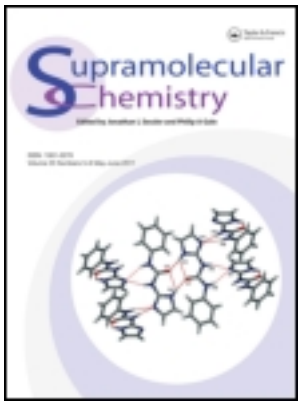

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[a] Department of Chemistry, University of West Georgia, Carrollton, GA, 30118, USA
[b] Department of Biology, University of West Georgia, Carrollton, GA, 30118, USA

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Direct binding of halide ions by valinomycin

J. Brett Kimbrell[a], Joshua R. Hite[a], Kristin N. Skala[a], Christopher M. Crittenden[a], Courtney N. Richardson[a], S. Swamy Mruthinti[b], Megumi Fujita[a]* and Farooq A. Khan[a]*

[a]*Department of Chemistry, University of West Georgia, Carrollton, GA 30118, USA;* [b]*Department of Biology, University of West Georgia, Carrollton, GA 30118, USA*

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The antibiotic valinomycin is a potassium-selective ionophore, which increases the transport of potassium ions across cell membranes and thereby causes damage to bacteria cells. Valinomycin has been extensively studied as an ionophore for cations. We report for the first time the direct binding of anions to valinomycin using electrospray ionisation mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The binding selectivity for halide ions is found to be in the order $Cl^- > Br^- \sim F^- \gg I^-$ based on electrospray ionisation mass spectrometry experiments in methanol. ^{1}H NMR studies in acetone-d_6 and CD_3CN reveal the binding selectivity of $Cl^- > Br^- \gg F^- \sim I^-$. NMR studies and density functional theory (DFT) calculations support a bracelet-like structure for the binding of a chloride ion to valinomycin. Association constants of $531 \pm 45\,M^{-1}$ and $57 \pm 2\,M^{-1}$ were obtained via NMR titrations in acetone-d_6 for chloride and bromide ions, respectively.

Keywords: valinomycin; anion; binding

Introduction

The transport of ions across biological membranes is a vital process in living systems. Of considerable interest are substances that mediate such ion transport. Over the past four decades, the natural ion-carrier valinomycin has drawn immense interest (*1*, *2*). It is an antibiotic produced by *Streptomyces fulvissimus* and is a dodecadepsipeptide composed of three identical fragments that form a 36-member macrocycle (Figure 1). Valinomycin binds to alkali (*3–15*), alkaline earth (*7*, *12*, *16*), transition metal (*12*, *17*) and ammonium ions (*8*) in solution. It is highly selective for potassium ions over sodium ions in solution, and its function as an antibiotic is based on selective transport of potassium over sodium ions across biological membranes (*1*, *2*). The interaction between valinomycin and metal ions (*3–17*), in particular alkali metal ions (*3–15*), has been studied extensively by a variety of experimental techniques, including NMR (*3–6*, *11*, *15*, *16*), infrared (IR) (*6*, *8*, *11*, *17*), circular dichroism (CD) (*15*, *17*) and Raman (*7*, *13*) spectroscopy as well as mass spectrometry (*9*, *10*, *12*). In recent years, computational studies (*18*) pertaining to the binding of valinomycin to cations have also been reported.

Valinomycin has been shown to transport anions across cell membranes. de Freitas and co-workers (*11*) have shown that both Cl^- and ClO_4^- ions form ion pairs with alkali metal complexes of valinomycin based on the measurement of ^{35}Cl line widths in NMR spectroscopy. They have also demonstrated the efflux of Cl^- ions in large unilaminar vesicles in the presence of valinomycin via ion pairing. In another study, Shirai et al. (*14*) used cyclic voltammetry to demonstrate valinomycin-mediated transport of not only alkali metal ions but also anions across lipid bilayer membranes.

The high structural flexibility of valinomycin and the presence of six electron-deficient NH groups pose the intriguing possibility that valinomycin can *directly* bind to anions. To the best of our knowledge, this has not been demonstrated before. In this report, we have explicitly examined the direct binding of halide ions to valinomycin using a combination of electrospray ionisation mass spectrometry (ESI-MS), ^{1}H NMR spectroscopy and computational studies.

Results and discussion

ESI-MS

To investigate the non-covalent interactions between valinomycin and halide ions, we first carried out experiments using ESI-MS. ESI-MS is a powerful technique that has been employed extensively to study the non-covalent interactions between cations and ionophores, in particular, crown ethers (*19*). The binding of valinomycin with alkali metal ions has also been reported (*9*). In contrast, only a few studies on the non-covalent interactions between ionophores and anions have been carried out using ESI-MS (*20*, *21*). In several studies, the binding of anions (including halide ions) with calix[4]pyrroles was examined, and the 1:1 stoichiometry

*Corresponding authors. Email: fkhan@westga.edu; mfujita@westga.edu

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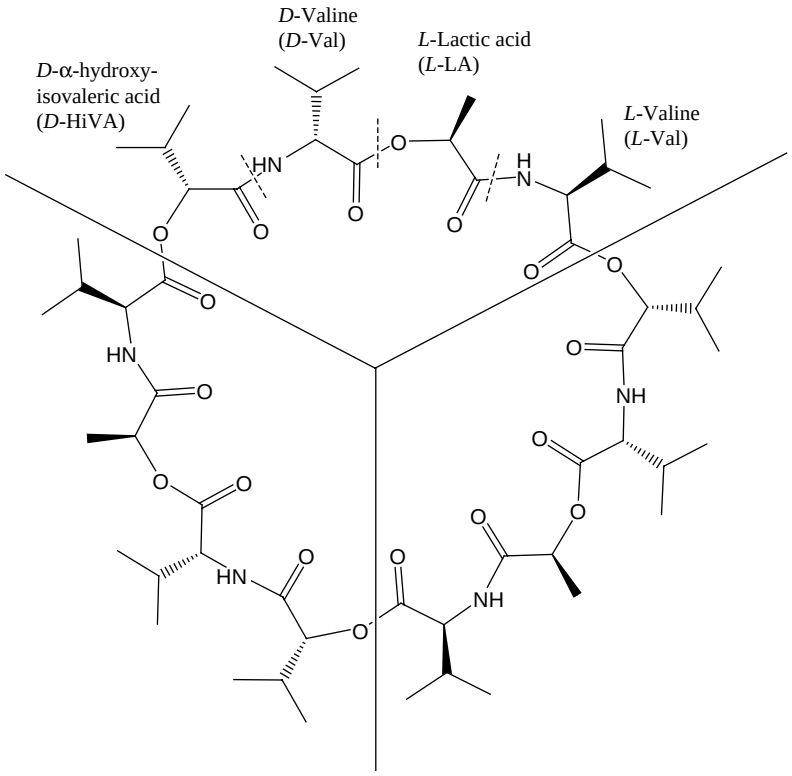

Figure 1. The antibiotic valinomycin.

of the neutral host and negatively charged guests in the mass spectra was conclusively demonstrated (*21*). These ESI-MS studies on calix[4]pyrroles, whose anion-binding properties are well established via other experimental techniques such as UV–vis and NMR spectroscopy (*22*), demonstrate the usefulness of mass spectrometry as a viable technique for probing anion binding in solution.

The negative ion electrospray ionisation (ESI) mass spectrum for a solution containing valinomycin (0.010 mM) and tetrabutylammonium fluoride (TBAF), tetrabutylammonium chloride (TBACl), tetrabutylammonium bromide (TBABr) and tetrabutylammonium iodide (TBAI; 0.010 mM each) in methanol is shown in Figure 2. Each of the halide ions forms a 1:1 adduct with valinomycin. The signal for deprotonated valinomycin is also observed. The intensity of the adduct of the chloride ion is the highest followed by those of bromide, fluoride and iodide ions. In additional competition experiments, a 2-, 5- and 10-fold excess of halide ions was used and the data are summarised in Table 1. In all instances, the intensity of the adduct of the chloride ion is the highest, and that of the iodide ion is the least, but the relative intensities of the adducts of fluoride and bromide and deprotonated valinomycin do vary. Similar experiments were also attempted in acetonitrile, but the overall signal intensities in acetonitrile are substantially smaller in general than in methanol, and such experiments in acetonitrile were not pursued further.

An interesting question in interpreting intensities in ESI mass spectra is the extent to which they reflect solution binding properties. In a careful study carried out by Brodbelt et al. for crown ethers and alkali metal ions (*23*), it was found that solvation is an important

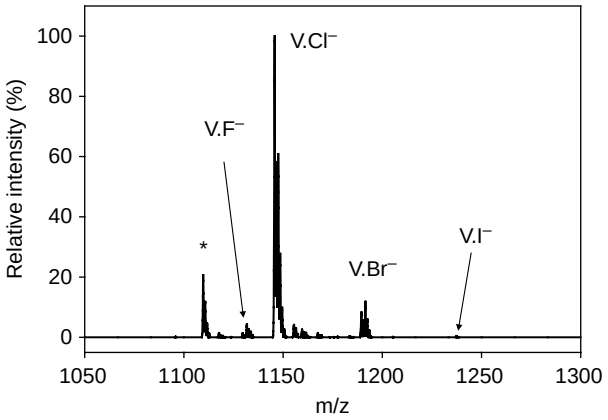

Figure 2. ESI mass spectrum of valinomycin (0.010 mM) in the presence of TBAF (0.010 mM), TBACl (0.010 mM), TBABr (0.010 mM) and TBAI (0.010 mM) in methanol. The peaks correspond to the 1:1 adducts formed for each of these halide ions with valinomycin, abbreviated as V. Deprotonated valinomycin is indicated with an asterisk.

Table 1. Normalised signal intensities of ESI mass spectra in methanol.[a]

Concentrations (mM)[b]		Signal intensities				
Valinomycin	TBAX	$V-H^-$	$V{\cdot}F^-$	$V{\cdot}Cl^-$	$V{\cdot}Br^-$	$V{\cdot}I^-$
0.010	0.010	15.5	1.0	100.0	12.5	0.3
0.010	0.020	89.4	13.4	100.0	15.7	0.0
0.010	0.050	46.5	21.5	100.0	7.7	0.0
0.010	0.10	43.3	13.5	100.0	4.6	0.0

Note: [a]The intensities reflect isotopic abundances of chloride and bromide ions and are normalised to 100 for the most abundant chloride ions.
[b]Each of the compounds, TBAF, TBACl, TBABr and TBAI, was present in equimolar quantity.

consideration in determining binding selectivities using ESI-MS quantitatively. It was shown that for a single host forming adducts with multiple guest ions, the intensities in ESI-MS are essentially reflective of quantitative binding constants in solution; presumably, the solvation energies are comparable for adducts consisting of a single host and different alkali metal ions. In another study, which focused on the binding of valinomycin to alkali metal ions, there was a good quantitative agreement between intensities in the ESI mass spectra and the logarithms of the known solution binding constants (*9*). Thus, with an underlying assumption of comparable solvation energies for each of the halide ions with valinomycin, the ESI mass spectra in our study strongly suggest that the relative solution anion affinity for valinomycin is the highest for chloride ions and the least for iodide ions.

NMR spectroscopy

To investigate the binding of halide ions quantitatively, we carried out ^{1}H NMR spectroscopy which has been used extensively as a powerful tool for probing the conformation of molecules as well as to determine association constants for anions. NMR spectroscopy has provided a wealth of information on the conformations of free valinomycin and of cation-bound valinomycin. Free valinomycin forms a 'bracelet' conformation (Figure 3(a)) in non-polar solvents (cyclohexane-d_{12} and chloroform-*d*) and in acetonitrile-d_3, in which all six amide NH groups are internally hydrogen bonded to amide carbonyls three residues away (L-Val NH to O═C of D-HiVA, and D-Val NH to O═C of L-LA, Figure 3(b)) (*16*, *24*, *25*). In more polar solvents such as a 3:1 mixture of CCl_4 and DMSO-d_6, the conformation is 'propeller' shaped in which three out of six internal hydrogen bonds are broken (*24*). The solution conformation of the valinomycin·K^+ complex is known to have a bracelet structure, and the K^+ ion is centrally located in the cavity, coordinated by all six ester carbonyls; this structure is also found in the solid state (*1*, *6*, *24*). The complexes of K^+, Rb^+ and Cs^+ are very similar in a variety of solvents. In contrast, the smaller cations Li^+ and Na^+ form weaker complexes with different structures in which there is lesser coordination with the ester carbonyls (*6*, *15*).

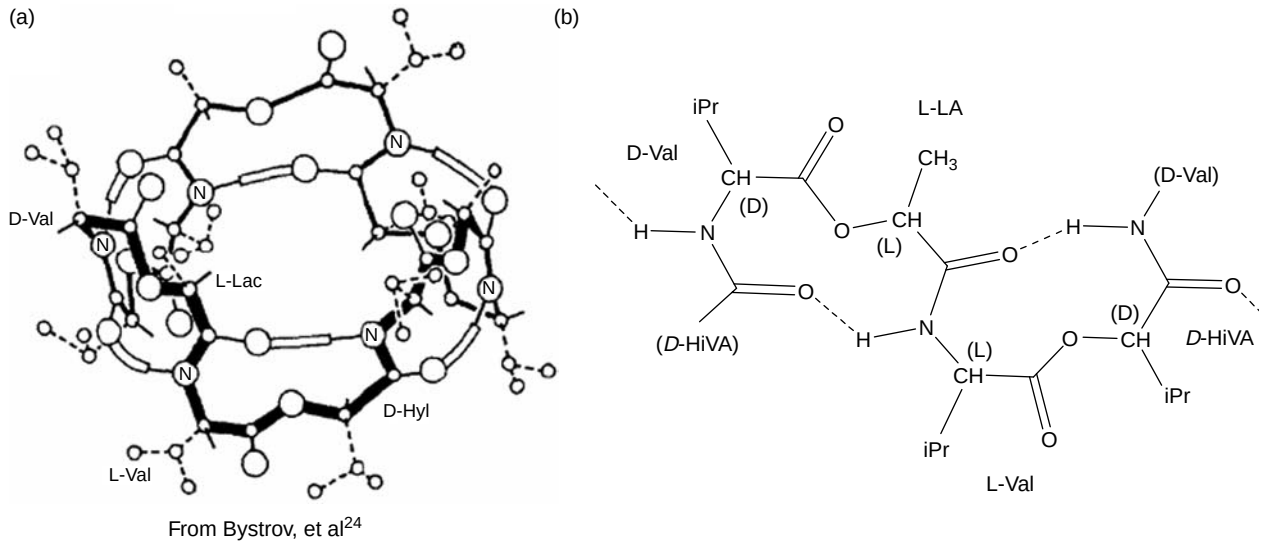

Figure 3. (a) The bracelet conformation of valinomycin [from Bystrov et al. (*24*), with permission from John Wiley and Sons]. (b) The internal hydrogen-bonding network in the bracelet conformation of valinomycin.

Table 2. A comparison of chemical shifts and coupling constants for valinomycin in various solutions.

Solvent (conformation)	Chemical shifts (ppm)						Coupling constants $HNC^{\alpha}H$ (Hz)		References
	D-Val NH	L-Val NH	L-LA $C^{\alpha}H$	D-HiVA $C^{\alpha}H$	D-Val $C^{\alpha}H$	L-Val $C^{\alpha}H$	D-Val	L-Val	
Cyclo-C_6D_{12} (bracelet)	8.03	7.89	5.34	5.04	4.05	3.94	8.5	7.0	24
$CDCl_3$ (bracelet)	7.80	7.67	5.31	5.03	4.03	3.91	8.7	6.8	24
CCl_4:DMSO-d_6, 3:1 (propeller)	7.48	8.41	5.09	4.82	4.26	4.36	10.1	7.5	24
CD_3CN	7.62	7.57	5.25	5.00	4.11	4.14	7.6	7.6	16 and present studies
Acetone-d_6	7.64	7.85	5.40	5.01	4.38	4.17	8.4	6.8	Present studies

In an initial screening for a suitable solvent for titration, ^{1}H NMR spectra were obtained for approximately 5 mM valinomycin first by itself, and then with approximately 5 equivalents of TBACl in the solvents $CDCl_3$, CD_3CN, acetone-d_6, DMSO-d_6 and CD_3OD. Significant downfield shifts of both L-Val NH and D-Val NH signals, along with minor movements of the α-H signals, were observed only in acetone-d_6 and CD_3CN. In DMSO-d_6, valinomycin signals were not affected by the presence of TBACl. In CD_3OD, the NH signals disappeared due to the fast H–D exchange, and is thus not suitable for titration studies. It was at first peculiar to see no movement of the NH signals in $CDCl_3$. However, in this less polar medium, the TBA^+ and Cl^- ions are presumably forming an intact ion pair and the chloride ions are not available for binding with valinomycin. In other words, in the competition of TBA^+ and valinomycin, the former is probably binding Cl^- much more strongly in a non-polar medium.

As stated earlier, valinomycin takes the 'bracelet' conformation in CD_3CN (*16*). In acetone-d_6, we conclude that valinomycin also takes the bracelet conformation based on the similarity of the ^{1}H chemical shifts and the HN—C^{α}H coupling constants to those in CD_3CN and in less polar solvents originally reported by Bystrov et al. (*24*) as shown in Table 2.

A series of titrations of 5.0 mM valinomycin with 250 mM TBAX (X = F, Cl, Br, I) were then carried out in acetone-d_6 and CD_3CN. TBACl caused the highest degree of downfield shifts of both L-Val NH and D-Val NH signals among all halides in both solvents. This strongly indicates that incoming Cl^- is forming hydrogen bonding with these amide NH bonds. Similar changes were observed in the titration with TBABr, but with smaller extent compared to TBACl. Hardly any changes were observed with TBAF and TBAI. This selectivity for Cl^- binding, followed by Br^- binding and much weaker binding with F^- and I^-, is in general agreement with the observations in the ESI-MS studies in methanol.

The association constants K_a for the valinomycin·Cl^- complex were calculated using the program WinEQNMR2 (*26*) based on the titration curves of the L-Val NH signal. Although both L-Val and D-Val NH peaks were affected by incoming halide ions and both titration curves fit well to a 1:1 valinomycin·X^- complex formation, the L-Val NH signal exhibited a larger peak shift (see discussion later). The K_a values in acetone-d_6 and CD_3CN are summarised in Table 3. The K_a values were significantly higher in acetone-d_6 than in CD_3CN; the K_a for Cl^- binding was approximately 75 times higher in the former ($531 \pm 45\,M^{-1}$) than in the latter ($7.1 \pm 0.6\,M^{-1}$). Nevertheless, in both solvents, Cl^- binding was the most prominent, followed by Br^- binding, and the peak movements were too small for F^- and I^- to calculate K_a.

Table 3. K_a values (M^{-1}) of titration of valinomycin (5 mM) with TBAX (250 mM) in two solvents based on the shift of the L-Val NH peak in ^{1}H NMR.

	X^- in TBAX			
Solvents	F^-	Cl^-	Br^-	I^-
Acetone-d_6	–	531 ± 45	57 ± 2	–
CD_3CN	–	7.1 ± 0.6	–	–

Note: '–' indicates that the K_a value could not be calculated because the NH peak shifts were not significant enough.

In the titration experiments described here, some caution must be exercised because of the possible cation dependence, as noted by a reviewer. In their study, Gokel and co-workers have explicitly examined the cation dependence of chloride ion complexation for a number of synthetic peptides (*27*). Such interactions affect the magnitude of the calculated binding constants to some extent, and have not been investigated explicitly in this work, predominantly undertaken to demonstrate for the first time that valinomycin binds anions. We will explore this avenue in future work.

Figure 4 shows the ^{1}H NMR titration curves of 5.0 mM valinomycin with 250 mM TBACl in acetone-d_6. The movement of the two NH and the four α-H signals is plotted against the equivalent of Cl^-. The L-Val NH goes through larger downfield shift than the D-Val NH upon the addition of Cl^-, indicating that the L-Val NHs have

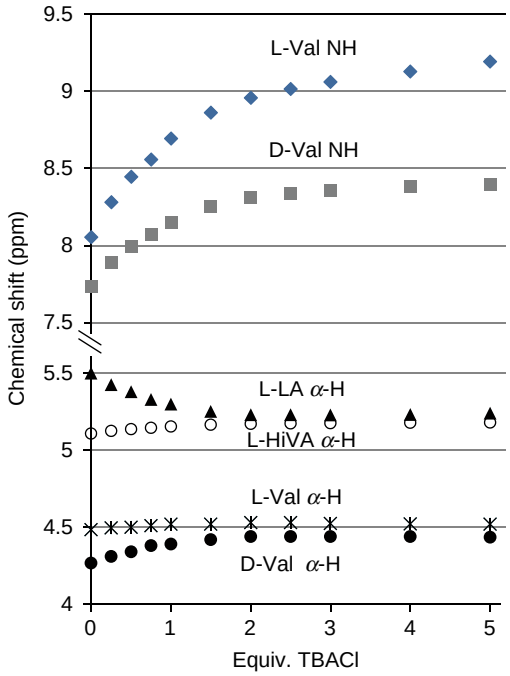

Figure 4. ^{1}H NMR titration curve of the selected protons of valinomycin (5.0 mM) upon titration with TBACl (250 mM) in acetone-d_6.

stronger hydrogen-bonding interaction with Cl^-. The changes of the four α-H signals are minimal upon the addition of Cl^-; the largest change was the Δ0.20 ppm downfield shift of the L-LA α-H peak upon 24 equivalent of Cl^- in acetone-d_6. The minimal changes in the α-H signals indicate that there is no significant structural change in the 'bracelet' conformation of valinomycin upon binding of Cl^-. These observations strongly suggest that both Cl^- and Br^- are incorporated in the central cavity of the 'bracelet' valinomycin with symmetrical interaction with all three four-residue subunits.

Computational studies

As noted in the previous section, ^{1}H NMR observations strongly suggest that both Cl^- and Br^- are incorporated in the central cavity of the 'bracelet' conformation of valinomycin without significantly affecting the structure. We examined this scenario using Gaussian 09. For simplicity, we built a valinomycin molecule with all isopropyl groups replaced with methyl groups.

The initially built valinomycin was optimised using the semi-empirical method (PM6) with the six internal amide NH···O=C hydrogen-bonding network to form a bracelet conformation. The adjustment of the structure was repeated until the molecule achieved C3 symmetry. Since there are still some degrees of freedom in the orientations of the peripheral groups such as the ester groups and the side alkyl groups, four possible orientations of such groups on the bracelet framework were examined (Figure 5). If the internal hydrogen bonding is viewed from the side (left) C=O···H—N (right), three ester carbonyl groups (from D-Val) are on the top edge and the other three (from L-Val) are on the bottom edge. In Figure 5, the top row shows the scheme of four possible orientations based on whether these ester carbonyls are pointing inward or outward: namely (**5a**) top-in, bottom-in; (**5b**) top-in, bottom-out; (**5c**) top-out, bottom-in and (**5d**) top-out, bottom-out. After the optimisation of the corresponding input structures using the semi-empirical method (PM6), the structure **5d** converged to the structure **5b**. The middle and bottom rows of Figure 5 show the optimised structures from top and side angles.

Next, a Cl^- ion was placed in the middle of the bracelet ring of **5a–c**, and each structure was optimised by the semi-empirical (PM6) method. The optimised structures all held the Cl^- ion in the middle of the bracelet, equidistant from all the three subunits of valinomycin (deviation range is within 0.7%). The optimised structures and the average interatomic distances between L-Val N*H* to Cl^- and D-Val N*H* to Cl^- are summarised in Figure 6. In the ^{1}H NMR studies, it was clearly demonstrated that the L-Val NH had the stronger interaction with the incoming Cl^-. Structure **6b** had shorter L-Val NH···Cl^- distance than D-Val NH···Cl^-,

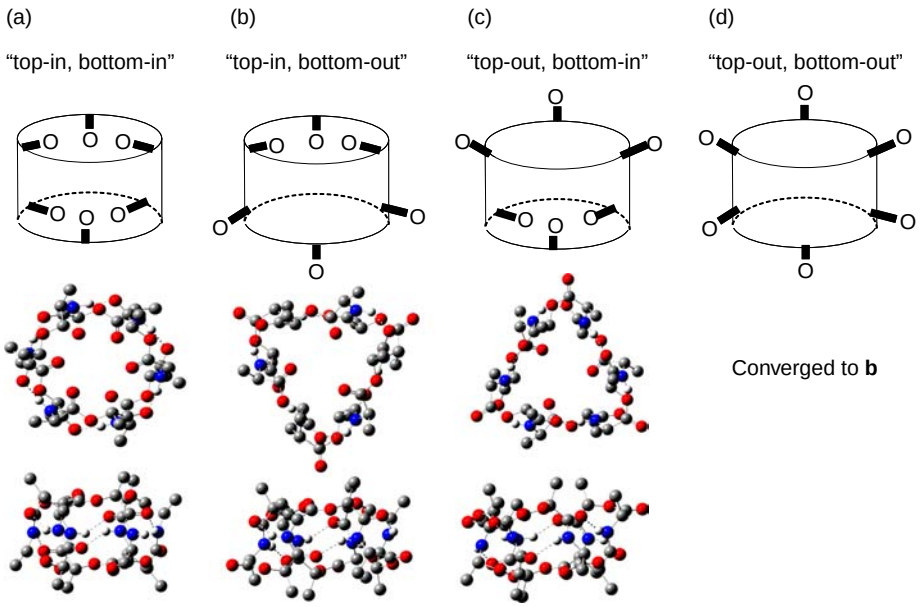

Figure 5. Four conformations of bracelet valinomycin. Top row: conformation scheme of bracelet valinomycin based on the orientation of the top and bottom ester carbonyl groups. Middle and bottom row: optimised structures of each conformation by semi-empirical method (PM6). Note: H atoms are omitted for clarity except for the N-bonded ones.

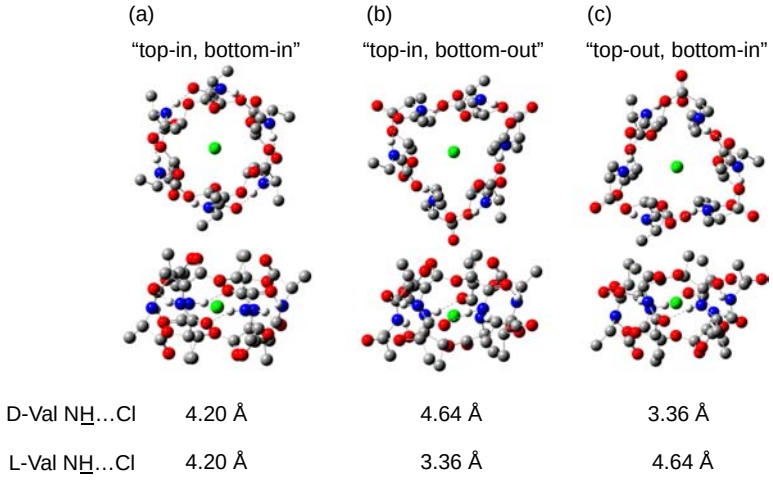

Figure 6. Valinomycin·Cl^- adduct structures from valinomycin with conformations **5a**–**c** optimised by the semi-empirical method (PM6).

consistent with the ^{1}H NMR observation. On the other hand, structure **6c** is eliminated from the possible conformation because this had shorter D-Val NH···Cl^- distance than L-Val NH···Cl^- distance. Structure **6a** had equal NH···Cl^- distances from both D-Val and L-Val moieties, and this does not correspond to the ^{1}H NMR observation either. Conformation **5a** is most suited for cation binding, as all the ester carbonyls are pointing inwards to coordinate to a cation placed in the middle of the bracelet.

We then examined whether the **6b** state, in which a Cl^- is situated in the middle of the bracelet ring of valinomycin with the top-in, bottom-out conformation, is indeed the most energetically stable state. The input structure was created by placing Cl^- *outside* of valinomycin on the bottom side of **6b** (Figure 7(i)). When this was optimised by the DFT method (B3LYP, 3-21G basis set), Cl^- was incorporated into the middle of the bracelet to take practically the same structure as **6b** (Figure 7(ii)). This result shows that the lowest energy

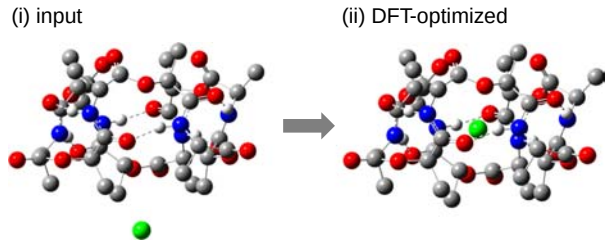

Figure 7. Incorporation of Cl^- into valinomycin with conformation **5b**. (i) Input structure. (ii) DFT-optimised structure.

place to incorporate Cl^- is in the middle of the bracelet, and this incorporation occurs without significantly perturbing the internal hydrogen-bonding network.

When analogous 'halide incorporation experiments' were carried out with other halides in the place of Cl^-, Br^- and I^- ions were incorporated into the middle of the valinomycin bracelet in a very similar manner as the Cl^- ion. However, the calculation with F^- failed to converge. This failure of convergence with F^- while successful completion with the bigger halides indicates that this size effect is similar to the one observed for the binding of cations, as noted in the first paragraph of the NMR spectroscopy section. Namely, the complexes of the larger cations K^+, Rb^+ and Cs^+ are very similar in a variety of solvents, with all six ester carbonyls coordinated; but the smaller cations Li^+ and Na^+ form weaker complexes with different structures in that there is lesser coordination with the ester carbonyls (*6*, *15*). Although F^- is generally the best hydrogen-bonding acceptor among the halides, the size of the ion is probably too small to interact with the NH groups in the bracelet valinomycin without significantly skewing the bracelet structure or breaking the internal hydrogen-bonding network, which may require too much energy to occur.

At first glance, it appears strange to see that a Cl^- ion is readily incorporated in the middle of the bracelet ring of valinomycin without breaking the internal hydrogen-bonding network in the computations described here. The distinct downfield shift of the ^{1}H NMR signals of both L-Val NH and D-Val NH was a clear indication of hydrogen-bonding interaction with Cl^-; yet, the DFT-optimised valinomycin·Cl^- complex structure does not seem to show a noticeable change in the orientation of the NH bonds. However, the L-Val NH$\cdots Cl^-$ distance of 3.36 Å in the structure **6b** is within the known range of N—H$\cdots Cl^-$ hydrogen-bonding distance (though on the longer side) (*28*). The bracelet-shaped valinomycin has *three* of L-Val NH groups, and altogether the attractive force for Cl^- can be strong enough to accommodate the ion into the cavity.

In structure **6b**, the D-Val NH$\cdots Cl^-$ distance (4.46 Å) is clearly out of the hydrogen-bonding range. However, the D-Val NH ^{1}H NMR signal had a clear downfield shift upon the addition of Cl^-. This can be explained as a result of the

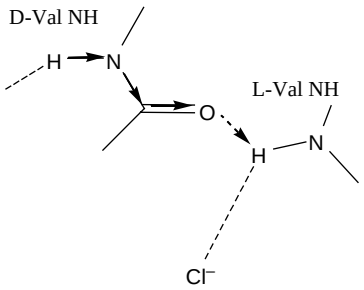

Figure 8. The inductive effect of the L-Val NH$\cdots Cl^-$ hydrogen bonding to the D-Val NH through the internal hydrogen bonding.

secondary influence from the L-Val NH hydrogen bonding to Cl^-. The additional hydrogen bonding of the L-Val NH to Cl^- makes the H atom more electron deficient, and this inductively draws more electron density from the hydrogen-bonded carbonyl group, which is part of the amide group that contains a D-Val NH (Figure 8). This causes the electron deficiency of D-Val NH which is consistent with the downfield shift of its ^{1}H NMR signal.

The above described computational studies narrowed down to the 'top-in, bottom-out' valinomycin bracelet structure as the most likely conformation to incorporate a halide ion in the middle of the bracelet. The relatively unchanged valinomycin bracelet framework and the shorter L-Val NH$\cdots Cl^-$ distance than D-Val NH$\cdots Cl^-$ distance are both consistent with the ^{1}H NMR observations.

Experimental

TBAF trihydrate, TBACl, TBABr and TBAI and valinomycin with a purity of 97% or higher were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. HPLC grade methanol and acetonitrile (Honeywell Burdick & Jackson, marketed by VWR International, Radnor, PA) were used in all ESI-MS experiments. Deuterated NMR solvents were purchased from Acros Organics, Fisher Scientific (Pittsburgh, PA) (acetone-d_6, CD_3CN and $CDCl_3$) or from Sigma-Aldrich (CD_3OD and DMSO-d_6). Standard solutions were freshly prepared prior to use.

ESI-MS in the negative ion mode was employed in all the experiments described here, using a Finnigan LXQ linear ion trap mass spectrometer (Thermo Electron Corporation, West Palm Beach, FL). Solutions were introduced into the mass spectrometer using the integrated syringe pump at a flow rate of 10 μL/min. Spraying was achieved with nitrogen as the nebulising gas with a probe voltage of 4.5 kV at a temperature of 270°C. The experimental conditions were sheath gas flow rate (10); auxiliary and sweep gas flow rates (0); and capillary voltage (9 V) and tube lens (90 V). The mass spectrum displayed is an average of 500 scans.

^{1}H NMR spectra were recorded using a Varian (Palo Alto, CA) 400 MHz NMR spectrometer at ambient

temperature. Gaussian calculations were carried out using Gaussian 09 on a computer with a quad-core processor.

Conclusions

In conclusion, we have demonstrated for the first time via ESI-MS that anions bind directly to valinomycin in methanol with the binding selectivity of $Cl^- > Br^- \sim F^- \gg I^-$. ^1H NMR studies in acetone-d_6 and CD_3CN revealed the binding selectivity of $Cl^- > Br^- \gg F^- \sim I^-$ while retaining the bracelet structure. The computational studies support the mode of halide binding to valinomycin as a single halide ion incorporation in the middle of the bracelet structure of valinomycin.

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