Synthesis, Structure Elucidation, in Vitro Biological Activity, Toxicity, and Caco-2 Cell Permeability of Lipophilic Analogues of α-Conotoxin MII

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The α-conotoxin MII is a two disulfide bridge containing, 16 amino acid long peptide toxin isolated from the marine snail *Conus magus*. This toxin has been found to be a highly selective and potent inhibitor of neuronal nicotinic acetylcholine receptors (nAChRs) of the subtype α3β2. To improve the bioavailability of this peptide, two lipidic analogues of MII have been synthesized, the first by coupling 2-amino-D,L-dodecanoic acid (Laa) to the N terminus (LaaMII) and the second by replacing Asn5 in the MII sequence with this lipoamino acid (5LaaMII). Both lipidic linear peptides were then oxidized under standard conditions. ^{1}H NMR shift analysis of these peptides and comparison with the native MII peptide showed that the tertiary structure of the N-conjugated analogue, LaaMII, was consistent with that of the native conotoxin, whereas the 5LaaMII analogue formed the correct disulfide bridges but failed to adopt the native helical tertiary structure. The N terminus conjugate was also found to inhibit nAChRs of the subtype α3β2 with equal potency to the parent peptide, whereas the 5LaaMII analogue showed no inhibitory activity. The active LaaMII analogue was found to exhibit significantly improved permeability across Caco-2 cell monolayers compared to the native MII, and both peptides showed negligible toxicity.

Introduction

Predatory marine snails belonging to the genus *Conus* use spectacularly complex cocktails of bioactive peptide toxins to immobilize their prey.[1] Many different classes of conotoxins have been characterized in terms of their structure and the receptors they target.[2] The α-conotoxin class antagonizes nicotinic acetylcholine receptors (nAChRs). The structure of these small peptides (12−19 residues) is constrained by disulfide bonds that stabilize the active conformer.

nAChRs are ligand-gated ion channel receptors expressed in neurons and skeletal muscle. The receptors are pentamers assembled from 16 different subunits designated α1−α9, β1−4, γ, δ, and ϵ. Neuronal nAChRs contain only subunits α2−7, α9, and β2−4.[3] The diversity this affords nAChRs presents a formidable challenge to the search for methods of distinguishing the various subtypes. Fortunately, various toxins from animal venoms including the α-conotoxins have shown remarkable selectivity for different subtypes of receptors.[4−7] These compounds have been invaluable as biochemical and pharmacological tools.[2,7,8] Neuronal nAChRs have been implicated in several disease states including Alzheimer's, Tourette's and Parkinson's diseases and schizophrenia.[3,9,10] Those receptors that incorporate a β2 subunit have been directly linked to nicotine addiction by their ability to stimulate the release of dopamine in the mesolimbic dopamine system, thus mediating the reinforcing properties of nicotine.[11]

The α-conotoxins have several characteristics that recommend them as drug candidates. They are small peptides that are particularly stable to enzymes and acidic conditions. The stable, well-defined structure of the α-conotoxins (subclasses α3/5, α4/7, and α4/3) is mediated by the formation of usually two, but occasionally three, disulfide bonds in these cysteine-rich peptides.[12,13]

α-Conotoxin MII is a potent and highly selective competitive antagonist of the α3β2 nAChR. The toxin was first isolated from *Conus magus* by Cartier et al.,[14] and the three-dimensional solution structure was described initially by Shon et al.[15] and later by Hill et al. in their study on the effects of different solvent conditions on the helicity of the peptide.[16] α-Conotoxin MII, a 16 amino acid peptide (Figure 1) belongs to the α4/7 subclass by virtue of the Cys2−Cys8 and Cys3−Cys16 disulfide bonds. The toxin exhibits an IC_{50} value of 0.5 nM toward *Xenopus* oocytes expressing α3β2 nAChRs and is several orders of magnitude less potent toward other subunit combinations.[14] The segments of the receptor responsible for this exceptional selectivity have been identified and include sequence segments 121−181 and 181−195, especially Lys185 and Ile188 on the α3 subunit, and 1−54, 54−63, and 63−80 on subunit β2, with particular importance placed on Thr59.[17]

For any peptide to progress to the clinic, the issues of bioavailability and membrane permeability must be addressed. Although MII is relatively stable under biological conditions, it would not be expected to readily cross intestinal mucosal membranes or the blood−brain barrier. Increasing the lipid solubility of a hydrophilic

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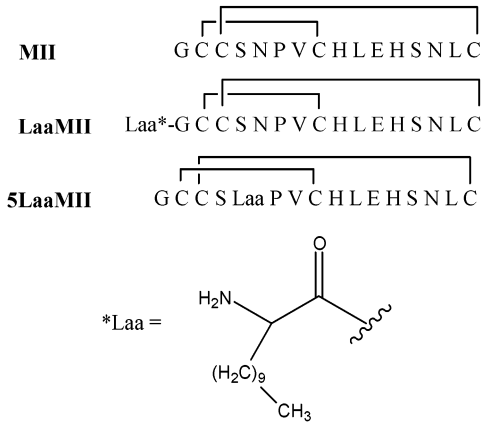

Figure 1. Sequence of α-conotoxin MII and the C_{12}−Laa conjugates LaaMII and 5LaaMII. All of the peptides have amidated C termini.

compound has been established as an important factor in improving absorption via passive transport across intestinal mucosal membranes.[18] There are numerous reports of peptides that have been coupled to various lipid units which have resulted in improved intestinal delivery characteristics.[19] For example, lauric acid (C12) was attached to the N terminus of thyrotropin-releasing hormone (TRH), which resulted in significantly increased penetration across the upper small intestine compared to the parent peptide in an in vitro everted sac experiment.[20] A captoyl−tetragastrin conjugate produced significantly increased acid secretion in rats compared to the native tetragastrin.[21] We have demonstrated previously that the conjugation of TRH and luteinizing hormone releasing hormone (LHRH) with one or two lipoamino acids (Laas) increased the peptides' half-lives in a homogenate of human intestinal epithelial cells by ∼30-fold from only 2−5 min to 1−3 h,[22] and in vivo experiments in rats demonstrated a significant increase in the stability and oral uptake of the peptide conjugates.[23]

The use of Laas to introduce lipidic groups into peptides allows the conjugation of one or more of these groups via standard peptide-coupling techniques and for them to be incorporated into the solid phase synthesis of a peptide. Thus, with a long-term goal of improving the bioavailability of conotoxins, our study aims to determine the effect of incorporating a C_{12} Laa into the MII sequence on the tertiary structure, in vitro activity, toxicity, and transepithelial transport of the peptide.

The human intestinal epithelial cell line, Caco-2, derived from a human colorectal carcinoma has become an increasingly important tool in drug delivery research as an in vitro model for intestinal absorption. Caco-2 cells spontaneously differentiate into monolayers of polarized enterocytes under conventional cell culture conditions, expressing high levels of several brush border hydrolases and forming tight cellular junctions.[24,25] The permeability of compounds through the Caco-2 monolayer can be related to the extent of oral absorption in humans,[26,27] and thus it is an important in vitro assay in assessing drug delivery systems.

Results and Discussion

Investigations of the solution structure of MII have determined that the peptide is helical over residues 6−11 in aqueous solution.[15,16] The study of the solvent accessibility of the residues by Hill et al. led them to suggest Pro6, Val7, Leu10, Glu11, and Asn14 as likely candidates for the residues that interact with the nAChRs.[16] Thus, we assumed that the formation of this helix which presents these residues to the surface of the peptide is essential for the activity of this peptide.

Synthesis. The synthesis of active, native MII has been achieved by oxidation in air of the deprotected linear peptide in a dilute solution of 0.1 M NH_4HCO_3 (pH 8).[16] Although this method afforded predominately the correctly folded MII conformer, it was not known if the presence of non-native groups such as lipoamino acids on the termini of the linear peptide or within the sequence would alter the outcome of this oxidation.

The solid phase synthesis of the MII sequence containing 2-amino-D,L-dodecanoic acid on the N terminus (LaaMII) required no alterations to the standard HBTU activation protocol used in the synthesis of native MII. The coupling efficiency of the lipoamino acid coupling was comparable with those of the natural amino acids.

The synthesis of an alternative lipophilic analogue of MII, which contained 2-amino-D,L-dodecanoic acid incorporated at position 5 of the MII sequence in place of the Asn residue (5LaaMII, Figure 1), was completed using the same solid phase synthetic techniques and purified and oxidized under the same conditions as the Laa−MII conjugate. Asn-5 was chosen as the residue to be replaced because it lies outside the important helical portion of the active molecule but is far enough removed from the Cys2 and Cys3 residues to minimize the possible adverse steric effects of the C_{12} side chain on the formation of the disulfide bonds. Unfortunately, to date there are no structure−function relationship data available for MII; thus, the importance of Asn-5, or any other residue in the structure, was not known. LC/MS analysis of the product of the oxidation showed the required loss of 4 mass units, suggesting the formation of two disulfide bonds.

The increase in lipophilicity of LaaMII and 5LaaMII necessitated the use of C4 stationary phase in the HPLC purification procedures, but this was the only minor change to the procedures used in the synthesis of the lipophilic MII conjugates. Both linear peptides were exposed to the oxidation conditions described above,[16] and the folded peptides were examined by NMR.

NMR Investigation. Proton shifts are sensitive to the local environment of the proton and are therefore useful indicators of structural change. The NH shifts (Figure 2A), αH shifts (plotted relative to random coil αH shifts in Figure 2B), and side-chain H shifts (not shown) for LaaMII were very similar to those for MII, indicating that overall there was little structural difference between the two peptides. Peaks for the three N-terminal residues were broadened probably as a result of movement of the lipid chain, with the NH peaks of Gly1 and Cys2 so broadened that they could not be identified in the spectra. The only LaaMII H shift that differed significantly from MII was the αH shift of Cys3, which is not surprising due to its proximity to the N-terminal lipid. Small changes in other shifts, such as the NH of His9 and the αHs of Cys8 and Glu11, may reflect some subtle structural changes. The negative deviation of the αH shifts of residues Pro6 to Glu11 of

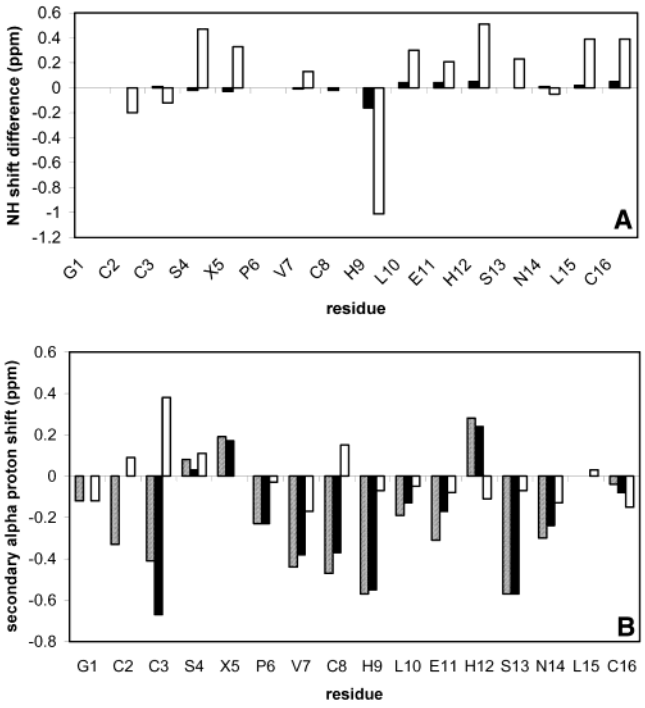

Figure 2. (A) Differences between the amide proton chemical shifts of LaaMII (black bars) or 5LaaMII (white bars) and MII. No values are given for Gly1 as its NH cannot be seen. Pro6 does not have an NH. The NH of Cys2 in LaaMII could not be identified in spectra so it could not be compared to MII and is absent in this figure. X5 represents Asn5 in MII and LaaMII and represents the lipoamino acid in 5LaaMII. (B) Secondary α proton chemical shifts of MII (striped bars), LaaMII (black bars), and 5LaaMII (white bars). Secondary αH shifts were calculated by subtracting the αH shift for the residue in a random coil peptide from the actual αH shift measured for that residue in MII and the MII analogues. The αHs of Gly1 and Cys2 were not identified for LaaMII and are absent in the graph. The two αHs of Gly1 in MII and in 5LaaMII were overlapped so only one Gly1 secondary αH shift was plotted for each of these peptides. 0.29 was subtracted from the secondary shifts for residue 5 as it precedes a Pro. X5 indicates Asn5 in LaaMII and MII and 5Laa in 5LaaMII.

MII from the αH shifts of similar residues in random coil peptides, indicative of helical secondary structure over those residues, was retained in LaaMII (Figure 2B). Overall the structural analysis showed that addition of the lipoamino acid to the N terminus of MII caused minimal change to the structure of the peptide.

Analysis of the NMR spectra of the 5LaaMII conjugate showed that its proton shifts differed dramatically from the proton shifts of MII (Figure 2). Analysis of the secondary αH shifts of 5LaaMII, that is, the αH shifts relative to those for residues in random coil peptides, indicated that it was not as well structured as MII and did not feature the helical region found in MII (Figure 2B). βH shift differences can also be used to assess how structured a peptide is as βHs tend to be well separated in structured peptides. Comparison of the βH shift differences, shown in Figure 3, confirmed that 5LaaMII was not as well structured as MII.

To investigate the cause of such a remarkable change in the tertiary structure of the 5LaaMII peptide, the precise disulfide connectivities were determined by partial reduction and alkylation of the oxidized peptide. In this experiment, the disulfide bonds in the peptide were partially reduced by tris(2-carboxyethyl)phosphine

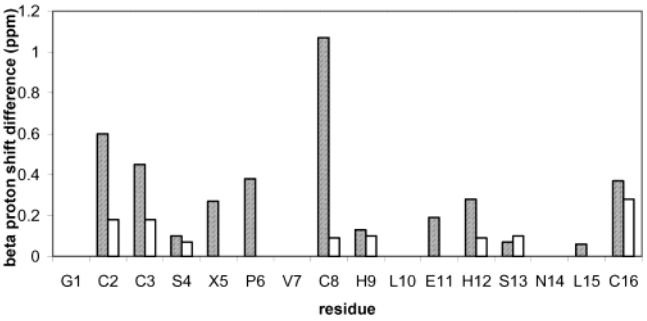

Figure 3. Differences in βH shifts for MII (striped bars) and 5LaaMII (white bars). X represents Asn in MII and the lipoamino acid in 5LaaMII. Gly residues do not have βHs, and Val residues have only one βH, so nothing is graphed for Gly1 or Val7.

and then the free thiol groups alkylated by maleimide. The resultant product ion spectrum from the LC/MS/MS analysis of the partially reduced and alkylated mixture revealed the ions at *m*/*z* 258 corresponding to the b_2 ion (Gly-Cys2-Mal), *m*/*z* 361 corresponding to the b_3 ion (Gly-Cys2-Mal-Cys3), and *m*/*z* 448 corresponding to the b_4 ion. The doubly charged ions b_{15}^{2+} (*m*/*z* 987.8), b_{14}^{2+} (936.3), b_{13}^{2+} (879.8), and b_{12}^{2+} (822.8) were also present with no maleinyl group present on Cys16. These data indicated that in the oxidized form of 5LaaMII, the disulfide bonds form between Cys2 and Cys8 and between Cys3 and Cys16, the same pattern of connectivity that is seen in native MII and in LaaMII.[28] It was clear, therefore, that the presence of the Laa in position 5 of the peptide sequence did not inhibit the formation of the correct disulfide connectivities but did prevent the formation of the required helix tertiary structure. This large structural change may be the result of the lipid chain associating with the hydrophobic residues of the peptide. The lipid may affect Pro6 which, with the exception of the cysteines, is the only conserved residue in α-conotoxins and is thought to be vital for the formation of the helix.

Biological Activity. The NMR studies of these two MII analogues showed that the tertiary structure of the two compounds were significantly different and the in vitro α3β2 nAChR inhibition activities reflect this result. Rapid focal application of ACh (500 μM) to dissociated neurons of the rat parasympathetic ganglia held at −60 mV, in the presence of 100 nM atropine, resulted in a characteristic biphasic inward current comprising an initial transient peak, which rapidly decayed to a steady-state current [Figure 4A(i)]. Native MII and the Laa−MII conjugate inhibited the ACh-evoked current in a dose-dependent manner. The Laa−MII conjugate at a concentration of 4.5 nM reduced the nACh-activated current to 92 ± 1% (n = 3) of control, whereas 4.5 nM native MII reduced the nACh-activated current to 90 ± 7% (n = 3) of the control response. At a concentration of 22 nM Laa−MII and native MII reduced the amplitude of the nACh-activated current to 57 ± 4% (n = 4) and 57% (n = 2) of control, respectively. Finally, 222 nM Laa−MII and 222 nM native MII reduced the amplitude to 41% (n = 2) and 47 ± 4% (n = 3) of control. These data corresponded to IC_{50} values of 12.8 nM for LaaMII and 10 nM for native MII. 5LaaMII, on the other hand, had no effect on the nACh-induced current amplitude at concentrations up to 10 μM (n = 3).

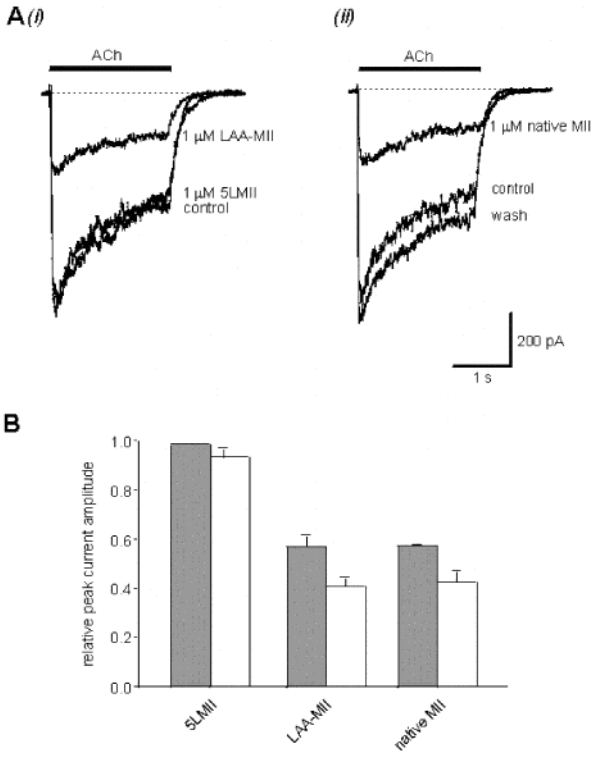

Figure 4. Effect of native MII and conjugates on ACh-evoked currents in parasympathetic neurons. (A) (*i*) 5LaaMII (1 μM) has no effect on the peak ACh-evoked current, whereas 1 μM LaaMII inhibits the current by ~50%. (*ii*) In the same neurone native MII reduces the current by a similar amount to Laa−MII and the current recovers completely on washout of the toxin. (B) Bar graph of the relative peak ACh-evoked current by 5LaaMII, LaaMII, and native MII. Shaded and white columns represent data for 100 nM and 1 μM, respectively. Data represent mean ± SEM, $n = 3-4$.

These results clearly showed that the presence of an Laa on the N terminus of MII had no effect on the potency of the peptide's inhibition of the $\alpha 3\beta 2$ nAChRs. Proton chemical shift analysis of the Laa−MII conjugate also showed that the lipidic group had no effect on the tertiary structure adopted by the peptide under the standard MII oxidation conditions. The 5LaaMII analogue, however, showed complete loss of inhibition toward the nAChRs at the concentrations examined, due to the undesired tertiary structure adopted during the oxidation of this peptide.

Caco-2 Cell Permeability. Caco-2 cell monolayers are being used in pharmaceutical research with increasing frequency to study the transepithelial transport of drugs. The ultimate purpose of conjugating MII to lipoamino acids is to improve its oral bioavailability. Thus, as an in vitro assessment of the merits of the strategy, the Caco-2 cell monolayer permeability of MII and the active analogue LaaMII were measured. As the integrity and permeability of monolayers can vary between experiments, a control compound, [^{14}C]mannitol, which is known to have negligible oral bioavailability, was also studied in the same experiment. The peptides were radiolabeled by acetylation using tritium-labeled acetic anhydride, and the amount of peptide present in the basolateral chamber was quantified by liquid scintillation. The average ($n = 4$) apparent permeability values (P_{app}) of the three compounds are listed in Table 1. The P_{app} for MII was similar to that of mannitol, indicating minimal permeability across the monolayer, as would be expected for a peptide of the size and hydrophilicity of MII. LaaMII, however, exhibited a 4-fold increase in the apparent permeability compared to that of the parent peptide (Figure 5). This significant increase in P_{app} for LaaMII indicated that this peptide is expected to show significant absorption through the human gastrointestinal tract.[27]

Table 1. Apparent Permeability Values for Mannitol, MII, and LaaMII

compound	$P_{app} \times 10^{-6}$ (cm/s)
mannitol	5.88 ± 0.87
MII	4.97 ± 1.47
LaaMII	20.5 ± 2

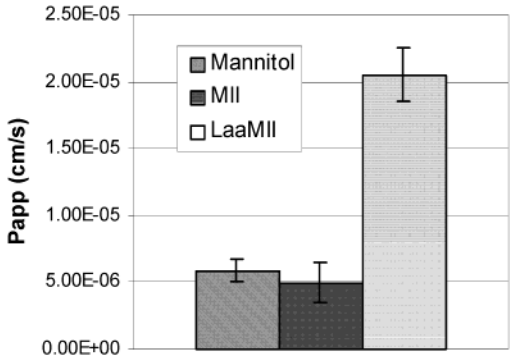

Figure 5. Graphical representation of the P_{app} values observed for mannitol, MII, and LaaMII.

Table 2. Percent Hemolysis Observed in Rat Red Blood Cells When Treated with SDS, MII, or LaaMII

	% hemolysis
max	100
min	0
SDS, 1 mM	12.25
SDS, 2 mM	51.54
SDS, 5 mM	62.66
MII, 1 mM	0.53
MII, 2 mM	0.83
MII, 5 mM	1.35
LM, 1 mM	1.05
LM, 2 mM	0.45
LM, 5 mM	0.83

Toxicity. Before in vivo experiments using these peptides can be initiated, some assessment of their toxicity is required. The integrity of the Caco-2 cell monolayer before and after treatment with the peptides, as measured by the transepithelial electrical resistance (TEER) across the monolayer, is one indication of toxicity. Neither MII nor LaaMII showed significant toxicity to Caco-2 cells under these conditions. As a further test of the peptides' toxicity, the effect of each compound on red blood cells isolated from a male rat was assessed. This assay measured the extent of hemolysis of the cells over 30 min. In addition to the two peptides, sodium dodecyl sulfate (SDS) was also examined in the experiment. SDS is known to be toxic to red blood cells at the concentrations used in the assay and therefore was included as a positive control. The results of the experiment are detailed in Table 2. Neither MII nor LaaMII caused appreciable hemolysis of red blood cells, even at the relatively high concentrations used in the assay.

Conclusions

The synthesis of two lipophilic analogues of the α-conotoxin MII was achieved via solid phase methods followed by oxidation of the linear peptide. The N-terminal substituted analogue retained both the tertiary structure and in vitro inhibitory potency of the parent peptide. The analogue bearing a lipoamino acid at position 5 in place of an Asn residue in the sequence failed to adopt the desired tertiary structure and showed no inhibitory activity toward nAChRs.

The active analogue LaaMII was also found to exhibit significantly enhanced Caco-2 cell permeability, indicating improved intestinal absorption, and was also shown to be nontoxic to both epithelial cells and red blood cells. These data clearly indicate that LaaMII is an excellent candidate for future in vivo biodistribution experiments.

Experimental Section

Synthesis. MBHA resin and protected amino acids were obtained from Novabiochem (Melbourne, Australia). DMF and TFA of peptide synthesis grade were purchased from Auspep (Parkville, Australia). HPLC grade acetonitrile was purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). Mass spectrometric measurements were performed using a triple-quadrupole PE Sciex API 3000 mass spectrometer with positive ion electrospray (ES). The mobile phase was a mixture of 50% solvent A (0.1% formic acid in water) and 50% solvent B (0.1% formic acid in 90% acetonitrile/water) at a flow rate of 0.1 mL/min.

(a) 2-(*tert*-Butoxycarbonylamino)-D,L-dodecanoic acid. Sodium (3.81 g, 166 mmol) was dissolved in ethanol (100 mL) under nitrogen, and diethyl acetamido malonate (30.00 g, 138 mmol) was added followed by 1-bromodecane (42.76 g, 193 mmol). The solution was refluxed overnight under a nitrogen atmosphere. Upon cooling, the mixture was poured onto crushed ice (600 mL) and stirred. The precipitated product was collected and air-dried. The crude product was refluxed overnight in a solution of HCl/DMF (9:1, 200 mL). Upon cooling, the precipitated product was collected, washed with ice water, and air-dried to afford α-aminododecanoic acid hydrochloride [37.57 g, 97% MS $[M + H]^+$ *m/z* 216 ($[M + H]^+$ of $C_{12}H_{25}NO_2$ requires 216)]. 2-Amino-D,L-dodecanoic acid hydrochloride (24.24 g, 96.3 mmol) was suspended in a solution of *tert*-butyl alcohol/water (2:3, 500 mL) and the pH adjusted to 13 with sodium hydroxide (5 M). Di-*tert*-butyl dicarbonate (31.52 g, 144 mmol) in *tert*-butyl alcohol (50 mL) was added. The solution was stirred overnight, maintaining the pH at 13. The mixture was diluted with water (200 mL), and solid citric acid was added to pH 3. The mixture was extracted with ethyl acetate (5 × 150 mL), and the combined extracts were dried ($MgSO_4$) and evaporated to yield a crude product (oil and crystals). This product was recrystallized from warm acetonitrile to afford α-(*tert*-butoxycarbonylamino)dodecanoic acid (21.98 g, 72%): mp 61−63 °C, lit. mp 62−64 °C;[29] MS, *m/z* $[M + H]^+$ 316 ($[M + H]^+$ of $C_{17}H_{33}NO_4$ requires 316), 260; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.55 (1H, br s, COOH), 5.09 (1H, d, amide NH), 4.29 (1H, m, α-CH), 1.9−1.5 (2H, m, β-CH_2), 1.43 [9H, s, $C(CH_3)_3$], 1.24 (16H, m, 8CH_2), 0.86 (3H, t, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.6, 155.6, 80.1, 53.4, 32.4, 31.9, 29.6, 29.5, 29.4, 29.3, 29.2, 28.3, 25.3, 22.7, 14.1.

(b) Peptide Chain Assembly and Cleavage. All of the peptides were assembled on p-MBHA resin (100−200 mesh, 0.67 mmol/g loading) using HBTU/DIEA activation and the in situ neutralization protocol for Boc chemistry.[30] The peptides were synthesized on a 0.5 mmol scale, and the following amino acid side-chain protection was used: Cys(MeBzl), Ser(Bzl), His(DNP), Glu(OcHx). Each residue was coupled for 15 min; the coupling efficiencies were determined by the quantitative ninhydrin reaction,[31] and recoupling was performed if the efficiency was <99.2%. Prior to removal of the N-terminal Boc group, the DNP protecting group was removed by treatment with a solution of 20% mercaptoethanol, 10% DIEA, and 70% DMF for 30 min with one repetition of this procedure. The N-terminal Boc group was then removed by treatment with TFA, and the resin was washed successively with DMF, DCM, and methanol and then dried under vacuum. The peptide was cleaved from the resin by treatment with 20 mL of liquid HF, 1 mL of *p*-cresol, and 1 mL of *p*-thiocresol at 0 °C for 1 h. After removal of the HF, the crude product was precipitated and washed in cold diethyl ether and then dissolved in 50% acetonitrile. This solution was lyophilized and the crude peptide purified by preparative RP-HPLC on a Vydac C18 22 × 250 mm column in the case of α-conotoxin MII and a Vydac C4 22 × 250 mm column in the case of LaaMII and 5LaaMII. Purification was performed using a linear solvent gradient from 100% solvent A to 80% solvent B (solvent A = 0.1%TFA in water, solvent B = 80% acetonitrile, 0.1% TFA in water) in 35 min at a flow rate of 5 mL/min. The presence of the desired peptides in the fractions was detected by ESMS analyzing for the appearance of the $[M + H]^+$ ion (MII = *m/z* 1716, LaaMII = *m/z* 1913, and 5LaaMII = *m/z* 1799) and the more abundant $[M + 2H]^{2+}$ ion (MII = *m/z* 858.5, LaaMII = *m/z* 957, and 5LaaMII = *m/z* 900). The purity of the preparative HPLC fractions was determined by analytical RP-HPLC using a Vydac C18 22 × 4.6 mm column with a flow rate of 1 mL/min and a linear gradient from 100% solvent A to 80% solvent B over 30 min. The fractions containing pure peptide were combined and lyophilized to afford the peptides as white solids (reduced MII yield = 40%, reduced LaaMII yield = 24%, reduced 5LaaMII yield = 28%).

(c) Oxidation. The purified peptides were oxidized by dissolving in 0.1 M NH_4HCO_3 to a final peptide concentration of 20 μM and stirring vigorously at room temperature for 5 days. The oxidized peptides were purified as described above. $[M + H]^+$: MII = *m/z* 1712, LaaMII = *m/z* 1909, 5LaaMII = *m/z* 1795. $[M + 2H]^{2+}$: MII = *m/z* 856.5, LaaMII = *m/z* 955, 5LaaMII = *m/z* 898. Oxidized MII yield = 34%, oxidized LaaMII yield = 10%, oxidized 5LaaMII yield = 67%.

Disulfide Connectivity 5LaaMII. Purified peptide was dissolved in NH_4OAc (pH4) to an approximate concentration of 0.1 mg/mL. This solution (5 μL) was treated with 2 μL of tris(2-carboxyethyl)phosphine solution (0.1 M) and the reaction monitored by LC/MS until ∼50% reduction had occurred. Maleimide solution (20 μL of 0.1 M) was then added and the reaction again monitored for the appearance of the dialkylated peptide ($[M + 2H]^{2+}$ = 996.3), at which time the solution was acidified by the addition of 1 μL of TFA. The resulting mixture was analyzed using an Applied Biosystems Qstar Pulsar electrospray Qqtof mass spectrometer running in information-dependent acquisition mode, MS/MS scanning being switched on by detection of the $[M + 2H]^{2+}$ *m/z* 996.3 ion. Interpretation of the data was assisted by use of Bioanalyst 1.1 from Applied Biosystems. The LC component consisted of a 2.1 × 150 mm Zorbax SB300 C3, 5 μm column using a solvent gradient of 0−40% solvent B over 40 min (solvent A = 0.1% formic acid in H_2O, solvent B = 90% acetonitrile, 0.1% formic acid) at a flow rate of 300 μL/min.

NMR Experiments. Samples for NMR experiments contained ∼1.5 mM peptide in 40% C^2H_3CN/60% ^1H_2O. NMR experiments were carried out on Bruker 500 and 750 MHz NMR spectrometers. These included double quantum filtered correlation spectroscopy (DQF-COSY),[32] total correlation spectroscopy (TOCSY) with a mixing time of 80 ms,[33] and nuclear Overhauser effect spectroscopy (NOESY) with mixing times of 200 and 350 ms.[34,35] Spectra were internally referenced to DSS at 290 K. Additional spectra were acquired at 280, 285, 295, and 300 K for LaaMII to allow assignment of resonances that were overlapped at 290 K. Spectra for 5LaaMII were also acquired at 288 and 298 K with the peptide dissolved in 30% C^2H_3CN/70% ^1H_2O.

Spectra were processed on a Silicon Graphics Indigo workstation using XWIN-NMR (Bruker).

nAChR Inhibition Studies. (a) Cell Preparation. Parasympathetic neurons were dissociated from neonatal (3−8-day-

old) rat intracardiac ganglia as described previously[36] and maintained in culture for 24−48 h.

(b) Electrophysiological Recording. Membrane currents were recorded using the whole-cell recording configuration of the patch clamp technique. Electrical access to the cell interior was obtained using the perforated-patch whole-cell recording configuration.[37] A final concentration of 240 μg/mL amphotericin B in 0.4% DMSO was used in the pipet solution. Patch electrodes were pulled from thin-wall borosilicate glass (Harvard Apparatus Ltd., Kent, U.K.) and had resistances of ∼1 MΩ. Access resistances using the perforated patch configuration were routinely 4−8 MΩ before series resistance compensation.

ACh-evoked currents were recorded using an Axopatch 200A patch clamp amplifier (Axon Instruments Inc., Union City, CA), filtered at 2 kHz and then digitized at 10 kHz (Digidata 1200A interface, Axon Instruments Inc.) and stored on the hard disk of a PC for viewing and analysis. Voltage protocols were applied using pClamp software (version 6.1.2, Axon Instruments Inc.). Numerical data are presented as the mean ± SEM (*n*, number of observations).

(c) Solutions and Reagents. The pipet filling solution for perforated patch experiments contained 75 mM K_2SO_4, 55 mM KCl, 5 mM $MgSO_4$, and 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), titrated with *N*-methyl-D-glucamine to pH 7.2. The control extracellular solution contained 140 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 7.7 mM glucose, and 10 mM HEPES−NaOH. Acetylcholine (500 μM) and atropine (100 nM, to inhibit muscarinic ACh receptor activation) were applied for a duration of 2 s using a rapid piezo application system to minimize the rapid desensitization of the α7 component of the whole-cell ACh-evoked current.[38] Peptides were applied to the extracellular pipet solution as well as the constant perfusing solution. Experiments were carried out at 22 °C. The osmolality of all solutions was monitored with a vapor pressure osmometer (Westcor 5500) and was in the range of 280−290 mmol/kg. All chemicals used were of analytical grade. Acetylcholine chloride and atropine sulfate were supplied by Sigma Chemical Co.

Caco-2 Cell Permeability Studies. (a) ^{3}H-Labeling of Peptides. Purified peptide was dissolved in a minimum volume of DMF, and $(^3H_3CCO)_2O$ (0.5 equiv) was added followed by DIEA (3 equiv). The solution was allowed to stand at room temperature overnight. Non-radioactive acetic anhydride (0.5 equiv) was then added and the reaction continued for 8 h. The volatiles were then removed under a steady stream of N_2 gas and the reaction lyophilized to dryness. The residue was redissolved in water and lyophilized again to yield a fluffy white powder. This powder was dissolved in Hank's balanced salt solution (HBSS) containing 0.5% DMSO in sufficient volume to produce a 200 μM solution.

(b) Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Transwell polycarbonate inserts were from Costar (Cambridge, MA), and cell culture reagents were purchased from Gibco-BRL (Grand Island, NY).

Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% nonessential amino acids at 95% humidity and 37 °C in an atmosphere of 5% CO_2. The medium was changed every second day. After reaching 80% confluence, the cells were subcultured using 0.2% EDTA and 0.25% trypsin. Approximately 5×10^5 cells (passage 57) were seeded onto polycarbonate cell culture inserts (Transwell, mean pore size = 0.45 μm, 6.5 mm diameter) and cultivated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were allowed to grow and differentiate for 21 days. The medium was changed every second day. Transepithelial electrical resistance (TEER) of the monolayers was measured using the Millicell-ERS system (Millipore Corp., Bedford, MA) before and after the drug transport experiments. All of the monolayers used had initial and final TEER values >400 Ω/cm^2.

(c) Permeability Assay. The assays were performed in HBSS containing 25 mM HEPES (pH 7.4) in air at 95% humidity at 37 °C. Prior to the study, the monolayers were washed in prewarmed HBSS−Hepes for 30 min. At the start of the experiments 100 μL of the peptide or mannitol solutions (200 μM) was added to the donor side of the monolayers and 600 μL of HBSS−Hepes without drug was added to the receiver side. The plates were shaken in a Heifolf Titramax 1000 at 400 rpm at 37 °C during the whole experiment. At regular intervals (30, 90, and 150 min) the Transwell insert containing the monolayer was lifted carefully and placed in a new receiver chamber containing fresh buffer. The entire contents of each receiver chamber were added to 4 mL of Wallac OptiPhase HiSafe 3 liquid scintillation cocktail, and the radioactivity was measured in a liquid scintillation spectrometer (Tri-Carb 2700 TR).

(d) Determination of Permeability Coefficients. The apparent permeability coefficients (P_{app}, cm s^{-1}) were determined according to the following equation:

$$P_{app} = \frac{dC}{dt} \times \frac{V_r}{AC_0}$$

d*C*/d*t* is the steady-state rate of change in the radiochemical concentration (dpm mL^{-1} s^{-1}) in the receiver chamber, V_r is the volume of the receiver chamber (mL), *A* is the surface area of the cell monolayers, and C_0 is the initial concentration of the donor chamber (dpm mL^{-1}). Four replicates of each compound were performed, and the P_{app} values in Table 1 represent an average of these values.

Hemolysis Assay. Solutions of MII, LaaMII, and SDS in phosphate-buffered saline (PBS) with 1% DMSO were prepared (2, 4. and 10 mM). Blood (9 mL) was collected from a male Sprague−Dawley rat via cardiac puncture and treated with 1 mL of a 4% sodium citrate solution. The red blood cells (RBC) were isolated from the whole blood by centrifugation at 800*g* for 10 min. The plasma was removed, and the cells were washed three times with (PBS). After washing, the cells were resuspended in 10 mL of PBS. The RBC preparation (100 μL) was placed in the required number of wells of a 96-well tissue culture plate, which was shaken in a Heifolf Titramax 1000 at 400 rpm at 37 °C for 30 min. A 100 μL aliquot of one of the test compound solutions was then added to each well. Six wells received only PBS. The plates were incubated for a further 30 min. Three concentrations of each compound (1, 2, and 5 mM) were assayed in triplicate. When incubation was complete, the solutions from three of the wells that received only PBS were removed. The plate was then centrifuged at maximum speed for 2 min to pellet any remaining whole cells. A 20 μL aliquot of the supernatant of each well, or the whole cell preparation from the three PBS wells removed prior to centrifugation, was added to 5 mL of Drabkin's reagent containing Brij35 detergent. These solutions were then wrapped in foil and placed in the dark for 30 min. The absorbance of each sample at 540 nm was then measured. The wells treated with only PBS that were not centrifuged represented the maximum hemolysis (100%), whreas the wells treated with PBS that were centrifuged represented the background or minimum hemolysis. The percentage of hemolysis caused by each concentration of compound was calculated using the average Abs_{540} in the following equation:

$$\% \text{ hemolysis} = \frac{\text{Abs540} - \text{minAbs540}}{\text{maxAbs540} - \text{minAbs540}} ¥ 100$$

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Supporting Information Available: Tables of the ^{1}H shifts of MII, LaaMII, and 5LaaMII in 40% C^2H_3CN/60% ^1H_2O, acquired at 290 K and internally referenced to DSS, and extracts of the product ion mass spectrum of the ES/LC/MS/MS analysis of the partially reduced and alkylated 5LaaMII

peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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