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The synthesis and structure of an n-terminal dodecanoic acid conjugate of α-conotoxin MII

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Summary

The α-conotoxin MII is a 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 of the subtype α3β2. To improve the bioavailability of this peptide, we have coupled to the N-terminus of conotoxin MII, 2-amino-D,L-dodecanoic acid (Laa) creating a lipidic linear peptide which was then successfully oxidised to produce the correctly folded conotoxinMII construct.

Key words: α-aminododecanoic, drug delivery, α-Conotoxin MII, lipoamino acid, peptide synthesis

Introduction

Predatory marine snails belonging to the species *Conus* use spectacularly complex cocktails of bioactive peptide toxins to immobilise their prey [1]. Many different classes of conotoxins have been characterised in terms of their structure and the receptors they target [2]. The α-conotoxin class antagonise nicotinic acetylcholine receptors (nAChRs). The structure of these small peptides (12–19 residues) is constrained by disulfide bonds which stabilise the active conformer. Members of the α-conotoxin class selectively antagonise specific subtypes of nAChRs, making them of particular interest as biochemical probes and potential pharmaceutics [3,4].

The α-conotoxins have several characteristics which 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 is mediated by the formation of usually two, but occasionally three, disulfide bonds in these cysteine rich peptides [5,6]. This family of peptide toxins are further classified by the number of residues between these disulfide bonds into the α3/5, α4/7 and α4/3 subclasses [3].

α-Conotoxin MII is a potent and highly selective competitive antagonist of the α3α2 neuronal nAChR [7]. The toxin was first isolated from *Conus magus* by Cartier et al. [7] and the three-dimensional solution structure described initially by Shon et al. [8] and later by Hill et al. in their study of the effects of solvent on the helicity of the peptide [9]. 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 IC50 value of 0.5 nM towards oocytes expressing α3α2 nAChRs and is several orders of magnitude less potent towards other subunit combinations [7].

In order for any peptide to progress to the clinic the issues of bioavailability and membrane permeability must be addressed. Although α-conotoxin 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 compound has been established as an important factor in improving absorption via passive transport across intestinal mucosal membranes [10]. There are numerous reports of peptides that have been coupled to various lipid units to improve intestinal delivery characteristics [11]. The use of lipoamino acids to introduce lipidic groups into peptides allows the conjugation of these groups using standard solution or solid phase peptide coupling techniques. To improve the absorption of α-conotoxinMII across biological barriers such as the gut and blood brain barrier, an MII conjugate bearing 2-amino-D,Ldodecanoic acid on the N-terminus (Figure 1) was synthesised and demonstrated to maintain the native disulfide connectivity.

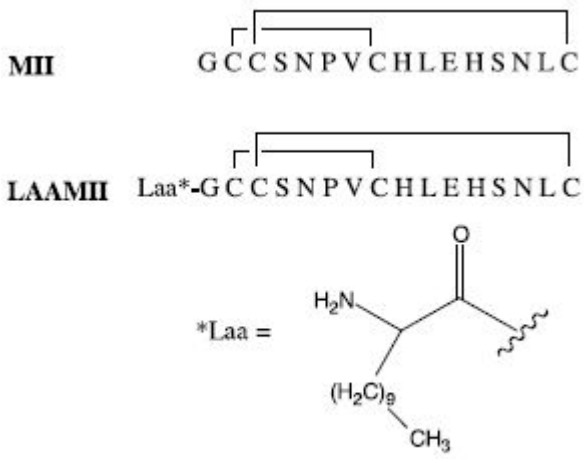

Figure 1. The sequence of α-conotoxin MII and LaaMII. Both peptides have an amidated C-terminus.

Results and Discussion

Native α-conotoxin MII was assembled, oxidised and purified and confirmed by comparison with authentic material to be the correctly folded conformer [9]. Previous investigations of the solution structure of MII have determined that the peptide is helical over residues 6–11 in aqueous solution. [8,9] A study by Hill et al. of the solvent accessibility of the residues led them to suggest Pro6, Val7, Leu10, Glu11 and Asn14 as likely candidates responsible for interaction with the nAChRs [9]. The formation of the helix with correct disulfide connectivities which presents these residues to the surface is thus essential for full activity. While the formation of the correct disulfide connectivity of α-conotoxin MII was readily achieved by air oxidation of the naked linear peptide in a dilute solution of 0.1 M NH4HCO3 (pH8) [9], it was not known if the presence of non-native groups such as lipoamino acids on the termini of the peptide or within the sequence would alter the outcome of this oxidation.

The solid phase chain assembly of the LaaMII sequence required no alterations to the standard HBTU activation protocol used in the synthesis of native MII [9]. The coupling efficiency of the Laa coupling was comparable with those of the natural amino acids. The increased lipophilicity of LaaMII necessitated the use of C4 stationary phase in the HPLC purification procedures. The linear peptide was exposed to the oxidation conditions described above [9] and LCMS analysis of the reaction indicated the presence of the expected product (the loss of four mass units). The oxidation was complete after 5 days. The yield of purified LaaMII is lower than that of MII under the same conditions, however it is suspected that this loss of product occurs during the purification procedures and it is anticipated that this loss will be reduced with further optimisation of the HPLC conditions

In an alternative solution phase synthetic route Boc protected 2-amino-D,L-dodecanoic acid was coupled to the N-terminus of oxidised, native α-conotoxin MII using DCC. After deprotection and purification this material was compared by analytical HPLC and electrospray mass spectrometry with the product of the oxidation of the LaaMII linear peptide and found to be identical (Figure 2). This indicates that the desired Cys2-Cys8 and Cys3-Cys16 disulfide bonds were formed during the oxidation of the LaaMII peptide. The successful solid phase synthesis of a lipidic analogue of the α-conotoxin MII is important in demonstrating that non-native groups, even bulky substituents on the termini of the peptide, do not alter the preferred disulfide bridge connectivity leading the way to the optimisation of substituted MII for improved absorption and bioavailablility. Future studies are underway to examine the precise tertiary structure of the LaaMII conjugate and the effect of dodecanoic acid and other non-native groups on nAChR antagonist activity.

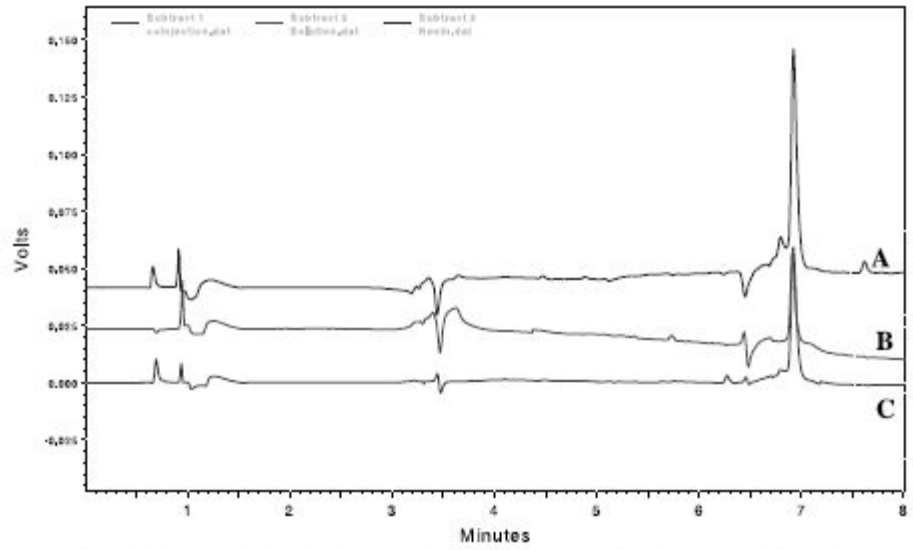

Figure 2. Comparison of the HPLC traces of: A: LaaMII formed by the oxidation of a linear LaaMII peptide (Y axis offset 0.04 V). B: LaaMII formed by the coupling of α-aminododecanoic with native, oxidised MII (Y axis offset 0.025 V). C: A co-injection of the two LaaMII products. HPLC performed on an Agilent Technologies Zorbax® SB-C_{18} 3.5μm, 4.6 × 150 mm column using a solvent gradient from 100% solvent A to 100% solvent B over 9 minutes and a flow rate of 2 mL/min. Detection was achieved using a Shimadzu® SPD-6A UV spectrometric detector monitoring at 214 nm.

Methods

General

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 operating in Q1 scan mode with positive ion electrospray. 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.

2-(t-Butoxycarbonylamino)-D,L-dodecanoic acid

Sodium (3.81 g, 166 mmol) was dissolved in ethanol (100 mL) under nitrogen in a round bottom flask fitted with a coil condenser. Diethyl acetamido malonate (30.00 g, 138 mmol) was then added followed by 1-bromodecane (42.76 g, 193 mmol) and the solution refluxed overnight under a nitrogen atmosphere. Upon cooling the mixture was poured onto crushed ice (600 mL) and stirred. The product precipitated, was collected and air dried. The crude product was refluxed overnight in a solution HCl:DMF (9:1, 200 mL). Upon cooling the product precipitated and was collected on a Buchner funnel, washed with ice water and air dried to afford α-aminododecanoic acid hydrochloride (37.57g, 97% M.S. [M+H]+ *m/z*: 216 ([M+H]+ of C12H25NO2 requires 216)). 2-Amino- D,L-dodecanoic acid hydrochloride (24.24 g, 96.3 mmol) was suspended in a solution of *t*-butanol:water (2:3, 500 mL) and the pH adjusted to 13 with sodium hydroxide (5 M). Di-*t*-butyldicarbonate (31.52 g, 144 mmol) in *t*-butanol (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 dried (MgSO4) and evaporated to yield a crude product (oil and crystals). The product was recrystallised from warm acetonitrile to afford α- (*tert*-butoxycarbonylamino) dodecanoic acid (21.98 g, 72%). M.p. 61-63 ◦C, literature m.p. 62–64 ◦C[12]. M.S. *m/z*: [M+H]+ 316 ([M+H]+ of C17H33NO4 requires 316), 260. 1H NMR. (300MHz, CDCl3): 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-CH2); 1.43 (9H, s, C(CH3)3); 1.24 (16H, m, 8CH2); 0.86 (3H, t, CH3). 13C NMR (75MHz, CDCl3): 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.

Peptide chain assembly and cleavage

Both peptides were assembled on p-MBHA resin (100–200 mesh, 0.67 mmol/g loading) usingHBTU/DIEA activation and the *in situ* neutralisation protocol for Boc-chemistry. [13] The peptides were synthesised 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 minutes, the coupling efficiencies were determined by the quantitative ninhydrin reaction[14] and recoupling 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, 70% DMF for 30 minutes with one repetition of this procedure. The N-terminal Boc group was then removed by treatment with TFA, the resin washed successively with DMF, DCM and methanol then dried under vacuum. The peptide was cleaved from the resin by treatment with 20 mL liquid HF, 1 mL *p*-cresol and 1 mL *p*-thiocresol at 0 ◦C for 1 hour. 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 lyophilised 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. The 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 minutes at a flow rate of 5 mL/min. The presence of the desired peptides in the fractions was detected by ESMS analysing for the appearance of the [M+H]+ ion (MII = 1716, LaaMII = 1913) and the stronger [M+2H]++ ion (MII = 858.5, LaaMII = 957). 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 1mL/min and a linear gradient from 100% Solvent A to 80% Solvent B over 30 minutes. The fractions containing pure peptide were combined and lyophilised to afford the peptides as white solids (reduced MII yield = 40%, reduced LaaMII yield = 24%).

Oxidation

The purified peptides were oxidised by dissolving in 0.1MNH4HCO3 to a final peptide concentration of 20 μM and stirring vigorously at room temperature for 5 days. The oxidised peptides were purified as described above. Analytical RP-HPLC (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 minutes) and ESMS analysing for the appearance of the [M+H]+ ion (MII = 1712 Da, LaaMII = 1909 Da) and the stronger [M+2H]++ ion (MII = 856.5 Da, LaaMII = 955 Da) confirmed the purity and molecular weight of the synthetic peptides. The fractions containing pure peptide were combined and lyophilised to afford the peptides as white solids (oxidised MII yield = 34%, oxidised LaaMII yield = 10%).

Solution phase synthesis of LaaMII

Crystalline 2-(t-butoxycarbonylamino)-D,L-dodecanoic acid (10 mg, 32 μmol) was dissolved in dry DCM (1 mL). DCC (16 μL of 1M solution, 16 μmol) was added and the solution stirred until no further precipitation could be observed (~15 minutes). This solution was filtered into a solution of purified, authentic α- conotoxinMII in dry DCM (5 mL) and a minimum of DMF under a nitrogen atmosphere. The reaction was allowed to continue overnight. The solution was then diluted with DCM and treated with TFA (10 mL) for 1 hour. The reaction was concentrated and the residue redissolved and concentrated several times to remove all residual TFA. The product was purified on a C4 semi-preparative Vydac®column using a flow rate of 2 mL/min and a linear gradient from 10% solvent B to 80% solvent B over 35 minutes. The product containing fractions were combined and lyophilised to afford LaaMII (8.7 mg, 78% yield).

Comparison of solid phase synthetic product and solution phase synthetic product

Samples of the LaaMII product from each of the synthetic methods (solid phase and solution phase coupling) were prepared by dissolving the peptide in a degassed solution of 50% H2O 50% CH3CN. An aliquot of each of these samples was removed and combined to create a third co-injection sample. These samples were examined by analytical HPLC on an Agilent Technologies Zorbax®SB-C18 3.5μm, 4.6×150 mm column using a solvent gradient from 100% solvent A to 100% solvent B over 9 minutes and a flow rate of 2 mL/min. Detection was achieved using a Shimadzu®SPD-6A UV spectrometric detector monitoring at 214 nm. All samples gave a single peak at a Rt = ~6.9 minutes.

Abbreviations

Laa, 2-amino-D,L-dodecanoic acid; LaaMII, 2-amino-D,L-dodecanoic acid conjugated MII; nAChR, nicotinic acetylcholine receptor; HBTU O-benzotriazol-1-yl-NNN_N_tetramethyluronium hexafluorophosphate; DMF, N_N_-dimethylformamide; TFA, trifluoroacetic acid; DCC, 1,3-dicyclohexylcarbodiimide; DCM, dichloromethane; p-MBHA, *p*-methylbenzhydrylamine; RP-HPLC, reverse phase high performance liquid chromatography; ESMS, electrospray mass spectrometry

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