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Highly Modified Cysteine-Containing Antibiotics. Chemical Structure and Configuration of Nosiheptide

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Abstract: The complete structure of a metabolite with a strong antibiotic activity, isolated from *Streptomyces actuosus*, has been established by x-ray methods to be a polythiazole-containing product related to the structures of thiostrepton and siomycin A. The crystals are tetragonal, $C_{51}H_{43}N_{13}O_{12}S_6$, space group $I4_1$, with $a = 36.05$ and $c = 11.44$ Å. The structure has been solved by direct methods. The configurations of all asymmetric centers were established, and the general macrocycle ring conformation is discussed in terms of thiostrepton and peptide chains analogies.

Many strains of *Streptomyces* produce antibiotic substances containing thiazole rings and peptide residues. A tentative classification of such metabolites has been formalized by Berdy.[1] In this study, about 20 known structures were compiled and, among them, only two were found to contain more than four thiazole rings: thiostrepton[2] (from *S. azureus*), whose chemical formula has been mostly established by an x-ray analysis,[3] and siomycin A (from *S. sioyaensis*),[4] a close parent of the former, as deduced from ^{13}C NMR comparisons.[5] Moreover, the thiostrepton antibiotic group contains some other compounds like multhiomycin,[6] thiopeptin,[7] and sporangiomycin;[8] they have been included on the basis of similarities in antibacterial activity and physicochemical properties, but their complete structures have not hitherto been reported. In Figure 1 are shown the chemical formulas of two different compounds of the general thiazole-containing group: a complex one, thiostrepton, and the somewhat simpler althiomycin[9] (from *S. althioticus* and *S. matensis*). These two compounds have the structure of peptide chains highly modified by intense enzymatic oxidations or dehydrogenations. Gram-positive bacteria are very sensitive to all of these compounds while gram-negative species and yeasts are naturally resistant to these agents. The main characteristic of the antibiotics of this group is a very low toxicity, which is, however, associated with a low water solubility. These compounds so far have not found a place in medicine except thiostrepton itself, which is used for the treatment of bovine mastisis.

Thiazole rings encountered in these structures are actually stated to arise from a *D*-cysteine residue.[10] The unusual configuration of this precursor is deduced from the absolute configuration of the remaining asymmetric center in thiazole rings (when they are present), and from its quantitative isolation in the thiazoline hydrolysis.

In the present study, we wish to report the complete structure of nosiheptide, a new metabolite isolated from *S. actuosus*, with a strong in vitro activity and we shall discuss its particular conformation.

Initial studies, by biological cross resistance experiments, strongly suggested that nosiheptide belonged to the thiostrepton class. Intense degradative studies[12] led to the trial skeleton given in Figure 2.

Nosiheptide, as obtained by precipitation, is a yellowish powder. It crystallizes in ethyl acetate or acetic acid at low temperature (40 °C). The mass spectrum did not show the M^+ ion and, as the field desorption technique was not available, the final $C_{51}H_{43}N_{13}O_{12}S_6$ formula was mostly deduced from combustion analyses and ^{13}C, ^1H, and ^{15}N NMR spectrometric experiments.[13]

Nosiheptide is very sensitive to hydrolysis. The acid hydrolysis (HCl) gives several well-identified fragments (Figure 2), 1 mol of H_2S, 1 mol of L-threonine, and compounds **1, 2,** and **3.** On the other hand, alkaline hydrolysis liberates 4 mol of ammonia and the indolic fragment **4** is obtained after subsequent esterifications.

Using analogies between these products and those initially obtained by Bodansky et al.[14] in their thiostrepton degradations, the backbone given in Figure 2 was the state of the structure at the beginning of this x-ray determination. Five thiazole rings were recognized in these fragments but the sixth sulfur atom location could not be deduced from these studies and, furthermore, it was not possible to connect the indolic part **4** to the rest of the macrocycle. It is evident that if it is bridged in a similar way as in thiostrepton, it would possess shorter linkages, as no other usual amino acid than the L-threonine residue was detected in the degradative products (see Figure 1 for the tetrapeptidic chain of the quinaldic precursor in thiostrepton).

Experimental Section

Nosiheptide crystallizes in acetate-containing solvents, but gives

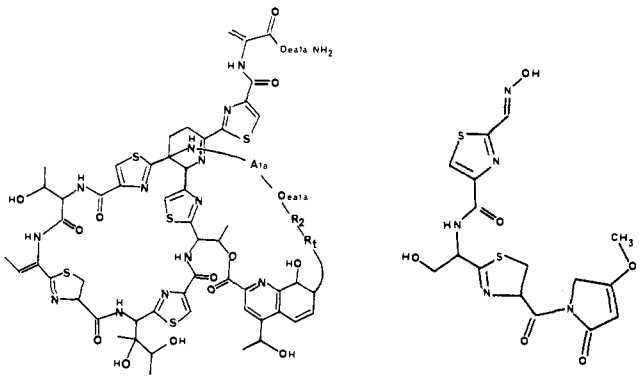

Figure 1. Two examples of cysteine oxidized antibiotics: thiostrepton (left), R_1-R_2 = Ile-Ala, and althiomycin (right). Dehydroalanine residue is noted Deala in the following.

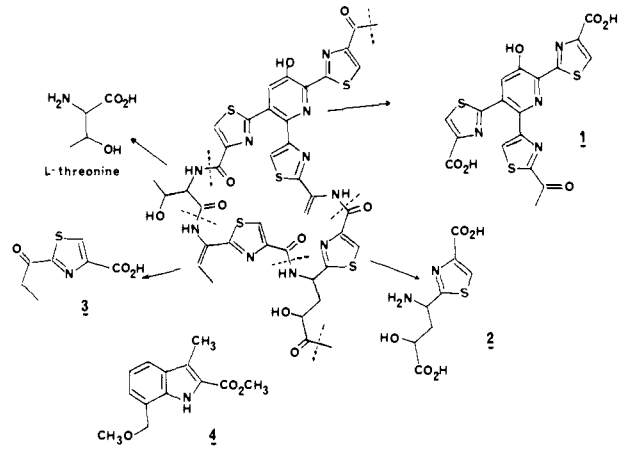

Figure 2. Fragment arrangement of the nosiheptide macrocycle (from ref 12).

unsuitable twinned crystals. Many attempts to find a good solvent led to the choice of a precise 2:1 $CHCl_3/C_2H_5OH$ mixture. Upon very slow evaporation of a solution of nosiheptide in this mixture, at a temperature of 30 °C, well-sized prisms develop. Any attempt to change the ratio of the two solvents or to accelerate the evaporation led to powdery precipitates. Crystals, when removed from the mother liquor, lost within 1 min their ability to diffract as single crystals. Obviously, the solvent is closely involved in the crystal structure. Owing to its extreme lability, the solvent was expected to freely diffuse in the crystal, like water in protein crystals. All transfer at room atmosphere in Lindemann capillaries causes microcrystalline precipitates from the mother liquor, and considerable increase of the background during data collection. The mounting operations were conveniently realized in a glove box, the atmosphere of which was saturated by continuous flooding of large chromatographic paper sheets disposed on the walls with the same solvent mixture as the mother liquor. Dimensions of the crystals were approximately 0.2 × 0.2 × 0.4 mm.

The crystals were mounted on a Philips PW 1100 computer-controlled diffractometer operating with the Cu Kα radiation (λ = 1.5418 Å), filtered by a graphite monochromator. In order to correct the data set from a slow decomposition, three standard reflections were monitored each hour. The data collection was performed up to θ = 65°, but a rapid decrease in intensities was found at elevated θ values, and only 2740 reflections were scanned in the $\theta/2\theta$ mode above the background (2σ probability level). These intensities were reduced to structure factors after corrections for decomposition and Lorentz polarization, but no absorption corrections were made.

Crystal Data. $C_{51}H_{43}N_{13}O_{12}S_6$, mol wt 1222, the system is tetragonal, space group $I4_1$, a = 36.05 Å, c = 11.44 Å, Z = 8, and V = 14 735 Å^3. The measured density is 1.41, while the calculated one is 1.07 for eight molecules in the cell. This important difference is attributed to the presence of solvent in the crystal and represents about 20% of the crystal mass.

Resolution of the Structure. Any attempt to solve the structure by the use of the Patterson function failed owing to considerable overlapping,[17] and no information could be obtained about the sulfur locations. Direct methods were then employed. Preliminary attempts to use the multisolution method[18] failed too: figures of merit were inconsistent and no phase expansion process could be accepted until five symbolic phases were introduced. The use of Riche's phase function,[19] following a careful analysis of the $\Sigma 2$ list, allowed a selection of the best starting set by trial and error calculations. An eight-dimensional phase function was then calculated with a 45° step in phase variations. It revealed that ten maxima could give good values for the initial starting phases, but the following phase refinements and the subsequent E maps failed to give correct atomic positions. At this stage, the normalized E factors were suspected to be imprecise at high θ values, and all the reflections with $I > 10$ were subsequently eliminated from the initial F_o list. Following a new normalization scheme, a second phase function was built with the same eight symbols, leading to a better contrast in figures of merit. Two solutions were then regarded as possible. The corresponding Fourier maps showed that translation of $a/2$ was the unique difference between them. The recycling procedure applied to the second E map, assuming the four highest peaks as sulfur atoms, revealed a long molecular chain with reasonable bond distances and angles. Several E cycles permitted to locate 20 atoms, assumed to be carbons, and five sulfur atoms. Subsequent F cycles resulted in the location of three thiazole rings surrounding the hydroxypyridine (fragment **4** in the degradative products, Figure 2), and later the complete structure. The refinement of the atomic positions and individual isotropic thermal parameters[20] led the R index to become 23% after two cycles. Sulfur atoms were then allowed to vibrate anisotropically while the rest of the molecule was kept isotropic. Several cycles of full-matrix least squares[21] gave a final R = 18% with all the reflections and a Cruickshank weighting scheme;[22] R was 13.3% when reflections with $\sin\theta/\lambda < 0.1$ are omitted (ca. 300 reflections). In view of the small number of data, no attempt was made to refine the whole molecule anisotropically. The general formula of nosiheptide obtained from this analysis is given in Figure 3; difference Fourier syntheses showed no peak greater than 1 e Å^{-3}, except one (height 2 eÅ^{-3}) situated at the center of the molecule and attributed to a water molecule. A $CHCl_3$ molecule was also located in a "bottleneck" of the structure packing, with a low occupation factor

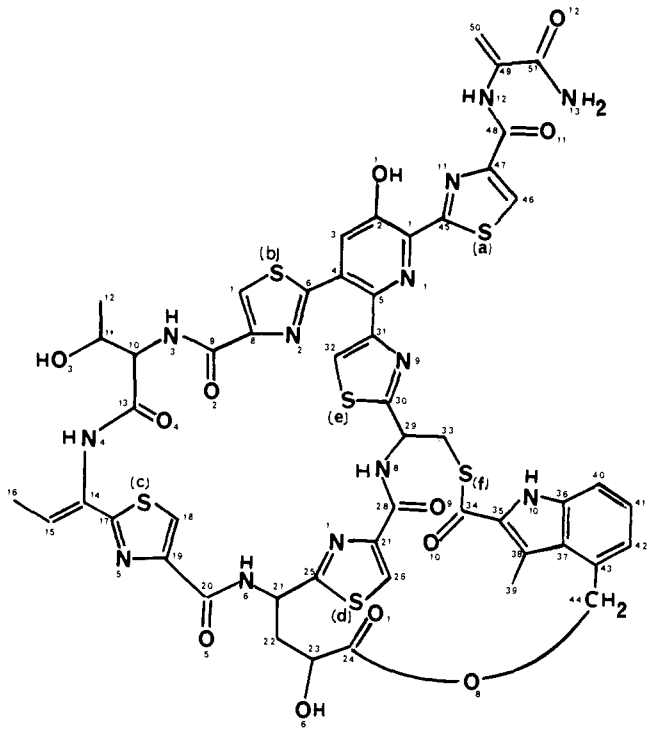

Figure 3. Formula of nosiheptide with the atomic numbering used in this study: macrocyclic ring is first labeled, followed by the indolic part and the upper extended chain. N and O atoms are separately numbered in the same way; S atoms are indicated by letters a, b, etc.

Table I. Positional Atomic Parameters ($\times 10^4$) and Isotropic Thermal Factors (in the Form $\exp(-B \sin^2 \theta/\lambda^2)$).

Atom	x	y	z	B^a	Atom	x	y	z	B^a
S(A)	709 (2)	1230 (2)	9471 (7)	7.7	C(11)	1564 (7)	−1255 (7)	4853 (26)	7.15
S(B)	2490 (1)	−34 (1)	8265 (7)	5.8	C(12)	1339 (6)	−935 (6)	4239 (22)	5.99
S(C)	942 (2)	25 (2)	5307 (7)	7.3	C(13)	1079 (6)	−1321 (6)	6385 (22)	6.66
S(D)	−65 (1)	−1872 (2)	4485 (7)	6.4	C(14)	500 (6)	−1658 (6)	5928 (24)	5.40
S(E)	819 (2)	−581 (2)	−543 (8)	9.4	C(15)	301 (7)	−1680 (7)	6840 (28)	7.77
S(F)	2157 (2)	−195 (2)	4200 (8)	7.3	C(16)	459 (10)	−1697 (10)	8180 (36)	13.32
O(1)	1797 (4)	895 (4)	11243 (15)	6.29	C(17)	362 (6)	−1707 (6)	4812 (23)	5.31
O(2)	2260 (4)	−1175 (4)	6602 (14)	5.80	C(18)	7 (6)	−1826 (6)	2981 (21)	6.57
O(3)	1458 (3)	−1589 (3)	4197 (13)	5.06	C(19)	346 (5)	−1667 (5)	2900 (19)	4.15
O(4)	941 (4)	−1072 (4)	7116 (16)	7.81	C(20)	477 (5)	−1550 (5)	1652 (20)	4.84
O(5)	312 (3)	−1611 (3)	748 (13)	5.54	C(21)	1003 (6)	−1229 (6)	658 (21)	5.65
O(6)	1194 (4)	−1968 (4)	−440 (16)	7.23	C(22)	1403 (5)	−1392 (5)	485 (19)	4.90
O(7)	1952 (7)	−2116 (7)	−428 (26)	14.52	C(23)	1414 (5)	−1812 (6)	396 (21)	5.37
O(8)	2044 (6)	−1784 (6)	924 (22)	10.79	C(24)	1851 (7)	−1945 (7)	348 (28)	8.65
O(9)	1089 (4)	392 (4)	1867 (17)	6.77	C(25)	1006 (5)	−848 (5)	643 (19)	4.33
O(10)	2167 (4)	−227 (4)	1863 (15)	7.83	C(26)	902 (8)	−158 (8)	125 (29)	9.26
O(11)	736 (3)	2132 (3)	12271 (12)	5.27	C(27)	1068 (5)	−250 (6)	1235 (22)	4.78
O(12)	2017 (4)	1813 (4)	13179 (14)	7.20	C(28)	1191 (6)	44 (6)	2055 (21)	5.30
N(1)	1260 (4)	683 (4)	8593 (14)	4.04	C(29)	1526 (6)	250 (6)	3867 (22)	5.76
N(2)	1855 (4)	−322 (4)	7791 (15)	4.45	C(30)	1385 (6)	198 (6)	5026 (20)	4.91
N(3)	1663 (5)	−985 (4)	6749 (16)	5.00	C(31)	1322 (6)	280 (5)	6923 (20)	4.34
N(4)	910 (4)	−1617 (4)	5943 (15)	4.48	C(32)	995 (7)	134 (6)	6875 (24)	7.30
N(5)	543 (4)	−1599 (4)	3854 (15)	4.61	C(33)	1941 (6)	237 (7)	3789 (25)	7.98
N(6)	839 (4)	−1380 (4)	1655 (17)	5.24	C(34)	2254 (6)	−400 (6)	2742 (22)	5.71
N(7)	1120 (4)	−623 (4)	1475 (14)	4.50	C(35)	2385 (6)	−782 (6)	2800 (22)	5.70
N(8)	1385 (5)	−50 (5)	3029 (17)	5.72	C(36)	2583 (6)	−1335 (6)	3644 (23)	5.53
N(9)	1543 (4)	304 (4)	6014 (17)	5.77	C(37)	2518 (6)	−1356 (7)	2411 (23)	5.81
N(10)	2475 (5)	−960 (5)	3919 (17)	6.25	C(38)	2405 (7)	−1025 (7)	1872 (29)	7.18
N(11)	1225 (4)	1366 (4)	10978 (15)	4.79	C(39)	2354 (8)	−941 (8)	601 (32)	10.57
N(12)	1331 (4)	1916 (4)	12603 (15)	4.56	C(40)	2724 (7)	−1572 (8)	4479 (28)	6.70
N(13)	2052 (5)	2252 (5)	14611 (19)	6.86	C(41)	2801 (7)	−1931 (7)	4061 (26)	8.00
C(1)	1373 (5)	845 (5)	9657 (19)	4.03	C(42)	2722 (6)	−2009 (6)	2786 (24)	6.10
C(2)	1688 (6)	737 (5)	10172 (19)	4.11	C(43)	2588 (7)	−1779 (7)	2034 (29)	7.57
C(3)	1904 (6)	468 (6)	9690 (21)	5.13	C(44)	2470 (8)	−1865 (8)	942 (32)	9.89
C(4)	1795 (5)	287 (6)	8689 (22)	4.35	C(45)	1134 (5)	1153 (5)	10028 (17)	3.75
C(5)	1459 (5)	416 (5)	8141 (19)	4.39	C(46)	647 (7)	1612 (6)	10431 (24)	7.26
C(6)	2014 (6)	−24 (6)	8154 (21)	5.11	C(47)	957 (5)	1622 (5)	11169 (19)	3.69
C(7)	2488 (5)	−462 (5)	7738 (19)	3.57	C(48)	988 (5)	1908 (5)	12055 (20)	4.37
C(8)	2148 (6)	−567 (6)	7505 (21)	5.20	C(49)	1452 (6)	2134 (6)	13512 (21)	5.15
C(9)	2019 (6)	−945 (6)	6850 (23)	5.84	C(50)	1267 (6)	2400 (6)	14182 (24)	7.09
C(10)	1510 (5)	−1291 (5)	6111 (21)	3.90	C(51)	1884 (6)	2052 (6)	13680 (24)	6.84

[a] For the sulfur atoms, the B factor is calculated from anisotropic factors.[20,23]

and high thermal parameters. These residual peaks were not introduced in the final refinement.

Results and Discussion

The complete structure of nosiheptide, given in Figure 3, obtained from this x-ray analysis is in fact closely related to the structures of thiostrepton (Figure 1, R_1-R_2 = Ile-Ala) and siomycin A (R_1-R_2 = Val-Deala), but some very interesting differences may be noted and will be presently discussed.

The main characteristic of the nosiheptide structure is a general tendency to be more oxidized than the thiostrepton one. No thiazoline, or hydrogenated quinaldic precursor nor tetrahydropyridine rings are present in nosiheptide. All the corresponding rings are unsaturated; however, this dehydrogenation is an important stabilizing factor governing the overall conformation and leads to fewer degrees of freedom in the thermal motion of the macrocyclic thiazole-containing ring. The restricted orientation of this ring is one of the reasons why a better precision in atomic positional parameters was obtained here (Table I). This was one of the major problems encountered in the thiostrepton resolution by Hodgkin et al.[3]

The bond distances and angles have been calculated and deposited.[24] The values are consistent with the proposed formula (Figure 3). The estimated standard deviations of bond lengths and angles are 2.10^{-2} Å and 2°, respectively. Figure 4 shows the ORTEP stereoscopic view of the molecule.

The assignment of the absolute configuration on the basis of anomalous scattering[25] by the sulfur atoms was not necessary, because of the presence of the L-threonine residue, which allows immediate attribution of the three other asymmetric centers in the structure. The S configuration is therefore deduced at C(21), C(23), and C(29), as well as a Z configuration for the C(14)-C(15) double bond (butyrine fragment). The numbering used in the present study is given in Figure 3.

The thiazolic macrocycle in nosiheptide and thiostrepton, despite large modifications of the original peptide residues, adopts orientations roughly similar to those of polypeptide or protein chains. More observations can be made along this basis.

The nosiheptide skeleton appears to be much simpler than the thiostrepton one; this is due (i) to the aromaticity of the central part of the molecule constituted by the pyridine ring, (ii) to the conjugation of the adjacent amide bonds with the thiazole rings. The 26-membered macrocyclic ring can be described as the joining of four twisted planes in an helix configuration, around the pyridine, leading to the formation of a large "figure of eight" firmly stabilized by van der Waals contacts at the L-threonine level. Such a spatial configuration has been observed, by NMR studies or x-ray crystallography, in macrocyclic peptide antibiotics, such as valinomycin, to promote their cation complexing properties;[26] however, no heavy metal complexes have hitherto been reported in the case of the thiostrepton class of products (in fact, such a complex with copper ions has been reported in the case of a closer par-

Table II. Best Planes[28] of the Nosiheptide Structure[a]

Plane	I		II		III		IV		V		VI	
	Atoms	Distance	Atoms	Distance	Atoms	Distance	Atoms	Distance	Atoms	Distance	Atoms	Distance
	C(5)	0.03	C(21)	−0.12	C(14)	−0.12	C(4)	0.11	C(33)	−0.24	C(1)	−0.09
	C(29)	0.28	C(25)	−0.03	C(15)	−0.40	C(6)	0.15	C(34)	0.17	C(2)	−0.29
	C(30)	0.05	C(26)	0.04	C(16)	−0.30	C(7)	−0.14	C(35)	0.16	C(3)	−0.23
	C(31)	0.01	C(27)	−0.02	C(17)	−0.01	C(8)	−0.03	C(36)	0.11	C(4)	−0.03
	C(32)	−0.04	C(28)	−0.00	C(18)	0.11	C(9)	0.05	C(37)	−0.02	C(5)	0.20
	C(33)	−0.27	C(29)	0.16	C(19)	0.05	C(10)	0.30	C(38)	0.03	C(6)	−0.02
	N(9)	−0.06	N(7)	−0.06	C(20)	−0.01	C(13)	0.02	C(39)	0.08	C(45)	−0.01
	S(e)	−0.02	N(8)	0.09	C(21)	−0.24	N(2)	0.07	C(40)	0.23	C(46)	0.04
			O(9)	−0.15	N(4)	0.27	N(3)	0.12	C(41)	0.11	C(47)	−0.06
	A = 0.3556		S(d)	0.04	N(6)	−0.08	N(4)	0.03	C(42)	−0.11	C(48)	−0.05
	B = 0.9166		A = 0.8871		O(5)	0.07	O(2)	−0.07	C(43)	−0.02	C(49)	0.00
	C = −0.1828		B = 0.055		S(c)	−0.00	O(4)	−0.32	C(44)	−0.52	C(50)	−0.16
	D = 2.217		C = 0.458		A = 0.373		S(b)	−0.01	N(10)	0.15	C(51)	0.29
			D = −2.74		B = −0.9222		A = −0.024		O(10)	0.16	N(1)	0.19
Torsional	I/II = 73°6′				C = −0.104		B = 0.434		S(f)	−0.05	N(11)	−0.12
angles	I/VI = 33°		II/III = 76°5′		D = −5.599		C = −0.901		A = 0.094		N(12)	0.03
					III/IV = 108°4′		D = 8.722		B = 0.324		N(13)	0.27
							IV/VI = 29°6′		C = −0.085		O(1)	−0.51
									D = −6.739		O(11)	−0.05
											O(12)	0.32
											S(a)	−0.01
											A = 0.389	
											B = 0.631	
											C = −0.671	
											D = 3.433	

[a] Distances are given in Å. The equations of the planes are of the form $AX + BY + CZ + D = 0$. (These planes are shown in Figure 5.)

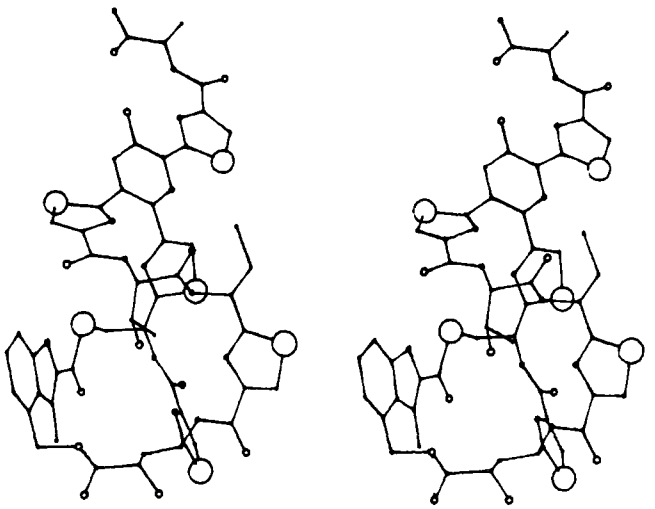

Figure 4. Stereoscopic view of the molecule along the z axis.

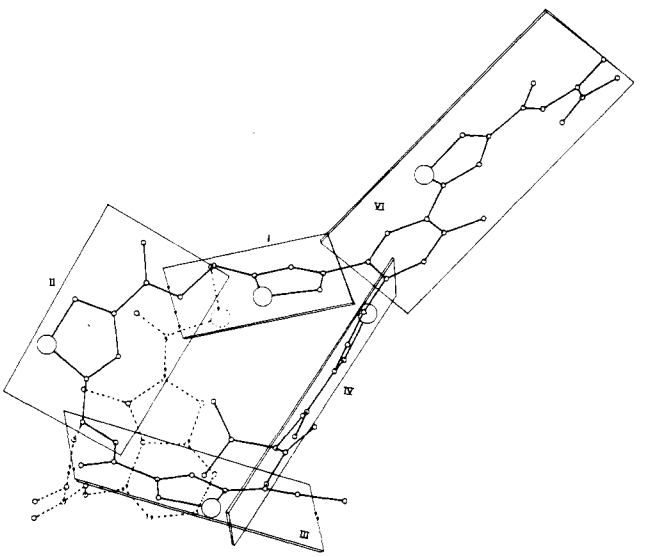

Figure 5. Schematic helicity of nosiheptide and mean plane description of the twisted macrocycle.

ent, bleomycin,[27] but the structure of this compound contains other different functional groups than the two present thiazole rings, especially two sugar moieties which could greatly enhance its chelating properties). The schematic junction of the planes in nosiheptide is given in Figure 5, and the mean deviations of the atoms from each plane, as well as the dihedral angles, are depicted in Table II.

A similar attempt to describe the macrocycle part of thiostrepton could not be easily realized, because of complex distortion. The solvent molecules appear to play a key role in the globular conformation. In both structures, a central residual peak is observed and attributed to a water molecule, strongly coordinated. This molecule has numerous short distances with the heteroatoms of the macrocycle, leading to a supplementary stabilization effect of its conformation.

In both the nosiheptide and thiostrepton structures, the thiazole-containing macrocycle is tightened by a base-containing bridge, but some differences can be noted in the composition of this bridge: in thiostrepton it consists of an hydrogenated quinoleine derivative (the quinaldic precursor) with an extended tetrapeptidic linkage (Ile-Ala-Deala-Ala) whereas the nosiheptide structure shows a methylindolic base, of similar biosynthetic origin, but closely bridged by ester linkages. No complete peptide residue can be recognized at all in these linkages but an unusual thiol ester function is observed. To our knowledge, this is the first thiol ester found in a macrocyclic antibiotic,[29] and this is probably the main special feature of nosiheptide as compared to the two other compounds of the class. The second ester linkage of the precursor, at the lower part of the molecule, is found to be the most thermally agitated part of the structure; if mean plane deviations (plane 5 in Table II) provide evidence of the double bond character of the ring

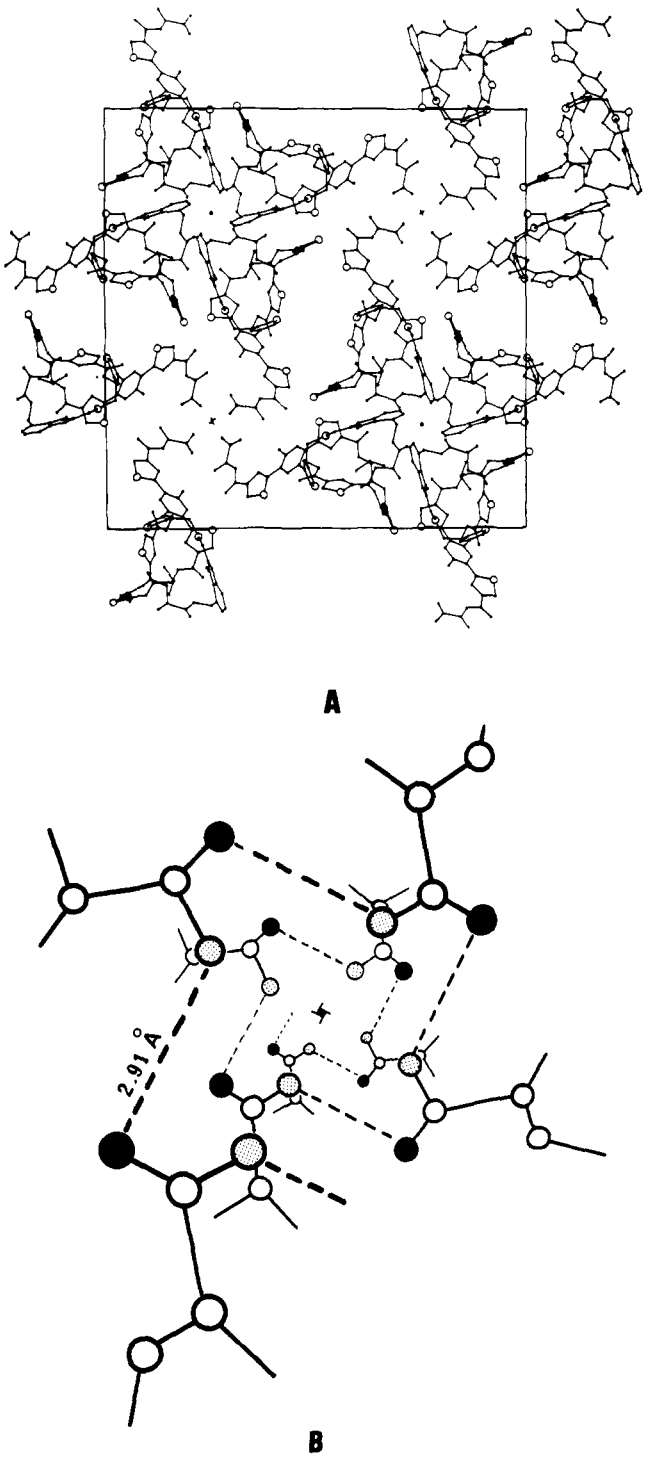

Figure 6. (A) Packing of the molecule, projected along the z axis. (B) Perspective view along one of the fourfold axis (¼, ¼, z), showing the hydrogen bond helix.

bonds, the C(44) atom is somewhat distorded from the idealized planarity. This could be attributed to the disorder encountered in this part of the molecule (see Table I for the high B values for C(24), C(44), O(7), and O(8) atoms). The corresponding plane 5 is not drawn on Figure 5, and the contained atoms of the precursor are dashed.

Both molecules of nosiheptide and thiostrepton contain an extended upper chain, topping the six-membered ring (tetrahydropyridine or pyridine). These chains consist of one or several dehydroalanine (Deala) residues. It is interesting to note that one of these residues, probably the one bonded to the thiazole ring, could not be identified properly in the degradation products: only two pyruvic fragments were reliably titrated in thiostrepton over the three present, while it was difficult to characterize by chemical means the single Deala residue of nosiheptide. This has not yet received an explanation but it could be attributed to a particular degradative pathway when this residue is bonded to a thiazole ring.

The intense conjugation which takes place in this upper part of the structure allows the complete skeleton to be observed. This can be compared to the difficulties encountered in the thiostrepton x-ray analysis[3] to identify the last Deala residue because of an important disorder, increasing with the distance from the thiostreptic part of the molecule. The presence of this supplementary residue has been confirmed by a recent ^{13}C NMR analysis.[5]

The mean plane corresponding to the 22 atoms of the almost entirely flat upper part of nosiheptide is also given in Table II (plane 6) and described in Figure 5.

Packing. No special hydrogen bond nor short distances can be observed between the different molecules of the cell, except the very particular helicoidal net of hydrogen bonds around two of the fourfold axis (O–N distances 2.9 Å) between the oxygen and the amide hydrogens of the Deala terminal residue of three different molecules, Figure 6B.

The poor hydrogen bond content of the crystal, as well as the large channels, apparently filled with solvent in a disordered fashion, which separate the molecules of the cell, are other similarities with protein crystals. The packing view of the molecule, projected along the z axis, is given in Figure 6A.

In conclusion, we have obtained the complete x-ray resolution of the molecular structure of the antibiotic compound nosiheptide. This study confirms that it belongs to the rare class of thiostrepton compounds. If thiostrepton was first considered as a curiosity, owing to its great complexity, it was recognized as a highly modified peptide-containing antibiotic by intense enzymatic dehydrogenation process. Many more differences than those found between thiostrepton and siomycin A (Figure 1) are presently observed in nosiheptide; particularly unexpected is the sulfur-containing bridge over the macrocycle ring. However, despite these large modifications, the nosiheptide molecule has nearly the same globular structure as the two former compounds, and it can be easily observed, as well as the helicoidal figure of the macrocycle, in the stereoscopic view, Figure 4.

Supplementary Material Available: Listing of observed and calculated structural factors, the bond lengths and angles (22 pages). Ordering information is given on any current masthead page.

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The Protonation Site of Vitamin B_1 as Determined from Natural-Abundance ^{15}N Nuclear Magnetic Resonance Spectra[1]

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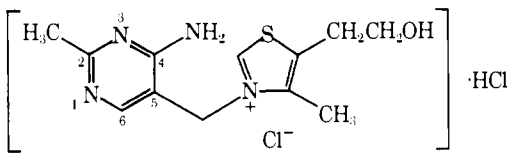
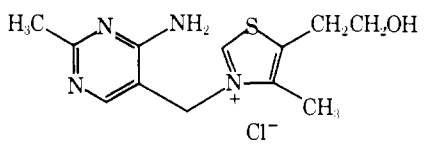
Abstract: Natural-abundance nitrogen-15 nuclear magnetic resonance spectroscopy has been used to determine the site of protonation in vitamin B_1. Selectively decoupling individual protons of the vitamin in water, D_2O, and ethylene glycol permitted assignment of the nitrogen resonances and thence the determination of the site of protonation, which was found to be N-1 of the pyrimidine ring.

Because of its important biological function, vitamin B_1 (thiamine hydrochloride, **1**) has been the subject of many



vitamin B_1 **1**

studies.[2] A problem that has never been solved is which nitrogen is protonated when thiamine (**2**) reacts with hydrogen



thiamine **2**

chloride. Common representations of the vitamin show protonation of the vitamin on N3 or the $-NH_2$ group.[3] These representations are used despite the spectroscopic studies of aminopyrimidines in acid solutions by Brown and co-workers,[4] from which it has been predicted that protonation of **2** would be most favorable on N1.[2] With the advent of practical natural-abundance nitrogen-15 spectroscopy, it should be possible to resolve the protonation question by examining the nitrogen chemical shift changes in going from thiamine to the conjugate acid.

Experimental Section

Proton spectra were taken using a Varian A-60 NMR spectrometer. Spectra in water and D_2O solutions used sodium 3-(trimethylsilyl)propanoate as reference, while those in ethylene glycol employed Me_4Si reference. No concentration dependence of the shifts was noted in dilute water solutions.

Nitrogen-15 spectra at 18.25 MHz were taken with a Bruker WH-180 NMR spectrometer using an external $H^{15}NO_3/H_2O$ capillary reference. Because of the poor solubility of thiamine in water, the protonation studies employed 0.9 M solutions in ethylene glycol with an internal Me_2SO-d_6 lock. Selective decoupling of vitamin B_1 was carried out in 2 M solutions in water with internal D_2O lock, or 2 M solutions in D_2O (solvent lock). The selective decoupling with thiamine employed a 1 M solution made up of 5.0 g of vitamin B_1, 13 mL of ethylene glycol, 1.0 mL of Me_2SO-d_6 (internal lock), and 0.75 mL of 18.3 M aqueous NaOH.

Results and Discussion

The ^{15}N NMR spectrum of thiamine hydrochloride in water solution taken at the natural-abundance level of ^{15}N without proton decoupling is shown in Figure 1. Four well-separated resonances were observed: singlets at 136, 166, and 208 ppm and a triplet at 268 ppm. The triplet (J = 91 Hz) can be assigned with certainty to the $-NH_2$ nitrogen (see Figure 1). If this nitrogen were protonated, its resonance should not be a triplet but a quartet. Furthermore, when the spectrum was run with broad-band proton decoupling, two of the nitrogen resonances had large nuclear Overhauser enhancements (NOE) and strong negative intensities as expected for *two* different nitrogens with attached protons. Finally, the nitrogen resonance which changed positions most prominently on addition of alkali was not the triplet at 268 ppm, but the broad singlet at 208 ppm (see Table I).

It should be noted that the addition of 1 equiv of base will result in deprotonation of **1** on the pyrimidine ring.[4] The pK_a of this titration is 4.85 (at 25 °C).[2] The second titration step of thiamine requires 2 equiv of base and occurs at pK_a =