or 402 K, demonstrating unambiguously the rigidity of the side chain.

Conclusions

Our study demonstrates the interest of a multifaceted approach in order to find out why some penicillins are resistant.

The kinetic tests on the degradation of cephalothin by β-lactamases from *Enterobacter aerogenes* allowed us to evaluate the resistance of six penicillins or analogues; they are classified by order of their decreasing inhibitory powers: dicloxacillin > cloxacillin > flucloxacillin > oxacillin > nafcillin > methicillin. Sulfoxides and methyl esters are always less active and show the importance of an unmodified penam nucleus and the necessity of a free acidic group. Since these characteristics are also required for the antibacterial activity, we can therefore suppose a similar mode of interaction with transpeptidases or carboxypeptidases.

The main nucleus and the side chains of these molecules were studied systematically by different techniques. The structure of the thiazolidine ring for both resistant and sensitive penicillins shows no significant difference. Two conformations were observed in the crystalline state which probably equilibrate in the solution. The distance of the β-lactam nitrogen atom is ca. 0.4 Å from the plane of its three substituents; this is an essential requirement to obtain antibacterial activity. Contrary to what was observed for the "3" side chain of a cephalosporin,[53,56] the "6" side chain of penicillins has no influence on the electronic properties of the nucleus, as shown by IR and ^{13}C NMR results.

The relative resistance power can only be explained by the nature and, thus, the structure of the side chain. Contrary to sensitive penicillins, the resistant ones seem to have a rigid side chain. Their conformation is mostly governed by the nonbonded Van der Waals interactions, and, in the oxacillins, partly by the conjugation between the exocyclic groups; in the solid state,the nonbonded groups approach the Van der Waals distances. It is significant to point out here that the side-chain conformations of oxacillin and its penicilloic acid are identical despite the profound modifications in the main penam nucleus and, consequently, in the crystalline environment.

These observations agree with the thesis of Samuni and Citri[57] that "the unique characteristics of an enzymic reaction may be traced to the effect of the substrate on the conformation of the enzyme". These authors[5,6,57] have shown, through a further study of the kinetic hydrolysis parameters of penicillins by β-lactamase from *Bacillus cereus 569/H*, that it is possible to distinguish two types of penicillins, i.e., A- and S-type substrates. The kinetics are linear for the S-type but biphasic for the A-type penicillins. Unlike the former, A-type penicillins are more resistant to enzymatic hydrolysis. It seems that the side chain of the A-type substrates induces a conformational change of the active site in the enzyme, which then becomes unfavorable to the catalytic reaction. The transition is reversible and slow enough to result in a biphasic kinetic; a decelerating phase is followed by a second linear phase in which both k_{cat} and K_m values remain constant.[52] Oxacillins, methicillin, and nafcillin belong to the A-type penicillins.[6]

The conformational map (Figure 5) concerning oxacillin, obtained by the PCILO method, shows that two regions are forbidden for the side chain of this molecule; the largest is centered at $\alpha = 0$, $\beta = 180°$, and the other at $\alpha = 0$, $\beta = 0$. The lower energies, correspond to the combined α and β values such as the exocyclic groups (phenyl, isoxazol and amide) are maintained at the Van der Waals distances. The lowest energy is close to the conformation observed in the crystalline state, which could favor the conjugation between the exocyclic groups. This lowest energy for oxacillin is only 2–3 kcal lower than those found in the region where the folded crystalline conformations of cloxacillin, dicloxacillin, and flucloxacillin are depicted. This is therefore not a forbidden zone and, as our ^{1}H NMR results indicate that in solution both open and folded conformations are possible, the only way to pass from one to the other is along the "valley" illustrated in Figure 5 and corresponding to Van der Waals contacts. This is most likely the case for the analogues of oxacillin also.

We can conclude that the lack of flexibility of the sterically restricted penicillins we have studied results in a distorting effect on the structure of the active site of β-lactamase, leading to the biphasic kinetics observed by Samuni and Citri. Similar changes have been already showed by X-ray work[58] in the active-site conformation of carboxypeptidase A upon binding a substrate.

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Supplementary Material Available: Table containing atomic coordinates and temperature factors of flucloxacillin and nafcillin (2 pages). Ordering information is given on any current masthead page.

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