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New Antitumour Cyclic Astin Analogues: Synthesis, Conformation and Bioactivity

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Abstract: Astins, antitumour cyclic pentapeptides, were isolated from the *Aster tataricus*. Their chemical structures, consist of a 16-membered ring system containing a unique β,γ-dichlorinated proline [Pro(Cl)$_2$], other non-coded amino acid residues and a *cis* conformation in one of the peptide bonds. The astin backbone conformation, along with the *cis* peptide bond in which the β,γ-dichlorinated proline residue is involved, was considered to play an important role in their antineoplastic activities on sarcoma 180A and P388 lymphocytic leukaemia in mice, but the scope and potential applications of this activity remain unclear. With the aim at improving our knowledge of the conformational properties influencing the bioactivity in this class of compounds, new astin-related cyclopeptides were synthesized differing from the natural products by the presence of some non-proteinogenic amino acid residues: Aib, Abu, -(S)β^3-hPhe and a peptide bond surrogate (-SO$_2$-NH-). The analogues prepared c(-Pro-Thr-Aib-β^3-Phe-Abu-), c[Pro-Thr-Aib-(S)β^3-hPhe-Abu], c[Pro-Abu-Ser-(S)β^3-hPheΨ(CH$_2$-SO$_2$-NH)-Abu] and c[Pro-Thr-Aib-(S)β^3-hPheΨ(CH$_2$-SO$_2$-NH)-Abu] were synthesized by classical methods in solution and tested for their antitumour effect. These molecules were studied by crystal-state x-ray diffraction analysis and/or solution NMR and MD techniques.

Keywords: astins; biology; conformation; non-coded amino acids

INTRODUCTION

Astins A–I, a family of cyclopentapeptides isolated from the medicinal plant *Aster tataricus* (Compositae), are characterized by a 16-membered ring containing several non-coded amino acids: a unique β,γ-dichlorinated proline residue, α-aminobutyric acid (Abu), modified phenylalanine and L-*allo* threonine [1–5]. Astins have been screened for antineoplastic activity using sarcoma 180A in mice, P388 lymphocytic leukaemia in mice and nasopharynx carcinoma (KB) cells [6]. A comparison of the natural astin congeners showed that variation of substituents on the proline ring results in significant differences in their biological profiles.

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A common feature to all astin structures is the presence of a *cis* peptide bond between the Abu[5] and Pro[1] residues [4]. As the crystal-state structure of astin B is stabilized by an intramolecular H-bond between Thr[2] and Ser[3] residues, it has been postulated that this feature could be related to its antineoplastic activity [1].

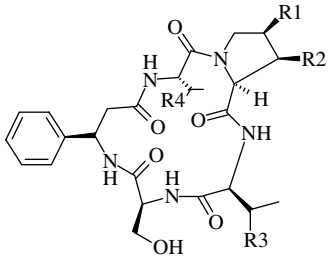
Astins have also provided challenges for the synthesis of a variety of non-proteinogenic amino acids. Recent highlights include efficient syntheses of *cis*-3,4-dihydroxyproline, *cis*-3,4-dichloroproline [4] and β-aminoethanesulfonyl derivatives [7]. With the aim at elucidating the influence of conformational flexibility on biological activity, several astin G analogues were designed [4] containing non proteinogenic residues able to modify the peptide backbone structure (Scheme 1). This paper reports the structure–activity studies of selected synthetic cyclopeptides corresponding to the sequences c(Pro-Thr-Aib-β^3-Phe-Abu) (peptide **I**), c[Pro-Thr-Aib-(S)β^3-hPhe-Abu] (peptide **II**), c[Pro-Abu-Ser-(S)β^3-hPheΨ(CH_2-SO_2-NH)-Abu] (peptide **III**), c[Pro-Thr-Aib-(S)β^3-hPheΨ(CH_2-SO_2-NH)-Abu] (peptide **IV**) prepared by classical solution methods and tested for their antitumour effects.

MATERIALS AND METHODS

Peptide Synthesis and Characterization

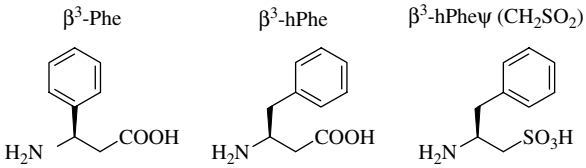
The N-benzyloxycarbonyl (Z) and methoxy (OMe) C-protected α-amino acids were purchased from Novabiochem (Switzerland); H-β^3-Xaa-OMe and H-(S)β^3-hXaa-OMe non-coded β-amino acids [8][†] were purchased from Fluka Chemie-GmbH (Switzerland). The C-activating agents: N,N′-dicyclohexylcarbodiimide (DCCI), 1-hydroxy-1,2,3-benzotriazole, (HOBt), isobutyl chloroformate (iBCCl) and O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyleneuronium hexafluorophosphate (HBPyU) were purchased from Sigma-Aldrich (Germany). The non proteinogenic derivative of Z-(S)β^3-hPheΨ(CH_2-SO_2-)Cl was obtained following the literature procedure [7]; its chemical characterization was in agreement with the data reported. Solvents were reagent grade; the other commercially available organic reagents were used without further purification. Silica gel plates 60F-254 (Merck) were visualized by an ultraviolet (UV) lamp and/or ninhydrin chromatic reaction. HPLC analyses were performed by a Shimadzu LC-10AD apparatus equipped with a SPD-M10AV diode array detector. Purifications were carried out on a Millipore-Waters Delta Prep 3000 HPLC system. MALDI-TOF mass measurements were performed on a Voyager-DE BioSpectrometry™ Workstation (PerSeptive Biosystems) using standard procedures for calibration: α-cyano-4-hydroxycinnamic acid (α-CHCA) at $[M + H]^+ = 190.094$ and by the substance P-amide at $[M + H]^+ = 1347.74$. The amino acid composition was determined using an HPLC system equipped with pumps, gradient-makers, injectors and UV-VIS variable wavelength detectors, autosampler-HPLC system, data recorder, fraction collector, columns for amino acid analysis, columns for RP-HPLC analysis of libraries, and a RF-551 spectrofluorimeter (used for Fmoc-amino acid detection: UV absorption at $\lambda = 263$ nm, fluorescence with $\lambda_{ex} = 263$ nm, $\lambda_{em} = 313$ nm).

The amino acids are identified by their retention times. Compositions were obtained by comparison of peak areas of the different Fmoc-amino acids. A correction factor was introduced in order to take into account the different reaction rates of amino acids toward Fmoc-Cl. No corrections were introduced to take into account incomplete hydrolysis of some particularly resistant amide bonds.



	R1	R2	R3	R4
astin A	Cl	Cl	H	OH
astin B	Cl	Cl	OH	H
astin C	Cl	Cl	H	H
astin G	H	H	H	H

a. Structures of astins A–G (see reference [4]).



b. Chemical formulas of non-coded amino acids inserted in the astin G related analogues.

Scheme 1

[†] The notation β^3 e (S)β^3-hXaa, where the numbers indicate the position of the side chain in the backbone of the β–amino acid, has been proposed.

Z-Abu-Pro-OMe (1)

To a solution of 3.77 g of Z-Abu-OH (15.9 mmol) in 50 ml CH_2Cl_2, 2.16 g (15.9 mmol) of iBCCl and 1.60 g (15.9 mmol) of N-methylmorpholine (NMM) were added at −10 °C under stirring. After 10 min the precooled solution of 2.05 g (15.9 mmol) of H-Pro-OMe in 30 ml CH_2Cl_2 was added. After 3 h stirring at room temperature the reaction mixture was washed with 0.5 M $KHSO_4$, saturated $NaHCO_3$ solution and water, dried over Na_2SO_4 and evaporated to give 5.20 g (94% yield) of TLC pure dipeptide (1). $R_f = 0.80$ ($CHCl_3$–CH_3OH 95 : 5).

Z-Abu-Pro-OH (2)

5.20 g of (**1**) (14.9 mmol) in 50 ml of CH_3OH was saponified at room temperature for 24 h after adding a solution of 0.720 g (18.0 mmol) of NaOH in 20 ml of water. After the evaporation of the organic solvent, the alkaline aqueous solution was extracted with diethyl ether, acidified to pH 3.0 with 0.5 M $KHSO_4$ and extracted with ethyl acetate. The organic phase, washed with water and dried over Na_2SO_4, was evaporated to afford 4.84 g (97% yield) of (**2**) as a foam. $R_f = 0.35$ (ethyl acetate–acetic acid 9 : 1).

Z-Thr(tBu)-Aib-OMe (3)

To a stirred solution of 2.32 g of Z-Thr(tBu)-OH (tBu, *tert*-butyl) (7.50 mmol) in 50 ml of CH_2Cl_2 was added at 0 °C 1.55 g (7.50 mmol) of DCCI, 2.03 g (15.0 mmol) of HOBt and 0.877 g (7.50 mmol) of H-Aib-OMe dissolved in 20 ml of CH_2Cl_2. After stirring 1 h at 0 °C and overnight at room temperature, the reaction mixture, cleared from *N*,*N*′-dicyclohexylurea by filtration, was worked up as described above for (**1**) affording a product that was chromatographed on a silica gel column in diethyl ether–hexane 6 : 4 as eluant. 1.42 g (46% yield) of TLC pure dipeptide (**3**) were obtained. $R_f = 0.80$ (diethyl ether–hexane 7 : 3).

Z-Abu-Pro-Thr(tBu)-Aib-OMe (4)

1.42 g of Z-dipeptide (**3**) (3.48 mmol) in 50 ml of CH_3OH was hydrogenated over 150 mg of 10% Pd on charcoal as catalyst. Hydrogenation was completed after 2 h. After removal of the catalyst, the filtrate was evaporated and the intermediate product, dissolved in 30 ml of CH_2Cl_2, was added to a mixed anhydride (MA) solution prepared at −10 °C in CH_2Cl_2 with 1.16 g of dipeptide (**2**) (3.48 mmol), 0.470 g iBCCl (3.48 mmol) and 0.350 g (3.48 mmol) of NMM. The reaction mixture, worked up as previously described, gave 1.5 g (73% yield) of TLC pure tetrapeptide (**4**). $R_f = 0.75$ ($CHCl_3$–CH_3OH 95 : 5).

Z-Abu-Pro-Thr(tBu)-Aib-OH (5)

1.50 g of (**4**) (2.54 mmol), dissolved in 20 ml of CH_3OH, was saponified with a solution of 120 mg of NaOH (3.00 mmol) in 5 ml of water for 2 days at room temperature. Following the procedure previously described for (**2**), 1.32 g (90% yield) of title compound was obtained. $R_f = 0.50$ (ethyl acetate–acetic acid 9 : 1).

Z-Abu-Pro-Thr(tBu)-Aib-(S)β^3-hPhe-OMe (6)

To a solution of 0.580 g of (**5**) (1.00 mmol) in 20 ml of CH_2Cl_2, 206 mg of DCCI (1.00 mmol), 270 mg HOBt (2.00 mmol) and 193 mg of H-β^3-hPhe-OMe (1.00 mmol) were added at 0 °C. After 1 h stirring at 0 °C and overnight at room temperature, the reaction mixture, cleared from *N*,*N*′-dicyclohexylurea by filtration, was worked up as described above for (**1**). The product, chromatographed on a Sephadex LH 20 column (2.5 × 250 cm) in CH_3OH as eluant, afforded 490 mg (65% yield) of pentapeptide (**6**). $R_f = 0.60$ ($CHCl_3$–CH_3OH 95 : 5).

H-Abu-Pro-Thr(tBu)-Aib-(S)β^3-hPhe-OH (7)

490 mg of (**6**) (0.652 mmol) in 10 ml of CH_3OH was saponified at room temperature for 24 h after adding a solution of 31 mg of NaOH (0.775 mmol) in 1 ml of water. After the usual work up, 480 mg (97% yield) of TLC pure pentapeptide acid were obtained. This intermediate product, dissolved in 10 ml of CH_3OH, was hydrogenated for 1 h at room temperature over 100 mg of 10% Pd on charcoal, affording 392 mg (quantitative yield) of the deprotected linear pentapeptide (**7**). $R_f = 0.30$ (butanol : acetic acid : water 4 : 1 : 1).

Z-Abu-Pro-Thr(tBu)-Aib-β^3-Phe-OMe (8)

576 mg of Z-tetrapeptide (**5**) (1.00 mmol) was reacted with 179 mg of H-β^3-Phe-OMe (1.00 mmol) by adopting the same procedure followed for the synthesis of Z-pentapeptide-OMe (**6**). After purification of the product on a Sephadex LH-20 column in CH_3OH as eluant, 500 mg (68% yield) of (**8**) was obtained. $R_f = 0.55$ ($CHCl_3$–CH_3OH 95 : 5)

H-Abu-Pro-Thr(tBu)-Aib-β^3-Phe-OH (9)

The *N*- and *C*- terminal protecting groups were removed from 500 mg of linear pentapeptide (**8**) by adopting the same procedure used to obtain the deprotected pentapeptide (**7**). 400 mg (quantitative yield) of title compound (**9**) was obtained. $R_f = 0.35$ (butanol : acetic acid : water 4 : 1 : 1).

c(-Abu-Pro-Thr-Aib-(S)β^3-hPhe-) (10) (peptide II) and c(-Abu-Pro-Thr-Aib-β^3-Phe-) (11) (peptide I)

0.580 mmol of deprotected linear pentapeptides (**7**) and (**9**) in 60 ml of THF and 90.0 ml of DMF was treated under stirring at $-10\,^{\circ}C$ with 66.0 mg of trifluoroacetic acid (TFA) and after 5 min with 79.0 mg of iBCCl. After 10 min stirring at $-10\,^{\circ}C$ a precooled solution of 200 mg of NMM in 90 ml of THF and 180 ml of DMF was added and the reaction mixture stirred overnight at room temperature. The solution was evaporated *in vacuo* and the crude product chromatographed on a silica gel column (2×30 cm) using the $CHCl_3$–CH_3OH (95 : 5) mixture as eluant. The TLC pure, side-chain protected cyclopentapeptides were kept 1 h in TFA. The title compounds were further purified by HPLC in a 0.1% TFA-containing CH_3CN–water linear gradient. Following the described procedure 60 mg of (**10**) (19% yield) and 55 mg of (**11**) (18% yield) were obtained, respectively.

For compound (**10**) $R_f = 0.30$ ($CHCl_3$–CH_3OH 9 : 1); for compound (**11**) $R_f = 0.31$ ($CHCl_3$–CH_3OH 9 : 1). Mass spectra analyses gave $[M + H]^+ = 530$ and $[M + H]^+ = 516$ values for compounds (**10**) and (**11**), respectively, as expected. The amino acid compositions for the title compounds (**10**) and (**11**) were: Abu 0.90; Pro 1.0; Thr 0.98; (S)β^3-hPhe 1.0; Aib 0.89 and Abu 0.92; Pro 0.98; Thr 1.0; β^3-hPhe 1.0; Aib 0.90, respectively.

Z-Abu-Pro-Abu-OMe (12)

As previously described for (**4**), 5.01 g of dipeptide (**2**) (15.0 mmol), preactivated at $0\,^{\circ}C$ by 2.0 g iBCCl (15.0 mmol) and 1.7 g (15.0 mmol) of NMM, were reacted with 1.75 g of H-Abu-OMe (15.0 mmol). After the usual work up, the product, chromatographed on a silica gel column using the $CHCl_3$–CH_3OH 95 : 5 mixture as eluant, afforded 4.20 g (65% yield) of TLC pure (**12**). $R_f = 0.50$ ($CHCl_3$–CH_3OH 95 : 5).

Z-Abu-Pro-Abu-OH (13)

4.20 g of (**12**) (9.70 mmol) in 50 ml of CH_3OH was saponified for 3 h at room temperature by the addition of 0.490 g of NaOH dissolved in 10 ml of water. Following the procedure already described for (**2**), 3.33 g (82% yield) of (**13**) was obtained. $R_f = 0.40$ (ethyl acetate–acetic acid 9 : 1).

Z-Abu-Pro-Abu-Ser(tBu)-OMe (14)

3.33 g of (**13**) (7.94 mmol) was reacted by the MA method with 1.39 g of H-Ser(tBu)-OMe (7.94 mmol). The product, obtained by the usual procedure, was purified by chromatography on a silica gel column using the $CHCl_3$–CH_3OH (95 : 5) mixture as eluant, affording 2.70 g (59% yield) of tetrapeptide (**14**). $R_f = 0.60$ ($CHCl_3$–CH_3OH 95 : 5).

Z-(S)β^3-hPhe-ψ(CH_2-SO_2-NH)-Abu-Pro-Abu-Ser(tBu)-OMe (15)

2.70 g of (**14**) (4.69 mmol) in 30 ml of CH_3OH was hydrogenated for 5 h at room temperature over 300 mg of 10% Pd on charcoal. The catalyst was removed by filtration and the organic solvent evaporated *in vacuo*. The intermediate product, dissolved in 20 ml of anhydrous THF, was added at room temperature to the stirred solution of 1.72 g of Z-(S)β^3-hPhe-Ψ(CH_2-SO_2)Cl (4.68 mmol) in 20 ml of THF. The reaction mixture was stirred overnight at room temperature, taken up in CH_2Cl_2 and worked up as previously described for (**1**). The product, chromatographed on a silica gel column using the $CHCl_3$–CH_3OH (95 : 5) mixture as eluant, afforded 1.00 g of (**15**) (27% yield). $R_f = 0.30$ ($CHCl_3$–CH_3OH 95 : 5)

c(-Pro-Abu-Ser-(S)β^3-hPhe-ψ(CH_2-SO_2-NH)-Abu-) (16) (peptide III)

1.00 g of (**15**) (1.27 mmol) was saponified with 62.0 mg of NaOH in 2 ml of water overnight at room temperature. The product of the saponification, obtained following the usual work-up procedure, was hydrogenated for 5 h at room temperature over 100 mg of 10% Pd on charcoal. To the stirred solution of the terminally deprotected linear pentapeptide in 1 l of DMF were added 602 mg (1.39 mmol) of HBPyU and 340 mg (2.6 mmol) DIEA (diisopropylethylamine). After stirring the mixture for 24 h at room temperature, the solvent was evaporated *in vacuo* and the residue, taken up in ethyl acetate, was washed with 0.5 M $KHSO_4$, saturated $NaHCO_3$ solution and water. The organic phase, dried over Na_2SO_4, was evaporated and the product chromatographed on a silica gel

column using the $CHCl_3$–CH_3OH (96 : 4) mixture as eluant. The obtained 160 mg of side-chain protected cyclopentapeptide was kept for 1 h in 2 ml of TFA. After evaporation and washing with diethyl ether, 140 mg (20% yield) of title compound (**16**) was obtained, which was recrystallized from ethyl acetate. $R_f = 0.50$ ($CHCl_3$–CH_3OH 9 : 1). The MALDI-TOF analysis for $[M + H]^+$ was 552, as expected. The amino acid composition was: Pro 1.0; Abu 2.0; Ser 0.89; β^3-hPhe-ψ(CH_2-SO_2-) undetected.

Z-Pro-Thr(tBu)-Aib-OMe (17)

1.36 g of Z-Pro-OH (5.46 mmol) was coupled with 1.49 g (5.46 mmol) of H-Thr(tBu)-Aib-OMe by the MA method as described above for (**4**) affording 1.52 g (55% yield) of (**17**). $R_f = 0.50$ ($CHCl_3$–CH_3OH 95 : 5).

Z-(S)β^3-hPhe-ψ(CH_2-SO_2-NH)-Abu-OMe (18)

To a solution of 0.810 g of H-Abu-OMe (7.00 mmol) and 0.800 ml of NMM in 20 ml of anhydrous THF was added under stirring at room temperature a solution of 2.56 g of Z-(S)β^3-hPhe-ψ(CH_2-SO_2)Cl (7.00 mmol) in 30 ml of THF. After overnight stirring at room temperature the solvent was evaporated and the residue, taken up in CH_2Cl_2, was worked up as usual to afford 1.17 g (37.0% yield) of TLC pure (**18**). $R_f = 0.50$ (diethyl ether–hexane 8 : 2).

Z-Pro-Thr(tBu)-Aib-(S)β^3-hPhe-Ψ(CH_2-SO_2-NH)-Abu-OMe (19)

To a stirred solution in 20 ml of CH_2Cl_2 1.27 g (2.60 mmol) of Z-Pro-Thr(tBu)-Aib-OH, obtained by saponification of (**17**) was added at 0 °C 0.535 g of DCCI (2.60 mmol) and 0.700 g of HOBt (5.20 mmol). After 5 min a solution in 20 ml of CH_2Cl_2 0.816 g of H-β^3-hPheψ(CH_2-SO_2-NH)-Abu-OMe (2.60 mmol), obtained by hydrogenation of (**18**), was added. After stirring for 1 h at 0 °C and overnight at room temperature, the reaction mixture, cleared from *N*,*N*′-dicyclohexylurea by filtration, was worked up as described above for (**1**). The product was chromatographed on a silica gel column using the $CHCl_3$–CH_3OH (95 : 5) mixture as eluant, affording 1.4 g (68% yield) of title compound. $R_f = 0.55$ ($CHCl_3$–CH_3OH 95 : 5).

c(-Pro-Thr-Aib-(S)β^3-hPheΨ(CH_2-SO_2-NH)-Abu-) (20) (peptide IV)

For saponification and hydrogenation of 1.40 g of (**19**) the previously described procedures adopted to deprotect pentapeptide (**15**) were followed. 606 mg (0.948 mmol) of deprotected linear pentapeptide dissolved in 1 l of DMF was treated with 449 mg (1.02 mmol) of HBPyU and 0.35 ml of DIEA as described for the synthesis of cyclopentapeptide (**16**). Purification of the crude product by HPLC with a 0.1% TFA–containing CH_3CN–water linear gradient and treatment with TFA for 1 h of the tBu side-chain protected intermediate afforded 190 mg (35% yield) of TLC pure title compound (**20**). $R_f = 0.50$ ($CHCl_3$–CH_3OH 9 : 1) MALDI-TOF analysis for $[M + H]^+$ was 566, as expected. The amino acid composition for compound (**20**) was: Abu 0.90; Pro 1.0; Thr 0.98; β^3-hPheΨ(CH_2-SO_2) undetected; Aib 0.90.

X-Ray Diffraction Analysis

Colourless single crystals of c[-Pro-Thr-Aib-(S)β^3-hPhe-Abu-] (peptide **II**) were obtained by slow evaporation at room temperature from an acetonitrile solution. Data collection was carried out on a CAD4 Enraf-Nonius X-ray diffractometer at the 'Istituto di Biostrutture e Bioimmagini', C.N.R., at the University of Naples 'Federico II'. Unit cell determinations were carried out by least-square refinement of the setting angles of 25 high angle reflections accurately centred. No significant variation was observed in the intensities of the standard reflections monitored at regular intervals during data collection, thus implying electronic and crystal stabilities. Lorentz and polarization corrections were applied to the intensities, but no absorption correction was made. Crystallographic data for peptide **II** are listed in Table 1. The structure was solved by direct methods using the SIR97 program [9]. The solution with the best figure of merit revealed the coordinates of most of the non-H atoms. Refinement was performed by full-matrix least-squares procedures, with the SHELXL97 program [10]. All non-H atoms were refined anisotropically. H-atoms were calculated and during the refinement they were allowed to ride on their carrying atoms, with U_{iso} set equal to 1.2 times the U_{eq} of the carrying atom. It is worth noting that the crystals of the cyclic molecule were of low quality with a resultant rather limited number of observed reflections having $I > 2\sigma(I)$. These experimental findings give an explanation for the relative high R factor of the refined structure.

The scattering factors for all atomic species were calculated from Cromer and Waber [11]. CCDC 208 351 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/

Table 1 Crystallographic Data and Structure Refinement Parameters for c[Pro-Thr-Aib-(S)β^3hPhe-Abu] (**II**)

Molecular formula	$C_{27}H_{39}N_5O_6{\cdot}H_2O$
M.w. (a.m.u.)	547.65
Crystal system	Trigonal
Space group	$P3_1$
Z (mol/unit cell)	3
a (Å)	13.577(2)
c (Å)	14.105(1)
V (Å^3)	2251.7(5)
Temperature (K)	293
θ range for data collection (°)	1–70
Density (calc.) (g/cm^3)	1.212
μ (mm^{-1})	0.726
Crystal size (mm)	0.2 × 0.2 × 0.3
Radiation (λ, Å)	Cu Kα (1.54178 Å)
Scan mode	$\theta/2\theta$
Number of reflections collected	4636
Number of unique reflections	2971
Observed reflections	1828[$I > 2\sigma(I)$]
Structure solved	SIR97
Structure refined by	SHELXL97
Final R indices [$I > 2\sigma(I)$]	R1 = 0.0977
	wR2 = 0.2206
R indices (all data)	R1 = 0.1303
	wR2 = 0.2442
Goodness of fit	1.404
Crystallization solvent	CH_3CN
$\Delta\rho_{max}/\Delta\rho_{min}$ (eÅ^{-3})	0.426/−0.496

conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ (England); fax: (internat.) +44-1223-336-033; E-mail: deposit@ccdc.cam.ac.uk].

NMR and MD Analyses

The samples for NMR analysis were prepared by dissolving the appropriate amount of peptide in deuterated acetonitrile (99.98% Carlo Erba) to a final concentration of 2 mM.

All spectra were recorded on a Bruker DRX-500 spectrometer at 300 K. All chemical shifts, in part per millions (ppm), are referred to the methyl resonance of acetonitrile ($\delta = 2.0$ ppm for ^{1}H spectra; $\delta = 1.5$ ppm for ^{13}C spectra). One dimensional (1D) NMR spectra were acquired using typically 32–48 scans with 32 K data size. Pulse programs of the standard Bruker software library were used for the two-dimensional (2D) experiments. All 2D spectra were acquired in the phase sensitive mode, with quadrature detection in both dimensions, by use of the time proportional phase increment (TPPI) [12]. Typically, 256 increments of 48 scans each were performed: relaxation delay 1 s; size 2048; spectral width in F2 6024 Hz. Data processing was performed with NMRPipe [13] and spectral analysis with NMRView [14] zero-filling to 1024 in F1; squared cosine or Gaussian multiplication were used in both dimensions before the Fourier transform. A mixing time of 70 ms was used for TOCSY experiments [15]. NOESY [16] experiments were run at mixing times from 400 to 600 ms. Temperature coefficients of the amide protons were measured in the 280–310 K range. The structural features of the peptides have been determined on the basis of the analysis of the chemical shift values, the quantitative evaluation of $^3J_{NH\text{-}CH}$ vicinal coupling constants, the temperature coefficients of the NH proton resonances, and the interproton distances as derived by the NOEs. All spin systems were identified by means of total correlation spectroscopy (TOCSY) experiments and all resonances were sequentially assigned by the combined use of TOCSY and NOESY experiments.

Chemical shift values in deuterated acetonitrile at 300 K are reported in the Supplementary Material. The $^3J_{NH\text{-}CH}$ vicinal coupling constant values and the NH proton temperature coefficients for all peptides are reported in Table 2.

The DYANA 1.5 program [17] was used for structure calculation from NMR data. Assigned NOE peak volumes [14] were translated into distance restraints with the CALIBA [18] procedure, obtaining 43 (peptide **I**) and 51 (peptide **II**) upper-limit restraints. 102 (peptide **I**) and 98 (peptide **II**) lower-limit restraints (4.0 Å) were added for interproton distances corresponding to unequivocally missing NOE peaks (so-called 'anti-NOEs'). Supplementary Materials contains a table showing the lower- and/or upper-limit values. In addition, the restraint for the torsional ϕ angle derived from the J-coupling constant value of residue 4 ($J < 5$ Hz) was used in the case of peptide **I**. Structures were calculated with the standard DYANA 'calc_all' simulated anneal macro and the default set of parameters. Library files for the non-standard residues were built by using residue covalent geometries from crystal structures.

Biological Tests

The antitumour activity test *in vitro* was performed on NPA human cell line. NPA cell lines, a kind gift of Dr J. Fagin, were derived from a human papillary thyroid carcinoma and were tumorigenic when injected into nude mice. The cells were cultured

Table 2 NH Proton Temperature Coefficients and $^3J_{NH\text{-}CH}$ Values

Peptide **I**	Peptide **II**	Peptide **III**	Peptide **IV**
NH Proton Temperature Coefficients ($\Delta\delta/\Delta T$) (ppb)			
Thr −3.7	Thr −1.0	Abu −4.6	Thr −1.2
Aib −3.0	Aib −2.7	Ser −0.8	Aib −1.0
β^3-Phe 0.2	(S)β^3-hPhe −1.3	(S)β^3-hPhe $\Psi(CH_2\text{-}SO_2)$ −3.0	(S)β^3-hPhe $\Psi(CH_2\text{-}SO_2)$ −1.0
Abu −4.3	Abu −3.8	Abu −3.6	Abu −3.0
$^3J_{NH\text{-}CH}$ Vicinal Coupling Constant Values (Hz)			
Thr 7.4	Thr 5.9	Abu 6.1	Thr 7
Aib –	Aib –	Ser 8.2	Aib –
β^3-Phe 4.7	(S)β^3-hPhe 4.7	(S)β^3-hPhe $\Psi(CH_2\text{-}SO_2)$ –	(S)β^3-hPhe $\Psi(CH_2\text{-}SO_2)$ 8.6
Abu 5.5	Abu 7.2	Abu -	Abu 9.5

in the Dulbecco's Modified Eagle Medium (DMEM) containing glutamine, streptomycin (50 units/ml) and penicillin (50 units/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. For these experiments 24 multiwells, each well containing 10 000 cells in 1 ml were used. After 24 h the medium was aspirated and the astins, dissolved in 0.5 ml of the medium, were added. After another 72 h the supernatant was aspirated and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide [MTT] was added (1 : 10) for 2–3 h. During this period precipitation of tetrazolium salts was controlled by microscope. Finally, a solubilizing solution containing dimethylsulfoxide was added and incubated overnight. The IC_{50} values (concentration of astins at which 50% of cell died) were obtained by the ratio between readings at 690 and 570 nm. Each experiment was performed in triplicate and the values are the mean of three different experiments.

RESULTS AND DISCUSSION

Biological Tests

The IC_{50} of astins obtained were astin A 18 μM; astin B 80 μM; peptide **II** 160 μM and peptide **III** 40 μM. The antitumour activity tested *in vitro* on the NPA human cell line showed an IC_{50} value for peptide **III** comparable with that of natural astins A and B. The *in vitro* biological experiment of the synthetic astin G related cyclopeptides in which the di-chlorinated proline was replaced by a proline residue showed, for the first time, a biological activity similar to that of natural astins A and B. No antitumour activity was observed using the linear precursors of peptides **I**–**IV**. These data are in agreement with those of other acyclic astins [19] which did not show antineoplastic properties, thus suggesting that the cyclic nature of astins play a crucial role in their antitumour activities.

Crystal-state Molecular Conformation

In Figure 1 a stereo drawing of the crystal-state structure of the cyclic peptide **II**, as derived from the x-ray diffraction analysis, is presented. Bond lengths and bond angles show values in agreement with literature data [20]. The conformation is described by the torsion angles listed in Table 3. A *cis* peptide bond occurs between Abu^5 and Pro^1, all other peptide bonds being *trans*. The occurrence of a *cis* conformation in a Xxx-Pro bond, where Xxx is a residue with a branched side chain, as Val, Leu, Ile, Thr, and the same chirality of the Pro residue, has been repeatedly observed in cyclic hexa- and pentapeptides [21–24]. The Thr^2 C=O group is intramolecularly hydrogen bonded to the Thr^2 N-H group with an N···O distance of 2.595 Å, giving rise to the formation of a C_5 ring [25] (Table 4). All other N-H groups are involved in intermolecular H-bonds with symmetry related C=O groups.

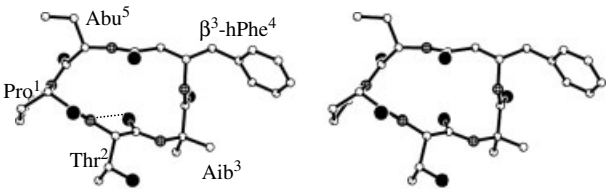


Figure 1 Stereo view of the x-ray diffraction structure of c[Pro-Thr-Aib-(S)β^3-hPhe-Abu] with residue numbering. The intramolecular H-bond is represented by a dotted line.

Table 3 Relevant Torsion Angles (°) (with e.s.d. in parentheses) for c[Pro-Thr-Aib-(S) β^3-hPhe-Abu] (**II**)

Residue	ϕ	μ	ψ	ω	$\chi^{1,1}$	$\chi^{1,2}$	$\chi^{2,1}$	$\chi^{2,2}$
Pro[1]	−83.7(10)		−11.9(12)	−175.6(8)				
Thr[2]	−151.3(8)		172.2(7)	−175.6(7)	65.3(9)	−170.2(7)		
Aib[3]	56.0(9)		43.0(10)	−178.5(7)				
β^3-hPhe[4]	67.9(10)	65.4(9)	−164.9(8)	−171.7(8)	−48.7(10)		−83.1(10)	96.4(11)
Abu[5]	−134.5(8)		124.5(8)	10.9(12)	−176.1(9)			

Table 4 Intra- and Intermolecular H-bond Parameters for c[Pro-Thr-Aib-(S)-β^3-hPhe-Abu] (**II**)

Donor D-H	Acceptor A	Symmetry equiv. of A	Distance (Å) D···A	Angle (°) D···A=C
N_2-H	O_2	x, y, z	2.595(9)	66.1(4)
N_3-H	O_5	$-x+y, -x, -1/3+z$	3.183(8)	168.4(5)
N_4-H	O_5	$-x+y, -x, -1/3+z$	3.002(8)	135.3(5)
N_5-H	O_4	$-y, x-y, 1/3+z$	2.945(8)	166.5(6)
O_2^{γ}-H	O_w	$-x+y, 1-x, -1/3+z$	2.99(2)	
O_w-H	O_1	$-x+y, -z, 2/3+z$	2.85(1)	123.4(6)
O_w-H	O_3	x, y, z	2.74(1)	135.9(7)

In detail, the Pro[1] residue shows a conformation in the helical region A of the ϕ,ψ map [26] with ϕ and ψ values −83.7°, −11.9°. Both Abu[5] and Thr[2] residues have conformations in the E region of the ϕ,ψ map. The Aib[3] residue is in the left-handed helical region (A*) ($\phi = 56.0°$, $\psi = -43.0°$). The (S)β^3-hPhe[4] residue assumes a conformation described by the ϕ, μ and ψ conformational angles which have values of 67.9°, 65.4° and −164.9°, respectively. This conformation does not correspond to any one of the most populated conformers of the (S)-β^3-hPhe residue [27], but it is one of the predicted accessible conformations having a μ angle equal to 60° (*gauche*$^+$).

The side-chain pyrrolidine ring of the Pro[1] residue has a Cγ-endo conformation [28] (q2 = 0.295 Å, $\varphi 2 = 89.9°$) [29]. The conformation of the Thr[2] side chain is described by the $\chi^{1,1}$ and $\chi^{1,2}$ torsion angles which are 65.3° and −170.2°, respectively. This conformation corresponds to one of the most populated conformers for this residue [30]. The Abu[5] side chain assumes a *trans* conformation ($\chi^1 = -176.1$). The (S)-β^3-hPhe[4] side-chain conformation defined by the χ^1 torsion angle, is *gauche*$^-$ ($\chi^1 = -48.7°$), while the χ^2 angles are sufficiently close to 90° (−83.1°, 96.4°) as expected for an aromatic residue [30]. The Thr[2], (S)-β^3-hPhe[4] and Abu[5] side chains extend away from the cyclic systems, parallel to the mean plane of the ring.

A structural comparison between natural astins and peptide **II** shows that the backbone conformational similarity is limited to the region of the *cis* Xxx-Pro peptide bond (Figure 2 and Table 5). The superposition of the backbones results in a RMS deviation of 0.90 Å. The analysis of the side-chain relative orientation reveals that in both compounds those of the Abu and (S)-β^3-h residues point out from the cyclic peptide.

In the crystal the peptide molecules are stabilized along the ***c*** axis by five intermolecular H-bonds (Table 4). Each independent molecule forms three intermolecularly H-bonds with a symmetry related molecule along the three fold axis (the Aib[3] NH is

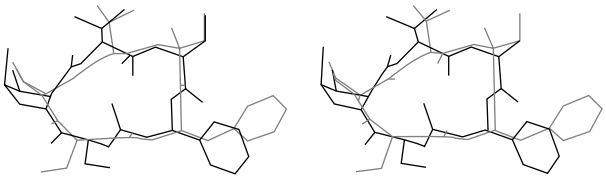


Figure 2 Stereo view of the x-ray diffraction structures of c[Pro-Thr-Aib-(S)β^3-hPhe-Abu] (thin lines) and astin B (bold lines) after backbone superposition.

Table 5 Backbone Torsion Angles (°) in Astin B and c[Pro-Thr-Aib-(S)-β^3-hPhe-Abu][a] **(II)**

Residue	Torsion angle	Astin B	**(I)**
Pro(Cl_2)[1]	ϕ	−96.2	−83.7
(Pro)	ψ	7.4	−11.9
	ω	169.2	175.6
αThr[2]	ϕ	−83.3	−151.3
(Thr)	ψ	−149.5	172.2
	ω	−171.4	−175.6
Ser[3]	ϕ	−69.0	56.0
(Aib)	ψ	−26.5	43.0
	ω	176.9	−178.5
β^3-Phe[4]	ϕ	−159.1	67.9
(S)β^3-hPhe	μ	56.6	65.4
	ψ	87.9	−164.9
Abu[5]	ω	−168.2	−171.7
(Abu)	ϕ	−71.4	−134.5
	ψ	140.7	124.5
	ω	15.4	10.9

[a] The amino acids in parentheses indicate those in c[Pro-Thr-Aib-(S)β^3-hPhe-Abu].

H-bonded to the Abu[5] C=O, the β^3-hPhe[4] NH is H-bonded to the Abu[5] C=O and the Abu[5] NH is H-bonded to the β^3-hPhe[4] C=O), and two intermolecular H-bonds along the same axis with the water molecule (the O_w molecule is H-bonded to the Pro[1] C=O and to the Aib[3] C =O groups). The co-crystallized water molecule does not form H-bonds with any NH group. This arrangement gives rise to the formation of cylinders of intermolecularly H-bonded peptide molecules along the ***c*** axis. These rows are kept together by a H-bond between the Thr[2] side-chain O^γ-H group and the water molecule along the ***b*** direction. In addition, in the ***ab*** plane the cylinders pack with each other in an hexagonal fashion by van der Waals interactions involving the side chains.

Solution Molecular Conformation

The NMR solution study has been carried out at 500 MHz in acetonitrile at 300 K. The structural data in solution for peptides **I** and **II** are compatible with an equilibrium in solution involving two or three major conformers, all of them characterized by the *cis* peptide bond between the Abu[5] and Pro[1] residues. This latter feature is supported by a strong Xaa-Pro $d_{\alpha\alpha}$ NOE and the observation of a large difference (ca.10 ppm) in the ^{13}C chemical shift [31] between β and γ carbons of the Pro residue [32].

Peptides **III** and **IV**, where the carbonyl in the (S)β^3-hPhe-Abu- peptide bond has been replaced by a -SO_2- group, show a largely increased backbone flexibility, resulting in the presence of two families of isomers characterized by a 60 : 40 *cis–trans* ratio for the Abu[5]-Pro[1] peptide bond.

For peptides **I** and **II** a semi-quantitative analysis of the NMR data, with identification of NOESY peaks corresponding to the major conformer, was possible. Twenty structures were then calculated for each peptide, by using the procedure described in the Materials and Methods section.

Cluster analysis by superposition of backbone atoms revealed the presence of three main conformer families for both peptides **I** and **II**. A comparison of the three conformers obtained for peptide **II** with the corresponding crystal structure shows that one of the conformations (**II-a**) in solution shares a great similarity with the crystal-state structure (Figure 3), with all backbone torsional angles in the same regions of the Ramachandran map.

A second conformer (**II-b**) exhibits differences in the region surrounding the (S)β^3-hPhe[4] residue, with formation of a γ-turn [25] involving the Aib[3] residue. The third conformer (**II-c**) differs more substantially from the other two, due to a change in the signs of the 'helical-like' conformation of Aib [3].

A comparison between the solution structures of peptides **I** and **II** (Table 6) shows that (**I-b**), one of the three representative conformers obtained for peptide **I**, is very similar to (**II-b**). (**I-a**) also shares a great similarity with the 'crystal-like' (**II-a**) conformation, but shows larger discrepancies on the average and, in particular, it exhibits a different conformation for the μ and ψ torsion angles of (S)β^3-hPhe[4] and for the ϕ torsion angle of Abu [5]. (**I-c**), in turn, is similar to (**II-c**), except for part of the Aib[3]-(S)β^3-hPhe[4] region. The (**I-c**) conformation resembles quite closely that observed for the crystal structure of astin B (the RMSD of backbone atoms

Figure 3 Stereo view of the x-ray diffraction (dark grey, stick and ball) and model (**IIa**) from NMR data in solution (light grey, stick) structures of c[Pro-Thr-Aib-(S)β^3-hPhe-Abu] after backbone superposition.

Table 6 Selected Backbone Torsion Angles (°) for Representative Solution Conformers of c-[Pro-Thr-Aib-β^3-Phe-Abu] (**I**), c-[Pro-Thr-Aib-β^3-hPhe-Abu] (**II**) and Astin B, and X-ray Diffraction Structure of (**II**)

Residue	Angle	(I)-a	(I)-b	(I)-c	Astin B	(II)-a	(II)-b	(II)-c	(II)-X-ray
Pro[1]	ϕ	−75.0	−75.0	−75.0	*−96.2*	−75.0	−75.0	−75.0	*−83.7*
	ψ	−43.4	−43.1	−27.0	*7.4*	−28.5	−46.0	−29.3	*−11.9*
Thr[2]	ϕ	−114.8	−123.4	−60.1	*−83.3*	−124.3	−120.7	−64.2	*−151.3*
	ψ	129.5	136.0	−124.8	*−149.5*	144.7	136.9	−123.1	*172.2*
Aib[3]	ϕ	84.7	85.5	−60.8	*−69.0*	86.0	84.7	−82.3	*56.0*
	ψ	72.3	−86.5	−134.0	*−26.5*	53.4	−85.4	−54.7	*43.0*
β^3-Phe[4]/β^3-hPhe[4]	ϕ	43.2	−57.9	−65.4	*−159.1*	33.0	−60.2	−60.7	*67.9*
	μ	50.9	−52.4	80.3	*56.6*	97.4	−51.4	−28.1	*65.4*
	ψ	143.2	−128.7	89.5	*87.9*	−148.8	−130.5	175.8	*−164.9*
Abu[5]	ϕ	−93.7	−62.0	−74.0	*−71.4*	−161.4	−59.9	−58.7	*−134.5*
	ψ	146.8	139.9	151.1	*140.7*	130.5	138.3	153.2	*124.5*

is 0.47 Å), with the exception of a swapping in the ψ(Aib[3])− ϕ((S)β^3-hPhe[4]) pair of torsion angles.

The occurrence in solution of the same conformation detected in the crystal state for peptide **II** suggests an intrinsic stability of this conformation, which apparently does not depend upon packing or other stabilizing effects peculiar to the crystal. This finding supports its potential role in the structure–activity relationship of these peptides.

CONCLUSIONS

The *in vitro* biological experiments of our synthetic astin G-related cyclopeptides, in which the dichlorinated proline was replaced by a proline residue, showed for the first time a biological activity similar to that of natural astins A and B. No antitumour activity was observed using the linear precursors of peptides **I**–**IV**. These data are in agreement with those of other acyclic astins [19] which did not show antineoplastic properties, suggesting that the cyclic nature of astins plays a crucial role in their antitumour activities.

A careful comparison of the structural (x-ray diffraction and NMR) data and biological results shows that the conformation observed in the crystal state and in solution for peptide **II** is not the bioactive one. On the contrary, the antitumour activity of peptide **III**, comparable to that of natural astins A and B, indicates that this peptide, with a large increased backbone flexibility, introduced by the -SO_2- group, could easily assume a conformation resembling that observed for the crystal structure of astin B.

Although the mechanism of antineoplastic action of astins remains unknown, it seems reasonable to evoke a crucial role in producing the biological active conformation for both the cyclic nature and the backbone flexibility of these new cyclic astin-related peptides containing non-coded amino acids and the (SO_2-NH) peptide bond surrogate.

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REFERENCES

1. Morita H, Nagashima SH, Takeya K, Itokawa H, Iitaka Y. Cyclic peptides from higher plants. 4. Structures and conformation of antitumor cyclic pentapeptides, astins A, B and C from *Aster tataricus*. *Tetrahedron* 1995; **51**: 1121–1132.
2. Morita H, Nagashima SH, Shirota O, Takeya K, Itokawa H. Two novel monochlorinated cyclic pentapeptides, astins D and E from *Aster tataricus*. *Chem. Lett.* 1993; 1877–1880.
3. Morita H, Nagashima SH, Takeya K, Itokawa H. Cyclic peptides from higher plants. Part 8. Three novel cyclic pentapeptides, astins F, G, and H from *Aster tataricus*. *Heterocycles* 1994; **38**: 2247–2252.

4. Schumacher KK, Hauze DB, Jiang J, Szewczyk J, Reddy RE, Davis FA, Joullié MM. First total synthesis of astin G. *Tetrahedron Lett.* 1999; **40**: 455–458.
5. Morita H, Nagashima SH, Takeya K, Itokawa H. A novel cyclic pentapeptide with a β-hydroxy-γ-chloroproline from *Aster tataricus*. *Chem. Lett.* 1994; 2009–2010.
6. Itokawa H, Takeya K, Hitotsuyanagi Y, Morita H. Antitumor compounds isolated from higher plants. *J. Biochem. Mol. Biol. Biophys.* 2000; **4**: 213–222.
7. Brouwer AJ, Monnee MCF, Liskamp RMJ. An efficient synthesis of N-protected β-aminoethanesulfonyl chlorides: versatile building blocks for the synthesis of oligopeptidosulfonamides. *Synthesis* 2000; 1579–1584.
8. Hintermann T, Seebach D. Synthesis of a β-hexapeptide from (R)-2-aminomethyl alkanoic acids and structural investigations. *Synlett* 1997; **5**: 437–438.
9. Altomare A, Burla MC, Camalli M, Cascarano G, Giacovazzo C, Guagliardi A, Moliterni AGG, Polidori G, Spagna R. *A Program for Automatic Solution and Refinement of Crystal Structures*. 1997. University of Bari: Bari, Italy.
10. Sheldrick GM. *SHELXL97. Program for Crystal Structure Refinement*. 1997. University of Göttingen: Göttingen, Germany.
11. Cromer DT, Waber JT. *International Tables for X-ray Crystallography*, Vol. IV, Table 2.2B. Kynoch Press: Birmingham, 1974- (now distributed by D. Reidel Publ. Co.).
12. Marion D, Wüthrich K. Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of 1H-1H spin-spin coupling constants in proteins. *Biochem. Biophys. Res. Commun.* 1983; **113**: 967–974.
13. Delaglio F, Grzesiek S, Vuister G, Zhu G, Pfeifer J, Bax A. NMRPipe: multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 1995; **6**: 277–293.
14. Johnson BA, Blevins RA. NMR view: a computer program for the visualization and analysis of NMR data. *J. Biomol. NMR* 1994; **4**: 603–614.
15. Bax A, Davis DG. Mlev-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* 1985; **65**: 355–360.
16. Jeener J, Meyer BH, Bachman P, Ernst RR. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Chem. Phys.* 1979; **71**: 4546–4553.
17. Güntert P, Mumenthaler C, Wüthrich K. Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* 1997; **273**: 283–298.
18. Güntert P, Graun W, Wüthrich K. Efficient computation of three-dimensional protein structures in solution from nuclear magnetic resonance data using the program DIANA and the supporting programs CALIBA, HABAS, and GLOMSA. *J. Mol. Biol.* l991; **217**: 517–530.
19. Morita H, Nagashima S, Takeya K, Itokawa H. Structure of a new peptide, astin J, from *Aster tataricus*. *Chem. Pharm. Bull.* 1995; **43**: 271–273.
20. Benedetti E. In *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, vol. 6, Weinstein B (ed.). Dekker: New York, 1982; 105–184.
21. Kartha G, Bhandary KK, Kopple KD, Go A, Zhu PP. Synthesis and conformation studies by x-ray crystallography and nuclear magnetic resonance of cyclo(L-Phe-L-Pro-D-Ala)$_2$. *J. Am. Chem Soc.* 1984; **106**: 3844–3850.
22. Bhandary KK, Kopple KD. Conformation of cyclo-bis(-L-valyl-L-prolyl-D-alanyl-), a synthetic cyclic hexapeptide. *Acta Crystallogr.* 1991; **C47**: 1280–1283.
23. Morita H, Yun YS, Takeya K, Itokawa H, Shiro M. η3-π-Allylzirconocene derivatives: preparation of steroidal side chains. *Tetrahedron* 1995; **51**: 5987–6002.
24. Zanotti G, Saviano M, Saviano G, Tancredi T, Rossi F, Pedone C, Benedetti E. Structure of cyclic peptides: the crystal and solution conformation of cyclo(Phe-Phe-Aib-Leu-Pro). *J. Pept. Res.* 1998; **51**: 460–466.
25. Rose GD, Gierasch LM, Smith JA. Turns in peptides and proteins. *Adv. Protein Chem.* 1985; **37**: 1–109.
26. Zimmerman SS, Pottle MS, Némethy G, Scheraga HA. Conformational analysis of the 20 naturally occurring amino acid residues using ECEPP. *Macromolecules* 1977; **10**: 1–9.
27. Rossi F, Bucci E, Isernia C, Saviano M, Iacovino R, Romanelli A, Di Lello P, Grimaldi M, Montesarchio D, De Napoli L, Piccialli G, Benedetti E. Effect of lengthening of peptide backbone by insertion of chiral β-homo-amino acid residues: conformational behavior of linear peptides containing alternating L-leucine and β-homo-L-leucine residues. *Biopolymers* 2000; **53**: 140–149.
28. Ashida T, Kakudo M. Conformations of prolyl residues in oligopeptides. *Bull. Chem. Soc. Jpn.* 1974; **47**: 1129–1133.
29. Cremer D, Pople JA. General definition of ring puckering coordinates. *J. Am. Chem. Soc.* 1975; **97**: 1354–1358.
30. Benedetti E, Morelli G, Némethy G, Scheraga HA. Statistical and energetic analysis of side-chain conformations in oligopeptides. *Int. J. Pept. Protein Res.*1983; **22**: 1–15.
31. Bax A, Griffey RH, Hawkins BL. Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. *J. Magn. Reson.* 1983; **55**: 301–315.
32. Dorman ED, Bovey FA. ^{13}C Magnetic resonance spectroscopy. The spectrum of proline in oligopeptides. *J. Org. Chem.*1973; **38**: 2379–2383.

