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New synthesis of *cis* 5-*tert*-butyl-L-proline via cuprate. Evaluation as a *cis* proline mimetic in a biological active octapeptide

CARINE AUBRY,[a] HASSAN OULYADI,[b] GUILLAUME DUTHEUIL,[a] JEROME LEPRINCE,[c] HUBERT VAUDRY,[c] XAVIER PANNECOUCKE[a] and JEAN-CHARLES QUIRION[a]*

[a] Laboratoire d'Hétérochimie Organique, UMR CNRS 6014, IRCOF, INSA et Université de Rouen, F-76821 Mont-Saint-Aignan, France
[b] Laboratoire de Biologie Structurale UMR CNRS 6014, IRCOF, Université de Rouen, F-76821 Mont-Saint-Aignan, France
[c] Laboratoire de Neuroendocrinologie Cellulaire et Moléculaire, INSERM U413, Université de Rouen, F-76821 Mont-Saint-Aignan, France

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Abstract: *cis* 5-*tert*-butyl-L-proline (Cbp) was prepared rapidly and efficiently by the addition of low-valent *tert*-butyl cuprate to an aminal derived from proline. [Cbp^2, D-Leu^5]-OP was then synthesized, showing a predominant cis peptide bond conformation, as confirmed by NMR. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide mimic; proline analogue; *tert*-butyl proline; cuprate; acyliminium; peptide conformation

INTRODUCTION

The octadecaneuropeptide (ODN; QATVGDVNTDRP-GLLDLK), a proteolytic fragment of diazepam-binding inhibitor, was originally characterized as a selective ligand for central-type benzodiazepine receptor (BZR) [1,2]. We have previously reported that ODN induces a potent anorexigenic effect independent of BZR activation [3]. We have also demonstrated that ODN increases the intracellular calcium concentration in cultured rat astrocytes through the activation of a G-protein-coupled receptor (GPCR) and that the *C*-terminal octapeptide (OP, RPGLLDLK) is the shorter isoactive fragment, whereas [D-Leu^5]-OP acted as a poor antagonist of the cytosolic ODN-response [4,5]. More recently, we have designed a selective agonist and a full antagonist of the ODN-GPCR corresponding to the head-to-tail cyclization of OP (cyclo-OP) and [D-Leu^5]-OP (cyclo-[D-Leu^5]-OP), respectively [6]. Structural analysis by two-dimensional ^{1}H-NMR and molecular modeling showed that cyclo-OP exhibits a single conformation characterized by both a β-turn (-LDLK-) and a γ-turn (-PGL-), whereas cyclo-[D-Leu^5]-OP adopts two equimolar conformations resulting from a trans/cis isomerization of the Arg–Pro peptide bond [6].

In order to obtain information about the active conformation of prolyl peptides, conformationally rigid prolyl amide surrogates have emerged as important tools for probing the relationship between amide geometry and peptide bioactivity. A large number of cis and trans proline analogues has been described [7–11]. Recently, Lubell reported that *cis* 5-*tert*-butyl-L-proline (Cbp) augments the amide cis isomer population in prolyl peptides [7,12]. The *N*-acetyl-*N*′-methylamide derivative of *trans* 5-*tert*-butyl-L-proline (Tbp) also favours cis isomer population [7], but the incorporation of Tbp in a peptide chain has never been reported because of the difficulty of the peptide bond formation. We therefore decided to prepare the cis isomer of this compound for its simplicity of structure and less denaturing behaviour and to introduce it in position 2 of [D-Leu^5]-OP by automated peptide synthesis.

MATERIALS AND METHODS

General

^{13}C NMR (75.5 MHz) and ^{1}H NMR (300 MHz) spectra were recorded on a 300 MHz Bruker DPX and 600 MHz Bruker DMX spectrometers. All samples were taken in $CDCl_3$ solvent. All chemical shifts are reported in parts per million downfield (positive) of the standard: TMS for ^{1}H and ^{13}C. Coupling constants (J) are given in Hertz (Hz). ^{1}H- and ^{13}C-NMR peaks were assigned using standard COSY, HMQC and HMBC experiments. All reactions were carried out in oven-dried glassware that was placed under vacuum while hot and cooled under argon. Column chromatography was performed on silica gel Kieselgel 60 (32–63 μm SdS). All reagents were obtained from commercial sources, or prepared as in the literature. All commercial solvents were distilled before using. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under argon atmosphere. TLC was performed on Merck 60F-250 silica gel plates. Optical rotations

Abbreviations: As recommended in *J. Pept. Sci.* 2003; 9: 1–8 and URL: http://www.chem.qmw.ac.uk/iupac/AminoAcid/ with the following additions and variations: Cbp, *cis* 5-*tert*-butyl-L-proline; DIEA, diisopropylethylamine; ECF, ethyl chloroformate; FEP, 2-fluoro-1-ethylpyridinium tetrafluoroborate; HMBC, Heteronuclear Multiple Bond Correlation Experiment; HMQC, Heteronuclear Multiple-Quantum Coherence; Tbp, *trans* 5-*tert*-butyl-L-proline.

* Correspondence to: J.-C. Quirion, IFRMP23, LHO, UMR CNRS 6014, IRCOF, Mont-Saint-Aignan, France; e-mail: quirion@insa-rouen.fr

were measured on a Perkin-Elmer 341 polarimeter using a sodium lamp at room temperature and are reported as follows: $[\alpha]_\lambda = \alpha_{obs}/(c.l)$(c g/100 mL, l dm, λ 589 nm). Mass spectra were obtained on a HP5890 (electron impact 70 eV). MALDI-TOF mass spectra were recorded on a Voyager DE PRO in the reflector and positive modes with α-cyano-4-hydroxycinnamic acid as a matrix.

Gaseous chromatographies were performed with a VARIAN 3300 apparatus, equipped with a VARIAN 3400 integrator using β-cyclodextrine column. High-performance liquid chromatography was performed with a GILSON apparatus using VYDAC 218TP54 analytical column and VYDAC 218TP1022 preparative column.

Organic Synthesis

(2S)-1*-tert-butyl-*2-methylpyrrolidine-1,2-dicarboxylate, Boc-Pro-OMe 2. To a solution of L-Proline 1 (15 g, 130 mmol) in 90 ml of methanol under N_2 at 0 °C was added thionyl chloride (10.39 ml, 143 mmol) dropwise for 20 min. The mixture was heated for 1 h under reflux. Concentration afforded crude product (16.8 g, 130 mmol), which was diluted in 175 ml of *tert*-butanol under N_2. Triethylamine (37.11 ml, 783 mmol) was then added dropwise for 10 min, followed by di-*tert*-butyl dicarbonate (28.47 g, 130 mmol) in 35 ml of *tert*-butanol for 30 min. The mixture was stirred for 18 h and then filtered off. After evaporation, the crude product was diluted in CH_2Cl_2 and washed with 2 × 150 ml of 1N HCl, 150 ml of saturated $NaHCO_3$ solution and 150 ml of saturated NaCl solution. Removal of solvents afforded compound **2** (24.75 g, 83%) as a pale yellow oil. ^{1}H NMR *(two rotamers):* 1.3 (s, 9H, H_9 rotamer1), 1.4 (s, 9H, H_9 rotamer2), 1.7–2.2 (m, 4H, $H_{4,5}$), 3.3–3.5 (m, 2H, H_6), 3.6 (s, 3H, H_1), 4.1–4.2 (m, 1H, H_3). ^{13}C NMR *(two rotamers):* 23.9 and 24.6 (C_5), 27.7 and 28.5 (C_9), 28.6 and 28.7 (C_4), 46.6 and 46.9 (C_6), 52.2 and 52.4 (C_1), 59.0 and 59.4 (C_3), 80.0 and 80.1 (C_8), 154.1 and 154.7 (C_7), 173.8 and 174.1 (C_2).

(2S)-1*-tert-butyl-*2-methyl-5-methoxypyrrolidine-1,2-dicarboxylate, Boc-Pro(5-methoxy)-OMe 3. To a solution of compound **2** (15.25 g, 66.6 mmol) diluted in 135 ml of methanol and kept in a one-compartment cell, equipped with a carbon felt cathode ($S = 10$ cm^2, specific area $= 0.3$ m^2 g^{-1}) and, was added *p*-toluenesulphonate tetraethylammonium (20.05 g, 66.6 mmol). A 1-A constant current was applied to the mixture for 30 h at 0 °C. After evaporation, the mixture was poured into water (200 ml) and extracted with CH_2Cl_2 (3 × 200 ml). Concentration and flash chromatography (ethylacetate : cyclohexane–3 : 7) afforded compound **3** (12.76 g, 74%) as a pale yellow oil. ^{1}H NMR: 1.3–1.4 (m, 9H, H_{10}), 1.9–2.3 (m, 4H, $H_{4,5}$), 3.2–3.4 (m, 3H, H_7), 3.6–3.7 (m, 3H, H_1), 4.2 (m, 1H, H_3), 5.1–5.2 (m, 1H, H_6). ^{13}C NMR: *two diastereomers and two rotamers:* 27.4 and 28.4 (C_5), 28.5 and 28.7 (C_{10}), 30.5 and 31.1 (C_4), 52.3 and 52.4 (C_1), 55.4, 55.8, 56.3 and 56.5 (C_7), 59.1, 59.3, 59.6 and 59.9 (C_3), 80.7 and 80.9 (C_9), 88.4, 88.6, 89.3 and 89.4 (C_6), 154.1 and 154.3 (C_8), 173.1 and 173.4 (C_2).

(2S)-1*-tert-butyl-*2-methyl-5-tert-butylpyrrolidine-1,2-dicarboxylate, Boc-Pro(5-tBu)-OMe 4. . To a suspension of copper cyanide (0.346 g, 3.86 mmol) in 20 ml of diethylether under argon at −40 °C was added *tert*-butyl lithium 1.5 M in pentane (2.57 ml, 3.86 mmol) dropwise. The mixture was stirred for 30 min at −20 °C and then cooled to −78 °C. A solution of compound **3** (0.5 g, 1.93 mmol) in 10 ml of diethylether was added slowly, followed 30 min later by borontrifluoride diethyletherate (0.49 ml, 3.86 mmol) dropwise. The mixture was stirred for 1 h at −78 °C and warmed overnight to room temperature. The mixture was then quenched with 300 ml of NH_4Cl/NH_{3aq} (1/1) solution and stirring was continued until appearance of deep blue color. The mixture was then extracted with CH_2Cl_2 (3 × 100 ml) and dried over $MgSO_4$. Solvents were removed and the yellow oil was purified on column chromatography (ethylacetate : cyclohexane–1 : 9), affording compound **4** (0.357 g, 65%) as colorless oil. ^{1}H NMR *(two rotamers)*: 0.8 (s, 9H, H_8, rotamer 1), 0.9 (s, 9H, H_8, rotamer 2), 1.3 (s, 9H, H_{11}, rotamer 1), 1.4 (s, 9H, H_{11}, rotamer 2), 1.7–1.8 (m, 4H, $H_{4,5}$), 3.6 (s, 3H, H_1, rotamer 2), 3.7 (s, 3H, H_1, rotamer 1), 3.9 (m, 1H, H_6), 4.2 (m, 1H, H_3). ^{13}C NMR *(two rotamers and two diastereomers)*: 25.5 and 26.4 (C_5), 27.8, 27.9 and 28.0 (C_8), 28.5 and 28.6 (C_{11}), 29.4 and 30.5 (C_4), 36.3, 36.6 and 36.9 (C_7), 51.7 and 51.9 (C_1), 61.4 and 61.5 (C_3), 66.2 and 66.7 (C_6), 79.7, 79.9 and 80.3 (C_{10}), 155.4 and 156.2 (C_9), 173.8, 174.1 and 174.4 (C_2).

(2S,5R)-methyl 5-tert-butylpyrrolidine-2-carboxylate, H-Cbp-OMe 5a and (2S,5S)-methyl 5-tert-butylpyrrolidine-2-carboxylate, H-Tbp-OMe 5b. To a solution of compound **4** (0.837 g, 2.94 mmol) in 17 ml of CH_2Cl_2 was added trifluoroacetic acid, TFA (5.67 ml, 73.4 mmol), and the mixture was stirred for 2 h at room temperature. After concentration, the crude product was diluted in CH_2Cl_2 and washed three times with saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$, filtered and solvents were removed under reduce pressure. Flash chromatography (ethylacetate : cyclohexane–1 : 1) afforded 73% yield **5b** (0.198 g, Rf = 0.68) and **5a** (0.199 g, Rf = 0.33) separately as colorless oils. The enantiomeric purities were ascertained by chiral GPC. Analytical conditions were β-cyclodextrine column: 30 m length with 0.32 mm internal diameter and 1 µm deposit thickness. The oven, injector and FID detector were heated to 150 °C isotherm, 250 °C and 250 °C respectively.

Diastereomer 5a. $[\alpha]_D^{20} = -33.7$ (c = 1, MeOH). ^{1}H NMR: 0.8 (s, 9H, H_8), 1.2–1.4 (m, 1H, H_5), 1.5–1.6 (m, 1H, H_5), 1.7–1.8 (m, 1H, H_4), 1.9–2.0 (m, 1H, H_4), 2.1 (s, 1H, H_9), 2.8 (dd, $^3J_{H\text{-}H} = 9.5$ Hz, $^3J_{H\text{-}H} = 3.1$ Hz, 1H, H_6), 3.6 (s, 3H, H_1), 3.7 (dd, $^3J_{H\text{-}H} = 8.7$ Hz, $^3J_{H\text{-}H} = 3.1$ Hz, 1H, H_3). ^{13}C NMR:26.5 (C_5), 26.7 (C_8), 30.5 (C_4), 32.8 (C_7), 51.9 (C_1), 59.9 (C_3), 69.6 (C_6), 175.5 (C_2).

Diastereomer 5b. $[\alpha]_D^{20} = -48.9$ (c = 1, MeOH). ^{1}H NMR: 0.7 (s, 9H, H_8), 1.3–1.5 (m, 1H, H_5), 1.6–1.8 (m, 2H, $H_{4,5}$), 2.0–2.1 (m, 1H, H_4), 2.2 (s, 1H, H_9), 2.9 (dd, $^3J_{H\text{-}H} = 8.5$ Hz, $^3J_{H\text{-}H} = 1.6$ Hz, 1H, H_6), 3.6 (s, 3H, H_1), 3.6 (dd, $^3J_{H\text{-}H} = 18.7$ Hz, $^3J_{H\text{-}H} = 6.4$ Hz, 1H, H_3). ^{13}C NMR: 26.2 ($C_{5,8}$), 30.3 (C_4), 33.3 (C_7), 51.8 (C_1), 59.9 (C_3), 67.5 (C_6), 176.3 (C_2).

Boc–Arg(Boc)$_2$ –Cbp–OMe 6. To a solution of compound **5a** (0.152 g, 0.82 mmol), Boc–Arg(Boc)$_2$–OH (0.432 g, 0.91 mmol) and FEP (0.194 g, 0.91 mmol) in 10 ml of CH_2Cl_2 at −10 °C was added the DIEA (443 µl, 2.62 mmol). The mixture was stirred for 5 min at −10 °C and then 1 h at room temperature. The mixture was washed with 5% aqueous $NaHCO_3$ solution, saturated NaCl solution and 5% aqueous citric acid solution. The organic layer was dried over $MgSO_4$, filtered and solvents

were removed under reduce pressure. Flash chromatography (ethylacetate : cyclohexane–1 : 1) afforded **6** (0.331 g, 63%) as a colorless oil. ^{1}H NMR: 0.9 and 1.0 (2s, 9H), 1.3, 1.4 and 1.5 (3s, 3 × 9H), 1.6–2.4 (m, 8H), 3.1–3.6 (m, 1H), 3.6–3.7 (2s, 3H), 4.1–4.6 (m, 3H), 5.7 (m, 1H). ^{13}C NMR: 24.8, 26.8, 27.9, 28.3, 28.9, 29.8, 35.8, 44.4, 51.3, 51.8, 51.9, 52.6, 59.9, 60.3, 66.7, 78.6, 79.6, 83.6, 154.9, 160.5, 163.7, 170.9, 172.2.

Boc–Arg(Boc)$_2$ –CbpOH 7. To a solution of compound **6** (0.112 g, 0.175 mmol) in 1 ml of dioxane was added a 2N aqueous LiOH solution (349 µl, 0.7 mmol). The mixture was stirred at room temperature until the reaction was completed, as monitored by TLC, and then extracted with ethylacetate (3 ml). 1N aqueous KH_2PO_4 solution was added to aqueous layer until pH reached 4. This aqueous layer was then extracted with 3 × 3 ml ethylacetate. The organic layer was washed with saturated NaCl solution (3 × 3 ml) and a drop of 37% aqueous HCl solution was added to the pH = 4 aqueous solution. The aqueous layer was then extracted back with 3 ml ethylacetate. The combined organic layers were dried over $MgSO_4$, filtered and solvents were removed under reduce pressure, affording pure compound 7 (0.064 g, 59%) as a colorless oil. ^{1}H NMR: 0.9 and 1.0 (2s, 9H), 1.4 and 1.5 (3s, 3 × 9H), 1.5–2.5 (m, 8H), 3.1–4.6 (m, 4H), 5.5 (d, $^3J_{H\text{-}H}$ = 8.7 Hz, 1H). ^{13}C NMR: 22.3, 25.2, 26.6, 28.4, 28.7, 28.8, 29.5, 36.2, 36.9, 40.9, 41.8, 50.7, 51.4, 62.8, 67.2, 67.4, 77.7, 80.1, 80.4, 84.1, 154.1, 154.2, 154.4, 154.9, 155.6, 155.9, 156.6, 173.4, 176.2. MS : 628 (MH^+), 268, 172, 126.

Peptide Synthesis

The *C*-terminal hexapeptide of ODN (GLLDLK) was synthesized (0.1-mmol scale) on a Fmoc-Lys(Boc)-HMP resin using a 433A Applied Biosystems peptide synthesizer and the manufacturer's standard procedures. All Fmoc-amino acids (1 mmol, 10 equiv) were coupled by *in situ* activation with HBTU/HOBt (1 mmol, 10 equiv; 1 : 1, mol/mol) in DMF and DIEA (2 mmol, 20 equiv) in NMP. Reactive side-chains were protected as follows: Asp, *tert*-butyl ester (O*t*Bu) and Lys, *tert*-butyloxycarbonyl (Boc). After completion of the chain assembly, the dipeptide Boc–Arg(Boc)$_2$–Cbp–OH 7 (0.47 mmol, 4.7 eq.) was manually coupled by addition of HATU/HOAt (0.47 mmol, 4.7 equiv; 1 : 1, mol/mol) and DIEA (0.94 mmol, 9.4 equiv) in NMP for 18 hours at room temperature. The peptidyl-resin was filtered and washed twice with NMP, CH_2Cl_2 and iPrOH.

Peptide Cleavage, Purification and Characterization

[Cbp^2, D-Leu^5]-OP was deprotected and cleaved from the resin by adding 10 ml of the mixture TFA/phenol/H_2O/thioanisole/ethanedithiol (82.5 : 5 : 5 : 5 : 2.5, v/v/v/v/v; reagent K, [13]) for 90 min at room temperature. After filtration, the crude peptide was precipitated by addition of TBME, centrifuged (4500 rpm, 15 min), washed three times with cold TBME, and lyophilized. The peptide was purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a 2.2 cm × 25 cm Vydac 218TP1022 C_{18} column using a linear gradient (10–50% over 40 min) of acetonitrile : TFA (99.9 : 0.1, v/v) at a flow rate of 10 ml/min. Analytical RP-HPLC (1 ml/min) was performed on a 0.45 cm ×25 cm Vydac 218TP54 C_{18} column using a linear gradient (10–40% over 30 min) of acetonitrile/TFA. The purified peptide was characterized by MALDI-TOF mass spectrometry (exact mass: 966.6; MH^+ observed: 967.3).

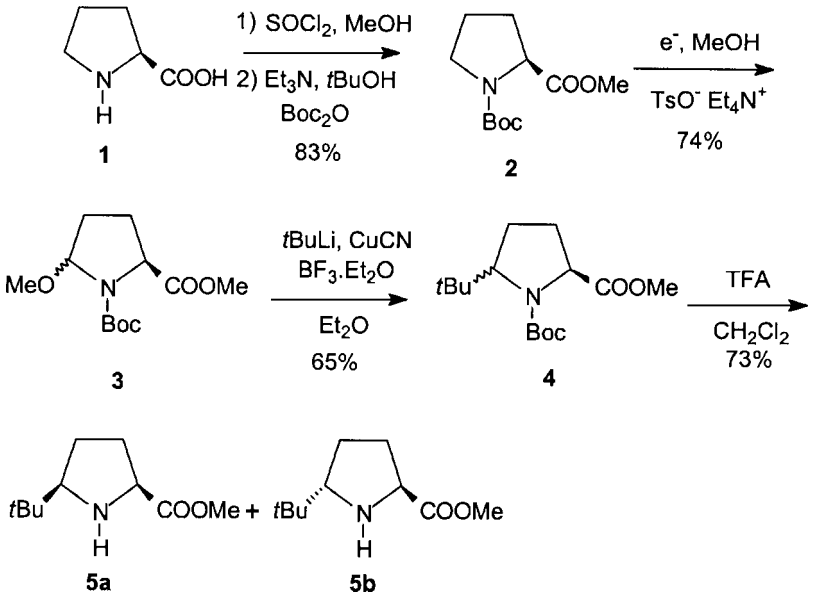
RESULTS AND DISCUSSION

Synthesis of *cis* 5-*tert*-butyl-L-proline

The synthesis of Cbp was first described in five steps starting from glutamic acid [12]. However, several attempts made by us failed to obtain products with the same good yield, mainly on account of purification problems. To circumvent this, we decided to study a more straightforward synthesis involving the direct introduction of a *tert*-butyl group on an acyliminium derivative of proline. The addition of alkylcopper reagent to chiral *N*-acyliminium ion has already been studied and used for the asymmetric synthesis of proline derivatives [14–17]. All the results showed a high level of selective *trans* addition explained by the formation of a complex between the *N*-acyliminium ion, the carbonyl group of the ester and the copper species [14,17]. However, at the beginning of our study, this highly selective process had never been applied to the preparation of *tert*-butylproline except once by Lubell, but in low yield (32%), and the reaction was described as not reproducible [18]. In order to obtain the cis isomer preferentially, we decided to study the influence of the nature of the copper reagent.

Aminal **3** (Scheme 1) was easily prepared by anodic oxidation of Boc–proline methyl ester **2** [19]. Our first attempts of addition of *tert*-butylcopper reagents were performed using the experimental conditions described in literature. We never obtained the desired compound in more than 32% yield (Table 1). Finally, using an excess of low-valent *tert*-butyl cuprate, prepared by addition of *tert*-butyllithium (2 equiv) to copper cyanide (2 equiv) at −78 °C, we were pleased to observe the formation of Boc–5-*tert*-butylproline methyl ester **4** with 65% yield as a 1 : 1 mixture of diastereoisomers (Table 1).

This result was rather surprising as all reported additions of alkyl copper reagents led to a highly selective *trans* addition as explained above [14,17]. Even when 4-substituted prolinates were used, it has been demonstrated that the stereochemistry was still controlled by this process. Furthermore, during the course of this study, a paper from the group of Wallén was published describing the selective synthesis of (2*S*, 5*S*) *trans* 5-*tert*-butylproline [19] (trans/cis ratio: 78 : 22), using an organocuprate prepared from *tert*-BuLi and CuBr.SMe$_2$. The mixture of diastereoisomers **5** could be easily separated after TFA deprotection of the Boc group, affording after column chromatography, the two isomers **5a** and **5b**. Their spectral and analytical data were in complete agreement with those in the literature [10]. The enantiomeric purity of *(2S, 5R)*- and (2*S*, 5*S*)-5-*tert*-butylproline methyl esters **5a** and



Scheme 1 H–Cbp–OMe and H–Tbp–OMe synthesis.

Table 1 Alkylation of aminal 3 with different *tert*-butyl electrophiles

Entry	Nucleophile	Equiv. of nucleophile	Yield (%)	*cis* : *trans*
1	*t*BuLi	1	10	50 : 50
2	$t\text{Bu}_2\text{CuCNLi}_2$	1	15	50 : 50
3	*t*BuCuCNLi	1	32	50 : 50
4	*t*BuCuCNLi	2	65	50 : 50
5	*t*BuCuCNLi	3	15	50 : 50

5b was next ascertained by chiral GPC and by Boc protection of the amine function to obtain **4a** (cis) and **4b** (trans) respectively. Comparison with α_D in the literature confirmed that both *cis* and *trans* isomers of **5** are configurationally stable [12]. Saponification of **5a** and**5b** can be achieved with LiOH (3 equiv) in a biphasic system water/CH_2Cl_2 affording H–Cbp–OH and H–Tbp–OH respectively.

Peptide Synthesis

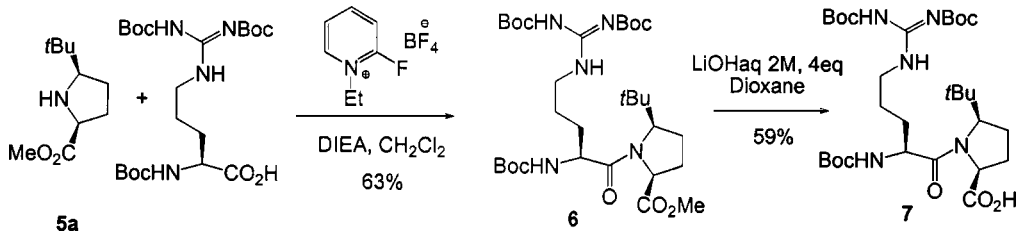
For the octapeptide synthesis, previous studies from Lubell's group [20] indicated that *N*-acylation of the sterically bulky (2*S*, 5*R*) 5-*tert*-butylproline was more effectively achieved in solution. For that reason, synthesis of dipeptide unit Arg–Cbp correctly protected was undertaken. As arginine is the *N*-terminal residue of OP and for practical reasons (resistance to basic reaction condition) we used a Boc group to protect the amine and the guanidine functions of arginine. Compound **5a** was coupled with Boc–Arg(Boc)$_2$–OH. After optimization of the reaction conditions (coupling agent TBTU, HATU, ECF or FEP and base DIEA or NMM), the best activating agent to promote the coupling was the FEP [21] with DIEA in CH_2Cl_2, yielding **6** in 63% yield. Saponification of the ester function afforded the desired dipeptide **7** in 59% yield (Scheme 2).

The *C*-terminal hexapeptide of ODN was assembled according to Figure 1. The dipeptide Boc–Arg(Boc)$_2$–Cbp–OH **7** (4.7 equiv) was then manually coupled. Cleavage of the peptide from the resin with concomitant side-chain deprotections was effected by treating the peptidyl-resin with reagent K [13] for 2 h at room temperature. After precipitation and centrifugation, [Cbp^2, DLeu^5]-OP could be obtained (Figure 1).

RP-HPLC analysis of the synthetic peptide revealed that the purity of [Cbp^2, DLeu^5]-OP was higher than 98%. The molecular weight observed by MALDI-TOF mass spectrometry agreed with the theoretical value (exact mass: 966.6; MH^+ observed: 967.3).

NMR Study

NMR spectra of octapeptide [Cbp^2, D-Leu^5]-OP were recorded on spectrometer BRUKER Advance DMX 600. Assignment of the proton NMR spectrum of octapeptide in pure water at 288 K was carried out by the sequential assignment strategy proposed by Wüthrich [22]. Firstly,



Scheme 2 Dipeptide Boc–Arg(Boc)$_2$–Cbp–OH synthesis.

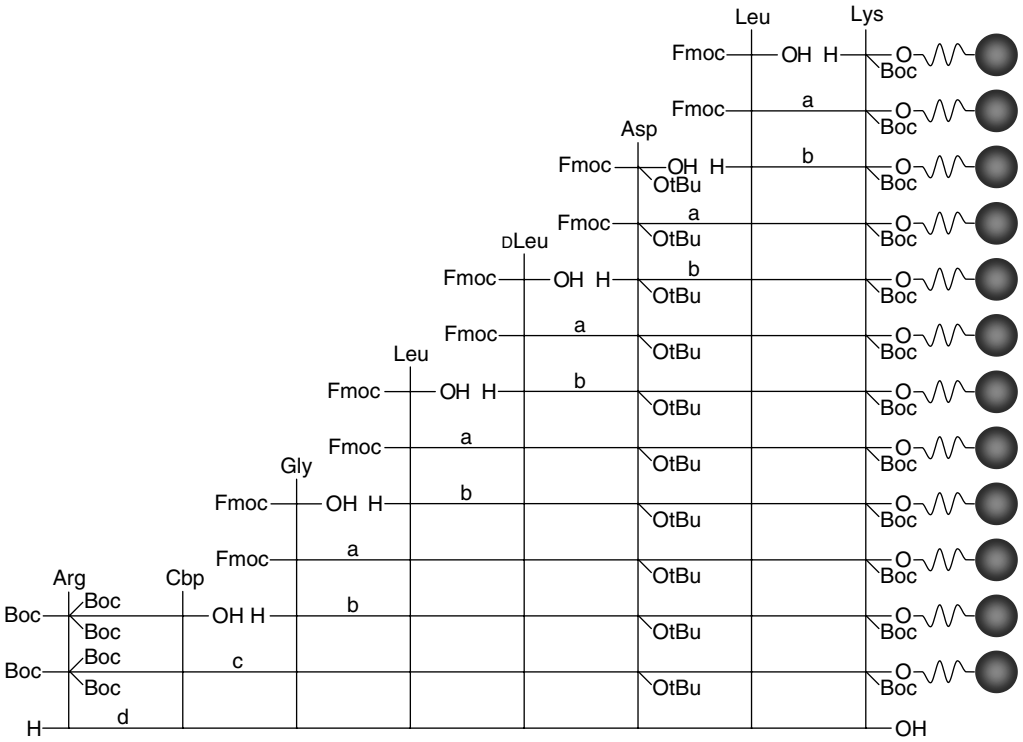


Figure 1 [Cbp^2, D-Leu^5]-OP synthesis.

coupling-based two-dimensional NMR spectra COSY and TOCSY were used for the identification of amino acid spin systems. At this stage, two glycine-type remote peaks were clearly observed in the COSY and TOCSY spectra of octapeptide instead of the one expected. This observation suggested the existence of two spectroscopically distinct molecular conformers. Secondly, the spin system residues were assigned to specific locations in their sequence by the observation in the NOESY spectrum of NOEs between resonances of sequentially adjacent residues. The observation, in the NOESY spectrum of the octapeptide, of repeated pattern of sequential cross peaks (Figure 2A) confirmed the occurrence in the solution of two distinct species corresponding to the same primary structure.

These data suggest that the sequence of octapeptide is sensitive to the Arg^1–Cbp^2 peptide bond *cis–trans* isomerism. Some of the spectral data are illustrated in Figure 2B, which shows a region of the NOESY spectrum containing the sequential $d\alpha\alpha$(Arg^1, Cbp^2) and $d\alpha\delta$ (Arg^1, Cbp^2) connectivities, characteristic of the *cis* and *trans* isomeric forms (Figure 3). The relative proportion, after integration of well-resolved NH amide of Asp^6 residue of *cis* and *trans* conformer, is 71 : 29. This result is in accordance with the observed results on dipeptide analogs [23] (from 70 to 90% of the *cis* isomer depending on the nature of the second amino acid) and on nonapeptide oxytocin mimics [20] (33% of the *cis* isomer compared to the 10% in the natural oxytocin).

In conclusion, we developed a method for the rapid synthesis of *cis tert*-butyl-L-proline, using a *tert*-butyl anion addition to an aminal species derived from proline. The absence of diastereoselectivity during the key-step is in disagreement with the previously reported mechanism. [Cbp^2, D-Leu^5]-OP was then synthesized showing a predominant *cis* conformation of the Arg^1-Cbp^2 peptide bond, as confirmed by NMR. This OP

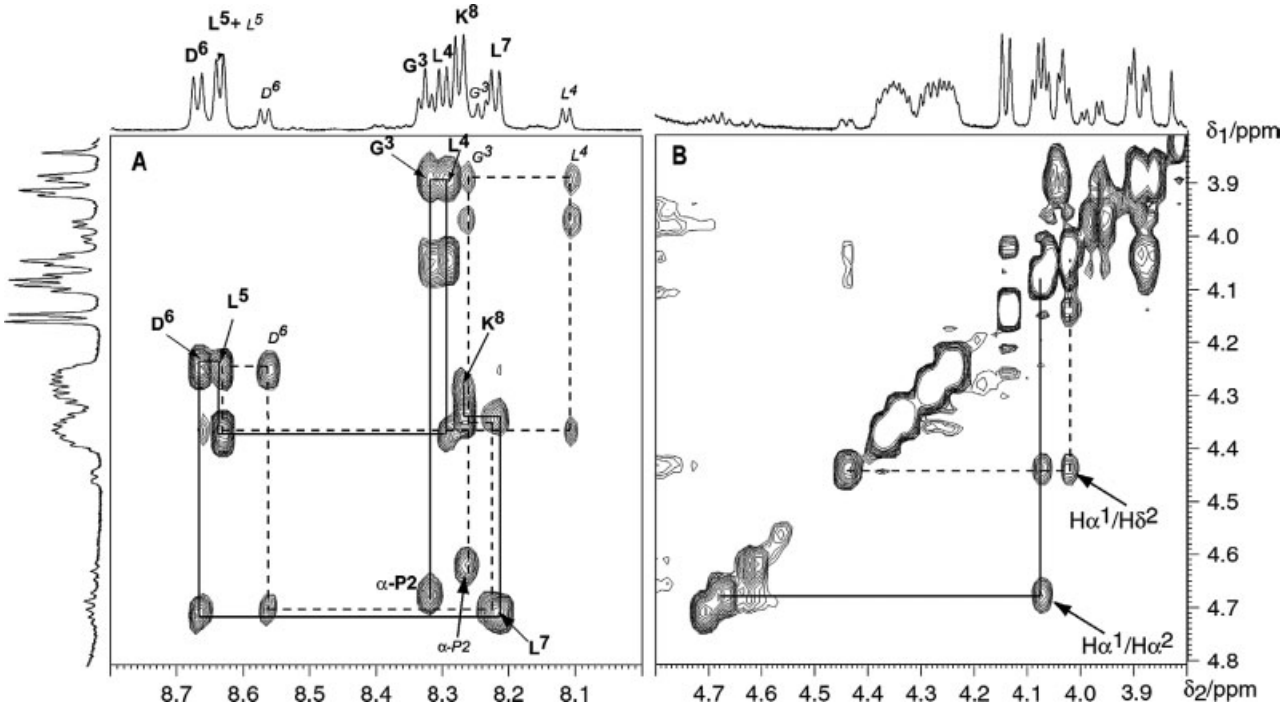


Figure 2 Regions of a 600-MHz NOESY spectrum of [Cbp^2, D-Leu^5]-OP recorded with a mixing time of 200 ms at 288 K in H_2O. (A) NH–αCH cross peaks: *cis* isomer bold letters, *trans* isomer, italic letters. (B) αCH–αCH cross peaks.

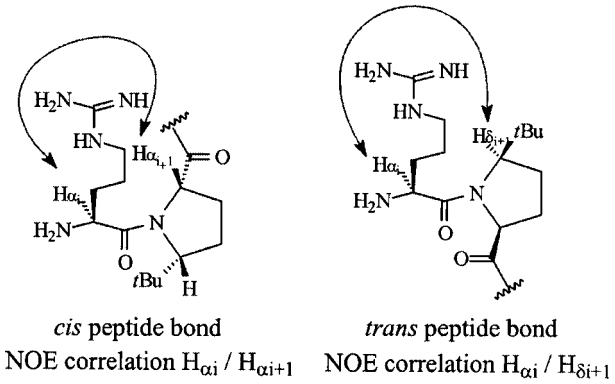


Figure 3 Specific NOE correlation of the *cis* and *trans* conformations of [Cbp^2, D-Leu^5]–OP.

analogue is presently tested *in vitro*, measuring the intracellular concentration of calcium ion in cultured rat astrocytes through a GPCR.

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