Potential Anticancer Agents, XI. X-Ray Structure Determination of Acantholide [1]

H. Lotter and H. Wagner

Institut für Pharmazeutische Arzneimittellehre der Universität München, Karlstr. 29, D-8000 München 2

and

A. A. Saleh, G. A. Cordell, and N. R. Farnsworth

College of Pharmacy, University of Illinois at the Medical Center, Chicago, Illinois, USA

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Acantholide, *Acanthospermum glabratum,* X-Ray Structure, Cytotoxic Principle

The X-ray structure of acantholide, a melampolide sesquiterpene lactone from *Acanthospermum glabratum* (DC.) Willd. was determined by the aid of direct methods. The compound was cytotoxic but displayed no antitumor activity. Evaluation of the X-ray and NMR data indicated that the same conformation exists in the solid state as in solution.

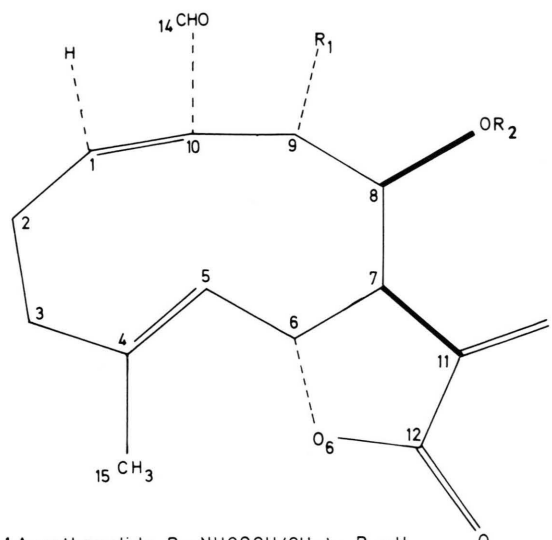
Introduction

In a previous paper [2] concerning the isolation of anticancer principles from the Tanzanian plant *Acanthospermum glabratum* (DC.) Willd. (Asteraceae), we described the isolation and structure elucidation of acanthamolide, a novel melampolide amide (**1**), see Fig. 1. The plant proved to be a rich source of melampolide derivates having the common structural features of a *cis*-1.10 unsaturated aldehyde, an α,β-unsaturated lactone and a 4,5-double bond substituted by a methyl group at the 4-position. The results of these studies will be presented elsewhere, for this paper is solely concerned with the structure of one member of this series, acantholide.

Results and Discussion

Acantholide was obtained by column chromatography of the benzene soluble fraction of *Acanthospermum glabratum* and crystallized as prisms, m. p. 208 °C, from methanol. The mass spectrum indicated a molecular ion at *m/e* 348, analyzing for $C_{19}H_{24}O_6$, and from the IR spectrum hydroxyl (ν_{max} 3500 cm^{-1}), α,β-unsaturated lactone (1780, 1760 cm^{-1}) saturated ester (1725 cm^{-1}) α,β-unsaturated carbonyl (1690, 1660 cm^{-1}) and olefinic (1625 cm^{-1}) groups could be deduced.

Some of the main features of the proton NMR spectrum included two, three proton doublets ($J = 7.0$ Hz) at 1.15 and 1.10 ppm an olefinic methyl group at 1.97 ppm, two doublets ($J = 3.0$ and 3.5 Hz) at 5.64 and 6.33 ppm for the *exo* protons of an *exo*-methylene moiety and a one-proton doublet ($J = 1.8$ Hz) at 9.41 ppm for an aldehyde on a *cis* double bond.



1 Acanthamolide, R_1 = NHCOCH $(CH_3)_2$, R_2 = H
2 Acantholide, R_1 = OCOCH $(CH_3)_2$, R_2 = H, 4,5 - trans
3 $R_1 = R_2$ = $OCOCH_3$

Fig. 1.

Reprint requests to Prof. Dr. H. Wagner, Inst. für Pharmazeutische Arzneimittellehre der Universität München, Karlstr. 29, D-8000 München 2.

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Comparison with the spectral data of acanthamolide (**1**) [2] indicated a very close structural similarity and in particular suggested a germacradienolide nucleus substituted with an isobutyroxy and a hydroxy group.

The presence of a secondary hydroxy group was substantiated when acantholide formed an acetate derivative, (M^+ 362) in which acetylation *and* ester exchange had taken place. Confirmation of this came from the proton NMR spectrum which indicated the presence of *two* acetate groups at 2.02 ppm and an absence of methyl doublets in the region of 1.1 ppm. More significantly from a structural aspect a proton from the region of 5.0 ppm had been shifted downfield to 6.65 ppm and exhibited coupling constants of 8.8 and 1.6 Hz. In addition a secound doublet of doublets ($J = 8.9$ and 1.75 Hz) was now apparent at 5.24 ppm. This signal had been obscured beneath a four proton complex in the region 4.75 – 5.37 ppm in the 60 MHz spectrum of acantholide. These data indicated that the substituents (hydroxy and butyroxy) were vicinally disposed at the 8- and 9-positions. It remained to deduce the relative location of these groups and determine the stereochemistry at each asymmetric centre.

Previous work [2 – 4] on closely related compounds has established that an H 8 proton having an α-stereochemistry is markedly deshielded compared with what might reasonable be expected for such a proton. Thus, in acanthamolide (**1**) [2] this proton appeared at 4.41 ppm and in related compounds bearing an acyloxy function at the 8-position, chemical shifts in the range 6.3 – 7.0 ppm were observed [3 – 7]. As noted by Herz [3] this deshielding is caused by coplanarity of the C 14 carbonyl and H 8. Consequently, C 9 is substituted by the isobutyroxy group and C 8 by the hydroxy group. In "acantholide acetate" therefore, the signals at 5.24 and 6.65 ppm may be assigned to H 9 and H 8 respectively.

The coupling constants of H 8 and H 9 could also be used to deduce the relative stereochemistry at C 7, C 8 and C 9. Coupling of the C 14 (aldehyde) proton is in itself an unusual phenomenon, and as shown with acanthamolide [2] is not due to coupling with the C 1 proton, but rather with the C 9 proton in α,β-configuration. Dreiding models indicate that such an arrangement produces a rather beautiful W relationship thereby permitting substantial ($J = 1.8$ Hz) long-range coupling to occur. This coupling was verified by double-resonance studies, and served to substantiate the chemical shift assignments made for H 8 and H 9 indicated above.

A coupling constant of 8.6 Hz observed between H 8 and H 9 in acantholide acetate suggested [4] that these protons were *trans*-disposed to each other and consequently that the hydroxy group at C 8 had a β-stereochemistry. Since H 8 exhibits a small ($J = 1.75$ Hz) coupling with H 7 it could be deduced that H 7 and H 8 are *cis*-oriented. Such an assignment is in agreement with prior data for this series of compounds. The C 7 – C 11 bond in all known sesquiterpene lactones invariably has the stereochemistry, and since germacradienolides obey Samek's rule [8] the C 6 proton is also β.

The 60 MHz spectrum of acantholide failed to resolve the complex pattern in the region 4.75 – 5.37 ppm for H 5, H 6, H 8 and H 9, but at 270 MHz, this region was clarified. A doublet of doublets ($J = 10.1$, 10.4 Hz) at 5.20 ppm could be assigned to the C 6 proton with H 5 as a slightly broadened doublet ($J = 10.4$ Hz) at 4.87 ppm. The C 8 and C 9 protons were assigned based on the magnitude of the coupling constant of H 9 with H 14 to be at 5.03 and 5.31 ppm respectively. These data confirmed that acantholide was substituted at C 8 with a hydroxy group and at C 9 with an isobutyroxy group, and that the stereochemistry of these groups was probably 8β and 9α-respectively. It could also be suggested that H 5 and H 6 had a similar spatial relationship to H 6 and H 7 and that the C 4, C 5-bond was *trans*. At this point therefore attention was focused on a determination of the complete structure by X-ray crystallography.

Initial efforts in this area were devoted to a related compound, acanthospermolide [7], but were unsuccessful for reasons which are at present obscure. Subsequent studies with acantholide using the Multan [9] and X-ray System [10] have proved more successful. The basic skeleton of acantholide was found to be comprised of a macrocyclic ten-membered ring *trans*-fused to a five membered lactone (see Fig. 2). The presence of two double bonds in the ten-membered ring at 1,10- and 4,5- positions was demonstrated by the short distances 1.37 and 1.34 Å respectively. In agreement with the NMR spectral data the X-ray structure indicated the ten-membered ring to be substituted at C 4, C 8, C 9 and C 10 by the groups methyl, hydroxyl, isobutroxy and carboxaldehyde respectively. The stereochemistry of the groups at C 8 and C 9 was in agreement with the assignments

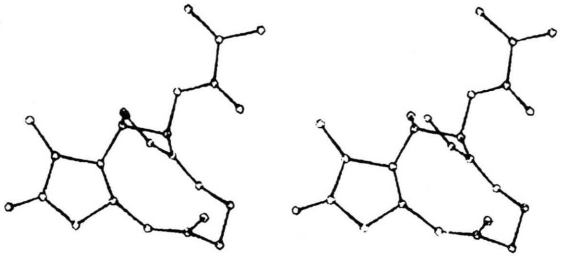

Fig. 2. Stereoscopic view of the molecular structure of acantholide.

made by examination of the coupling constants for the protons at C 7, C 8, C 9 and C 5, and additionally the *cis*-stereochemistry of the α,β-unsaturated aldehyde moiety was also confirmed.

The angle of H 8 with H 9 is 166 ° and is the reason for the substantial coupling constant of 8.9 Hz between these two protons. Similarly the angle (60 °) between H 8 and H 7 having a slightly twisted *cis* relationship of the substituents at these carbon atoms accounts for the small *J*-value. One interesting feature of the intramolecular angles determined for acantholide are the rather small angles C 1 – C 10 – C 15 and O 9 – C 9 – C 8 at 112.6 ° and 102.5 ° respectively. More importantly and relating to the initial aims of the work, the 4,5-stereochemistry is shown conclusively to be *trans* such that the aldehyde at C 10 and the methyl group at C 4 are on opposite faces of the molecule. In addition the C 6 – O 6 bond was shown to be *trans* to the C 7 – C 11 bond.

The "best plane" through the atoms C 1, C 10, C 14, O 14 and H 14 was determined using the program LSQPL of the X-ray System [10]. Almost perfect coplanarity was observed for these atoms, the maximum distance from the plane being only 0.07 Å. More importantly however the angle between this plane and the C 9 – H 9 bond was found to be only 2 °. Determination of this critical angle in the molecule accounts exceedingly well for the substantial (1.8 Hz) coupling constant observed between H 14 and H 9.

Indeed the close agreement between the stereochemical relationships of the vicinal hydrogen atoms, as determined from an evaluation of coupling constant data and the X-ray analysis, suggests that there is very close correspondence between the preferred conformation in solution and that observed in the crystalline state.

Acantholide therefore has the complete and absolute structure shown in **2** and is an additional member of the expanding group of melampolide compounds. Pursuant to our evaluation of *Acanthospermum glabratum* for anticancer activity acantholide (NSC – 277282) was found to be cytotoxic in the KB system (ED_{50} 2.2 μg/ml) but inactive in the P-388 lymphocytic leukemia system in mice.

Experimental

Melting points were determined using a Kofler hot plate and are uncorrected. The UV spectra were obtained with a Beckman model DB-G grating spectrophotometer. The IR spectra were determined with a Beckmanmodel 18-A spectrophotometer with polystyrene calibration at 1601 cm^{-1}. NMR spectra were recorded in $CDCl_3$ solution with Varian model T-60 A instrument, operating at 60 MHz with a Nicolet, model TT-7, Fourier Transform attachment or on a Bruker 270 MHz instrument. Tetramethylsilane was used as an internal standard and chemical shifts are reported in δ (ppm) units. Low resolution mass spectra were obtained with a Hitachi Perkin Elmer, model RMU-6 D, single-focusing spectrometer operating at 70 eV. High resolution mass spectra were obtained with a Varian 731 double-focusing spectrometer operating at 70 eV.

Plant material

The plant material was collected in the Mufindi District of Tanzania during November 1973, and supplied through the auspices of the Drug Research and Development Branch, National Cancer Institute, by the Medicinal Plant Resources Laboratory, Plant Genetics and Germplasm Institute, Agricultural Research Service, USDA, Beltsville, MD. A herbarium specimen documenting this collection (Spjut 3493) is deposited in the Herbarium of the National Arboretum, Agricultural Research Service, USDA Washington, DC.

Extraction and initial fractionation

After defatting a sample (5.5 kg) of the plant material was extracted thoroughly with benzene and the solution concentration *in vacuo* to 373 g of residue, which was partitioned between chloroform (1 l) and 2% aqueous hydrochlorid acid (1 l). After processing

the residue from the chloroform phase weighed 277 g.

Separating of the chloroform fraction

The chloroform fraction (60 g) was chromatographed on a column of silica gel PF-254 (2 kg) packed in benzene and eluted with mixtures of benzene amid ethyl acetate of increasing polarity. Fractions of 100 ml were collected.

Isolation of acantholide

Column fractions 620 – 691, which were eluted with benzeneethyl acetate (9 : 1), were combined and the residue (1,6 g) further chromatographed on silica (100 g) eluting with chloroform. The main constituent of the fraction, 48 – 53, was obtained by further chromatography on Florisil and crystallization from methanol to colorless needles of acantholide (**2**) (150 mg) having the following spectral properties: m. p. 208 °C; IR ν_{max} (KBr) 3500, 1780, 1760, 1725, 1690, 1660, 1625, 1240, 1190, 1140, 1045 and 960 cm^{-1}. NMR ppm (270 MHz, $CDCl_3$), 1.10 and 1.15 (d. $J = 7.0$ Hz. 3 H each, $-CH(CH_3)_2$), 1.97 (bs, 3 H. $C=CCH_3$), 4.87 (d, $J = 10.4$ Hz, 1 H, H 5), 5.03 (dd, $J = 8.6$, 1.0 Hz, 1 H, H 9), 5.2 (dd, $J = 10.1$, 10.4 Hz, 1 H, H 6), 5.31 b (dd, $J = 8.6$, 1.75 Hz, 1 H, H 8), 5.64 (d, $J = 3.0$ Hz, H 13a), 6.33 (d, $J = 3.5$ Hz, H 13b), 6.74 (bd dd, $J = 6.8$, 10.4 Hz, H 1) and 9.41 (d, $J = 1.8$ Hz, 1 H, H 14); MS, *m/e* 348 (M^+, 1.9%), 277 (M^+–71, 1.7), 260 (M^+–88, 40), 242 (30), 214 (20), 213 (15), 177 (56), 91 (29), 84 (56), 71 (79), 69 (53) and 43 (100). Mass measurement: found: 348.1571; calcd.: for $C_{19}H_{24}O_6$ 348.1571.

Acetylation of acantholide

Acantholide (20 mg) was treated with acetic anhydride: Pyridine (1 : 1, 1 ml) at room temperature overnight. Work-up in the usually way afforded an acetate derivative identified as **3**, and exhibiting the following physical properties: δ ppm (60 MHz $CDCl_3$) 1.97 (bs, 3 H, $C-CCH_3$), 2.02 (s, 6 H, 2 x $-OCOH_3$), 5.02 (m, 2 H, H_5, H_6), 5.24 (dd, $J = 8.9$, 1.75 Hz, 1 H, H 9), 5.72 (d, $J = 3.1$ Hz, 1 H, H 13a), 6.21 (d, $J = 3.1$ Hz, 1 H, H 13b), 6.65 (dd, $J = 9.0$, 1.6 Hz, 1 H, H 8), 6.68 (dd, $J = 7.7$, 10.6 Hz, 1 H, H 1), and 9.46 (d, $J = 1.6$ Hz, 1 H, H 14); MS *m/e* 362 (M^+, 0.5%), 320 ($M^+ - 42.4$), 302 ($M^+ - 60.2$), 277 (2), 260 (8), 242 (25), 214 (10), 213 (15), 177 (24), 91 (13) and 43 (100).

Table I. Atomic Parameters for the nonhydrogen atoms ($\times 10^4$).
Positional parameters are given as fractions of the unit cell edges and anisotropic thermal parameters as coefficients to conform to the exponent $-(h^2\beta_{11} + k^2\beta_{22} + l^2\beta_{33} + 2hk\beta_{12} + 2hl\beta_{13} + 2kl\beta_{23})$. Standard deviations are given, on the same scale, in parentheses.

	x/a	*y/b*	*z/c*	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
C (1)	8139 (7)	2744 (18)	−3157 (9)	77 (7)	324 (27)	152 (11)	−17 (12)	4 (7)	29 (15)
C (2)	8613 (7)	3987 (19)	−4110 (8)	107 (8)	365 (25)	125 (10)	−30 (13)	−24 (8)	− 6 (15)
C (3)	9762 (7)	4876 (18)	−3695 (8)	89 (7)	361 (27)	148 (10)	23 (12)	−23 (7)	− 25 (15)
C (4)	9399 (7)	5996 (17)	−2557 (9)	84 (7)	261 (19)	184 (12)	−24 (11)	− 18 (7)	2 (15)
C (5)	9423 (6)	5367 (16)	−1317 (8)	71 (6)	287 (21)	158 (10)	− 7 (10)	3 (6)	− 3 (13)
C (6)	8667 (7)	6082 (17)	− 180 (7)	100 (7)	269 (19)	134 (9)	−18 (11)	20 (7)	10 (13)
C (7)	2319 (6)	9890 (16)	− 249 (7)	87 (6)	250 (19)	115 (8)	11 (10)	9 (6)	− 10 (12)
C (8)	3468 (7)	143 (16)	−9564 (8)	81 (7)	281 (20)	135 (10)	−18 (10)	4 (6)	− 14 (12)
C (9)	3345 (6)	9585 (16)	−8078 (8)	52 (5)	333 (24)	128 (9)	−18 (10)	10 (6)	− 11 (13)
C (10)	7280 (6)	2993 (15)	−2213 (7)	66 (6)	218 (16)	127 (9)	−17 (9)	− 5 (6)	10 (11)
C (11)	7542 (8)	5232 (16)	−8275 (9)	136 (9)	235 (19)	154 (11)	− 1 (12)	17 (8)	3 (13)
C (12)	1284 (9)	834 (18)	−2081 (9)	178 (12)	254 (22)	142 (11)	24 (15)	37 (10)	− 23 (14)
C (13)	6601 (9)	5064 (18)	−7418 (10)	147 (10)	349 (26)	171 (13)	29 (15)	−18 (10)	13 (17)
C (14)	8879 (10)	7672 (18)	−2905 (11)	169 (12)	262 (25)	214 (17)	5 (15)	−25 (11)	− 88 (17)
C (15)	6946 (8)	1498 (18)	−1497 (10)	98 (9)	355 (29)	181 (13)	−17 (13)	− 6 (9)	19 (17)
C (16)	5098 (8)	4977 (20)	−3385 (9)	105 (8)	449 (33)	150 (12)	21 (16)	12 (8)	− 6 (19)
C (17)	6219 (8)	−440 (21)	−6442 (10)	100 (8)	624 (41)	171 (13)	−16 (17)	53 (9)	− 22 (22)
C (18)	3037 (8)	5193 (27)	−2477 (13)	80 (8)	980 (66)	298 (20)	62 (21)	20 (11)	223 (33)
C (19)	3477 (10)	5105 (30)	−4846 (13)	148 (13)	1134 (84)	259 (19)	−47 (30)	81 (13)	−110 (39)
O (6)	577 (5)	1168 (0)	− 984 (6)	122 (6)	331 (17)	178 (8)	50 (9)	34 (6)	− 23 (11)
O (8)	6211 (5)	6828 (13)	− 443 (7)	109 (6)	261 (15)	192 (10)	52 (8)	16 (6)	20 (9)
O (9)	5397 (4)	4340 (13)	−2243 (5)	68 (4)	358 (16)	133 (6)	2 (7)	13 (4)	− 6 (9)
O (12)	911 (6)	1050 (15)	−3127 (6)	218 (9)	402 (20)	165 (8)	56 (13)	64 (7)	− 26 (13)
O (15)	6118 (6)	1440 (14)	− 634 (7)	131 (7)	343 (18)	223 (10)	−36 (9)	−18 (7)	− 37 (12)
O (16)	5797 (6)	5648 (18)	−4150 (7)	118 (7)	890 (38)	177 (9)	− 3 (14)	30 (6)	−168 (17)

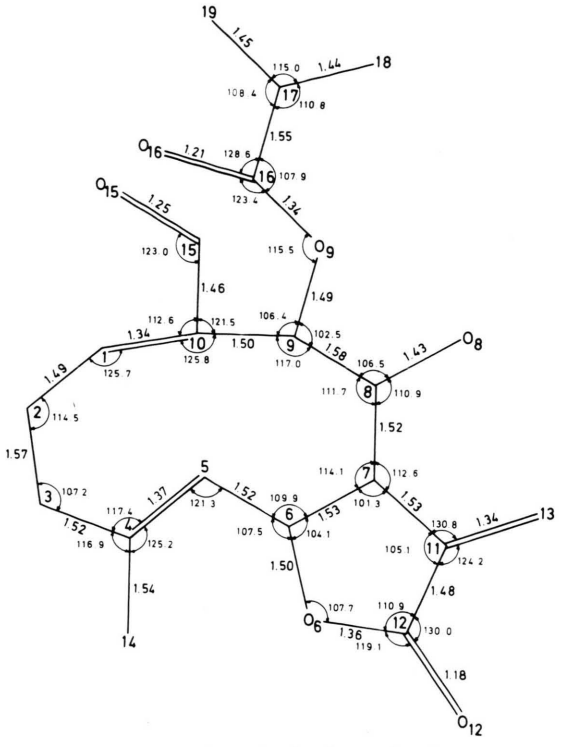

Fig. 3. Distances and angles in the molecular structure of acantholide.

X-ray analysis

Acantholide was crystallized from methanol and gave colorless prisms of size 1.0×0.4 mm. Cell units, as determined from preliminary Weissenberg and precession photographs are $a=11.293$ Å, $b=8.194$ Å, $c=10.186$ Å, $\alpha=\gamma=90°$, $\beta=93.33°$ in the monoclinic space group $P2_1$ with b unique axis. The density, measured by flotation in KI/H_2O, was 1.23 g/cc and gave two molecules in the unit cell. Single crystal data were collected on an automated Siemens diffractometer with CuK_α radiation up to $\sin\theta/\lambda=0.56$ ($\theta-2\theta$ scan range ± 1°, scan speed 1° min^{-1}). From a total of 1504 independent reflections 1347 were recorded as observed ($>2\sigma(I)$). Data were scaled by Wilson statistics.

The structure was solved by direct methods using Multan [9]. Three origin and three additional starting reflections were selected (one fixed the enantiomorph) and 32 possible phase sets calculated. An E map with the best of these, obtained by means of the "combined figure of merit" using 200 E gave positions for 22 of the 25 nonhydrogen atoms. Two subsequent Fourier maps with the complete data set were calculated with the X-ray System [10] and revealed all atoms.

In order to compare NMR data with the X-ray results a Difference Fourier synthesis was calculated, and revealed all hydrogens that are bound or close to the skeleton. The positions of the methyl group hydrogens at C 15, C 18 and C 19 could not be determined, probably because of thermal motions in these side chains. The angles between spin-interacting hydrogens have been calculated by the program LSQPL of the X-ray System and are defined according to the well known Karplus-Conroy equation. Refinement to convergence was carried out by a full matrix least-squares approach. A final *R*-factor of 7.6% resulted based on the observed reflections. The function minimized was $\Sigma w \Delta^2$. Fig. 2 is a stereoscopic view of the molecule and bond lenghts and angles are given in Fig. 3. Fractional coordinates of the nonhydrogen atoms are listed in Table I, and the hydrogen coordinates in Table II.

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Table II. Positional parameters for the found hydrogens (× 10^4) and isotropic temperature factors.

Atom	Bound to	*x/a*	*y/b*	*z/c*	B
H (1)	C (1)	8361 (41)	1429 (70)	−3429 (47)	7.7
H (21)	C (2)	8854 (44)	3198 (70)	−4917 (48)	4.8
H (22)	C (2)	7939 (43)	4441 (70)	−4406 (45)	7.4
H (31)	C (3)	168 (42)	3901 (73)	−3542 (46)	7.2
H (32)	C (3)	310 (42)	5841 (72)	−4495 (45)	7.5
H (5)	C (5)	9643 (42)	4176 (74)	−1250 (45)	6.3
H (6)	C (6)	8443 (43)	7518 (69)	− 373 (50)	7.7
H (7)	C (7)	8031 (43)	3694 (70)	+ 142 (46)	7.1
H (8)	C (8)	5934 (42)	4240 (74)	+ 56 (47)	8.8
H (81)	O (8)	4550 (41)	2217 (68)	+ 3 (53)	8.9
H (9)	C (9)	7091 (42)	5594 (71)	−2622 (47)	6.6
H (15)	C (15)	7512 (42)	408 (70)	−1615 (46)	7.2

Cancer Treatment, National Cancer Institute, US Department of Health, Education and Welfare, Betheseda, MD. 20014. The authors would like to thanks Ms. I. Handy for the 270 MHz pmr spectrum of acantholide obtained at the University of Chicago.

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