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Astins K–P, six new chlorinated cyclopentapeptides from *Aster tataricus*

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ABSTRACT

During further investigation on the methanol extract of roots and rhizomes of *Aster tataricus*, six new chlorinated cyclopentapeptides, designated as astins K–P (**9**–**14**), together with eight known derivatives, astins A–H (**1**–**8**), were isolated. Structures of the new cyclopeptides were established using extensive spectroscopic methods, and the absolute configurations were determined by the advanced Marfey's method. Astin P (**14**) represents a unique cyclopentapeptide linking as *cyclo*-(L-Pro (Cl_2)^1-L-*allo*-Thr^2-L-Ser^3-L-β-Phe^4-L-Ava^5). Astin B (**2**) showed cytotoxicity against BGC-823 cell with IC_{50} value of 19.2 μg/ml. Astin C (**3**) exhibited cytotoxicity on HCT-116 and BGC-823 cells with IC_{50} values of 13.4 and 3.3 μg/ml, respectively.

1. Introduction

In the frame of our long-term research program directed at discovery of structurally unique and potentially useful cyclopeptide drugs from traditional Chinese medicines (TCMs),[1] we had the opportunity to investigate the TCM *Aster tataricus* Linn. f. (Compositae, Chinese name 'zi-wan'). *A. tataricus*, widespread in China, has been used for relieving cough and eliminating phlegm, and documented in *Chinese Pharmacopoeia*.[2] It has been described to produce many natural compounds of biological interest, such as triterpenes, sterols and cyclopeptides.[3] Previous chemical investigation on this plant has led to the isolation of nine cyclopentapeptides (astins A–I),[4] and two cyclotetrapeptides (tataricins A and B).[5] These cyclopeptides belong to a family of Compositae-type cyclopeptides or astins, and share remarkable structural similarity, which are characterized by the presence of four nonproteinogenic amino acids (L-β-Phe, L-Abu, L-*allo*-Thr, chlorinated L-Pro derivatives) and one proteinogenic amino acid (L-Ser).[6,7] Besides their interesting structures, these cyclopeptides also show potential biological activities. Astins A–C (**1**–**3**) have shown the potent antitumor activity by using Sarcoma 180A in mice.[4a,8] Moreover, it is noteworthy that astin C (**3**) was recently found to have immunosuppressive activity by inducing activated T-cell apoptosis.[9] Intrigued by the distinctive chlorinated proline feature and their potential antitumor and immunosuppressive activities,[9,10] six new chlorinated cyclopentapeptides, designated as astins K–P (**9**–**14**), together with eight known derivatives, astins A–H (**1**–**8**), were isolated from the methanol extract of roots and rhizomes of the titled plant (Fig. 1). In this paper, we reported their structure elucidation, absolute configuration assignments, and biological activities (cytotoxic, anti-HBV and immunosuppressive activities).

2. Results and discussion

2.1. Structure determination

The methanol extract of the air-dried and powdered roots and rhizomes of *A. tataricus* was partitioned between EtOAc and *n*-butanol. The EtOAc portion was subjected to a series of silica gel, Sephadex LH-20, RP-18 column chromatography (CC) and HPLC to afford six new cyclopentapeptides, astins K–P (**9**–**14**), together with eight known ones, astins A–H (**1**–**8**). The known compounds were identified by comparing to literature data.[4] Astin C (**3**) was further confirmed by an X-ray experiment (CCDC 933250, see S8, Supplementary data (SM)).

Astin K (**9**), obtained as an amorphous powder, was a cyclopeptide as evidenced by its positive reaction with ninhydrin using a thin layer chromatography (TLC) protosite acidic hydrolysis.[11] Its

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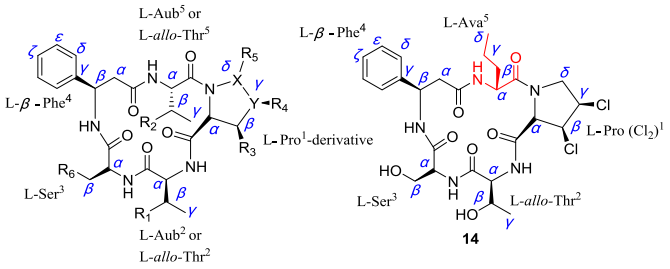

	R_1	R_2	R_3	R_4	R_5	R_6	X-Y
1	H	OH	Cl	Cl	H	OH	CH-CH
2	OH	H	Cl	Cl	Cl	OH	CH-CH
3	H	H	Cl	Cl	H	OH	CH-CH
4	H	H	H	H	Cl	OH	C=C
5	OH	H	H	H	Cl	OH	C=C
6	H	H	Cl	H	H	OH	CH-CH
7	H	H	H	H	H	OH	CH-CH
8	H	OH	H	H	Cl	OH	C=C
9	OH	OH	Cl	Cl	H	OH	CH-CH
10	OH	OH	H	H	Cl	OH	C=C
11	H	H	H	Cl	H	OH	CH-CH
12	H	H	H	Cl	H	OH	C=C
13	H	H	Cl	Cl	H	OAc	CH-CH

Fig. 1. Structures of compounds **1**–**14**.

molecular formula, $C_{25}H_{33}N_5O_8Cl_2$, was deduced from the HRESIMS at *m*/*z* 602.1788 $[M+H]^+$, indicating 11 degrees of unsaturation. Its UV spectrum peaks at 203 and 266 nm suggested the existence of a phenyl group. The IR absorption bands at 3404 and 1649 cm^{-1} indicated the presence of OH, NH, and C=O groups. In the 1D NMR spectra, four amide protons at δ 8.21, 9.56, 10.13 and 10.21 ppm and five amide carbonyls at δ 168.7, 171.4, 171.8, 172.7 and 175.2 ppm were clearly observed, which supported **9** could be a cyclopentapeptide with a proline residue. Detail analysis of the 1D NMR spectra, two methyls [$\delta_H/_C$ 1.73/23.2 (*allo*-Thr^2-γ), 1.77/22.6 (*allo*-Thr^5-γ)], two methylenes [$\delta_H/_C$ 2.63 and 3.27/43.9 (β-Phe^4-α); 3.94 and 5.12/52.2 (Pro $(Cl_2)^1$-δ)], one *O*-methylene [$\delta_H/_C$ 4.21 and 4.40/61.5 (Ser^3-β)], seven methines [$\delta_H/_C$ 4.73/59.4 (Ser^3-α), 4.78/56.1 (Pro $(Cl_2)^1$-γ), 5.30/58.6 (*allo*-Thr^2-α), 5.53/60.4 (*allo*-Thr^5-α), 5.55/66.8 (Pro $(Cl_2)^1$-β), 5.73/53.0 (β-Phe^4-β), 6.42/66.8 (Pro $(Cl_2)^1$-α)], two *O*-methines [$\delta_H/_C$ 4.57/69.0 (*allo*-Thr^5-β), 4.95/67.6 (*allo*-Thr^2-β)] and one monosubstituted benzene ring [$\delta_H/_C$ 7.10/127.4 (β-Phe^4-ζ), 7.14/128.9 (β-Phe^4-ε), 7.40/126.4 (β-Phe^4-δ), δ_C 143.2 (β-Phe^4-γ)] were showed. The five amino acid residues were ascribed to one serine (Ser), two threonines (Thr), one β-phenylalanine (β-Phe) and one dichloroproline (Pro (Cl_2)) on the basis of the observations above together with the 2D NMR spectra (^{1}H–^{1}H COSY, HSQC and HMBC, Fig. 2). In addition, the Pro residue containing two chlorine atoms was disclosed to be 2,3-dichloroproline by the ^{1}H–^{1}H COSY correlations of $H_\alpha/H_\beta/H_\gamma/H_\sigma$ (2H) in Pro $(Cl_2)^1$ residue as shown in Fig. 2. The relative configuration of the two chlorines in Pro (Cl_2) was established to *cis* like that of **3**, which was confirmed by the similar coupling constants in Pro (Cl_2) of the astins, the NOE correlation observed between H_β and H_γ of Pro (Cl_2) in the ROESY spectrum, and X-ray crystallographic analysis (Figs. 2 and 3, see S8, SM). In the HMBC spectrum the correlations were showed from *allo*-Thr^5-N*H* to β-Phe^4-*C*O, β-Phe^4-N*H* to Ser^3-*C*O, Ser^3-N*H* to *allo*-Thr^2-*C*O, *allo*-Thr^2-N*H* to Pro $(Cl_2)^1$–*C*O, and Pro $(Cl_2)^1$-H_α and Pro $(Cl_2)^1$-H_δ to *allo*-Thr^5-*C*O, which established the sequence of *cyclo*-(Pro $(Cl_2)^1$-*allo*-Thr^2-Ser^3-β-Phe^4-*allo*-Thr^5).

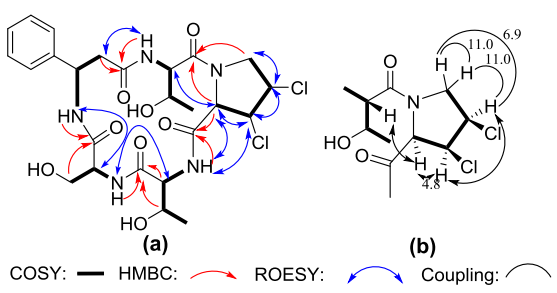

Fig. 2. a. Selected ^{1}H–^{1}H COSY, HMBC, ROESY correlations of **9**. **b**. Selected coupling (Hz) and ROESY correlations in Pro $(Cl_2)^1$ of **9**.

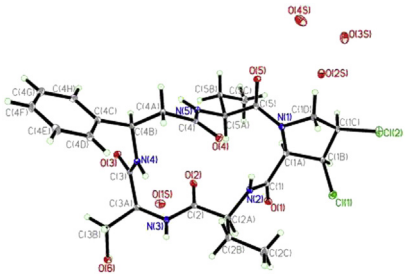

Fig. 3. X-ray structure of astin C (**3**).

The absolute configuration of **9** was assigned using advanced Marfey's method[12] and LC–MS analysis. The results indicated that the absolute configurations at the α-carbons were the L configuration for Ser, Thr, β-Phe residues (see Table S1, SM). In the ROESY spectrum, the presence of a strong cross peak between H_α in Pro $(Cl_2)^1$ and H_α in Thr^5 indicated the L configuration of Pro $(Cl_2)^1$ and a cis peptide bond like that of astin A–H.[4,6] Because the threonine residue has two stereogenic centers, the L-*allo*-Thr and L-Thr standards were carried out and subjected to the advanced Marfey's analysis using HPLC. The identical retention times of the L-Marfey derivatized L-*allo*-Thr and Thr in the hydrolyzates of **9** suggested an L-configuration of the *allo*-Thr residue. Therefore, **9** was established as depicted in Fig. 1.

Astin L (**10**) was obtained as amorphous powder. Its molecular formula was determined as $C_{25}H_{32}N_5O_8Cl$ according to the $[M+Na]^+$ peak at 588.1847 in the HRESIMS spectrum. Analysis of the 1D NMR spectra revealed that all proton and carbon signals of **10** closely resembled to those of **9** except for the signals in the Pro residue. In the Pro residue of **10**, the presence of a trisubstituted double bond was established by the chemical shifts of $\delta_H/_C$ 5.95/124.7 (Pro^1-γ) and δ_C 127.8 (Pro^1-σ). The ^{1}H–^{1}H COSY correlations of H_α/H_β (2H)/H_γ and HMBC correlation of H_β with *allo*-Thr^2-CO indicated that the double bond was located at C_γ and C_σ, and the only chlorine atom was attached to C_σ (see S3, SM). Thus, the planar structure of **10** was determined as *cyclo*-($^{\triangle 4\,(5)}$ Pro (Cl)^1-*allo*-Thr^2-Ser^3-β-Phe^4-*allo*-Thr^5).

Astin M (**11**) showed a pseudomolecular ion peak at *m*/*z* 558.2089 $[M+Na]^+$ in the HRESIMS spectrum, indicating a molecular formula of $C_{25}H_{34}N_5O_6Cl$. Compared the 1D NMR data and molecular formula of **11** with those of **3**,[4a] it showed that the only difference between them was also happened in the Pro^1 residue, in which the chlorine atom in C_β of **3** was displaced with one hydrogen atom in **11**. The change was further supported by the ^{1}H–^{1}H COSY correlations of H_α/H_β (2H)/H_γ/H_σ (2H) in the Pro residue. The configuration of H_γ was α-oriented as evidenced by the ROESY correlation of H_γ with H_α (see S4, SM). Thus, **11** was established as *cyclo*-(Pro (Cl)^1-Abu^2-Ser^3-β-Phe^4-Abu^5).

Astin N (**12**) had the molecular formula $C_{25}H_{32}N_5O_6Cl$ based on the HRESIMS spectrum ($[M+Na]^+$ *m*/*z* 556.1955); 2 mass units less than that of **11**. Comparison of the 1D NMR data of **12** with those of **11** strongly suggested a similar structure of both compounds. One apparent change in **12** was one more double bond (δ_H 6.41 (Pro-σ) and δ_C 130.0 (Pro-γ), 124.5 (Pro-σ)) appearance in the Pro residue. It was

located between the C_γ and C_σ based on the HMBC correlation of H_σ with Pro (Cl)^1–CO (see S5, SM). Thus the planar structure of **12** was elucidated as *cyclo*-($^{\triangle 4\,(5)}$ Pro (Cl)^1-Abu^2-Ser^3-β-Phe^4-Abu^5).

The molecular formula ($C_{27}H_{35}N_5O_7Cl_2$) of Astin O (**13**) was deduced from the HRESIMS analysis of the pseudomolecular ion peak at *m*/*z* 634.1802 [M+Na]$^+$. Its 1D NMR spectra were quite consistent with those of **3** except for the resonance of Ser^3 residue.[4a] In the ^{13}C NMR spectrum of **13**, a characteristic acetyl group [δ_C 20.9 (CH_3CO–) and 171.0 (CH_3CO–)] was observed. It was attached to the hydroxyl group of Ser^3 residue as evidenced by the HMBC resonances from $H_{\beta1}$ 4.97 (dd, 11.4, 4.2) and $H_{\beta2}$ 5.12 (dd, 11.4, 9.0) to [δ_C 171.0 (CH_3–*C*O–O–)] (see S6, SM). Accordingly, **13** was elucidated to be an analogue of **3** in which Ser^3 was substituted by acetoxylation (Fig. 1). So, the planar structure of **13** was *cyclo*-(Pro (Cl$_2$)^1-Abu^2- (Ac-Ser)^3-β-Phe^4-Abu^5).

Astin P (**14**) was isolated as an amorphous powder. It showed a [M−H]$^-$ peak at *m*/*z* 598.1820 in the HRESIMS spectrum, which was consistent with the molecular formula of $C_{26}H_{35}N_5O_7Cl_2$ accounting for 11 degrees of unsaturation. Comparison of the 1D NMR spectra of **9** and **14** revealed that a methine [$\delta_H/_C$ 4.57/69.0 (*allo*-Thr^5-β)] in **9** was replaced with two methylene signals [$\delta_H/_C$ 1.65/20.1 (Ava^5-γ), 1.86/32.9 (Ava^5-β)] in **14**, indicating that the *allo*-Thr residue in **9** was replaced by the Ava residue in **14**. This change was further supported by the ^{1}H–^{1}H COSY data of H_α/H_β (2H)/H_γ (2H)/H_σ (3H) in Ava^5 residue (see S7, SM) and the acidic hydrolysis experiment. It is should be noteworthy that this is the first time for having found an Ava residue in the Compositae-type cyclopentapeptides.[4,6,7] Thus, **14** was identified as a unique cyclopentapeptide linking as *cyclo*-(Pro (Cl$_2$)^1-*allo*-Thr^2-Ser^3-β-Phe^4-Ava^5) (Fig. 1).

Except the variable proline derivatives, **9** and **10** have the same residues and **11**–**13** also have the same residues. Since small amounts of **10**, **12** and **13** were obtained, only **9**, **11**, **14** were subjected to establish their absolute configuration of the amino acid residues by the advanced Marfey's method described above. The results were depicted in the SM (Table S1) and suggested that all the amino acid residues were L configurations. Furthermore, the strong cross peaks between H_α of Pro^1 residue and H_α of the fifth residue in the ROESY spectrum of **11** and **14** revealed the Pro residue was also L configuration and cis peptide bond like that of **9**. The configuration of the Thr residues in **10** and **14** was indicated as L-*allo*-Thr by using HPLC method as described in **9**. Therefore, the structures of **9**–**14** were finally established and shown in Fig. 1.

2.2. Biological assays

In the present study, compounds **1**–**14** were tested for cytotoxic activity against BEL-7402, BGC-823, A549, HeLa, and HCT-116 cell lines by SRB or MTT method.[13] Only **2** showed cytotoxicity against BGC-823 cell with IC_{50} value of 19.2 μg/ml, and **3** exhibited cytotoxicity on HCT-116 and BGC-823 cells with IC_{50} values of 13.4 and 3.3 μg/ml, respectively. In addition, **1**–**14** were tested for anti-HBV activity on Hep G 2.2.15 cell;[14] but they exhibited no inhibitory activity against the secretion of HBsAg and HBeAg. Moreover, **2**, **4**, **9** were also examined for immunosuppressive activity against activated lymph node cells from mice by the MTT method.[9] Unfortunately, none of them exhibited obvious immunosuppressive activity.

3. Conclusions

To the best of our knowledge, astins are the only chlorinated cyclopeptides in higher plants and obtained only from *A. tataricus*. Therefore, discovery of these six new chlorinated cyclopeptides astins K–P (**9**–**14**) expands the cyclopeptide diversity in *A. tataricus* and could be considered as one of characteristic constituents for this TCM quality control. Furthermore, cytotoxic activity results indicated that the *cis* dichlorinated proline residue and the number of hydroxyl groups in the side chains play important roles. These findings are beneficial to further synthesize new astin analogues and discover drug candidates.

4. Materials and methods

4.1. General experimental procedures

Optical rotations were measured on a Horiba SEPA-300 polarimeter. IR spectra were obtained on a Tensor 27 spectrometer with KBr pellets. UV spectra were recorded using a Shimadzu UV-2401A spectrophotometer. 1D and 2D NMR spectra were performed on a Bruker AVANCE III-600 spectrometer with TMS as the internal standard. Mass spectra were measured on a VG Auto Spec-3000 or API-Qstar-Pulsar instrument. Analytical or semi-preparative HPLC was performed on Agilent 1100 with Zorbax Eclipse-C_{18} (4.6 mm×150 mm, 1 mL/min; 9.4 mm×250 mm, 3 mL/min; 5 μm). Column chromatography was performed using silica gel (100–200 and 200–300 mesh, Qingdao Yu-Ming-Yuan Chemical Co. Ltd., Qingdao, P. R. China), Sephadex LH-20 (Pharmacia Fine Chemical Co., Uppsala, Sweden) or Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany). Fractions were monitored by TLC (GF254, Qingdao Yu-Ming-Yuan Chemical Co. Ltd., Qingdao, P. R. China), and spots were visualized by the chemical detection of cyclopeptides.[11]

4.2. Plant material

Roots and rhizomes of *A. tataricus* were commercially purchased from the Yunnan Lv-Sheng Pharmaceutical Co. Ltd., P. R. China, and identified by Professor Xi-Wen Li at the Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 200704) was deposited in the Herbarium of Kunming Institute of Botany.

4.3. Extraction and isolation

The air dried and powdered roots and rhizomes of *A. tataricus* (50 kg) were extracted three times with methanol (3×50 L). The extract was concentrated to 13 kg, and then was suspended in water and partitioned successively with ethyl acetate (EtOAc) and *n*-butanol, respectively. The EtOAc fraction (2 kg) was subjected to silica gel CC and eluted with gradient $CHCl_3$/MeOH (20:1, 10:1, 85:15, 8:2, and 0:1) to yield six fractions (Fr.1–Fr.6).

Fr.1 (320 g) was chromatographed over silica gel using gradient petroleum ether/acetone (10:1–1:1) to furnish three subfractions (Fr.1-1–Fr.1-3). Fr.1-2 (50 g) was subjected to silica gel column using gradient $CHCl_3$/acetone (15:1–5:1) to afford subfractions (Fr.1-2-1–Fr.1-2-3). Fr.1-2-1 (2 g) was applied to Sephadex LH-20 using $CHCl_3$/MeOH (1:1), and then silica gel CC ($CHCl_3$/EtOAc, 10:1–5:1) to yield two subfractions (Fr.1-2-1-1–Fr.1-2-1-2). Astin L (**10**) (5 mg) was afforded from subfraction Fr.1-2-1-1 (34 mg) by a further purification using semi-HPLC (20% CH_3CN, 0.5% TFA). Additionally, Fr.1-2-2 (4 g) was separated by a silica gel column eluting with $CHCl_3$/EtOAc (1:3) and then purified by repeated silica gel CC eluting with $CHCl_3$/MeOH (9:1) to obtain astin K (**9**) (30 mg).

Fr.2 (75 g) was subjected to silica gel CC and eluted with gradient petroleum ether/acetone (2:1–1:2) to yield six subfractions (Fr.2-1–Fr.2-6). Fr.2-2 (14 g) was separated into four subfractions (Fr.2-2-1–Fr.2-2-4) on a silica gel column using $CHCl_3$/MeOH (7:1). Fr.2-2-1 (282 mg) was firstly purified by Sephadex LH-20 ($CHCl_3$/MeOH, 1:1), and then by semi-HPLC (35% CH_3OH, 0.5% TFA) to yield astin F (**6**) (10 mg), astin H (**8**) (20.9 mg), and astin M (**11**) (7 mg). Fr.2-3 (21 g) was separated by Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) and followed by silica gel CC ($CHCl_3$/MeOH, 20:1–5:1) to afford five subfractions (Fr.2-3-1–Fr.2-3-5). Fr.2-3-4 (3 g) was purified by semi-HPLC to obtain astin N (**12**) (5 mg). Fr.2-5 (4 g) was separated

by a silica gel column ($CHCl_3$/MeOH, 20:1–5:1) to get astin D (**4**) (1 g). Fr.2-6 (1.2 g) was subjected to Sephadex LH-20 eluted with $CHCl_3$/MeOH (1:1) to give two subfractions (Fr.2-6-1–Fr.2-6-2). Fr.2-6-1 (88.7 mg) was further fractionated by silica gel CC eluting with EtOAc/MeOH (25:1) to afford astin A (**1**) (13.6 mg). Fr.2-6-2 (535 mg) was repeated submitted to CC ($CHCl_3$/MeOH, 20:1), then purified by silica gel CC ($CHCl_3$/acetone) to afford astin O (**13**) (3 mg).

Fr.3 (55 g) was separated into five subfractions (Fr.3-1–Fr.3-5) by silica gel CC using elution with EtOAc/$CHCl_3$ (3:1-5:1). Fr.3-2 (40 g) was further purified by Sephadex LH-20 ($CHCl_3$/MeOH, 1:1), and then by recrystallization ($CHCl_3$/MeOH) to yield astin C (**3**) (8 g).

Fr.4 (85 g) was divided into two subfractions (Fr.4-1–Fr.4-2). Then Fr.4-2 (60 g) was separated by repeated RP-18 (MeOH/H_2O, 25%–70%) and led to obtain three subfractions (Fr.4-2-1–Fr.4-2-3). Fr.4-2-2 (14 g) was passed through Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) and repeated silica gel CC ($CHCl_3$/MeOH, 20:1-5:1) to afford four subfractions (Fr.4-2-2-1–Fr.4-2-2-4). Astin P (**14**) (15.1 mg) was purified by semi-HPLC (45% CH_3OH, 0.5% TFA) from subfraction Fr.4-2-2-1 (300 mg). Fr.4-2-2-2 (4 g) was submitted to repeated silica gel CC, and purified by Sephadex LH-20 to afford astin E (**5**) (1.1 g). Astin G (**7**) (20 mg) was given from Fr.4-2-2-4 (292 mg) by semi-HPLC (15% CH_3OH, 0.5% TFA).

Fr.5 (146 g) was subjected to a silica gel CC using $CHCl_3$/MeOH (15:1–5:1) to furnish two subfractions (Fr.5-1–Fr.5-2). Fr.5-2 (50 g) was purified by Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) to yield astin B (**2**) (15 g).

*4.3.1. Astin K (**9**).* Amorphous powder; $[\alpha]_D^{17.3}$ −88.7 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.11), 266 (4.40) nm; IR (KBr) ν_{max} 3404, 2935, 1649, 1533, 1423, 1312, 1080, 758, 700, 588 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 1; positive ESIMS *m*/*z* 602 $[M+H]^+$; HRESIMS *m*/*z* 602.1788 $[M+H]^+$ (calcd for $C_{25}H_{34}N_5O_8Cl_2$, 602.1784).

*4.3.2. Astin L (**10**).* Amorphous powder; $[\alpha]_D^{24.6}$ −357.6 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.70), 284 (2.97) nm; IR (KBr) ν_{max} 3421, 2930, 1646, 1434, 1204, 1080, 703, 527 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 1; negative ESIMS *m*/*z* 564 $[M-H]^-$, 1129 $[2M-H]^-$; HRESIMS *m*/*z* 588.1847 $[M+Na]^+$ (calcd for $C_{25}H_{32}N_5O_8ClNa$, 588.1837).

*4.3.3. Astin M (**11**).* Amorphous powder; $[\alpha]_D^{24.2}$ −52.2 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.01) nm; IR (KBr) ν_{max} 3430, 2934, 1636, 1549, 1451, 1288, 1204, 1132, 702, 517 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 2; positive ESIMS *m*/*z* 536 $[M+H]^+$; 1093 $[2M+Na]^+$; HRESIMS *m*/*z* 558.2089 $[M+Na]^+$ (calcd for $C_{25}H_{34}N_5O_6ClNa$, 558.2095).

*4.3.4. Astin N (**12**).* Amorphous powder; $[\alpha]_D^{24.4}$ −105.8 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.12) nm; IR (KBr) ν_{max} 3406, 2972, 1639, 1533, 1431, 1319, 1207, 1140, 1079, 700 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 2; positive ESIMS *m*/*z* 556 $[M+Na]^+$; 1089 $[2M+Na]^+$; HRESIMS *m*/*z* 556.1955 $[M+Na]^+$ (calcd for $C_{25}H_{32}N_5O_6ClNa$, 556.1938).

*4.3.5. Astin O (**13**).* Amorphous powder; $[\alpha]_D^{23.7}$ −113.3 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.10), 262 (3.10) nm; IR (KBr) ν_{max} 3422, 2923, 2853, 1646, 1530, 1461, 1239, 701 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 3; positive ESIMS *m*/*z* 634 $[M+Na]^+$, 1247 $[2M+Na]^+$; HRESIMS *m*/*z* 634.1802 $[M+Na]^+$ (calcd for $C_{27}H_{35}N_5O_7Cl_2Na$, 634.1811).

*4.3.6. Astin P (**14**).* Amorphous powder; $[\alpha]_D^{24.7}$ −21.7 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.81), 254 (3.73) nm; IR (KBr)

Table 1
^1H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds **9** and **10** in pyridine-d_5 (δ in ppm, *J* in Hz)

	9		**10**	
	δ_C	δ_H, mult	δ_C	δ_H, mult
Pro[1]				
α	66.8	6.42 (d, 4.8)	70.4	6.37 (m)
β_1	66.8	5.55 (overlapped)	53.3	4.41 (overlapped)
β_2				4.75 (m)
γ	56.1	4.78 (m)	124.7	5.95 (m)
δ_1	52.2	3.94 (t, 11.0)	127.8	
δ_2		5.12 (dd, 11.0, 6.9)		
C=O	168.7		169.3	
allo-Thr[2]				
α	58.6	5.30 (t, 9.8)	59.8	5.36 (overlapped)
β	67.6	4.95 (m)	69.3	4.65 (overlapped)
γ	23.2	1.73 (d, 6.0)	22.2	1.63 (d, 6.6)
C=O	171.8		172.4	
NH		9.56 (d, 9.8)		8.77 (d, 9.0)
Ser[3]				
α	59.4	4.73 (m)	59.8	4.61 (m)
β_1	61.5	4.21 (dd, 11.1, 3.3)	61.1	4.47 (dd, 10.5, 4.5)
β_2		4.40 (dd, 11.1, 5.1)		4.65 (overlapped)
C=O	171.4		170.4	
NH		10.13 (d, 4.2)		10.47 (d, 6.0)
β-Phe[4]				
α_1	43.9	2.63 (t, 12.8)	42.1	2.75 (dd, 13.5, 7.2)
α_2		3.27 (dd, 12.8, 5.1)		3.09 (dd, 13.5, 5.4)
β	53.0	5.73 (m)	52.8	5.47 (overlapped)
γ	143.2		143.6	
δ	126.4	7.40 (d, 7.2)	127.3	7.48 (d, 7.6)
ε	128.9	7.14 (t, 7.2)	129.2	7.28 (t, 7.6)
ζ	127.4	7.10 (t, 7.2)	127.5	7.17 (t, 7.6)
C=O	172.7		172.2	
NH		8.21 (d, 6.0)		8.21 (d, 6.0)
allo-Thr[5]				
α	60.4	5.53 (overlapped)	59.6	5.36 (overlapped)
β	69.0	4.57 (m)	69.0	4.43 (overlapped)
γ	22.6	1.77 (d, 6.0)	22.2	1.55 (d, 6.0)
C=O	175.2		172.9	
NH		10.21 (d, 3.6)		10.09 (d, 6.6)

Table 2
^1H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds **11** and **12** in pyridine-d_5 (δ in ppm, *J* in Hz)

	11		**12**	
	δ_C	δ_H, mult	δ_C	δ_H, mult
Pro[1]				
α	60.9	5.10 (overlapped)	69.0	5.71 (m)
β_1	41.2	2.78 (m)	56.9	4.58 (m)
β_2		3.29 (d, 8.4)		4.78 (d, 16.2)
γ	57.1	4.73 (t, 5.1)	130.0	
δ_1	58.3	4.13 (d, 14.3)	124.5	6.41 (br s)
δ_2		4.33 (dd, 14.3, 5.1)		
C=O	172.3		170.1	
Abu[2]				
α	55.9	5.15 (m)	56.1	5.10 (m)
β_1	24.7	2.26 (m)	25.7	2.04 (m)
β_2				2.14 (m)
γ	11.8	1.11 (t, 7.2)	11.3	0.98 (t, 7.6)
C=O	172.8		172.8	
NH		9.30 (d, 9.0)		8.91 (d, 8.4)
Ser[3]				
α	60.4	4.76 (m)	60.3	4.70 (m)
β_1	61.5	4.29 (dd, 11.1, 3.9)	61.2	4.44 (dd, 11.1, 4.5)
β_2		4.46 (dd, 11.1, 5.1)		4.54 (m)
C=O	171.2		170.8	
NH		8.49 (d, 5.4)		9.19 (d, 6.0)
β-Phe[4]				
α_1	43.8	2.65 (t, 12.6)	42.8	2.80 (dd, 13.4, 9.6)
α_2		3.22 (dd, 12.6, 4.8)		3.10 (dd, 13.4, 5.1)
β	53.3	5.67 (m)	52.8	5.60 (m)
γ	143.6		143.5	
δ	126.7	7.39 (d, 7.2)	127.1	7.45 (d, 7.2)
ε	129.1	7.14 (t, 7.2)	129.2	7.24 (overlapped)

(*continued on next page*)

Table 2 (*continued*)

	11		**12**	
	δ_C	δ_H, mult	δ_C	δ_H, mult
ζ	127.6	7.09 (m)	127.6	7.16 (t, 7.2)
C=O	172.8		172.4	
NH		8.40 (d, 6.0)		9.05 (d, 6.6)
Abu[5]				
α	55.2	5.10 (overlapped)	53.9	5.03 (m)
β_1	24.6	1.81 (m)	24.9	1.77 (m)
β_2		1.87 (m)		1.90 (m)
γ	10.9	1.01 (t, 7.5)	10.9	1.77 (d, 6.0)
C=O	172.9		172.1	
NH		10.12 (d, 3.6)		9.94 (d, 4.8)

Table 3
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds **13** and **14** in pyridine-d_5 (δ in ppm, J in Hz)

	13		**14**	
	δ_C	δ_H, mult	δ_C	δ_H, mult
Pro[1]			Pro[1]	
α	66.2	5.47 (d, 6.6)	66.9	5.76 (overlapped)
β	63.6	5.63 (dd, 6.6, 4.8)	66.4	5.69 (d, 5.4)
γ	57.5	5.06 (m)	55.8	5.13 (m)
δ_1	53.2	4.14 (dd, 12.2, 7.5)	52.3	3.99 (t, 10.5)
δ_2		4.69 (dd, 12.2, 6.0)		5.04 (m)
C=O	167.3		168.2	
Abu[2]			*allo*-Thr[2]	
α	55.8	5.29 (m)	58.5	5.26 (t, 9.8)
β_1	26.4	2.19 (m)	67.4	4.99 (m)
β_2		2.31 (m)		
γ	10.6	1.15 (t, 7.5)		1.74 (d, 6.0)
C=O	172.9		23.3	
NH		8.43 (d, 7.2)	171.7	9.38 (d, 9.8)
Ser[3]			Ser[3]	
α	56.7	4.64 (m)	59.6	3.79 (m)
β_1	63.1	4.97 (dd, 11.4, 4.2)	61.5	4.23 (dd, 10.8, 3.6)
β_2		5.12 (dd, 11.4, 9.0)		4.41 (dd, 10.8, 5.4)
OCO*CH*$_3$	20.9	1.98 (s)	—	—
O*CO*CH$_3$	171.0		—	—
C=O	168.4		171.3	
NH		10.16 (d, 5.4)		10.13 (d, 4.2)
β-Phe[4]			β-Phe[4]	
α_1	41.6	2.86 (dd, 13.5, 6.9)	44.0	2.65 (t, 12.5)
α_2		3.03 (dd, 13.5, 5.7)		3.35 (dd, 12.5, 4.8)
β	52.6	5.38 (m)	53.1	5.76 (overlapped)
γ	143.1		143.5	
δ	127.4	7.47 (d, 7.7)	126.4	7.46 (d, 7.4)
ε	129.2	7.33 (t, 7.7)	128.9	7.17 (t, 7.4)
ζ	127.5	7.23 (overlapped)	127.3	7.12 (t, 7.4)
C=O	171.9		172.9	
NH		9.44 (d, 6.0)		8.21 (d, 6.0)
Abu[5]			Ava[5]	
α	53.2	5.16 (m)	53.6	5.35 (m)
β_1	25.6	1.62 (m)	32.9	1.86 (m)
β_2		1.77 (m)		—
γ	10.9	0.89 (t, 7.2)	20.1	1.65 (m)
δ	—	—	13.8	0.73 (t, 7.2)
C=O	173.4		174.1	
NH		9.90 (d, 7.2)		10.21 (d, 3.6)

ν_{max} 3426, 2932, 1680, 1385, 1289, 1206, 1136, 801, 723 cm^{-1}; ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR, see Table 3; negative ESIMS m/z 598 [M−H]$^-$, 634 [M+Cl]$^-$; HRESIMS m/z 598.1820 [M−H]$^-$ (calcd for $C_{26}H_{34}N_5O_7Cl_2$, 598.1835).

4.4. Advanced Marfey's analysis of the absolute configuration of amino acid residues

Each solution of **9**, **11**, **14** (each 0.5 mg) in 6 N HCl (1 mL) was heated at 110 °C for 24 h, respectively. The hydrolyzate solution was evaporated to dryness, and the residue was redissolved in 100 μL of acetone. To each a half portion (50 μL) were added 20 μL $NaHCO_3$ (1 M) and 100 μL 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide or 1-fluoro-2,4-dinitrophenyl-5-D-leucinamide (L-FDLA or D-FDLA, 1% in acetone), and the mixture was heated at 45 °C for 1.5 h. Reaction was cooled to room temperature, and then acidified with 2 N HCl (10 μL), dried and dissolved in 50% aqueous CH_3CN. About 5 μL of each solution of FDLA derivatives were analyzed by LC/MS.

The analysis of the L- and D,L-FDLA (mixture of D- and L-FDLA) derivatives was performed using an Agilent Eclipse XDB-C_{18} column (4.6×150 mm, 5 μm) maintained at 40 °C. Acetonitrile/0.1% HCOOH/H_2O was used as the mobile phase under a linear gradient elution mode (acetonitrile, 30%–50%, 50 min) at a flow rate of 1 mL/min. A Waters Xevo TQ-S mass spectrometer was used for detection in ESI (negative) mode. The capillary voltage was kept at 2.5 KV, and the ion source at 350 °C. Nitrogen gas was used as a sheath gas at 400 L/h. A mass range of m/z 100–1000 was scanned in 0.2 s. The retention times (t_R, min) of the Marfey-derivatized amino acids are summarized in Table S1 (see SM). However, this was not conclusive since the *allo*-Thr standard was not used. The Thr residues in compounds were subsequently confirmed on the basis of chemical manipulation using the advanced Marfey's method as follows.

4.5. Advanced Marfey's analysis of the configuration of Thr

L-Thr or D,L-*allo*-Thr standard was derivatized with L,D-FDLA or L-FDLA with the advanced Marfey's method to produce four stereoisomers of Thr, which were analyzed using the HPLC Waters 1525-2707-2998 system [column, Eclipse XDB-C_{18} column (4.6×150 mm, 5 μm); mobile phase, CH_3CN in 0.1% HCOOH/H_2O; flow rate, 1 mL/min using a linear gradient (30–50% CH_3CN over 50 min)]. The retention times of the L/D-FDLA-derivatized amino acids were as follows: L-Thr-L-FDLA (10.470), L-Thr-D-FDLA (=D-Thr-L-FDLA, 17.537), L-*allo*-Thr-L-FDLA (11.940), D-*allo*-Thr-L-FDLA (=L-*allo*-Thr-D-FDLA, 14.268). The t_R of the Thr residue in the hydrolyzates of **9**, **10**, and **14** was similar to the L-*allo*-Thr-L-FDLA standard at 11.930, 11.913, 11.902 min.

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Supplementary data

Detailed 1D and 2D NMR spectra, MS, IR, UV, $[\alpha]_D$ of compounds **9**–**14**, X-ray crystallographic data of **3** and bioassay protocols used are supplied. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tet.2013.07.006. These data include MOL files and InChiKeys of the most important compounds described in this article.

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