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Synthesis of Deuterated Aminocaproyl Linkers

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Gramicidin A (gA) analogues with a biotin attached to the C-terminus by an aminocaproyl linker are used as ion channels in the AMBRI® biosensor and are referred to as biotinylated gA. In order to examine the conformation and dynamics of the aminocaproyl linker by ^{2}H solid-state nuclear magnetic resonance, the synthesis of deuterated aminocaproic acid is required. We report on the synthesis of (D_{10})-6-aminocaproic acid from commercially available perdeuterated cyclohexanol and its covalent attachment to the C-terminal group of gA to form gAX_DXB.

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Introduction

Biotinylated gramicidins are an important component of the AMBRI® biosensor[1] created by Ambri Pty Ltd, Sydney, N.S.W., Australia (Fig. 1). The AMBRI® biosensor has a vast range of potential applications, from medical diagnosis to the development of pharmaceutical products and environmental monitoring systems. The biosensor consists of a receptor attached to gramicidin A (gA, a peptide ion channel) embedded in a lipid membrane. The gA ion channel functions as a dimer, while the receptor has the ability to bind to a ligand. When binding takes place, dimer formation is disrupted and, therefore, ions do not readily flow across the lipid membrane. The receptor is linked to gA by a biotin group, which is covalently attached to the C-terminus of gA by an aminocaproyl linker (X). These modified gA molecules or biotinylated gramicidins (Fig. 2) function best in the AMBRI® biosensor with five aminocaproyl groups (gA5XB), but not with shorter linkers (gA2XB).[2] A knowledge of the conformation and dynamics of these linker groups in lipid bilayers is essential in order to determine the optimum linker groups for biotinylated gA used in the AMBRI® biosensor. A means of obtaining this information would utilize deuterium (^{2}H) nuclear magnetic resonance (NMR) of specifically deuterated linkers. This paper reports on the synthesis of deuterated aminocaproyl linkers and their attachment to gA.

Vogt and coworkers[3] have reported the synthesis and structural studies of gA analogues using specifically deuterated and perdeuterated acyl gramicidins. The chain order and dynamics of these derivatives were investigated in aligned lipid bilayers using solid-state ^{2}H NMR.[4] Using similar methods, we propose to investigate the conformation and dynamics of the aminocaproyl groups, in oriented bilayers, as a function of linker length. We report the

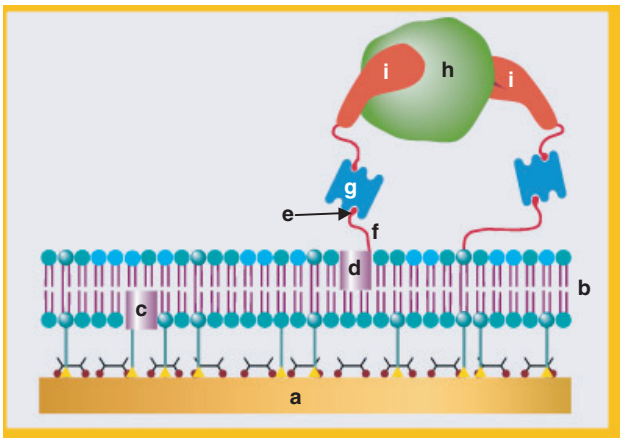

Fig. 1. Diagrammatic representation of the AMBRI® biosensor,[1] consisting of a gold electrode (a) with an attached lipid bilayer (b). Embedded in the lipid bilayer are two gA analogues, one fixed (c) and a mobile gA (d) with biotin (e) attached through the aminocaproyl linker (f). Biotin binds strongly to streptavidin (g). When a molecule of a desired analyte (h) binds to the biotinylated receptor (i), the ion channel is disrupted and interrupts ion current flow to the gold electrode below.

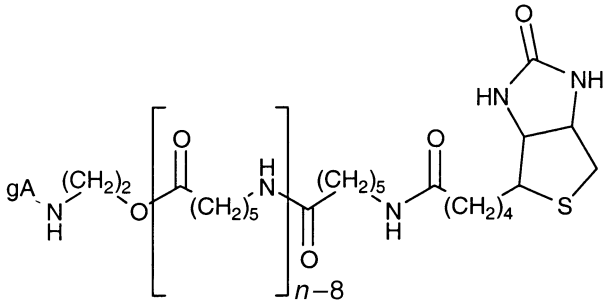

Fig. 2. Biotinylated gA, consisiting of a gramicidin A molecule with an attached biotin moiety linked by *n* aminocaproic acid groups (*n* = number of aminocaproyl groups).

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synthesis of deuterated aminocaproic acid necessary for ^2H NMR studies, and its covalent attachment to the C-terminal group of gA to form gAX_DXB (Fig. 3).

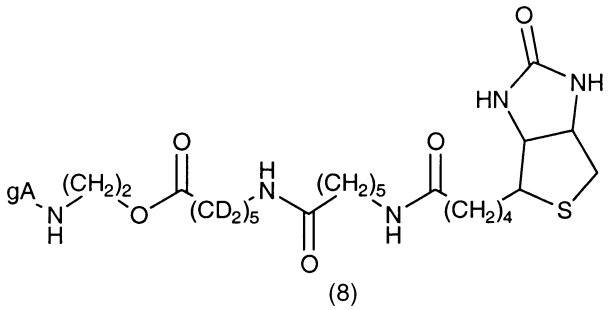
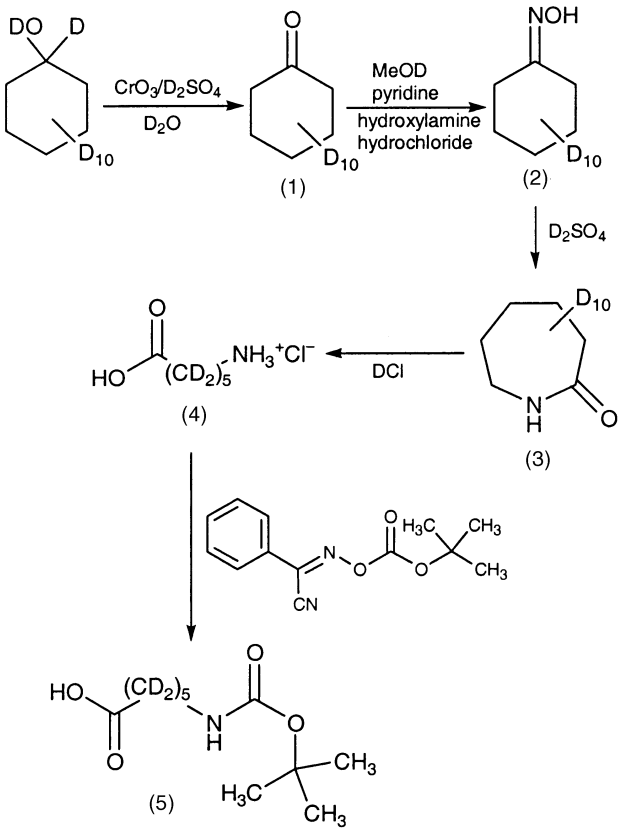


Fig. 3. Deuterated biotinylated gA derivative gAX_DXB (8).

Results and Discussion

Deuterated aminocaproic acid was prepared from commercially available perdeuterated cyclohexanol in four steps as summarized in Scheme 1. All the intermediates were characterized by ^2H and ^{13}C NMR spectroscopy and electrospray (EI) and matrix-assisted laser-desorption ionization (MALDI) mass spectrometry (MS). The perdeuterated ketone (1) was synthesized by the oxidation of (D_{12})cyclohexanol using pyridinium chlorochromate (PCC) absorbed on alumina.[5] The product showed a molecular ion at 108 consistent with the presence of 10 deuterons in the formula. In order to optimize the conditions for the synthesis of (D_{10})cyclohexanone, protonated cyclohexanone was first synthesized following the literature procedure.[5] When the same conditions were used for the preparation of (D_{10})cyclohexanone, the reaction was incomplete. Longer reaction times and more vigorous conditions (the reaction mixture was heated to reflux overnight rather than left stirring at room temperature for 2 h) were required to oxidize (D_{12})cyclohexanol. This apparent difference in reactivities is presumably a result of the deuterium isotope effect.[6] The ketone (1) was then converted into the oxime derivative (2) by modifying a previously recorded method.[7] Oxime (2) was heated in deuterated sulfuric acid (D_2SO_4),[8,9] affording the Beckmann product, deuterated ϵ-caprolactam* (3). D_2SO_4, rather than H_2SO_4, was used to avoid proton incorporation into the product. Hydrolysis of (3)[10] in 30% DCl/D_2O gave (D_{10})-6-aminocaproic acid† as its deuterochloride salt (4). The ^2H and ^{13}C NMR spectra of all of these deuterated derivatives were compared with the ^1H and ^{13}C NMR of the equivalent protonated compounds and they were in good agreement.

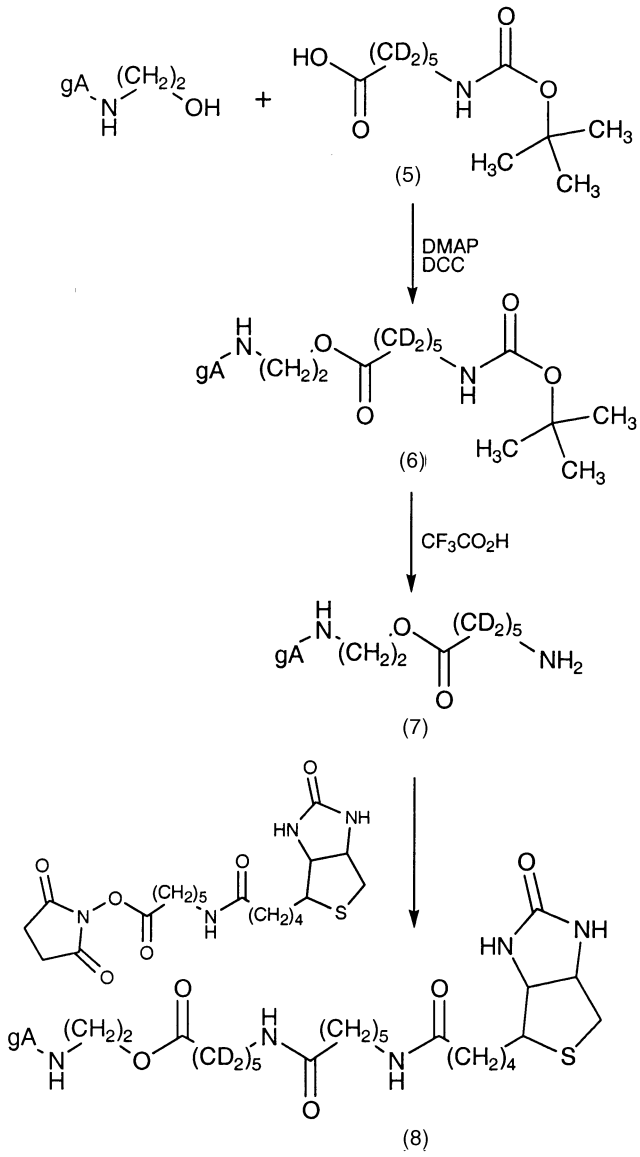
The primary amino group in (4) was Boc protected with [2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) to form 6-[*N*-(*tert*-butoxycarbonylamino)](D_{10})-caproic acid, X_DBOC (5).[11] This was then coupled to the ethanolamine moiety at the C-terminus of gA to form [6-[*N*-(*tert*-butoxycarbonylamino)](D_{10})caproyl]gramicidin,



Scheme 1. Synthesis of deuterated aminocaproyl linker.

gAX_DBOC (6), as shown in Scheme 2. The conjugate was purified by Sephadex gel-permeation chromatography[12] followed by silica gel chromatography. ^1H and ^2H NMR spectra confirmed covalent attachment of the deuterated linker to gA. Deprotection of gAX_DBOC (6) using trifluoroacetic acid gave (*N*-6-amino(D_{10})caproyl)gramicidin, gAX_D (7). Biotinamidocaproate-*N*-hydroxysuccinimide was coupled onto this deprotected derivative (7) to form [6-(aminocaproyl)-*N*-biotinyl(D_{10})(aminocaproyl)]gramicidin, gAX_DXB (8), which was purified by Sephadex gel-permeation chromatography followed by silica gel chromatography. The final product (8) was characterized by ^1H, ^2H NMR and MALDI MS which showed the presence of an ion at 2346, which corresponded to the molecular weight of gAX_DXB. ^1H NMR as well as thin-layer chromatography (TLC) data for the final product were almost identical to native gA, confirming that gAX_DXB had similar structural characteristics. Two-dimensional NMR spectroscopy has already shown that gA2XB has the same backbone conformation and function as native gA.[13] ^1H NMR confirmed that deuteration of the linker in the final product was greater than 98%. The conformation and dynamics of the linker reconstituted into aligned phospholipid bilayers will be studied by solid-state NMR techniques[14] and reported elsewhere.

* ϵ-Caprolactam refers to 2-oxohexamethyleneimine.
† Caproic acid refers to hexanoic acid.



Scheme 2. Synthesis of gAX_DXB.

Experimental

Instrumentation

^2H and ^{13}C NMR experiments were carried out using a Varian Unity 400 MHz (Palo Alto, CA, U.S.A.) spectrometer. Spectra were acquired in chloroform ($CDCl_3$), unless otherwise specified. The ^1H NMR spectrum of each deuterium derivative showed no evidence of proton exchange. Therefore, 98+ atom % D was assumed. EI MS was obtained on a micromass Quattro II instrument recorded at 30 V (solvent system used: acetonitrile/water, 50 : 50). MALDI MS experiments were recorded on a PerSeptive Voyager D.E. spectrometer, Perkin Elmer (MA, U.S.A.).

Reagents

(D_{12})Cyclohexanol (98+ atom % D) was purchased from Aldrich (St Louis, MO, U.S.A.) and used without further purification. Deuterated solvents were used throughout the synthesis to prevent deuterium–hydrogen exchange. All were purchased from Aldrich. BOC-ON, 1,3-dicyclohexylcabodiimide (DCC), 4-dimethylamino-pyridine (DMAP) and alumina were also purchased from Aldrich (St Louis, MO, U.S.A.). Gramicidin D (gD) was obtained from Sigma (St Louis, MO, U.S.A.). Sephadex LH20 was purchased from Amershaw Pharmacia Biotech AB (Uppsula, Sweden).

Pyridinium Chlorochromate (PCC) on Alumina

To a suspension of chromium trioxide (6 g, 39.5 mmol), in 6N deuterated hydrochloric acid (11 mL), at 40°C, was added pyridine (4.75 g, 60.1 mmol) within 10 min. The mixture was cooled to 10°C then reheated to 40°C and aluminium oxide (50 g, 490.4 mmol) was added. A yellow solid was obtained and dried under vacuum.

(D_{10})Cyclohexanone (1)

To a solution of (D_{12})cyclohexanol (2.5 g, 22 mmol) in hexane (56 mL), fresh PCC on alumina was added (56 g). The mixture was heated to reflux overnight. The solid was filtered off and washed with diethyl ether (5 × 50 mL). The diethyl ether fractions were combined and evaporated under vacuum to yield (D_{10})cyclohexanone (1) (1.53 g, 14 mmol, 64%) as a yellow oil. Mass spectrum *m/z* 108 [MH^+]. ^2H NMR ($CDCl_3$) δ 2.24, m, 4D; 1.77, m, 4D; 1.61, m, 2D. ^{13}C NMR ($CDCl_3$) δ 211.7, CO; 40.1 **C**D_2CO; 23.5, 22.0, CD_2.

(D_{10})Cyclohexanone Oxime (2)

(D_{10})Cyclohexanone (1) (1.53 g, 14 mmol) was dissolved in (D_4)methanol (34 mL), followed by the addition of hydroxylamine hydrochloride (0.48 g, 6.9 mmol) and pyridine (0.48 g, 6.1 mmol). The mixture was stirred for 5 h under N_2. D_2O (43 mL) was added and the product was extracted with ether (3 × 43 mL) and the extracts were washed with saturated copper sulfate solution to remove pyridine. The combined ether solutions were dried with magnesium sulfate, filtered and dried under high vacuum to yield (D_{10})cyclohexanone oxime (2) (0.94 g, 7.6 mmol, 55%) as white crystals. Mass spectrum *m/z* 124 [MH^+]. ^2H NMR ($CDCl_3$) δ 1.86, m, 2D; 1.64, m, 4D; 0.97, m, 4D. ^{13}C NMR ($CDCl_3$) δ 166.3, CO; 30.7, **C**D_2CO; 23.7, 22.0, CD_2.

(D_{10})-ϵ-Caprolactam (3)

To (D_{10})cyclohexanone oxime (1) (0.34 g, 2.76 mmol) was added 85% D_2SO_4 in D_2O (1 mL). The mixture was heated until bubbles first appeared and then allowed to cool down to 0°C. The mixture was made slightly alkaline with the addition of 24% potassium hydroxide solution. Potassium sulfate precipitated out and was filtered off and washed with deuterated chloroform. The solution containing (3) was then extracted with chloroform (5 × 10 mL). The combined chloroform solutions were washed with deuterated water and then evaporated under vacuum to yield (D_{10})-ϵ-caprolactam (3) (0.18 g, 1.46 mmol, 53%) as a yellow oil. Mass spectrum *m/z* 124 [MH^+]. ^2H NMR ($CDCl_3$) δ 3.20, m, 2D; 2.40, m, 2D; 1.60, m, 6D. ^{13}C NMR ($CDCl_3$) δ 180.1, CO; 43.1, CD_2NH; 36.0, **C**D_2CO; 29.4, 28.3, 22.0, CD_2.

(D_{10})-6-Aminocaproic Acid·DCl (4)

To (D_{10})-ϵ-caprolactam (3) (0.142 g, 1.15 mmol) was added 30% deuterated hydrochloric acid. The mixture was heated to reflux for 2 h. The solution was then evaporated under vacuum to yield (D_{10})-6-aminocaproic acid·DCl (4) (0.11 g, 0.78 mmol, 68%) as white crystals. Mass spectrum *m/z* 141 [MH^+]. ^2H NMR (D_2O) δ 3.08, m, 2D; 2.32, m, 2D; 1.60, m, 2D; 1.46, m, 2D; 1.34, m, 2D. ^{13}C NMR (D_2O) δ 174.2, CO; 34.8, CD_2NH; 20.9, 19.4, 18.2, CD_2.

6-[N-(tert-Butoxycarbonylamino)](D_{10})caproic Acid (5)

(D_{10})-6-Aminocaproic acid (4) (70 mg, 0.39 mmol) was dissolved in 50% aqueous dioxan (1.6 mL) and triethylamine (82 μL). The mixture was stirred at room temperature and BOC-ON (107 mg, 0.43 mmol) was added. The mixture was then stirred for 1 h at 45°C. H_2O (5 mL) was added and the mixture was washed with ethyl acetate (3 × 10 mL). The aqueous layer was acidified with HCl (3 M). The product was then extracted with ethyl acetate (3 × 10 mL), the solution dried with sodium sulfate and then evaporated under vacuum to yield deuterated 6-[*N*-(*tert*-butoxycarbonylamino]caproic acid (5) (45 mg, 0.19 mmol, 48%) as a yellow oil. Mass spectrum *m/z* 241 [MH^+]. ^2H NMR ($CDCl_3$) δ 3.08, m, 2D; 2.32, m, 2D; 1.60, m, 2D; 1.46, m, 2D; 1.34, m, 2D. ^{13}C

NMR δ 174.2, CO_2H; 150.05, CO; 85.87, **C**$(CH_3)_3$; 34.8, **C**CO; 27.7, $(CH_3)_3$; 20.9, 19.4, 18.2, CD_2.

*[6-[*N-*(*tert-*Butoxycarbonylamino)](D$_{10}$)caproyl]gramicidin (6)*

gA was isolated from the natural mixture gD using a flash silica column eluted with dichloromethane/methanol/water (800 : 60 : 4), R_F 0.6 (modified procedure).[12,15] TLC plates were developed in dichloromethane/methanol/water/acetic acid (400 : 60 : 4 : 4) and visualized with the tryptophan reagent, *N*,*N*-dimethylaminobenzaldehyde.[16] To the purified gA (22 mg, 0.02 mmol) was coupled 6-[*N*-(*tert*-butoxycarbonylamino](D$_{10}$)-6-caproic acid (19.6 mg, 0.07 mmol) and DMAP (3 mg, 0.02 mmol). Dry dichloromethane (5 mL) and DCC (13 mg, 0.06 mmol) were added.[15] The solvent was evaporated, then the crude product was dissolved in methanol and passed down a Sephadex LH20 column. The eluent was dried and then further purified on a flash silica column and eluted with dichloromethane/methanol/water (800 : 60 : 6) to afford a major fraction of [6-[*N*-(*tert*-butoxycarbonylamino)](D$_{10}$)caproyl]gramicidin (6) (15 mg, 7.07×10^{-3} mmol, 36%), R_F 0.44. ^{1}H NMR (CD_3OD) δ 0.3–1.8, m, 72H (note: a sharp singlet at 1.5 from BOC methyl ^{1}H was difficult to integrate due to overlap of aliphatic proton resonances from gA); 2.05–2.40, m, 9H; 3.0–3.7, m, 16H; 3.8–4.8, m, 14H; 6.8–7.6, m, 20H; 8.15, s, 1H.

*(*N*-6-Amino(D$_{10}$)caproyl)gramicidin (gAX$_D$) (7)*

[6-[*N*-(*tert*-Butoxycarbonylamino)](D$_{10}$)caproyl]gramicidin (6) was deprotected using trifluoroacetic acid (3 mL) and the solution was evaporated to dryness and dried under high vacuum to yield (*N*-6-amino(D$_{10}$)caproyl)gramicidin (gAX$_D$) (7). ^{1}H NMR (CD_3OD) δ 0.3–1.8, m, 72H; 2.05–2.40, m, 9H; 3.0–3.7, m, 16H; 3.8–4.8, m, 14H; 6.8–7.6, m, 20H; 8.15, s, 1H.

*[6-(Aminocaproyl)-*N*-Biotinyl(D$_{10}$)(aminocaproyl)]gramicidin (gAX$_D$XB) (8)*

To gAX$_D$ (7) (15 mg, 7.07×10^{-3} mmol) was added dichloromethane/methanol (2 : 1) (2 mL) and biotinamidocaproate-*N*-hydroxysuccinimide (8.4 mg, 0.02 mmol). The reaction mixture was stirred overnight at room temperature. The solvent was evaporated, and then the crude product was dissolved in methanol and passed down a Sephadex LH20 column. The eluent was dried and then further purified on a flash silica column eluted with dichloromethane/methanol/water (800 : 100 : 8) to afford a major fraction of [6-(aminocaproyl)-*N*-biotinyl(D$_{10}$)(aminocaproyl)]gramicidin (gAX$_D$XB) (8) (6.3 mg, 2.69×10^{-3} mmol, 39%), R_F 0.35. MALDI MS 2346. ^{1}H NMR (CD_3OD) δ 0.3–1.8, m, 80H; 2.05, m, 4H; 2.05–2.20, m, 8H; 2.78, d, 1H; 2.90, dd, 1H; 3.0–3.7, m, 19H; 3.88, dd, 2H; 3.90, s, 2H; 3.9–4.8, m, 16H; 6.8–7.6, m, 20H; 8.15, s, 1H.

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