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Effective synthesis of kynurenine-containing peptides *via* on-resin ozonolysis of tryptophan residues: synthesis of cyclomontanin B†

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The preparation of Kyn-containing peptides is difficult, owing to the low reactivity of Kyn in the coupling reaction. In this report, Kyn-containing peptides were efficiently obtained *via* on-resin ozonolysis of the corresponding Trp-containing peptide. In addition, a Kyn-containing cyclic peptide, cyclomontanin B, has been synthesized by this strategy in the combination with serine/threonine ligation (STL)-mediated cyclization.

Introduction

Kynurenine (Kyn) is a metabolite amino acid produced biologically from the degradation of tryptophan by a series of enzymatic reactions.[1] The enzymes, including tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), use superoxide to perform oxidative metabolism on tryptophan. These enzymes are important in the regulation of cell growth, division and inflammation.[2,3] Kyn itself plays a central role in the kynurenine pathway, which includes three distinct pathways to form kynurenic acid, 3-hydroxy-L-kynurenine and anthranilic acid using a series of tightly controlled enzymes. As a result, variations of the Kyn concentration in the body could indicate diseases, including cancers, HIV, rheumatoid arthritis, diabetics and CNS disorders.[4–8] On the other hand, Kyn itself was reported to be a neuro-protective molecule on the CNS but at the same time immunosuppressive.[9]

Although tryptophan and Kyn are highly similar in terms of size and hydrophobicity, the occurrence of Kyn in natural products suggests that Kyn could have specificity toward their receptors or targets. For instance, daptomycin (Fig. 1), an FDA approved cyclic Kyn-containing lipodepsipeptide isolated from *Streptomyces roseoporus*, has been used in treatment of skin infections caused by Gram-positive pathogens. It has been shown that the mutation of Kyn to Trp causes decreased antibacterial activity.[10,11] Moreover, Malik *et al.* have discovered a Kyn-containing decapeptide hormone from Vietnamese stick insects that has hypertrehalosemic and cardioacceleratory activities.[12] Bowie and co-workers have isolated a Kyn-containing opioid tetrapeptide from the skin of the Australian red tree frog.[13] In addition, a cyclic Kyn-containing peptide, cyclomontanin B, isolated from seed of *Annona montana* has exhibited promising anti-inflammatory activity (Fig. 1).[14] Therefore, the presence of Kyn residues in peptide sequences could possibly alter the properties and therapeutic index of the peptides.

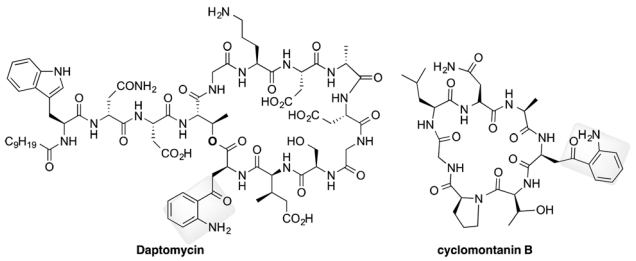

Fig. 1 The structure of daptomycin and cyclomontanin B.

As a result, an efficient synthesis method for Kyn-containing peptides and proteins would be valuable to perform functional and structural studies as well as screening of novel Kyn bioactive peptides. An earlier preparation of Kyn compounds relied on ozonolysis of Trp-OH to produce Kyn(CHO)-OH; however, the yield from this transformation was very low (~20–33%).[15] Hoffmann and co-workers reported the synthesis of Kyn from Trp *via* oxindolealanine (Oia) kynurenine with an overall yield of 35%.[16] Another multistep preparation of Kyn *via* β-3-oxindolyl-alanine was recently developed.[17]

Recently, we have disclosed an easy solution to the problem of Kyn preparation, where an *N*-Boc-protected Trp building block [*e.g.*, Fmoc-Trp(Boc)-OH] was efficiently ozonolyzed to provide Fmoc-Kyn(Boc, CHO)-OH in a quantitative yield (Fig. 2a).[18] However, we also observed an abnormally low reactivity of Fmoc-Kyn(Boc)-OH in the coupling reaction.[18] Herein,

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‡CTTW and HYL with equal contribution.

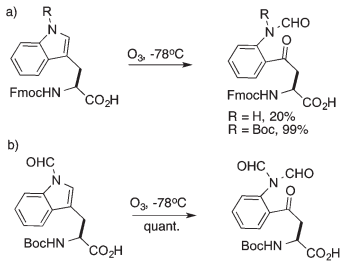

Fig. 2 The synthesis of Kyn building blocks from tryptophan *via* ozonolysis.

we report an efficient strategy to synthesize Kyn-containing peptides, including cyclic peptides.

Results and discussion

To overcome the low reactivity of the Kyn moiety in the coupling reaction, we sought to examine the feasibility of on-resin ozonolysis of Trp-containing peptides to obtain the corresponding Kyn moieties. To this end, we first synthesized, *via* the standard Fmoc-SPPS either on a rink amide resin or a 2-chlorotrityl chloride resin, an array of Trp-containing peptides composed of 4–5 amino acids (Table 1). The resulting resin-bound peptides were directly subjected to ozonolysis conditions (O_3, −78 °C, DCM, 5 min; subsequently, Me_2S, 1 h, rt), followed by TFA cleavage. The LCMS analysis indicated that all the peptides could be converted into Kyn containing peptides directly from tryptophan *via* ozonolysis without noticeable side-products (Table 1). For peptides containing methionine, under our conditions with ozone treatment, approximately 26% of the amino acid was converted into sulfone, 54% became sulfoxide and less than 12% remained unreacted. Other ozone-sensitive amino acids reported by Sharma and Berlett,[19] including the conversion of histidine into 2-amino-*N*-formylureido-succinimic acid and oxidation of tyrosine to 2-amino-3-(3,4-dioxo-cyclohexa-1,5-dienyl)-propionic acid, were not observed under our reaction conditions.

Having demonstrated the effectiveness of the on-resin ozonolysis of Kyn-containing peptides, we next undertook the synthesis of a Kyn-containing cyclic peptide, cyclomontanin B. Two synthetic strategies were devised (Fig. 3).

First, we examined the feasibility of traditional cyclization at the Gly–Leu conjunction, followed by in-solution ozonolysis to convert Trp into Kyn (Fig. 3a). It started with 2-chlorotrityl

Table 1 Synthetic Kyn-containing peptide models and the percentages of conversion

Entry	Sequence	Obs. mass	%[a]
1	H-**S-Kyn-E-Q-F**-OH	701.01	75
2	Ac-**H-Y-Kyn-F-P**-NH_2	794.00	89
3	H-**H-Kyn-Y-A**-OH	580.02	99
4	H-**F-P-Kyn-L**-NH_2	564.92	99
5	H-**R-V-N-Kyn-M**(O)-NH_2	723.91	54

[a] Conversion based on LC-MS analysis.

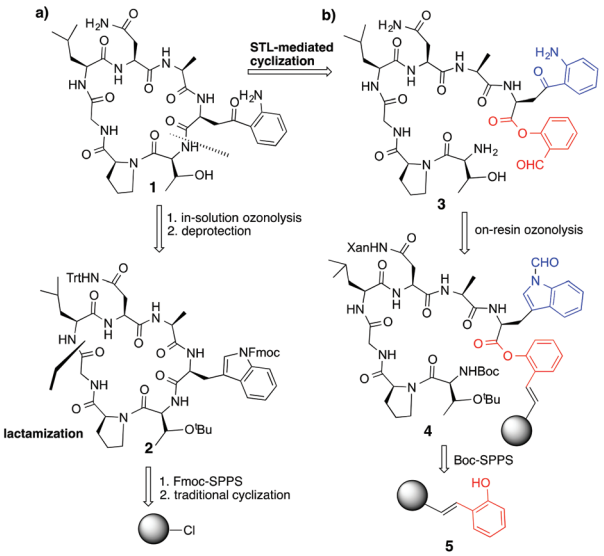

Fig. 3 Retrosynthetic strategies of cyclomontanin B.

chloride resin to be preloaded with Fmoc-Gly-OH using the standard conditions. Then the functionalized resin was subjected to Fmoc-SPPS to assemble the linear peptide. The resulting peptide was released from the resin with a mild acid mixture (TFE–DCM–AcOH), whilst the side-chain protecting groups were kept intact. With this protected linear peptide precursor in hand, we then applied some conditions commonly used for peptide lactamization. Under the conditions attempted (PyBOP, DIEA, DMF, and DEPET, DIEA, DMF), only traces of the desired cyclic product together with dimerized and trimerized cyclic peptides were observed by LC-MS analysis. Instead of probing other coupling reagents or other cyclization sites, we decided to take up an alternative strategy (Fig. 3b).

Recently, we have reported a serine/threonine ligation (STL) enabling protein synthesis and peptide cyclization, in which a peptide with a C-terminal salicylaldehyde (SAL) ester could chemoselectively react with another or the same peptide containing an N-terminal serine or threonine residue to form an *N*,*O*-benzylidene acetal linkage intermediate, affording the natural peptide linkage Xaa-Ser/Thr upon acidolysis.[18,20] According to the retrosynthesis of cyclomontanin B, the cyclization could be performed at the conjunction of Kyn-Thr using threonine ligation. As observed previously, Kyn-OH was resistant to the coupling reaction;[18] thus cyclization at the Kyn-Thr site could be likely challenging, and could serve as a good model to test the constraint of serine/threonine ligation (STL).

We planned to use Boc-SPPS together with the on-resin ozonolysis approach to prepare the requisite linear peptide precursor (**3**) amenable to cyclization *via* threonine ligation-mediated cyclization. As shown in the retrosynthetic analysis (Fig. 3b), we envisioned that the peptide could be assembled on a resin linked with 2-vinylphenol *via* Boc-SPPS. Thus synthesized peptide precursor (**4**) was subjected to on-resin ozonolysis; the peptide would be released from the resin. At the same time,

the Kyn moiety would be obtained from the corresponding Trp.

To enable Boc-SPPS synthesis, Boc-Trp(CHO)-OH needs to be used. Thus, we first studied the effectiveness of ozonolysis of Boc-Trp(CHO)-OH. Indeed, the reaction proceeded equally well as Fmoc-Trp(Boc)-OH (Fig. 2b). Next, an aminomethyl resin derivatized with a 2-hydroxylphenyl-acrylamide linker (**6**) was subjected to Boc-SPPS, with Boc-Trp(CHO)-OH, Boc-Ala-OH, Boc-Asn(Xan)-OH, Boc-Leu-OH, Boc-Gly-OH, Boc-Pro-OH, and Boc-Thr(Bzl)-OH, sequentially. After the resin bound peptide (**7**) was synthesized, TMSOTf/TFA/thioanisole is used to remove the side chain protecting groups while keeping the peptide intact on the resin. Then, on-resin ozonolysis (O_3, −78 °C, DCM/TFA) released the peptide from the resin with the generation of the required peptide salicylaldehyde ester; at the same time, the embedded Trp(CHO) residue was converted into Kyn(CHO, CHO). This time, the formyl groups on Kyn fell off to afford peptide **8** (72%) together with mono-formylated Kyn product (28%), due to the presence of TFA during on-resin ozonolysis (Fig. 4).

With linear cyclomontanin B precursor in hand, we continued to examine the peptide cyclization *via* a threonine ligation. Towards the end, the peptide SAL ester was dissolved in a pyridine–acetic acid mixture (1 : 2, mole : mole) at a concentration of 1 mM. Gratifyingly, the peptide SAL ester underwent cyclization smoothly to completion within 4 hours. Then, after acidolysis with TFA/H_2O to remove the *N*,*O*-benzylidene acetal, followed by the basic treatment to remove the CHO group on Kyn, the desired cyclomontanin B was obtained in 16% yield after reverse-phase HPLC purification based on the resin loading (Fig. 5). It is worthwhile to note that Kyn possesses very low reactivity in the coupling reaction; however, the cyclization at Kyn-Thr *via* threonine ligation is apparently not retarded, demonstrating the efficiency of STL.

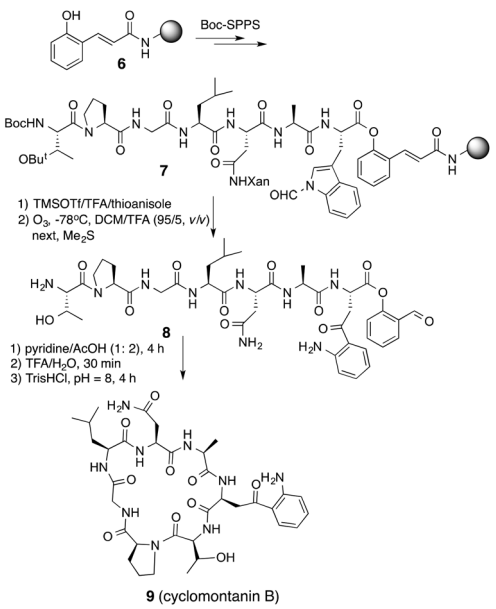

Fig. 4 Synthetic scheme of cyclomontanin B.

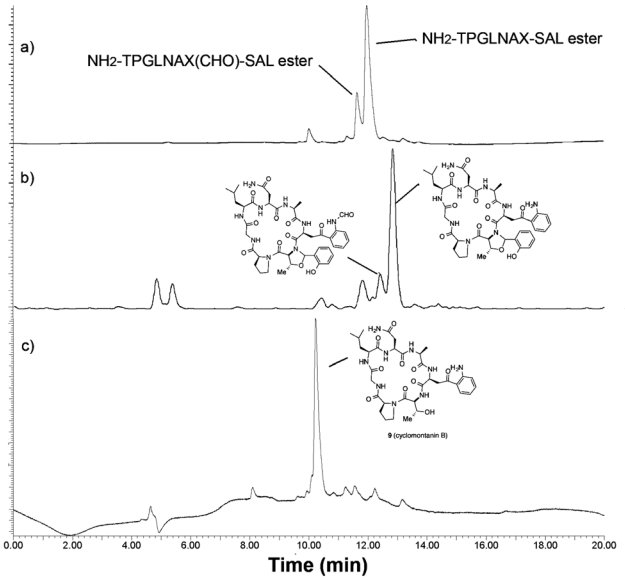

Fig. 5 Analytical HPLC traces of the peptide cyclization to synthesize cyclomontaninin B. (a) The crude peptide SAL ester after on-resin ozonolysis; (b) threonine ligation-mediated cyclization; (c) the crude product after acidolysis and removal of the formyl group. X = Kyn.

Conclusions

In summary, we have presented a new approach to the synthesis of Kyn-containing peptides *via* on-resin ozonolysis of the corresponding suitably protected Trp-containing peptides. This strategy is found to be very effective with easy operation in this regard, allowing the Kyn-containing peptide to be accessed readily. Furthermore, we have combined this strategy and STL-mediated cyclization to prepare a Kyn-containing cyclic peptide, cyclomontanin B.

Experimental

General

All commercial materials (Aldrich, Chemimpex and GL Biochem) were used without further purification. All solvents were of reagent grade or HPLC grade (RCI or DUKSAN). Dry dichloromethane (CH_2Cl_2) was distilled from calcium hydride (CaH_2). 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(O*t*Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-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) at wavelength 190–400 nm using a Vydac 218TP™ C18 column (5 μm, 300 Å, 4.6 × 250 mm) at a flow rate of 0.6 mL min^{-1} for analytical HPLC and XBridge™ and a Prep C18 10 μm OBD™ column (10 μm, 300 Å, 30 × 250 mm) at a flow rate of 15 mL min^{-1} for preparative HPLC. Low-resolution mass spectral analyses were performed using a Waters 3100 mass spectrometer.

Typical procedure for solid phase peptide synthesis of linear peptides

Fmoc-rink-amide resin: the Fmoc-rink-amide resin (GL Biochem, loading 0.49 mmol g^{-1}, 300–500 mg) was swollen in CH_2Cl_2 for 30 min followed by deprotection of the Fmoc group using 20% piperidine in DMF. The resin was washed with DMF (10 ml × 3) and CH_2Cl_2 (10 ml × 3). A solution of Fmoc-Xaa-OH (4.0 equiv. relative to the 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 × 3) and CH_2Cl_2 (10 mL × 3). 2-Chlorotrityl chloride resin: 2-chlorotrityl chloride resin (GL Biochem, loading 0.79 mmol g^{-1}, 300–500 mg) was swollen in CH_2Cl_2 for 15 min. The first Fmoc amino acids (2.0 equiv.) were 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 h. The unreacted resin was capped by CH_3OH. A solution of Fmoc-Xaa-OH (4.0 equiv. relative to the 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 × 3) and CH_2Cl_2 (10 mL × 3).

Typical procedure for on-resin ozonolysis

The resin bound peptide was swollen in CH_2Cl_2 at −78 °C. After the mixture was treated with O_3 at −78 °C for 5 min, dimethyl sulfide (10.0 equiv. relative to the 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 × 3) and CH_2Cl_2 (10 ml × 3). A mixture of TFA–H_2O–TIS (9 : 0.5 : 0.5, v/v/v) was used for global deprotection of the peptide for 1.5 h at room temperature. The peptide was precipitated with Et_2O and was confirmed by analytical LCMS (5–95% ACN–H_2O over 15 min).

Synthesis of cyclomontanin B

Synthesis of NH_2-Thr-Pro-Gly-Leu-Asn-Ala-Kyn-SAL ester. The aminomethyl resin derivatized with a 2-hydroxylphenylacrylamide linker (**6**) (loading, 0.9–1.0 mmol g^{-1}) was washed with DMF (10 mL × 3). A solution of the first amino acid [Boc-Trp(CHO)-OH] to be coupled (4.0 equiv. relative to the 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. The Boc group was removed with neat TFA (2 × 5 min) followed by sequential washing with CH_2Cl_2 (10 mL × 3), DMF (10 mL × 3) and CH_2Cl_2 (10 mL × 3). A solution of Boc-Xaa-OH (4.0 equiv. relative to the 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 × 3) and CH_2Cl_2 (10 mL × 3). Next, 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_2Cl_2 (10 mL × 6). Then, the resin bound peptide was swollen in CH_2Cl_2–TFA (95 : 5, v/v) at −78 °C. After the mixture was treated with O_3 at −78 °C for 5 min, dimethyl sulfide (10.0 equiv. relative to the 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 filtrate was concentrated under vacuum to afford *crude* NH_2-Thr-Pro-Gly-Leu-Asn-Ala-Kyn-SAL ester. ESI calcd for $C_{41}H_{56}N_9O_{12}$ $[M + H]^+$ m/z = 866.40; found 866.54 (containing 28% NH_2-Thr-Pro-Gly-Leu-Asn-Ala-Kyn (CHO)-SAL ester). ESI calcd for $C_{42}H_{56}N_9O_{13}$ $[M + H]^+$ m/z = 894.95; found 895.43.

Peptide cyclization. Crude NH_2-Thr-Pro-Gly-Leu-Asn-Ala-Kyn (For)-SAL ester (130 mg, 0.15 mmol) obtained from the above 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 vacuum. 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_3CN–H_2O over 30 min) followed by lyophilization afforded cyclomontanin B as a white powder (35 mg, 16% from resin loading). ^1H NMR and ^{13}C NMR spectra were recorded and were found to be the same as the natural cyclomontanin B.[14] ESI calcd for $C_{34}H_{50}N_9O_{10}$ $[M + H]^+$ m/z = 744.36; found 743.67. ^1H NMR (400 MHz, pyridine-d5) δ 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). ^{12}C NMR (100 MHz, pyridine-d5) δ 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.

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