NEW *N*-ALKYL DERIVATIVES OF AMPHOTERICIN B

SYNTHESIS AND BIOLOGICAL PROPERTIES

ANDRZEJ CZERWIŃSKI, WILFRIED A. KÖNIG†, TERESA ZIENIAWA, PAWEŁ SOWIŃSKI, VOLKER SINNWELL†, SŁAWOMIR MILEWSKI and EDWARD BOROWSKI

Department of Pharmaceutical Technology and Biochemistry,
Technical University of Gdańsk,
80-952 Gdańsk, Poland
†Institut für Organische Chemie, Universität Hamburg,
Martin Luther King Platz 6, D 2000 Hamburg, FRG

(Received for publication February 8, 1991)

The synthesis of new *N*-alkyl amphotericin B derivatives obtained in the Michael addition reaction of the antibiotic with *N*-substituted maleimides is described and *in vitro* biological data are presented.

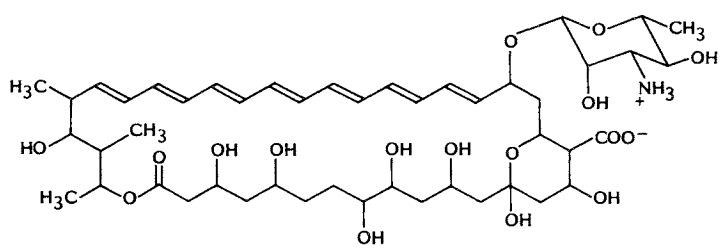
Amphotericin B (AMB), Fig. 1, the polyene macrolide antifungal antibiotic of high clinical importance, is the drug of choice for the treatment of deep-seated disseminated mycotic infections in humans[1,2]. However, the therapy is associated with toxic side effects, the most serious being the nephrotoxicity[3]. These undesirable effects are consequences of the poor selectivity of AMB interaction with the animal and fungal cellular sterol targets[4]. To improve the biological and physico-chemical properties of the antibiotic, a program of derivative synthesis has been undertaken. It resulted in a number of AMB modification products with increased selective toxicity and solubility in water[5,6]. In this report, we describe the preparation and *in vitro* biological properties of a novel group of *N*-alkyl AMB derivatives.

Materials and Methods

General

AMB was obtained from Hoffmann-La Roche Ltd. (Basel, Switzerland). *N*-Ethylmaleimide (NEM) was purchased from Fluka (Buchs, Switzerland). *N*,*N*'-Hexamethylenedimaleimide and *N*-(3-dimethylaminopropyl)maleimide hydrochloride were synthesized according to known methods[7,8]. TLC was carried out on precoated silica gel plates (Kieselgel 60, Merck). UV spectra were measured in methanol using a Beckman Model 3600 spectrophotometer. ^{1}H NMR spectra were obtained with a Bruker WM-400 spectrometer operating at 400.13 MHz. Solutions of the compounds in DMSO-d_6 (internal standard, 2.49 ppm) were used. The OH's were exchanged with CD_3OD which was subsequently removed by

Fig. 1. Structure of AMB.



evaporation *in vacuo*. FAB mass spectra were recorded on a VG 70-250S mass spectrometer (VG Instruments, Manchester, UK) fitted with a saddle field FAB gun (Ion Tech, UK) operated with xenon at 8 keV and 1 mA. 3-Nitrobenzyl alcohol was employed as the matrix.

Biological activity of the compounds synthesized was determined as described previously[9].

Synthesis

In a typical synthesis, to a stirred suspension of AMB (277 mg, 0.3 mmol) in DMF (5 ml) triethylamine (0.042 ml, 0.3 mmol) and then NEM (225 mg, 1.8 mmol) were added. The mixture was stirred at room temperature for 27 hours. After the addition of anhydrous ethyl ether (250 ml), the precipitate formed was separated by centrifugation, washed with ether and dried *in vacuo* to yield 248 mg of a crude product. This was purified by column chromatography (SiO_2; $CHCl_3$-MeOH-H_2O, 13:6:1) to give 137 mg (yield 43.6%) of *N*-(*N'*-ethylsuccinimido)amphotericin B (**1**). The other derivatives were obtained in a similar manner. In regard to the purification procedure, the exception was compound **2**, which was purified by counter-current distribution in a Craig apparatus ($CHCl_3$-MeOH-0.5% NaCl aq solution, 2:2:1, 140 transfers, partition coefficient K=2.18).

Results and Discussion

Synthesis

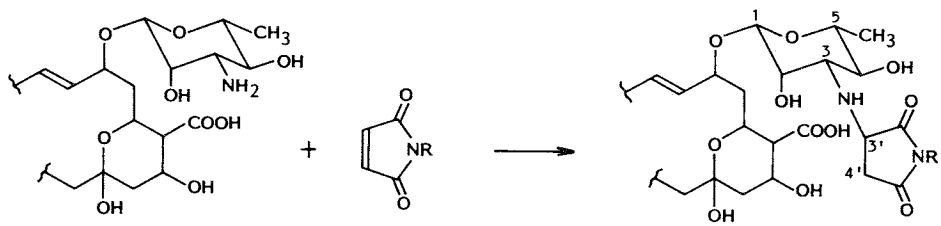
According to the molecular model of polyene-sterol interaction[10], the nitrogen atom in *N*-substituted polyene macrolide derivatives should be protonable to ensure the formation of a hydrogen bond with the oxygen atom of a sterol hydroxyl group, indispensable for the proper mutual orientation of both reacting molecules. The compounds unable to form such a bond, like *N*-acetylamphotericin B, exhibit poor antifungal activity[6]. The *N*-alkyl derivatives retain the basic character of the nitrogen atom fulfilling the aforementioned requirement. However, *N*-alkylation of polyene macrolides is rather difficult. The Michael addition reaction[11] with *N*-substituted maleimides, described here, provides a novel and general route for the synthesis of a variety of polyene macrolides *N*-alkyl derivatives. The corresponding

Table 1. Rf values of AMB and its derivatives on silica gel.

Compound	A	B	C	D
AMB	0.24	0.19	0.54	0.56
1	0.78	0.38	0.77	0.82
2	0.09	0.09	0.15	0.19
3	0.86	0.54	0.83	0.84

A: $CHCl_3$-MeOH-water (10:6:1).
B: EtOAc-AcOH-water (4:1:1).
C: 1-Butanol-AcOH-water (3:1:1).
D: 1-Butanol-pyridine-water (3:2:1).

Fig. 2. The site of the Michael addition of AMB to *N*-substituted maleimides and the resulted products.



1 R=$-C_2H_5$
2 R=$-(CH_2)_3-N(CH_3)_2$
3 R=$-(CH_2)_6-N$

Table 2. Major ions in the positive ion FAB mass spectra of compounds **1**~**3**.

Compound	Formula	MW	Major ions (*m/z*)
1	$C_{53}H_{80}N_2O_{19}$	1,049	1,072 $(M+Na)^+$, 1,050 $(M+H)^+$, 761 $(M+H-aminosugar)^+$
2	$C_{56}H_{87}N_3O_{19}$	1,106	1,107 $(M+H)^+$, 1,089 $(M+H-H_2O)^+$, 761 $(M+H-aminosugar)^+$, 743 $(M+H-aminosugar-H_2O)^+$
3	$C_{61}H_{89}N_3O_{21}$	1,200	1,223 $(M+Na)^+$, 1,201 $(M+H)^+$, 761 $(M+H-aminosugar)^+$, 743 $(M+H-aminosugar-H_2O)^+$

Table 3. ^1H NMR spectral data of AMB derivatives **1**~**3**[a].

Assignment	**1**		**2**		**3**	
	δ (ppm)	$J_{H,H}$ (Hz) (coupling partner)	δ (ppm)	$J_{H,H}$ (Hz) (coupling partner)	δ (ppm)	$J_{H,H}$ (Hz) (coupling partner)
Aminosugar:						
1	4.44,		4.31,		4.43,	
		~0		~0		~0
	4.35		4.33		4.35	
2	3.76,		3.68,		3.76,	
		d 1.5 (3)		d 2.9 (3)		d 2.3 (3)
	3.63		3.58		3.63	
3	2.34,		2.35,		2.33,	
		dd 1.5 (2), 9.8 (4)		dd 2.9 (2), 9.6 (4)		dd 2.3 (2), 9.8 (4)
	2.59		2.47		2.54	
4	2.89,		2.93,		2.90,	
		dd 9.8 (3), 9.8 (5)		dd 9.6 (3), 9.6 (5)		dd, 9.4 (3), 9.4 (5)
	3.03		3.02		3.03	
5	3.10,		3.08	m 9.6 (4), 6.0 (6)	3.10,	
		m 9.8 (4), 6.0 (6)				m 9.4 (4), 6.0 (6)
	3.13				3.13	
6	1.131,		1.149,		1.134,	
		d 6.0 (5)		d 6.0 (5)		d 6.0 (5)
	1.128		1.141		1.130	
Dioxopyrrolidine:						
3′	3.87,		3.88,		3.87,	
		dd 8.1 (4a), 4.5 (4b)		dd 8.1 (4a), 4.9 (4b)		dd 8.0 (4a), 4.5 (4b)
	3.96		3.96		3.96	
4′a	2.91,		2.90,		2.91,	
		dd 8.1 (3), 17.7 (4b)		dd 8.1 (3), 17.8 (4b)		dd 8.0 (3), 17.8 (4b)
	2.92		2.91		2.93	
4′b	2.48,		2.55,		2.48,	
		dd 4.5 (3), 17.7 (4a)		dd 4.9 (3), 17.8 (4a)		dd 4.5 (3), 17.7 (4a)
	2.57		2.65		2.54	
N-Alkyl:						
1″	3.38	m	3.40	m	3.36	m
2″	1.043,		1.97	m	1.45	m
		t 7.2 (1)				
	1.039					
3″	—		2.56	m	1.21	m
4″	—		—		1.21	m
5″	—		—		1.45	m
6″	—		—		3.36	m
$N(CH_3)_2$	—		2.38	br s	—	
CH=CH	—		—		6.97	s

[a] The analyzed compounds were a mixture of diastereoisomers that resulted in pairing of some proton signals observed in their ^1H NMR spectra.

addition products are shown in Fig. 2.

Chromatographic properties of the products are given in Table 1. The derivatives exhibited UV absorption maxima at 363, 382 and 406 nm, the same as the parent antibiotic. $E^{1\%}_{1cm}$ values at 382 nm for compounds **1**, **2** and **3** were 1,370, 1,210 and 1,200, respectively. FAB-MS spectrometry of the synthetic derivatives showed MW's in accordance with the proposed structures. The corresponding data are presented in Table 2. Moreover, in the spectra the ion at m/z 761 ($(M+H-\text{aminosugar})^+$) was found indicating the presence of unchanged aglycone and modified mycosamine in the derivatives examined. ^1H NMR data of *N*-alkylated aminosugar derivatives are listed in Table 3. The signals were assigned on the basis of a 2D NMR experiment.

Biological Activity

Antifungal and hemolytic properties of the AMB derivatives were tested on *Saccharomyces cerevisiae* and *Candida albicans*, as models of ergosterol-containing fungal cells and on human red blood cells (RBC), representative of cholesterol-containing host cells, respectively. The antifungal, hemolytic and membrane-permeabilizing activities (MIC, EH_{50} and EK_{50}, respectively) of derivatives **1**~**3** and AMB, as a reference compound, are reported in Table 4. Of the derivatives tested, only compound **2** retained the comparable antifungal activity of the parent antibiotic. Compound **1** was somewhat less active than AMB while compound **3** was significantly less active. On the other hand, all derivatives exhibited decreased hemolytic and RBC-permeabi-

Table 4. Biological properties of AMB and its derivatives (**1**~**3**).

Compound	MIC (μg/ml)		EH_{50} (μg/ml)	EK_{50} (μg/ml)
	Saccharomyces cerevisiae ATCC 9763	*Candida albicans* 2047		
AMB	0.15	0.13	2.0	0.4
1	0.50	0.38	>200	>10
2	0.18	0.15	38	4.1
3	3.50	2.75	>200	>10

EH_{50}: Concentration of compound which induced 50% hemolysis of human erythrocytes.

EK_{50}: Concentration of compound causing 50% of intracellular potassium release from human erythrocytes.

Table 5. The antifungal spectra of AMB, its derivative **2** and AMA.

Test organisms	MIC (μg/ml)		
	AMB	**2**	AMA
Saccharomyces cerevisiae ATCC 9763	0.15	0.18	0.20
Candida albicans ATCC 26278	0.08	0.10	0.10
C. albicans[a] (3)	0.18	0.18~0.23	0.50
C. arborea PCM 1427	0.38	0.50	0.75
C. mycoderma Łock 2	0.25	0.38	0.50
C. tropicalis[a]	0.25	0.23	0.50
Geotrichum candidum[a]	0.15	0.18	0.25
Torulopsis candida PCM 282	0.75	0.75	1.50
Trichophyton nanum[a]	0.15	0.13	0.25
Aspergillus niger	60	50	75
A. nidulans 590[a]	50	38	50
Penicillium cytrinum	55	45	75
Mucor mucedo[a]	35	30	50

[a] Clinical isolates.

( ): No. of strains.

MIC values of AMB, compound **2** and AMA were obtained in a side by side experiment. The data for AMB and AMA have been also reported elsewhere[15].

lizing activities when compared to AMB. We focused our interest on compound **2**, which displayed high antifungal activity as well as much better selective toxicity than the parent antibiotic. Moreover, it formed a water soluble salt upon addition of triethylamine. We compared the antifungal activity of compound **2** with that of amphotericin B 3-dimethylaminopropylamide diaspartate (AMA). The latter compound, being optimal among the first generation AMB derivatives[6], has been shown to exhibit *in vitro*[6] and *in vivo*[12] biological properties much improved as compared to the parent antibiotic. The MIC values of AMB, derivative **2** and AMA are summarized in Table 5. Compound **2** was essentially as active as AMB and appeared to have somewhat higher activity than AMA against most fungi tested. Furthermore, it was less hemolytic than AMA and showed an RBC-permeabilizing activity ten times less than AMB and comparable to that of AMA (AMA; $EH_{50}=11\,\mu g/ml$, $EK_{50}=4.5\,\mu g/ml$; the data for compound **2** can be seen in Table 4).

As mentioned previously, compounds **1** and **3** exhibited diminished antifungal activity, as compared to AMB. This might be explained by the decrease of a basic character of the modified amino group in the aminosugar moiety and/or a steric hindrance effect of the dioxopyrrolidine ring, both influencing the polyene-sterol target interaction. Derivative **2** retained the high antifungal activity of the parent antibiotic. This retention can be attributed to the presence of the additional, basic nitrogen atom in the modified aminosugar moiety. It has been shown previously that the activity of a polyene decreased by *N*-substitution resulting in loss of basicity of the nitrogen atom (*e.g.* *N*-acylation). The activity can be restored upon incorporation of a new basic amino group (*e.g.* aminoacylation) which takes over the biological function of the native polyene amino group in the process of a polyene-sterol complex formation[6,13,14]. The high antifungal activity of compound **2** indicates that the similar shift of the amino group in this *N*-alkyl derivative gives the same effect as in *N*-aminoacyl derivatives.

Recently it has been demonstrated that improvement of selective toxicity of AMB may be attained by blocking the carboxyl group, resulting in the absence of a carboxylate anion[6,10]. The biological properties of derivative **2** indicate that it is possible to retain the antifungal activity of AMB and to decrease significantly its toxicity towards red blood cells, as measured by hemolytic and potassium release criteria, also by the modification of the amino group with retention of the zwitterionic character of the parent antibiotic.

Acknowledgments

This work was supported by the Ministry of National Education, Warsaw (Grant P/01/161/90-2) and in part by the University of Hamburg, FRG. We are also indebted to Miss A. MEINERS for FAB-MS measurements.

References

1) HERMANS, P. E. & T. F. KEYS: Antifungal agents used for deep-seated mycotic infections. Mayo Clin. Proc. 58: 223~231, 1983
2) MCKINSEY, D. S.; M. R. GUPTA, S. A. RIDDLER, M. R. DRIKS, D. L. SMITH & P. J. KURTIN: Long-term amphotericin B therapy for disseminated histoplasmosis in patients with the acquired immunodeficiency syndrome (AIDS). Ann. Intern. Med. 111: 655~659, 1989
3) SCHAFFNER, C. P.: Chapter 12. Polyene macrolides in clinical practice: Pharmacology and adverse and other effects. *In* Macrolide Antibiotics. Chemistry, Biology, and Practice. *Ed.*, S. ŌMURA, pp. 457~507, Academic Press, 1984
4) GALE, E. F.: Chapter 11. Mode of action and resistance mechanisms of polyene macrolides. *In* Macrolide Antibiotics. Chemistry, Biology, and Practice. *Ed.*, S. ŌMURA, pp. 425~455, Academic Press, 1984
5) SCHAFFNER, C. P.: Amphotericin B derivatives. *In* Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents. *Ed.*, R. A. FROMTLING, pp. 595~618, J. R. Prous Science Publishers, 1987

6) CHÉRON, M.; B. CYBULSKA, J. MAZERSKI, J. GRZYBOWSKA, A. CZERWIŃSKI & E. BOROWSKI: Quantitative structure-activity relationships in amphotericin B derivatives. Biochem. Pharmacol. 37: 827~836, 1988
7) KOVACIC, P. & R. W. HEIN: Cross-linking of polymers with dimaleimides. J. Am. Chem. Soc. 81: 1187~1190, 1959
8) BRANTLEY, R. K.; L. HAEFFNER-GORMLEY & D. E. WETLAUFER: Preparation of a positively charged maleimide and its application to the high-performance liquid chromatographic separation of the tryptic peptides of lysozyme. J. Chromatogr. 295: 220~225, 1984
9) CZERWIŃSKI, A.; W. A. KÖNIG, P. SOWIŃSKI & E. BOROWSKI: Amides of polyene macrolide aureofacin. Synthesis and biological properties. J. Antibiotics 40: 1023~1027, 1987
10) HERVÉ, M.; J. C. DEBOUZY, E. BOROWSKI, B. CYBULSKA & C. M. GARY-BOBO: The role of the carboxyl and amino groups of polyene macrolides in their interactions with sterols and their selective toxicity. A ^{31}P-NMR study. Biochim. Biophys. Acta 980: 261~272, 1989
11) SMYTH, D. G.; O. O. BLUMENFELD & W. KONIGSBERG: Reactions of *N*-ethylmaleimide with peptides and amino acids. Biochem. J. 91: 589~595, 1964
12) ZAREMBA, M.; J. BOROWSKI, K. ZAREMBA, E. BOROWSKI & J. GRZYBOWSKA: Comparative antifungal activity and pharmacokinetic studies of a new amphotericin B derivative (AMA) and Fungizone in animal models. Abstracts of the 8th Int. Symp. on Future Trends in Chemotherapy, p. 54, Tirrenia, Italy, Mar. 28~30, 1988
13) WRIGHT, J. J. K.; J. A. ALBARELLA, L. R. KREPSKI & D. LOEBENBERG: *N*-Aminoacyl derivatives of polyene macrolide antibiotics and their esters. J. Antibiotics 35: 911~914, 1982
14) CZERWIŃSKI, A.; J. GRZYBOWSKA & E. BOROWSKI: *N*-Dimethylaminoacyl derivatives of polyene macrolide antibiotics. J. Antibiotics 39: 1025~1027, 1986
15) CZERWIŃSKI, A.; T. ZIENIAWA, E. BOROWSKI & L. G. MICOSSI: Amphotericin B 2-morpholinoethylamide diaspartate, a new water soluble derivative of the antibiotic synthesis and biological properties. J. Antibiotics 43: 680~683, 1990