Supporting Information – 33 Pages

Solid Phase Syntheses of Polyamine Toxins HO-416b and PhTX-433. Use of an Efficient Polyamide Reduction Strategy That Facilitates Access to Branched Analogues

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Contents:

(1) Experimental section. General.

All reagents used are commercially available and were employed without further purification. Fmoc-protected amino acids and PS-Trityl chloride resin (200-400 mesh, 1% DVB, 0.80 mmol/g) were purchased from Novabiochem (San Diego, California) and Rapp-Polymere (Tuebingen, Germany) respectively. All glassware used in solid-phase reactions had been silanized by treatment with 20% chlorotrimethylsilane/toluene for 12h and then dried under vacuum. Polypropylene(PP) filter vessels were obtained from Bio-Rad. THF was dried by distillation over sodium/benzophenone ketyl, CH_2Cl_2 over sodium hydride. Anhydrous DMF was obtained commercially from Aldrich. NMR spectra were recorded at Bruker AM-400 and AM-300, Varian Inova 300 or Varian Unity 500 MHz. Low and high resolution ES-MS were done on a Hewlett-Packard 1100 MSD and ZabSpecETOF respectively. Compound purity analysis was carried out by RP-HPLC on a Hewlett-Packard 1100 system using conditions described in the manuscript. Abbreviations: HBTU – 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexa-fluorophosphate, HOBt – N-Hydroxybenzo-triazole, DIPEA – Diisopropylethyl-amine, γ-Abu – 4-Aminobutyric acid, Tyr(t-Bu) – O-t.-butyl L-tyrosine, Dde-OH – 2-Acetyl-dimedone, $(Boc)_2O$ – Di-t.-butyl-dicarbonate, TFA – Trifluoroacetic acid.

(2) Experimental details for the synthesis of 1.

PS-Trityl-NH$(CH_2)_3NH_2$ resin (4) 1,3-Diaminopropane (4.0 mL, 48.0 mmol) was dissolved in 4 mL of dry CH_2Cl_2 in a PP filter vessel. PS-Trityl chloride resin (1.20g, 0.96 mmol, 0.80 mmol/g) was then added to the solution in 4 portions over one hour with vortexing in between additions. After vortexing for an additional hour, 2 mL of methanol was added followed by another 20 min. of vortexing. The resin was then filtered and rinsed with MeOH, 1:4 Et_3N/DMF, MeOH and CH_2Cl_2 (3 times each), and dried under high-vacuum for over 12 hours to give PS-Trityl-NH$(CH_2)_3NH_2$ resin **4** (1.22g, 0.73 mmol/g, as determined by Fmoc release U.V. assay after derivatizing with Fmoc-Cl). A ninhydrin assay gave a positive result.

PS-Trityl-NH$(CH_2)_3NH_2$ resin-bound triamide (5) To the resin **4** (0.382g, 0.279 mmol, 0.73 mmol/g) in a PP filter vessel was added N-Fmoc-ßAlanine (0.349g, 1.12 mmol) as a solution in 3 mL of dry DMF. The vessel was shaken for 10 min. before a solution of HBTU (0.43g, 1.12 mmol) and HOBt (0.16g, 1.12 mmol) in 5 mL of dry DMF, and DIPEA (0.39 mL, 2.24 mmol) were added. After vortexing for an hour, the resin was filtered and rinsed with DMF, MeOH and CH_2Cl_2 (3 times each). A ninhydrin assay gave a negative result. The resin was rinsed with dry DMF (3 times) and then treated with 20% piperidine/DMF (3 mL for 3 min. then 3 mL for 25 min.). After washing with MeOH, CH_2Cl_2 and DMF (3 times each), the coupling procedure was

exactly repeated followed by the second repetition as above with 3 mL solution of Fmoc-γAbu (0.37g, 1.12 mmol), a 5 mL DMF solution of HBTU (0.43g, 1.12 mmol) and HOBt (0.16g, 1.12 mmol), and DIPEA (0.39 mL, 2.24 mmol). Subsequently, the resin was again rinsed with dry DMF, MeOH and CH_2Cl_2 (3 times each), and dried under vacuum overnight to afford the resin-bound triamide **5** (0.430g, 0.59 mmol/g, calculated from the loading of its Fmoc-protected precursor, obtained from Fmoc release U.V. assay). A ninhydrin assay gave a negative result. A portion of the Fmoc-protected precursor of **5** was cleaved from the resin and its ES-MS analysis validated the efficiency of synthesis of the tripeptide **5**.

PS-Trityl resin-bound tetraamine (6) The resin-bound triamide **5** (0.276g, 0.163 mmol, 0.59mmol/g) was weighed into a 25 mL silanized round bottom flask and swelled in dry THF (1.5 mL) under nitrogen. The diborane solution (1M in THF, 6.0 mL, 6.0 mmol) was added dropwise at rt over 2 min. The flask was then equipped with a condenser and the suspension was gently refluxed at 65°C for 48h. Upon cooling to rt, the suspended resin was rapidly transferred into a PP filter vessel via a silanized pipette using dry THF to rinse out the flask and to wash the resin extensively. Then, dry THF (2.0 mL), anhydrous DIPEA (0.3 mL) and glacial AcOH (0.6 mL) were added successively. After shaking the suspension the iodine was added (1.53g, 6.0 mmol, as a concentrated THF solution) and the vessel was vortexed for 4h. The resin was then filtered and rinsed with THF, 1:3 Et_3N/DMF, MeOH and CH_2Cl_2 (3 times each) and dried under high-vacuum overnight to give the resin-bound tetraamine **6** (0.220g). A portion of the tetraamine **6** was acetylated (6 eq. Et_3N, 22 eq. Ac_2O, DMF, rt, 12h) and then cleaved from the resin. The ES-MS analysis of the acetylated derivative confirmed the efficiency of the reduction reaction (see attached MS spectra).

PS-Trityl resin-bound Boc-protected tetraamine (7) To the resin-bound tetraamine **6** (0.184g) in a PP filter vessel was added Dde-OH (40 mg, 0.22 mmol) as a solution in 2 mL of dry DMF. After vortexing for 2h, the resin was filtered and rinsed with DMF, MeOH and CH_2Cl_2 (3 times each). A ninhydrin assay gave a negative result. Then, a solution of DIPEA (0.24 mL, 1.32 mmol) in 1 mL dry CH_2Cl_2 was added, after shaking for an minute, followed by addition of 1 mL dry CH_2Cl_2 solution of $(Boc)_2O$ (0.58g, 2.64 mmol). The suspension was vortexed overnight. Then, the resin was filtered and rinsed with CH_2Cl_2, MeOH and DMF (3 times each) and treated with 2% hydrazine in DMF (3 mL for 10 min. then 3 mL for 30 min.) to remove the Dde-protecting group. The resin was rinsed with DMF, MeOH and CH_2Cl_2 (3 times each) and dried under vacuum for over 12h to afford the PS-Trityl resin-bound selectively Boc-protected tetraamine **7** (0.195g). A ninhydrin assay gave a positive result.

PS-Trityl resin-bound Boc-protected HO-416b (9) The resin-bound selectively Boc-protected tetraamine **7** (182 mg) was swelled in 1 mL CH_2Cl_2 in a PP filter vessel. Et_3N (24 μL, 0.15 mmol) and DMAP (20mg, 0.15 mmol) as a solution in 1 mL of dry CH_2Cl_2 were added successively. After vortexing for a few minutes, a CH_2Cl_2 solution of mixed anhydride **8** was added, prepared by reacting 3-indoleacetic acid (0.14g, 0.80 mmol) in 2 mL dry CH_2Cl_2 with trimethylacetyl chloride (99 μL, 0.80 mmol) in the presence of Et_3N (0.12 mL, 0.80 mmol) at rt for 1h. After shaking overnight the resin was filtered and rinsed with DMF, MeOH and CH_2Cl_2 (4 times each) and dried under high-vacuum for over 12h to give the PS-Trityl resin-bound selectively Boc-protected HO-416b **9** (184 mg). A ninhydrin assay gave a negative result.

HO-416b (1) The resin-bound Boc-protected HO-416b **9** (41 mg) was weighed into a 5 mL silanized round bottom flask and stirred in a freshly prepared TFA/H_2O/i-Pr_3SiH (95:2.5:2.5) cleavage cocktail (2 mL) for 2h at rt. After removing the solution by a pipette from the flask, the above-mentioned cleavage cocktail (2 mL) was added to the resin left. The suspension was then stirred for an additional 2h. The contents were filtered through a glasswool plug and the resin rinsed extensively with TFA/MeOH/CH_2Cl_2 (5:30:65). The combined filtrates from two rounds of cleavage were evaporated and dried over high-vacuum for >12h to give crude HO-416b **1** as a penta(trifluoroacetate) ammonium salt (13.5 mg. 57% from tripeptide **5**). Its purity was estimated to be 81% according to RP-HPLC analysis. Two rounds of precipitation with methanol/ether finally afforded HO-416b **1** (8.1 mg) of 89% purity with a 37% overall yield from tripeptide **5**.

<u>Analytical data for 1</u>:

^{1}H NMR (400 MHz, CD_3OD):

1, ·5TFA salt: δ= 1.50-1.85 (4H, m), 2.01-2.22 (6H, m), 2.94-3.27 (16H, m), 3.65 (2H, s), 7.02 (1H, t, J=7.0Hz), 7.11 (1H, t, J=7.0Hz), 7.18 (1H, s), 7.36 (1H, d, J=8.0Hz), 7.53 (1H, d, J=8.0Hz).

ES-MS ($C_{23}H_{40}N_6O$): m/z 416.3 $(M+H)^+$

RP-HPLC: (a) Retention time: 11.70 min;

(b) Conditions: Column Zorbax SB-C18 (4.6X150mm, 5μm);
Eluent (Isocratic) 12.5% MeCN (0.1% TFA) and 87.5% H_2O (0.1% TFA);
Flow rate 1.5 mL/min.; Detection 279 nm.

Selected NMR, ES-MS and HPLC spectra are shown on pages 11 to 15.

(3) Experimental details for the synthesis of PhTX-433 (2)

PS-Trityl-NH(CH_2)$_3$$NH_2$ resin-bound diamide (10) To the resin **4** (0.338g, 0.247mmol, 0.73 mmol/g) in a PP filter vessel was added N-Fmoc-ßAlanine (0.314g, 1.0

mmol) as a solution in 3 mL of dry DMF. The vessel was shaken for 10 min. before a solution of HBTU (0.38g, 1.0 mmol) and HOBt (0.14g, 1.0 mmol) in 5 mL of dry DMF, and DIPEA (0.35 mL, 2.0 mmol) were added. After vortexing for an hour, the resin was filtered and rinsed with DMF, MeOH and CH_2Cl_2 (3 times each). A ninhydrin assay gave a negative result. The resin was rinsed with dry DMF (3 times) and then treated with 20% piperidine/DMF (3 mL for 3 min. then 3 mL for 25 min.). After washing with MeOH, CH_2Cl_2 and DMF (3 times each), the coupling procedure was repeated except for the use of Fmoc-γAbu (0.33g, 1.0 mmol) instead of Fmoc- ßAlanine. Subsequently, the resin was again rinsed with dry DMF, MeOH and CH_2Cl_2 (3 times each), and dried under vacuum overnight to afford the resin-bound diamide **10** (0.385g, 0.63 mmol/g, calculated from the loading of its Fmoc-protected precursor, obtained from Fmoc release U.V. assay). A ninhydrin assay gave a negative result. A portion of the Fmoc-protected precursor of **10** was cleaved from the resin and its ES-MS analysis validated the efficiency of synthesis of the dipeptide **10**.

PS-Trityl resin-bound triamine (11) The resin-bound diamide **10** (0.316g, 0.20 mmol, 0.63mmol/g) was weighed into a 25 mL silanized round bottom flask and swelled in dry THF (1.5 mL) under nitrogen. The diborane solution (1M in THF, 5.0 mL, 5.0 mmol) was added dropwise at rt over 2 min. The flask was then equipped with a condenser and the suspension was gently refluxed at 65°C for 48h. Upon cooling to rt, the suspended resin was rapidly transferred into a PP filter vessel via a silanized pipette using dry THF to rinse out the flask and to wash the resin extensively. Then, dry THF (2.0 mL), anhydrous DIPEA (0.3 mL) and glacial AcOH (0.6 mL) were added successively. After shaking the suspension the iodine was added (1.27g, 5.0 mmol, as a concentrated THF solution) and the vessel was vortexed for 4h. The resin was then filtered and rinsed with THF, 1:3 Et_3N/DMF, MeOH and CH_2Cl_2 (3 times each) and dried under high-vacuum overnight to give the resin-bound triamine **11** (0.269g). A portion of the triamine **11** was acetylated (6 eq. Et_3N, 22 eq. Ac_2O, DMF, rt, 12h) and then cleaved from the resin. The ES-MS analysis of the acetylated derivative confirmed the efficiency of the reduction reaction (see attached MS spectra).

PS-Trityl resin-bound Boc-protected triamine (12) To the resin-bound triamine **11** (0.152g) in a PP filter vessel was added Dde-OH (38 mg, 0.21 mmol) as a solution in 2 mL of dry DMF. After vortexing for 2h, the resin was filtered and rinsed with DMF, MeOH and CH_2Cl_2 (3 times each). A ninhydrin assay gave a negative result. Then, a solution of DIPEA (68 µL mL, 0.38 mmol) in 1 mL dry CH_2Cl_2 was added, after shaking for an minute, followed by addition of 1 mL dry CH_2Cl_2 solution of $(Boc)_2O$ (0.17g, 0.76 mmol). The suspension was vortexed overnight. Then, the resin was filtered and rinsed with CH_2Cl_2, MeOH and DMF (3 times each) and treated with 2% hydrazine in DMF (3 mL for 10 min. then 3 mL for 30 min.) to remove the Dde-protecting group. The resin

was rinsed with DMF, MeOH and CH_2Cl_2 (3 times each) and dried under vacuum for over 12h to afford the PS-Trityl resin-bound selectively Boc-protected triamine **12** (0.185g). A ninhydrin assay gave a positive result.

PS-Trityl resin-bound Boc-protected PhTX-433 (13) To the resin-bound selectively Boc-protected triamine **12** (83 mg) in a PP filter vessel was added N-Fmoc-Tyr(t-Bu) (88 mg, 0.19 mmol) as a solution in 2 mL of dry DMF. The vessel was shaken for 10 min. before a solution of HBTU (73 mg, 0.19 mmol) and HOBt (27 mg, 0.19 mmol) in 3 mL of dry DMF, and DIPEA (68 μL mL, 0.38 mmol) were added. After vortexing for an hour, the resin was filtered and rinsed with DMF, MeOH and CH_2Cl_2 (3 times each). A ninhydrin assay gave a negative result. The resin was rinsed with dry DMF (3 times) and then treated with 20% piperidine/DMF (3 mL for 3 min. then 3 mL for 25 min.). After washing with MeOH, CH_2Cl_2 and DMF (3 times each), the coupling procedure was repeated except for the use of butyric acid (18 μL, 0.19 mmol) instead of Fmoc-Tyr(t-Bu). Subsequently, the resin was again rinsed with dry DMF, MeOH and CH_2Cl_2 (3 times each), and dried under vacuum overnight to afford the resin-bound selectively Boc-protected PhTX-433 **13** (86 mg). A ninhydrin assay gave a negative result.

PhTX-433 (2) The resin-bound Boc-protected PhTX-433 **13** (38 mg) was weighed into a 5 mL silanized round bottom flask and stirred in a freshly prepared TFA/H_2O/i-Pr_3SiH (95:2.5:2.5) cleavage cocktail (2 mL) for 2h at rt. After removing the solution by a pipette from the flask, the above-mentioned cleavage cocktail (2 mL) was added to the resin left. The suspension was then stirred for an additional 2h. The contents were filtered through a glasswool plug and the resin rinsed extensively with TFA/MeOH/CH_2Cl_2 (5:30:65). The combined filtrates from two rounds of cleavage are evaporated and dried over high-vacuum for >12h to give crude PhTX-433 **2** as a tris(trifluoroacetate) ammonium salt (14.0 mg. 77% from dipeptide **10**). Its purity was estimated to be 80% according to RP-HPLC analysis. Two rounds of precipitation with methanol/ether finally afforded PhTX-433 **2** (8.5 mg) of 92% purity with a 54% overall yield from dipeptide **10**.

Analytical data for 2:

^{1}H NMR (500 MHz, CD_3OD):

Synthetic product **2**, ·3TFA salt: δ= 0.84 (3H, t, J=7.4Hz), 1.54 (2H, q, J=7.4Hz), 1.46-1.64 (4H, m), 2.04-2.18 (4H, m), 2.16 (2H, dt, J_1=1.5Hz, J_2=7.4Hz), 2.80 (2H, dd,

J_1=8.5Hz, J_2=14Hz), 2.93-3.20 (12H, m), 4.39 (1H, dd, J_1=7.0Hz, J_2=8.5Hz), 6.70 (2H, d, J=8.5Hz), 7.04 (2H, d, J=8.5Hz).

Natural product **2**, ·3TFA salt: δ= 0.84 (3H, t, J=7.4Hz), 1.56 (2H, q, J=7.4Hz), 1.46-1.64 (4H, m), 1.98-2.08 (4H, m), 2.16 (2H, dt, J_1=1.5Hz, J_2=7.4Hz), 2.80 (2H, dd, J_1=8.5Hz, J_2=14Hz), 2.94-3.20 (12H, m), 4.38 (1H, dd, J_1=7.0Hz, J_2=8.5Hz), 6.70 (2H, d, J=8.5Hz), 7.04 (2H, d, J=8.5Hz).

^{13}C NMR (75 MHz, CD_3OD) for Synthetic product **2**, TFA salt: δ= 13.90, 20.24, 24.11, 24.29, 25.36, 26.01, 27.24, 27.57, 37.79, 38.20, 38.71, 39.25, 45.65, 45.99, 56.86, 116.23, 129.08, 131.27, 157.31, 174.13, 176.10.

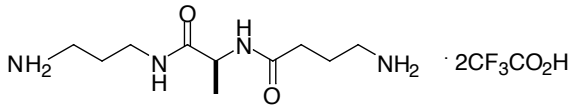
ES-MS ($C_{23}H_{41}N_5O_3$): Synthetic product **2**: m/z 436.3 $(M+H)^+$
Natural product **2**: m/z 436.3 $(M+H)^+$

RP-HPLC: (a) Retention time: synthetic product **2** 11.83 min., natural product **2** 11.83 min., co-injection of synthetic and natural products eluted as one peak at 11.83 min.
(b) Conditions: Column SB-C18 (4.6X150mm, 5μm);
Eluent 10% MeCN (0.1% TFA) and 90% H_2O (0.1% TFA); Flow rate 1.5 mL/min.; Detection 274 nm.

Selected NMR, ES-MS and HPLC spectra are shown on pages 16 to 23.

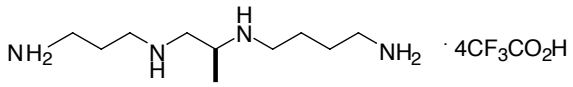
(4) Characterization of 3, an ethylenediamine structural analogue of PhTX-433.

Synthesis of **3** was similar to that outlined for **2** except for the use of L-Ala instead of β-Ala in the first amino acid coupling.



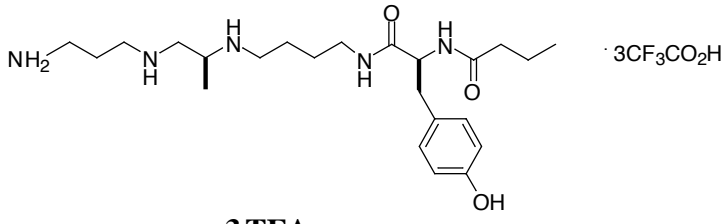
14

H_2N-$(CH_2)_3$-NH-LAla-γAbu (14). ^{1}H NMR (300 MHz, CD_3OD) δ 4.22 (q, J = 7.2 Hz, 1H), 3.30 (t, J = 7.0 Hz, 2H), 2.97 (t, J = 7.0 Hz, 2H), 2.94 (t, J = 7.0 Hz, 2H), 2.37 (t, J = 7.1 Hz, 2H), 1.92 (quintet, J = 7.2 Hz, 2H), 1.84 (quintet, J = 7.2 Hz, 2H), 1.35 (d, J = 7.2 Hz, 3H). ESMS M+H 231.1.



15

Tetraamine tetrakis(fluoroacetate) salt (15). Cleavage from the resin followed by precipitation from methanol/ether gave the salt as a white solid in 77 % yield from the diamide. ^{1}H NMR (300 MHz, CD_3OD) δ 3.71 (multiplet, 1H), 3.48 (dd, J = 5.9 Hz, 13.4 Hz, 1H), 3.31 (dd, J = 5.9 Hz, 13.4 Hz, 1H), 3.24 – 2.94 (multiplet, 8H), 2.12 (quintet, J = 7.8 Hz, 2H), 1.90 – 1.65 (multiplet, 4H), 1.46 (d, J = 6.9 Hz, 3H). ^{13}C NMR (75.5 MHz, CD_3OD) δ 163.4 (C), 163.0 (C), 52.9 (CH), 50.8 (CH_2), 46.9 (CH_2), 45.9 (CH_2), 39.9 (CH_2), 37.9 (CH_2), 25.5 (CH_2), 24.3 (CH_2), 15.0 (CH_3). ESMS 203.2 (M+H), 102.2 (M+2H)/2. ESMS of acetylated tetraamine 329.2 (M+H).



3·TFA

PhTX-433 analogue (3·3TFA). Two rounds of precipitation from methanol/ether afforded **3** as a yellow amorphous solid in 88 % purity, as determined by HPLC, with a 75 % overall yield from the diamide. ^{1}H NMR (300 MHz, CD_3OD) δ 7.04 (d, J = 8.1 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 4.40 (dd, J = 6.9 Hz, 8.5 Hz, 1H), 3.62 – 3.50 (broad s, 1H), 3.28 – 2.96 (multiplet, 10H), 2.97 (dd, J = 6.6 Hz, 13.8 Hz, 1H), 2.80 (dd, J = 8.7 Hz, 13.8 Hz, 1H), 2.16 (t, J = 7.2 Hz, 2H), 2.12 – 1.98 (m, 2H), 1.89 – 1.73 (m 2H), 1.70 – 1.46 (m, 8H), 1.42 (d, J = 6.6 Hz, 3H), 0.84 (t, J = 7.5 Hz, 3H). ^{13}C NMR (75.5 MHz, CD_3OD) δ 176.1 (C), 174.1 (C), 163.4 (C), 163.0 (C), 157.3 (C), 131.3 (CH), 129.1 (C), 120.0 (CF_3), 116.2 (CH), 116.2 (CF_3), 56.8 (CH), 52.7 (CH), 50.6 (CH_2), 46.9 (CH_2), 39.2 (CH_2), 38.7 (CH_2), 37.8 (CH_2), 27.2 (CH_2), 25.5 (CH_2), 24.5 (CH_2), 20.2 (CH_2), 15.5 (CH_3), 13.9 (CH_3). ES-MS 436.3 (M+H). HRMS-ES-MS M+H for $C_{23}H_{42}N_5O_3$ calcd. 436.328766, obsd. 436.329035.

Selected NMR, ES-MS and HPLC spectra are shown on pages 24 to 33.