STRUCTURE OF THE NATURAL ANTIBIOTIC IONOPHORE CP-54,883

JON BORDNER, PAUL C. WATTS and EARL B. WHIPPLE*

Central Research, Pfizer Inc.,
Groton, Connecticut 06340, U.S.A.

(Received for publication April 15, 1987)

The structural formula of the novel antibiotic ionophore CP-54,883 is deduced by systematic reduction from its ^{13}C and ^{1}H NMR spectra. The molecule consists of a polyether ring network and side chain terminated by an aromatic ring containing a phenoxy and two chlorine substituents. Based partly on an assumed analogy to corresponding regions of the similar structure nigericin-A_1, the configurations about the sixteen asymmetric carbons are also derived. A belated crystal structure determination confirms, with the exception of one configuration assumed from nigericin-A_1, the conclusions drawn, and shows that the anionic charge is in the phenoxy group, rather than the carboxylic acid function.

While the capability of NMR spectroscopy to provide sufficient information to permit the logical deduction of rather complex chemical structures has been demonstrated many times[1], before the advent of two-dimensional methods a virtual precondition for this task was the ability to resolve the ^{1}H spectrum. Shift correlation experiments in two dimensions effectively remove this restriction insofar as the determination of atom connectivities is concerned, and the deductive process can be reduced to an almost formal one. Moreover, with the higher dispersion available on modern instruments, one can make substantial progress toward the more ambitious goal of deriving a complete three-dimensional structure. The following example of a novel antibiotic ionophore[2] serves to illustrate many of these points.

Experimental

The experimental data on which the formal constructions were based are contained in columns 2, 3, and 5~8 of Table 1, using a format that modern NMR data systems should be able to generate automatically. They were obtained on a Bruker WM-250 spectrometer equipped with an Aspect-3000 data system, using 50 mg samples dissolved in 0.5 ml of chloroform-*d* in a 5-mm dual $^{13}C/^{1}H$ probe. The ^{13}C peak registration and distortionless enhancement by polarization transfer (DEPT)[3] editing in columns 2 and 3 of Table 1 were used to derive the data summary and structure units in Table 3 from the chemical analysis in Table 2, there being no parent ion from the electron impact mass spectrum (EI-MS). These data were also used to generate, using nominal chemical shift ranges and parity rules, the information in columns 4 and 9 of Table 1. The latter describes the number of units to which the table entry is connected, and the former the number of connections through singly bonded oxygen units.

Since the ^{1}H and ^{13}C shifts can be correlated accurately through two-dimensional experiments[4], it is no longer advantageous to index both spectra independently. These units are accordingly indexed in order of ^{13}C chemical shift in Table 1, and the shifts of the attached protons, measured directly from the bonded ^{1}H, ^{13}C shift-correlated spectrum, entered in column 5 of the Table 1. These values were then used to cross index the correlation peaks in the correlation spectroscopy (COSY)[5] spectrum (column 6), and in heteronuclear shift-correlated spectra with delays adjusted to reflect long-range spin couplings of the order of 5 Hz (column 8). The data in columns 6 and 8 were then used to build the sequences in Table 4. While straightforward in principle, the interpretation of the latter experi-

Table 1. NMR data and assignments.

Index	^{13}C Shift[a]	No. of proton	No. of oxygen	^{1}H Shift	^{1}H-^{1}H Connections	Isotope shift[b]	^{13}C-^{1}H Connections[c]	No. of connections	T_1[d]	Assignment
1	180.9	0	1				37	2	3.90	1
2	173.1	0	1				15	2	7.50	25
3	158.9	0	1				5	3	9.00	31
4	130.3	0	0				5, 36	3		27
5	130.2	1	0	7.20				2	0.38	29
6	125.7	0	0				36, 5	3		26
7	121.0	0	0				5	3	8.30	30
8	116.8	0	0				5, 36	3	8.00	28
9	108.7	0	2				39, 38, 25	4	5.40	13
10	86.9	0	1				33, 14	4	3.90	20
11	86.3	1	1	4.07	31		34, 31	3	0.38	17
12	85.3	0	1				34, 11	4	3.80	16
13	79.2	1	1	3.29	28		38	3	0.38	11
14	78.8	1	1	3.78			41, 20	3	0.37	22
15	77.2	1	1	5.13	41, 14		41, 14	3		23
16	75.1	1	1	3.20	14	−0.11	33, 14	3	0.40	21
17	73.9	1	1	3.85	29, 32		35	3	0.38	7
18	72.1	1	1	3.79	22		40	3	0.38	3
19	61.1	1	1	4.33	28			3	0.35	9
20	60.0	3	1	3.40			14	1	1.71	22-OCH_3
21	59.0	3	1	3.31			13	1	1.63	11-OCH_3
22	42.4	1	0	2.42	18, 37			3	0.40	2
23	40.5	2	0	1.63	24			2	0.21	15
				1.63	23, 24					
24	38.3	1	0	2.30	39, 23		39	3	0.38	14
25	36.4	1	0	1.68	38		38	3	0.38	12
26	36.0	2	0	2.10				2	0.32	19
				1.77	26					
27	34.7	2	0	1.68	32			2	0.22	5
				1.40	27, 32					
28	31.6	2	0	2.19	13, 19			2	0.21	10
				0.96	28, 13, 19					
29	29.5	2	0	2.21	17, 19			2	0.20	8
				0.99	29, 19, 17					
30	28.3	1	0	1.80	40		40	3	0.40	4
31	27.9	2	0	2.00	11			2	0.28	18
				1.55	31, 11					
32	27.0	1	0	2.19	35, 27		35	3	0.39	6
33	19.9	3	0	1.06			16	1	0.53	20-CH_3
34	19.0	3	0	1.24				1	0.53	16-CH_3
35	17.9	3	0	0.73	32			1	0.32	6-CH_3
36	17.3	3	0	2.20				1	1.80	27-CH_3
37	13.6	3	0	0.98	22			1	0.54	2-CH_3
38	12.9	3	0	0.92	25			1	0.81	12-CH_3
39	12.7	3	0	0.90	24			1	0.69	14-CH_3
40	11.9	3	0	0.95	30			1		4-CH_3
41	11.9	3	0	1.32	15		14	1		23-CH_3
				3.10	16					

[a] In ppm from TMS in $CHCl_3$-*d* solution.
[b] Entered only for lines split by H_2O - D_2O (1 : 1) addition.
[c] Proton unit.
[d] By inversion-recovery.

ments in crowded spectra was quite laborious, requiring a repetition of the preliminary assignments of the structure units in benzene-d_6 solution in order to circumvent ambiguities caused by overlap of correlation peaks in the two-dimensional maps from a single solvent.

Some of the steps in the formal construction have been programmed in RS-1 code on a VAX computer.

Structure Units

The formal procedure consists of first establishing a complete atom set (molecular formula) and then building it stepwise into larger units until such a time that it becomes more convenient to switch to a strategy of elimination from an enumerable set of alternative structures. In the absence of a reliable parent ion in the mass spectrum, it began with the chemical analysis (Table 2) and a ^{13}C count of n=41 to estimate a molecular weight of M=845 based on the analytical expression:

$$p_i = 100\, n_i A_i / M$$

and then proceeded to derive the remaining atom counts as shown in Table 2. The molecular formula of $C_{41}H_{61}O_{12}Cl_2Na$ derived from the chemical analysis meets the usual checks and balances applied to such problems, with the discrepancies in hydrogen and oxygen indicating about 0.3 equivalents of occluded water in the analytical sample. The double bond equivalent is ten, and the edited DEPT[3] spectrum gives 59 protons attached to carbon, leaving two hydroxyl groups in addition to the carboxylate-ONa function.

While not yielding a molecular ion, the EI-MS does provide a valuable shortcut in the form of a fragment cluster of principal mass *m/z* 202.9719, corresponding to the elemental composition $C_8H_5O_2Cl_2$, and consisting of an aromatic ring containing the two chlorine and one each phenoxyl, methyl and carbonyl substituents. By thus relegating the chlorine substituents to a known region of the molecule, the remainder of the problem could be treated as one involving only carbon, hydrogen and oxygen atoms, where ^{13}C chemical shift ranges can be used reliably to complete the set of structure units in Table 3. Since there are two carbonyl units (lines 1 and 2) and three incipient hydroxyls, the number of oxygen links is seven, and there must be a total of 2(7)+3=17 single bonds between oxygen and carbon (excluding peroxides). Two are to the carbonyls, and there are twelve alkoxy units, leaving three in the overlapping ranges of carbonate plus vinyloxy or vinyl plus ketal. Parity thus requires at least one vinyloxy unit, and the ^{13}C chemical shifts allow only one (line 3). The total number of lines in the vinyl range is odd, so that is must encompass one ketal unit (line 9). The molecule thus contains five double bonds and five rings, one of which is known independently to be aromatic. Of the seven ether oxygen, two are in methoxyl groups and one connects the aromatic ester, leaving one for each of the four rings remaining to be closed in the polyether ionophore†.

Table 2. Chemical analysis and molecular formula.

Atom	A_i	p_i	n_i
C	12.010	58.29	41[a]
H	1.008	7.35	61.60
N	14.007	0.12	0.07
Cl	35.453	8.11	1.93
Na	22.99	2.72[b]	1[c]
O	15.999	23.41[d]	12.36

[a] From ^{13}C count (Table 1).
[b] Reverse calculation.
[c] Assumed.
[d] Analytical defect less calculated Na.

† In molecules containing only carbon, hydrogen and oxygen, it is usually possible to relate the numbers of ether oxygen and rings prior to determining the molecular formula, since one can express both the double bond equivalent (given the DEPT editing) and the number of single bonded oxygen receptors in terms of the number of hydroxyl groups, and cancel this unknown term.

Table 3.

(A) NMR summary.

Lines	Shift range	Type	No.	No. of attached protons			
				0	1	2	3
1~2	173~181	Carboxy	2	2			
3~8	116~159	Alkene	6	5	1		
9	109	Ketal	1	1			
10~21	59~87	Alkoxy	12	2	8		2
22~41	11~43	Alkyl	20		5	6	9
Total			41	10	14	6	11

(B) Structure units.

Unit	Type	No.
OH[a]	Terminal	3
Cl	Terminal	2
CH_3	Terminal	11
C=O	Link	2
O	Link	7
CH_2	Link	6
=CH	Link	1
C=	Branch	5
CH	Branch	13
C	Intersection	3

[a] Includes carboxylate-ONa.

Table 4. Sequences.

Sequence	Description[a]
A	O ‖ –2–O–15(41)–14(O20)–16(OH)–10(33)– |
B	| –27–32(35)–17–29–19–28–13(O21)–25(38)–9–24(39)–23– | | |
C	O ‖ –30(40)–18–22(37)–1(ONa) |
D	–12(34)–11–31– | |

[a] Vertical open connectors denote attachment to oxygen.

Sequences

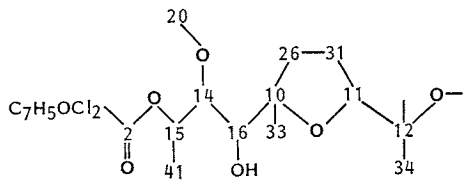
Using the information in columns 6 and 8 of Table 1, the structure units in Table 3 are first grouped into clusters of connected units, and then into the sequences shown in Table 4. In the second reduction, it is assumed that COSY correlations between protons on saturated carbons occur over a maximum of three bonds, and therefore serve, apart from the trivial connections between methylene proton pairs, to directly connect units. The ^{13}C-^{1}H correlations in column 8 of Table 1 occur over the same ranges, but in this case do not yield immediate sequences. Methyl proton multiplicities and isotope shifts of ^{13}C lines on replacement of the labile protons with deuterium provide the additional information needed for the constructions. In the case of the aromatic ring, there is insufficient information

to reduce the cluster to a sequence, but one can deduce that the ring is attached to unit 2 at the beginning of sequence A in Table 4, reducing it to a short link with a long side chain.

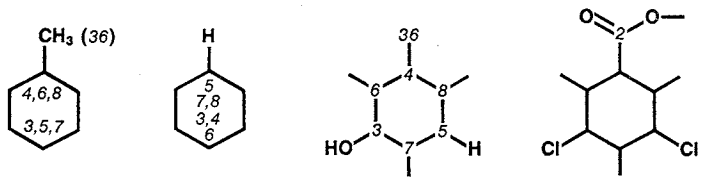
Relaxation Times

In addition to its immediate use in optimizing experiments, knowledge of the spin-lattice relaxation times can provide important insight into the structure[6]. The methine carbon T_1 values are all very nearly equal, implying that the molecule, including the side chain in sequence A, rotates as an isotropic rigid body. This is not unexpected, since for a polyether of this size to function as an ionophore, some of the functional groups in the side chain must participate in the ligand bonding. The methylene carbon relaxation times, with two exceptions, are about half as long as the methine carbon T_1 average, again conforming to the rigid body model. Methylene carbon 31 at the end of sequence D and carbon 26 constituting the one unit not assigned to a sequence both have anomalously long relaxation times, and one of several otherwise ambiguous interpretations of the COSY spectrum is that these units are connected. It is also necessary by now that, in the absence of small rings (as indicated by normal $^1J_{C,H}$ couplings), virtually all the units in sequences B through E be incorporated into the four remaining rings. To connect units 26 and 31 in such a way that only they can undergo librational averaging implies a [5] ring, which by virtue of oxygen substitution on unit 11 is a furan ring. Saturation of the methyl proton singlet from unit 33 at the end of sequence A gives, in addition to some subsequently useful nuclear Overhauser polarizations in the side chain, a 5%-enhancement of the methine proton on unit 11 in sequence D and a small enhancement of the 1.77 ppm proton in methylene unit 26, completing the assemblage AD' below.

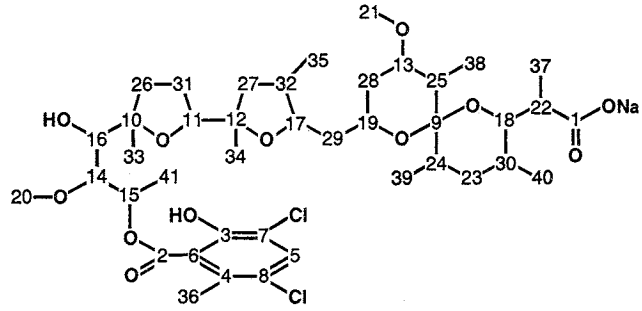
The methyl carbon relaxation times, with three exceptions, are in the range between one-third and three times the methine carbon value, corresponding to the model for internal rotation about a single axis attached to a rigid body. Two of the exceptions are the methoxyl carbons, which have an extra degree of freedom, and consequently longer T_1's. The third is the methyl group on the benzene ring, whose T_1 is 57% above the maximum allowable value for the model, hence requiring that the benzene ring as a whole undergo an additional fast motion. On the other hand, the relaxation time of the protonated aromatic carbon is normal. This permits the positioning of substituents on the aromatic ring to be completed as follows. Analysis of the ^2J and ^3J couplings between the aromatic carbons and the substituent protons serves to group the ring carbons into the two independent sets **1a** and **1b** below, whose combination gives the assignments in **1c**. The problem is thus reduced to one of three possible superimpositions of **1d** onto **1c**. In order for the aromatic methyl group to undergo an internal motion of the ring which does not affect the ring proton, the C-H bond must be parallel to the internal axis of the motion; *i.e.*, the unit must be *para* to the bond by which the ring is connected to the structure. The carboxy carbon must therefore be attached to carbon 6, which completes the aromatic ring system.



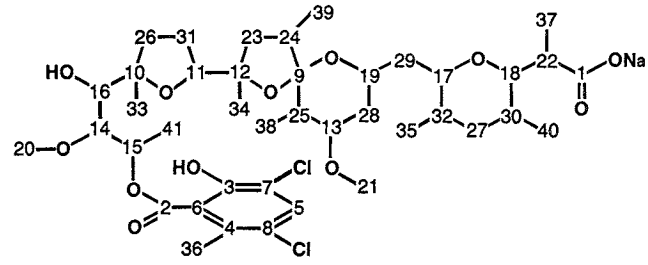
AD'



1a 1b 1c 1d



2



3

Structural Formula

With the AD' assemblage derived above, the connectivity problem is reduced to attaching the links C and AD' to two of the six open bonds on sequence B, followed by the closure of three rings, the last of which is trivial. Of the five bonds to be closed in the process, two are between carbon units, and the other three insert ether oxygens. Since one C, C bond terminates on each of the links, the alternatives are to connect both to sequence B by this means, which can be done in either of two possible ways, or to connect them together and then form an [11] carbocyclic ring within unit B. Reasoning on the basis of a polyether ionophore structure with a continuous carbon chain, one has the two choices presented by the first alternative, followed by $4!/2!2!=6$ possible ways of closing two rings through oxygen links onto spiroketal unit 9, which logically completes the structure. One of these results in an oxetane [4] ring between units 17 and 19 in sequence B, which can be excluded by the absence of abnormally large $^{1}J_{C,H}$ coupling constants. There are hence ten possible, and only two reasonable, polyether ionophore structures remaining. These are shown in **2** and **3**.

Free Acid Form

To make this final choice required a third perturbation of the ^{1}H shifts in order to circumvent ambiguities from overlapping $^{3}J(H, H)$ correlation peaks, accomplished this time through the spectrum

Fig. 1. Nigericin-A_1.

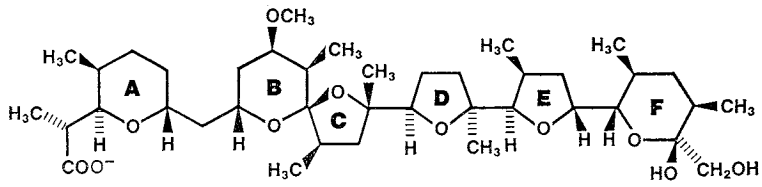
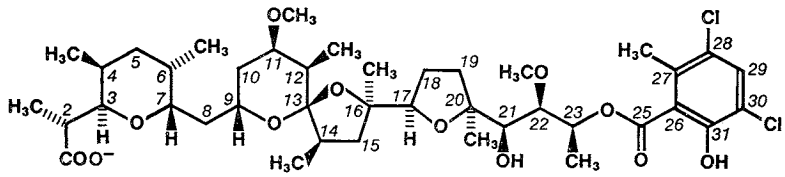


Fig. 2. CP-54,883.



of the free acid form of the ionophore in chloroform-*d* solution. This spectrum gave a resolved proton band from unit 30 at the end of sequence C in Table 4, which enabled its connection to the methylene protons on unit 27 at the beginning of sequence B to be singularly established in the COSY spectrum. This connection is unique to structure **3**, and therefore completes the assembly.

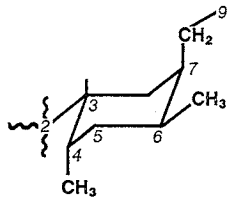
Configurations and Conformation

Since the topographic structure **3** contains sixteen asymmetric carbons, the structure set has only been reduced to 65,536 members. However, three of the rings (B, C and D) correspond to the known[7] antibiotic nigericin-A_1 (Fig. 1), and a fourth (A) differs only by one methyl substituent. It therefore seems reasonable to assume, as a working model, stereochemical analogy to nigericin-A_1 in the three common rings which contain half the asymmetric centers. A few preliminary checks on ^3J(H, H) couplings resolvable in the 250 MHz spectrum verify the analogy to the extent that the B-ring has a chair conformation in which C-8 is equatorial (9-H axial) and the 11-methoxyl group is axial. Since 11-H is equatorial, the position of 12-H is undefined by ^3J(H, H) coupling constants, but saturation of the axial proton 10-H_b gives a nuclear Overhauser polarization of 12-H, showing that it is also axial. The relative configurations at positions 9, 11 and 12 in the B-ring thus correspond to nigericin, along with the ring conformation. Also, the previously mentioned nuclear Overhauser enhancement of 17-H by the 20-methyl group requires correspondence of the relative configurations within the D-ring. The configurations of the three centers in the C-ring, and that of ring D relative to B, have not been established experimentally, but are assumed by analogy.

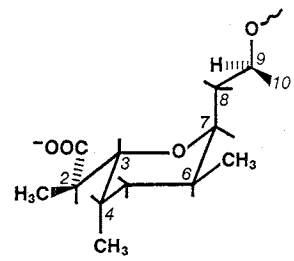
A-ring

The configurations in the A-ring were incorrectly assigned until ^{1}H data at 500 MHz became available†. At 250 MHz, the protons on positions 2, 3, 5b, 7, and the 6-methyl group were the only resolvable ones in the region of interest. Under the usual assumption that the shift of the higher field methylene proton on position 5 resulted from an axial position, its observed hyperfine splitting into a 13.4-Hz doublet of 3 Hz triplets would indicate that 4-H and 6-H were both equatorial. At 500 MHz, the bands from 4-H and the low-field 5-H_a proton were also resolved in benzene-d_6 solution, and the

† Provided during the course of NMR instrument demonstrations by Drs. GEORGE GRAY (Varian Associates), JERRY DALLAS (General Electric) and WOLFGANG BERMEL (Bruker Instruments, Inc.).



4a



4b

Table 5. Observed equilibrium NOE polarizations involving side chain protons.

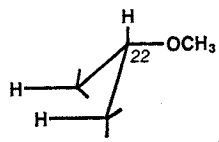
Observed nucleus	$f_d(s)$[a]							
	Saturated nucleus							
	23-CH_3	23-H	22-H	21-H	21-OH	22-OCH_3	20-CH_3	16-CH_3
23-CH_3	—	0.084		0.019	0.017	0.026		
23-H	0.132	—	0.058	0.148	0.015			0.044
22-H	0.023	0.068	—	0.037	0.026	0.095	0.123	0.007
21-H	0.020	0.118	0.021	—				0.024
21-OH	0.043	0.008	0.016	?[b]	—	0.024	0.076	
22-OCH_3	0.041		0.103		0.024	—	0.075	
20-CH_3			0.049		0.038	0.039	—	
16-CH_3		0.025						—

[a] s: Saturated nucleus, d: observed nucleus. See ref 11.
[b] Unmeasurable due to proximity of signals.

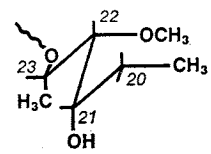
band from 6-H overlapped only one other from 10-H, which caused no confusion. 5-H_a is a 13.4-Hz triplet of 4.4 Hz doublets, which is inconsistent with its assignment to an equatorial position. Decoupling 4-H and 6-H establish that the large vicinal coupling is to 6-H, which completes the assignment of the A-ring as shown in **4a** above. Furthermore, the protons on positions 7 and 9 are *trans* to different methylene protons on position 8, and this information, together with the constraint that the ionophoric property requires that the carboxyl group be situated around the ligand cavity, is sufficient to force the conclusion that the configurations at positions 7, 4, 3 and 2 must either all or none correspond to those in nigericin-A_1. Reasoning by analogy that all do, the arrangement in **4b** results.

Side Chain

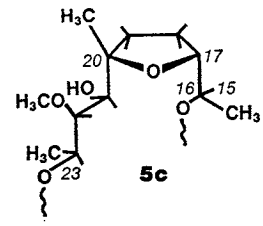
22-H is weakly coupled to both 21-H (1.4 Hz) and 23-H (0.9 Hz), which implies *gauche* conformations about the (21 and 22) and (22 and 23) single bonds. The strong nuclear Overhauser effect (NOE) between 21-H and 23-H (Table 5) further implies that these protons be near their distance of closest approach, establishing the side chain conformation approximately as shown in **5a** below. The methyl group on position 20 gives no Overhauser enhancement of 21-H, but does give sizeable enhancements of 22-H, the 22-methoxyl group, and the 21-hydroxyl proton, establishing the relative configurations at positions 21 and 22 and constraining the torsion angle about the (20 and 21) bond to two nominal values depending on the choice of configuration at position 21. The 23-methyl group gives no polarization of 22-H, but does enhance the 22-methoxyl and 21-hydroxyl signals by small amounts. The only staggered chain model that is consistent with these results is that in **5b**, or its mirror image.



5a



5b



5c

In nigericin-A_1, five oxygen atoms are at distances (2.3~2.7 Å) indicative of ligand bonding to the metal atom, and only four of these (carboxylate, 11-methoxy, C and D ring ethers) are present in CP-54,883. The rigidity of the side chain shown by the T_1 measurements suggests that at least one of its functional groups is involved in ligand bonding to the metal ion, and this is reinforced by the observation that ^{13}C shifts in excess of 1 ppm between the salt and acid forms are confined to the regions near both ends of the molecule plus a few interior carbons at positions directly attached to ligand oxygens in nigericin-A_1. A weak (2%) but reproducible long range nuclear Overhauser polarization of the aromatic proton by the 11-methoxyl group is also observed. Since the 11-methoxyl oxygen is one of the ligands, the NOE implies that the aromatic ring terminating the side chain is folded into a limited space surrounding the ionophoric cavity. Molecular models incorporating these various features led us to initially postulate the final arrangement shown in **5c**, and further suggest that the 25-carbonyl oxygen may function as the extra ligand, and that the 31-OH group forms a hydrogen bond to the 1-carboxylate oxygen not involved with the metal ion.

A subsequent crystal structure (see Note in Proof) confirms all the deductions made directly from the NMR evidence, but shows that one of the configurations assumed by analogy to nigericin-A_1 (position 16 in the C-ring) is inverted. This change was in fact indicated by the data available; *i.e.*, the methyl group on position 16 gives weak, but reproducible polarizations of 23-H and 21-H in the side chain (the latter in a D_2O exchanged sample not included in Table 5), but no discernible polarization of 22-H whose signal overlaps broader ones from 3-H and 17-H. The explanation for the small nuclear Overhauser polarization of 22-H relative to 21-H and 23-H by the 16-methyl protons is that the oxygen and methyl substituents on position 16 in **5c** are permuted.

Note in Proof

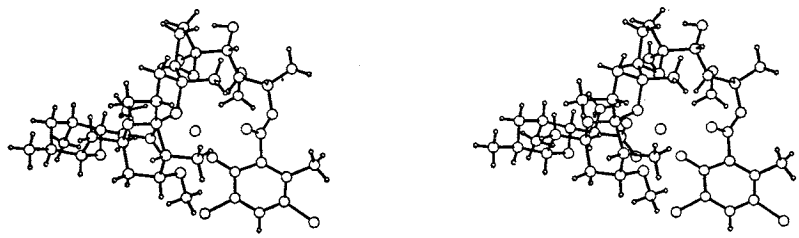
Following the submission of this manuscript, a crystal structure of CP-54,883 was finally completed (J. BORDNER), the details of which are attached as Appendix A. The crystal structure shows that the configuration at position 16 in the C-ring, originally assigned by analogy to nigericin-A_1, in fact differs, while the rest of the structure, including the identification of the ligands, is confirmed. The crystal structure further shows that the anionic charge in CP-54,883 resides on the phenoxide oxygen, with a normal carboxylic acid function at position 1. The phenoxide, both carbonyl, 11-methoxyl, and ether oxygens from the C and D rings surround the sodium ion in a distorted octahedral array, at distances ranging from 2.32 to 2.64 Å. The carboxylic acid proton also forms an internal hydrogen bond with the phenoxide oxygen atom.

Appendix A

X-Ray Crystal Structure

A representative crystal grown by slow evaporation from hexane solution was surveyed and a 1 Å data set (maximum $\sin\theta/\lambda=0.5$) was collected on a Nicolet R3m/μ diffractometer. Atomic scat-

Fig. 3. CP-54,883 Na salt.



tering factors were taken from the International Tables for X-ray Crystallography[8]. All crystallographic calculations were facilitated by the SHELXTL[9] system. All diffractometer data were collected at room temperature using copper radiation (λ=1.5418 Å).

A trial structure was obtained by direct methods, and refined routinely. Hydrogen positions were calculated wherever possible. The methyl hydrogens and hydrogens on oxygens were located by difference Fourier techniques. The hydrogen parameters were added to the structure factor calculations but were not refined. The shifts calculated in the final cycle of least squares refinement were all less than 0.1 of their corresponding standard deviations. The absolute configuration was determined using the method of IBERS and HAMILTON and was correct at the 99.5% level of confidence[10]. The final R-index was 0.069. A final difference Fourier revealed no missing or misplaced electron density.

The refined structure was plotted using the SHELXTL plotting package (Fig. 3). Coordinates, anisotropic temperature factors, distances and angles have been forwarded to the Crystallographic Data Center, Cambridge, England.

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