Analog of Monensin with a Modified C₂₅–C₂₆ Moiety: Na⁺/K⁺ Selectivity and Biological Activity

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C₂₅–C₂₆ chemical derivatives of monensin (1) were prepared: 25-dehydroxymethyl, 25-oxo (2); 25,26-isopropyliden (3); 25-*O*-methyl (4); 26-*O*-acyl (5); 26-*O*-[*N*-chlorophenyl]carbamoyl (6); and 26-*O*-[*N*-chlorophenyl, *N*-methyl]carbamoyl (7). Measurements of Na⁺ and K⁺ extractions in a water/toluene–butanol (70:30) system by these analogs showed the key role of the C_[26]H₂–OH arm for the stability and selectivity of 1:1 complexes formed by monensin. The antibiotic activity and toxicity in mice were determined in relation to the Na⁺/K⁺ cationic affinity.

Monensin A (1) (Fig. 1a) is of special interest as it was the first member to be described¹⁾ of the large class of carboxylic polyether antibiotics.²⁾ It has rapidly found extensive applications in poultry farming for its anticoccidial activity, and for improving the efficiency of feed utilization in ruminant animals.³⁾

This ionophore presents a rather unique selectivity of complexation⁴⁾ and transport^{5–7)} in favor of Na⁺ *versus* K⁺. This selectivity, which is linked to the structure of the polyether, has been investigated in particular by a thorough structural analysis of its various alkaline complexes by X-ray crystallography.⁸⁾

Several studies on the chemical modification of the monensin skeleton have been reported, either on the C₁–C₅ carboxylic moiety,^{9,10)} or at the C₂₆-hydroxyl group,¹¹⁾ following the isolation of antibiotics X-14667A and B¹²⁾ (monensin C₂₆-phenethyl urethans). We have shown that the C₂₆-chloro-4 phenyl urethan

derivative had a transport selectivity, with K⁺ > Na⁺ in a chloroform phase.⁷⁾ ΔG , ΔH and ΔS of complexation measured simultaneously in methanol confirmed the change of complexing properties,¹³⁾ in agreement with results obtained with X-14667.¹²⁾ Very recently, a 26-deoxymonensin has been isolated from the blocked mutant of a monensin-producing strain.¹⁴⁾

In the natural metabolite, the terminal hemiketal moiety interacts by chelation with the carboxylic group, and brings one site to the coordination sphere of the cation. This molecular arrangement, which is specific to monensin, is achieved by a well-defined orientation of the C₂₆-hydroxyl arm afforded by the hydrogen bond network⁸⁾ (Fig. 1b). In the present work, we focused our investigations on the role of the two hydroxyl groups borne by carbon atoms 25 and 26; appropriate chemical modifications were carried out for this purpose.

Fig. 1(a). Monensin A (1).

Results are reported on the extraction of Na^+ and K^+ in a water/organic phase system by the analogs prepared; the antibiotic activity on *B. cereus* ATCC 14575 and toxicity in mice are presented in relation to the cationic affinity. Modifications of rumen fermentation by these compounds in *in vitro* studies have been reported separately.¹⁵⁾

Results

1. Chemical modifications

Hemisyntheses carried out on the monensin A free acid under gentle experimental conditions are summarized in Fig. 2. Agtarap *et al.*¹⁾ proposed, without experimental details, the preparation of lactone **2** by sodium periodate oxidation of **1**. This reaction was achieved quantitatively under nitrogen, in a mixed water-dioxane medium. The 25,26-isopropylidene derivative (**3**) was obtained in high yield, using the Chittenden method¹⁶⁾ with dimethoxy-2,2 propane and SnCl_2 in a

catalytic amount.

Methylation of the anomeric OH to give **4** was achieved with trimethyl orthoformate and camphorsulfonic acid, following the protective reaction used by Kishi *et al.*¹⁷⁾ in the course of their first total synthesis of monensin A. ^{13}C -NMR spectra showed that we had obtained a 75:25 mixture, which was not separable by chromatography, of $\text{OMe}_{\text{ax}}/\text{OMe}_{\text{eq}}$.

Regioselective esterifications (**5a-d**) of the $\text{C}_{[26]}\text{H}_2\text{-OH}$ were performed by adding the acid anhydrides in pyridine at 0°C and then maintaining the reaction for one day at room temperature. Urethan **6** was prepared according to Liu and Westley.¹⁸⁾ It was *N*-methylated to **7** in dry THF by sodium hydride and then methyl iodide.¹⁹⁾

For all the structures, positive and negative FAB mass spectrometry was used for confirmation, and ^1H and ^{13}C -NMR spectra were totally assigned using 2D-NMR spectroscopy.

2. Na^+/K^+ selectivity in a biphasic system

The ionic exchange technique in a water-organic phase system was first proposed by Pressman²⁰⁾ to characterize ionophores, and it was used subsequently with some modifications by Pfeiffer and Lardy for calcimycin.²¹⁾ More recently, we have adopted this method for analogs of calcimycin using the water/toluene-butanol (70:30, v/v) system.²²⁾ We kept this system, which gave us satisfactory results, for screening several related compounds.

Fig. 1(b). Simplified Representation of a 1:1 Neutral Complex between Monensin and a Monovalent Cation, Following X-Ray Studies.⁸⁾

Fig. 2. Analogs Prepared.

Fig. 3. Extraction Curves Representing the $[AM]/[AH]_{init.}$ Ratio in the 70:30 Toluene-Butanol Phase (—, K^+ ; ---, Na^+) versus the Aqueous Phase pH.

The heterogeneous equilibrium studied can be written as

where AH stands for the protonated form of the ionophore.

Extraction curves representing the $[AM]/[AH]_{init.}$ ratio, in the organic phase, versus the aqueous phase pH are shown in Fig. 3. These fitted a 1:1 stoichiometry for the metal-ionophore complex. Equilibrium constants K_e were determined for half-complexation of Na^+ and K^+ , without correcting the molar activity coefficient (Table I).

Equilibrium (Eq. 1) is the sum of two steps at the interface between water and the organic phase, i.e.,

Table I. K_e VALUES FOR EQUILIBRIUM (Eq. (1))

Compound	Na^+	K^+
1	$5.6 \pm 0.9 \cdot 10^{-5}$	$3.5 \pm 0.6 \cdot 10^{-6}$
2	*	*
3	*	*
4	*	*
5a	$4.5 \pm 0.7 \cdot 10^{-7}$	$1.6 \pm 0.3 \cdot 10^{-6}$
5b	$4.5 \pm 0.7 \cdot 10^{-7}$	$1.6 \pm 0.3 \cdot 10^{-6}$
5c	$5.6 \pm 0.9 \cdot 10^{-7}$	$2.1 \pm 0.3 \cdot 10^{-6}$
5d	$7.7 \pm 1.2 \cdot 10^{-7}$	$2.6 \pm 0.4 \cdot 10^{-6}$
6	$1.1 \pm 0.2 \cdot 10^{-6}$	$1.1 \pm 0.2 \cdot 10^{-5}$
7	$4.2 \pm 0.7 \cdot 10^{-7}$	$1.5 \pm 0.3 \cdot 10^{-6}$

* Not determined.

For all the compounds, the carboxylate formation (Eq. (2)) remains nearly unaffected compared with monensin. This has been proved by pK_a measurements for 6.¹³⁾ Variations recorded in the extraction experiments are thus linked to modifications to the equilibrium (Eq. (3)).

The graphs allowed us to divide the ligands into two groups. The first, compounds **5** to **7**, kept the complexing ability of natural metabolite **1**, but with a reversed selectivity in favor of K^+ . This was especially marked for **6**, in good agreement with our previous work done under different experimental conditions.⁷⁾ For all the derivatives, there was a marked reduction of affinity for Na^+ compared with monensin. The other group, compounds **2-4**, did not show the ionophorous behaviour of derivatives **5-7** in the pH range 6-10, only a very small transfer of cation to the organic phase being recorded. The K_c constants were not determined for these compounds.

3. Antibacterial activity and toxicity in mice

The minimal inhibitory concentration (MIC) for the strain of *B. cereus* ATCC 14575 and the toxicity in mice are listed in Table II. LD₅₀ values were measured for a few compounds only as this required large amounts of material. However, the number of deaths at 100 mg/kg was systematically determined. This afforded an indication of the toxicity range for all the compounds prepared.

There was a correlation between the extracting properties of the compounds, the antibacterial activity and the toxicity in mice. Very poor complexing systems **2-4** showed weak antibacterial activity and also reduced toxicity in mice, whereas ligands exhibiting a net selec-

tivity for potassium were antibiotic and highly toxic. This was especially true for **6** and also for esters **5**. It is worth noting here that these results were obtained by only a slight modification affecting the hydroxyl group on carbon 26, apparently a key site for this ionophore. Monensin itself with its specific affinity for sodium was situated between the two extremes.

Discussion

We have shown that modifications of the head-to-tail chelation system in monensin led to marked changes in the complexing properties of the skeleton and, consequently, in its biological activity.

Drastic effects were observed when the ability of the chain to close was suppressed in lactone **2**, acetonide **3**, and also when the anomeric C₂₅-OH was alkylated in **4**.

Further, acylation of the C₂₆-hydroxyl group changed the ionic selectivity. Association with potassium was not greatly affected (see Table I), but K_c values for sodium were markedly decreased. Thus, this part of the molecule appears to be essential for the re-

Table III. COUPLING CONSTANTS
IN CDCl₃ (300 MHz)

J_{Hz}	1 (Acid)	2 (Acid)	6 (Acid)	6 (K ⁺ salt)
2-3	10.1	6.0	10.0	10.2
2-36	6.7	6.5	6.5	6.5
3-4	2.1	4.0	2.0	2.2
4-5	11.7	10.5	11.4	11.5
4-34	7.2	7.0	7.0	6.5
5-6	2.0	2.0		1.5
6-33	7.4	7.0		7.0
7-8A	3.4	3.5		3.5
13-14A	10.8	9.0		11.0
13-14B	5.4	6.0		5.0
17-18	3.9	4.0	4.0	3.0
19A-20	5.9	6.5		6.0
19B-20	13.8	10.0		10.0
20-21	2.9	4.0	2.8	2.5
21-22	10.5	9.0	10.0	10.5
22-28	6.7	6.5	6.5	6.5
24-27	5.1	7.0	6.5	6.5
30-31	7.5	7.0	7.5	7.5

Table II. ANTIMICROBIAL ACTIVITY (*B. cereus*)
AND TOXICITY (IN MICE) FOR MONENSIN
AND ITS ANALOGS

Compound	M.I.C. (μ g/ml)	Number of deaths at 100 mg/kg	LD ₅₀ (mg/kg)
1	1.56	2/10	150-175
2	50	1/10	500-700
3	6.25	0/10	
4	1.56	0/10	
5a	1.56	10/10	<50
5b	0.78	10/10	
5c	0.78	10/10	
5d	0.2	7/10	
6	<0.05	10/10	10-25
7	0.1	n.d.	

markable stability of the 1:1 sodium complex in monensin. To our knowledge, this point has never been mentioned before, and might be linked to results obtained in the lariat ether field, where it was shown that modifications to the side arms can improve not only the complexing ability but also the Na⁺/K⁺ selectivity.²³⁾

³J scalar coupling constants available from the ¹H-NMR spectra, in CDCl₃, for three chosen examples (Table III) revealed that the conformation of lactone **2** (acid form) was different from urethan **6** and monensin **1**, in the terminal parts of the chain. Respective values for H₂-H₃ (6.0 Hz), H₃-H₄ (4.0 Hz), H₄-H₅ (10.5 Hz) and H₂₀-H₂₁ (4.0 Hz) were more compatible with an increased rotational mobility of the C-C bonds concerned, in comparison with **1** and **6** (acid and K⁺ salt), which showed invariant coupling constants corresponding to a globular conformation with head-to-tail interaction. The lactone conformation was close to that proposed for monensin in methanol,²⁴⁾ where the protic solvent competed with the intramolecular chelating system and broke up to hydrogen bond network. The absence of a preformed cavity and its poor stabilization in the 1:1 complex with cations, when formed, are consistent with low ionophorous properties for the lactone.

We have already proposed a structure for urethan **6**⁷⁾ with the implication of -NH in the chelating system, without participation of the modified arm in the cation coordination sphere (Fig. 4). Methylation of -NH in **7** led to a net destabilization of both the K⁺ and Na⁺ complexes. This observation supported our starting structural hypothesis.

Concerning the antibiotic mode of action of

Fig. 4. Postulated Head-to-Tail Interaction for 4-Chlorophenyl Urethan Derivative **6**.

monensin, several studies have been carried out on ruminal bacteria and have recently been reviewed.²⁵⁾ The authors appropriately underlined that the selectivity of an ionophore measured in a model system was just an index of ion-binding preference, but the direction of induced fluxes in bacteria and the final distribution of ions were determined by the initial concentrations of H⁺, K⁺ and Na⁺ ions across the cell membrane. For the example of *S. bovis*, the following movements were observed: a large efflux of intracellular K⁺ (K_{in}⁺ ↔ H_{out}⁺ antiport), partially compensated for by Na⁺ influxes in the cell (Na_{out}⁺ ↔ H_{in}⁺ antiport), with resulting acidification inside the cell. We recently reached the same conclusion for an *S. faecalis* strain by NMR measurements.²⁶⁾ These ionic modifications strongly affected the microbial metabolism and consequently inhibited the cell growth. We are thus led to propose that K⁺-selective derivatives, especially urethan **6**, are highly antibiotic and toxic in mice because the efficient K_{in}⁺, H_{out}⁺ exchange is not partially compensated for by the Na_{out}⁺, H_{in}⁺ movement, as the affinity for Na⁺ is markedly decreased compared with monensin. This, of course, needs further experimental support.

In conclusion, it appears that the -C_[26]H₂-OH arm is a main site modulating the ionophorous and biological properties of monensin. Other modifications are currently under investigation.

Experimental

Melting points (mp) were determined on Reichert hot-plate apparatus and are uncorrected. Optical rotations were measured with a Perkin Elmer model 141 polarimeter in chloroform at 1.5 g/100 ml with λ = 578 nm (Hg) and T = 25°C. NMR spectra were recorded on a Bruker 300 MSL spectrometer in a CDCl₃ solution with TMS as an internal standard. All the resonances were assigned for the ¹H and ¹³C spectra by ¹H-¹H chemical shift correlation and ¹H-¹³C correlation. Only characteristic peaks for the acid form are given here. FAB mass spectra were determined with a V.G. 70-70F spectrometer at the Service Central d'Analyse du C.N.R.S. Vernaion. Values in the text for positive FAB correspond to (M + Na)⁺, and for negative FAB to (M - H)⁻. pH values were measured with

a digital Orion 701A pH meter. Cation concentrations were obtained by atomic absorption with a Perkin Elmer 420 instrument.

TLC analysis was performed with Schleicher and Schüll 60 PF plastic silica gel plates, using vanillin-sulfuric acid in methanol as a spray reagent. Column chromatography was carried out with Merck Kieselgel (60/70, 230 mesh ASTM).

Pure monensin A was produced in our laboratory from the *Streptomyces cinnamomensis* ATCC 15413 strain. It was strictly identical (TLC, NMR, mass) to a Sigma sample of this antibiotic, after separating co-metabolite monensin B, of which a few percent is always present.²⁷⁾

25-Dehydroxymethyl, 25-oxo-monensin (2). To a solution of the acid form of **1** (500 mg, 0.75 mmol) in 8 ml of dioxane under nitrogen and protected from light, sodium metaperiodate (400 mg, 1.9 mmol) dissolved in 4.5 ml of water was added. The mixture was stirred for 1 hr at room temperature and left for 12 hr without stirring, before being diluted with water and extracted with CHCl_3 . The organic phase was stirred with 0.5 N HCl and then rinsed with deionized water until neutral pH was obtained. The organic phase was evaporated and dried by several consecutive dissolutions and evaporations with high-grade acetone. By following this procedure, the reaction was quantitative in giving lactone **2** (acid form) with good purity (>95%). Final purification was achieved by column chromatography (white powder), mp 68–70°C. $[\alpha]_{578}^{25}$: +43° ($c=1.5$, CHCl_3). $^{13}\text{C-NMR}$ δ C1: 178.3; C25: 174.5; no C26. FABMS positive m/z : 661.4; negative m/z : 636.9 ($M=638.4$ for $\text{C}_{35}\text{H}_{58}\text{O}_{10}$). *Anal.* Found: C, 61.75; H, 9.00. Calcd. for $\text{C}_{35}\text{H}_{57}\text{O}_{10}\text{Na} \cdot \text{H}_2\text{O}$: C, 61.93; H, 8.76%.

25,26-Isopropyliden-monensin (3). To a solution of monensin (1 g, 1.5 mmol) in 50 ml of diethyl ether, 0.75 ml of 2,2-dimethoxypropane (6 mmol) and 1–2 mg of SnCl_2 were added. The mixture was refluxed for 2 hr and then diluted with ether. The organic phase was washed with distilled water and evaporated. The product was purified by column chromatography with a solvent gradient of cyclohexane-ethyl acetate running from 15:85 to 25:75. 60% yield after purification (white powder), mp 90–92°C. $[\alpha]_{578}^{25}$: +49° ($c=1.5$, CHCl_3). $^{13}\text{C-NMR}$ δ C1: 179.0; C25: 107.4; C26: 73.8, C_{gem} -diether: 106.2; C methyls (isopropyl): 26.6 and 24.1. FABMS positive m/z : 733.4; negative m/z : 709.4 ($M=710.5$ for $\text{C}_{39}\text{H}_{66}\text{O}_{11}$). *Anal.* Found: C, 65.74; H, 9.36. Calcd. for $\text{C}_{39}\text{H}_{66}\text{O}_{11}$: C, 65.89; H, 9.36%.

25-O-Methyl-monensin (4). To a solution of monensin (1 g, 1.5 mmol) in 40 ml of methanol-methylene chloride (1:1), 320 mg (3 mmol) of $(\text{MeO})_3\text{CH}$ were added together with few crystals of (*d,l*)camphorsulfonic acid. The mixture was left for 6 hr at room temperature with stirring and protected from light. The solvent was evaporated, and the solid phase was dissolved in chloroform, which was then washed with distilled water. After evaporating the solvent,

the product was purified by column chromatography (eluent: gradient of CHCl_3 to $\text{CHCl}_3 + 1\%$ methanol). 50% yield after purification (white powder), mp 74–76°C. $[\alpha]_{578}^{25}$: +60° ($c=1.5$, CHCl_3). As already mentioned, complete assignment of the $^{13}\text{C-NMR}$ spectra showed that it was a 75:25 mixture of epimers $\text{OMe}_{\text{ax}}/\text{OMe}_{\text{eq}}$ with the respective chemical shifts given here for a few resonances: C1, 179.1 and 177.4; C25, 99.2 and 97.2; C26, 63.9 and 67.9; -OMe, 48.6 and 48.7. FABMS positive m/z : 707.0; negative m/z : 683.1 ($M=684.4$ for $\text{C}_{37}\text{H}_{64}\text{O}_{11}$). *Anal.* Found: C, 63.42; H, 9.46. Calcd. for $\text{C}_{37}\text{H}_{64}\text{O}_{11} \cdot \text{H}_2\text{O}$: C, 63.22; H, 9.46%.

26-O-Acyl-monensin (5). The general procedure for the four esters prepared was for monensin (1 g, 1.5 mmol) to first be dissolved in 15 ml of anhydrous pyridine. The solution was then cooled to 0°C in an ice-water bath and stirred, while 3 mmol of the acid anhydride was added drop by drop. The mixture was left for 48 hr, the bath progressively reaching room temperature. The solvent was carefully evaporated with a rotary evaporator equipped with a vacuum pump to give a viscous liquid, which was dissolved in acetone, before the solvent was evaporated. This operation was carried out three times until a white foam was obtained. This foam, dissolved in diethylether, was washed twice with 0.5 N HCl, and then deionized water, before the ether solution was dried over MgSO_4 . The product was purified chromatographically with an eluent gradient of cyclohexane-ethyl acetate of 15:85 to 25:75. The yield after purification was close to 70% (white powder).

Monensin 26-O-C(=O) CH_3 (**5a**): mp 78–80°C. $[\alpha]_{578}^{25}$: +70° ($c=1.5$, CHCl_3). $^{13}\text{C-NMR}$ δ C1: 176.7; C26: 67.1; C=O: 170.9; CH_3 : 21.2. FABMS positive m/z : 735.0; negative m/z : 712.1 ($M=712.4$ for $\text{C}_{38}\text{H}_{64}\text{O}_{12}$). *Anal.* Found: C, 63.81; H, 9.04. Calcd. for $\text{C}_{38}\text{H}_{64}\text{O}_{12}$: C, 64.02; H, 9.05%.

Monensin 26-O-C(=O) CH_2CH_3 (**5b**): mp 70–72°C. $[\alpha]_{578}^{25}$: +67° ($c=1.5$, CHCl_3). $^{13}\text{C-NMR}$ δ C1: 176.9; C26: 67.0; C=O: 174.1; CH_2 : 27.6, CH_3 : 9.6. FABMS positive m/z : 749.0; negative m/z : 725.1 ($M=726.5$ for $\text{C}_{39}\text{H}_{66}\text{O}_{12}$). *Anal.* Found: C, 62.71; H, 9.00. Calcd. for $\text{C}_{39}\text{H}_{65}\text{O}_{12}\text{Na} \cdot \text{H}_2\text{O}$: C, 62.53; H, 8.75%.

Monensin 26-O-C(=O) $\text{CH}_2(\text{a})\text{CH}_2(\text{b})\text{CH}_3$ (**5c**): mp 58–60°C. $[\alpha]_{578}^{25}$: +60° ($c=1.5$, CHCl_3). $^{13}\text{C-NMR}$ δ C1: 176.9; C26: 66.9; C=O: 173.4; $\text{CH}_2(\text{a})$: 36.2, $\text{CH}_2(\text{b})$: 18.5; CH_3 : 13.7. FABMS positive m/z : 763.0; negative m/z : 739.1 ($M=740.5$ for $\text{C}_{40}\text{H}_{68}\text{O}_{12}$). *Anal.* Found: C, 63.11; H, 9.07. Calcd. for $\text{C}_{40}\text{H}_{67}\text{O}_{12}\text{Na}$: C, 62.95; H, 8.86%.

Monensin 26-O-C(=O) $\text{CH}(\text{CH}_3)_2$ (**5d**): mp 70–72°C. $[\alpha]_{578}^{25}$: +68° ($c=1.5$, CHCl_3). $^{13}\text{C-NMR}$ δ C1: 176.6; C26: 66.8; C=O: 176.5; CH: 34.1; (2) CH_3 : 19.1. FABMS positive m/z : 763.5; negative m/z : 739.1 ($M=740.5$ for $\text{C}_{40}\text{H}_{68}\text{O}_{12}$). *Anal.* Found: C, 63.63; H, 9.05. Calcd. for $\text{C}_{40}\text{H}_{68}\text{O}_{12} \cdot \text{H}_2\text{O}$: C, 63.30; H, 9.30%.

26-O-[N-4-Chlorophenyl, N-methyl] carbamoyl-monensin

(7). The 4-chlorophenylurethan of monensin **6** was first described by Liu and Westley.¹⁸⁾ We have already reported the assignment of its ¹³C-NMR spectrum.⁷⁾ The *N*-methylation was carried out on a solution of 250 mg of **6** (0.31 mmol) in 5 ml of dry tetrahydrofuran, to which 20 mg (0.83 mmol) of NaH was added with stirring. After 15 min, 2 ml of CH₃I (0.32 mmol) was added, and the reaction mixture was left for 30 h at room temperature, after which time no further evolution was observed. The methylation was not complete. The THF was evaporated off and the product purified by TLC (eluent of cyclohexane–ethyl acetate, 80:20). The yield was 40%. Its optimization was not studied (white powder), mp 73–75°C. [α]_D²⁵: +80° (*c* = 1.5, CHCl₃). ¹³C-NMR δ C1: 176.8; C26: 67.7; C=O: 155.3; C(N-methyl): 32.9; C arom: 142.1, 131.3, 128.7 (2), 127.1 (2). FABMS positive *m/z*: 860.1; negative: *m/z*: 836.2 (*M* = 837.5 for C₄₄H₆₈O₁₂NCl).

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