Anal. Chem. **2003,** *75,* 152−156

Synthesis of an Ammonium Ionophore and Its Application in a Planar Ion-Selective Electrode

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A modular technique was used to synthesize an ammonium-selective ionophore based on a cyclic depsipeptide structure. The ionophore was incorporated into a planar ion-selective electrode sensor format and the selectivity tested versus a range of metal cations in a commercial clinical diagnostic "point-of-care" instrument. Four sensor membrane formulations were tested, all of which consisted of plasticized PVC. Formulations differed as to the type of plasticizer used and whether an ionic additive was present. It was found that the membrane containing the polar plasticizer nitrophenyl octyl ether in the absence of ionic additive exhibited near-Nernstian behavior (slope, 60.1 mV/decade at 37 °C) and possessed high selectivity for ammonium ion over lithium and the divalent cations, calcium and magnesium (log $K^{POT}_{NH_4^+,j} = -7.3, -4.4,$ and -7.1 for lithium, calcium, and magnesium ions, respectively). The same membrane also exhibited sodium and potassium selectivity that was comparable to that reported for nonactin (log $K^{POT}_{NH_4^+,j} = -2.1$ and -0.6 for sodium and potassium, respectively, compared to -2.4 and -0.9 in the case of nonactin). Membranes containing the less polar plasticizer, dioctyl phthalate, showed sub-Nernstian behavior (slope, <50 mV/decade at 37 °C). In all cases, the presence of the ionic additive potassium tetrakis(4-chlorophenyl)borate substantially reduced the selectivity observed. The flexible modular synthetic technique developed and reported here will allow the cyclic depsipeptide structure to be tuned for optimum selectivity.

Metabolites such as urea and creatinine are important disease indicators[1] and for this reason, considerable effort has been expended in developing diagnostic tools for their detection. Since the ammonium cation is a byproduct of various enzymatic reactions involving these substrates, much work has focused on fabricating sensors for selective ammonium ion detection.[2−9] To date, the most common strategy involves the use of ion-selective electrodes (ISEs) that employ the natural antibiotic nonactin as an ionophore doped into plasticized poly(vinyl chloride) (PVC) membranes.[10] However, it is well known that nonactin-based ammonium ISEs are limited in their utility because while nonactin has reasonable ammonium ion/sodium ion selectivity (log $K^{pot}_{NH_4^+,Na^+} \sim -2.4$), it has poor selectivity over potassium ions (log $K^{pot}_{NH_4^+,K^+} \sim -0.9$).[1] Consequently, significant effort is now being made to develop new synthetic ionophores with higher ammonium ion selectivity.[11−14]

In early work, Lehn et al. synthesized macrotricyclic cryptands that exhibited a substantial enhancement in NH_4^+/K^+ selectivity, as determined by NMR studies.[11] Greater selectivity was attributed to the tetrahedral geometry provided by the ionophore and its ability to donate four hydrogen bonds to stabilize the cation. This result pointed out the particular importance of hydrogen bonding and symmetry considerations in the design of ammonium ion recognition sites. Another consideration especially important in sensor applications is the reversibility of ion binding as indicated by the magnitude of the ion/ionophore complex dissociation constant. In the systems studied by Lehn, the dissociation constant was several orders of magnitude smaller than for nonactin, which leads to the conclusion that such systems cannot be used for rapid reversible detection of ammonium ion in an ISE format. In effect, the cryptands described were ammonium ion sinks.

Chin and co-workers synthesized 1,3,5-tri(3,5-dimethylpyrazol-1-ylmethyl)-2,4,6-triethylbenzene in which the three pyrazole groups provide hydrogen-bonding sites.[12] An ISE incorporating this molecule showed improvement in ammonium ion selectivity over potassium ion as compared to nonactin (log $K^{pot}_{NH_4^+} = -2.6$), again illustrating the importance of hydrogen bonding and symmetry. However, the limit of detection for this ionophore is 2 orders of magnitude higher than for nonactin, and therefore, it is not sufficiently sensitive for some applications. Kim et al. investigated the use of thiazole-containing benzocrown ethers as

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10.1021/ac0257851 CCC: $25.00
Published on Web 11/22/2002

ammonium ionophores and reported potassium selectivity comparable to nonactin and enhanced selectivity for ammonium over sodium ion (log $K^{pot}_{NH_4^+} = -3.9$).[13] Similarly, others have used 19-crown-6 structures with decalino blocking groups to control selectivity, reporting increased selectivity for ammonium over both smaller and larger cations.[14]

Our approach to the design and synthesis of ammonium ionophores has, as with Lehn and others, focused on the incorporation of hydrogen bond donors in tetrahedrally symmetric complexation sites. Given the structural complexity of some of the synthetic ionophores reported, we have used a molecular motif that both lends itself to straightforward synthesis and allows structural modifications to be incorporated without substantial changes in synthetic strategy. Our experience to date, as well as that of others,[15−19] has shown that ionophores based on cyclic peptide and depsipeptide structures, i.e., those that are similar to natural ionophores, can be readily synthesized in high yield by either solution- or solid-phase methods.

In the work reported here, we have taken valinomycin as our inspiration for the design of a new ammonium ion-specific ionophore. Valinomycin is a naturally occurring antibiotic having high selectivity for potassium ions. It has a cyclic depsipeptide structure consisting of alternating amide and ester units (6 of each, 12 in total) and has been synthesized on a solid-phase support.[19] Valinomycin preorganizes through hydrogen bonding of its amide carbonyl groups to form a pocket that presents its six ester carbonyl groups as sources of electrostatic stabilization for potassium ions.[20] Thus, the pocket provides an octahedral-type complexation site with a size that is a close match to the estimated ionic radius of potassium (1.33 Å). We report here the synthesis of an ammonium ion-specific ionophore, **IV**, which has some of the same structural elements as valinomycin. **IV** is a cyclic depsipeptide consisting of alternating amide and ester groups (three of each, six in all) which is in effect, half of the valinomycin structure. **IV** does not fold onto itself and therefore it provides a complexation site that is approximately the same size as valinomycin, a necessary feature because the ammonium ionic radius (1.43 Å) is comparable to that of potassium.[11] An important difference though, is that **IV** is not capable of providing an octahedral binding site. However, it has hydrogen bond donors arranged tetrahedrally (necessary for ammonium complexation), and it is this distinction that we expected to allow the ionophore to discriminate efficiently between potassium and ammonium ions.

Below we describe the synthesis of **IV**, the incorporation of this ionophore into a planar ISE sensor format, testing of the potentiometric response of the electrode in a commercial clinical diagnostic "point-of-care" instrument, and the results of selectivity studies for ammonium versus other metal cations.

EXPERIMENTAL SECTION

Reagents. Mass spectra were performed by Synpep Corp. (Dublin, CA). Melting points are measured in a Mel-Temp capillary melting point apparatus and are not corrected. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 400 in $CDCl_3$ unless otherwise noted. All solvents and reagents were analytical reagent grade and used as supplied from Aldrich Chemical Co. PVC, nitrophenyl octyl ether (NPOE), dioctyl phthalate (DOP), potassium tetrakis(4-chlorophenyl)borate (KtpClPB), and D-hydroxyisovaleric acid were purchased form Fluka AG (Buch, Switzerland). Amino acids L- and D-valine-*N*-fmoc were purchased from Calbiochem-Novabiochem Corp. Buffers were prepared with deionized water (18 MΩ·cm).

Molecular Modeling Calculations. Molecular modeling was performed on an SGI 320 running Windows NT. Calculations were carried out using the Molecular Operating Environment version 2000.02 computing package (Chemical Computing Group Inc., Montreal, PQ, Canada). Structures were minimized first using the AMBER94 potential control under a solvent dielectric of 5. PEF95SAC was used to calculate partial charges. Minimized structures were then subjected to a 30-ps molecular dynamics simulation employing the NVT statistical ensemble. The structures were heated to 400 K, equilibrated at 310 K, and cooled to 290 K in the dynamics thermal cycle at a rate of 10 K/ps. The lowest energy structures obtained from these dynamics calculations were then minimized again. Using the minimized structures, docking energies of the ammonium and potassium cations were calculated by employing the default parameters supplied with the program.

Synthesis of Ionophore (Scheme 1). A detailed description of the synthesis and purification of the cyclodepsipeptide **IV** is provided in the Supporting Information. Briefly, two building blocks, **I** and **II,** were formed in solution from the corresponding L-lactic acid, D-valine-*N*-fmoc residues (**I**) and D-hydroxyisovaleric acid, L-valine-*N*-fmoc residues (**II**). These were coupled sequentially as **I**−**II**−**I** onto a Wang resin, then cleaved to give **III,** and subsequently cyclized to give the title compound, **IV**.

ISE Membrane and Electrode Preparation. Four membrane cocktails were prepared to test **IV**. The specific formulations are as follows: M1, 69/30/1 wt % of NPOE/PVC/**IV**; M2, same as M1 with 50 mol % of KtpClPB to **IV**; M3, 69/30/1 wt % of DOP/PVC/**IV**; M4, same as M3 with 50 mol % KtpClPB to **IV**. Membrane cocktails were prepared as 10 wt % solutions in THF. The base electrodes were constructed in a thick-film planar format[21] using a polymeric internal electrolyte layer.[22,23] A single wafer composed of 100 individual electrode elements was used for the sensor construction. The polymer, methacrylamidopropylmethylammonium chloride (MAPTAC), for the internal electrolyte was prepared as a 10 wt % solution in EtOH, spun on to the planar wafer at 750 rpm for 30 s, and allowed to dry for 1 h before membrane deposition. Internal electrolyte thickness was ∼3.5 μm. The wafer was then quartered giving 4 wafers of 25 sensors each. Membrane cocktails were deposited (0.9 mL) onto the wafers and allowed to cure for 24 h before use, giving a membrane thickness of ∼105 μm. The planar wafers were singulated by hand, giving 25 sensors for each formulation.

ISE Testing. The sensors were housed in the proprietary flow-through cell used with the Bayer Diagnostics Rapidpoint 400 critical care system. This system uses a saturated Ag/AgCl reference cell. Two flow cells were constructed, which contained

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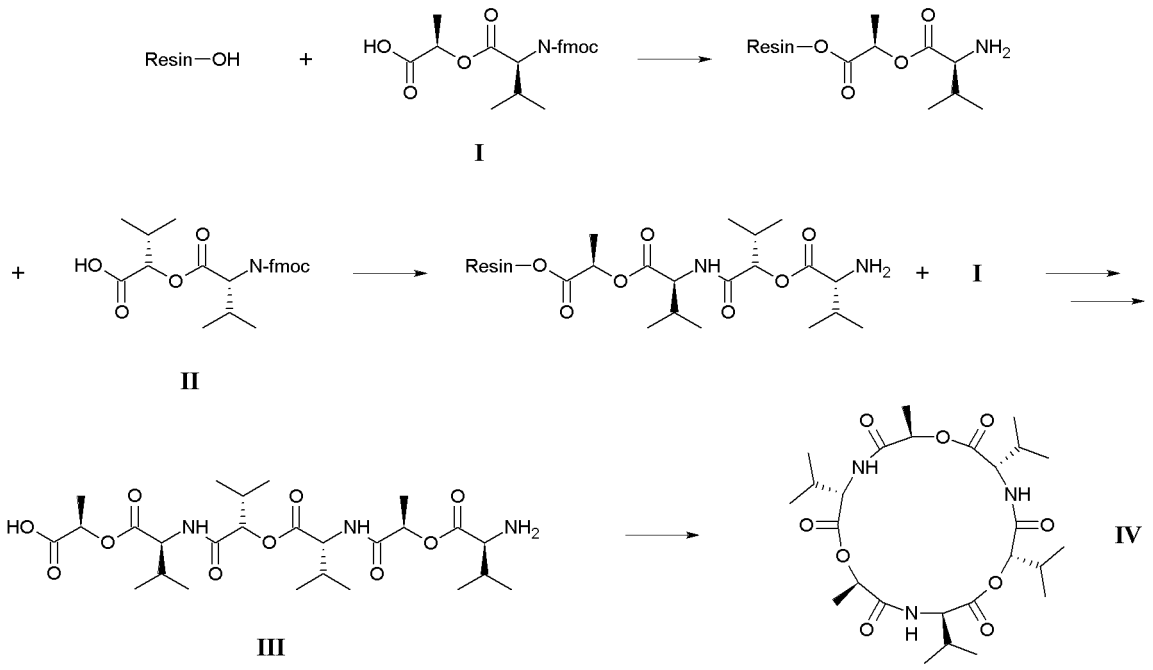
Scheme 1



3 sensors of each formulation for a total of 12 tested sensors. Each cell was tested individually on the Rapidpoint system that maintains a 37 °C temperature for the cell. The sensors were tested using solutions containing NH_4Cl (0.5−100 mM), 100 mM Tris buffer (pH 7.2), and 0.05 g/L Brij 700. Selectivity testing was based on the separate solution method (SSM),[24,25] where $i = j =$ 0.1 M.

RESULTS AND DISCUSSION

Ionophore Modeling and Synthesis. As we have noted, **IV** possesses some structural similarities to valinomycin in that it provides an appropriately sized binding pocket for complexation of ammonium or potassium cations (ionic radii: 1.43 and 1.33 Å, respectively).[11] However, instead of providing an octahedral complexation geometry like valinomycin, **IV** is only able to stabilize ions with tetrahedral binding requirements such as ammonium ion. It is on this basis that we predicted enhanced ammonium ion/potassium ion selectivity for **IV** over that of valinomycin. This is supported by Figure 1, which shows the results of modeling the binding of ammonium and potassium ions with **IV**. These minimized structures (obtained as described above) show the ammonium cation centrally located within the pocket and able to hydrogen bond with at least five of the carbonyl groups in **IV**. In contrast, the potassium cation adopts a position that is shifted to one side, and while not shown in the figure, it lies well above the plane of the disklike structure of **IV** and therefore provides an unfavorable binding site for potassium.

Modeling of **IV** also indicates that it may offer enhanced ammonium/potassium selectivity over nonactin, the ammonium ionophore commonly used in ISE applications. Minimized structures of nonactin with ammonium and potassium ions are shown in Figure 2. The crown ether backbone of nonactin is quite flexible

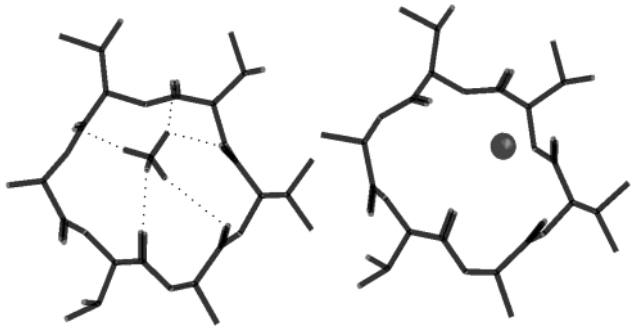


Figure 1. Minimized structures of **IV** complexed with NH_4^+ (left) and with K^+ (right).

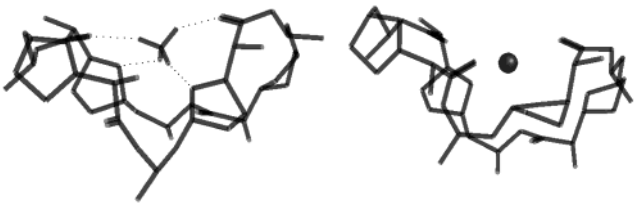


Figure 2. Minimized structures of nonactin complexed with NH_4^+ (left) and with K^+ (right).

and allows for the formation of wrapping-type complexes with both ions. In such complexes, the ions are enveloped by the nonactin structure and have multiple binding opportunities with the ethereal or carbonyl oxygen atoms present. It is important to note that formation of this envelope is essential for binding potassium ions because it is only in this conformation that an octahedral binding geometry is provided. On the other hand, the cyclodepsipeptide **IV** possesses a more rigid backbone structure that cannot easily form a wrapping-type structure. As a result, an octahedral binding site is not provided and potassium ion binding will not be favorable. However, since a tetrahedral complexation geometry is available, ammonium ion binding can occur.

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To estimate the efficiency of ammonium binding in **IV** compared with that in nonactin, docking energies were obtained for the ion/ionophore complexes in each case. In the case of **IV**, the difference in docking energies between ammonium ion and potassium ion was calculated to be 6 kcal/mol more negative than that calculated for nonactin. While these calculations give relative values only, they indicate qualitatively that **IV** should be at least comparable to nonactin in terms of its NH_4^+/K^+ selectivity.

Scheme 1 shows the strategy used for the synthesis of **IV**. The same solution- and solid-phase techniques reported previously for the synthesis of valinomycin[19] have been used here with the exception that an Fmoc protection strategy was employed. This strategy allows the synthesis to be carried out on a commercially available solid-phase support (Wang resin). Also, cleavage can be carried out under mild conditions. Thus, block components **I** and **II** were synthesized in solution; **I** was loaded on a Wang resin coupled with **II** and then again with **I** to yield **III**, which was subsequently cleaved from the resin and cyclized to form **IV**. Although **IV** was synthesized from the same component groups found in valinomycin (L-lactic acid, D-hydroxisovaleric acid, L- and D-valine), it is clear that a variety of hydroxylated amino acid derivatives and natural amino acids can be used in order to produce an ionophore with the appropriate binding site size and symmetry. (We note that the stereochemistry of the individual groups in valinomycin has been preserved in the design of **IV**.) This approach, then, represents a flexible strategy that will allow future systematic investigations of the effects of structure on the efficiency and selectivity of ammonium ion complexation.

Sensor Fabrication and Testing. With the advent of microfabrication techniques, planar-type electrodes have become an attractive alternative to traditional Phillips body ISEs due to the ability to construct and test many sensors at once. In addition, planar-type electrodes make use of polymeric solid internal contacts, which allow for the construction of an all-solid-state ISE. The all-solid-state format has advantages over traditional Phillips body ISEs such as ease of construction, cost-effectiveness, and ability for miniaturization. Systems such as these have been shown to be quite stable and have been shown to give potentiometric selectivities that are comparable to traditional ISEs.[26] In particular, ammonium sensors have been constructed using the all-solid contact concept and have been shown to possess selectivities that are typical of nonactin-based Phillips body ISEs.[27]

These advances have led to a substantial increase in the use of the planar format and have prompted manufacturers to offer clinical diagnostic instrumentation that utilizes planar ISEs.[28,29] Following this trend, **IV** was incorporated into a planar ISE structure employing a polymeric solid contact material[21−23] and tested in a commercially available point-of-care clinical diagnostic system. Four membrane formulations were tested in order to determine which environment would yield the best potentiometric properties of sensors constructed with **IV**. Each sensor membrane consisted of plasticized PVC. Formulations differed as to the type of plasticizer used and whether an ionic additive was present. NPOE and DOP were used as plasticizers since they have been used in other ammonium-sensitive electrodes and yielded good results.[10,13] We also investigated the effect of a lipophilic ionic additive, i.e., KtpClPB, in combination with the two plasticizers.

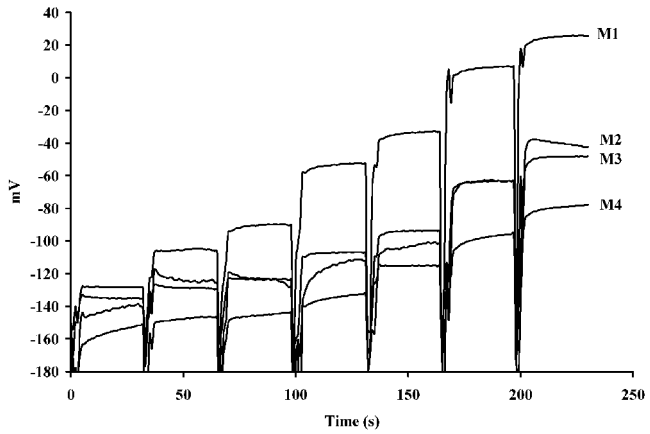

Figure 3. Potentiometric responses of planar ISEs to NH_4^+ (10^{-4}−10^{-1} M) for membranes 1−4 based on **IV**.

Table 1. Selectivity of **IV** versus Other Cations

	selectivity coefficients, log $K^{POT}_{NH_4^+,j}$					
membrane[a]	Li^+	Na^+	K^+	Ca^{2+}	Mg^{2+}	slope[b]
M1	−7.3	−2.1	−0.6	−4.4	−7.1	60.1
M2	−1.9	−1.5	−1.0	−1.3	−1.5	55.8
M3	−5.0	−1.9	−0.6	−3.9	−7.3	45.8
M4	−3.2	−1.5	−0.4	−3.4	−3.9	39.4
nonactin[d]	−4.4[4]	−2.4[1]	−0.9[1]	−2.5[4]	−4.2[4]	59.3[c]

[a] M1, 69/30/1 wt % of NPOE/PVC/**IV**; M2, same as M1 with 50 mol % of KtpClPB to **IV**; M3, 69/30/1 wt % of DOP/PVC/**IV**; M4, same as M3 with 50 mol % KtpClPB to **IV**. [b] Determined between10^{-3} and 10^{-1} M cation, at 37 °C. [c] At 25 °C. [d] Data for nonactin taken from references indicated.

Figure 3 shows the potentiometric responses of the four membrane formulations to increasing concentrations of aqueous NH_4Cl. Two effects can be observed in the data, one that we attribute to the plasticizer and a second due to the ionic additive. It is clear that membranes containing the plasticizer NPOE, both in the presence and absence of KtpClPB, consistently produced sensors with the highest slopes, i.e., the closest to the ideal Nernstian condition (see Table 1). We attribute this effect to the higher polarity that NPOE imparts to the membrane compared to DOP. It is known that depsipeptide structures such as valinomycin (and likely **IV**) experience intramolecular hydrogen-bonding (H-bonding) interactions. In the case of **IV**, these interactions would be expected to interfere with the complexation of ammonium ions (since this complexation also requires H-bonding). In a polar environment such as that provided by NPOE, the intramolecular H-bonding will probably be decreased, thus allowing for more efficient ammonium ion complexation.[30,31] Such would not be the case in lower polarity environments such as those produced by the presence of DOP. In fact, our NMR dilution

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studies support this suggestion. In this study, NMR spectra of **IV** were obtained over a concentration range of 2−20 mM in nonpolar ($CDCl_3$) and polar (MeCN-d_3) solvents. This concentration range brackets the concentration of **IV** in the membranes tested. In the nonpolar environment, spectra of **IV** at each concentration exhibited broad and structureless NH resonances symptomatic of intramolecular H-bonding, while in the polar medium, NH resonances were sharp, suggesting the disruption of H-bonding.

In addition to this apparent polarity effect, it was observed that a 50 mol % loading of the ionic additive, KtpClPB (in combination with either the NPOE or DOP), results in significant deviations from Nernstian behavior and substantially reduces the selectivity, particularly over the divalent cations (see selectivity data in Table 1). Although it has been shown previously that modest numbers of anionic sites within liquid polymeric membranes can improve the potentiometric properties of ISEs, a low molar ratio of ionophore to ionic sites can substantially degrade selectivity and decrease the slope.[24,32,33] This behavior is confirmed in the present study. It is likely that the deleterious effect on selectivity of the ionic additive, which is present in the membrane in a 1:2 ratio relative to **IV**, is due to nonspecific ion exchange, likely through the formation of ion pairs.[34,35]

Table 1 shows the results of selectivity studies that were carried out using the separate solution method[24,25] on four membrane preparations. It is clear from these data that the optimum potentiometric characteristics are obtained using the NPOE plasticizer in the absence of added ionic sites. Membranes produced with this formulation gave near-Nernstian responses (slope, 60.1 mV/decade). Taking membrane M1 as the best example, this formulation exhibited excellent selectivity for ammonium ion over the divalent cations calcium and magnesium as well as lithium and good selectivity over sodium and potassium ($\log K^{POT}_{NH_4^+,j} = -2.1$ and -0.6, respectively) that is comparable to nonactin ($\log K^{POT}_{NH_4^+,j}$ −2.4 and −0.9 for sodium and potassium, respectively).[1,4] This selectivity dropped considerably for most ions with the addition of ionic additive and is reflected in the non-Nernstian slopes as previously noted. Membranes that were formulated using DOP as the plasticizer exhibited substantial sub-Nernstian behavior, making comparisons of selectivity less meaningful. As indicated above, this effect is likely induced by the low polarity of the polymeric solvent inducing intramolecular hydrogen bonding and thus reducing the ability of the ionophore to complex the cations. However, again, potassium selectivity was comparable to that of nonactin.

The ionic selectivity pattern for M1, $NH_4^+ > K^+ > Na^+ \gg Ca^{2+}$, $\gg Mg^{2+} \sim Li^+$, indicates a substantial improvement over that of nonactin with respect to the divalent cations as well as lithium ion. The high ammonium/lithium selectivity can be attributed to a size fit effect. As noted, the pocket of **IV** is designed to accommodate the larger cations and this, coupled to the fact that **IV** cannot form wrapping-type complexes, precludes the possibility of favorable binding to lithium ion whose ionic radius (0.68 Å) is much smaller than that of ammonium ion.

High selectivity of **IV** for ammonium ion over the divalent cations is likely due to two effects, a size fit effect for both calcium and magnesium ions and, in the case of calcium ion, a low coordination geometry. The size fit effect is straightforward since calcium and magnesium ions have radii of 0.99 and 0.82 Å, respectively, and thus are too small to be effectively complexed. The second effect specific to calcium ion can be attributed to the fact that this ion has been shown to favor ligands with six coordinating groups such as ETH 1001.[10] Compound **IV** cannot present all six carbonyl groups at one face due to dipole−dipole repulsion and therefore would be not be able to stabilize the calcium ion.

The data presented show that new ammonium ionophores based upon cyclodepsipeptide motifs are an attractive alternative to others presented in the literature. While the potentiometric data are comparable to nonactin, modifications to the ionophore backbone are expected to enhance its selectivity. In particular, it has been shown that the addition of bulky substituents to other ionophores has improved selectivities through steric effects.[14] This approach also has the added advantage of increasing the lipophilicity of the compounds, thereby making them more compatible with nonpolar membrane environments. Due to the facile nature of depsipeptide synthesis, the introduction of bulky groups such as phenyl or long alkyl chains will allow for additional tuning of the ionophore's potentiometric properties.

CONCLUSIONS

We have reported here the modular synthesis of a new ammonium-selective ionophore based upon a cyclic depsipeptide motif. This approach yielded an ionophore that, when incorporated into an ISE format, provides selectivity for ammonium ion over potassium and sodium that is comparable to nonactin. We believe that the flexible modular approach used will enable us to tune the structure of similar molecules to achieve higher selectivity and sensitivity characteristics.

SUPPORTING INFORMATION AVAILABLE

Detailed description of the synthesis and purification of the cyclodepsipeptide **IV**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review May 20, 2002. Accepted October 23, 2002.

AC0257851