# Effective Synthesis of Kynurenine-containing Peptide via On-resin Ozonolysis of Tryptophan Residues; Synthesis of Cyclomontanin B

Clarence T. T. Wong<sup>†</sup>, Hiu Yung Lam<sup>†</sup> and Xuechen Li\* Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, P. R. China xuechenl@hku.hk

## **Materials and Methods**

All commercial materials (Aldrich, Chemimpex and GL Biochem) were used without further purification. All solvents were reagent grade or HPLC grade (RCI or DUKSAN). Dry dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) was distilled from calcium hydride (CaH<sub>2</sub>). The following Boc amino acids were purchased from GL Biochem and Chemimpex and used in the solid phase synthesis: Boc-Ala-OH, Boc-Asn(Xan)-OH, Boc-Leu-OH, Boc-Phe-OH, Boc-Thr(Bzl)-OH, Boc-Pro-OH, Boc-Trp(For)-OH, Boc-Gly-OH. Fmoc amino acids were purchased from GL Biochem and used in solid phase synthesis: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH. All separations involved a mobile phase of 0.05% TFA (v/v) in acetonitrile (solvent A)/0.05% TFA (v/v) in water (Solvent B). HPLC separations were performed with a Waters HPLC system equipped with a photodiode array detector (Waters 2996) wavelength 190-400 nm using a Vydac 218TP<sup>TM</sup> C18 column (5 µm, 300 Å, 4.6 x 250 mm) at a flow rate of 0.6 mL/min for analytical HPLC and XBridge<sup>TM</sup> Prep C18 10 µm OBD<sup>TM</sup> column (10 µm, 300 Å, 30 x 250 mm) at a flow rate of 15 mL/min for preparative HPLC. Low-resolution mass spectral analyses were performed with a Waters 3100 mass spectrometer. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on Bruker Avance DRX 400 FT-NMR spectrometer at 400 MHz for <sup>1</sup>H NMR and 100 MHz for <sup>13</sup>C NMR and Bruker Avance DRX 300 FT-NMR spectrometer at 300 MHz for <sup>1</sup>H NMR and 75 MHz for <sup>13</sup>C NMR.

## Experimental

Fmoc-Kyn(Boc, For)-OH :



Fmoc-Trp(Boc)-OH (1.0 g, 1.93 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 ml) and cooled to -78°C. O<sub>3</sub> was bubbled into the reaction for 5 min using ozone generator (Ruiquing, China) connected to oxygen tank. Dimethyl sulfide (0.5 ml) was added into the reaction at -78°C. The reaction mixture was allowed to warm to room temperature over 1 h. The reaction mixture was concentrated under *vacuo* to give Fmoc-Kyn(Boc, For)-OH as a pair of rotamers without further purification. <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) mixture of rotamers δ 9.21 (1H, s), 9.20 (1H, s), 7.99 – 7.00 (2H, m), 7.86 – 7.88 (4H, m), 7.56 – 7.69 (10H, m), 7.37 – 7.41 (4H, m), 7.29 – 7.32 (6H, m), 4.45 – 4.48 (2H, m), 4.27 – 4.29 (4H, m), 4.19 – 4.22 (2H, m), 1.36 (9H, s), 1.33 (9H, s) <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD) mixture of rotamers δ 207.7, 207.6, 183.5, 173.6, 171.8, 166.4, 162.0, 154.3, 151.2, 144.9, 143.5, 141.3, 140.3, 139.5, 138.2, 137.6, 135.7, 133.9, 131.7, 130.7, 94.3, 94.2, 76.2, 70.3, 60.3, 59.9, 57.1, 52.0, 41.8, 37.9 ESI calcd. for C<sub>31</sub>H<sub>31</sub>N<sub>2</sub>O<sub>8</sub> [M+H]<sup>+</sup> m/z = 559.21 found 558.92

Boc-Kyn(CHO, CHO)-OH:



Boc-Trp(For)-OH (1.0 g, 3.01 mmol) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 ml) and cooled to -78 °C. O<sub>3</sub> was bubbled into the reaction for 5 min using ozone generator (Ruiquing, China) connected to

oxygen tank. Dimethyl sulfide (0.5 ml) was added into the reaction at -78°C. The reaction mixture was allowed to warm to room temperature over 1 h. The reaction mixture was concentrated under *vacuo* to give Boc-Kyn(For, For)-OH without further purification. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD)  $\delta$  9.16 (2H, s), 7.97 (1H, d, J = 7.4 Hz), 7.59 – 7.72 (2H, m), 7.28 – 7.30 (1H, m), 7.02 (1H, d, J = 8.1 Hz), 4.37 (1H, q, J = 7.6 Hz), 3.21 – 3.42 (2H, m), 1.35 (9H, s) <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD)  $\delta$  198.2, 173.8, 166.5, 166.5, 155.8, 135.9, 133.4, 133.4, 130.8, 130.0, 130.0, 78.7, 50.8, 42.1, 28.7 ESI calcd. for C<sub>17</sub>H<sub>21</sub>N<sub>2</sub>O<sub>7</sub> [M+H]<sup>+</sup> *m/z* = 365.13 found 364.62

#### General procedure for solid phase peptide synthesis of linear peptide models

Fmoc-rink-amide resin: The Fmoc-rink-amide resin (GL Biochem, loading 0.49 mmol/g, 300-500 mg) was swollen in  $CH_2Cl_2$  for 30 min followed by deprotection of Fmoc group using 20 % piperidine in DMF. The resin was washed with DMF (10 ml x 3) and  $CH_2Cl_2$  (10 ml x 3). A solution of Fmoc-Xaa-OH (4.0 equiv. relative to resin capacity), HATU (4.0 equiv.) and DIPEA (8.0 equiv.) in anhydrous DMF (10 mL) was added to the resin. The mixture was gently agitated for 45 min followed by sequential washing with DMF (10 mL x 3) and  $CH_2Cl_2$  (10 mL x 3).

2-chlorotrityl chloride resin: 2-chlorotrityl chloride resin (GL Biochem, loading 0.79 mmol/g, 300-500 mg) was swollen in  $CH_2Cl_2$  for 15 min. The first Fmoc amino acids (2.0 equiv.) was mixed with DIPEA (4.0 equiv.) for 5 min in  $CH_2Cl_2$ . Then the solution and the resin were mixed and gently agitated for 1 hr. The unreacted resin was capped by  $CH_3OH$ . Peptide coupling: A solution of Fmoc-Xaa-OH (4.0 equiv. relative to resin capacity), HATU (4.0 equiv.) and DIPEA (8.0 equiv.) in anhydrous DMF (10 mL) was added to the resin. The mixture

was gently agitated for 45 min followed by sequential washing with DMF (10 mL x 3) and  $CH_2Cl_2$  (10 mL x 3).

Ozonolysis: After peptide elongation was completed, the solvent was drained and the resin was washed with DMF (10 ml x 3) and  $CH_2Cl_2$  (10 ml x 3). The resin bound peptide was swollen in  $CH_2Cl_2$  at -78 °C. After the mixture was treated with O<sub>3</sub> at -78°C for 5 min, Dimethyl sulfide (10.0 equiv. relative to resin capacity) was then added at -78°C. The reaction mixture was allowed to warm to room temperature over 1 h. The solvent was drained and the resin was

washed with DMF (10 ml x 3) and  $CH_2Cl_2$  (10 ml x 3).

Global deprotection: A mixture of TFA/H<sub>2</sub>O/TIS (9:0.5:0.5, v/v/v) was used for the global deprotection of the peptide for 1.5 h at room temperature. The peptide was precipitated with Et<sub>2</sub>O and was confirmed by analytical LCMS (5 – 95 % ACN/H<sub>2</sub>O over 15 min).

Peptide 1



Sequence: NH<sub>2</sub>-Ser-Kyn-Glu-Gln-Phe-OH. ESI calcd. for  $C_{32}H_{42}N_7O_{11}[M+H]^+ m/z = 700.71$ ; found 701.01.



**Figure S1**: UV trace (190-400 nm) from HPLC analysis of a crude reaction mixture of  $NH_2$ -Ser-Kyn-Glu-Gln-Phe-OH: gradient 5-95% ACN/H<sub>2</sub>O containing 0.05% TFA over 15 min at a flow rate of 0.6 ml/min. Elution time = 9.84 min.



Figure S2. ESI-MS of NH<sub>2</sub>-Ser-Kyn-Glu-Gln-Phe-OH.

Peptide 2



Sequence: Ac-His-Tyr-Kyn-Phe-Pro-NH<sub>2</sub>. ESI calcd. for  $C_{41}H_{48}N_9O_8 [M+H]^+m/z = 794.36$ ; found 794.00.



**Figure S3**: UV trace (190-400 nm) from HPLC analysis of a crude reaction mixture of Ac-His-Tyr-Kyn-Phe-Pro-NH<sub>2</sub>: gradient 5-95% ACN/H<sub>2</sub>O containing 0.05% TFA over 15 min at a flow rate of 0.6 ml/min. Elution time = 11.82 min.



Figure S4. ESI-MS of Ac-His-Tyr-Kyn-Phe-Pro- NH<sub>2</sub>.

Peptide 3



Sequence: NH<sub>2</sub>-His-Kyn-Tyr-Ala-OH. ESI calcd. for  $C_{28}H_{34}N_7O_7[M+H]^+ m/z = 580.25$ ; found 580.02.



**Figure S5**: UV trace (190-400 nm) from HPLC analysis of a crude reaction mixture of  $NH_2$ -His-Kyn-Tyr-Ala-OH: gradient 5-95% ACN/H<sub>2</sub>O containing 0.05% TFA over 15 min at a flow rate of 0.6 ml/min. Elution time = 8.86 min.



Figure S6. ESI-MS of NH<sub>2</sub>-His-Kyn-Tyr-Ala-OH.

Peptide 4



Sequence: NH<sub>2</sub>-Phe-Pro-Kyn-Leu-NH<sub>2</sub>. ESI calcd. for  $C_{30}H_{41}N_6O_5[M+H]^+ m/z = 565.31$ ; found 564.92



**Figure S7**: UV trace (190-400 nm) from HPLC analysis of a crude reaction mixture of  $NH_2$ -Phe-Pro-Kyn-Leu-NH<sub>2</sub>: gradient 5-95% ACN/H<sub>2</sub>O containing 0.05% TFA over 15 min at a flow rate of 0.6 ml/min. Elution time = 12.21 min.



Figure S8. ESI-MS of NH<sub>2</sub>-Phe-Pro-Kyn-Leu-NH<sub>2</sub>.





Sequence: NH<sub>2</sub>-Arg-Val-Asn-Kyn-Met[O]-NH<sub>2</sub>. ESI calcd. for  $C_{30}H_{50}N_{11}O_8S [M+H]^+ m/z = 724.35$ ; found 723.91.



**Figure S9**: UV trace (190-400 nm) from HPLC analysis of a crude reaction mixture of NH<sub>2</sub>-Arg-Val-Asn-Kyn-Met[O]-NH<sub>2</sub>: gradient 5-95% ACN/H<sub>2</sub>O containing 0.05% TFA over 15 min at a flow rate of 0.6 ml/min. Peak at 9.10 min = Met[O], 8.80 min = Met [2O].



Figure S10. ESI-MS of NH<sub>2</sub>-Arg-Val-Asn-Kyn-Met[O]-NH<sub>2</sub>.

Synthesis of cyclomontanin B



**Scheme S1.** Synthesis of SAL ester linker for SPPS. Key: (a) Ac<sub>2</sub>O, pyridine, 100%; (b) THF, 85%; (c) 95% TFA; (d) PyBOP, DIEA, DMF, aminomethyl resin; (e) 20% piperidine in DMF.

#### Synthesis of NH<sub>2</sub>-Thr-Pro-Gly-Leu-Asn-Ala-Kyn-SAL ester:

NH<sub>2</sub>-Thr-Pro-Gly-Leu-Asn-Ala-Kyn-SAL ester was prepared according to Scheme S1.

**Loading of the resin:** The hydroxyl group of salicylaldehyde was first protected with an acetyl group, followed by the Wittig reaction with triphenylphosphorane to form compound **6a**. Compound **6a** was treated with 95% TFA to deprotect the *t*Bu group to liberate the carboxylic acid followed by removal of the solvent by a stream of condensed air. Aminomethyl resin (Chemimpex, loading 1.15 mmol/ g, 1 g) was swollen in anhydrous  $CH_2Cl_2$  for 30 min. A solution of deprotected compound **6a** (2.0 equiv. relative to resin capacity), PyBOP (2.0 equiv.) and DIPEA (4.0 equiv.) in anhydrous DMF (10 mL) was added. The mixture was gently agitated for 12 h. The solvent was drained and the resin was washed with DMF (10 mL x 3) and  $CH_2Cl_2$  (10 mL x 3). The unreacted resin was capped by acetic anhydride. The resin was dried under *vacuo*. The loading of the resin was determined by the mass difference to be 0.9 - 1.0 mmol/g. **First amino acid coupling:** A solution of 20 % piperidine in DMF (10 mL) was added to the loaded resin (300 mg, 0.9 - 1.0 mmol/g) and the mixture was gently agitated for 1 h to remove

the acetyl group. The solvent was drained and the resin bound SAL ester linker 6 was washed with DMF (10 mL x 3). A solution of the first amino acid to be coupled (4.0 equiv. relative to resin capacity), PyBOP (4.0 equiv.) and DIPEA (8.0 equiv.) in anhydrous DMF (10 mL) was added. The mixture was gently agitated for 12 h.

**Boc deprotection:** The Boc group was removed with neat TFA (2 x 5 min) followed by sequential washing with  $CH_2Cl_2$  (10 mL x 3), DMF (10 mL x 3) and  $CH_2Cl_2$  (10 mL x 3).

**Peptide coupling:** A solution of Boc-Xaa-OH (4.0 equiv. relative to resin capacity), HATU (4.0 equiv.) and DIPEA (8.0 equiv.) in anhydrous DMF (10 mL) was added to the resin. The mixture was gently agitated for 45 min followed by sequential washing with DMF (10 mL x 3) and  $CH_2Cl_2$  (10 mL x 3).

**Global deprotection:** A mixture of TMSOTf/ TFA/ thioanisole (1:8.5:0.5, v/v/v) was added to the resin bound peptide at 0°C and the mixture was gently agitated for 1 h at 0°C. The resin was then washed with CH<sub>2</sub>Cl<sub>2</sub> (10 mL x 6).

**Cleavage from the resin:** The resin bound peptide was swollen in CH<sub>2</sub>Cl<sub>2</sub>/ TFA (95:5, v/v) at - 78°C. After the mixture was treated with O<sub>3</sub> at -78°C for 5 min, Dimethyl sulfide (10.0 equiv. relative to resin capacity) was then added at -78°C. The reaction mixture was allowed to warm to room temperature over 1 h. The mixture was then filtered and the filtrated was concentrated under *vacuo* to afford *crude* NH<sub>2</sub>-Thr-Pro-Gly-Leu-Asn-Ala-Kyn-SAL ester. ESI calcd. for C<sub>41</sub>H<sub>56</sub>N<sub>9</sub>O<sub>12</sub> [M+H]<sup>+</sup> m/z = 866.40; found 866.54. (containing 28% NH<sub>2</sub>-Thr-Pro-Gly-Leu-Asn-Ala-Kyn (CHO)-SAL ester. ESI calcd. for C<sub>42</sub>H<sub>56</sub>N<sub>9</sub>O<sub>13</sub> [M+H]<sup>+</sup> m/z = 894.95; found 895.43 ).



**Figure S11**. UV trace (190-400 nm) from HPLC analysis of a crude reaction mixture of the peptide SAL ester.



 $\label{eq:Figure S12} Figure \ S12. \ ESI-MS \ of \ NH_2-Thr-Pro-Gly-Leu-Asn-Ala-Kyn-SAL \ ester$ 



Figure S13. ESI-MS of NH2-Thr-Pro-Gly-Leu-Asn-Ala-Kyn(CHO)-SAL ester

#### Synthesis of cyclomontanin B:



*Crude* NH<sub>2</sub>-Thr-Pro-Gly-Leu-Asn-Ala-Kyn(For)-SAL ester (130 mg, 0.15 mmol) was dissolved in 150 mL pyridine/acetic acid (1:2, mole: mole) at a concentration of 1 mM at room temperature. The reaction mixture was stirred at room temperature for 4 h. The solvent was removed under *vacuo*. The crude cyclized product was then treated with 95% TFA for 30 min to give the native cyclic peptide. The solvent was removed by a stream of condensed air. Next, the crude reaction mixture was treated with Tris HCl buffer (pH 8) for 4 h to remove the formyl group on Kyn. Preparative HPLC purification (10-60% CH<sub>3</sub>CN/H<sub>2</sub>O over 30 min) followed by lyophilization afforded cyclomontanin B as a white powder (35 mg, 16% from resin loading). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded and were found to be the same as the natural cyclomontanin B<sup>1</sup>. ESI calcd. for C<sub>34</sub>H<sub>50</sub>N<sub>9</sub>O<sub>10</sub>[M+H]<sup>+</sup> *m/z* = 744.36; found 743.67

<sup>1</sup>H NMR (400 MHz, pyridine-d5)  $\delta$  10.26 (1H, m), 9.27 – 9.28 (2H, m), 9.00 (1H, d, J = 8.0 Hz), 8.76 (1H, d, J = 9.4 Hz), 8.60 – 8.64 (1H, m), 8.14 (1H, d, J = 9.4 Hz), 7.72 (1H, d, J = 7.2 Hz), 7.08 – 7.12 (2H, m), 6.80 – 6.82 (2H, m), 6.35 – 6.39 (2H, m), 5.86 – 5.90 (1H, m), 5.25 – 5.29 (2H, m), 4.94 – 4.95 (1H, m), 4.65 – 4.72 (1H, m), 4.25 – 4.62 (2H, m), 4.33 (1H, t, J = 7.8 Hz), 3.52 – 4.02 (7H, m), 1.77 – 1.95 (3H, m), 1.60 – 1.69 (3H, m), 1.50 (3H, d, J = 6.2 Hz), 1.22 – 1.23 (4H, m), 0.76 – 0.79 (6H, m) <sup>12</sup>C NMR (100 MHz, pyridine-d5)  $\delta$  200.6, 175.9, 173.9, 173.8, 173.7, 173.5, 170.9, 170.8, 165.5, 153.5, 135.7, 133.0, 118.9, 118.6, 116.2, 70.5, 62.9, 59.1, 55.5, 54.2, 52.6, 52.3, 49.7, 45.4, 44.9, 43.5, 37.3, 30.7, 26.0, 25.9, 23.6, 23.5, 21.2, 18.1



Figure S14: UV (190-400 nm) and MS traces from LCMS analysis of the synthetic cyclomontanin B: gradient 5-95% ACN/H<sub>2</sub>O containing 0.05% TFA over 15 min at a flow rate of 0.6 ml/min. Elution time = 11.01 min.



Figure S15. ESI-MS of the synthetic cyclomontanin B

## **References:**

1. P. H. Chuang, P. W. Hsieh, Y. L. Yang, K. F. Hua, F. R. Chang, J. Shiea, S. H. Wu and Y. C. Wu, *J. Nat. Prod.*, 2008, **71**, 1365-1370

## <sup>1</sup>H and <sup>13</sup>C NMR spectra

### <sup>1</sup>H spectrum of Fmoc-Kyn(Boc, CHO)-OH (crude product after ozonolysis):





## <sup>13</sup>C NMR spectrum of Fmoc-Kyn(Boc, CHO)-OH (crude product after ozonolysis):

#### <sup>1</sup>H spectrum of Boc-Kyn(CHO, CHO)-OH (crude product after ozonolysis):



