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Solid phase synthesis of novel α/β-tetrapeptides, electrospray ionization mass spectrometric evaluation of their metal cation complexation behavior, and conformational analysis using density functional theory (DFT)

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Thirty-four novel α/β-tetrapeptides (1–34) have been prepared employing solid-phase and in-parallel synthetic protocols. α/β-Tetrapeptides 1–34 were prepared by a combination of three α-amino acid residues (alanine (Ala), phenylalanine (Phe), and isoleucine (Ile)) with one β-amino acid residue (β^3-homophenylglycine). The corresponding complexes of several selected α/β-tetrapeptides with alkali, alkaline earth, and transition metals, [tP + M^+], were evaluated using ion electrospray-ionization mass spectrometry (ESI-MS). According to the results from analysis of mixtures, we can conclude that the position of the β-amino acid is determinant in the affinity toward different metal cations. Computational modeling (DFT, B3LYP 6-311++G) provided useful information regarding the most likely coordination sites of the metal ions on the receptor α/β-tetrapeptide 12, HO_2C-α-Phe-α-Phe-α-Ile-β^3-hPhg-NH_2, as well as the conformational changes induced by the metal upon [tP + M^+] complex formation. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: α/β-tetrapeptides; solid phase synthesis; coordination; metal ions; electrospray-ionization mass spectrometry

INTRODUCTION

As a consequence of the fundamental importance of peptides and proteins in physiological events, the interest in peptide synthesis as well as in the structural characterization of peptides has increased exponentially in recent years. Although the vast majority of natural peptides and proteins are constituted by α-amino acids, the pioneering and systematic studies of Seebach[1] and Gellman[2] have shown that the presence of β-amino acids instead of α-amino acids drastically modifies the activity and increases the hydrolytic stability of several bioactive natural peptides. Furthermore, it has been demonstrated that inclusion of β-amino acids in natural peptides leads in some cases to an increase in inhibitory ability of platelet aggregation,[3] or to increased affinity toward opioid receptors.[4,5] It is then very important to gain knowledge on the nature of the interactions that allow supramolecular recognition between the peptide's secondary structure and potential substrates, since such understanding paves the way to potential developments of pharmacologically promising peptidomimetics.[6,7]

In this regard, the binding of metal ions to certain functional groups in the peptide chain can induce the formation of stable helix or turn conformations.[8–13] For example, Searle and coworkers[14] synthesized one β-peptide with two and three histidine (His) fragments close to the *N*- and *C*-terminal and studied the effect of the addition of Zn^{2+} ions, finding a hairpin stabilizing effect in the peptide's secondary structure as a result of the interaction between Zn^{2+} and the His residues. In a particular example with β-peptides, Seebach and coworkers[15] discovered that the addition of Zn^{2+} salts stabilizes the secondary structure of β-peptides containing β^3-hCys and β^3-hHis residues. Furthermore, peptides are able to transport metals through cellular walls;[16] hence, evaluation of peptidic affinity toward metal ions is fundamental for the understanding of catalytic processes by proteins.

Electrospray ionization-mass spectrometry (ESI-MS) has proved to be a versatile method for the analysis of supramolecular complexes formed in solution and transported into the gas phase

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for detection. In particular, electrospray ionization is sufficiently gentle that non-covalent complexes can be transferred from the solution to the gas phase without disruption of the binding interactions of the complexes.[17–30] Furthermore, based upon the intensities of complexes in the resulting mass spectra, it is possible to estimate the relative binding affinities of different hosts toward different guests.[21–27] This general method provides the basis for the examination of the metal ion binding selectivities and stoichiometries of several novel α/β-tetrapeptides reported in the present study.

EXPERIMENTAL

Instrumentation and materials

Wang resin, reagents, solvents, and amino acids employed in this work were high-purity reagents (>99.5%) and were purchased from commercial suppliers. Glassware, needles, and magnetic bars were dried in an oven at 150°C for 24 h before their use. Anhydrous solvents were obtained by conventional methods. The purification of organic products was achieved by means of flash chromatography on 230–400 mesh silica gel, followed by recrystallization or Kugelrohr distillation. Melting points were measured on an Electrothermal apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin Elmer FT-IR 1600 spectrometer. ^{1}H and ^{13}C-NMR spectra were recorded on a JEOL GSX-270 at 270 MHz, JEOL GSX-400 at 400 MHz, and Bruker WM300 at 300 MHz Spectrometers in $CDCl_3$ or DMSO-d_6 with TMS as internal standard. The optical rotations were determinated in a Perkin-Elmer polarimeter model 241 at $\lambda = 589$ nm and 25–28°C of temperature. The β-amino acid (*S*)-3-amino-3-phenylpropionic acid [(*S*)-β^3-homophenylglycine, (*S*)-β^3-hPhg] was prepared according to the described procedure[31,32] via the highly diastereoselective conjugate addition of *N*-benzyl-α-phenylethylamine to *t*-butylcinnamate.[33,34] The syntheses of α/β-tetrapeptides were performed on a Vantage aapptec Automation reactor from Advanced Chem. Tech. Inc. or under microwave irradiation using a CEM Discover SPS or a CEM Liberty reactor and were obtained with 90% of purity (determined by HPLC analysis with a column C18 and 0.1 or 0.05% TFA in H_2O—CH_3CN with gradient). Mass spectra were recorded on an Agilent 1100 series LC/MSD Trap SL spectrometer under the following conditions: normal scanning mode, 50–2200 m/z range, 13 000 m/z/seg speed, 14.0 psi nebulizer pressure, drying gas (nitrogen), flow of 7.0 L/min, 325°C temperature, normal optimization of data with a −4500.0 to −1500.0 V gradient and positive polarity and the samples were analyzed by direct insertion. Relative abundances were determined in quintuplicate measurements.

For computational modeling, molecular mechanics conformational distribution searches were performed using MMFF force fields method[38] using PC Spartan Pro software (Wavefunction Inc.[39]). The equilibrium geometries of the lowest energy conformers were optimized using semiempirical PM3 calculations and the lowest energy conformer was then subjected to higher level methods in *ab initio* calculations using the Hartree–Fock Model, initially at the 3-21G level and subsequently at the 6-31G level of theory using Gaussian 03W software (Gaussian Inc.[41]). Finally, the lowest energy conformers were subjected to DFT B3LYP 6-311++G//B3LYP 6-31G calculation. To ensure the validity of the final structures, all the conformers obtained were subject of a frequency analysis.

Methods

(S)-3-(9H-fluoren-9-ylmethoxycarbonylamino)-3-phenylpropionic acid, (S)-β^3-hPhg[31–37]

A round-bottom flask equipped with magnetic stirrer was charged with tert-butyl (*S*)-3-amine-3-phenylpropionate (2.18 g, 9.9 mmol) in 3 ml of CH_2Cl_2 at 0°C and was added 4 ml of TFA, and stirred for 16 h at that temperature. After that, the temperature was increased to room temperature and the solvent was totally removed under vacuum. The lightly brown solid [(*S*)-3-amino-3-phenylpropionic acid] was dissolved in a THF/H_2O (92 ml 1:1 v/v) mixture and cooled to 0°C, then, 3.3 g (9.77 mmol) of FmocSuc and 8.21 g (97.74 mmol) of $NaHCO_3$ was added. The reaction was stirred for 3 h at room temperature and then the solvent was removed under vacuum. The aqueous phase was washed with diethyl ether and acidified with HCl 1N before the extraction with CH_2Cl_2; the organic phases were dried under anhydrous Na_2SO_4 filtered and evaporated under vacuum. The product was purified by recrystallization from ethyl acetate obtaining a white solid (73% yield after two steps, 2.8 g, 7.23 mmol); mp = 185–187°C (lit.[42] mp = 184°C). $[\alpha]_D = -22.8$ ($c = 1.1$, DMF). Lit.[42] $[\alpha]_D = -22.2$ ($c = 1$, DMF).

IR (KBr) C=O carbamate 1704 cm^{-1} (lit.[42] 1705 cm^{-1}).

^{1}H NMR (DMSO-d_6, 270 MHz) δ 2.61–2.78 (m, 2H), 3.44–3.56 (b, 1H), 4.17–4.33 (m, 3H), 4.92–5.00 (m, 1H), 7.16-7.99 (m, 13H), 1.27 (b, 1H).

^{13}C NMR (DMSO-d_6, 68 MHz) δ 41.1, 46.7, 51.6, 65.3, 120.1, 125.1, 126.3, 127.0, 127.6, 128.3, 140.7, 142.9, 143.7, 143.9, 155.3, 171.7.

Coupling of the first amino acid to the Wang resin

In a round-bottom flask was placed the resin and suspended in 9:1 v/v of CH_2Cl_2/DMF (approximately 15 ml of solution per gram of resin) and stirred for 30 min. Separately, 3–5 equivalents of the *N*-Fmoc protected β^3-amino acid were dissolved in the minimum amount of DMF. One equivalent of HOBt was then added and the resulting mixture was stirred before addition of the solution containing the resin. The reaction mixture was stirred for 10 min before the addition of 0.1 equiv. of DMAP dissolved in the minimum amount of DMF as well as 3–5 equiv. of DIC. The reaction mixture was stirred for 24 h at ambient temperature and then the resin was separated by means of a glass-sintered filter and washed three times with DMF, three times with CH_2Cl_2, three times with MeOH, and three times with Et_2O. The complete procedure was repeated and the modified resin was treated for 30 min with 2 equiv. of acetic anhydride and 2 equiv. of pyridine in order to protect the unreacted sites. The amino acid-containing resin was separated by means of a glass-sintered filter and washed three times with DMF, three times with CH_2Cl_2, three times with MeOH, and three times with Et_2O and dried under vacuum. A small amount of the resin was treated with a 20% solution of piperidine in DMF in order to liberate the amino acid, whose presence was confirmed by means of the ninhydrin test.[43] The degree of substitution was determined according to the procedure of Hamper *et al.*[44]

Deprotection in the Vantage reactor

A 3-ml solid-phase reactor was charged with the Wang-AA_1-NH-Fmoc (50 mg, 0.03 mmol) product, which was suspended in a solution of DCM and NMP (9:1 v/v, 1 ml). The resulting suspension was stirred for 3 min at 600 rpm. The Fmoc group

bound to the amino acid was removed with 20% of piperidine in NMP (2 ml, stirring for 40 min) and the amino acid was filtered, washed with MeOH (2×2 ml) and CH_2Cl_2 (2×2 ml), and dried under vacuum.

Deprotection by means of microwave irradiation

In a solid-phase reactor was placed the Wang-AA_1-NH-Fmoc resin and treated with 10 ml of DMF for 15 min before treatment with a solution of 20% piperidine in DMF. The resulting mixture was irradiated at 50 W and at 75°C during 12 min. The modified resin was washed with CH_2Cl_2 and dried under vacuum.

Amino acid coupling reactions in the Vantage reactor

Three-milliliter solid-phase reactors were charged with the amino acid-containing resin Wang-AA_1-NH_2 (50 mg, 0.03 mmol) and a CH_2Cl_2-NMP (9:1 v/v, 1 ml) solution. The resulting mixture was stirred for 3 min at 600 rpm before addition of the corresponding Fmoc-AA-OH (0.036 mmol) amino acid and HOBt (0.036 mmol) in NMP (0.75 ml). The reaction mixture was stirred for 2 min before the addition of DMAP (0.003 mmol) and DIC (0.036 mmol) in NMP (0.1 ml) and the resulting mixture was stirred for 3 h at ambient temperature. The modified resin was filtered and washed with NMP (1×2 ml), MeOH (1×2 ml) and CH_2Cl_2 (1×2 ml).

Amino acid coupling by means of microwave irradiation (on a 0.1 mmol scale)

In a solid-phase reactor was placed Wang-AA_1-NH_2 resin and 10 ml of DMF and the resulting mixture was treated with 5 equiv. of the Fmoc-AA-OH protected amino acid and 5 equiv. of HBTU. The coupling reaction was realized at 40 W and 75°C for 20 min, and the modified resin was washed with CH_2Cl_2. The addition of the subsequent amino acids was performed using the same coupling and deprotection procedures in order to obtain the desired α/β-tetrapeptide chain.

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Liberation of HO_2C-α/β-tetrapeptide-NH_2 in the Vantage reactor

Liberation of the modified Wang resin was achieved by treatment with 2 ml of a TFA-triisopropylsilane-H_2O (95:2.5:2.5) mixture during 90 min. The resin was recovered by filtration and the liberated product was collected in a 10 ml vials and treated with cold anhydrous ether to precipitate the product, then allowed to stand for 12 h at 0–4°C. The supernatant liquid was decanted and centrifuged and the product dried under vacuum.

Liberation of HO_2C-α/β-tetrapeptide-NH_2 by means of microwave irradiation

A flask containing the resin-α/β-tetrapeptide material was suspended in TFA-triisopropylsilane-H_2O (95:2.5:2.5) and irradiated for 18 min at 20 W, 38°C. The resin was separated by filtration and the filtrate was concentrated under vacuum before addition of diethyl ether to induce precipitation of the peptide (12 h at 0–4°C). The supernatant liquid was decanted and centrifuged and the product dried under vacuum.

Identification of the 34 synthesized α/β-tetrapeptides was performed by ESI mass spectrometry and the purity was determinated by HPLC analysis.

Sample preparation for affinity studies

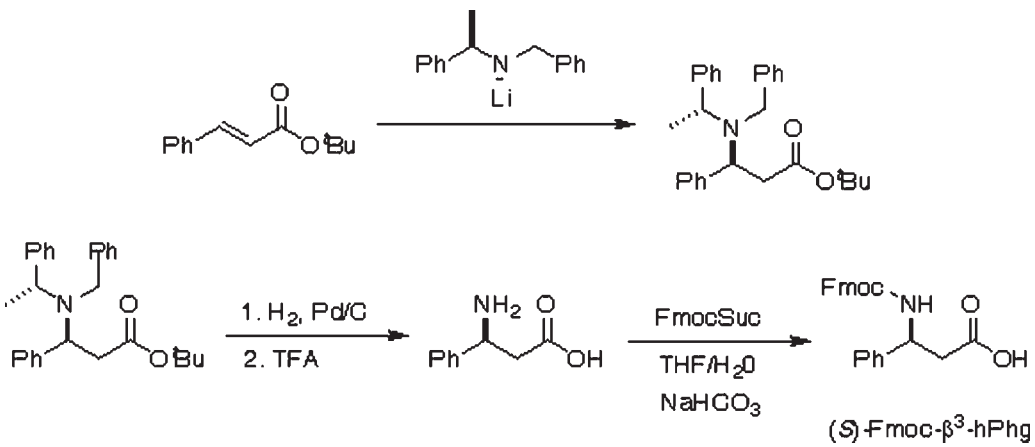
Two saline solutions with mixtures of LiCl-NaCl-KCl-$CuCl_2$-$ZnCl_2$ and LiCl-$MgCl_2$-$CaCl_2$-$CuCl_2$-$ZnCl_2$, and one of the α/β-tetrapeptide in methanol (1.5×10^{-5} M) were prepared. Mixtures (1:1) of the peptide with each of the saline solutions were prepared and then let stand for 30 min. The resulting solutions were analyzed by direct insertion in the mass spectrometer with electrospray ionization, using a syringe pump (Kd Scientific, model 100), at 240 µl/h.[45]

RESULTS AND DISCUSSION

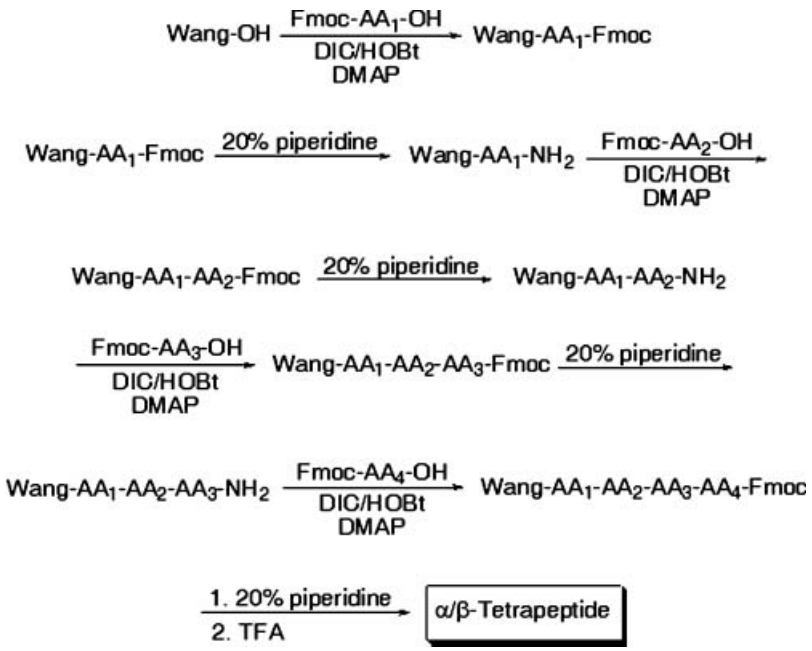
Solid-phase in-parallel synthesis of novel α/β-tetrapeptides 1–34

Initially, the key β-amino acid (*S*)-3-amino-3-phenylpropionic acid [(*S*)-β^3-homophenylglycine, (*S*)-β^3-hPhg] was prepared according to the described procedure[31,32] via the highly diastereoselective conjugate addition of *N*-benzyl-α-phenylethylamine to *t*-butylcinnamate[33,34] (Scheme 1).

β-Amino acid (*S*)-β^3-hPhg was *N*-protected using 9-fluorenylmethoxycarbonyl succinimide (FmocSuc)[35–37] to give derivative (*S*)-Fmoc-β^3-hPhg in 73% yield (Scheme 1). The synthesis of the



Scheme 1. Synthesis of enantiopure (*S*)-Fmoc-β^3-hPhg



Scheme 2. General scheme for amino acid coupling

desired α/β-tetrapeptides was achieved by means of the Wang resin,[46–47] which was selected because the liberation of the peptides is readily carried out under mild conditions, allowing for smooth liberation in 30 min. The incorporation of β-amino acid (*S*)-β^3-hPhg to the Wang resin was accomplished by means of a standard protocol employing 1-hydroxybenzotriazole (HOBt), 4-dimethylaminopyridine (DMAP), and diisopropylcarbodiimide (DIC) in *N*-methypyrrolidone (NMP) or *N*,*N*-dimethylformamide (DMF).[48] The deprotection was performed with piperidine at 20% in NMP or DMF. This coupling–deprotection method was also used to incorporate the other three α-amino acids Ala, Phe, and Ile, until achievement of the desired amino acid sequence in the final α/β-tetrapeptide (Scheme 2).

The reactions were carried out in an instrument for combinatorial chemistry by using a solid-phase reactor or under microwave irradiation using a manual or automatic peptide synthesizer, so that α/β-tetrapeptides **1**–**7**, **8**–**19**, and **33**, and **20**–**26** were obtained from commercially available resin-supported Ala, Phe, and Ile, respectively. Finally, α/β-tetrapeptides **27**–**32** and **34** were prepared in parallel from resin bound (*S*)-β^3-hPhg. Thus, the library design involved three α-amino acids (Ala, Phe, and Ile) and one β-amino acid (β^3-hPhg) to give a total number of possible combinations equal to 256. We synthesized the 34 linear α/β-tetrapeptides shown in Table 1, which includes the corresponding molecular ions obtained by mass spectrometry.

Analysis of mass spectra

Binding properties of alkali/alkaline earth and transition metal ions to several selected α/β-tetrapeptides (α/β-tP **12**, **18**, and **31**) were evaluated via ESI-MS methods. These α/β-tetrapeptides were selected as representative of peptides presenting the β-amino acid residue in different positions of the α/β-tetrapeptide. Salts in the form of chlorides for the following metal ions were used: Li^+, Na^+, K^+ (alkali metals), Mg^{2+}, Ca^{2+} (alkaline earth metals), and Cu^{2+}, Zn^{2+} (transition metals).

As discussed in the introduction, ESI-MS was deemed as the most convenient analytical tool to determine the potential formation of metal ion-α/β-tetrapeptide complexes [tP · M^+] in solution. The method by which binding selectivities are determined via ESI-MS relies upon comparison of signal intensities obtained upon spraying solutions containing a single host molecule and multiple metal ions.[49] A competitive equilibrium is established in solution, thus leading to a distribution of α/β-tetrapeptide-metal complexes that reflects the relative binding constants of the α/β-tetrapeptide-metal complexes or the selectivity of the peptide host.[23] Upon electrospraying the solutions, the complexes generated in solution are transported to the gas phase.[50]

Nevertheless, comparison of the ion intensities for the [tP · M^+] complexes in the mass spectra requires consideration of the relative ionization efficiencies, which are estimated from the analysis of the relative response attained by ionization of individual solutions containing a single α/β-tetrapeptide and a single metal ion.[50] Thus, the relative spray efficiencies of different α/β-tetrapeptide-metal complexes were estimated based on spraying solutions containing a single peptide and a single metal and comparing the observed intensities for the different complexes.

In particular, tP **12** (HO_2C-α-Phe-α-Phe-α-Ile-β^3-hPhg-NH_2) in a 2.6×10^{-5} M methanolic solution was treated with individual salt solutions of LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $CuCl_2$ y $ZnCl_2$ at three different concentrations in MeOH to afford 1:1 mixtures of α/β-tP and salt. The results from ESI-MS are shown in Fig. 1, which includes the R^2 and *m* parameters for each salt.

A lineal correlation between the ratio of α/β-tetrapeptide and metal ion in a range of 0.5 to 1.0 equiv. of salt is clearly found. More important, the substantial difference in ion abundances for individual complexes confirm rather significant differences in spray efficiencies. This observation confirms the need for calibration of measured abundances in competition experiments (as described below) in order to estimate binding affinities. Because the slopes for each individual metal ion in Fig. 1 are also different, we used the relative abundances obtained at a 1:1 $\alpha/$

Table 1. Molecular ions of the 34 α/β-tetrapeptides synthesized in this work

HO_2C-α/β-tetrapeptide-NH_2	$[M_{tP} + H]^+$
1, α-Ala-α-Phe-α-Ala-β3-hPhg	455
2, α-Ala-α-Phe-α-Ile-β3-hPhg	497
3, α-Ala-α-Phe-β3-hPhg-α-Ile	497
4, α-Ala-α-Ile-α-Phe-β3-hPhg	497
5, α-Ala-α-Ile-β3-hPhg-α-Phe	497
6, α-Ala-β3-hPhg-α-Phe-α-Ile	497
7, α-Ala-β3-hPhg-α-Ile-α-Phe	497
8, α-Phe-α-Ala-α-Phe-β3-hPhg	531
9, α-Phe-α-Ala-α-Ile-β3-hPhg	497
10, α-Phe-α-Ala-β3-hPhg-α-Ile	497
11, α-Phe-α-Phe-α-Ala-β3-hPhg	531
12, α-Phe-α-Phe-α-Ile-β3-hPhg	573
13, α-Phe-α-Ile-α-Ala-β3-hPhg	497
14, α-Phe-α-Ile-α-Ile-β3-hPhg	539
15, α-Phe-α-Ile-β3-hPhg-α-Ala	497
16, α-Phe-β3-hPhg-α-Ala-α-Phe	531
17, α-Phe-β3-hPhg-α-Ala-α-Ile	497
18, α-Phe-β3-hPhg-α-Phe-α-Ile	573
19, α-Phe-β3-hPhg-α-Ile-α-Ala	497
20, α-Ile-α-Ala-α-Phe-β3-hPhg	497
21, α-Ile-α-Ala-β3-hPhg-α-Phe	497
22, α-Ile-α-Phe-α-Ala-β3-hPhg	497
23, α-Ile-α-Phe-β3-hPhg-α-Ala	497
24, α-Ile-β3-hPhg-α-Ala-α-Phe	497
25, α-Ile-β3-hPhg-α-Phe-α-Ala	497
26, α-Ile-β3-hPhg-α-Phe-α-Phe	573
27, β3-hPhg-α-Ala-α-Phe-α-Ile	497
28, β3-hPhg-α-Ala-α-Ile-α-Phe	497
29, β3-hPhg-α-Phe-α-Ala-α-Ile	497
30, β3-hPhg-α-Phe-α-Ile-α-Ala	497
31, β3-hPhg-α-Ile-α-Ala-α-Phe	497
32, β3-hPhg-α-Ile-α-Phe-α-Ala	497
33, α-Phe-α-Ile-β3hPhg-α-Phe	573
34, β3-hPhg-α-Phe-α-Pheα-Ile	573

β-tP to M^+ ratio equal to calculate the normalization factor that was subsequently used before interpretation of data recorded when α/β-tetrapeptide **12** was exposed to mixtures of the salts of interest.

Accordingly, the relative ESI efficiencies for α/β-tetrapeptide 12 complexes among the series Na^+, Mg^{2+}, K^+, Ca^{2+}, Li^+, Cu^{2+}, and Zn^{2+} are 13.0, 4.25, 4.1, 3.2, 1.9, 1.3, and 1.0, respectively. Similarly, the relative ESI efficiencies for metal complexes of α/β-tetrapeptides **18** and **31** were estimated and the results are collected in Tables 2 and 3.

Calibration of the experimentally obtained abundances recorded from competitive experiments where a particular α/β-tetrapeptide is exposed to mixtures of metal ions dissolved in methanol (α/β-tetrapeptides **1**–**34** are insoluble in water) affords the data collected in Tables 4 and 5.

In the case of α/β-tetrapeptides **12** and **18**, where the β-amino acid is located at the amino group terminus or in the inner section, it is observed that the relative affinity among the alkali metal mixture is $Na^+ > K^+ > Li^+$, whereas the relative abundances of adducts produced in the presence of divalent alkaline earth metals were $Mg^{2+} > Ca^{2+}$. On the other hand, for transition metals Cu^{2+} and Zn^{2+} α/β-tetrapeptide **12** showed similar affinity whereas the trend for tetrapeptide **18** was $Cu^{2+} > Zn^{2+}$.

In the case of α/β-tetrapeptide **31**, where the β-amino acid is located at the carboxylic group terminus the relative affinity

Table 2. Relative ESI efficiencies for α/β-tetrapeptides **12**, **18**, and **31** with alkali metals

	α/β-Tetrapeptide		
Adduct	**12**	**18**	**31**
$[M + Li]^+$	1.88	1.00	1.00
$[M + Na]^+$	13.01	11.77	15.85
$[M + K]^+$	4.13	2.36	4.13

Table 3. Relative ESI efficiencies for α/β-tetrapeptides **12**, **18**, and **31** with alkaline earth metals

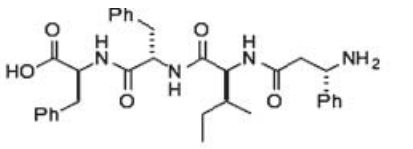
	α/β-Tetrapeptide		
Adduct	**12**	**18**	**31**
$[M–H + Mg]^+$	4.25	6.78	7.16
$[M–H + Ca]^+$	3.19	1.06	1.33
$[M–H + Cu]^+$	1.30	6.37	1.03
$[M–H + Zn]^+$	1.00	2.37	1.22

Table 4. Relative abundance (estimated affinities, %, multiplied by ESI efficiencies) for α/β-tetrapeptides **12**, **18**, and **31** with alkali metals

	α/β-Tetrapeptide		
Adduct	**12**	**18**	**31**
$[M + Li]^+$	5.6	5	24
$[M + Na]^+$	1157.9	211.9	317
$[M + K]^+$	78.5	33	66

Table 5. Relative abundance (estimated affinities, %, multiplied by ESI efficiencies) for α/β-tetrapeptides **12**, **18**, and **31** with alkaline earth metals

	α/β-Tetrapeptide		
Adduct	**12**	**18**	**31**
$[M–H + Mg]^+$	357	67.8	608.6
$[M–H + Ca]^+$	47.8	6.4	95.7
$[M–H + Cu]^+$	3.9	114.7	568.6
$[M–H + Zn]^+$	3	18.9	420.9



α/β-tP **12**, HO_2C-α-Phe-α-Phe-α-Ile-β^3-hPhg-NH_2

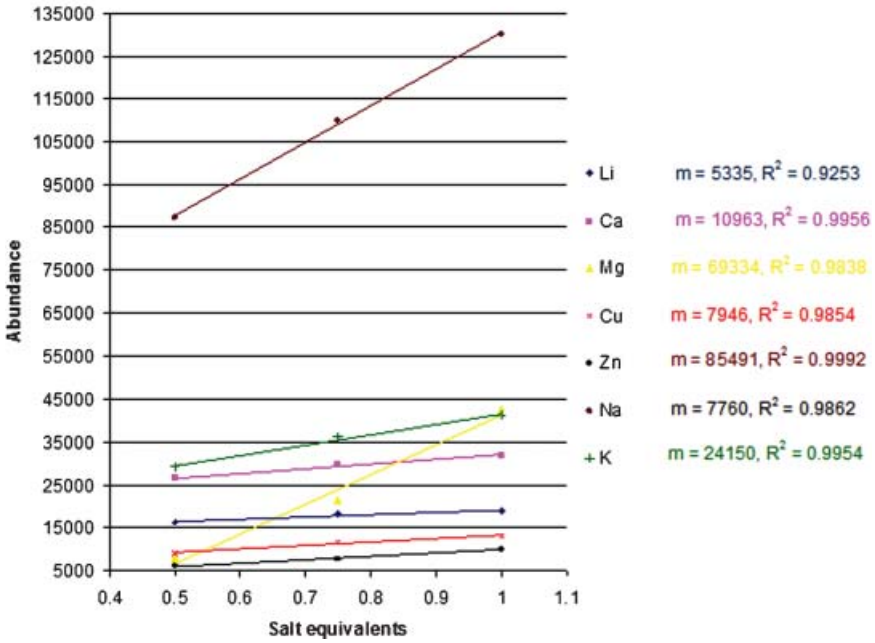


Figure 1. Ion abundances of complexes formed from α/β-tetrapeptide **12** in the absence of competition with other metals. Range of values from 0.5 to 1 equivalent of salt. *m*, slope.

among the alkali metal mixture is again $Na^+ > K^+ > Li^+$. Interestingly, among divalent metal ions a very large affinity toward Cu^{2+} and Zn^{2+} is observed.

Computational analysis

In addition to methodologies such as X-ray diffraction crystallography and NMR spectroscopy that are commonly used to determine the structural characteristics of peptides and proteins, molecular modeling using computational methods is becoming increasingly reliable to predict their lower-energy conformations and behavior.[51,52]

Conformational analysis in the absence of metal ions

In this computational study, α/β-tetrapeptide **12** (HO_2C-α-Phe-α-Phe-α-Ile-β^3-hPhg-NH_2) was selected as model system in view of the well-defined behavior exhibited in the ESI-MS affinity measurements (as described above). Three initial conformations, chosen after consideration of the structural behavior already reported in representative peptides,[53–56] were used as starting structures in the iterative energy-minimization process. These starting structures were then subjected to a conformational analysis where the conformers were distributed according to their relative energy, using the PC Spartan Pro (Wavefunction Inc.) program. This conformational analysis is based in MMFF molecular mechanics MonteCarlo methods that have proved quite efficient in similar conformational searches.[40]

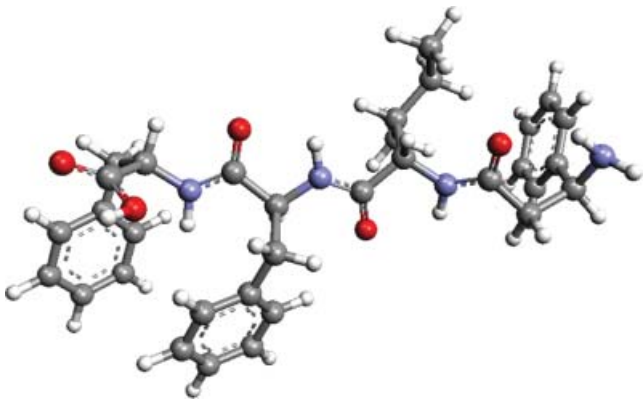


Figure 2. Conformation of lowest energy obtained for α/β-tetrapeptide **12** (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, and blue for nitrogen.

The structure of nearly forty conformers of low energy obtained from this process were then optimized by *ab initio* Hartree–Fock methods at the 3-21G level, and then the three lowest energy structures obtained at this level were re-optimized at the DFT B3LYP 6-31G level of theory and then realized a single point calculation at B3LYP 6-311++G level by means of the Gaussian 03 (Gaussian Inc.) software package.[41] Figure 2 depicts the conformation of lowest energy calculated for α/β-tetrapeptide **12**. That this is a true minimum was confirmed by frequency analysis.

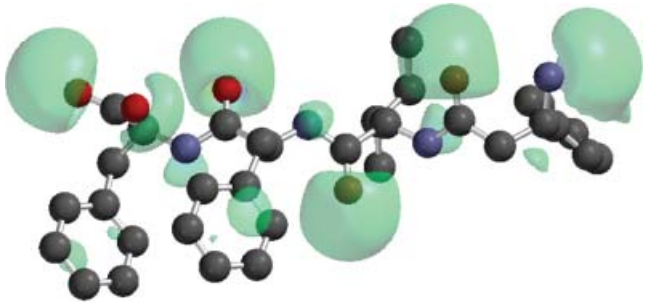


Figure 3. Electronic potential surface calculated for α/β-tP **12** The relative size of the green contours correlates with the relative concentration of electron density. The hydrogen atoms were removed for clarity.

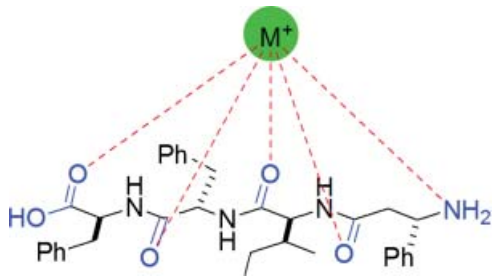


Figure 4. Most likely metal coordination sites on α/β-tP **12**.

It can be appreciated in Fig. 2 that the lowest energy conformation obtained for α/β-tP **12** corresponds to a linear conformation, where steric repulsion among substituents is minimum. On the other hand, the arrangement adopted by the β-amino acid residue β^3-hPhg (far right in Fig. 2) allows for hydrogen-bond formation between the terminal amino group and the carbonyl group in the same amino acid. Finally, the lowest energy conformation of α/β-tP **12** depicted in Fig. 2 allows for π–π interaction between the aromatic groups of the phenylalanine segments.

Conformational analysis in the presence of metal ions

The potential coordination sites in α/β-tetrapeptide **12** (the terminal amino and carboxylic groups, internal amide segments and aromatic phenyl substituents[57]) are highlighted in Fig. 3, they were derived from the electronic potential surface calculation using for that purpose the PC Spartan Pro software.

According to the information provided by Fig. 3, one may anticipate that the most likely binding sites in α/β-tetrapeptide **12** are the carbonyl groups on the peptidic segments, as well as the terminal amino and carboxylic groups (Fig. 4).

Consideration of the potential coordinating sites depicted in Fig. 4 suggested[57–63] the use of nine starting structures for the complexes generated from α/β-tP **12** and each one of the seven metal ions studied in this work. The nine starting structures for each cation were then optimized following the same protocol described for the optimization of α/β-tetrapeptide **12** (as described above). The most stable complexes α/β-tP **12** $\cdot M^+$ presented in Figs 5–11 were obtained in this fashion.

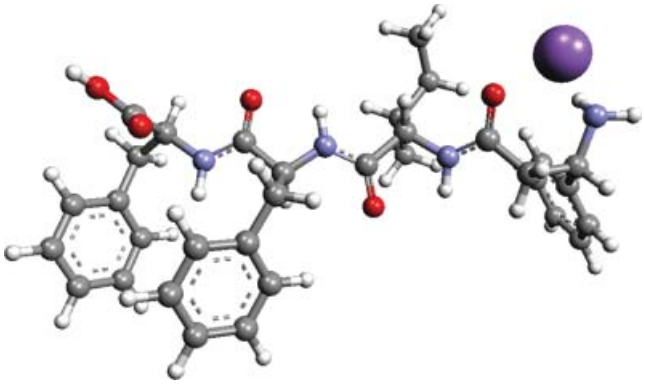


Figure 5. Optimized conformation and molecular structure for α/β-tP **12** $\cdot Na^+$ complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and purple for sodium.

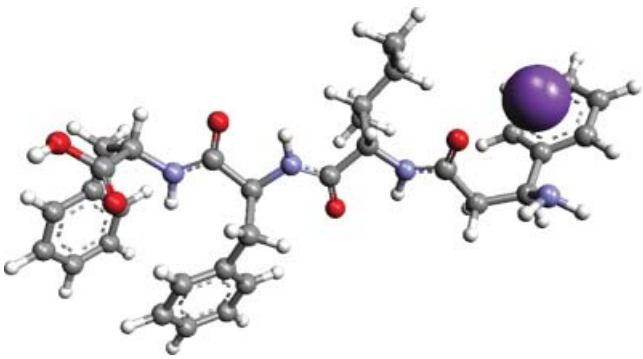


Figure 6. Optimized conformation and molecular structure for α/β-tP **12** $\cdot K^+$ complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and purple for potassium.

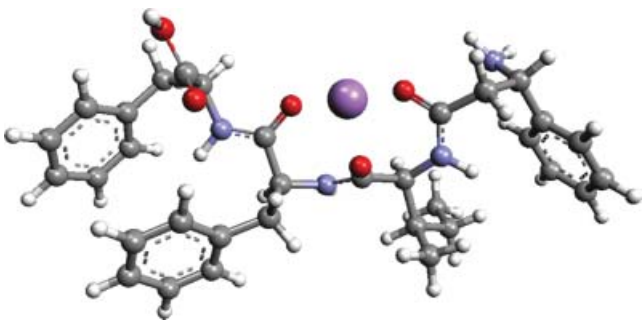


Figure 7. Optimized conformation and molecular structure for α/β-tP **12** $\cdot Li^+$ complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and purple for lithium.

Significant conformational differences are appreciated among the complexes of α/β-tP **12** and the three alkali metals studied. For example, with Na^+ the resulting complex preserves a linear conformation with the metal dicoordinated to the amino and carbonyl groups of the β-amino acid residue, β^3-hPhg (Fig. 5). In the case of K^+ (softer and bigger), there is an additional coordination with the aromatic ring in the same residue[58–63] (Fig. 6). By contrast, with Li^+ (smaller and harder) a tricoordinated species is predicted, where the metal ion induces a folded

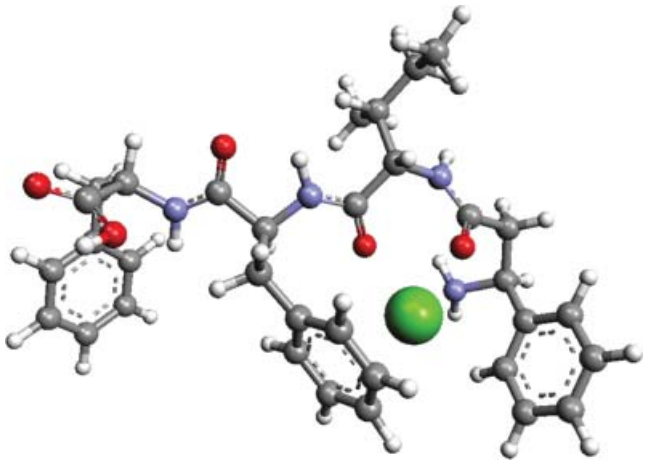


Figure 8. Optimized conformation and molecular structure for α/β-tP **12** · Ca^{2+} complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and green for calcium.

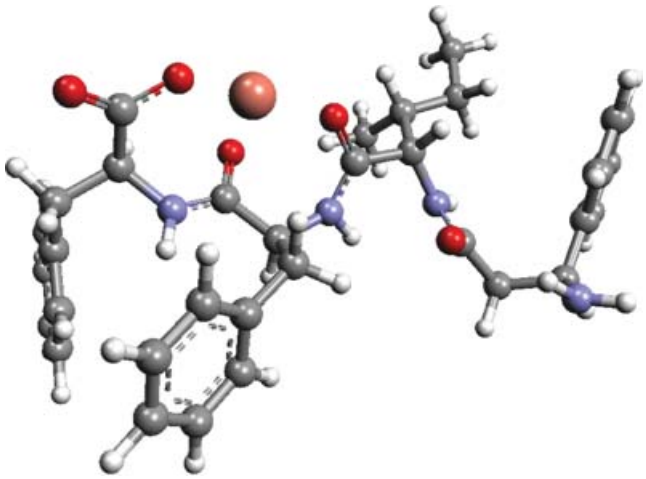


Figure 10. Optimized conformation and molecular structure for α/β-tP **12** · Cu^{2+} complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and pink for copper.

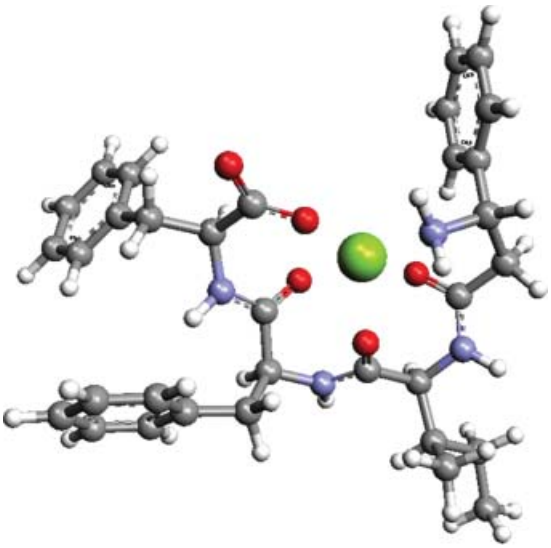


Figure 9. Optimized conformation and molecular structure for α/β-tP **12** · Mg^{2+} complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and green for magnesium.

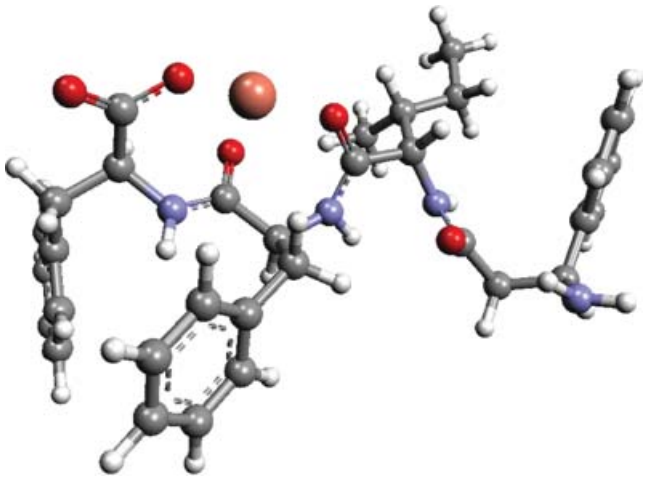


Figure 11. Optimized conformation and molecular structure for α/β-tP **12** · Zn^{2+} complex (B3LYP 6-311++G//B3LYP 6-31G level). Colour code: gray for carbon, white for hydrogen, red for oxygen, blue for nitrogen, and pink for zinc.

conformation via association with the α-Phe-α-Ile-β^3-hPhg segment (Fig. 7). It is apparent then that the ionic radius of the metal is determinant, where the larger Na^+ y K^+ ions (102 y 138 pm, respectively)[64] bind more easily to the terminal β-amino acid residue inducing only small changes in the peptidic conformation, whereas the electrostatic stabilization afforded by the smaller and harder Li^+ ion (76 pm radius)[50] upon coordination to the three carbonyls of the α-Phe-α-Ile-β^3-hPhg segment compensates for the otherwise increased energy of the folded peptide.

The contrasting binding modes for Na^+ and K^+ noticed in Figs 5 and 6 do not modify substantially the original conformation of α/β-tP **12**, since the three α-amino acid residues remain unaltered. Interestingly, complexation to Li^+ does provoke significant folding of the original, 'lineal' conformation of the free peptide (Fig. 7), but the metal affinity studies reported in the first part of this report indicate that complexation to lithium cation is not so favorable. That is, coordination to sodium cation causes little conformational changes in the original peptide, and the resulting complex seems to be most stable by comparison with those where metal coordination affects the receptor peptide's native conformation. This observation might be relevant in examination of related substrate–receptor phenomena such as in enzymatic activity where conformational changes of the native protein structure probably lead to modified structures in the active adducts.[65]

The complex formed by coordination of the soft Ca^{2+} ion and α/β-tP **12** (ionic radius for $Ca^{2+} = 100$ pm)[64] is calculated to present the metal ion associated to the α-Phe-α-Ile-β^3-hPhg segment, apparently via coordination to both carbonyl groups, to the amino group, and to both aromatic rings.[58–63] This coordination mode gives rise to a folded conformation of the

peptide where the β-amino acid residue constitutes a 'side chain' (Fig. 8).

By the same token, tight Mg^{2+} ion (ionic radius for $Mg^{2+} = 72$ pm)[64] affords a pentacoordinated adduct with α/β-tetrapeptide **12**. In particular, Mg^{2+} binds to the four available carbonyl groups as well as the amino group on the β-amino acid residue, β^3-hPhg. This coordination gives rise to a drastically folded conformation (Fig. 9).[66,67]

Transition metals Cu^{2+} and Zn^{2+} are predicted to form complexes with α/β-tP **12** that resemble those with Ca^{2+} and Mg^{2+}. Thus, adduct α/β-tP **12** $\cdot Cu^{2+}$ presents partial folding as a consequence of the coordination of the Cu^{2+} ion with the carbonyl groups on the terminal α-Phe and the internal peptidic carbonyls. Interestingly, the calculated conformation of the α/β-tP **12** $\cdot Cu^{2+}$ complex exhibits significant steric hindrance among the substituents on the peptide, and the anticipated repulsive interaction may be responsible for the low affinity of the complex observed in the experimental ESI-MS study (as described above).

Finally, the adduct arising from coordination between α/β-tetrapeptide **12** and transition metal Zn^{2+} shows a distorted tetrahedral core with the metal bound to both the carbonyl and amino groups of the β-amino acid residue in addition to the carbonyl of the inside phenylalanine residue as well as the carboxylate group of the terminal phenylalanine residue (Fig. 11). This molecular structure suggests several unfavorable steric interactions among the α-Phe-α-Phe segment, and such repulsive effects may be responsible for the experimentally observed low abundance of [α/β-tP **12** $\cdot Zn^{2+}$] complex.

Summary

Thirty-four novel α/β-tetrapeptides containing both α and β amino acid residues (α/β-tetrapeptides **1**–**34**) have been prepared by solid-phase synthesis and using in-parallel methodology. According to the results from analysis of mixtures of representative α/β-tetrapeptides **12**, **18**, and **31** in a methanolic solution containing a mixture of metal ions, it was confirmed that the ESI-MS method allows the determination of α/β-tetrapeptide-metal complex formation and the relative affinity toward specific metal ions. In the case of α/β-tetrapeptides **12** and **18**, where the β-amino acid is located at the amino group terminus or in the inner section, it is observed then that the relative affinity among the alkali metal mixture is $Na^+ > K^+ > Li^+$, whereas the relative abundances of adducts produced in the presence of divalent alkaline earth metals were $Mg^{2+} > Ca^{2+}$. On the other hand, for transition metals Cu^{2+} and Zn^{2+} α/β-tetrapeptide **12** showed similar affinity whereas the trend for α/β-tetrapeptide **18** was $Cu^{2+} > Zn^{2+}$. In the case of α/β-tetrapeptide **31**, where the β-amino acid is located at the carboxylic group terminus the relative affinity among the alkali metal mixture is again $Na^+ > K^+ > Li^+$. Interestingly, among divalent metal ions a very large affinity toward Cu^{2+} and Zn^{2+} is observed. Computational modeling (DFT, 6-311++G level) provides useful information regarding the likely coordination sites as well as the conformational changes induced by the metal.

Acknowledgements

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