Ionophore Properties of Monensin Derivatives Studied on Human Erythrocytes by ^{23}Na NMR and K^+ and H^+ Potentiometry: Relationship with Antimicrobial and Antimalarial Activities

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Eight derivatives of monensin with a modified C25−C26 moiety were synthesized. Their ionophore properties were studied on human erythrocytes by measuring Na^+ influx with ^{23}Na NMR and concomitant K^+ and H^+ efflux by potentiometry. Modification of OH-26 led to inversion of selectivity of transport in favor of K^+/Na^+ in comparison with monensin. This selectivity disappeared by suppression of the C26-OH moiety. Finally the ionophore ability was lost if the head-to-tail chelation of the monensin skeleton was prevented by blocking the terminal OH-25 and -26 functions. All the compounds were inactive on Gram-negative bacteria and fungi. MIC measured on *Bacillus cereus* showed that derivatives with increased K^+/Na^+ selectivity were clearly the most active against *Bacillus* growth. Most of the compounds showed potent antimalarial properties in the nanomolar range when tested *in vitro* against *Plasmodium falciparum*. The IC_{50}s measured were correlated with the whole Na^+ and K^+ transport efficiency rather than with the ionic selectivity. In both cases determination of initial fluxes of transport for both cations (Na^+ and K^+) was necessary to investigate the relationship between biological and ionophore properties.

Introduction

Monensin (**1**) is a well-known carboxylic polyether antibiotic, isolated from *Streptomyces cinnamonensis*, which transports cations across biological membranes, as a mobile carrier, by a proposed mechanism of H^+/cation exchange.[1,2] Its selectivity of complexation and transport in favor of sodium versus potassium[3−9] makes this molecule especially interesting for biochemists as a tool to modify sodium concentration in cells. Its ionophore ability also results in a variety of biological properties that include antimicrobial activity against Gram-positive bacteria and anticoccidial activity against *Eimeria* for poultry, and it is used as a growth promoter in ruminants.[10−12] In addition, carboxylic polyether antibiotics appear to be potential antimalarial reagents as shown by Liu[13] and recently by Gumila (unpublished data). The intraerythrocytic development of *Plasmodium*, responsible for malaria infection, induces modifications of membrane permeability of erythrocytes. As a result, the potassium level in host cytosol decreases whereas that of sodium increases; conversely the parasite cytosol contains a relatively high concentration of K^+ and low concentration of Na^+.[14−16] Membrane effectors such as monensin can thus be expected to disturb these specific ionic gradients present in infected red cells with a resulting antiparasitic effect.

Structure−activity relationship studies have been conducted by different groups on derivatives of ionophores such as salinomycin,[17] nigericin,[18−19] and monensin.[20−24] We previously showed for monensin that chemical modifications of the C26-OH moiety affected the Na^+/K^+ selectivity measured on a physicochemical model, and a related change in the antibiotic activity was observed.[7] This prompted us to examine this question in greater detail.

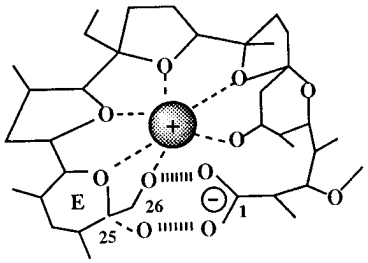

Figure 1. Schematic representation of the monensin globular structure (vertical hash marks, head-to-tail hydrogen bonding; dashes, cation coordination).

In the work reported here, new derivatives of monensin with a modified structure on heterocycle E at the C25 and/or C26 site were synthesized; this part of the molecule is involved in the chelating interactions with the carboxylic group, to give the well-documented globular structure of the transporting system (Figure 1). The chemical reactions carried out in gentle conditions had to be regio- and chemoselective with high yields and provide compounds with high chemical stability after purification by column chromatography. Of 20 derivatives obtained by this approach, seven were chosen on the basis of the above properties (**2**−**5** and **7**−**9**; see Scheme 1), and a complete study was made to compare their biological activities in connection with their ionophore properties.

Na^+, K^+, and H^+ transports were investigated in a relevant model, namely, human red blood cells (RBCs). Other groups have already used this cell model.[20−22,25−27] Modifications of the Na^+ gradients induced by deriva-

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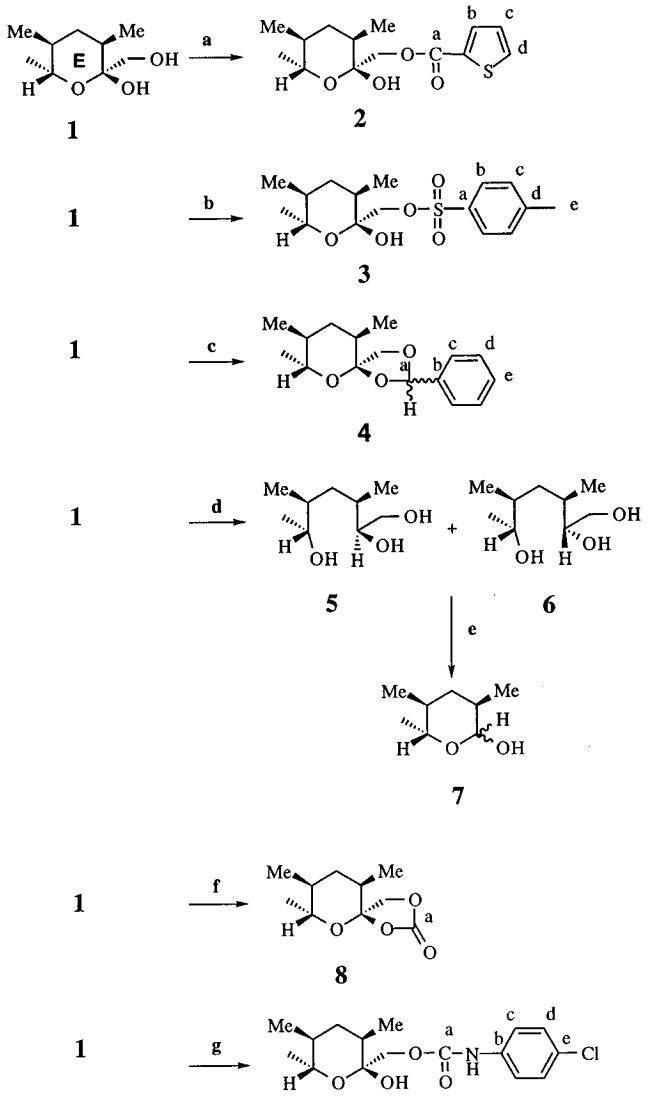
Scheme 1. Synthesis of Monensin Derivatives[a]



[a] (a) 2-Thiophenecarbonyl chloride/benzene; (b) *p*-toluenesulfonyl chloride/benzene; (c) benzaldehyde/$ZnCl_2$; (d) $NaBH_4$/EtOH; (e) $NaIO_4$/dioxane; (f) 1,1′-diimidazolecarbonyl/CH_2Cl_2; (g) *p*-chlorophenyl isocyanate/benzene.

tives **1**−**5** and **7**−**9** were studied by ^{23}Na NMR in the presence of $Dy^{3+}(PPPi)_2^{7-}$ as external shift reagent. This technique has been successfully applied for the study of erythrocytes.[20−22,24,28−33] Simultaneously, it was necessary to know if concomitant K^+ and H^+ movements were induced in RBCs by these transporting systems. Potentiometric measurements in the external medium proved to be the most convenient method to follow K^+ and H^+ fluxes. The growth inhibitions of *Plasmodium falciparum* and Gram-positive bacteria were studied in connection with these ionic exchanges.

Results and Discussion

Chemical Synthesis of Monensin Derivatives. Derivatives prepared on the E cycle of monensin A (**1**) free acid under gentle experimental conditions are summarized in Scheme 1. Regioselective esterification of monensin (C26-OH) with 2-thiophenecarbonyl chloride in benzene afforded compound **2**. Tosylate **3** was obtained by reaction with *p*-toluenesulfonyl chloride in well-established conditions. Reaction of monensin with benzaldehyde in the presence of zinc chloride gave **4**; analysis of ^1H and ^{13}C NMR spectra indicated that the reaction was stereoselective, but the absolute configuration of the acetal carbon formed was not determined. Reduction of monensin by $NaBH_4$ in pure ethanol afforded two diastereoisomers of dihydromonensin, which were separated by chromatography. The *S*-configuration enantiomer **5** was obtained in 40% yield and used in further studies. The *S*-configuration was attributed by direct analogy with N1 obtained from reduction of nigericin.[34] Oxidation by sodium metaperiodate of the diastereomeric mixture of dihydromonensins **5** + **6** gave compound **7**: In this case ^1H and ^{13}C NMR spectra showed that the reaction afforded a ratio of epimers OH_{ax}/OH_{eq} estimated to be 80/20. They were not separable. This result was unusual as the aldehyde intermediate formed during the oxidation was expected to give OH_{ax} preferentially during hemiacetal cyclization, due to the anomeric effect. To our knowledge, this result was not described previously in monensin chemistry. Carbonate **8** was prepared by treatment with 1,1′-diimidazolecarbonyl in methylene chloride. Finally urethane **9** was synthesized by reaction with 4-chlorophenyl isocyanate as previously described.[13]

Modifications of Na^+, K^+, and H^+ Gradients on Human Erythrocytes. Under physiological conditions, human erythrocytes show ionic gradients on both sides of the membrane that are the same as those encountered for living cells: $[Na^+]_{in}$ (∼10 mM) ≪ $[Na^+]_{out}$ (∼150 mM); $[K^+]_{in}$ (150 mM) ≫ $[K^+]_{out}$ (<1 mM). They make interesting models to study ionophore properties of compounds such as **1**−**9**, especially for cation-carrying efficiency, Na^+/K^+ transport selectivity, and pH modifications.

For several technical reasons, mainly linked to the NMR method (e.g., sensitivity, homogeneity of the medium), experimental conditions for ^{23}Na NMR studies (hematocrit 40%, [ionophore] = 4×10^{-7} M, corresponding to 0.87×10^{-10} mol of ionophore for 10^9 cells) were slightly different from those for potentiometric measurements (hematocrit 20%, [ionophore] = 3×10^{-7} M, corresponding to 10^{-10} mol of ionophore for 10^9 cells). For the sake of consistency, we thus determined in all experiments initial fluxes (J_i) for sodium and potassium in nmol min^{-1} with respect to 10^9 cells and for 10^{-10} mol of ionophore; in this case J_i values were taken to be proportional to the ionophore concentrations in the narrow range considered (1−0.87×10^{-10} mol). As previously established by us,[24] the very low ionophore concentrations used here clearly showed Na^+/K^+ transport selectivity, while for higher concentrations it was masked.

The time dependency for the variation of internal sodium concentration (ΔNa^+) and external potassium concentration (ΔK^+) in the presence of the monensin derivatives is reported in Figure 2A,B, respectively. Note that in the absence of ionophore, erythrocytes maintained fairly constant Na^+ and K^+ gradients.

These experiments clearly help to characterize Na^+- and K^+-transporting abilities of compounds **1**−**9**. All the compounds tested transport Na^+ less efficiently than monensin (**1**) (see Figure 2A). However, **3** and **9** still remain efficient sodium carriers, while a marked reduction of this property is observed for **2**, **8**, and **7**. Derivatives **4** and **5** were totally inactive in Na^+ transport. **5** is also inefficient for K^+ (Figure 2B). **8**, **4**,

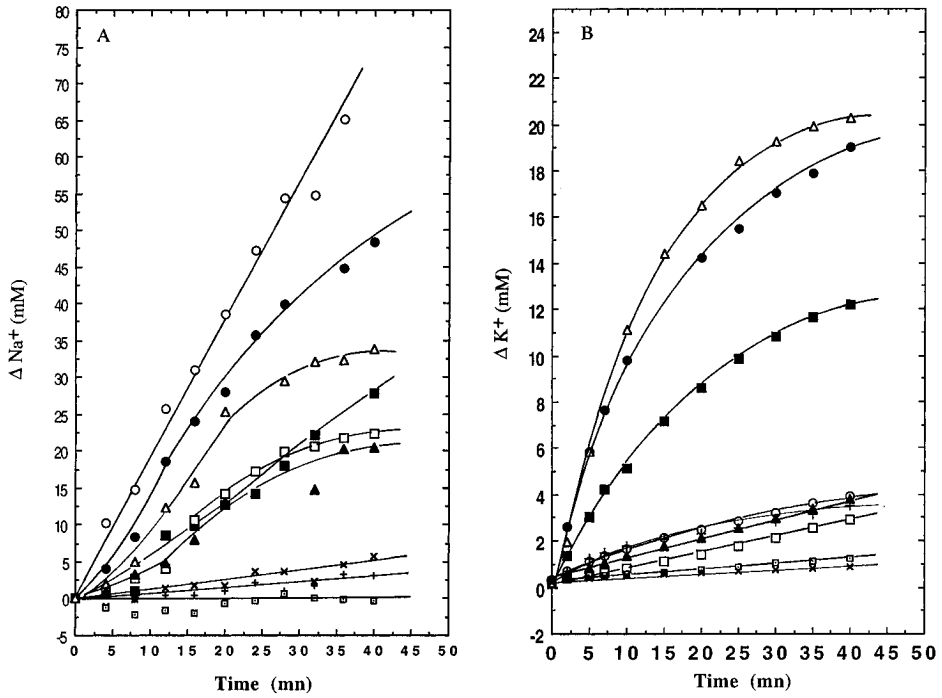


Figure 2. Effect of monensin derivatives on the intraerythrocytic sodium content and ionophore-induced K^+ release. (A) Time dependency of the variation of Na^+ internal concentration (ΔNa^+) determined from ^{23}Na NMR spectra recorded every 4 min at 79.39 MHz for 40 min. RBCs (2 mL) (hematocrit 40%) were incubated at 37 °C with 4×10^{-7} M ionophores in a buffer containing 80×10^{-3} M NaCl, 6×10^{-3} M $Na_7Dy^{3+}(PPPi)_2^{7-}$, 30×10^{-3} M sucrose, and 5×10^{-3} M NaH_2PO_4, pH 7.4. $\Delta Na^+ = [Na^+]_{time_t} - [Na^+]_{time0}$. (B) Time dependency of the variation of K^+ concentration (ΔK^+) in the supernatant measured by potentiometry every 5 min for 45 min. Erythrocytes (5 mL) (hematocrit 20%) were incubated at 37 °C in the presence of ionophores at a final concentration of 3×10^{-7} M in sodium buffer containing 140×10^{-3} M NaCl, 30×10^{-3} M sucrose, and 5×10^{-3} M NaH_2PO_4, pH 7.4, adjusted with choline hydroxide. $\Delta K^+ = [K^+]_{time_t} - [K^+]_{time0}$. Symbols: (○) **1**, (■) **2**, (●) **3**, (+) **4**, (⊡) **5**, (▲) **7**, (□) **8**, (△) **9**, and (×) control sample.

Table 1. Sodium Influx and Potassium Efflux Induced by Monensin Derivatives[a]

compd	$J_i(Na^+)/J_i(K^+)$	$J_i(Na^+)*J_i(K^+)$
1	4.68	2266
2	0.75	728
3	0.48	13 689
4	0	0
5	0	0
7	2.26	510
8	5.6	273
9	0.40	11 424

[a] Initial fluxes (J_i, nmol/min) are calculated for 10^9 cells and 10^{-10} mol of ionophore.

and **7** are poor K^+ carriers, comparable with **1**. On the contrary, **9**, **3**, and **2** are much better K^+ carriers than natural compound **1**.

Potency of the various monensin derivatives to exchange sodium and potassium through the erythrocyte membrane was more precisely characterized by the initial fluxes of ion movements induced by their presence. Ratios of $J_i(Na^+)/J_i(K^+)$ reported in Table 1 quantify the ionic selectivity of derivatives (J_i values of transport were determined from Figure 2). Thus inversion of selectivity in favor of potassium for **9**, **3**, and **2** is evident, while **1** and **8** are selective for sodium; **7** transports Na^+ and K^+ with almost the same rate but with a small preference for Na^+. For (4-chlorophenyl)-urethane derivative **9**, this is consistent with previous results obtained in model systems.[35,36]

The time dependency of pH in the supernatant was measured for each ionophore with a glass electrode in the sodium buffer (data not shown). Under these conditions, only very slight pH variations were detected

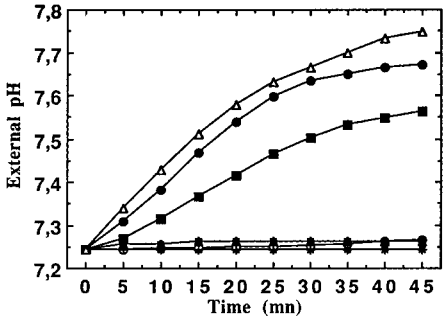


Figure 3. Time dependency of the pH in the supernatant measured with a glass electrode. Erythrocytes (5 mL) (hematocrit 20%) were incubated with monensin derivatives at a final concentration of 3×10^{-7} M in buffer containing choline$^+$ instead of Na^+. Symbols: (○) **1**, (■) **2**, (●) **3**, (+) **4**, (⊡) **5**, (▲) **7**, (△) **9**, and (×) control sample.

($\Delta pH \leq 0.08$), which may result from an efficient buffering of the external medium but also from the fact that Na^+/H^+ and K^+/H^+ exchanges take place in opposite directions, not necessarily with symmetrical effects. This last point is corroborated by the following experiment.

The same measurement of external pH was made in buffer containing choline$^+$ instead of Na^+ in the presence of monensin derivatives (Figure 3). **9**, **3**, and **2** induced positive variations of the external pH (ΔpH = 0.51, 0.44, and 0.33, respectively), while the other compounds had almost no effect. When Na^+ was replaced by choline$^+$, no Na^+ was available to be transported inside the cells, though intracellular K^+ was the main internal cation which can be expelled from the cells. Thus efficient K^+ carriers gave rise, by a K^+/H^+

antiport mechanism, to an alkalinization of the external medium. This experiment clearly confirmed the affinity of **9**, **3**, and **2** for K^+.

In conclusion, **9**, **3**, and **2** having a modified C26-OH moiety proved to be well-characterized K^+ carriers. **3** still kept affinity for Na^+ but less so than **1**. Clearly, suppression of the head-to-tail-chelating possibility between $C_1(OO^-)$ and C26-OH induced a loss of Na^+ selectivity compared to monensin with increased selectivity toward K^+. A prior study of monensin esters of the type C26-O-C(O)-R (R = methyl, ethyl, butyl, and isobutyl) carried out in a physicochemical system led to similar change for the sodium/potassium selectivity.[7] All this confirms the central role played by the C26-OH moiety in the selectivity of monensin for sodium.

When the C25-OH of the terminal hemiacetal (E cycle) cannot interact specifically by chelation with the carboxylic group, Na^+ is no longer complexed and is thus not transported. This is the case for **4** (the two hydroxy functions of the E cycle are protected) and **5** (the E ring is opened). K^+ is very weakly transported by **4** but not by **5**. This loss of ionophore properties is consistent with previous results reported for lactone and acetonide derivatives of monensin.[7] The case of carbonate **8** is also interesting as its ionophore properties are still detectable though rather weak, and its selectivity in favor of Na^+ is preserved. For **7**, when the C26-OH moiety was replaced by H, in this case, K^+ was no longer transported efficiently as observed with **9**, **3**, and **2**. In parallel, Na^+ transport was lowered; as a result no selectivity was observed for this compound, but a lower ionophore activity was found.

As RBCs contain ATP,[24] the competition of the erythrocyte intrinsic carriers[37] with ionophore action cannot be excluded in the resulting ionic movements. However, we have checked that the addition of ouabain (up to 1 mM), a well-known inhibitor of Na^+/K^+ ATPase, had no influence on Na^+ and K^+ transport with or without monensin (data not shown). This is in good agreement with the pioneering work of Pressman,[25] who reported that erythrocytes behave almost like inert model membranes even though they are energized. The ion transports induced by the ionophores, in the time range considered, do not seem to be efficiently compensated for by the erythrocyte systems and especially the Na^+/K^+ ATPase.

In conclusion, the ionophore properties determined for molecules **1**−**9** in a biological membrane, and not in a physicochemical model system, can be more validly linked to observed biological activities.

Antimicrobial Activity. Antibiogram tests (data not shown) show that derivatives **1**−**9**, as usually observed for carboxylic polyether antibiotics, were inactive on Gram-negative bacteria and fungi[10] as a result of nonaccessibility of the cytoplasmic membrane. This last point was experimentally demonstrated by us for calcimycin using a tritiated sample of this divalent ionophore.[38]

Most of the compounds (**1**−**3**, **8**, and **7**) were active on the three Gram-positive bacteria strains studied (data not shown): *Bacillus cereus*, *Bacillus sphaericus*, and *Micrococcus luteus*; **5** was totally inactive. Surprisingly, **4** showed a peculiar activity on *B. cereus* in comparison with the two other strains.

Table 2. Biological Activities of Monensin Derivatives

ionophores	MIC (μg/mL), *B. cereus* ATCC 14579	IC_{50} (ng/mL), *P. falciparum*
1	1.56	1.65
2	0.02	2.7
3	0.78	1.25
4	6.25	750
5	> 50	5250
7	0.39	11.93
8	1.56	0.75
9	0.05	1.25

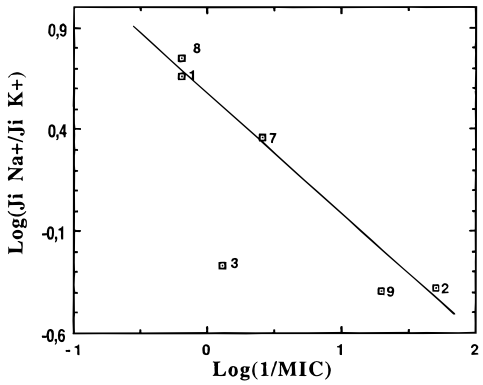


Figure 4. Relationship between sodium and potassium transport of monensin derivatives and antibacterial activity: plot of the $\log(J_i(Na^+)/J_i(K^+))$ vs $\log(1/MIC)$ for *B. cereus* ATCC 14579. *R* (correlation coefficient) = 0.98 if data for compound **3** is left out.

The minimum inhibitory concentrations (MIC) were determined on *B. cereus* ATCC 14575 strain (Table 2). Clearly, **5** had no antibiotic properties; this result may be connected with the loss of ionophore activity of this compound. The significant MIC for **4** (6.25) was unexpected with regard to its poor ionophore ability; in addition it was active only on *B. cereus*. As we have already shown that natural compounds such as nigericin or grisorixin can be bioconverted by bacteria,[34,39] we suspected that *B. cereus* bioconverted **4** into a more active compound. This possibility was checked on tubes seeded with *B. cereus* containing 50, 15, 6.25, and 1.25 μg/mL **1** and **4** after 24-h cultures at 27 °C, corresponding to standard conditions for MIC determinations, by extracting tubes with organic solvents and chromatographic analysis. No degradation of **1** or **4** occurred under these conditions. Consequently the activity of **4** on *B. cereus* could not have resulted from a bioconversion but must arise from some other unidentified mechanism.

Ionophore properties and antibacterial activity were closely correlated by plotting $\log(J_i(Na^+)/J_i(K^+))$ vs $\log(1/MIC)$ (Figure 4). Except for **3**, a fair correlation was obtained. The activity on *B. cereus* increased with the selectivity in favor of K^+ compared with Na^+. This quantifies our previous observation made with other C26-OH monensin derivatives for which a high antibiotic activity was found in the case of K^+ selective systems.[7]

Interestingly, when $\log(J_i(Na^+))$ or $\log(J_i(K^+))$ alone was plotted vs $\log(1/MIC)$, no relationship was observed. This indicates that in the antibiotic mode of action both cation movements must be considered together. To our knowledge, this point has never been shown before.

Antimalarial Activity. The antimalarial potentialities of derivatives **1**−**9** were evaluated by measuring the

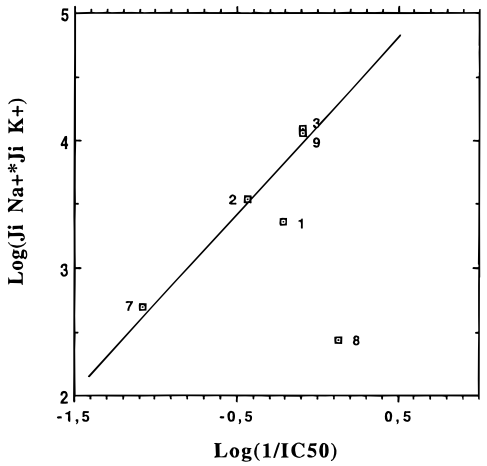


Figure 5. Relationship between sodium and potassium transport of monensin derivatives and antimalarial activity: plot of the $\log(J_i(Na^+)*J_i(K^+))$ vs $\log(1/IC_{50})$ for *P. falciparum*. *R* (correlation coefficient) = 0.90 if data for compound **8** is left out.

in vitro growth inhibition of *P. falciparum*. IC_{50} values thus obtained are reported in Table 2. Compound **5**, previously shown as nonantibiotic, did not inhibit parasite growth. **4** was also very weakly active, consistent with its transporting and antibacterial abilities (except on *B. cereus*). This agreed with the result obtained for the lactone derivative of monensin tested under the same conditions on *P. falciparum* (unpublished data), which was neither ionophoric nor antibiotic.[7] On the other hand, monensin and all other derivatives exhibited a potent activity against *P. falciparum*, the human malaria parasite. Concentrations that inhibited 50% of the parasite growth were from 0.75 to 12 ng/mL, i.e., in the nanomolar range.

As previously shown for antibacterial activity, when $\log(J_i(Na^+))$ or $\log(J_i(K^+))$ was plotted vs $\log(1/IC_{50})$, no correlation was observed; this was also true with $\log(J_i(Na^+)/J_i(K^+))$; in contrast, a relationship was apparent if $\log(J_i(Na^+)*J_i(K^+))$ was plotted vs $\log(1/IC_{50})$ (Figure 5). These results can be interpreted as follows: (i) movements of both cations (Na^+ and K^+) must be considered together for correlation, which means that we observed an overall effect for this system with several compartments, (ii) the selectivity of transport of these compounds is not the determining factor, and (iii) the antimalarial activity can result in a cumulative effect related to the efficiency of transport for both Na^+ and K^+. This was illustrated by considering the product of the initial rates ($J_i(Na^+)*J_i(K^+)$) mentioned in Table 1. In this case, **8** presented an unexpectedly high activity on *P. falciparum*, given its low ionophore activity.

These results strongly suggest that the mode of action of such ionophores on *P. falciparum* is different from that on Gram-positive bacteria. This is not surprising as the infected red cells possess several compartments. In addition, during development in erythrocytes, Na^+ and K^+ intraerythrocytic concentrations are modified; the parasite creates a gradient between its own cytosol and the erythrocyte cytosol. Very likely the ionophores investigated disturb the ionic gradients established between the different compartments as they can reach all the membrane phases available. We showed, in a different system using tritiated calcimycin, that this ionophore was distributed not only in the cytoplasmic membrane but also in the intracellular compartment membranes.[40] It would be of interest to measure Na^+ and K^+ fluxes in malaria-infected red cells to confirm this result. Work is in progress in our laboratory to overcome technological problems related to these measurements.

Conclusion

Molecules closely related to monensin, with a modified C25−C26 moiety, were synthesized; ionophore abilities were investigated on erythrocytes in connection with antimicrobial and antimalarial activities. The first conclusion is that the C26-OH moiety is a major site in the polyether skeleton that modulates the ionophore and biological properties of monensin; this part of the molecule appears to be essential for the remarkable stability of the 1:1 sodium complex in the natural ionophore. When the C26-OH hydroxyl function is masked by a protecting group (**9**, **3**, **2**), efficiency of Na^+ transport decreases while K^+ transport increases in comparison with monensin, leading to a net inversion of selectivity. If this moiety is masked by a proton (**7**), Na^+ transport is still reduced but no enhancement of K^+ transport is observed; in this case almost no selectivity of transport is evidenced. Finally, when the head-to-tail chelation of monensin is prevented, the ionophore ability is lost (**5**, **4**).

The second main conclusion is that it is necessary to measure Na^+ and K^+ variations in parallel to characterize ionophore derivatives of monensin. Measuring only the Na^+ movements, as usually reported, does not give enough information on the mode of action of these cation carriers. Study of ionophore properties in living cells such as erythrocytes affords a more relevant structure−activity relationship than Na^+/K^+ selectivities measured on physicochemical model membranes. This approach takes into account ion gradients present on both sides of the membrane: $[Na^+]_{in} \ll [K^+]_{out}$, $[Na^+]_{out} \gg [Na^+]_{in}$, $[K^+]_{out} \ll [Na^+]_{out}$, and $[K^+]_{out} \ll [K^+]_{in}$, whereas these conditions are not reproduced with model systems.

The third emerging conclusion is that biological activities are closely related to the membrane accessibility for the ionophore systems. Thus, all the derivatives tested were inactive on Gram-negative bacteria and fungi. Further, in the case of Gram-positive sensitive strains, inversion of Na^+/K^+ selectivity in favor of K^+ induces higher antibacterial activities.

To our knowledge, our work is one of the first investigations of structure−activity relationships in the antimalarial domain for polyether ionophores; for that purpose precise information on both ionophore abilities on red cells and IC_{50} on *P. falciparum* were determined. We can conclude that antimalarial activities are clearly related to ionophore properties; the whole efficiency of transport for sodium and potassium seems to be involved rather than the ionic selectivity. We are continuing the study of some of these derivatives to determine their selectivity for infected erythrocytes compared with other mammalian cells.

Experimental Section

Synthesis. General. Melting points were determined on a Reichert hot-plate and are uncorrected. Optical rotations were measured with a Perkin Elmer model 141 polarimeter in chloroform at 1.5 g/100 mL with l = 578 nmHg and $T = 25$

Table 3. Physical Constants and Spectral Data of Compounds **2**−**9**

compd	formula	MW	obsd mass FAB$^+$ (MNa$^+$)	mp (°C)	α^{25}_D (deg) (c = 1.5, $CHCl_3$)	characteristic ^{13}C NMR data (C_6D_6) (δ, in ppm) (carbon position in parentheses)
2	$C_{41}H_{64}O_{12}S$	780.4	803.0	124−125	+65	183.40 (1), 162.3 (a), 134.16 (b), 132.85 (c), 131.14 (d), 132.61 (e)
3	$C_{43}H_{68}O_{13}S$	824.4	847.4	83−85	+40	176.90 (1), 144.3 (a), 128.8 (b), 130.3 (c), 134.9 (d), 21.6 (e)
4	$C_{43}H_{66}O_{11}$	758.5	781.0	78−79	+39	179.04 (1), 103.67 (a), 138.26 (b), 129.87 (c), 127.79 (d), 129.87 (e)
5	$C_{36}H_{64}O_{11}$	672.4	695.4	120−121	+45	178.61 (1), 78.15 (21), 77.23 (25), 65.05 (26)
7	$C_{35}H_{65}O_{10}$	640.4	663.3	77−78	+64	180.08 (1), 95.22 (25)
8	$C_{37}H_{60}O_{12}$	696.4	719.4	94−96	+49	179.44 (1), 154.09 (a)
9	$C_{43}H_{66}O_{12}NCl$	846.4	823.4	104−106	+78	178.6 (1), 153.1 (a), 137.4 (b), 119.7 (c), 128.6 (d), 127.4 (e)

°C. NMR spectra were recorded on a Bruker MSL 300 or AC400 spectrometer in a C_6D_6 solution with TMS as internal standard; some compounds were unstable in $CDCl_3$ solvent. All the resonances were assigned for the ^1H and ^{13}C NMR spectra by $^1H-^1H$ chemical shift correlation and $^1H-^{13}C$ correlation. Only characteristic peaks are given here. FAB mass spectra were determined with a VG 70-70F spectrometer at the Service Central d'Analyse du C.N.R.S. (Vernaison, France). Values in the text for positive FAB correspond to $(M + Na)^+$. TLC analysis was performed with Merck (ref 1.05735) plastic silica gel plates using vanillin−sulfuric acid in ethanol as a spray reagent. Column chromatography was carried out with Merck silica gel 60 (70−230 mesh ASTM).

Pure monensin A (**1**) was produced by us from the *S. cinnamonensis* ATCC 15413 strain. The physicochemical properties of the derivatives are summarized in Table 3.

26-*O*-(2-Thiophenylcarbonyl)monensin (2). Monensin (500 mg, 0.75 mmol) was dissolved in 8 mL of benzene at 0 °C under argon and protected from light. 2-Thiophenecarbonyl chloride (4 mmol) was added. After 3 h at 0 °C the mixture was left for 20 h at room temperature with stirring. The benzene was evaporated, and the solid phase was dissolved in chloroform (20 mL), which was then washed with distilled water (20 mL). Drying over $MgSO_4$ was followed by evaporation. Chromatography on silica gel with cyclohexane−ethyl acetate gradient from 20:80 to 5:95 gave compound **2** with a yield of 349 mg (60%).

26-*O*-(*p*-Tolylsulfonyl)monensin (3). Monensin (500 mg, 0.75 mmol) was dissolved in 8 mL of benzene and protected from light. *p*-Toluenesulfonyl chloride (229 mg, 1.2 mmol) were added. The mixture was stirred for 96 h at room temperature. The benzene was evaporated, and the solid phase was dissolved in ether (50 mL) and then washed twice with 0.2 N HCl and deionized water (2 × 50 mL). The ether solution was dried over $MgSO_4$, and the product was purified with an eluent gradient of cyclohexane−ethyl acetate (95:5−0:100). After purification compound **3** was obtained with a yield of 491 mg (80%).

25,26-*O*-Benzylidenemonensin (4). Monensin (500 mg, 0.75 mmol) was dissolved in 2 mL of freshly distilled benzaldehyde. $ZnCl_2$ (350 mg) was added. The mixture was stirred at 40 °C under argon for 24 h and then cooled to 0 °C in an ice−water bath. Deionized water (3 mL) was added. The mixture was stirred at 0 °C for 1.5 h. Water and benzaldehyde were carefully evaporated with a vacuum pump to give a viscous liquid, which was dissolved in ethyl acetate (20 mL). The organic phase was washed with 0.1 N HCl (20 mL) and deionized water (20 mL), dried over $MgSO_4$, and evaporated. The product was purified chromatographically with an eluent gradient of cyclohexane−ethyl acetate (90:10−85:15). Compound **4** was obtained with a yield of 420 mg (80%).

(21*S*,25*S*)-Dihydromonensin (5). Monensin (500 mg, 0.75 mmol) dissolved in 15 mL of ethanol was reduced by $NaBH_4$ (135 mg, 3.5 mmol). The mixture was stirred for 48 h at room temperature. The reaction was quenched carefully with water (50 mL) followed by addition of 0.1 N HCl (50 mL). The reaction mixture was extracted three times with 30 mL of chloroform, and the organic phase was washed with 60 mL of water. The organic solvent was dried over $MgSO_4$ and evaporated in vacuo. Chromatography of the residue on silica gel with $CHCl_3$−MeOH (98:02−93:07) gave two diastereoisomers which were separated with a yield of 200 mg (40%) for **5**.

25-De(hydroxymethyl)monensin (7). Two diastereoisomers of 21,25-dihydromonensin (**5** and **6**; 440 mg, 0.65 mmol) obtained as described previously were dissolved in 7 mL of dioxane. Sodium metaperiodate (400 mg, 1.9 mmol) dissolved in 7 mL of water was added. The mixture was stirred for 12 h at room temperature and then diluted with water (20 mL) and extracted three times with 10 mL of $CHCl_3$. The organic phase was washed twice with 10 mL of water and dried over $MgSO_4$. The solvent was evaporated in vacuo and the residue chromatographed on silica gel with cyclohexane−ethyl acetate (80:20−0:100). After purification compound **7** was obtained with a yield of 208 mg (50%). The ratio of epimers OH_{ax}/OH_{eq} was 80/20.

25,26-*O*-(Oxomethylene)monensin (8). To a solution of monensin (500 mg, 0.75 mmol) in 10 mL of methylene chloride under argon and protected from light was 1,1′-diimidazolecarbonyl (311 mg, 1.6 mmol). The mixture was stirred for 48 h at room temperature. After addition of 0.3 mL of triethylamine, the mixture was stirred for 24 h. The solvent was evaporated, and the reaction mixture was dissolved in chloroform (50 mL). The organic phase was washed three times with 0.2 N HCl and three times with 20 mL of distilled water. Drying over $MgSO_4$ was followed by evaporation. Chromatography on silica gel with chloroform−acetone from 50:50 to 0:100 gave compound **8** with a yield of 290 mg (56%).

26-*O*-[(4-Chlorophenyl)carbamoyl]monensin (9). This synthesis is described elsewhere.[13]

For measurement of biological activities, unless otherwise stated, ionophores were dissolved in DMSO, and the final concentration in the cell suspension was 0.5%. We checked that this concentration was nontoxic for the cells by ^{31}P NMR. For ^{23}Na NMR experiments, the final ionophore concentration in the cell suspension was 4×10^{-7} M, while it was 3×10^{-7} M for potentiometry experiments.

Antibiogram Tests and MIC Determinations. Five strains were tested, three Gram-positive bacteria (*B. cereus* ATCC 14579, *B. sphaericus* ATCC 1038, and *M. luteus* ATCC 4698), a Gram-negative bacterium (*Pseudomonas aeruginosa* ATCC 10145), and a yeast (*Candida albicans* 444; from Pasteur Institute). Antimicrobial activity was determined by the conventional paper disk (Durieux No. 268; 6 mm in diameter) diffusion method using the following nutrient media: Mueller-Hinton (Difco) for bacteria and Sabouraud agar (Difco) for *C. albicans*. Growth inhibition was examined after 24-h incubation at 27 °C. Monensin derivatives were dissolved in benzene (1 mg/mL), and a paper disk containing each of the products (5 μg) was placed on agar plates, after evaporation of the solvent benzene.

MICs of monensin derivatives were determined classically on *B. cereus* ATCC 14579 in Mueller-Hinton broth, pH 7.4 (Difco), after 24-h incubation at 27 °C. Ionophores diluted in DMSO were added to 18 tubes; the concentration range was from 100 μg/mL to 1.56 ng/mL. In order to check for any bioconversion of **4** and **1** by *B. cereus*, tubes containing 50, 15, 6.25, and 1.25 μg/mL **4** and **1** were extracted. The tubes (5 mL) were saturated with $(NH_4)_2SO_4$ and supplemented with 5 mL of ethyl acetate. After vigorous mixing, the tubes were centrifuged for 5 min at 4000*g*. The supernatant corresponding to the ethyl acetate phase was saturated with $MgSO_4$ and evaporated to dryness. This extraction procedure was repeated twice. The evaporated fractions were dissolved in 100 μL of ethyl acetate and spotted on silica gel plates (60F-254, 0.25 mm; Merck ref 5.747). The plates were developed in pure ethyl acetate at room temperature, dried, sprayed with vanillin−sulfuric acid reagent, and heated at 85 °C. Control tests were also performed in tubes containing the same concentration of ionophores but unseeded with *B. cereus*.

***In Vitro* Antimalarial Activity against *P. falciparum*.** The nigerian strain of *P. falciparum*[41] was maintained by serial passages in human erythrocyte culture at 7% hematocrit in complete medium at 37 °C using the petri dish candle-jar method.[42] G-[^{3}H]hypoxanthine was purchased from Amersham Corp. (Les Ulis, France), and RPMI 1640 medium was from Gibco Laboratories (France). Complete medium consisted of RPMI 1640 supplemented with 25 mM Hepes buffer, pH 7.4, and 10% AB^+ serum.

Drug effects on *P. falciparum* growth were measured *in vitro* in microtiter plates according to Desjardins.[43] Stock solutions of drugs were in DMSO. They were further diluted in culture medium so that final solvent concentration never exceeded 0.25%. The final volume in each well was 200 μL, consisting of 50 μL of complete medium with or without the drug (control) and 150 μL of *P. falciparum*-infected erythrocyte suspension (1−2% final hematocrit and 0.3−0.8% parasitemia). After 48 h of incubation at 37 °C, 30 μL of complete medium containing 0.8 μCi of [^{3}H]hypoxanthine was added to each well, and candle-jar incubations were continued for an additional 12−18-h period. Cells were then subsequently lysed and the parasite macromolecules including radioactive nuleic acids recovered on glass fiber filters (Whatman GF/C) using an automatic cell harvester (Skatron Macro 96). Filters were then counted for radioactivity after adding 2 mL of scintillation cocktail in a Beckman 5801 liquid scintillation spectrometer. Radioactive background was obtained from incubations of normal erythrocytes under the same conditions of hematocrit and medium. Growth inhibition in the presence of the various compounds was expressed as percent of control parasitemia observed without drug. IC_{50} values, which represent the drug concentration required to inhibit parasite growth by 50%, were evaluated from the plot of log dose versus parasite growth expressed as percent of the control and are means of at least two independent experiments, each in triplicate, using different stock drug solutions.

RBC Preparation. Blood samples were collected from healthy adults in 50-mL heparinized tubes and immediately centrifuged to remove plasma and buffy coat (4 °C, 3000*g*, 15 min). Cells were washed three times with sodium buffer containing 140 mM NaCl, 30 mM sucrose, and 5 mM NaH_2PO_4, pH 7.4, adjusted with sodium hydroxide. For ^{23}Na NMR experiments, the third wash was performed with choline buffer containing 80 mM NaCl and 6 mM $Na_{10}Dy(PPPi)_2$. For some specific H^+ potentiometry experiments, the cells were washed in choline buffer containing 140 mM choline chloride, 30 mM sucrose, and 5 mM NaH_2PO_4, pH 7.4, adjusted with choline hydroxide. Packed cells (hematocrit ∼ 80%) were kept at 0 °C and diluted just before use in the buffer; all the experiments were carried out at 37 °C (a preincubation of about 15 min was necessary for temperature equilibration).

The hematocrit was adjusted to ca. 40% for ^{23}Na NMR experiments. These are typical values for human whole blood hematocrit. For K^+ and H^+ potentiometry experiments, the final hematocrit was ∼20%.

Cell viability was confirmed by ^{31}P NMR analysis; ATP was clearly detectable on the spectra.[24] We checked that neither ATP nor pH evolved during a 45-min kinetic run (and even after 12 h). Another indication of cell energization was the maintenance of Na^+ and K^+ gradients in the absence of an ionophore.

^{23}Na NMR Experiments. ^{23}Na NMR experiments were performed on a Bruker MSL 300 spectrometer at 79.39 MHz and 37 °C. Cell suspension (2 mL) was transferred to 10-mm diameter tubes; a coaxial capillary (1-mm diameter) containing 7×10^{-3} M $Na_{10}Dy(PPPi)_2$ in D_2O was used as an external intensity reference, and its calibration gave an equivalence for 9.14 mM sodium. Shimming and field frequency locking were carried out with D_2O.

$Dy^{3+}(PPPi)_2^{7-}$ present in the cell suspension allowed us to distinguish Na^+_{in} from Na^+_{ex}; it was shown to be nontoxic for erythrocytes by Ogino et al.[30] It was prepared from $Na_7(PPPi)_2$ (Sigma) and $DyCl_3 \cdot 6H_2O$ (Aldrich) (molar ratio 2:1). ^{23}Na NMR spectra were accumulated in 4-min blocks over 40 min (90° pulse, 16.5 μs, repetition time 0.4 s, 600 scans, 5DB, 2K data points). No line broadening was applied, thus allowing direct measurements of the sodium areas from computer integration of the NMR signals. For the various kinetics recorded in the presence of ionophores, the sum of the NMR integrations of Na_{in} and Na_{ex} signals remained constant. This clearly indicates that Na_{in} was 100% visible, in agreement with other work.[24,30−33] Na^+ concentrations are given for a hematocrit of 40%; internal sodium content [Na_{in}] was calculated as follows:

$$[Na_{in}] = (A_{in}/A_{ref}) \times 9.14 \text{ mM}$$

A_{in} and A_{ref} are the integrals of internal sodium and sodium in the capillary determined from ^{23}Na NMR spectra. Each experiment was repeated three to five times, and the mean values were plotted (the relative error did not exceed 10%).

K^+ Potentiometry. The experiments were performed in a thermostated cell with magnetic stirring, and the K^+ concentrations were measured with a selective electrode (Ingold type 15 221 3000) previously calibrated. Typically, 1.66 mL of the packed cells and 5 mL of buffer were incubated at 37 °C for 15 min, 20 μL of a 10^{-4} M solution of ionophore in DMSO was added, and data were recorded for 45 min. Each experiment was repeated three to five times, and the mean values were plotted (the relative error did not exceed 10%).

The initial rates of Na^+ influx into the cells were determined graphically from ^{23}Na NMR experiments (see Figure 2A). The initial rates of K^+ efflux were determined graphically from potentiometry measurements (see Figure 2B). The number of cells/mL was calculated from the normal mean cell volume of 87 μm^3.[44] Finally, the initial fluxes (J_i) in nmol/min were calculated from the initial rate values for 10^9 cells and 10^{-10} mol of ionophore.

H^+ Potentiometry. The external pH was measured with a glass electrode (Ingold, U-402-2-S7) previously calibrated; 5 mL of the RBC suspension (hematocrit 20%) was incubated at 37 °C for 15 min. The external pH shifted from 7.40 to 7.24, in agreement with the observations of Moins.[45] Ionophore (3×10^{-7} M) was then added to the cell suspension. Each pH measurement was performed three to five times, and the mean values were plotted.

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