POLYENE ANTIBIOTICS. VII

CARBON-13 NUCLEAR MAGNETIC RESONANCE EVIDENCE FOR CYCLIC HEMIKETALS IN THE POLYENE ANTIBIOTICS AMPHOTERICIN B, NYSTATIN A_1, TETRIN A, TETRIN B, LUCENSOMYCIN, AND PIMARICIN[1,2]

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Carbon magnetic resonance establishes conclusively that six polyene macrolide antibiotics containing keto groups (the heptaene amphotericin B, the tetraene-diene nystatin A_1, and the tetraenes tetrin A, tetrin B, pimaricin, and lucensomycin) exist in the hemiketal form in solution. Their spectra all contain a hemiketal carbon's absorption near 97 ppm but lack a keto carbon's absorption near 210 ppm. The non-polyenic macrolide erythromycin, on the other hand, exists in the keto form.

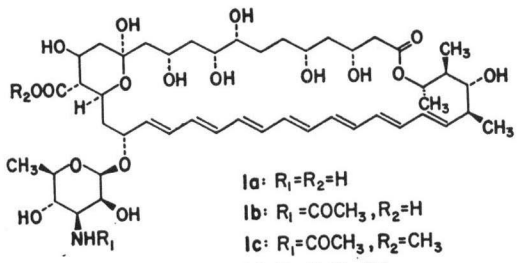
Interest in the polyene macrolide class of antibiotics, always strong due to their potent antifungal properties[3,4], has been heightened by reports of their activity in controlling benign prostatic hyperplasia[5] and hypercholesterolemia[6]. The structures of about 20 polyene macrolide antibiotics have been reported thus far[2,7]. Of these, all contain poly-β-hydroxy units and about 12 contain ketone functions in the macrolide ring. Although the latter were originally portrayed in the carbonyl form, the X-ray structure[8] of amphotericin B (**1a**) showed its ketone group to be present in the crystalline state as a hemiketal, as shown. This raised the possibility that the keto groups in the macrolide rings of other polyene antibiotics may also exist as hemiketals, and ORD data[9] have been reported which support this possibility. However, there has until now been no direct evidence for the presence of hemiketals in these polyenes and none for amphotericin B itself except in the crystalline state.

We wish to report here that carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy provides conclusive direct evidence for the nature of the keto group in polyene antibiotics. Though the ^{13}C NMR spectra of polyenes are very complex, particularly in the olefinic and carbinol regions, they are highly characteristic in the regions between 90 and 110 ppm and between 160 and 220 ppm. In the present paper we shall report results for polyene antibiotics of previously assigned structures—amphotericin B[8], a heptaene; nystatin A_1[10], a tetraene-diene (related to heptaenes by saturation of one double bond); and the tetraenes tetrin A[11], tetrin B[12], pimaricin[13], and lucensomycin[14]. In future papers we shall describe the application of similar ^{13}C NMR observations in the structural assignments of rimocidin[2], the hamycins, and the eurocidins, all of which contain keto groups in the hemiketal form[15].

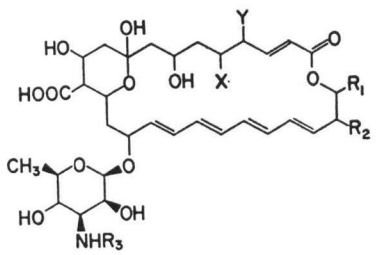
Amphotericin B.

Each of the polyene antibiotics discussed here (Fig. 1) has been assigned a structure which contains one lactone, one carboxyl, and one keto group. To enhance the solubility of the antibiotics in the present investigation the antibiotics were converted to their N-acetyl or methyl ester derivatives; thus, additional acetamide or carbomethoxy groups were present in some derivatives studied. By comparison of individual spectra with one another and with standard shift values[16~18] it was possible to assign all the carbonyl carbons (Table 1). Thus, the lactone absorption at 170.6 ± 0.1 ppm did not vary

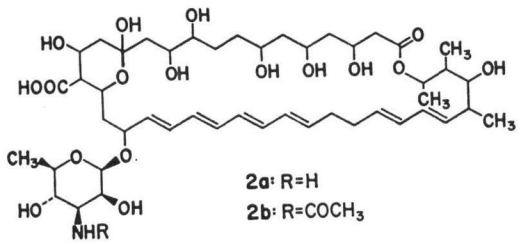
Fig. 1. Structures of polyene antibiotics and their derivatives: amphotericin B (**1a**) and its N-acetyl (**1b**), N-acetyl methyl ester (**1c**), and methyl ester (**1d**) derivatives; nystatin A_1 (**2a**) and its N-acetyl derivative (**2b**); tetrin A (**3a**), tetrin B (**4a**), lucensomycin (**5a**), pimaricin (**6a**) and their respective N-acetyl derivatives (**3b, 4b, 5b, 6b**).



1a: $R_1=R_2=H$
1b: $R_1=COCH_3, R_2=H$
1c: $R_1=COCH_3, R_2=CH_3$
1d: $R_1=H, R_2=CH_3$



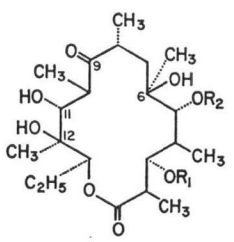
3a: $R_1=R_2=CH_3, R_3=H, X=OH, Y=H$
3b: $R_1=R_2=CH_3, R_3=COCH_3, X=OH, Y=H$
4a: $R_1=R_2=CH_3, R_3=H, X=Y=OH$
4b: $R_1=R_2=CH_3, R_3=COCH_3, X=Y=OH$
5a: $R_1=(CH_2)_3CH_3, R_2=R_3=H, X+Y=-O-$
5b: $R_1=(CH_2)_3CH_3, R_2=H, R_3=COCH_3, X+Y=-O-$
6a: $R_1=CH_3, R_2=R_3=H, X+Y=-O-$
6b: $R_1=CH_3, R_2=H, R_3=COCH_3, X+Y=-O-$



2a: R=H
2b: $R=COCH_3$

among amphotericin B (**1a**) and three of its derivatives (**1b**, **1c**, **1d**), while the amide carbonyl absorption near 169.5 ppm was found only in the N-acetyl derivatives **1b** and **1c** and the carbomethoxy carbonyl absorption of **1c** and **1d** (172.8 ppm) is upfield from those of the carboxyl absorption of **1a** (177.6 ppm) and **1b** (174.3 ppm). The latter carbon presumably exists as the carboxylate anion in **1a** but as a free carboxyl in **1b**. These values are near those expected for medium and large-ring lactones (172.2~173.8 ppm),[16] acetamido (dimethylacetamide, 169.6 ppm)[16], carbomethoxy (172.1~175.7 ppm)[16], carboxyl (179.6~182.8 ppm) and carboxylate (184.3~185.1 ppm) carbonyl carbons[16]. Since there is no ketone carbonyl carbon (near 210 ppm)[16], the ketone carbon may be assumed to be in the hemiketal form. This assumption is substantiated by the absorption of two carbons in the region expected for hemiacetal or hemiketal carbons (92.5~97.5 ppm)[16,19]. One of these, at 96.5±0.5 ppm, is a doublet in the off-resonance spectrum and must be a hemiacetal carbon, C-1 of mycosamine. The other, at 97.3 ppm, is a singlet in the off-resonance spectrum and must be a he-

Fig. 2. Structures of erythromycin A (**7**) and its derivatives, anhydroerythromycin A (**8**) and 8,9-anhydroerythromycin A 6,9-hemiketal (**9**).



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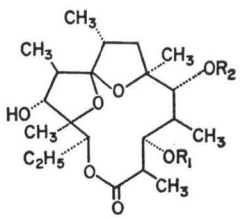

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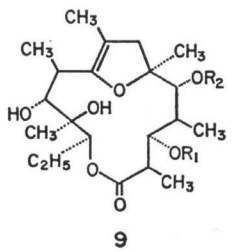

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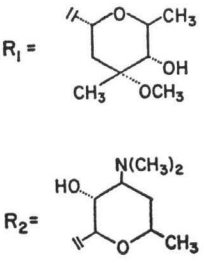

Table 1. Selected carbon magnetic resonance absorptions in polyene antibiotics

Carbon	δ, ppm[a,b,c]													
	1a	**1b**	**1c**	**1d**	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**	**5a**	**5b**	**6a**	**6b**
–C=O |														
lactone	170.6s	170.5s	170.6s	170.6s	170.1	170.5s	164.5	164.6	165.0s	164.7	164.8	164.8	164.6	164.4
$-COO^-$	177.6s				176.3		177.2		177.1s		177.7		175.4	
–COOH		174.3s				174.2s		174.5		174.3		174.1		174.3
$-\underline{C}OOCH_3$			172.8s	172.9s										
$-NH\underline{C}OCH_3$		169.6s	169.3s			169.5s		169.7		169.9		169.7		169.5
–C–O– | O–														
hemiketal	97.1s	97.3s	97.3s	97.3s	97.1	97.3s	97.0	97.2	97.2s	97.3	96.9	97.2	97.0	97.2
hemiacetal (mycosamine C-1)	95.9d	97.0d	96.6d	96.5d	97.6	98.1d	95.5	96.8	95.5d	96.8	95.7	96.9	96.9	96.8

[a] **1a**=amphotericin B, **1b**=N-acetylamphotericin B, **1c**=N-acetylamphotericin B methyl ester, **1d**=amphotericin B methyl ester, **2a**=nystatin A_1, **2b**=N-acetylnystatin A_1, **3a**=tetrin A, **3b**=N-acetyltetrin A, **4a**=tetrin B, **4b**=N-acetyltetrin B, **5a**=lucensomycin, **5b**=N-acetyllucensomycin, **6a**=pimaricin, **6b**=N-acetylpimaricin.

[b] Ppm from TMS, DMSO-d_6 solutions.

[c] Signals marked s and d refer to their multiplicity in off-resonance spectra: s=singlet, d=doublet.

miketal carbon. Thus, C-13 of amphotericin B exists in the hemiketal form in solution as well as in the crystalline state[8]. It is of interest that, while the hemiketal carbon appears in a relatively narrow chemical shift range (97.2±0.1 ppm), the hemiacetal carbon is sensitive to the nature of the 2-amino group of mycosamine and shifts upfield on protonation (as in **1a**).

Nystatin A_1.

The same arguments can be applied to other polyene antibiotics as well. Thus, the ^{13}C NMR spectrum of N-acetylnystatin A_1 (**2b**) contains a lactone carbon at 170.5 ppm, a carboxyl carbon at 174.2 ppm, and an acetamide carbonyl carbon at 169.5 ppm. Free (unacetylated) nystatin A_1 (**2a**), as expected, lacks the acetamido carbon, while the lactone carbon is in the same region and the carboxyl carbon is shifted downfield to 176.3 ppm. Neither compound **2a** nor **2b** contains absorption for a ketonic carbon. Instead, a hemiketal carbon (singlet in the off-resonance spectra) is found at 97.1 ppm.

Tetrin A, Tetrin B, Pimaricin, Lucensomycin.

The ^{13}C NMR spectra of the tetraene antibiotics tetrins A and B, lucensomycin, and pimaricin, and those of their N-acetyl derivatives are remarkably similar in their chemical shift values. They all contain carboxylate (or carboxyl) and acetamido carbonyl carbon absorptions in the same regions found for amphotericin B and nystatin A_1–176.9±0.6 ppm ($-COO^-$), 174.3±0.2 ppm (–COOH), 169.7±0.2 ppm ($-NHCOCH_3$). However, the lactones are all conjugated and the lactone carbonyl carbons are found, as expected[16~18], at *ca.* 6 ppm higher field (164.7±0.3 ppm). Again, no ketone carbons are observed, but hemiketal carbons (singlets) are found at 97.1±0.2 ppm, along with the hemiacetal (mycosamine C-1) carbons (doublets) at 95.6±0.1 or 96.8±0.1 ppm (ammonium salt or N-acetyl derivative, respectively).

Erythromycin A.

All of the polyene antibiotics we have examined exist in the hemiketal form if they contain only one keto group (although a second keto group in some other polyenes can exist in the carbonyl form)[2,15]. Some polyene antibiotics, *e.g.* filipin[20], chainin[21], and dermostatin[1] do not contain keto groups and thus cannot exist as hemiketals. On the other hand, a whole group of nonpolyene macrolide antibiotics exists[2,22,23] and most of these contain keto groups. The most important of these clinically is erythromycin A (**7**, Fig. 2, originally called erythromycin). Erythromycin A contains a secondary hydroxyl *beta* to the ketone in one direction (11-hydroxyl) and tertiary hydroxyl groups *gamma* to the ketone in both directions (6- and 12-hydroxys). In spite of the γ-hydroxyls, the X-ray structure[24] does not show erythromycin A in the hemiketal form in the solid state, although derivatives of erythromycin A are known in which the keto group at C-9 exists in the ketal (**8**)[25] or anhydrohemiketal (**9**)[25] form. We have examined the ^{13}C NMR spectra of erythromycin A and its derivatives for evidence of a hemiketal structure in erythromycin. In the spectrum of **8** (anhydroerythromycin A) C-9 appears at 116.0 ppm and in that of **9** (8, 9-anhydroerythromycin A 6, 9-hemiketal) C-9 appears at 151.9 ppm; both signals are singlets in the off-resonance spectra. The spectrum of erythromycin A (**7**) shows a keto carbon (C-9) at 221.9 ppm whose intensity is comparable to that of the lactone carbon (C-1) at 176.1 ppm. Two dioxygenated carbons appear at 103.3±0.2 and 95.7±0.9 ppm but these are doublets in the off-resonance spectrum and must be due to the anomeric carbons of the two sugars, β-D-desosaminyl and α-L-cladinosyl, respectively[26,27]. In view of the keto carbon's absorption at 221.9 ppm and the lack of a ketal carbon's absorption near 100 ppm, erythromycin A is concluded to exist in the keto form. Similar conclusions have been reached by others for keto carbons in non-polyene macrolide antibiotics,[26,27]

including erythromycin A[27].

Experimental

General. Melting points, determined on a Kofler micro hot stage or a Fisher-Johns melting point apparatus, are uncorrected. Infrared spectra were recorded on Perkin-Elmer Infracord, Model 137, and Beckman IR 12 infrared spectrophotometers. Ultraviolet spectra were taken in methanol, employing Beckman, Model DB, and Cary, Model 115, spectrophotometers. Optical rotations were measured on a Zeiss polarimeter. Proton magnetic resonance (^{1}H NMR) spectra were obtained by Mr. R. THRIFT and associates on Varian HA–100 and HR–220 spectrometers, employing TMS or DSS as the internal standard. ^{13}C NMR spectra were obtained by Mr. R. THRIFT, Dr. S. E. ULRICH and their associates on a Varian XLFT–100 spectrometer with Digilab computer. The spectra were measured in 12-mm sample tubes, employing DMSO-d_6 as solvent for polyene antibiotics and $CDCl_3$ for erythromycin A and its derivatives, TMS as internal standard, and a deuterium lock. The purity of the samples was checked by tlc and uv spectrum. N-Acetyl derivatives are more stable than the underivatized antibiotics, which slowly decompose in DMSO solution. Thin-layer chromatography was carried out on precoated thin-layer chromatography plates (silica gel GF, 250 microns) from Analtech, Inc. Developing solvent 1 consisted of chloroform - methanol - 0.05 M borate buffer, pH 8.3 (2: 2: 1, v/v, lower layer), developing solvent 2 of 1-butanol - acetic acid - water (4: 1: 5, v/v, upper layer). The spots were visualized either by exposure to iodine vapors or by spraying with sulfuric acid - glacial acetic acid (1: 1, v/v).

Amphotericin B (Squibb, #NN046NC) used in the present investigation turned brown at 200°C and decomposed above at 280°C [lit.[28] mp>170°C]; the sample had Rf 0.45 in solvent 2. The uv spectrum had $\lambda\lambda_{max}$ 405, 382, 364, 346 nm, $E^{1\%}_{1cm}$ 1282 at 382 nm [lit[28]. $E^{1\%}_{1cm}$ 1650 at 382 nm].

N-Acetylamphotericin B, prepared by the method reported earlier for N-acetyltetrin A,[11] had mp >160°C (d), Rf 0.26 in solvent 1, Rf 0.58 in solvent 2, $\lambda\lambda_{max}$ 406, 382, 364, 346 nm. Its ir spectrum (KBr) had bands at 3450, 2940, 1725, 1640, 1562, 1545, 1385, 1300, 1190, 1132, 1115, 1075, 1015, 855, and 795 cm^{-1}. The ^{1}H NMR spectrum (220 MHz, DMSO-d_6) showed an acetyl methyl at δ 1.06 (s, 3 H) in addition to the other methyl groups present in amphotericin B [δ 0.90 (d, 3 H), 1.00 (d, 3 H), 1.12 (d, 3 H), 1.15 (d, 3 H), J=6 Hz for each doublet].

Amphotericin B methyl ester, prepared by the reported method[29] employing diazomethane in tetrahydrofuran, darkened at 145°C, decomposed above 165°C, and had Rf 0.67 in solvent 1, Rf 0.65 in solvent 2, $\lambda\lambda_{max}$ 404, 380, 362, 344 nm. The ^{1}H NMR spectrum (220 MHz, DMSO-d_6) showed a signal at δ 3.68 assignable to a carbomethoxyl group, in addition to the other methyl signals present in the original molecule [δ 0.94 (d, 3 H), 1.07 (d, 3 H), 1.16 (d, 3 H), 1.20 (d, 3 H), J=6 Hz for each doublet].

N-Acetylamphotericin B methyl ester was prepared from N-acetylamphotericin B with diazomethane in tetrahydrofuran using methanol as the solvent instead of dimethyl sulfoxide[29]. The derivative darkened at 165°C, decomposed above 190°C, and had Rf 0.67 in solvent 1, Rf 0.62 in solvent 2, $\lambda\lambda_{max}$ 404, 380, 360, 344 nm. The ^{1}H NMR spectrum (220 MHz, DMSO-d_6) showed N-acetyl and carbomethoxyl methyl groups at δ 1.90 (s, 3 H), and 3.70 (s, 3 H), respectively. The other methyl groups appeared at δ 0.94 (d, 3 H), 1.06 (d, 3 H), 1.14 (d, 3 H) and 1.20 (d, 3 H), J=6 Hz for all doublets.

Nystatin (Upjohn, U–6908, Lot #4983), whose principal component[30] is nystatin A_1, darkened at 160°C and decomposed above 300°C [lit[31]. mp >160°C (d)]; it had $\lambda\lambda_{max}$ 318, 304, 290, 278 (sh), 228 nm, $E^{1\%}_{1cm}$ 868 at 304 nm].

N-Acetylnystatin, prepared by the method reported earlier for N-acetyltetrin A[11], softened at 130°C, decomposed above 180°C, and had $\lambda\lambda_{max}$ 319, 304, 291, 280 (sh), and 228 nm.

Tetrin A had mp >360°C (d) [lit[32]. mp >350°C(d)], Rf 0.48 in solvent 2, $\lambda\lambda_{max}$ 320, 306, 294, 280 (sh), 210 nm, $E^{1\%}_{1cm}$ 1013 at 306 nm [lit[29]. $E^{1\%}_{1cm}$ 1150 at 303 nm].

N-Acetyltetrin A, prepared as described earlier[11], had mp 161~165°C (d) [lit.[11,32] mp 167~171°C], Rf 0.35 in solvent 1, Rf 0.56 in solvent 2, $\lambda\lambda_{max}$ 320, 306, 294, 280 (sh) and 210 nm.

Tetrin B darkened at 160°C, melted above 360°C (d) [lit[12]. mp >360°C], and had Rf 0.26 in solvent

1, Rf 0.47 in solvent 2, $\lambda\lambda_{max}$ 320, 306, 292, 280 (sh), 210 nm, $E^{1\%}_{1cm}$ 876 at 306 nm [lit.[32] $E^{1\%}_{1cm}$ 1128 at 303 nm].

N-Acetyltetrin B, prepared as described earlier[12], darkened at 140°C, melted above 180°C(d) [lit[12]. mp 190°C], and had Rf 0.30 in solvent 1, Rf 0.63 in solvent 2, $\lambda\lambda_{max}$ 320, 306, 294, 280 (sh) and 210 nm.

Lucensomycin darkened at 210°C, decomposed above 300°C [lit[33]. mp 150°C (d)], and had Rf 0.36 in solvent 1, Rf 0.45 in solvent 2, $\lambda\lambda_{max}$ 318, 304, 290, 280 (sh), and 313 nm, $E^{1\%}_{1cm}$ 1184 at 304 nm[lit[33]. $E^{1\%}_{1cm}$ 1390 at 304~305 nm].

N-Acetyllucensomycin, prepared by a procedure described earlier,[12] softened at 140°C, decomposed above 160°C [lit[34]. mp 170°C (d)], and had Rf 0.37 in solvent 1, Rf 0.63 in solvent 2, $\lambda\lambda_{max}$ 318, 304, 288, 278 and 216 nm. The ^{1}H NMR spectrum (100 MHz, C_5D_5N) showed the acetyl methyl group at δ 2.05 (s, 3 H) in addition to the other methyl groups present in lucensomycin [δ 1.48 (d. 3 H, J=6 Hz), 0.85 (t, 3 H)].

Pimaricin (Lederle, 4007B53A), crystallized from methanol, darkened at 180°C, decomposed above 300°C [lit[32]. mp *ca.* 200°C (d)], and had Rf 0.33 in solvent 1, Rf 0.45 in solvent 2, $\lambda\lambda_{max}$ 318, 304, 290, 280 (sh) and 216 nm, $E^{1\%}_{1cm}$ 1020 at 304 nm [lit[36]. $E^{1\%}_{1cm}$ 1100 at 302 nm]. The methyl groups in the ^{1}H NMR spectrum (100 MHz, C_5D_5N) appeared at δ 1.28 (d, 3 H) and 1.46 (d, 3 H), J=6 Hz for both doublets.

N-Acetylpimaricin, prepared as described earlier[12], had mp 178~180°C (d) [lit[37]. mp 200°C], Rf 0.64 in solvent 2. The ^{1}H NMR spectrum (100 MHz, C_5D_5N) had the acetyl methyl group at δ 2.04 (s, 3 H), in addition to the other methyl groups in pimaricin [δ 1.29 (d, 3 H) and 1.46 (d, 3 H), J=6 Hz for both doublets].

Erythromycin (Upjohn U-4364, base) had mp 135~137°C, $[\alpha]_D^{26}$ −73.0° (*c* 3.22, MeOH) [lit[38]. mp 135~140°C [lit[39]. $[\alpha]_D^{25}$ −78° (*c* 1.99, EtOH)].

Anhydroerythromycin A was prepared according to the reported method[40] and crystallized from methylene chloride-hexane[25], mp 140~146°C, $[\alpha]_D^{26}$ −65.3° (*c* 1.24, MeOH), ε_{209} 923 [lit[25]. mp 130~140°C].

8, 9-Anhydroerythromycin A 6, 9-hemiketal was prepared by the reported method[25] and crystallized from carbon tetrachloride, mp 133~135°C, $[\alpha]_D^{26}$ −42.7° (*c* 1.24, MeOH), λ_{max}^{MeOH} 209 nm (ε 6035). [lit[25] mp 133~135°C; $[\alpha]_D^{25}$ −43° (*c* 1.19, MeOH), λ_{max}^{MeOH} 209 nm (ε 6640)].

Acknowledgment

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