N-Methyl-*N*-D-fructopyranosylamphotericin B Methyl Ester, New Amphotericin B Derivative of Low Toxicity

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The membrane active polyene macrolide antibiotics belong to the most interesting antifungal agents due to their high activity, broad spectrum, fungicidal action and reluctance to induce resistance. Among them only amphotericin B (AMB) is used in therapy for the treatment of systemic mycoses. However, its application in clinical practice is significantly limited because of poor selective toxicity and low water solubility. Many efforts have been made to remove these undesirable features by chemical modifications of the native antibiotic[1,2] as well as by the search for its new formulations[3]. Substantial reduction of amphotericin B renal toxicity in humans was recently achieved by the introduction of its lipid based preparations to the clinical use.

In our antifungal drug design program aimed at the improvement of the therapeutic properties of AMB by chemical modifications we have shown that the water solubility of its derivatives depends on net charge of the molecule[4].

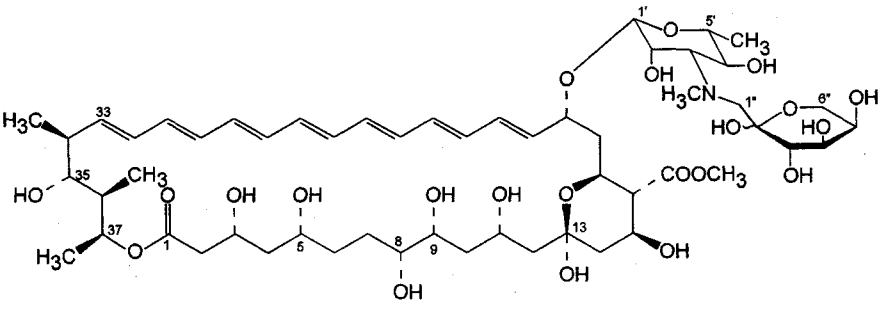
The structure-activity relationship established for AMB derivatives[5] as well as the results of biophysical studies allowed us to develop the concept of the molecular model of AMB-membrane sterol primary complex[6]. The recognition of molecular forces participating in complex formation points to the substitution at the AMB amino group as most promising way to achieve the increase of selective toxicity of a derivative. The *N*-substituent should be of the type which would allow the protonation of amino group essential for the interaction with sterol.

Recently, we have synthesized several new compounds fulfilling the above requirements for the improved selective toxicity and water solubility. These are *N*-methyl-*N*-glycosyl derivatives of polyene macrolides methyl esters[7]. Among them the most advantageous compound which we present in this paper appeared to be *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (MF-AME, Fig. 1). The derivative retained most of the antifungal activity of parent antibiotic and exhibited dramatically decreased animal toxicity due to the very improved selectivity of its biological action. The MF-AME is of a basic character and forms water soluble salts with organic and inorganic acids.

The *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester was obtained from the earlier synthesized *N*-D-fructopyranosylamphotericin B formed in the reaction of the amphotericin B and D-glucose occuring with simultaneous Amadori rearrangement[8]. The next step was the methylation of the derivative with diazomethane. The best yield of *N*-methylated product was achieved if the substrate used was in zwitterionic form well preserved in solid state. Thus, 1 g of *N*-D-fructopyranosylamphotericin B was dissolved in 10 ml of DMF and 60 ml of dry diethyl ether was added with vigorous stirring to precipitate the fine solid.

The flask was cooled in ice bath to 0~2°C and diethyl ether solution of diazomethane was added in ratio 2.5 mmol of CH_2N_2/1 mmol of the substrate. The reaction mixture was stirred for 1~2 hours at 0°C. The course of the reaction was followed by TLC on silica gel with $CHCl_3$ - MeOH - H_2O, 10 : 6 : 1 solvent system. The mixture contained several components. The MF-AME was the product with R_f value 0.5~0.54. After completion of the reaction an excess of the diazomethane was destroyed with acetic acid and then diethyl ether was

Fig. 1. The structure of *N*-methyl-*N*-D-fructopyranosylamphotericin B methyl ester (MF-AME).



evaporated under reduced pressure. The crude product (0.95 g) was precipitated with ether, centrifuged, washed with ether and *n*-hexane and dried in vacuum. The pure MF-AME was isolated by column chromatography on silica gel 60, 70~230 mesh, using $CHCl_3$ - MeOH - H_2O, 20 : 8 : 1 solvent system to give 0.137 g of final product.

The compound exhibited electronic absorption maxima of the same wavelengths as amphotericin B at $\lambda = 363,382$ ($E^{1\%}_{1\,cm}$ 1300) and 406 nm in MeOH. The oscillation bands at $\nu = 1730\,cm^{-1}$ and lack of $1590\,cm^{-1}$ revealed in IR spectrum the presence of ester bond. The structure of MF-AME was derived from NMR data upon ^1H (DQF-COSY, ROESY), ^{13}C (DEPT) and a heterocorrelated spectra (300 MHz). The most significant ^1H and ^{13}C information are listed in Table 1, 2 and 3. The NMR data of aglycone were in full agreement with those for amphotericin B[9,10]. ^1H chemical shifts (in DMSO/MeOD solvent system) of $-CH_3$ ($\delta = 2.35$ ppm) and H1″ (2.30 and 3.15 ppm) are characteristic for influence of an amino group substituent. After acidification δ changes to 2.92 ppm for $-CH_3$ and to 3.40; 3.64 for H1″ due to protonation of amino group (δ of the H3′ changes to 3.19 ppm). These evidences are supported also by ROE's NCH_3/3′, NCH_3/2′, NCH_3/1″b and 1″a/3′.

Coupling constants and ROE's reflect $^1C_1$ conformation of the mycosamine moiety as it was found for AMB. The comparison of ^{13}C data (Table 2) for the fructosyl fragment and literature data for fructoses[11] suggested the pyranoside form of the sugar substituent.

For the biological studies we have used MF-AME L-aspartate as the most convenient water soluble salt. To obtain the salt 0.137 g of MF-AME was suspended in 2.5 ml of water and 0.017 g of L-aspartic acid was added in 0.5 ml of water dropwise to give a clear solution. The MF-AME L-aspartate was then precipitated with an excess of acetone, centrifuged, washed twice with acetone and diethyl ether and dried in vacuum to yield 0.13 g of final product ($E^{1\%}_{1\,cm}$ 1100 at 382 nm, MeOH).

The biological properties of the MF-AME L-aspartate are presented in Table 3. The antifungal activity (IC_{50})

Table 1. ^1H Chemical shifts and ROE effects of MF-AME disaccharide fragment.

	Pyridine-d_5/methanol-d_4 =9:1		DMSO-d_6/methanol-d_4 =4:6	
Proton*	δ (ppm)	ROE to protons	δ (ppm)	ROE to protons
1′	4.77	2′, 3′, 5′, 18b, COOMe	4.48	3′, 5′
2′	4.41	1′, 3′, NMe, 17, COOMe, 1″a?	4.04	3′, NMe, COOMe
3′	2.06	1′, 2′, 5′, NMe, 1″a, COOMe	1.89	1′, 2′, NMe, 1″b
4′	4.38	6′	3.80	6′
5′	3.61	1′, 3′, 6′	3.45	1′, 3′, 6′
6′	1.23	4′, 5′	1.27	4′, 5′
1″a	2.56	3′, 3″ (2′)	2.30	
1″b	3.58	NMe	3.15	3″, 3′
3″	4.41	1″a?	3.65	1″b
4″	4.76		3.97	
5″	4.41		3.63	
6″a	4.21		3.57	
6″b	4.36		3.76	
NMe	2.29	2′, 3′, 1″b, COOMe	2.35	2′, 3′, 1″a, 1″b, COOMe
COOMe	3.70	1′, 2′, 3′, NMe, 16	3.77	2′, NMe

* Numbering is illustrated in Fig. 1.

Table 2. ^{13}C Chemical shifts of MF-AME disaccharide fragment and their comparison with fructoses data.

Carbon*	δ (ppm)	Data for fructoses, δ (ppm)		
		β-D-Fructopyranose	α-D-Fructofuranose	β-D-Fructofuranose
1′	98.3			
2′	72.4			
3′	66.4			
4′	69.8			
5′	72.3			
6′	18.2			
1″	62.2	64.1	62.1	63.9
2″	98.1	99.1	105.3	102.4
3″	66.9	70.5	83.0	76.5
4″	71.2	68.4	77.0	75.5
5″	72.2	70.0	82.2	81.5
6″	64.6	64.7	62.1	63.3
NMe	40.9			
COOMe	51.6			

* Numbering is illustrated in Fig. 1.

Table 3. $J_{H,H}$ coupling constants* within disaccharide fragment of MF-AME.

(Py-d_5/methanol-d_4 9 : 1)			
Protons	J [Hz]	Protons	J [Hz]
1′, 2′	0	1″a, 1″b	11.60
2′, 3′	2.2	3″, 4″	1.05
3′, 4′	9.5	4″, 5″	8.09
4′, 5′	9.2	5″, 6″a	5.76
5′, 6′	6.0	5″, 6″b	3.61
		6″a, 6″b	−10.86
		4″, 6″a	− 0.10

* Coupling constants and chemical shifts of fructose moiety's protons were refined iteratively by spins simulation due to occuring of a closely coupled spin systems (3″~6″).

Table 4. Biological data for MF-AME L-aspartate.

Compound	IC_{50} *Candida albicans* (μg/ml)	EH_{50} (μg/ml)	EK_{50} (μg/ml)	LD_{50} i.v. (mg/kg)
MF-AME L-aspartate	0.15	>250	40	>400
AMB	0.07	2	0.5	6 (Fungizone)

IC_{50}: The concentration of compound tested causing 50% inhibition of the growth of *Candida albicans* ATCC 26278 in Sabouraud liquid medium determined spectrophotometrically (λ=660 nm) after 24 hours incubation at 30°C.

EH_{50}: The concentration of compound tested causing 50% release of hemoglobin from human erythrocytes in saline after 30 minutes incubation at 37°C determined spectrophotometrically at λ= 550 nm.

EK_{50}: The concentration of compound tested causing 50% intracellular potassium release from human erythrocytes in saline after 30 minutes incubation at 37°C determined by flame photometry.

LD_{50}: Acute i.v. 50% lethal dose test carried out with groups of 5 female mice (Swiss Webster) weighing 20 g.

of the derivative was only slightly diminished when compared to the parent antibiotic. On the other hand, hemolytic and membrane-permeabilizing activities (EH_{50} and EK_{50} respectively) in regard to human red blood cells were very significantly reduced - in the case of hemolysis test this reduction was by up to two orders of magnitude. In consequence, the very great improvement of selective toxicity was observed. The compound is dramatically less toxic than AMB (Fungizone) in terms of acute toxicity in mice (LD_{50}, i.v., >400 mg/kg and 6 mg/kg, respectively). Among all known amphotericin B derivatives MF-AME L-aspartate remains the only compound exhibiting such a markedly diminished toxicity and for that reason was selected for broad pharmacological studies, which are under progress.

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