THE STRUCTURE OF AMPHOTERICIN A

II. THE COMPLETE STRUCTURE OF THE ANTIBIOTIC

PAWEŁ SOWIŃSKI, JAN K. PAWLAK and EDWARD BOROWSKI

Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, 80-952 Gdańsk, Poland

TAKASHI IWASHITA

Suntory Institute for Bioorganic Research, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

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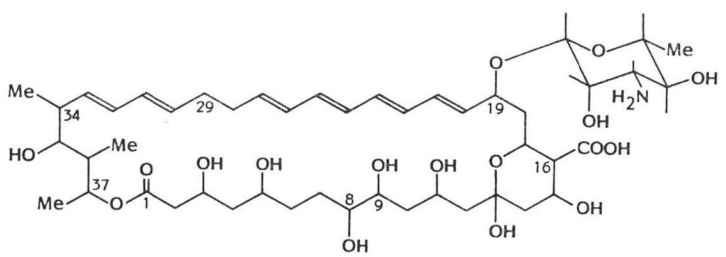
The structure of amphotericin A without the configuration of asymmetric carbon atoms has been elucidated. The stereochemistry of the sugar moiety has been determined. On the basis of homoscalar correlated 2D ^1H spectra of amphotericin A the position of the hemiketal moiety has been located, and the chemical shifts of all the protons in the antibiotic molecule have been determined.

In the first part of the report[1] the constitution of the carbon skeleton in amphotericin A as well as the location of oxygen functions were determined on the basis of mass spectra of suitable derivatives. The next stage of our research was to determine the location of the lactone linkage and chromophore system as well as identification of the sugar moiety together with its place of attachment to the aglycone.

The position of the sugar moiety in relation to the polyenic system was determined on the basis of its elimination (2% solution of hydrogen chloride in methanol) characteristic of an allylic position[2], with liberation of mycosamine and not mycosaminide. Simultaneously, the tetraenic UV spectrum (λ 291, 304, 318 nm) converts into that of pentaene (λ 318, 332, 381 nm)[3].

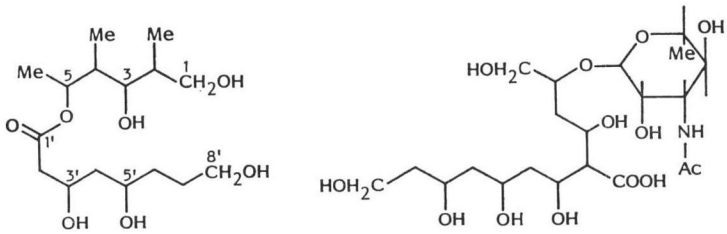
Ozonolysis of the double bonds in *N*-acetylamphotericin A and subsequent cleavage of the C-8–C-9 bond with sodium periodate followed by reduction with sodium borohydride gave ester **IV** and glycoside **V**. The formation of ester **IV** provided direct evidence for the position of the lactone linkage between C-1 and C-37 of amphotericin A (**I**). The NMR examination of ester **IV** proved the existence of coupling of the acyloxy group proton with C-4 methine proton, and C-6 methyl protons (Fig. 2). The signal assigned to the most unshielded proton at the carbon atom with the acyloxy group (C-5) appeared

Fig. 1. The structure of amphotericin A.



I

Fig. 2. Structure of ester IV and glycoside V.



IV V

Table 1. ^{13}C NMR spectral data of amphotericin A and their comparison with amphotericin B and nystatin A_1.*

Carbon	δ (ppm)		
	Amphotericin A	Amphotericin B	Nystatin A_1
$-COO^-$	176.10	177.6	176.3
Lactone	170.34	170.6	170.1
Acetal	97.33	95.9	97.6
Hemiketal	97.00	97.1	97.1
$=CH^-$	129~136		
CH–	66~76		
	55~60		
	38~39		
$-CH_2-$	40~45		
	28~35		
$-CH_3$	12.09		
	16.65		
	16.92		
	17.85		

* Data for amphotericin B and nystatin A_1 after PANDEY and RINEHART[4].

at δ 5.27 ppm (dq), and the coupling constants were J=6.5 Hz for C-6 methyl group protons and J=4.1 Hz for C-4 methine proton.

The formation of ester **IV** together with the formation of the pentaene chromophore as a result of the elimination of mycosamine from **I** unambigously localizes all double bonds in the molecule of amphotericin A. The tetraene chromophore was localized between C-20–C-27 atoms and the diene chromophore, between C-30–C-33 carbon atoms of **I**. In the glycoside mycosamine is attached at carbon atom C-19 (allylic to the chromophore).

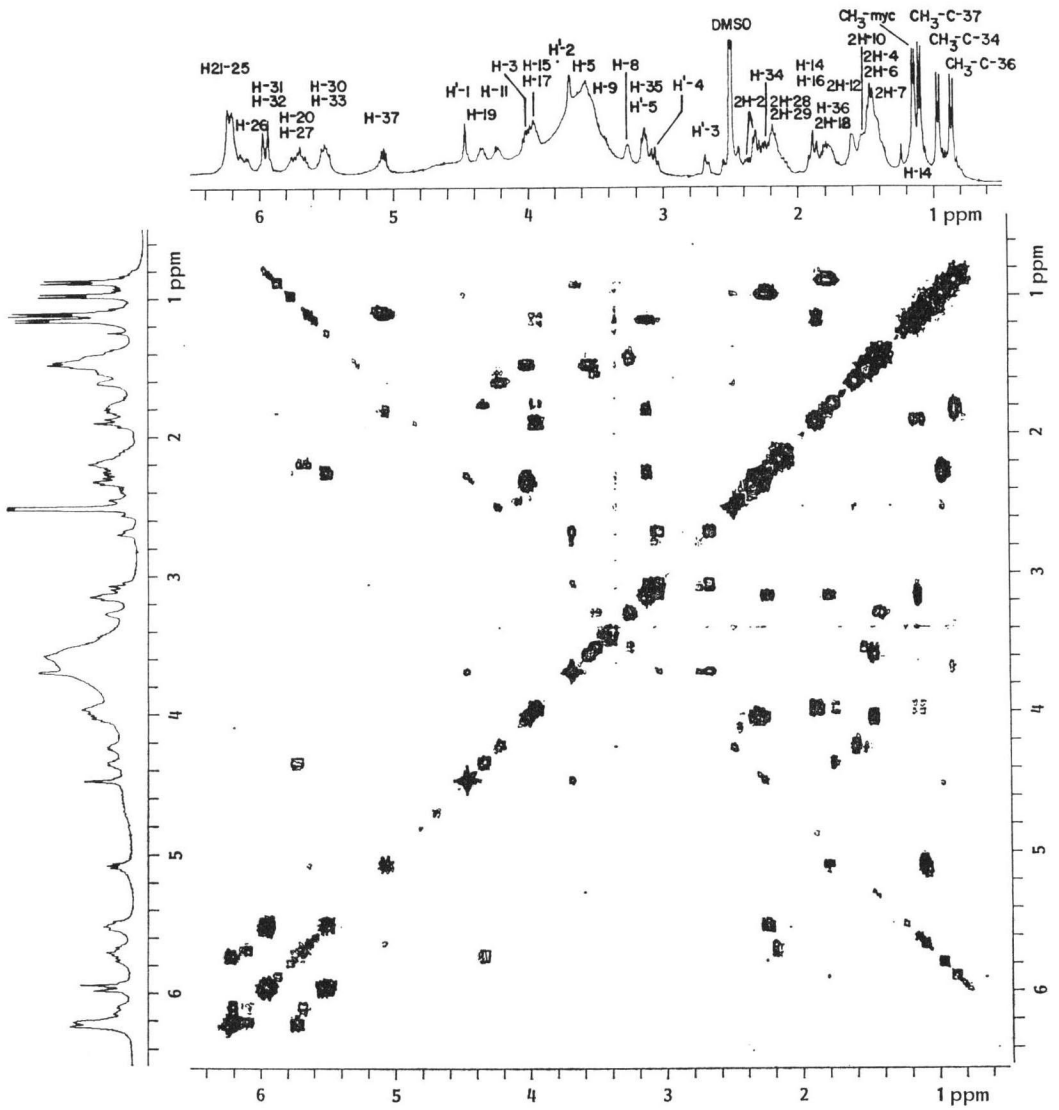
The identity of mycosamine was confirmed by direct comparison (GLC) of the *N*-acetyl trimethylsilyl derivative of the aminosugar resulting from the degradation of the glycoside **V** with the corresponding derivative of a standard mycosamine. The optical rotation of the *N*-acetyl derivatives of both aminosugar samples was identical.

Amphotericin A has been also examined by ^{13}C and ^1H NMR. The ^{13}C NMR spectrum allows simple determination of signals originating from the lactone, carboxylic anion, hemiketal and acetal (mycosamine) as well as four methyl groups.

The remaining signals are difficult to interpret because of minute differences in chemical shifts and mutual superposition.

Chemical shifts of carbon atoms of various orders were obtained on the basis of ^{13}C subspectra, determined by means of modification of INEPT method[5] by generation of spectra of CH; CH_3, CH and counter-phasically CH_2 as well as without quarternary carbon atoms. The ^{13}C NMR spectra of this type do not allow, however, the determination of location of structural elements of interest to us. Applying the correlated-2D technique at 360 MHz we were able to gain from one experiment the information on chemical shifts of all protons in the molecule and we could mutually correlate them by scalar coupling (Fig. 3). In this way we determined the position of lactone linkage, and the positions of double bonds and all methyl groups.

Fig. 3. Contour plot of a 2D correlated ^1H NMR spectrum at 360 MHz recorded in a 0.043 M solution of amphotericin A in DMSO-d_6.



The location of hemiketal is indicated on the one hand by cleavage of coupling system at C-13 atom, and on the other hand by the characteristic pattern of proton signals at C-14. These protons reveal high non-equivalence $\Delta\delta$=0.75 ppm indicating that the rigid six-membered hemiketal ring is linked to C-17 atom and not to the alternative C-9 atom.

Chemical shifts of all protons of amphotericin A are presented in Table 2.

Because of relatively large half width of the line at 3.2 Hz and strong superposition of signals of individual protons it was impossible in most cases to determine the coupling constants.

For sugar protons the determined coupling constants were: $J_{1,2}$=0, $J_{2,3}$=2.6, $J_{3,4}$=9.5, $J_{4,5}$=9.5, $J_{5,6}$=6 Hz (±0.5 Hz), which determined $^4C_1$ conformation[7]. In comparison, the respective values for amphotericin B are[6]: 0, 2.75, 9.25, 9.25, 5.75 (±0.5 Hz).

Table 2. ^{1}H NMR spectral data of amphotericin A and their comparison with amphotericin B.*

Proton	δ (ppm)		Proton	δ (ppm)	
	Amphotericin A	Amphotericin B		Amphotericin A	Amphotericin B
H-2, 2′	2.27, 2.34	2.12	H-26	6.12***	
H-3	4.03	4.03	H-27	5.69***	
2H-4	1.48	1.36	2H-28, 2H-29	2.05, 2.25	
H-5	3.58	3.46	H-30	5.51***	
2H-6	1.48		H-31	5.96***	
2H-7	1.42		H-32	5.96	
H-8	3.26	3.05**	H-33	5.51	5.41
H-9	3.54	3.46**	H-34	2.25	2.25
2H-10	1.50		H-35	3.13	3.06
H-11	4.22	4.22	H-36	1.82	1.73
2H-12	1.60		H-37	5.07	5.19
H-14, 14′	1.13, 1.88	1.10, 1.80	CH_3-C-34	0.97	1.07
H-15	3.95	3.96	CH_3-C-36	0.87	0.88
H-16	1.88	1.86	CH_3-C-37	1.09	1.00
H-17	3.95	4.17			
H-18, 18′	1.75, 1.77	1.47, 2.18			
H-19	4.34	4.28	Mycosamine		
H-20	5.75	5.94	H-1	4.47	4.46
H-21, H-22, H-23, H-24, H-25	6.22		H-2	3.70	3.79
			H-3	2.68	2.96
			H-4	3.07	3.18
			H-5	3.14	3.24
			CH_3	1.13	1.15

* Data for amphotericin B after BROWN and SIDEBOTTOM[6].
** These resonances may have crossed assignments.
*** The protons H-26 and H-31 as well as H-27 and H-30 may have simultaneously crossed assignments.

Data presented in Table 2 were obtained on the basis of two-dimensional spectrum. Without reference to further structural information the coupling sequences for the following parts the molecule can be correctly interpreted: C-2–C-4, C-7–C-12, C-17–C-20, C-33–C-37 and the protons of mycosamine.

Comparing the values of chemical shifts of protons in amphotericins A and B (differing only in decoupling of polyenic chromophore) a striking difference can be noticed in the latter case in the behavior of methylene protons on C-2 and C-18 carbon atoms. In the case of amphotericin B the protons on C-2 atom are chemically equivalent, while on C-18 atom they exhibit high non-equivalence. In amphotericin A the protons on C-2 atom reveal non-equivalence and differ highly in chemical shift, while the protons on C-81 are nearly equivalent. This may indicate that the lactone ring in amphotericins A and B adopt a different conformation.

Experimental

General Procedures

^{13}C NMR spectra of amphotericin A were measured on Jeol FX-100 instrument using 0.043 M DMSO-d_6 solution and employing DMSO as the internal standard and deuterium lock.

For ^{13}C NMR subspectra generation a variation of the INEPT method was applied[5]. The following subspectra were generated: for $\Delta=1/4J$ without C_q, for tertiary carbons $\Delta=1/2J$ and for $\Delta=3/4J$ CH_3, CH and CH_2 in negative phase.

^{1}H NMR spectra were measured on Nicolet FT 360 MHz instrument using 0.043 M DMSO-d_6 solution. Digital resolution was 0.3 Hz.

2D correlated spectra were developed on data matrix in the frequency domain 1024. The digital resolution in both dimensions was 5.1 Hz.

^{1}H NMR spectra of the compound **IV** was determined on 60 MHz Tesla BS 487 spectrometer.

UV spectra were recorded on Beckman 3600 spectrometer. Optical rotations were measured with a Hilger & Watts polarimeter.

Analytical thin-layer chromatography was carried out on Merck precoated silica gel 60 plates employing the following solvent systems: a) EtOAc - MeOH - H_2O, 30: 15: 4; b) EtOAc - MeOH - H_2O, 150: 15: 2; c) EtOH - BuOH - 28% NH_4OH, 5: 3: 3.

Gas chromatography was carried out with a Chromatron GCHF 18.3 gas chromatograph, using 2 m×4 mm I.D. column with 3% SE 30 on Chromosorb W-AW OMCS, 60~80 mesh. Temperature programing of 2°C/minute from 160 to 230°C and argon flow of 40 ml/minute were employed.

Ozonolysis of Double Bonds and Cleavage of *vic*-Glycol System with Sodium Periodate

Ester (**IV**) and Glycoside (**V**): *N*-Acetylamphotericin A (270 mg) dissolved in methanol (30 ml) was cooled to −78°C and ozone gas was passed for 30 minutes. The excess of ozone was removed by a flow of nitrogen, and the ozonide was hydrogenated over 5% palladium on asbestos catalyst for 2 hours at ambient temperature. The catalyst was separated, and the solution was evaporated to dryness. The crude residue was dissolved in water (25 ml) and reduced with sodium borohydride for 40 minutes at 0°C. The reaction mixture was neutralized with Dowex 50(H^+). The resin was then separated, and filtrate was exhaustively evaporated with methanol to remove borates. The crude product (220 mg) was obtained after precipitation with diethyl ether from methanol.

To a vessel containing the above material dissolved in water (50 ml) was added sodium periodate (150 mg) in water (15 ml) stepwise over 25 minutes.

After excess periodate was destroyed with ethylene glycol over a period of 30 minutes, the mixture was the neutralized with barium hydroxide solution, and the iodates were precipitated with barium chloride. The solids were filtered off, and the filtrate was cooled to 0°C. Sodium borohydride was then added, and the reductive medium was maintained for 30 minutes. The reaction mixture was adjusted to pH 4.5 with Dowex 50(H^+). The resin was then filtered, and the filtrate was evaporated to dryness. The residue was dissolved in methanol (0.5 ml), and the glycoside (**V**, 87 mg) was precipitated with diethyl ether - hexane, 3: 1.

The supernatant was evaporated to dryness yielding ester (**IV**, 55 mg). The ^{1}H NMR spectrum (acetone-d_6) was recorded after exchange of hydroxylic protons with deuterium atoms: δ 5.27 (1H, dq, H-5, $J_{5,6}$=6.5 Hz, $J_{4,5}$=4.1 Hz), 3.2~4.5 (bs, CHOD and CH_2OD), 2.38 (2H, d, H-2′, $J_{2',3'}$=6 Hz), 1.8~2.1 (2H, bs, H-2 and H-4), 1.3~1.8 (6H, bs, H-4′, H-6′, H-7′), 1.07 (3H, d, H-6, $J_{5,6}$=6.5 Hz), 0.78 (6H, d, CH_3C-4 and CH_3C-2, J=7 Hz).

Mycosamine: Isolation and Derivatization

Glycoside (**V**, 31 mg) and 2 ml of 4 N hydrochloric acid were placed in sealed ampule and kept for 3 hours at 100°C. The resultant solution was cooled and evaporated to dryness. The residue was redissolved in water 3 ml and extracted with butanol (3×3 ml). The water layer was evaporated to dryness yielding 13 mg of crude mycosamine hydrochloride. The TLC analysis with solvent system "c" established its identity with authentic mycosamine. The aminosugar was purified on a Dowex 50 WX8 (H^+) column (1×14 cm), employing 1 N hydrochloric acid as an eluent. The evaporation of collected fractions gave 11 mg of pure mycosamine hydrochloride.

The product obtained above (11 mg) was dissolved in water (1.5 ml), and 1 g of Dowex 1X8 (HCO_3^-) was added. The mixture was cooled to 0°C, and 80 μl of acetic anhydride was added. The reaction mixture was stirred for 2 hours maintaining the temperature at 5°C. The resin was separated and washed with water. The combined filtrates were evaporated to dryness. The crude product was dissolved in 0.5 ml of water and then passed through a Dowex 50WX8 (H^+) column (1×15 cm), water using as an eluent. Evaporation of the water gave 7 mg of *N*-acetylmycosamine.

Silylation of *N*-acetylmycosamine was carried out according to the BROBST[8] procedure, employing hexamethyldisilazane and trifluoroacetic acid. The OTMS derivatives of *N*-acetylaminosugars were subjected to GLC analysis. Both aminosugar derivatives obtained from amphotericin A and the one

prepared from an authentic mycosamine sample, showed the presence of α and β anomers with relative retention indexes of 0.72 and 0.82 respectively (with the *O*-TMS derivative of mannitol as a reference).

Acknowledgments

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