SEMISYNTHESIS OF A23187 (CALCIMYCIN) ANALOGS
II. INTRODUCTION OF A METHYL GROUP ON THE BENZOXAZOLE RING

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Semisynthesis of two demethylamino A23187 with a methyl group in the 4- or 5-position on the benzene ring were carried out *via* the cleavage of A23187 oxazole ring and rebuilding of modified benzoxazoles. These compounds were shown to release Ca^{++} and Mg^{++} from mitochondria and to keep in part the antibacterial activity of the natural metabolite.

A23187 (calcimycin, **1**) is a unique divalent cation ionophore[1] isolated from a strain of *Streptomyces chartreusis* NRRL 3882[2]. Its peculiar structure has attracted much interest from synthetic chemists, and since 1979 an extensive effort has been made towards achieving stereocontrolled total synthesis of this antibiotic[3,4]. Some modifications of the natural skeleton have been reported either by chemical methods[5] or by microbial bioconversion[6]. Recently, from cultures of the strain NRRL 3882 by addition of L-tryptophan to the culture medium, a demethylamino A23187 (cezomycin) has been obtained in our laboratory[7].

We have recently developed a semisynthetic method in several steps[5] for obtaining analogs with a modified benzoxazole ring. This is likely to be of interest for structure-activity studies, since the benzoxazole moiety plays a prominent part in determining the ionophorous properties of the molecule[3,9]. We report here the production of two semisynthetic demethylamino A23187 with a methyl group in the 4- or 5-position on the benzene ring. The transporting abilities of these compounds were characterized in rat liver mitochondria. The antibacterial activity was then determined in comparison with A23187.

Chemistry

Our semisynthetic approach outlined in Scheme 1 was based on the cleavage of the oxazole ring of **1** to obtain the synthon **2** *via* the methylation of the $NHCH_3$ group. This part has been already described[5] and will not be discussed here. However, we would like to comment briefly on the high field ^1H NMR spectrum of **2**. In comparison with A23187[10], the main coupling constants in the spiroketal part remain unchanged as shown in Table 1, which confirms that the stereochemistry is not modified in the cleavage process. Rebuilding of the benzoxazole was achieved in two steps: coupling of substituted 3-hydroxy anthranilic methyl esters **10a**, **b** with **2** by using BOP reagent[11], cyclization into the oxazoles **4a**, **b** carried out according to KANAOKA *et al.*[12] with ethyl polyphosphate. The last step was the deprotection of the acid group giving methylcezomycins **5a**, **b**.

As already pointed out by TABER[13], the production of substituted 3-hydroxyanthranilic acids requires a special route for each case. Compound **10a**, which participates in the structure of the actinomycins was prepared according to ANGYAL *et al.*[14]. The preparation of **10b** is described in Scheme 2, the free hydroxylamine **8** underwent rearrangement with $TsCl/NEt_3$ to give the crystalline tosylate **9** in good yield, from which the aminophenol **10b** was easily obtained by sodium naphthalene cleavage in

Scheme 1.

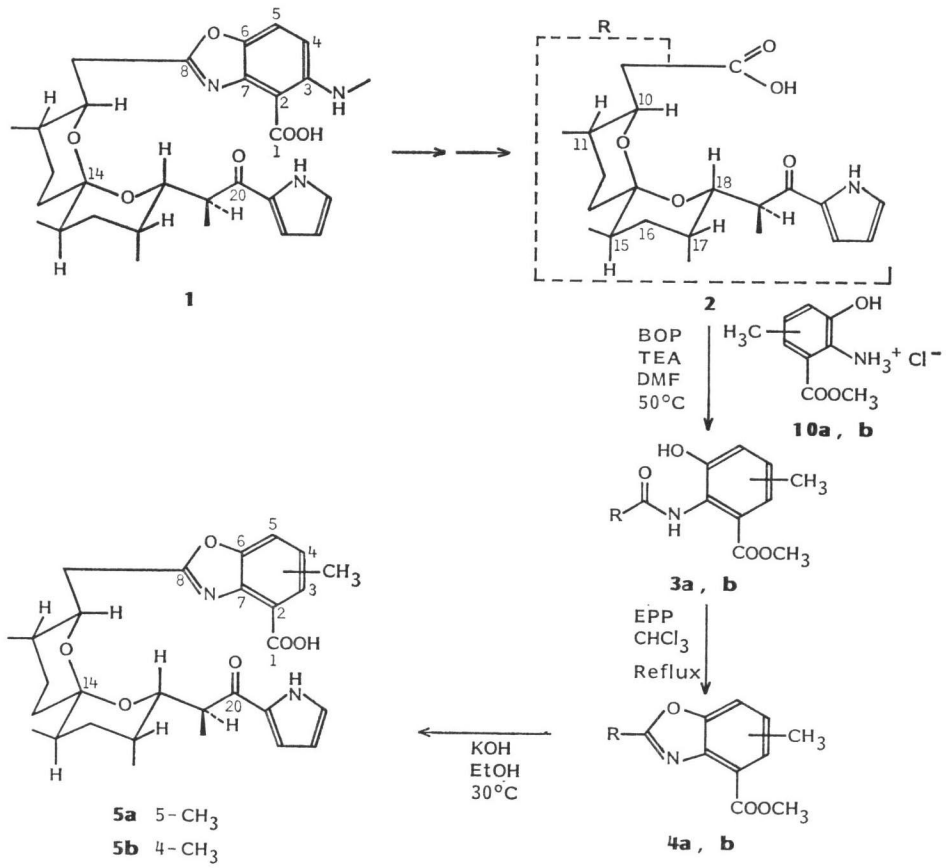


Table 1. Comparison of coupling constants in **1**[9] and **2** (C_6D_6, 400 MHz) in the spiroketal part*.

Compounds	$^3J_{10,11}$	$^3J_{15,16A}$	$^3J_{15,16B}$	$^2J_{16A,16B}$	$^3J_{16A,17}$	$^3J_{16B,17}$	$^3J_{17,18}$	$^3J_{18,19}$
1	2.2	12.4	4.2	−13.3	4.5	2.65	2.3	10.3
2	2.1	12.3	4.6	−13.4	4.4	2.9	2.2	10.5

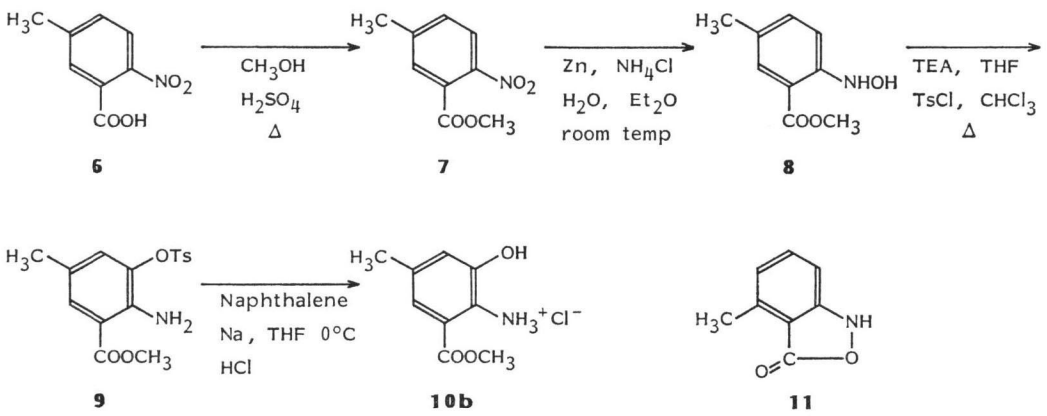
* Assignment of resonances made by homonuclear decoupling and *J* resolved 2D ^{1}H NMR.

THF[15]. The hydroxylamine rearrangement has already been explored by TABER[13] for 3-hydroxyanthranilic acid itself and discarded because of its low yield. However it could be an efficient synthesis when the *p*-position to the hydroxylamine is occupied; complementary studies will be necessary to prove this.

Our attempt to obtain the ester **10c** with a methyl in the *o*-position (6-CH_3) by the Scheme 2 synthesis was unsuccessful because the reduction of the nitro-intermediate led to a cyclization giving the isoxazolone **11**. New synthetic pathways are under investigation.

The E.I. mass spectra of all the compounds were examined. For the cyclized molecules **4a, b**; **5a, b**, in addition to the parent peak M^+ which was always present, the main fragmentations of A23187[1] shown on Fig. 1 were identified (Table 2), which was in agreement with the overall structures proposed for the synthetic products.

Scheme 2.



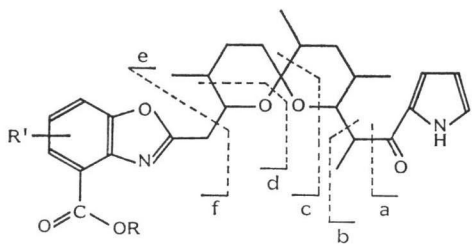
6 **7** **8** **9** **10b** **11**

Table 2. Main fragmentations (*m/z*, %) observed for **1**, **4a**, **4b**, **5a** and **5b**.

Compounds	Fragmentations						M^+
	a	b	c	d	e	f	
1	94 (100)	123 (23)	318 (31)	235 (20)	318 (31)	206 (65)	523 (74)
4a	94 (100)	123 (32)	318 (75)	234 (15)	318 (79)	205 (70)	522 (60)
4b	94 (65)	123 (10)	318 (100)	234 (15)	318 (100)	205 (31)	522 (77)
5a	94 (100)	123 (57)	304 (6)	220 (23)		191 (18)	508 (15)
5b	94 (100)	123 (40)	304 (20)	220 (5)		191 (34)	508 (17)

Comparison of ^{13}C NMR data (Table 3) further corroborates the structures. The resonance positions for the carbons of the benzoxazole moiety were assigned by using previous studies on A23187[10], cezomycin[7], substituent effects and *J*-modulated spin-echo. For the rest of the spectra, the chemical shifts were found constant for the five molecules listed which indicated no modification in the stereochemistry of the spiroketal.

Fig. 1.

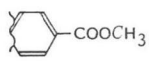
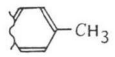
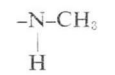


Semisynthetic acids exhibited the well-resolved high field ^1H NMR spectrum of A23187 in $CDCl_3$[10]. As expected the only modification were observed in the aromatic region for the benzoxazole resonances, there were two doublets (7.92 ~ 7.25 ppm, AB system, $J=8$ Hz) for **5a** and two singlets (7.92 ~ 7.60 ppm) for **5b**, aromatic methyl resonances were respectively observed at 2.66 and 2.54 ppm. The other resonances were very similar in their positions to A23187 ones. A conformational study of **5a, b** compared to A23187 will be published.

Ca^{++} and Mg^{++} Efflux in Rat Liver Mitochondria

The results given in Fig. 2a, b were obtained as follows. The mitochondria were incubated in the presence of increasing amounts of compounds **1**, **5a, b**. Reaction was stopped after 1 minute by rapid centrifugation; longer incubation proved unnecessary. The calcium or magnesium content of the pellet was

Table 3. ^{13}C NMR of **1**, **4a**, **4b**, **5a** and **5b** ($CDCl_3$, TMS).

Assignment	**1**[16]	**4a**	**4b**	**5a**	**5b**
C-1	168.1	166.9	166.6	167.4	167.0
C-2	98.2	119.0	121.1	117.6	119.4
C-3	150.8	124.8	127.4	126.2	127.6
C-4	108.4	126.5	134.1	126.9	135.6
C-5	116.7	126.2	115.1	127.1	115.5
C-6	140.8	150.5	151.7	149.6	150.8
C-7	141.7	141.0	139.5	140.1	138.6
C-8	166.1	165.9	166.0	165.1	165.0
C-9	32.4	32.6	32.7	32.7	32.5
C-10	68.4	68.2	68.2	68.7	68.6
C-11	28.8	28.8	28.7	29.2	29.2
C-12	25.7	25.7	25.8	25.8	25.7
C-13	25.4	25.5	25.6	25.5	25.5
C-14	98.5	98.7	98.9	98.7	98.6
C-15	32.3	32.4	32.4	32.5	32.5
C-16	35.0	35.5	35.6	35.3	35.3
C-17	28.3	28.5	28.6	28.4	28.4
C-18	72.9	74.0	74.2	72.9	73.0
C-19	42.5	42.6	42.8	42.8	42.6
C-20	193.7	194.5	194.7	194.0	194.1
C-21	133.5	133.7	133.9	133.2	133.3
C-22	116.3	116.4	116.4	116.6	116.7
C-23	110.1	109.8	109.9	110.3	110.3
C-24	124.3	124.8	124.6	124.5	124.5
C-15′	16.1	16.2	16.2	16.3	16.2
C-19′	13.0	12.4	12.4	13.0	12.9
C-11′	11.3	11.2	11.2	11.5	11.4
C-17′	10.7	10.6	10.7	10.9	10.8
		52.1	52.3		
		15.5	21.5	15.8	21.6
	30.0				

determined by atomic absorption after digestion. The percentage of cation released is plotted *versus* ionophore concentration. The three compounds induced a Mg^{++} efflux parallel to the Ca^{++} efflux. As shown in Fig. 2a, under our experimental conditions without Ca^{++} added to the medium, the addition of less than 1 nmol/mg protein of A23187 released 75% of endogenous calcium, the same result was obtained with 2 nmol of **5b** whereas 5 nmol of **5a** were necessary to release 50% of endogenous calcium.

In this test system, the two analogs prepared were thus able to transport divalent cations like A23187[17] but less efficiently. This transport of the carrier-type would involve the formation of a neutral lipophilic ionophore-cation complex in the membrane. From the known structures of these complexes for A23187[9,10], it is evident that **5a** and **5b** are likely to form similar associations with divalent cations since the coordinating sites ($\rangle$N oxazole, $-\rangle$C–O carboxylate, $\rangle$C=O acetylpyrrole) occupy the same position in the chain. The presence of the $-NHCH_3$ group in the natural metabolite apparently increases the ionophoric activity by a specific effect not yet explained.

Cationic extractions carried out in the two phase system water/toluene: 70, butanol: 30 did confirm

Fig. 2. Effects of increasing amounts of ionophores **1**, **5a**, **5b** on calcium (a) and magnesium (b) efflux in rat liver mitochondria.

The incubation medium was composed of 200 mM sucrose, 20 mM HEPES - NaOH buffer (pH 7.4 ~ 7.6), 5 mM glutamate. Mean ion contents without ionophore added were in nmol per mg of protein: Ca^{2+} 19±4, Mg^{2+} 26±4.

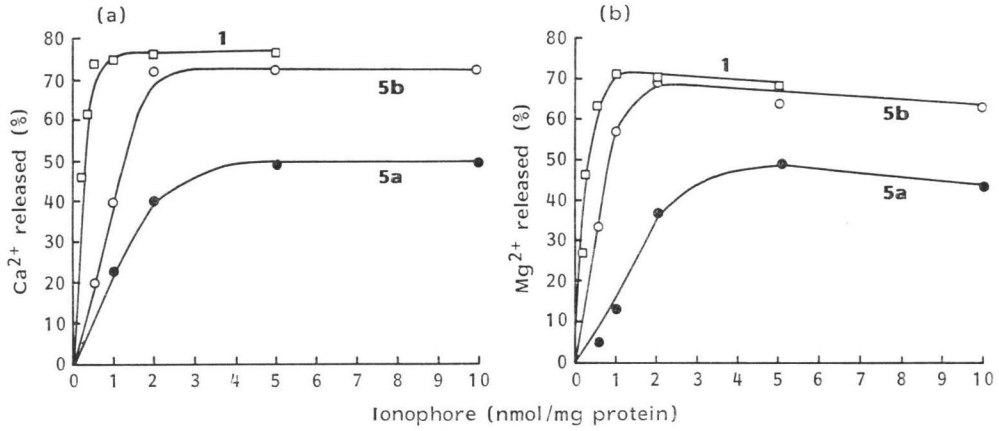


Table 4. *In vitro** antimicrobial activity (MIC μg/ml).

Compounds	*Bacillus cereus* ATCC 14575	*Bacillus megaterium* ATCC 14581	*Micrococcus luteus* ATCC 4698	*Streptomyces rimosus* NRRL 2234	*Penicillium decumbens* NRRL 742
1	0.024	0.0015	0.012	50	12.5
5a	0.39	<0.0015	0.006	25	6.25
5b	0.39	0.024	0.012	50	12.5

* Broth dilution test.

that compounds **5a**, **b** were able to transport Ca^{++} and Mg^{++} from the aqueous phase to the organic phase (to be published).

Antimicrobial Activity and Discussion

The "*in vitro*" antimicrobial activities of **5a** and **5b** in comparison with A23187 are shown in Table 4. Except for the strain of *Bacillus cereus* where the activity is ten times lower for the semisynthetic antibiotics, the activities are preserved. A strict relationship between the transporting abilities as shown in Fig. 2 and the MIC results cannot be established, but it is noteworthy that the antibiotic activity is kept along with the ionophorous properties.

The antibacterial mechanism of action for A23187 is not yet clearly elucidated. BAKER proposed that the inhibitory effect on microorganisms was probably due to a loss of Mg^{++} from the cell[18]. This hypothesis can explain our first results obtained with closely related analogs, but additional chemical and biochemical investigation is required.

We are now synthesizing structures with substituents in place of the $-NHCH_3$ group. This approach was recently stimulated by the isolation of X14885A[19], a novel calcimycin with an –OH group in the -3 position and the 15-methyl group missing. Starting from the synthon **2**, we recently achieved the semisynthesis of the X14885A analog (to be published).

Experimental

NMR spectra were recorded on a Bruker WM-400 spectrometer operating at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C, the two dimensional proton J-spectrum was recorded on the same instrument, the pulse sequence was ($\Pi/2$)-$t_{1/2}$-(Π)-$t_{1/2}$-(FID, t_2)[20]. Mass spectra were determined with A.E.I. MS 902 spectrometer at 70 eV using direct probe insert; they were performed by the Service Central d'Analyse du CNRS, Vernaison, France. The exact mass was measured when indispensable sample drying for C, H, N analysis was difficult to achieve (small amounts, decomposition, . . .). Optical rotations were measured with a Perkin-Elmer model 141 polarimeter. Melting points were determined on a Reichert hot-plate apparatus and are uncorrected. TLC was performed on Schleicher and Schüll plastic Silica gel plates (F 1500/LS 254) for analytical purposes, home made glass plates with Merck Kieselgel 60 PF 254+366 were used for the preparative scale. Column chromatography was carried out under low pressure using Merck Kieselgel 60 (70~230 mesh ASTM). A23187 (calcimycin, **1**) was from the stock sample of our laboratory, its production[21] and that of **2**[8] have been described previously. In the following experimental section the title compounds are named according to IUPAC nomenclature.

Methyl 3-Hydroxy-4-methyl Anthranilate, Hydrochloride (**10a**)

10a was prepared from commercial 3-hydroxy-2-nitro *p*-toluic acid according to ref 14.

Methyl 5-Methyl-2-nitro Benzoate (**7**)

A mixture of commercial compound **6** (10 g), methanol (70 ml) and concd H_2SO_4 (18 ml) was heated under reflux for 2 hours. After concentration of the solution, the residue was diluted with water, adjusted to pH 7 with aq NaOH, extracted with chloroform, dried over Na_2SO_4 and evaporated to yield 10.7 g of **7**; mp 77~78°C.

Methyl 2-(Hydroxyamino)-5-methyl Benzoate (**8**)

To a stirred mixture of **7** (5 g) in ether (100 ml) and NH_4Cl (5 g) in water (150 ml) zinc dust was added gradually. The reaction was followed by TLC (eluted with cyclohexane - ethyl acetate, 90: 10) until **7** had completely disappeared. About 2 hours were necessary for the addition. The mixture was then rapidly filtered and purified by column chromatography to give 3.6 g of **8** which was crystallized from hexane - chloroform; mp 110~112°C. ^{1}H NMR (60 MHz, DMSO-d_6) δ ppm/TMS 2.4 (3H, s, Ar-CH_3), 3.8 (3H, s, $COOCH_3$), 4~5 (2H, broad, NHOH), 6.9~8.0 (3H, m, Ar).

Methyl 2-Amino-5-methyl-3-tosyloxy Benzoate (**9**)

To a stirred solution of **8** (3.6 g), triethylamine (TEA, 2.1 g) and THF (250 ml) at 0°C was added dropwise a solution of *p*-toluene sulfonyl chloride (3.9 g) in chloroform (150 ml). The mixture was heated under reflux for 5 hours, washed with water, evaporated to give 4.3 g of **9** which was crystallized from water - ethanol as a white solid; mp 130~131°C. *m/z* (M), Found 335.0830, Calcd 335.0827 for $C_{16}H_{17}NO_5S$. ^{1}H NMR (60 MHz, $CDCl_3$) δ ppm/TMS 2.2 (3H, s, *p*-CH_3–Ar–SO_2), 2.4 (3H, s, CH_3Ar), 3.8 (3H, s, $COOCH_3$), 5.9 (2H, broad, NH_2), 6.9~7.9 (6H, m, Ar).

Methyl 3-Hydroxy-5-methyl Anthranilate, Hydrochloride (**10b**)

A concentrated solution of **9** (200 mg) in dry THF was injected into a stirred solution containing 6 equiv of naphthalene sodium in THF under nitrogen at 0°C Disappearance of the intense green color of the anion radical indicated the end of the reaction. The mixture was then diluted with water, extracted with ether to remove naphthalene and adjusted to pH 6. The hydroxyanthranilate was then extracted with ether and saturated with dry HCl gave ~100 mg of **10b** as a white solid, mp 75~76°C. ^{1}H NMR (60 MHz, $CDCl_3$) δ ppm 2.1 (3H, s, CH_3Ar), 3.8 (3H, s, $COOCH_3$), 5.4 (2H, broad, NH_2), 6.1 (1H, s large, Ar), 7.2 (1H, s large, Ar).

Methyl 2-*N*-[[3,9,11-Trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]-undec-2-yl]acetyl]-3-hydroxy-4-methyl Anthranilate (**3a**)

A light-protected solution of **2** (155 mg) in DMF (20 ml), triethylamine (170 mg), **10a** (100 mg) and benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (200 mg) or BOP added as a condensating agent[10] was stirred in a water-bath at 50°C for 6 hours, poured into water, extracted with ether, dried over Na_2SO_4 and purified by TLC (elution with cyclohexane - ethyl acetate,

50: 50) to yield **3a** (104 mg) as a white foam; mp 65~66°C; $[\alpha]_{578}^{25}$ +66° (*c* 0.030, $CHCl_3$),

Anal Calcd for $C_{30}H_{40}N_2O_7$: C 66.65, H 7.45, N 5.18.
Found: C 66.72, H 7.61, N 5.4.

Mass *m/z* 540; ^{13}C NMR (100.6 MHz, $CDCl_3$, TMS) 17.3 (CH_3Ar), 42.1 (C_9), 52.3 ($COOCH_3$), 98.6 (C_{14}), 168.5, 172.6 (COOH, C_8), 194.4 (C_{20}).

Methyl 2-*H*-[[3,9,11-Trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]-undec-2-yl]acetyl]-3-hydroxy-5-methyl Anthranilate (**3b**)

The same method as for **3a** was applied but stirring at 50°C was continued for 12 hours. Starting from **2** (150 mg) and **10b** (100 mg) yield **3b** (95 mg) as a white foam; mp 160~161°C, $[\alpha]_{578}^{25}$ +67° (*c* 0.043, $CHCl_3$); *m/z* (M), Found 540.2850, Calcd 540.2830 for $C_{30}H_{40}N_2O_7$. ^{13}C NMR (100.6 MHz, $CDCl_3$, TMS) 20.8 (CH_3Ar), 42.1 (C_9), 52.4 ($COOCH_3$), 98.5 (C_{14}), 168.5, 172.4 (COOH, C_8), 194.4 (C_{20}).

Methyl 7-Methyl-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro-[5.5]undec-2-yl]methyl]-4-benzoxazole Carboxylate (**4a**)

A light-protected solution of **3a** (160 mg), ethyl polyphosphate (2.2 g) in chloroform (8 ml) was stirred in a water-bath at 66°C for 3 hours, diluted with water, extracted with ether, dried over Na_2SO_4 to yield **4a** (103 mg) as a white foam; mp 59~60°C; Mass *m/z* 522;

Anal Calcd for $C_{30}H_{38}N_2O_6$: C 68.95, H 7.32, N 5.36.
Found: C 68.36, H 7.26, N 5.36.

^{13}C NMR given in Table 2.

Methyl 6-Methyl-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro-[5.5]-undec-2-yl]methyl]-4-benzoxazole Carboxylate (**4b**)

The same method as for **4a** from **3b** (42 mg), ethyl polyphosphate (EPP) (1 g) in chloroform (8 ml) refluxing for 2 hours yielded **4b** (38 mg) as a white foam; mp 59~60°C; $[\alpha]_{578}^{25}$ +3° (*c* 0.004, $CHCl_3$); *m/z* (M), Found 522.2724, Calcd 522.2730 for $C_{30}H_{38}N_2O_6$; ^{13}C NMR given in Table 2.

7-Methyl-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazole Carboxylic Acid (**5a**)

A light-protected mixture of **4a** (103 mg) in ethanol (100 ml) and 10% potassium hydroxide (7 ml) was stirred at 30°C for 2 hours, poured into water (200 ml), adjusted to pH 4.5 with 0.1 N HCl, extracted with ether, dried over Na_2SO_4. After ether evaporation the residue was purified by TLC (elution with cyclohexane - ethyl acetate, 50: 50). The product was then dissolved in acetone and acidified with 10% H_3PO_4[22] in water to give **5a** as the free acid (60 mg) as a white foam; mp 80~81°C; $[\alpha]_{578}^{25}$ +27° (*c* 0.0056, $CHCl_3$); *m/z* (M), Found 508.2564, Calcd 508.2572 for $C_{29}H_{36}O_6N_2$; ^{13}C NMR given in Table 2.

6-Methyl-2-[[3,9,11-trimethyl-8-[-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazole Carboxylic Acid (**5b**)

The same method as above gave from **4b** (103 mg) 62 mg of **5b** as a white foam; mp 77~78°C; $[\alpha]_{578}^{25}$ +13° (*c* 0.007, $CHCl_3$); *m/z* (M), Found 508.2577, Calcd 508.2572 for $C_{29}H_{36}O_6N_2$; ^{13}C NMR given in Table 2.

Mitochondria

Rat liver mitochondria were isolated according to JOHNSON and LARDY[23] in 0.25 M sucrose, 2.7 mM Tris (pH adjusted to 7.4~7.6). The protein concentration was measured by the Biuret method. Mitochondria (2 mg protein) suspended in 1 ml medium indicated in Fig. 2 were incubated with increasing amounts of ionophores (dissolved in DMSO) for 1 minute and then centrifuged. The digestion of the pellet was carried out with formic acid and the ion content measured with a Perkin Elmer 420 atomic absorption spectrophotometer.

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