Solid-Phase Synthesis and Biological Activity of a Thioether Analogue of Conotoxin G1

Jon Bondebjerg,[a] Morten Grunnet,[b] Thomas Jespersen,[b] and Morten Meldal*[a]

A bicyclic thioether analogue of α-conotoxin G1, a neurotoxin found in the venom of cone snails, was synthesized on solid phase. Two successive intramolecular on-bead cyclizations between a cysteine residue and a chloroacetylated reduced peptide bond are the key steps in the synthesis. The first reduced peptide bond was introduced by a reductive alkylation with a 9-fluorenylmethoxycarbonyl protected amino aldehyde, and the second by coupling of a dipeptide building block containing an allyloxycarbonyl protected reduced peptide bond. The desired bicyclic product was obtained as a mixture of two isomers, which were evaluated for their ability to inhibit the muscular nicotinic acetylcholine receptor expressed in Xenopus *oocytes. The two isomers were found to have IC_{50} values (inhibitory activities) of 144 μM and 48 μM, compared to 0.18 μM for native conotoxin G1.*

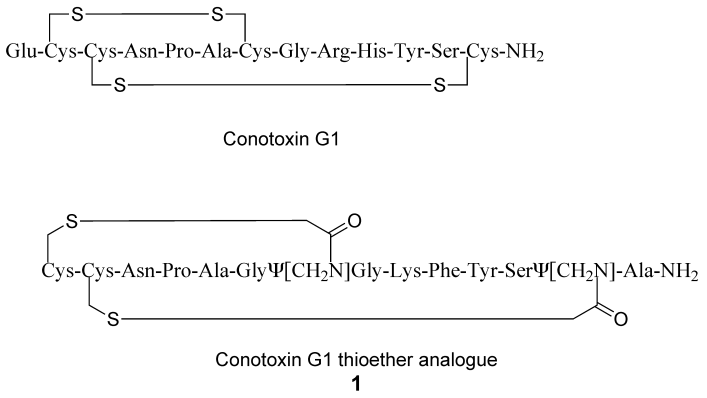
KEYWORDS:

cyclization · inhibitors · macrocycles · peptidomimetics · solid-phase synthesis

Introduction

Cyclization is an important tool for introducing conformational restriction into peptides, which leads to increased in vivo stability, bioavailability, receptor selectivity, and potency.[1–3] By introducing multiple disulfide bridges into short peptide sequences, nature has devised a unique class of biologically active molecules that are able to adopt well-defined three-dimensional structures because of the conformational stability conferred by these disulfide bridges. These highly potent and specific toxins, found in the venom of cone snails,[4] spiders,[5] scorpions,[6] snakes,[7] and lizards,[8] target the ion channels[9] and cell-surface receptors in the neurological system, which results in reduced or blocked neurotransmission and causes paralysis and sometimes death. These neurotoxins have become interesting as biological probes and drug discovery leads[10–15] because ion channels in particular are crucial to many biological processes, such as nerve transmission, hormone secretion, and the generation of cellular energy.[16] A number of potential therapeutic targets include multiple sclerosis, diabetes, and cystic fibrosis.[16] Neurex's prototype drug Ziconotide (SNX-111, Neurex Corp/Elan Pharmaceuticals), an ω-conotoxin from the venom of *conus magus* that targets calcium channels, has recently entered the final clinical trials before its intended commercial release as a potent analgesic for chronic pain.[15] This drug has paved the way for research and development of conotoxins and synthetic analogues. It was recently demonstrated that replacement of the smaller disulfide bridge in α-conotoxin S1 with a lactam group resulted in complete loss of activity, whereas replacement of the larger disulfide bridge gave a 60-fold reduction in affinity for one subsite of the muscular nicotinic acetylcholine receptor (nAchR), and a 70-fold increase in affinity for the other.[17] Thus, moderate modifications of the knotted disulfide framework are allowed and are in some cases beneficial from a biological standpoint.

The present report describes the design of a conotoxin analogue based on thioether bridges, previously demonstrated as disulfide bond mimetics that offer advantages under physiological conditions.[18, 19] The thioether bond is formed between a cysteine residue and an internally *N*-haloacetylated peptide. This highly selective reaction has previously been used for intermolecular ligation of large, unprotected peptide fragments.[20] It has also been used to form intramolecular thioether



Scheme 1. *Structures of conotoxin G1 and the thioether analogue* ***1****.*

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bridges,[18, 21, 22] however, in these reports the haloacetylation is performed on the N-terminal end of the peptide, thus effectively terminating the sequence after the first cyclization.

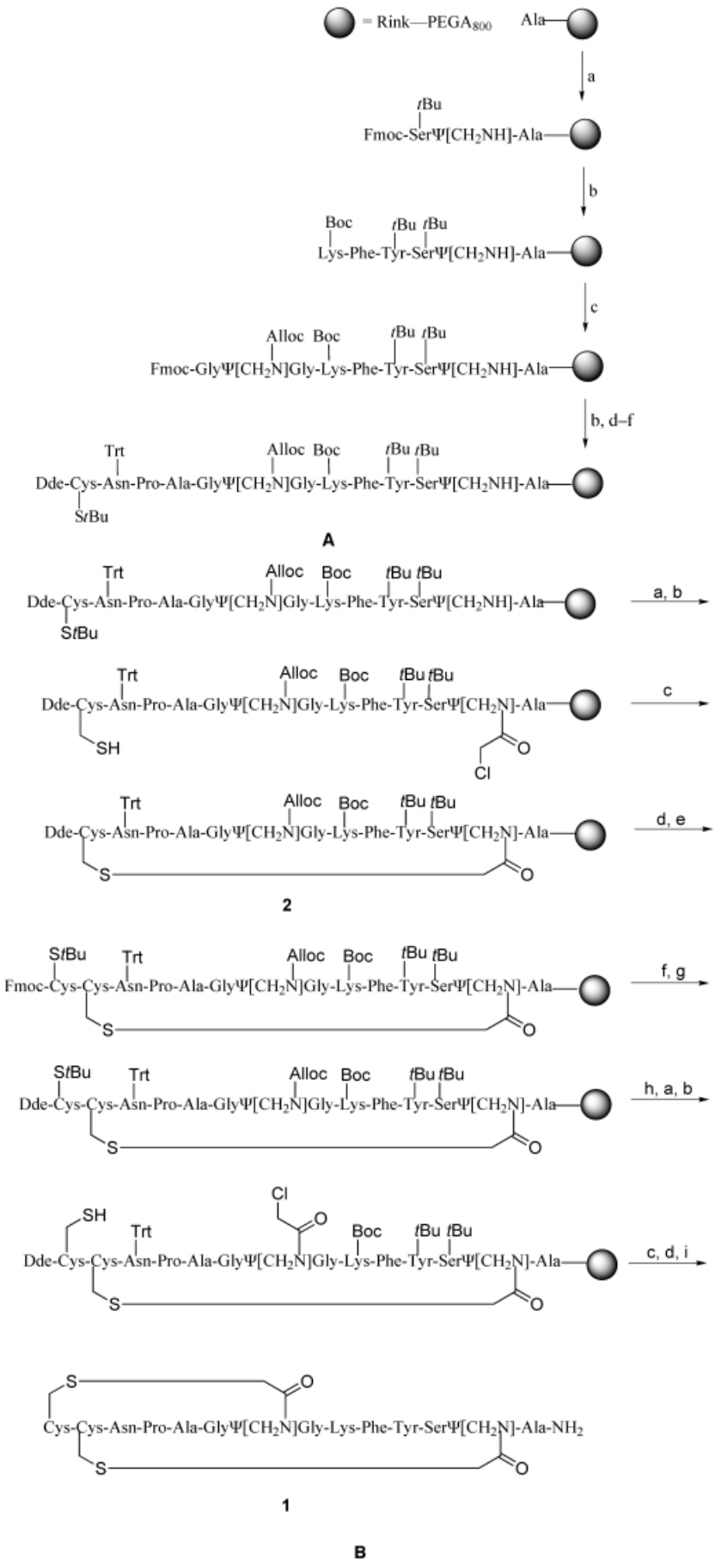
Herein, we report a solid-phase approach to the synthesis of an analogue of conotoxin G1[23–25] in which *both* the disulfide bridges have been replaced with thioether bridges (Scheme 1). The synthetic methodology (Scheme 2), which is based on intramolecular on-bead cyclization between a cysteine residue and a chloroacetylated reduced peptide bond, is applicable to continous solid-phase peptide synthesis of knotted bicyclic structures that resemble the structural framework found in conotoxins.

A previous report on modification of conotoxin G1 demonstrated that neither truncation of the sequence to the des-Glu analogue nor substitution of His10 with a Phe residue results in significantly reduced potency.[26] The conotoxin G1 thioether analogue was synthesized as the des-Glu sequence. To facilitate synthesis, a Lys residue was used instead of Arg9 and a Phe residue instead of His10. Arg9 has recently been identified as the key residue in α-conotoxin G1,[27] however, Lys is found in this position in the equally potent α-conotoxins M1 and S1A.[27]

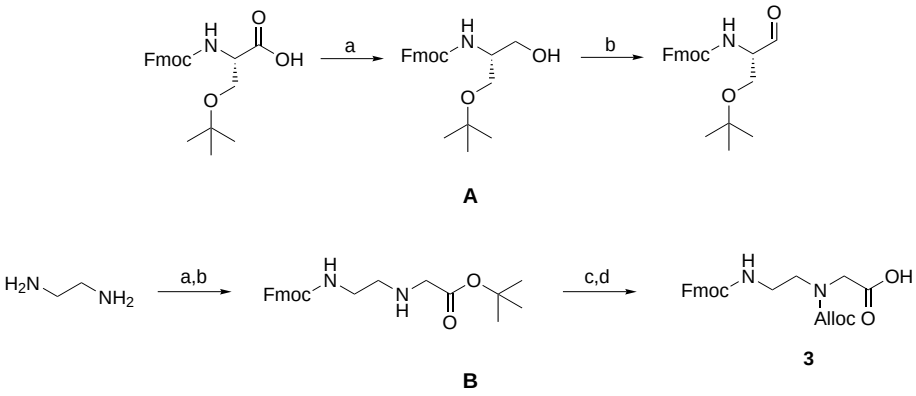
Results and Discussion

Synthesis of a thioether conotoxin G1 analogue

The chemistry was developed on poly(ethylene glycol)-poly(acryl amide) copolymer ($PEGA_{800}$) resin with the Rink amide linker (*p*-[(*R*,*S*)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid; Scheme 2a, b). An Ala residue was attached to the linker by standard 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis



Scheme 2. A) *Solid-phase synthesis (SPPS) of the thioether analogue of conotoxin G1: assembly of the peptide chain. a) Fmoc-Ser(tBu)-H (3 equiv), $NaBH_3CN$ (6 equiv), AcOH (20 equiv), DMF; b) Several cycles of SPPS; c) Fmoc-GlyΨ[CH_2N(Alloc)]Gly-OH (3 equiv), TBTU (2.88 equiv), NEM (4 equiv), DMF; d) Fmoc-Cys(StBu)-OH (3 equiv), TBTU (2.88 equiv), NEM (4 equiv), DMF; e) 20% (v/v) piperidine in DMF; f) 2-acetyldimedone (3 equiv), DMF.* ***B)*** *Formation of macrocyclic rings: a) $(ClCH_2CO)_2O$ (3 equiv), NEM (3 equiv), DCM; b) Bu_3P (100 equiv), THF, H_2O; c) NEM (3 equiv), DMF; d) 3% (v/v) NH_2NH_2, DMF; e) Fmoc-Cys(StBu)-OH (3 equiv), TBTU (2.88 equiv), NEM (4 equiv), DMF; f) 20% (v/v) piperidine in DMF; g) 2-acetyldimedone (3 equiv), DMF; h) $(Ph_3P)_4Pd$ (3 equiv), NEM, AcOH, $CHCl_3$; i) TFA/TIPS (95:5).*



Scheme 3. *A) Preparation of Fmoc-Ser(tBu)-H: a) iBuOCOCl, NEM, THF, 82%; b) Dess–Martin periodinane, DCM, 77%. B) Preparation of building block* ***3****: a) $ClCH_2COOtBu$, DCM, 80%; b) Fmoc-OSu, DIPEA, DCM, 90%; c) Alloc-Cl, DIPEA, DCM, quant.; d) TFA, TIPS, 56%. DIPEA = diisopropylethylamine.*

(SPPS). After Fmoc deprotection, the free amine was reductively alkylated with Fmoc-Ser(*t*Bu)-H by reaction with $NaBH_3CN$ in DMF/AcOH (DMF = *N*,*N*-dimethylformamide; Ac = acetyl). The reductive alkylation proceeds quantitatively to the monoalkylated product in 3–4 h at room temperature, as indicated by a negative result from the Kaiser test.[28] Fmoc was removed and the Tyr(*t*Bu), Phe, and Lys(Boc) residues (Boc = *tert*-butyloxycarbonyl) were then coupled as their Fmoc-protected pentafluorophenyl (Pfp) esters by standard SPPS. According to HPLC and MS analysis, the sterically congested reduced peptide bond was not acylated under these conditions. The inherent problem of dialkylation when Gly is used as a reactant in the reductive alkylation led us incorporate the next two Gly residues by coupling of the dipeptide building block **3** (Scheme 3B), which contains an allyloxycarbonyl (Alloc) protected reduced bond, to the growing peptide. This building block was prepared in solution, in part based on a previous report,[29] and coupled through *N*-[(1*H*-benzotriazol-1-yl)-(dimethylamino)-methylene]-*N*-methylmethan-aminium tetrafluoroborate *N*-oxide (TBTU) activation. Ala, Pro, and Asn(Trt) residues were coupled as their Fmoc-protected Pfp esters, followed by attachment of Fmoc-Cys(S*t*Bu)-OH (S*t*Bu = *tert*-butylthio) by TBTU activation. The Fmoc group was then exchanged for the more base-stable N^{ε}-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde)[30, 31] protecting group. The Ser reduced bond

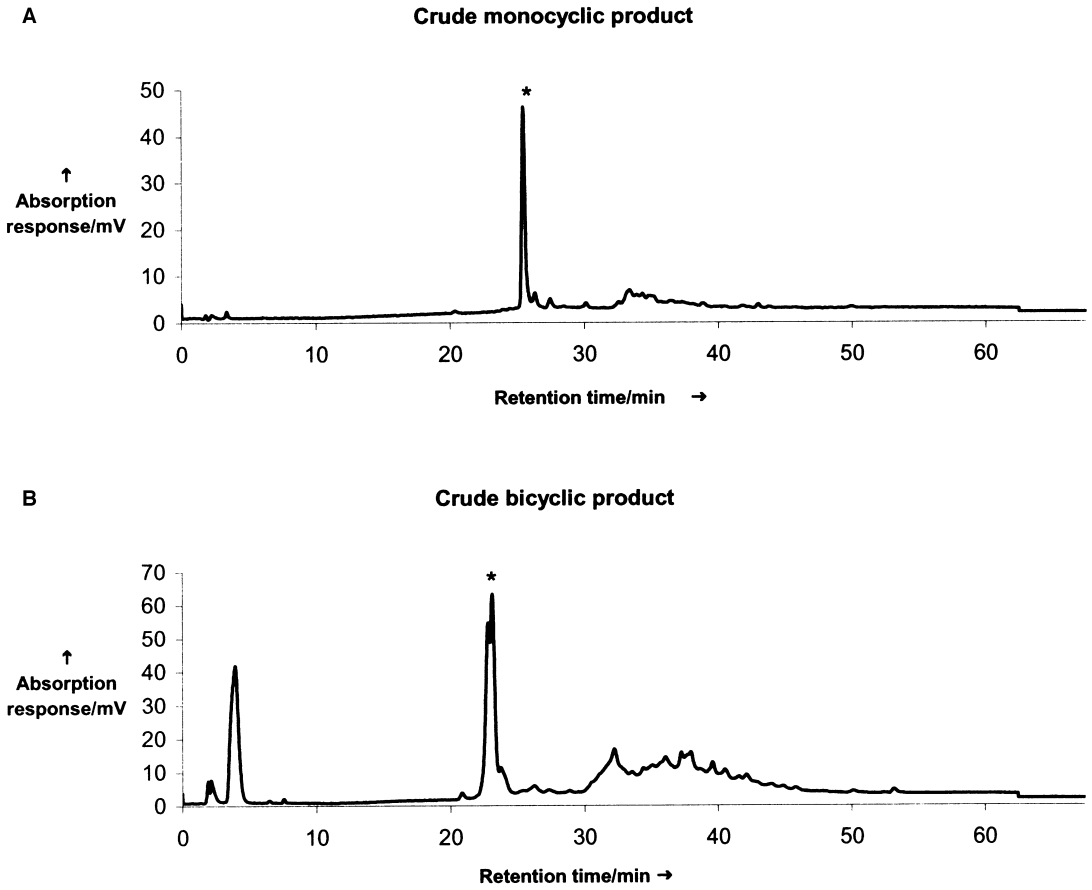


Figure 1. *RP-HPLC chromatograms of: A) crude monocyclic product; B) crude bicyclic product. The peaks corresponding to the desired products are marked with a*.*

was acylated quantitatively with chloroacetic anhydride in dichloromethane/*N*-ethylmorpholine (DCM/NEM) in less than 30 minutes, as indicated by a negative result from the Chloranil test for secondary amines.[32] The S*t*Bu cysteine protection group was removed with Bu_3P in tetrahydrofuran (THF)/H_2O to give the free thiol cyclization precursor. Cyclization to form the resin-bound monocyclic intermediate **2** was achieved by treatment with NEM in DMF at room temperature overnight and the presence of **2** was confirmed by a negative result from the Ellman test for free thiols.[33] The purity of the crude monocyclic product **2** was determined by cleaving an aliquot of the peptide from the resin and analyzing it by reversed-phase HPLC (RP-HPLC; Figure 1 A) and ES-MS. The resin-bound monocyclic intermediate **2** was treated with 3% hydrazine in DMF to remove the Dde group and coupled to Fmoc-Cys(S*t*Bu)-OH by TBTU activation. The Fmoc group was again replaced with the Dde group, and the Alloc group was subsequently removed by treatment with $(Ph_3P)_4Pd$ in $CHCl_3$/NEM/AcOH. The liberated reduced bond was acylated with chloroacetic anhydride, and the thiol was deprotected by treatment with Bu_3P. The second thioether bridge was formed by treatment with NEM in DMF for 7 days to give the bicyclic resin-bound peptide. The Dde group was removed and the product was cleaved from the resin by treatment with trifluoroacetic acid/triisopropylsilane (TFA/TIPS). The crude bicyclic product **1** (Figure 1 B) was obtained as a mixture of two distinct isomers, which were isobaric according to high-resolution MALDI-TOF MS results. The two isomers were separated by RP-HPLC to give the pure isomers **1 A** and **1 B** in yields of 3.4% and 3.1%, repectively, based on the initial loading of Fmoc – Rink – PEGA. Chiral HPLC of the two isomers did not reveal any isomeric peaks. Racemization of the Ser α carbon atom, which could potentially occur during the reductive alkylation, was not detected by 2D NMR studies of both isomers; this result indicates that **1 A** and **1 B** are not epimers. However, the 2D TOCSY spectra showed the presence of many (8 for **1 A**) conformers, which indicates that the two isomers are in fact slowly exchanging conformers that arise from rotation around the three tertiary amide bonds. NMR temperature experiments showed that heating the sample to 50 °C in D_2O/H_2O did not cause coalescence of the isomeric resonances, which indicates that the different conformers are quite stable.

Biological results

1 A and **1 B** were tested for their ability to inhibit the conducting property of the adult muscular nAchR expressed in *Xenopus* oocytes. For an initial characterization of the muscular nicotinic acetylcholine receptor, the four subunits $\alpha 1$, $\beta 1$, δ, and ε were injected into *Xenopus* oocytes in approximately equal molar amounts. Oocytes were clamped at -50 mV and acetylcholine receptors were activated by application of acetylcholine (10 μM). This process gave rise to an inward current with rapid activation and stable activity as long as the agonist was applied, as shown in Figure 2 A. Current activity was easily terminated by washing out the agonist, and receptor activity could be repeated after 3 min washing with Kulori medium without any noticeable difference in the magnitude of the observed current activity ($n = 5$). An inward current could not be observed when 10 μM acetylcholine was applied to non-injected oocytes (data not shown). For a more complete examination of acetylcholine activation, oocytes were clamped at -50 mV and superfused with different concentrations of agonist interrupted by washes with Kulori medium (Figure 2 B). Application of a solution of acetylcholine elicited a concentration-dependent activation of the acetylcholine receptor. When high concentrations of acetylcholine were applied (100 and 300 μM) a reduction in current amplitude could be observed even in the presence of agonist, which indicates that the muscular acetylcholine receptor under-

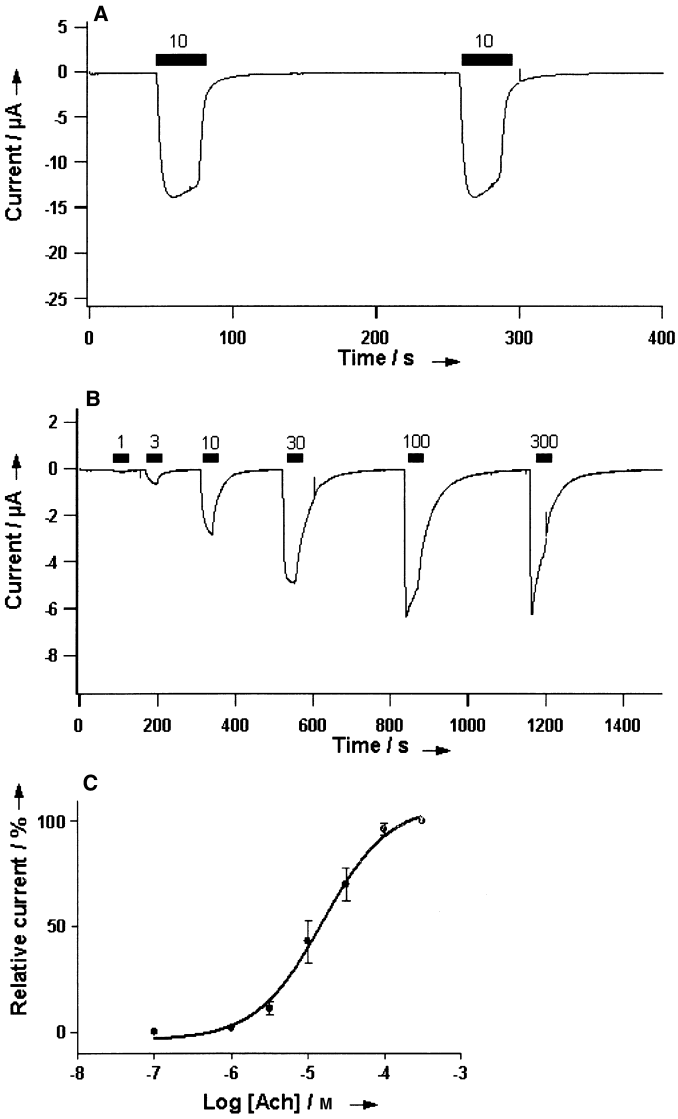


Figure 2. *Initial characterization of adult muscular nicotinic acetylcholine receptor expressed in* Xenopus *oocytes. A) Oocytes expressing muscular acetylcholine receptors were clamped at -50 mV and activated by a 30-s application of acetylcholine (10 μM; black bars), which resulted in a sustained inward current. B) Concentration-dependent activation of acetylcholine receptors. Oocytes clamped at -50 mV were superfused for 30 s with increasing concentrations of acetylcholine (black bars) followed by washes. Acetylcholine concentrations are given in μM in the figure. C) Summary of acetylcholine activation. The IC_{50} value was calculated to be 15.4 ± 6μM and $R^2 = 0.99$ (n = 6).*

goes a partial desensitization at high acetylcholine concentrations. The observed current amplitude was therefore recorded as the peak current. A summary of these activation experiments is presented in Figure 2C. The IC_{50} value for acetylcholine activation was calculated to be 15.4 ± 6 μM (linear regression, where $R^2 = 0.99$ and $n = 6$). Once the normal expression of the muscular nicotinic acetylcholine receptor in oocytes had been established, the inhibitory properties of **1A** and **1B** were tested. In addition, the potency of the crude, unpurified mixture of the two isomers, named **1C**, was also tested. Wild-type conotoxin G1 was used as a reference compound. Application of **1A** was performed in the following way: Oocytes clamped at −50 mV were initially activated by treatment with 10 μM acetylcholine for 30 s, followed by at least 3 min wash with Kulori medium. This activation was followed by 30 s application of 10 μM acetylcholine in the presence of toxin then at least 3 min wash. Finally oocytes were again activated by 30 s application of acetylcholine. Application of agonist both before and after application of **1A** made it possible to determine the degree of desensitization of the receptor. The inhibitory impact of the applied antagonist was established by measuring the magnitude of the observed desensitization. Once the potency of a specific concentration of **1A** had been confirmed, the same oocyte was exposed to the other toxin isomers in a similar manner before the experiment was terminated by application of wild-type conotoxin G1. A representative current trace from such experiments is shown in Figure 3A. When toxins were applied in high enough concentrations to inhibit more than 50% of the current, the current trace showed an initial peak followed by a reduced and stable current level. In these cases, the degree of inhibition was determined from the stable plateau current. The observations indicated that the on-rate for toxin binding is slow. On- and off-rates of toxin inhibition were not investigated further. A summary of the inhibitory activities of **1A**, **1B**, and **1C** is presented in Figure 3B. Application of antagonists were limited to 100 μM because of the limited availability of the compounds. Calculated IC_{50} values for inhibition and linear regression coefficients for the different isomers were as follows: **1A** = 144 ± 210 μM ($R^2 = 0.70$); **1B** = 48 ± 55 μM ($R^2 = 0.97$); **1C** = 1.5 ± 0.7 μM ($R^2 = 0.99$). The IC_{50} value of the reference compound conotoxin GI was calculated to be 0.176 ± 0.004 μM ($R^2 = 1.00$). For all experiments $n = 4$. Thus, the two isomers **1A** and **1B** were found to be 800-fold and 244-fold less potent, respectively, than native conotoxin, which indicates a slight preference for isomer **1B**. The crude mixture of the two isomers was found to be only eightfold less potent than conotoxin G1; this indicates that impurities present in the mixture are highly potent. Further studies may determine if the thioether modification is biologically tolerated in conotoxin G1. Furthermore, changing the orientation of the thioether bridges could have a significant impact on potency and selectivity, as previously reported for lactam analogues of conotoxin S1.[17]

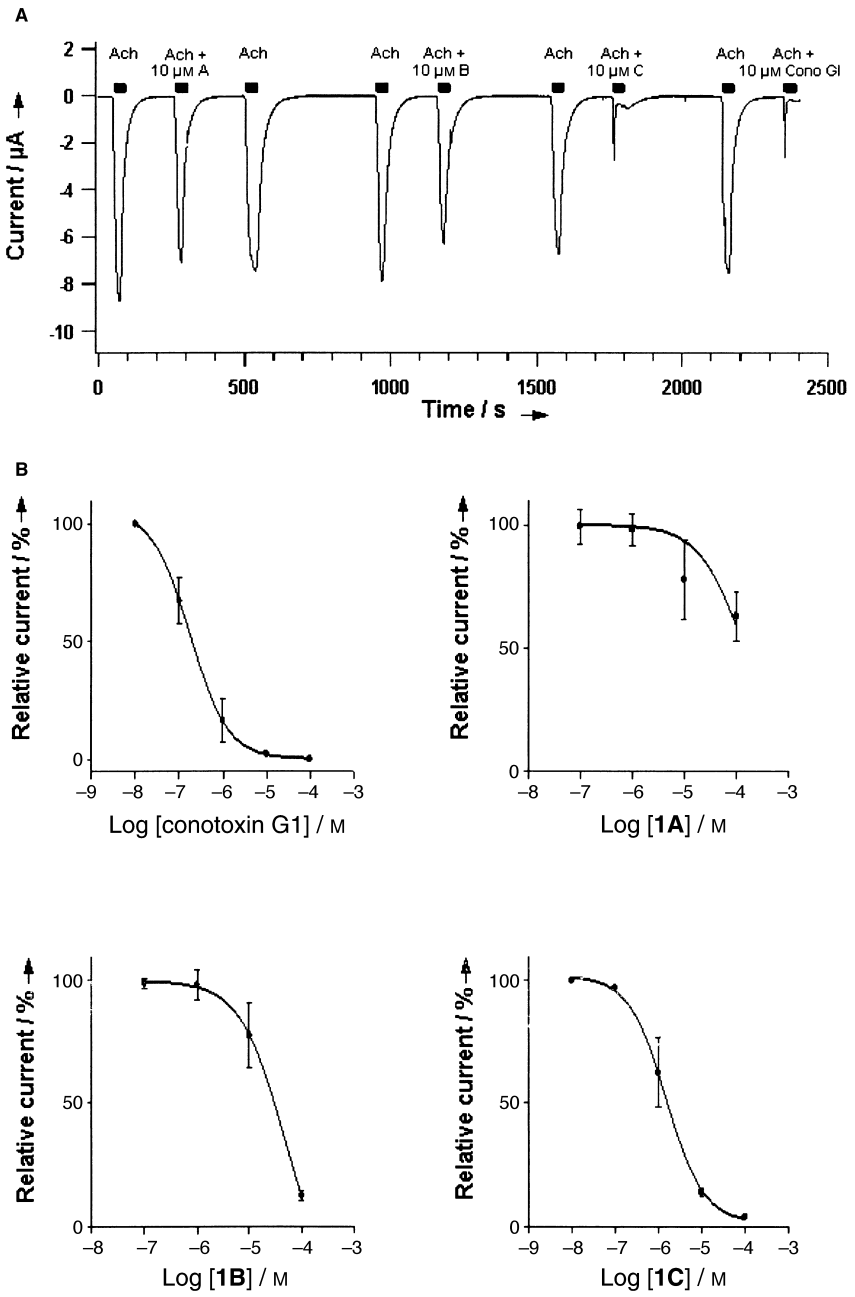


Figure 3. *Inhibitory properties of different conotoxin G1 derivates. A) Oocytes expressing adult rat muscular nicotinic acetylcholine receptors were clamped at −50 mV and exposed to acetylcholine (10 μM) or acetylcholine + different derivates of conotoxin (overall 10μM) for 30 s (black bars). Experiments were terminated by application of wild-type conotoxin G1 to confirm the nature of the expressed current. B) Dose–response curves for the inhibitory property of conotoxin G1, **1A**, **1B**, and **1C** (crude). Calculated IC_{50} values and regression coefficients are given in the text.*

In conclusion, the solid-phase synthesis of a bicyclic thioether analogue of conotoxin G1 has been described. The thioether bridges were formed by intramolecular reactions between a cysteine residue and a chloroacetylated reduced bond. The synthetic methodology presented here should be perceived as a general tool for introducing conformational constraints into a peptide to mimic the disulfide-knotted motif found in naturally occurring toxins. The two isomers tested herein were found to be significantly less potent than native conotoxin G1. However, the observed activity provides hope that other analogues may be as potent as, or more potent than the parent toxin.

Experimental Section

Reagents and general methods: All solvents were HPLC grade. Anhydrous solvents were obtained by storing the solvents over 4-Å activated molecular sieves. Degassed solutions were obtained by bubbling with Ar for 10 minutes. PEGA resin was purchased from Polymer Laboratories, England. Fmoc amino acids, Rink linker, and TBTU were purchased from Bachem. 2-Acetyldimedone was prepared according to a previous procedure.[34] Dess–Martin periodinane was purchased from Relakem (Riga, Latvia). All other starting materials were purchased from Fluka and Sigma–Aldrich and used without further purification. Solid-phase reactions were performed in flat-bottomed polyethylene syringes equipped with sintered Teflon filters (50-μm pores), Teflon tubing, Teflon valves for flow control, and a suction device to drain the syringes from below. Fmoc deprotection was performed with 20% piperidine in DMF (2 + 10 min). TBTU couplings were performed by dissolving the acid (3 equiv) in DMF with NEM (4 equiv), followed by addition of TBTU (2.88 equiv). The resulting solution was preactivated for 10 min before use (reaction time 2 h). Pfp esters were coupled by using the ester (3 equiv) with 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH; 1 equiv) present. The disappearence of the bright yellow color indicated complete reaction of the resin-bound amino groups. Unless otherwise noted, solid-phase reactions were generally run in sufficient solvent to cover the resin. Resin loadings were determined by Fmoc cleavage and optical density measurements at 290 nm by using a calibration curve. Routine NMR data were acquired on a Bruker Avance DRX 250 spectrometer. Chemical shifts are reported in ppm downfield, relative to internal solvent peaks (2.49 ppm for dimethylsulfoxide (DMSO-d_6), 7.25 ppm for $CDCl_3$). ES-MS spectra were obtained in the positive mode on a Fisons VG Quattro 5098 spectrometer. LC-MS spectra were obtained in the positive mode on a Hewlett Packard MSD1100 LC-MS apparatus. MALDI-TOF spectra were recorded on a Bruker Reflex III MALDI-TOF mass spectrometer with α-cyano-4-hydroxycinnamic acid as the matrix. High-resolution MALDI-TOF MS spectra were recorded at the University of Odense, Denmark on a 4.7-T Ionspec FT-ICR mass spectrometer, with 2,5-dihydroxybenzoic acid as both matrix and internal reference (substance P (RPKPQQFF GLM-NH_2) was used for **1A** and **1B**). The TLC plates used were Merck silica gel 60F_{254} on aluminum. Visualization was achieved with UV light when applicable, or developed by staining with the AMC reagent (21 g $(NH_4)_6Mo_7O_{24}$, 1 g $Ce(SO_4)_2$, 31 mL H_2SO_4, 500 mL water). Analytical HPLC was performed on: 1) a Waters system (490E detector at 215 and 280 nm, two 510 pumps with gradient controller, and a Zorbax RP-18 column, 300 Å, 0.45 × 50 mm, with eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile/water 9:1) used in a linear gradient (0% B → 100% B in 25 min.)), or 2) a Merck–Hitachi D7000 system (L-4250 UV/Vis detector 215 nm, L-6250 intelligent pump, with eluents A (0.1% TFA in water) and B (0.1% TFA:MeOH 1:9) used in a linear gradient (0% B → 100% B in 25 min.)). Retention times refer to the designated system. Preparative HPLC was performed on a Waters 600E system (Waters 991 photodiode array detector at 215 and 280 nm, FOXY fraction collector) connected to a Millipore Delta Pak RP-18 column (47 × 300 mm), with eluents A (0.1% TFA in water) and B (0.1% TFA in MeCN/H_2O 9:1) in a linear gradient starting at 100% A, with a slope of 0.5% min^{-1} and a flow of 20 mL min^{-1}.

***tert*-Butyl *N*-(2-aminoethyl)glycinate**:[29] A solution of *tert*-butyl chloroacetate (10 mL, 63 mmol) in DCM (100 mL) was added dropwise to a solution of ethylenediamine (45 mL, 830 mmol) in DCM (100 mL) at 0 °C over a period of 2 h. The mixture was warmed slowly to room temperature and then stirred for a period of 18 h. The reaction mixture was washed with water 3 times, and the combined aqueous wash was back-extracted with DCM. The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo to give a clear oil. Yield: 10 g (82%). ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 1.37$ (br, 3H), 1.40 (s, 9H), 2.57–2.62 (m, 2H), 2.69–2.74 (m, 2H), 3.22–3.23 (s, 2H) ppm.

***tert*-Butyl *N*-[2-(*N*-9-fluorenylmethoxycarbonyl)aminoethyl]glycinate hydrochloride**:[29] A solution of *N*-(9-fluorenylmethoxycarbonyl)succinimide (9.5 g, 29 mmol) in DCM (50 mL) was added to a solution of *tert*-butyl *N*-(2-aminoethyl)glycinate (5 g, 29 mmol) and DIPEA (4.8 mL, 29 mmol) in DCM (100 mL) at room temperature over a period of 1 h. The reaction mixture was stirred for 18 h and then washed with HCl (1 M; × 5) and once with brine. The organic layer was dried (Na_2SO_4) and partially concentrated (to about 50 mL) in vacuo. Cooling to −20 °C for a period of 18 h gave the desired compound as a white solid, which was isolated by filtration, washed with ice-cold DCM, and dried. Yield: 11.1 g (88%). R_f (petroleum ether/ethyl acetate 1:3, + a few drops of AcOH) ≈ 0.27. ^{1}H NMR and ^{13}C NMR as reported;[29] LC-MS calcd for $C_{23}H_{29}N_2O_4Cl$: 432.18 $[M]^+$; found (negative mode): *m/z* 431.1 $[M-H]^+$.

***N*-[2-(*N*-9-Fluorenylmethoxycarbonyl)aminoethyl]-*N*-(allyloxycarbonyl)]glycine (3)**: *tert*-Butyl-*N*-[2-(*N*-9-fluorenylmethoxycarbonyl)-aminoethyl]glycinate hydrochloride (2 g, 4.65 mmol) was dissolved in DCM (30 mL). DIPEA (1.6 mL, 9.3 mmol) and allyl chloroformate (750 μL, 7 mmol) were added. The mixture was stirred for 2 hours, washed with 1 M HCl and brine, dried ($MgSO_4$), and concentrated in vacuo to give a clear oil. TLC (petroleum ether/ethyl acetate 2:1) showed one spot ($R_f \approx 0.39$). Efforts to crystallize the intermediate failed. The oil was redissolved in DCM (30 mL) and TIPS (0.5 mL) was added, followed by 95% TFA (10 mL). The slightly yellow solution was stirred for 4 hours then concentrated to give a yellow residue, which was coevaporated with chloroform twice to give a yellow oil. The crude product was dissolved in the minimum amount of ethanol (96%), diluted with water until the solution became hazy, and then cooled to −20 °C overnight. The white solid was isolated by filtration, washed with water, and dried in vacuo. The solid was then dissolved in a minimum of ethyl acetate, diluted with petroleum ether until the solution became hazy, and cooled to −20 °C for a few hours. Compound **3** was isolated by filtration, washed with petroleum ether (× 3), and dried. Yield: 0.9 g. A further 0.2 g could be isolated from the filtrate by dilution with additional petroleum ether. Total yield: 1.1 g (56%). R_f (chloroform/methanol 5:1) ≈ 0.43; ^{1}H NMR (DMSO-d_6, 250 MHz): $\delta = 3.17–3.20$ (m, 2H, FmocNHCH_2), 3.35 (br, 2H, FmocNHCH$_2$CH_2), 3.94–3.97 (app d, $J = 6.6$, 2H, CH_2COOH), 4.22–4.25 (t, $J = 6.2$, 1H, CH(Fmoc)), 4.30–4.33 (d, $J = 6.2$, 2H, CH_2(Fmoc)), 4.51–4.52 (br, 2H, OCH_2CH=CH$_2$), 5.12–5.33 (m, 2H, CH=CH_2), 5.79–5.97 (m, 1H, C*H*=CH$_2$), 7.31–7.45 (m, 5H, Ar(Fmoc) + N*H*), 7.68–7.70 (d, $J = 7.2$, 2H, Ar(Fmoc)), 7.88–7.91 (d, $J = 7.3$, 2H, Ar(Fmoc)), 12.77 (br, 1H, COO*H*) ppm. ^{13}C NMR (DMSO-d_6, 62.9 MHz; 2 rotamers, rotameric signals given in parentheses):

$\delta = 38.8$((39.2); FmocNHCH$_2$), 47.1 (CH(Fmoc)), 47.4((48.1); FmocNHCH$_2$CH$_2$), 49.2((49.4); CH$_2$COOH) 65.6 (CH$_2$(Fmoc)), 65.8 (OCH$_2$CH=CH$_2$), 116.7 ((117.1); CH=CH$_2$), 120.5 (Ar(Fmoc)), 125.5 (Ar(Fmoc)), 127.4 (Ar(Fmoc)), 128.0 (Ar(Fmoc)), 133.5 (CH=CH$_2$), 141.1 (Ar(Fmoc)), 144.3 (Ar(Fmoc)), 155.6((155.8); CO(Alloc)), 156.5 (CO(Fmoc)), 171.5((171.6); COOH) ppm. HR-MS (MALDI): calcd for $C_{23}H_{24}N_2O_6Na$: 447.1527 [*M*Na]$^+$; found: *m/z* 447.1519. HPLC purity: 97%. R_t (A) = 15.06.

(*S*)-*N*-α-(Fluorenylmethyloxycarbonyl)-*O*-(*tert*-butyl)-serinol: Minor modifications were made to a previous procedure for preparation of amino alcohols.[35] (*S*)-*N*-α-(Fluorenylmethyloxycarbonyl)-*O*-(*tert*-butyl)-serine (3 g, 7.8 mmol) was dissolved in THF (45 mL) and cooled in an ice bath. NEM (990 μL, 1 equiv) was added, followed by isobutylchloro formate (1120 μL, 1.1 equiv). After 10 min, the precipitate was filtered off and washed with THF. The combined filtrate and washings were cooled to 0 °C and a solution of $NaBH_4$ (480 mg, 1.6 equiv) in water (5 mL) was added dropwise. The mixture was stirred for 15 min after the evolution of gas had subsided. After dilution with water (250 mL), the crude product was isolated by extraction with ethyl acetate (× 3). The combined organic layers were washed with saturated $NaHCO_3$ (× 3), 1 M HCl (× 2), and brine, then dried ($MgSO_4$). Concentration in vacuo gave the product as a clear oil, which was found to be sufficiently pure for further use. Yield: 2.36 g (82%). $R_f = 0.50$ (petroleum ether/ethyl acetate 1:1). ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 1.10$ (s, 9H), 2.83 (br, 1H) 3.50 (br, 2H), 3.61 – 3.79 (m, 3H), 4.11 – 4.17 (t, $J = 6.84$, 1H), 4.30 – 4.32 (d, $J = 6.85$, 2H), 5.44 (br, 1H), 7.17 – 7.34 (m, 4H), 7.50 – 7.53 (d, $J = 7.30$, 2H), 7.66 – 7.68 (d, $J = 7.35$, 2H) ppm. ^{13}C NMR ($CDCl_3$, 62.9 MHz): $\delta = 27.8$, 47.7, 52.1, 63.8, 64.8, 67.2, 74.2, 120.4, 125.5, 127.5, 128.1, 141.7, 144.4 ppm. ES-MS: mass calcd for $C_{22}H_{28}NO_4$ 370.20 [*M*H]$^+$; found: *m/z* 370.29.

(*S*)-*N*-α-(Fluorenylmethyloxycarbonyl)-*O*-(*tert*-butyl)-serinal: The amino alcohol obtained above was dissolved in DCM (50 mL). The solution was cooled in an ice bath and Dess – Martin periodinane (4 g, 1.5 equiv) was added in one portion. The mixture was stirred for 30 min then warmed to room temperature and stirred for 1 h. The reaction was quenched by addition of saturated aq. $NaHCO_3$ and 10% aq. $Na_2S_2O_3$ (20 mL each). After 10 min vigorous stirring the organic layer was separated, washed with brine, and dried ($MgSO_4$). Filtration and evaporation of the solvent gave the crude aldehyde as a yellowish oil. This was dissolved in chloroform and left in a freezer for 1 h. The precipitate was filtered off and the filtrate concentrated in vacuo. The residue contained only one major component according to TLC (petroleum ether/ethyl acetate 1:1), however, minor impurities (< 5%) were observed by ^{1}H NMR spectroscopy. This material was used directly without further purification. Yield: 1.8 g (77%). $R_f = 0.74$ (petroleum ether/ethyl acetate 1:1). ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 1.07$ (s, 9H), 3.51 – 3.57 (dd, $J = 9.5$, 4.4, 1H), 3.84 – 3.89 (dd, $J = 9.4$, 3.0, 1H), 4.12 – 4.18 (t, $J = 6.80$, 1H), 4.24 – 4.40 (m, 3H), 5.58 (br, 1H), 7.19 – 7.33 (m, 4H), 7.51 – 7.53 (d, $J = 7.30$, 2H), 7.65 – 7.69 (d, $J = 7.35$, 2H), 9.53 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 62.9 MHz): $\delta = 27.6$, 47.6, 60.4, 60.9, 67.6, 74.2, 120.4, 125.5, 127.5, 128.2, 141.7, 144.2, 156.7, 199.6 ppm. ES-MS: mass calcd for $C_{22}H_{26}NO_4$: 368.19 [*M*H]$^+$; found: *m/z* 368.32.

Synthesis of the thioether analogue of conotoxin G1: Lyophilized Fmoc – Rink – PEGA$_{800}$ resin (1 g, $L \approx 0.27$ mmol g^{-1}) was Fmoc deprotected and washed with DMF (× 5). Fmoc-Ala-OPfp (3 equiv) was coupled onto the resin and the product washed with DMF (× 5). The Fmoc group was removed and the resin was washed with DMF (× 5). AcOH (325 μL, 20 equiv), $NaBH_3CN$ (115 mg, 6 equiv), and Fmoc-Ser(*t*Bu)-H (3 equiv) were added in DMF (7 mL). After 3.5 h, the Kaiser test gave a negative result and the resin was washed with DMF (× 3), 96% EtOH, and DMF (× 3). The Fmoc group was removed and the resin washed with DMF (× 5). Fmoc-Tyr(*t*Bu)-OPfp, Fmoc-Phe-OPfp, Fmoc-Lys(Boc)-OPfp, Fmoc-Glyψ[CH$_2$N(Alloc)]Gly-OH (3 equiv of building block **3**; coupled by TBTU activation), Fmoc-Ala-OPfp, Fmoc-Pro-OPfp, Fmoc-Asn(Trt)-Opfp, and Fmoc-Cys(S*t*Bu)-OH (coupled by TBTU) were then coupled sequentially by using standard procedures. The Fmoc group was removed and the resin was washed with DMF (× 5) and treated overnight with 2-acetyldimedone (147 mg, 3 equiv) in DMF (7 mL). The resin was washed with DMF (× 5) and DCM (× 5). Chloroacetic anhydride (139 mg, 3 equiv) and NEM (103 μL, 3 equiv) were added in DCM (10 mL), and after 30 min a negative Chloranil test for secondary amines indicated complete acylation of the reduced bond. The resin was washed with DCM (× 5) and treated with Bu_3P (6.7 mL, 100 equiv) in THF/H_2O (19:1; 7 mL) for 1 h. The resin was then washed with DCM (× 5) and DMF (× 5) and left in DMF (7 mL) and NEM (103 μL, 3 equiv) until an Ellman test for free thiols was no longer positive, which indicates complete formation of the first thioether bridge (32 h). An aliquot (a few mg) of the resin was removed, treated with DMF/allyl alcohol/80% hydrazine hydrate (46:3:1; 3 × 3 min), washed with DMF (× 5), and cleaved by treatment with TFA/TIPS (95:5) for 2 h. The cleavage mixture was concentrated to dryness by using a speed vac apparatus. The residue was dissolved in water (500 μL) and extracted with diethyl ether (× 4). The aqueous phase (20 μL) was subjected to HPLC analysis. The identity of the desired monocyclic intermediate **2** was verified by ES-MS: calcd for $C_{55}H_{81}N_{14}O_{15}S$: 1209.57 [*M*H]$^+$; found: *m/z* 1209.3, 605.3 [*M*+2H]$^{2+}$. The rest of the resin was treated with DMF/allyl alcohol/80% hydrazine hydrate (46:3:1; 3 × 3 min) and washed with DMF (× 5). Fmoc-Cys(S*t*Bu)-OH was then coupled by TBTU activation. The resin was washed with DMF (× 5) and the Fmoc group was removed, followed by washing with DMF (× 5). 2-Acetyldimedone (147 mg, 3 equiv) was added in DMF (7 mL) and the resin was left for a period of 18 h. The resin was washed with DMF (× 5). The Alloc group was removed by treatment with $(Ph_3P)_4Pd$ (1 g, 3 equiv) under Ar in a degassed solution of $CHCl_3$/AcOH/NEM (92.5:5:2.5; 7 mL) for 7 h. The resin was washed with DCM (× 5) and DMF (× 5). The reduced bond was acylated with chloroacetic anhydride (139 mg, 3 equiv) and NEM (103 μL, 3 equiv) in DCM (10 mL) for 30 min then washed with DCM (× 5). The *tert*-butylthio group was removed by treatment with Bu_3P (6.7 mL, 100 equiv) in THF/H_2O (19:1; 7 mL) for 1 h. The resin was washed with DCM (× 5) and DMF (× 5). The resin was left in DMF (7 mL) and NEM (103 μL, 3 equiv) for 7 days, after which time an Ellman test gave a negative result (the reaction was not monitored during this time). The resin was washed with DMF (× 5). The Dde protection group was removed by treatment with 3% hydrazine hydrate in DMF (3 × 3 min). The resin was washed with DMF (× 5). The bicyclic peptide was cleaved from the resin by treatment with TFA/TIPS (95:5) for 2 h. The resin was subsequently washed with TFA (× 10) and the combined TFA fractions were concentrated in vacuo. The oily residue was precipitated with ice-cold diethyl ether to give the crude product **1** as an off-white solid. Yield: 162 mg. This crude product was purified by preparative HPLC to give two isomers:

1A: White solid. Yield: 11.5 mg (3.4%). RP-HPLC purity > 98%. R_t (1) = 9.07; (2) = 10.79. HRMS (MALDI) exact mass calcd for $C_{56}H_{81}N_{15}O_{15}S_2Na$: 1290.5375 [*M*Na]$^+$; found: 1290.5374.

1B: White solid. Yield: 10.6 mg (3.1%). RP-HPLC purity > 98%. R_t (1) = 9.20; (2) = 11.29. HRMS (MALDI) exact mass calcd for $C_{56}H_{81}N_{15}O_{15}S_2Na$: 1290.5375 [*M*Na]$^+$; found: 1290.5403.

Inhibition of acetylcholine binding to adult muscular nAchR expressed in *Xenopus* oocytes:

Molecular biology: The cDNA coding for rat α_1AChR, β_1AChR, δAchR, and εAChR (kindly provided by Veit Witzemann, Max-Planck Institute,

Heidelberg, Germany) were subcloned into the duel-function expression vector pXOOM to obtain robust expression in *Xenopus laevis* oocytes.[36] All four receptor subunits were amplified by PCR with oligonucleotides introducing a Kozak consensus translation initiation sequence (5′-end)[37] and the appropriate restriction enzyme recognition sites (EcoRI, NotI). The integrity of all PCR-generated DNA fragments was confirmed by sequencing.

In vitro transcription and capping were performed according to the manufacturer's instructions by using the mCAP mRNA capping kit (Stratagene, La Jolla, CA, USA). mRNA was phenol/chloroform extracted, ethanol precipitated, and dissolved in tris(hydroxymethyl)aminomethane-HCl/ethylenediaminetetraacetate (TE) buffer (pH 8) to a concentration of approximately 0.1 $\mu g\,\mu L^{-1}$. The integrity of the transcripts was confirmed by agarose gel electrophoresis, and mRNA was stored at $-80\,^{\circ}C$ until injection.

Expression in Xenopus oocytes: *Xenopus laevis* surgery and oocyte treatment were done as previously described.[38] Oocytes were kept in Kulori medium (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.4) for 24 hours at $19\,^{\circ}C$ before injection of mRNA (50 nL, approximately 5 ng). mRNA coding the four subunits $\alpha 1$, $\beta 1$, ε, and δ was mixed in an approximate 1:1:1:1 molar ratio. All injections were performed with a Nanoject microinjector (Drummond, USA). Oocytes were kept at $19\,^{\circ}C$ in Kulori medium for 2 – 4 days before measurements were performed. The Kulori medium was changed once a day.

Electrophysiology: The experimental set-up is depicted in Figure 4. Measurements were performed on a conventional two-electrode voltage-clamp set-up with a Dagan amplifier (Dagan CA-1B, Minneapolis, MN, USA). Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller (DMZ universal puller, Zeitz instruments, München, Germany) and had tip resistance between 0.3 and 2.0 $m\Omega$ when filled with 1 M KCl. During the experiments oocytes were placed in a small chamber (volume: 200 μL) and continually superfused (flow: 6 $mL\,min^{-1}$) with a gravity-driven perfusion system. Activators and inhibitors were applied by this system. For all recordings the solution for superfusion was Kulori medium consisting of NaCl (90 mM), KCl (1 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1 mM), HEPES (5 mM, pH 7.4). Experiments were performed at room temperature. The condition of each single oocyte was controlled before the membrane potential was recorded. Only oocytes with membrane potentials below -20 mV were used for current recordings. Oocytes were clamped at -50 mV and adult muscular nAch receptors were activated by application of acetylcholine alone or of acetylcholine and various amounts of inhibitors. Agonist application both in the presence and in the absence of antagonist was carried out over 30 s, followed by at least 3 min superfusion of Kulori medium.

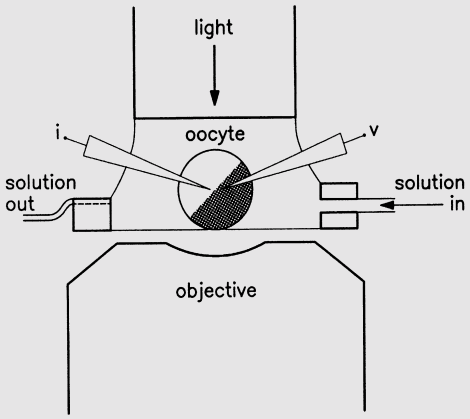


Figure 4. *Experimental setup for oocyte assay. "i" is the voltage tip and "v" is the tip used to measure the oocyte membrane potential.*

Data Analysis: Results of dose-response studies were subjected to a sigmoidal dose-response analysis with the equation:

$$Y = \text{bottom} + \frac{\text{top} - \text{bottom}}{(1 + 10_{(\text{Log}IC_{50} - X)}}$$

where Y = relative current (%), bottom = level of current in the presence of the highest inhibitor concentration used, top = level of current in the presence of the lowest inhibitor concentration, IC_{50} is the inhibitor concentration required for half the maximum inhibition of current, and X is the logarithm of the inhibitor concentration. In situations where the maximum concentration of inhibitor was insufficient to obtain a complete block of the current, the top and bottom values were fixed at 1 and 0, respectively. Unless otherwise stated, all data points represent the average of the results from four different oocytes. Results are given as the mean value ± standard deviation.

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