THE STRUCTURE OF AMPHOTERICIN A

I. MASS SPECTROMETRIC ANALYSIS OF DODECAHYDROAMPHOTERICIN A

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Amphotericin A had been transformed into dodecahydroamphotericin A by hydrogenation. Subsequently, the product obtained was reduced with metal borohydrides or borodeuterides and derivatized to volatile compounds which were analyzed by mass spectrometry.

Amphotericin A, a tetraene macrolide antibiotic, is formed by *Streptomyces nodosus* in coproduction with a heptaene macrolide, amphotericin B. Both compounds have been isolated in pure form[1]. The structure of amphotericin B, one of the most commonly used agents in antifungal therapy, has been elucidated[2,3]. Amphotericin A has been chemically and biologically partially characterized[1], but no data on its chemical constitution have been reported.

In this report we present the evidence for the structure of dodecahydroamphotericin A. The pure, crystalline amphotericin A with $E^{1\%}_{1cm}$ 1,350 at 304 nm (methanol) used in these studies originated from E. R. Squibb and Sons, New Brunswick, N.J., USA.

The molecular weight of amphotericin A (**I**) determined by a MS method was found to be 925. The SIMS method gave the *m/z* 926 for ion $(M+H)^+$. For *N*-acetyl methyl ester of **I** the *m/z* 1,004 for ion $(M+Na)^+$ was obtained by FD method.

The constitution of the carbon skeleton and assignment of oxygen functions were determined on the basis of the mass spectra of *N*-acetyl-*N*-methylteradeca-*O*-methylheptadecahydroamphotericin A (**II**) and its deuterio analogue (**III**).

Compounds **II** and **III** were obtained by the following reactions: *N*-acetylamphotericin A underwent smooth catalytic hydrogenation of the polyene chromophore, followed by reduction of the carbonyl groups with sodium borohydride or borodeuteride, respectively. Subsequently the carboxyl group was esterified with diazomethane and furthemore, together with the lactone linkage was reduced with lithium borohydride or lithium borodeuteride in anhydrous tetrahydrofuran. Methylation with methyl iodide in tetrahydrofuran in the presence of sodium hydride completed the preparation of compounds **II** and **III**.

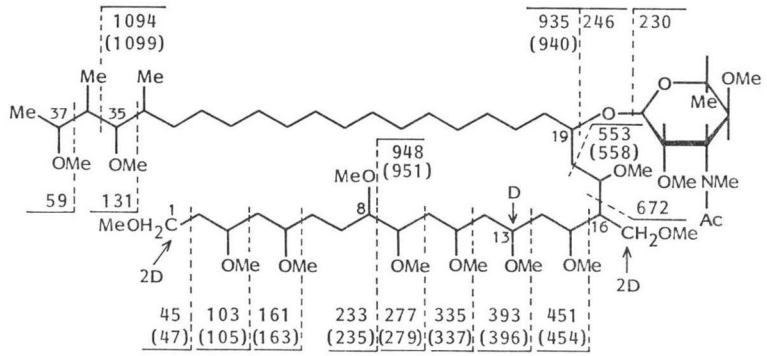
The products of the above reactions were purified by column chromatography and analyzed by mass spectrometry. The MS data for compounds **II** and **III** are given in Table 1.

The molecular ions were *m/z* 1,181 for **II** and *m/z* 1,186 for **III**, respectively. A series of elimination ions originating from the parent ions were observed: a: $M-CH_3$, *m/z* 1,166 (1,171)*; $M-CH_3-CH_3OH$, *m/z* 1,134 (1,139), b: $M-CH_3CO-CH_3OH$, *m/z* 1,106 (1,111), c: $M-n\,CH_3OH$, *m/z* 1,149 (1,154); *m/z* 1,117 (1,122); *m/z* 1,085 (1,090); *m/z* 1,053 (1,058) *etc.*, d: M−MycO**−n

* Values in brackets are related to deuterio analogue **III**.

** Myc means *N*-acetyl-*O*-trimethylmycosaminyl residue, *m/z* 230.

Fig. 1. Diagnostic fragmentations of compounds **II** (*m/z* 1,181) and its deuterio analog **III** (*m/z* 1,186).



II (**III**)

CH_3OH, *m/z* 935 (940); *m/z* 903 (908); *m/z* 871 (876); *m/z* 839 (844); *m/z* 807 (812); *m/z* 775 (780). The fragmentation patterns for compounds **II** and **III** are given in Fig. 1.

The fragment ions at *m/z* 59, 131, 672, 103, 161, 233, 277, 335, 393, 451, 553 formed by ether-type cleavage indicated the location of oxygen functions in **II**. Elemental composition of the following ions: 59.0494 (C_3H_7O), 131.1069 ($C_7H_{15}O_2$), 103.0755 ($C_5H_{11}O_2$), 233.1750 ($C_{12}H_{25}O_4$), 277.2017 ($C_{14}H_{29}O_5$), 335.2437 ($C_{17}H_{35}O_6$), 451.3273 ($C_{23}H_{47}O_8$) was determined by HRMS, and confirmed the proposed fragmentation pattern. Although, the fragment ions at *m/z* 161, 393, 553 exhibited low relative intensity, the ions at *m/z* 129, 329, 489 formed by loss of methanol from the appropriate fragment ions were clearly measurable and assigned as 129.0916 ($C_7H_{12}O_2$), 329.2324 ($C_{18}H_{33}O_5$), 489.3425 ($C_{26}H_{49}O_8$). A more detailed discussion of the fragmentation and elimination patterns for analogous polymethoxy ethers was published earlier[4,5].

Two types of elimination of mycosamine from the fragmentation ions were also observed: from ion *m/z* 948 the aminosugar moiety was eliminated with simultaneous transfer of one hydrogen atom from the carbohydrate ring to the resultant ion. Subsequent elimination of methanol gave the following ions:

1. *m/z* 607.5313 ($C_{38}H_{71}O_5$); 948−(MycO−H)−3×CH_3OH
   m/z 575.5035 ($C_{37}H_{67}O_4$); 948−(MycO−H)−4×CH_3OH
   m/z 543.4769 ($C_{36}H_{63}O_3$); 948−(MycO−H)−5×CH_3OH
2. Another type of elimination of the aminosugar was found for ion *m/z* 672 (cleavage of C-16–C-17 carbon bond). In this case a hydrogen atom is eliminated from the fragmentation ion together with the aminosugar moiety. The following ions of this series were detected:

   m/z 425.3990 $C_{27}H_{53}O_3$; 672−MycOH

and the ions, *m/z* 393.3734; *m/z* 361.3471 and *m/z* 329.3206 were formed as a result of the successive elimination of methanol molecules.

The ion at *m/z* 425 is a doublet, the second component (*m/z* 425.29057, $C_{24}H_{41}O_6$) was formed from the ion *m/z* 553 (553−4×CH_3OH).

In the region of lower ion values were found ion *m/z* 230 (base peak) resulting from the cleavage of the glycosidic linkage, and ion of *m/z* 246 (Myc−O). Ions originating from the fragmentation of the aminosugar moiety were also found[6]: *m/z* 201, 198, 186, 166, 156, 142, 129, 101, 87.

The location of mycosamine was determined on the basis of the fragment ions at *m/z* 1,094, 672,

Table 1. Ions observed in the mass spectra of *N*-acetyl-*N*-methyltetradeca-*O*-methylheptadecahydroamphotericin A (**II**) and its heptadeuterio analogue (**III**).

m/z	Relative intensity (%)		*m/z*	Relative intensity (%)		*m/z*	Relative intensity (%×10)		*m/z*	Relative intensity (%×100)	
	II	III		II	III		II	III		II	III
*			149	20	—	337	—	62	744	8	—
59	84	58	155	21	—	351	40	—	745	6	—
67	14	12	156	27	30	355	90	—	746	—	6
69	19	18	157	14	12	358	—	42	747	—	8
71	58	29	166	14	11	361	70	33	748	—	10
72	21	33	168	16	13	367	40	—	749	—	7
73	20	23	169	59	—	387	30	—	759	7	—
75	88	54	170	22	20	393	60	24	760	6	—
76	—	16	171	12	39	399	40	—	773	7	—
79	14	—	172	—	11	425	66	32	774	53	—
81	19	15	175	12	—	***			775	189	—
82	—	10	181	19	—	430	—	23	776	116	—
83	14	14	186	13	12	451	71	—	777	60	50
84	—	10	198	10	—	452	22	—	778	—	62
85	24	20	201	67	42	453	—	20	779	—	96
87	21	24	202	16	14	454	—	43	780	—	104
89	—	12	203	—	34	455	—	18	781	—	79
91	12	—	204	—	10	457	34	—	788	56	—
93	18	—	207	16	—	462	—	21	789	58	—
95	18	16	213	14	—	489	22	—	791	71	—
97	42	20	215	—	10	494	—	20	792	53	—
98	—	22	230	100	100	543	32	—	805	67	—
99	24	25	231	38	30	546	—	15	806	64	—
100	—	12	232	80	—	547	—	10	807	191	—
101	56	40	233	42	—	575	28	—	808	131	—
102	—	15	235	—	30	576	12	10	809	78	54
103	58	—	239	13	—	577	—	13	810	64	62
104	—	12	245	11	—	578	—	17	811	—	87
105	20	42	246	44	35	579	—	13	812	—	146
107	12	12	247	—	11	607	22	—	813	—	104
109	17	14	271	18	—	****			814	—	54
111	16	12	273	—	10	610	—	15	815	53	—
112	—	12	**			623	11	—	820	60	—
114	—	20	277	6	—	626	—	6	821	64	—
115	19	16	279	—	4	640	6	—	823	60	54
117	19	12	291	7	—	647	10	—	824	53	54
121	13	—	294	—	3	655	10	—	827	—	50
123	19	—	297	14	—	672	8	6	828	—	54
125	13	—	298	3	—	679	12	—	829	—	63
126	—	10	300	—	5	683	—	6	838	53	—
127	19	—	303	10	3	711	13	—	839	111	—
128	—	19	305	—	5	712	10	—	840	76	—
129	67	54	323	11	—	713	8	—	843	—	58
130	18	22	324	3	—	716	—	8	844	—	79
131	27	27	326	—	4	717	—	5	845	—	54
135	11	—	329	12	4	719	6	—	850	62	—
137	23	—	330	3	—	720	6	—	852	253	—
139	—	13	331	4	3	741	6	—	853	178	58
142	25	21	332	—	4	742	6	—	854	104	83
143	15	—	335	10	—	743	14	—	855	53	216

Ions of relative intensities above * 10%, ** 2.5%, *** 1%, **** 0.5% and ***** 0.2% have been tabulated.

Table 1. Continued.

m/z	Relative intensity (%×100)		m/z	Relative intensity (%×100)		m/z	Relative intensity (%×100)		m/z	Relative intensity (%×100)	
	II	III		II	III		II	III		II	III
856	—	171	954	—	21	1064	27	—	1122	—	38
857	—	83	966	28	—	1065	—	20	1123	—	33
858	—	79	988	38	—	1066	—	22	1124	27	—
859	—	54	990	27	—	1067	—	41	1133	29	—
871	64	—	991	27	—	1068	—	25	1134	42	—
876	—	58	1008	29	—	1085	24	—	1135	45	—
884	64	—	1020	24	—	1090	27	23	1136	27	—
887	—	104	1021	24	—	1091	27	20	1138	—	20
888	—	58	1025	—	27	1092	40	—	1139	—	33
889	—	50	1026	—	21	1093	27	23	1140	—	25
902	56	—	1030	21	—	1094	74	—	1149	30	—
903	80	—	1032	24	—	1095	49	—	1150	36	—
904	67	—	1036	27	—	1096	22	25	1151	27	—
907	—	50	1041	—	20	1097	—	29	1152	21	—
908	—	83	1046	24	—	1098	—	44	1154	—	24
934	82	—	1048	25	—	1099	—	81	1155	—	20
935	102	—	1049	29	—	1100	—	51	1166	44	—
936	71	—	1051	33	—	1101	—	20	1167	36	—
937	—	58	1052	38	—	1105	27	—	1168	20	—
938	—	50	1053	27	—	1106	60	—	1170	—	22
939	—	75	1054	27	—	1107	33	—	1171	—	35
940	—	125	1055	—	20	1108	24	—	1172	—	26
941	—	67	1056	—	25	1110	—	20	1179	20	—
*****			1057	—	27	1111	—	28	1180	20	—
948	32	—	1058	—	21	1112	—	17	1181	20	—
949	27	—	1059	—	20	1117	40	—	1185	—	20
950	27	—	1060	29	—	1118	42	—	1186	—	20
951	—	25	1061	24	—	1119	27	—			
952	—	33	1062	58	—	1120	27	—			
953	—	37	1063	42	—	1121	—	20			

553, 131 (**II**, Fig. 1). The ions at *m/z* 1,094 and 672, containing mycosamine in their structure, limited the positions for mycosamine to C-17, C-19 and C-35. However, the ions at *m/z* 553 and 131 which do not contain mycosamine in their structures left only one possibility for the location of the mycosamine moiety — at C-19.

The comparison of the mass spectra of compounds **II** and **III** identified the site of incorporation of two deuterium atoms at C-1 of **III**, another two deuterium atoms at the methoxymethyl group attached to C-16 and only one deuterium atom at carbon atom C-13. The position of deuterium labeling and the number of deuterium atoms attached to the individual carbon atoms in the deuterio analogue indicate that the ketone function is present at C-13 of the antibiotic, while the carboxyl groups are linked with C-2 and C-16 carbon atoms, respectively.

Thus, upon the results presented above the structure of dodecahydroamphotericin A has been elucidated and appears to be identical with the structure of tetradecahydroamphotericin B[2,3].

The evidence for the other amphotericin A structural features such as the lactone linkage, hemiketal ring fusion and location of polyene chromophore will be discussed in the second part of this report.

Experimental

General Procedures

EI and FD mass spectra were obtained on a Varian MAT 711 spectrometer employing the direct probe technique. The conditions for electron impact were as follows: electron energy, 70 eV, emission current, 0.8 mA; ions source temperature, 250°C; resolution, 1,000 and 10,000 for higher mass values. The conditions for field desorption were as follows: heating current, 14~18 mA; ions source temperature, 70~100°C; acceleration voltage, 8 kV; extraction voltage, 4 kV. SIMS spectrum was obtained on a modified Hitachi RMU-6 spectrometer.

Analytical thin-layer chromatography was carried out on Merck precoated silica gel 60 plates employing the following solvent systems:

a) EtOAc - H_2O - AcOH, 4: 1: 1.

b) C_6H_6 - EtOAc - EtOH, 5: 1: 1.

N-Acetylamphotericin A

To amphotericin A (200 mg) suspended in a mixture of *N*,*N*-dimethylformamide (3 ml) and water (1 ml) was added triethylamine (104 μl) followed by acetic anhydride (71 μl). The mixture was stirred for 25 minutes under nitrogen and additional portions of triethylamine (52 μl) and acetic anhydride (35 μl) were added. After another hour of continuous stirring, water was removed under vacuum, methanol (2.5 ml) was added, and the product was precipitated with a mixture of diethyl ether - hexane (2: 1). The precipitate was dissolved in 2 ml of methanol and precipitated again with diethyl ether, washed with fresh diethyl ether, centrifuged and dried to give 170 mg of *N*-acetylamphotericin A.

N-Acetylamphotericin A Methyl Ester

A solution of diazomethane in diethyl ether was added to a solution of 5 mg of *N*-acetylamphotericin A in 5 ml of methanol at 0°C. After 10 minutes, excess diazomethane and solvents were removed from the mixture under vacuum, and the product was dissolved in methanol (1 ml) and precipitated with 20 ml of diethyl ether, yielding 5 mg of the methyl ester. Solvent system "a" was employed for chromatographic analysis.

N-Acetyldodecahydroamphotericin A

N-Acetylamphotericin A (165 mg) was hydrogenated over 10% palladium on barium sulfate catalyst in 5 ml of methanol - water (9: 1) at room temperature and atmospheric pressure. After the reaction had ceased (18 hours), the catalyst was filtered and washed with methanol. Solvents were removed, and the residue was precipitated with diethyl ether. The yield of *N*-acetyldodecahydroamphotericin A was 140 mg. Progress of the reaction was monitored by TLC (system "a") and by UV analysis.

N-Acetyl-*N*-methyltetradeca-*O*-methylheptadecahydroamphotericin A (II) and its 1,1,13,16′,16′-Pentadeuterio Analogue (III)

A mixture of *N*-acetyldodecahydroamphotericin A (70 mg), tetrahydrofuran (2 ml) and water (1 ml) was cooled to 5°C, and sodium borohydride in water was added portionwise, maintaining reductive medium for 5 hours. The reaction mixture was diluted with methanol (2 ml) and Dowex 50 (H^+) was added until pH 4.5. The resin was removed by suction filtration and washed with methanol. The filtrate was evaporated four times to dryness with the addition of methanol. The foamy residue was dissolved in methanol (3 ml) at 0°C, and a solution of diazomethane in diethyl ether was added until the yellow color persisted. The mixture was left for 5 minutes and evaporated to dryness. The residue was dissolved in a small amount of methanol and precipitated with diethyl ether - hexane (2: 1) mixture. The precipitate was separated, washed twice with hexane and dried, yielding 53 mg of ester. The crude ester was dissolved in dry tetrahydrofuran(7 ml), and lithium borohydride (10 mg) was added. The reaction mixture was refluxed for two hours; then after cooling, acetone was added to destroy excess reducing agent. The flask contents were diluted with water (5 ml), adjusted with Dowex 50 (H^+) to pH 4.5 and filtered. The filtrate was evaporated to dryness, and the residue was redissolved in methanol and evaporated four times. The crude polyol (30 mg) was precipitated with diethyl ether - hexane mixture (2: 1) from methanol. A 50% oil dispersion of sodium hydride (50 mg) was twice

washed with anhydrous hexane (2×2 ml) and added to a solution of crude polyol in tetrahydrofuran (6 ml).

Methyl iodide (100 μl) was added in two portions, and the mixture was stirred for 20 hours. The suspension was then filtered with a glass sinter funnel. The resultant solids were washed with benzene, and the filtrates were combined and evaporated to dryness. The oily residue was redissolved in benzene (10 ml), washed with water (3×5 ml) and the benzene layer was evaporated to dryness yielding 24 mg of a crude product, **II**.

Crude **II** was applied to a silica gel 60 column (90×12 mm) and developed with benzene - ethyl acetate - ethanol, 25: 25: 3. A chromatographically pure product of **II** (16 mg) was obtained.

The pentadeuterio analogue (**III**) was prepared employing identical reactions and the work-up procedures cited above for preparation of **II**, except that sodium borodeuteride, lithium borodeuteride and deuterium oxide, respectively were used.

Acknowledgments

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