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Photomodulation of ionic current through hemithioindigo-modified gramicidin channels

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Incorporation of photo-switchable amino acids into peptides and proteins offers prospects for the control of complex biochemical processes using light. Currently, only a few photo-switchable amino acids are known. We report the design and synthesis of a novel hemithioindigo-based amino acid and its incorporation into the model ion channel gramicidin. Photoisomerization of the hemithioindigo moiety between *E* and *Z* isomeric forms is shown to modulate ionic current through the channel in a predictable way. This new amino acid thus expands the possibilities for photo-control in diverse systems.

Introduction

Reversible photo-control of protein structure and function can be achieved *via* photoisomerization of attached chromophores. Retinal chromophores in rhodopsins, linear tetrapyrrole chromophores in phytochromes and *p*-hydroxycinnamic acid chromophores are examples of naturally occurring photoisomerizable moieties found in photosensory proteins.[1] Use of these chromophores in engineered systems is complicated by the fact that the photochemistry is substantially different when the protein environment is changed or the chromophore is removed from the protein (*e.g.* ref. 2). In engineered systems, azobenzene based chromophores have been most widely used.[3,4] Amino acid derivatives of azobenzene are relatively easy to synthesize and display robust photochemistry.[5,6]

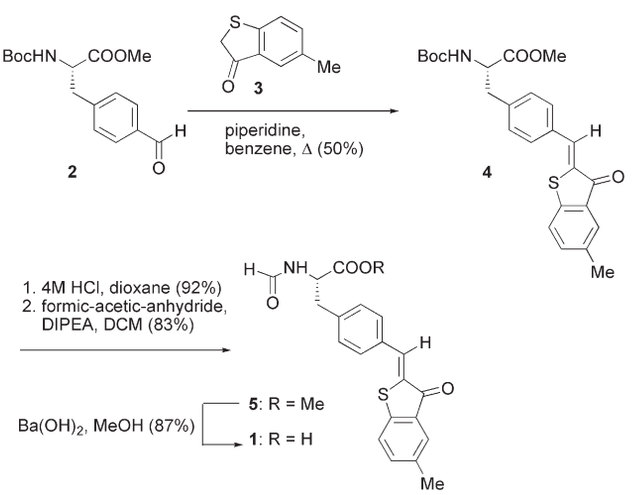
In an attempt to expand the range of photoisomerizable chromophores that can be readily incorporated into proteins we have explored the synthesis of hemithioindigo-based amino acids. Hemithioindigos, although less well studied than azobenzenes, are appealing candidates as components of photoswitchable biomolecules.[7–14] They are photochemically robust over thousands of cycles, isomerization occurs at wavelengths significantly longer than many azobenzene derivatives[7] and the more extended planar chromophore means that the end-to-end distance changes accompanying isomerization are substantial, making transduction of the isomerization event into a biochemical effect more likely. Finally isomerization causes a significant change in the orientation of the dipole moment of the chromophore.

The primary aims of the present study are (i) the synthesis of an amino acid derivative that can be incorporated into proteins *via* standard solid-phase synthesis methods, and (ii) a test of the ability of the derivative to function as a photoswitch in a biological environment. As a test case, we investigate the incorporation of hemithioindigo chromophores into the peptide ion channel gramicidin. Photo-control of ion channels offers the prospect of external control of cellular excitability. In previous work we installed the azobenzene-based amino acid phenylazo-phenylalanine as the N-terminal residue of gramicidin so that two azobenzene groups would be positioned at the center of the bilayer membrane in the dimeric active form of the channel.[6] In this configuration, *trans*-to-*cis* isomerization introduces a dipole at the center of the membrane oriented such that the positive end of the dipole is directed at the cation path. This causes a decrease in cation flux. As noted above, isomerization of the hemithioindigo chromophore is also expected to produce a dipole moment change. We examine the effects of this change on Na^+ and Cs^+ ion flux through the hemithioindigo-modified gramicidin channels.

Results and discussion

Synthesis

The hemithioindigo-based amino acid derivative **1** was prepared from *N*-Boc-4-formyl-phenylalanine methyl ester **2**. Subjecting **2** to an aldol condensation with thioindoxyl **3** using piperidine as catalyst gave the hemithioindigo **4**. After removal of the Boc-protecting group subsequent treatment with formic-acetic anhydride furnished the *N*-formyl-amino acid methyl ester **5**. Hydrolysis of the methyl ester under basic conditions ($Ba(OH)_2$, MeOH) gave the amino acid **1** (Scheme 1). The formylated hemithioindigo amino acid was then coupled to des(formylvalyl)-gramicidin using established methods.[15]



Scheme 1 Preparation of the hemithioindigo-based formylated amino acid **1**.

Photoisomerization and dipole moment changes

For the hemithioindigo-based *N*-formyl amino acid methyl ester **5** photoisomerization was readily observed in deutero-acetonitrile at room temperature (25 °C). Irradiation at 406 nm produced *Z*-to-*E* isomerization leading to a photostationary state that was 72% *E* as determined by ^{1}H NMR spectroscopy. Irradiation at 480 nm produced ~100% *Z* isomer. Thermal *E*-*to*-*Z* isomerization was found to proceed with a half-life of ~5 h. This behaviour is similar to that observed for underivatized hemithioindigo compounds.[7,14,16]

To estimate dipole moments for the *Z* and *E* forms of the hemithioindigo side chain the structure was built using SPARTAN with coordinates based on the X-ray structure determined for an analogue

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Table 1 Conductance properties of hemithioindigo-gramicidin channels

Channel type	Single channel current (200 mV) (1 M NaCl)	Single channel lifetime (1 M NaCl)	Single channel current (200 mV) (1 M CsCl)	Single channel lifetime (1 M CsCl)
Z/*Z* (dark)	1.33 pA	0.42 s	6.0 pA	0.34 s
E/*Z*; *Z*/*E*	1.50 pA	0.41 s	6.5 pA	0.33 s
E/*E* (irrad)	1.75 pA	0.40 s	7.0 pA	0.32 s

of the *Z* compound.[17] Dipole moments were calculated using *ab initio* 6–31G** methods in SPARTAN. Dipole moments of 2.8 D and 1.2 D were calculated for the *Z* and *E* forms respectively with the orientations shown in Fig. 1.

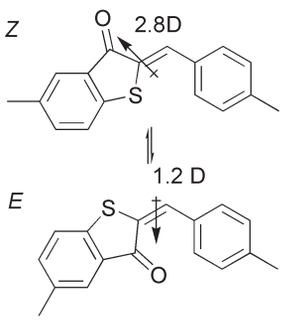

Fig. 1 Dipole moments of *Z* and *E* hemithioindigo side-chains.

Models of gramicidin A were then built with the hemithioindigo amino acid replacing valine at position 1 of the sequence. These models used the NMR derived membrane-bound structure of gramicidin A (Protein Data Bank Code 1MAG) as a starting point. Representative structures of the *Z* and *E* forms of hemithioindigo gramicidin are shown in Fig. 2.

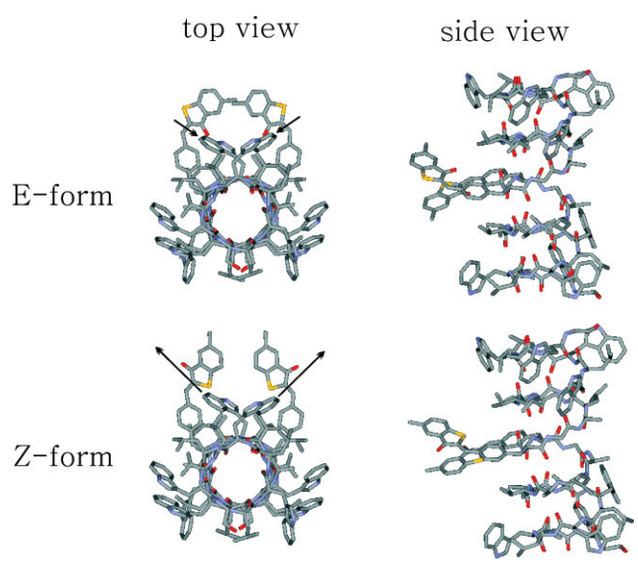

Fig. 2 Models of the dimeric gramicidin A channel with hemithioindigo amino acids incorporated at position 1. The top view shows the open ion-conduction pore through the structure. Dipole moments are indicated with the arrowhead at the negative end of the dipole.

The energy of interaction of these dipole moments with a cation in the pore depends on the torsion angles associated with the single bonds linking the hemithioindigo side chain to the peptide backbone (Fig. 3).

To estimate the energy of interaction, the distances from an ion at the centre of the channel to the carbonyl oxygen atom and to the double bond C atom of the thioindoxyl ring were measured during systematic conformational searches in which rotatable bonds were varied in 10° increments (Fig. 3). These atoms approximate the positions of the negative and positive ends of the hemithioindigo dipole. The distance between the C atom and an ion in the pore varies between 10.5 and 11.8 Å and does not depend on the isomeric

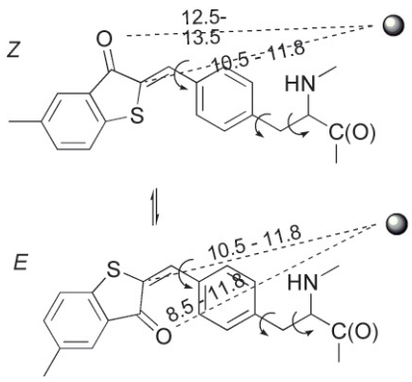

Fig. 3 Hemithioindigo amino acids incorporated into gramicidin A. Rotatable bonds are indicated. Distance ranges between an ion in the pore and the C atom and O atom that define the approximate direction of the dipole moment are indicated.

state. In contrast, the O atom is between 12.5 and 13.5 Å away from the ion in the *Z* isomer but moves to between 8.5 and 11.8 Å in the *E* isomer. Thus, for the *E* form of the hemithioindigo-modified channel, the negative end of the dipole is much closer to the permeant ion than in the *Z* form of the channel. Under conditions where the translocation of the permeant cation (*e.g.* Na^+) through the centre of the channel is the rate limiting step for transport[6] these models would predict that the *E* form of the channel showed higher conductance than the *Z* form.

Properties of ion channels formed by hemithioindigo-gramicidin

The hemithioindigo-gramicidin peptide was purified by HPLC and its ion channel forming properties were characterized using single-molecule planar bilayer methods. Single channel current histograms are shown in Fig. 4. Conductance properties are summarized in Table 1.

Dark-adapted channels showed one predominant conductance state which we assign to channels formed by the association of two *Z*-form hemithioindigo-gramicidin monomers. As is predicted by analysis of the dipole moments, photoisomerization from the dark-adapted *Z* form to the *E* form produces an increase in the single channel current. As was the case with azobenzene-modified gramicidin channels, two new conducting levels appear with relative probability that depends on the degree of photoisomerization. Since there is a thermal back reaction, the distribution of conducting states after irradiation is slowly time dependent (τ½ of several hours). By analogy with the azobenzene-gramicidin case and with other experiments involving mixtures of gramicidin species[18,19] we assign the highest single channel conductance to channels with two *E*-form hemithioindigo amino acids and the intermediate conductance level to those bearing one *E* and one *Z* isomer.

As predicted by the dipole analysis, the direction of the conductance change upon photoisomerization is opposite to that observed previously with gramicidin channels with the azobenzene chromophore at position 1 in the sequence. In that case irradiation produced a decrease in the single channel current and thermal relaxation to the dark-adapted *trans* isomer led to an increase in single channel current.

The effect of dipole switching is more pronounced when Na^+ is the permeant cation rather than Cs^+. This presumably reflects differences in the detailed free energy profiles for these ions; a similar

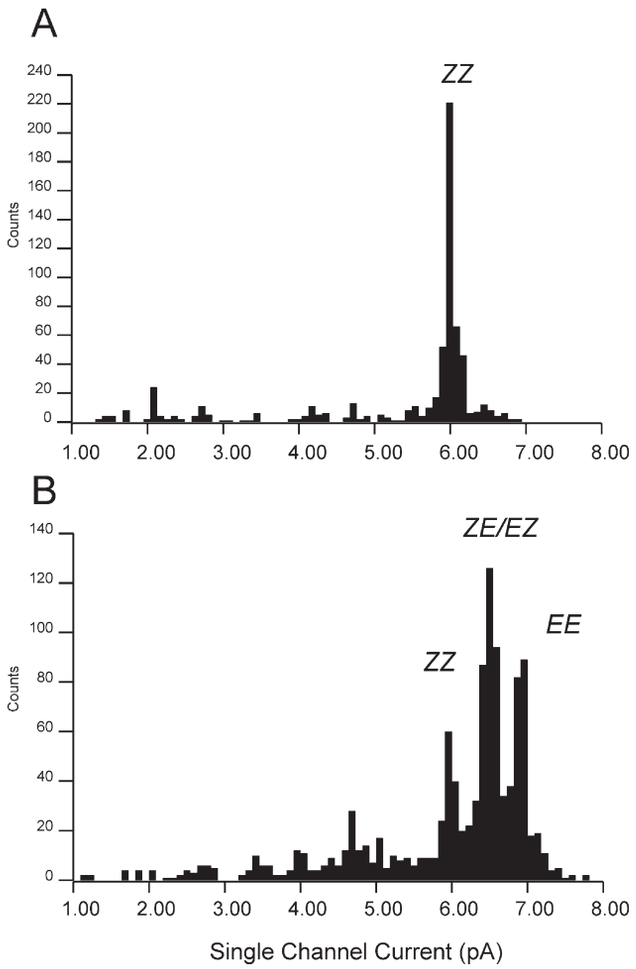

Fig. 4 Histograms showing frequency of occurrence of different conducting states on hemithioindigo-gramicidin channels (1 M CsCl, 200 mV) (A) dark-adapted channels (*Z* form); (B) irradiated at 406 nm.

pattern has been seen with other position 1 mutations that alter the dipole moment of the side chain.[19]

In summary, these results indicate that the hemithioindigo amino acid described here can act as a functional switch in a biochemical context and can be used to produce predictable effects on function in a system for which the structure/function relationships are well understood.

Experimental

Synthesis

N-Boc-4-formyl-phenylalanine methyl ester **2** was prepared by Pd-catalyzed carbonylation of *N*-Boc-L-tyrosine triflate methyl ester following the procedure described by Morera *et al.*[20] The synthesis of 5-methyl-benzo[*b*]thiophen-3-one (**3**) followed the procedure published by Yamaguchi.[16]

Preparation of *N*-Boc-[4-(5-methyl-3-oxo-3*H*-benzo[*b*]-thiophen-2-ylidenemethyl)]-phenylalanine methyl ester (4). To a solution of 0.14 g thioindoxyl (**3**) (0.9 mmol) and 0.27 g of aldehyde (**2**) (0.9 mmol) in 10 mL benzene, 0.06 mL piperidine (0.6 mmol, 0.67 equiv.) was added. The reaction mixture was refluxed for 3 h and then stirred at room temperature overnight (TLC monitoring). After addition of 100 mL diethyl ether and 100 mL water, the organic layer was separated and washed with saturated aqueous sodium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and the solvent was evaporated to yield 0.50 g of crude **4**. The remainder was treated with light petroleum/ethyl acetate (1 : 2) to remove impurities and then purified by flash-chromatography with light petroleum/ethyl acetate (15 : 1) on silica gel. Yield: 200 mg (50%, 0.4 mmol), yellow oil, R_f = 0.14 (light petroleum/ethyl acetate (4 : 1)); ^{1}H NMR (500 MHz, C_6D_6): δ (ppm) = 8.08 (s, 1H), 7.74 (s, 1H), 7.30 (d, 2H, J = 7.91 Hz), 6.91–6.68 (m, 4H), 4.93 (d, 1H, J = 7.69 Hz), 4.68–4.65 (m, 1H), 3.18 (s, 3 H), 2.92–2.71 (m, 2H), 1.89 (s, 3H), 1.39 (s, 9H); ^{13}C NMR (125.75 MHz, C_6D_6): δ (ppm) = 187.85, 171.73, 154.97, 142.78, 138.61, 135.98, 135.44, 133.32, 132.59, 131.03, 129.87, 128.81, 128.27, 127.30, 123.36, 79.38, 54.44, 51.37, 38.09, 28.07, 20.96, 20.22. IR (ATR): 3355, 2977, 1745, 1712, 1606, 1588, 1510, 1470, 1365, 1279, 116, 1080 cm^{-1}. HR MS (70 eV, EI): calcd for $C_{25}H_{27}NO_5S$: 453.1609, found: 453.1609.

Preparation of *N*-formyl-[4-[5-methyl-3-oxo-3*H*-benzo[*b*]-thiophen-2-ylidenemethyl)]-phenylalanine methyl ester (1). Starting from compound **4** (302 mg, 0.66 mmol) the removal of the Boc-protecting group followed the procedure described by Kemp using hydrogen chloride in dioxane (4.0 M).[21] Yield: 240 mg (92%, 0.61 mmol), brown solid, the compound was not further purified; R_f = 0.37 (diethyl ether/methanol (12 : 1)); ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 7.92 (s, 1H), 7.75 (s, 1H), 7.65 (d, 2H, J = 8.30 Hz), 7.40 (br s, 2H), 7.35 (d, 2H, J = 8.30 Hz), 3.37 (s, 3H), 3.62–3.54 (m, 1H), 3.19–3.01 (m, 1H), 2.96–2.86 (m, 1H), 2.41 (s, 3H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ (ppm) = 190.12, 175.06, 157.80, 144.52, 141.81, 138.11, 137.41, 134.33, 134.04, 132.18, 131.61, 131.24, 127.73, 125.00, 80.59, 56.00, 38.70, 28.75, 20.82. IR (ATR): 2941, 2859, 1751, 1642, 1453, 1243, 1186, 1139 cm^{-1}. HR MS (70 eV, EI): calcd for $C_{20}H_{19}NO_3S$: 353.1085, found: 353.1089.

Formic-acetic anhydride was prepared by the procedure previously described.[6] Treatment of the amino acid hydrochloride (96 mg, 0.25 mmol) with the latter anhydride gave the *N*-formyl amino acid methyl ester **5**. Yield: 80 mg (83%, 0.21 mmol), yellow solid, the compound was not further purified; R_f = 0.18 (pentane/ethyl acetate (1 : 1)); ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 8.21 (s, 1H), 7.90 (s, 1H), 7.75 (s, 1H), 7.65 (d, 2H, J = 7.81 Hz), 7.44–7.36 (m, 2H), 7.25 (d, 2H, J = 8.3 Hz), 6.08 (d, 1H, J = 6.84 Hz), 5.06–4.96 (m, 1H), 3.77 (s, 3H), 3.32–3.03 (m, 2H), 2.41 (s, 3H); ^{13}C NMR (50.3 MHz, $CDCl_3$) δ (ppm) = 188.85, 171.43, 160.62, 143.03, 137.99, 136.74, 135.91, 133.50, 132.77, 131.31, 130.92, 130.57, 130.12, 127.33, 123.70, 52.78, 51.79, 37.81, 20.98. IR (ATR): 3317, 3026, 2861, 1737, 1676, 1658, 1586, 1530, 1418, 1376, 1277, 1083, 909, 804 cm^{-1}. HR MS (70 eV, EI): calcd for $C_{21}H_{19}NO_4S$: 381.1035, found: 381.1041.

Hydrolysis of the methyl ester **5** (60 mg, 0.16 mmol) was accomplished by following the procedure described previously.[22] Yield: 48 mg (84%, 0.13 mmol), yellow oil, the compound was not further purified; R_f = 0.67 (methanol/dichloromethane/triethylamine (5 : 5 : 0.2)); ^{1}H NMR (200 MHz, [d_6]DMSO): δ (ppm) = 12.85 (br s, 1H), 8.43 (d, 1H, J = 7.32 Hz), 7.98 (s, 1H), 7.90 (s, 1H), 7.73–7.66 (m, 2H), 7.57 (d, J = 7.81 Hz, 2H), 7.42 (d, 2H, J = 7.82 Hz), 7.30 (s, 1H), 4.67–4.48 (m, 1H), 3.20–2.84 (m, 2H), 2.38 (s, 3H); ^{13}C NMR (50.3 MHz, [d_6]DMSO) δ (ppm) = 187.53, 172.26, 160.93, 142.02, 140.32, 137.06, 135.94, 132.64, 132.09, 130.73, 130.11, 129.68, 126,53, 124.19, 51.59, 36.64, 20.28. IR (ATR): 3352, 1717, 1673, 1603, 1469, 1380, 1280, 1224, 1080 cm^{-1}. MS (70 eV, EI): *m*/*z* (%): 367 (M^+) (21), HR MS (70 eV, EI): calcd for $C_{20}H_{17}O_4NS$: 367.0878, found: 367.0877.

Preparation of formyl-hemithioindigo des(formylvalyl)-gramicidin A. The preparation of formyl-hemithioindigo des(formylvalyl) gramicidin closely followed that of Weiss and Koeppe II.[15] Des(formylvalyl)-gramicidin (2.0 mg, 1.14 μmol) and formyl-hemithioindigo (1.0 mg, 2.7 μmol) were dissolved in 1 mL of anhydrous dimethylformamide containing 1 μL of freshly distilled diisopropylethylamine. The resulting mixture was cooled to 0 °C to which 5 μL (29 μmol) of diphenyl phosphoryl azide (Fluka) was added *via* syringe. After 1 hour the reaction was concentrated to dryness under high vacuum. The crude mixture was resuspended in methanol and the gramicidin products precipitated by addition of excess water. The peptides were lyophilized to yield a fine white powder which was further purified by gel filtration using Sephadex LH-20 (2.0 cm × 30 cm gravity column). Column fractions containing UV-Vis absorbances at both 280 and 450 nm were pooled. The product was verified by MALDI-MS. Formyl-hemithioindigo

des(formylvalyl) gramicidin A, MS data: calcd for $C_{113}H_{146}N_{20}O_{18}S$ $(MH)^+$: 2104.09, found = 2104.87; TLC (chloroform/methanol/water (65 : 25 : 4)): $R_f = 0.66$. The product was further purified using reverse-phase HPLC. Separation was achieved with a linear gradient starting at 80% methanol 20% water (containing 0.1% TFA, pH adjusted to 3.0) and ending at 100% methanol with a flow rate of 1 mL min^{-1} using a Zorbax RX-C8 column (4.6 × 250 mm). The UV-Vis detector was set to either 280 or 330 nm to monitor both tryptophan and hemithioindigo absorbances. The coupled product had a retention time of 10.4 minutes and was further verified by MALDI-MS. After each injection, the column was purged with 100% methanol for 10 minutes to remove residual gramicidin derivatives retained on the column. Double HPLC purification was performed prior to single-channel measurements.

Single-channel measurements

The general techniques for single channel recording of gramicidin channels have been described previously.[6] Peptides (~10 nM in MeOH) were added to membranes formed from diphytanoylphosphatidylcholine/decane (50 mg mL^{-1}). The *Z* state of hemithioindigo-gramicidin was obtained by dark adaptation of a stock solution of the peptide in MeOH. Photoisomerization to the *E* state was achieved by irradiation of the stock solution with a mercury lamp coupled to a monochromator ($\lambda = 406$ nm) for ~1 hour. Although isomerization *in situ* is also possible with our experimental arrangement,[23] isomerization of stock solutions was performed to permit monitoring of the *E*-isomer content by UV absorbance spectroscopy. Lipid bilayers were formed across a ~100 μm hole in a polypropylene pipette tip by painting a solution of lipid in decane. The pipette tip was mounted in a Teflon cell through a small hole in the back face. The front face of this cell had a removable circular glass window. Silver/silver chloride electrodes were placed in the pipette tip and in a cylindrical well drilled from the top of the cell. Symmetrical, buffered (5 mM BES, pH 7) CsCl (1 M) and NaCl (1 M) solutions were used. All measurements were made at room temperature. The current through lipid bilayers containing the gramicidin derivatives was measured and voltage was set using an Axopatch 1D patch-clamp amplifier (Axon Instruments) controlled by Synapse (Synergistic Research Systems) software. Single-channel events were recorded for a period of several hours for each set of experimental conditions. Data was filtered at 50 or 100 Hz, sampled at 1 kHz, stored directly to disk and analyzed using Synapse and Igor (Wavemetrics, Inc.) software. Mean lifetimes and current amplitudes were determined by manual fitting of appropriate functions to the corresponding histograms using the program Mac-Tac (Version 2.0, Instrutech Corp.).

Molecular modelling

All molecular modelling was performed on a Silicon Graphics Octane system (Irix 6.4) using the SYBYL (Tripos) and SPARTAN (Wavefunction) software packages. The gramicidin channel structure taken as a starting point for conformational searches was obtained from the Protein Data Bank (PDB code: 1MAG). A single Na^+ ion was positioned in the center of the channel. The amino acid at position one of the channel was replaced by the hemithioindigo amino acid (**1**), with coordinates based on the X-ray crystal structure of a closely related derivative.[22] Systematic searches were performed for both *Z* and *E* isomers varying the angles indicated in Fig. 3. For each conformation the distance between the O atom and the Na^+ ion and the C atom and the Na^+ were evaluated.

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17 The X-ray structure was measured at the Technical University Berlin on an *AXS Bruker* 3-circle-diffractometer with Mo-K_α-radiation with graphite monochromator. The structure was solved by direct methods using the program SHELXS-97 and refined against F2 on all data by full-matrix least squares with SHELXL-97. All non-hydrogen atoms were refined anisotropically. $C_{20}H_{16}O_6S$, Mr = 384.39, crystal size: 0.16 mm × 0.18 mm × 0.48 mm, monoclinic, space group $P2_1/c$, a = 1120.62(2) pm, b = 951.58(2) pm, c = 1731.690(10) pm, $\alpha = 90°$, β = 107.0850(10)°, $\gamma = 90°$, $V = 1.76511(5)$ nm^3, $Z = 4$, $\rho_{calcd} = 1.446$ mg m^{-3}, $F(000) = 800$, $\lambda = 71.073$ pm, $T = 293(2)$ K, μ (MoK_α) = 0.219 mm^{-1}. Total number of reflections measured 13132, unique reflections 4060 ($R_{int} = 0.1168$). Data/restraints/parameters 4060/0/245, data collection range $1.90° \leq \theta \leq 27.50°$. Final R indices: $R1 = 0.0445$, $wR2 = 0.1003$ on data with $I > 2\sigma(I)$ and $R1 = 0.0854$, $wR2 = 0.1202$ on all data; goodness of fit $S = 0.689$; extinction coefficient 0.0021(8); largest difference peak and hole: 362 and −444 e·nm^{-3}. CCDC reference number 233572. See http://www.rsc.org/suppdata/ob/b4/b408485c/ for crystallographic data in .cif or other electronic format.
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