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for

Monitoring Chemical Reactions by Using Ion Channel-Forming Peptides

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General methods: All HPLC experiments were performed using a C18-RP column (9.6 µm x 25 cm, Biotage, Inc.) using a 90-minute gradient from 60% Methanol (MeOH) in deionized water to 100% MeOH at room temperature unless otherwise noted. All reagents were purchased from Sigma-Aldrich, Inc. and used without further purification unless stated otherwise.

Characterization of gramicidin A: Gramicidin A (gA) was purchased from Cal Biochem (97% purity). ^{1}H NMR (400 MHz, CD_3OD): d = 0.33-0.48 (m, 18H), d = 0.6 (br d, 3H, 8 Hz), d = 0.7-1.0 (m, 28H), d = 1.1-1.2 (m, 4H), d = 1.23-1.4 (m, 9H), d = 1.6 (br s, 2H), d = 1.95 (br s, 2H), d = 2.01-2.03 (m, 4H), d = 3.35 (s, 6H), d = 3.47-3.57 (m, 2H), d = 3.84 (s, 1H), d = 3.90 (br s, 1H), d = 3.95 (br s, 1H), d = 4.14 (br d, 2H, 12 Hz), d = 4.22 (br d, 2H, 12 Hz), d = 4.28-4.42 (m, 3H), d = 4.54-4.68 (m, 4H), d = 6.87-7.1 (m, 11H), d = 7.23-7.36 (m, 5H), d = 7.4 (d, 2H, 8 Hz), d = 7.49-7.59 (m, 4H), d = 8.12 (s, 1H). gA was also characterized by HPLC (retention time = 61.5 minutes). ESI-MS revealed $[M + Na]^+$ and $[M + 2Na]^{2+}$ fragments at 1904.0 and 964.0 for gA, respectively.

N-(*tert*-butyloxycarbonyl)glycylgramicidin (1)

Preparation: Gramicidin A (24.5 mg, 0.013 mmol), (*N*-BOC) glycine (9.11 mg, 0.052 mmol), dicyclohexylcarbodiimide (DCC) (26.8 mg, 0.13 mmol) and dimethylamino-

pyridine (DMAP) (7.9 mg, 0.065 mmol) were dried separately in vacuo. N-BOC glycine, DCC, and DMAP were each dissolved in 2 mL freshly-distilled dichloromethane. Each solution was then added to gA in the order of (*N*-BOC) glycine, DCC, and DMAP. The resulting solution was stirred at room temperature for 6 hrs under N_2. The solvent was evaporated and **1** was purified from the crude mixture on silica with dichloromethane (DCM) / MeOH (9:1) as eluent to give 23 mg (87% yield) of the purified compound.

Characterization: The retention time of **1** on reversed-phase HPLC was 67.7 min. ESI-MS reveals a $[M + 2Na]^{2+}$ fragment at 1042.3. ^{1}H NMR (400 MHz, CD_3OD): d = 0.33-0.48 (m, 18H), d = 0.6 (br d, 3H, 8 Hz), d = 0.7-1.0 (m, 28H), d = 1.1-1.2 (m, 4H), d = 1.23-1.4 (m, 9H), d = 1.43 (s, 9H), d = 1.6 (br s, 2H), d = 1.95 (br s, 2H), d = 2.01-2.03 (m, 4H), d = 3.35 (s, 6H), d = 3.47-3.57 (m, 2H), d = 3.84 (s, 1H), d = 3.90 (br s, 1H), d = 3.95 (br s, 1H), d = 4.14 (br d, 2H, 12 Hz), d = 4.22 (br d, 2H, 12 Hz), d = 4.28-4.42 (m, 3H), d = 4.54-4.68 (m, 4H), d = 6.87-7.1 (m, 11H), d = 7.23-7.36 (m, 5H), d = 7.4 (d, 2H, 8 Hz), d = 7.49-7.59 (m, 4H), d = 8.12 (s, 1H).

Glycylgramicidin (2)

Preparation: A solution of 2 mL trifluoroacetic acid (TFA) / DCM (1:1, *v*/*v*) with 0.02 mL dimethylsulfide and 0.01 mL ethanedithiol was cooled to 0 °C. The TFA solution was then added to 23 mg of dried **1** and stirred while warming to room temperature for 4 h. After evaporating to dryness, the crude solid was dissolved in 0.75 mL of DCM / MeOH (2:1). This solution was added dropwise into a beaker containing 80 mL of deionized, stirring water at 0 ºC and **2** was precipitated out and filtered to yield 20.1 mg (90.2 % yield) of pure material.

Characterization: ESI-MS reveals three fragments: $[M]^+$ = 1938.7, $[M + Na]^+$ = 1961.9, $[M + 2Na]^{2+}$ = 992.4. ^{1}H NMR (400 MHz, CD_3OD): d = 0.33-0.48 (m, 18H), d = 0.6 (br d, 3H, 8 Hz), d = 0.7-1.0 (m, 28H), d = 1.1-1.2 (m, 4H), d = 1.23-1.4 (m, 9H), d = 1.6 (br s, 2H), d = 1.95 (br s, 2H), d = 2.01-2.03 (m, 4H), d = 3.35 (s, 6H), d = 3.47-3.57 (m, 2H), d = 3.84 (s, 1H), d = 3.90 (br s, 1H), d = 3.95 (br s, 1H), d = 4.14 (br d, 2H, 12 Hz), d = 4.22 (br d, 2H, 12 Hz), d = 4.28-4.42 (m, 3H), d = 4.54-4.68 (m, 6H), d = 6.87-7.1 (m, 11H), d = 7.23-7.36 (m, 5H), d = 7.4 (d, 2H, 8 Hz), d = 7.49-7.59 (m, 4H), d = 8.12 (s, 1H).

O-trityl glycolic acid

Preparation: A solution of 800 mg (10.5 mmol) of glycolic acid and 2.75 g (9.9 mmol) of diisopropylethylamine (DIEA) was prepared in 5 mL of DCM. The resulting mixture was cooled to 0°C. Then a solution of 1.62 g (37.6 mmol) of trityl chloride in DCM was added dropwise to the cooled solution of glycolic acid and DIEA. The reaction was allowed to stir for 1 h at 0°C, then 18 h at RT. The solution was then concentrated in vacuo. Reaction mixture was purified by silica chromatography using DCM as the eluent until all of the unreacted trityl chloride eluted out. The polarity of eluent was increased to 45:4 (DCM/MeOH) to elute *O*-trityl glycolic acid. Upon concentration in vacuo, a white solid (902 mg, 22% yield) of *O*-trityl glycolic acid was obtained.

Characterization: ^{1}H NMR (400 MHz, $CDCl_3$): d = 3.89 (s, 2H), d = 7.27 – 7.36 (m, 9H), d = 7.47 (d, 8 Hz)

Gramicidyl O-trityl glycolate

Preparation: A solution of 3.18 mg (0.01 mmol) of O-trityl glycolic acid and 5.3 mg (0.026 mmol) of DCC in 2 mL of dry DCM was stirred for 10 min. Then a 1 mL solution containing 5 mg (2.6 μmol) of gA and a 1 mL solution containing 1.6 mg (0.013 mmol) of DMAP were subsequently added in that order. The reaction was allowed to stir at RT under N_2 for 14 h. Upon purification by silica chromatography with DCM / MeOH (9:1) as eluent, 3.2 mg (55% yield) of Gramicidyl *O*-trityl glycolate was obtained.

Characterization: Reversed-phase HPLC retention time was 78.8 min (99% purity). ESI-MS reveals a $[M + 2Na]^{2+}$ fragment at 1113.8 and a $[M\text{-H}+3Na]^{2+}$ fragment at 1124.9. ^{1}H NMR (400 MHz, CD_3OD): d = 0.33-0.48 (m, 18H), d = 0.6 (br d, 3H, 8 Hz), d = 0.7-1.0 (m, 28H), d = 1.1-1.2 (m, 4H), d = 1.23-1.4 (m, 9H), d = 1.6 (br s, 2H), d = 1.95 (br s, 2H), d = 2.01-2.03 (m, 4H), d = 3.35 (s, 6H), d = 3.47-3.57 (m, 2H), d = 3.84 (s, 1H), d = 3.90 (br s, 1H), d = 3.95 (br s, 1H), d = 4.14 (br d, 2H, 12 Hz), d = 4.22 (br d, 2H, 12 Hz), d = 4.28-4.42 (m, 3H), d = 4.54-4.68 (m, 4H), d = 6.87-7.1 (m, 8H), d = 7.23-7.36 (m, 17H), d = 7.49-7.59 (m, 15H), d = 8.12 (s, 1H).

Gramicidyl glycolate (3)

Preparation: A solution of 1 mL TFA / DCM (1:1) with 0.02 mL dimethylsulfide and 0.01 mL ethanedithiol along with separately dried 2 mg of Gramicidyl O-trityl glycolate is cooled to 0°C. The TFA solution is then added to Gramicidyl O-trityl glycolate while stirring and allowed to warm to room temperature for 2 hours. Upon purification by silica chromatography with DCM / MeOH (9:1) as eluent, 1.8 mg (98% yield) of **3** was obtained.

Characterization: Reversed-phase HPLC retention time was 62.3 minutes (99% purity). ESI-MS reveals a $[M + Na]^+$ fragment at 1962.8 and a $[M + 2Na]^{2+}$ fragment at 992.7. ^{1}H NMR (400 MHz, CD_3OD): d = 0.33-0.48 (m, 18H), d = 0.6 (br d, 3H, 8 Hz), d = 0.7-1.0 (m, 28H), d = 1.1-1.2 (m, 4H), d = 1.23-1.4 (m, 9H), d = 1.6 (br s, 2H), d = 1.95 (br s, 2H), d = 2.01-2.03 (m, 4H), d = 3.35 (s, 6H), d = 3.47-3.57 (m, 2H), d = 3.84 (s, 1H), d = 3.90 (br s, 1H), d = 3.95 (br s, 1H), d = 4.14 (br d, 2H, 12 Hz), d = 4.22 (br d, 2H, 12 Hz), d = 4.28-4.42 (m, 3H), d = 4.54-4.68 (m, 6H), d = 6.87-7.1 (m, 11H), d = 7.23-7.36 (m, 5H), d = 7.4 (d, 2H, 8 Hz), d = 7.49-7.59 (m, 4H), d = 8.12 (s, 1H).

Monitoring the reaction of glycine with nitrite by NMR

Experiment: Glycine (7.5 mg, 0.1 mmol) and KCl (74 mg, 1 mmol) were dissolved in 10-mL of 0.4 M potassium acetate / acetic acid buffer in D_2O (pD = 3.8). Sodium nitrite (20.7 mg, 0.3 mmol) was added to a 1 mL aliquot of the buffered solution containing glycine and ^{1}H NMR's were taken at various time points to monitor the conversion of glycine to glycolic acid. ^{1}H NMR (D_2O, 400 MHz): Glycine methylene: d = 3.42 (s, 2H), Glycolic acid methylene: d = 3.90 (s, 2H). The chemical shift of glycolic acid was confirmed using an authentic commercial sample. Upon completion of the reaction (60 min), the pD was 3.8.

Ion channel measurements: Planar lipid bilayers were formed with the "painting technique". We pretreated each side of a pore (diameter 0.25 mm) in a bilayer cup (Warner Instruments, Delrin perfusion cup, volume 1 mL) with ~ 3 µL of a 20 mg mL^{-1} solution of 1,2-Diphytanoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DiPhyPC, Avanti Polar Lipids) in hexane. After adding recording buffer to both compartments of the bilayer setup, we "painted" a solution of 20 mg mL^{-1} DiPhyPC in n-decane over the pore by using a paint brush with a fine tip. Before addition of the gA derivatives, we followed the thinning of

the decane droplet to a planar bilayer by monitoring the capacitance of the bilayer. In case the decane droplet did not thin out, we bubbled air in one chamber underneath the pore. The rise of these air bubbles in the vicinity of the pore usually helped to thin out the decane/lipid droplet. After bilayer formation we stirred both chambers with a small stir bar (using a Spin-2 bilayer stirplate from Warner Instruments) at all times except during the 60 s recording intervals (to minimize electrical current noise). We performed single ion channel recordings in "voltage clamp mode" using a Ag/AgCl pellet electrode (Warner Instruments) in each compartment and a Geneclamp 500 amplifier (Axon Instruments) with a CV-5B 100GU PROBE headstage (Gain: 100mV pA^{-1}). We selected a filter cutoff frequency of 1 kHz on the amplifier (sampling frequency 25 kHz). The current traces shown in Figure S1 were filtered further using a digital Gaussian filter with a cutoff frequency of 0.2 kHz.

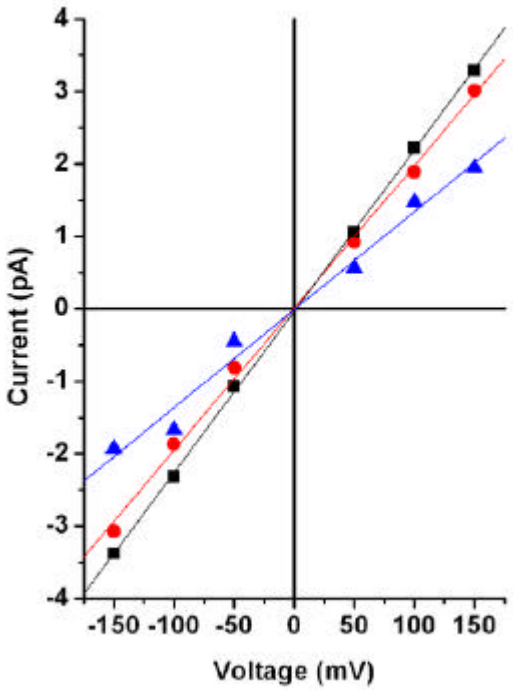

Figure S1. Conductance measurements of derivatives of gramicidin A carrying a *tert*-butoxycarbonyl protected glycine **1** (¦), glycine **2** (?), and glycolic acid **3** (?) in aqueous buffered solution (1 M KCl, 0.01 M HEPES, pH 7.4). The slopes of the linear fits were: 2.23 ± 0.02 pA $(0.1\ V)^{-1}$, 1.35 ± 0.08 pA $(0.1\ V)^{-1}$, 1.96 ± 0.05 pA $(0.1\ V)^{-1}$.

We performed the analysis of the single channel current traces by computing histograms of the current with ClampFit 9.2 software from Axon Instruments. From these histograms we extracted the main channel conductances for each of the derivatives **1**-**3**. All three derivatives typically showed a predominant single channel conductance but also sub-conductance states (i.e. a single–channel current that were somewhat smaller than the main conductance). These sub-conductance states were less frequent than the main conductance state but in some cases (e.g. with derivative **2**), we had to analyze several traces to establish the main conductance state since these molecules fluctuated between several single channel conductance states.