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Article

Solid-Phase Peptide Synthesis of Dipeptide (Histidine-β-Alanine) as a Chelating Agent by Using Trityl Chloride Resin, for Removal of Al^{3+}, Cu^{2+}, Hg^{2+} and Pb^{2+}: Experimental and Theoretical Study

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Solid-phase peptide synthesis of dipeptide (histidine-β-alanine) as a chelating agent examined by common *N*-9-fluorenylmethyloxycarbonyl-*N*-trityl-*L*-histidine and *tert*-butyloxycarbonyl-β-alanine-OH amino acid derivatives. Trityl chloride resin was used as a carrier resin. The molecular structure of the dipeptide was definite by using different methods such as ultraviolet visible (UV-Vis), Fourier transform infrared (FTIR), proton (^{1}H) nuclear magnetic ressonance (NMR) and liquid chromatography-mass spectrometry (LC-MS) and the chelating property of synthesized dipeptide was investigated for removing of metal ions Al^{3+}, Cu^{2+}, Hg^{2+} and Pb^{2+} *in vitro*. In addition, the pharmacological and biological activities of dipeptide were examined by prediction of activity spectra for substances (PASS) program.

Keywords: dipeptide, solid-phase peptide synthesis, trityl chloride resin, chelating property, PASS program

Introduction

Peptides are one of the best candidates for drug development due to their high specificity and low toxicity. Peptides are mostly obtained by biological technology or chemical synthesis. The chemical method, especially solid-phase peptide synthesis (SPPS), is usually used for the large-scale production of peptides because of its simplified reaction procedure and easy purification/isolation steps for the target products.[1] SPPS can be defined as a process in which amino acid bound by its C-terminus to an insoluble polymer.[2] This method has made peptide synthesis simple, rapid, and easily subject to automation.[3] Protection of α- and β-amino functionality of amino acids is one of the most important issues in peptide chemistry and is efficient to prevent polymerization of the amino acid once it is activated. Because most peptide synthesis, both in solution and on solid-phase, are carried out in the C to N direction, α- and β-amino protecting groups are removed several times during the synthesis, and therefore, removal must be done in mild conditions that do not affect the remaining protecting groups or even the peptide chain.[4] The two most commonly amine-protecting groups in solid-phase peptide synthesis are 9-fluorenylmethyloxycarbonyl (Fmoc) and *tert*-butyloxycarbonyl (Boc).

The success of the solid-phase method is closely dependent on the efficiency of the solid support. Trityl chloride resin is perhaps one of the most useful resins for the solid-phase synthesis of C-terminal peptide acids. It can be used for the preparation of both protected and unprotected peptides. The main advantages of its use are: (*i*) allows to cleave the peptide under mild acidic conditions [1% trifluoroacetic acid (TFA)]; (*ii*) minimizes the formation of diketopiperazine (DKP); (*iii*) minimizes racemization during the attachment of the first amino acid; and (*iv*) minimizes the formation of side chain.[5]

The ability of dipeptides to chelate of various metal ions should be investigated.[6,7] Complexation of this dipeptide with numerous cations (such as Ru^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Cd^{2+}, Mg^{2+}, and Ca^{2+}) have been studied.[8-11]

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In this paper, we report the synthesis of dipeptide histidine-β-alanine as a chelating agent with chemical method, solid-phase peptide synthesis. Molecular structure and chelating property of synthesized dipeptide were investigated. In addition, the pharmacological and biological activities of dipeptide were examined by prediction of activity spectra for substances (PASS) program.

Experimental

Chemicals

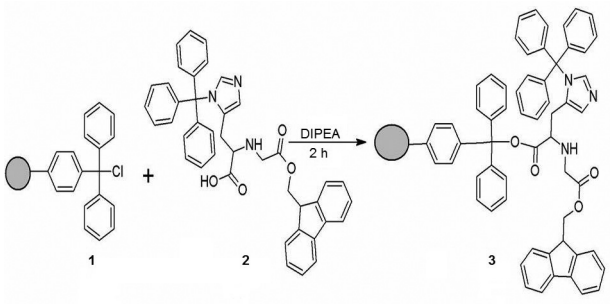
Trityl chloride resin (ca. 200-400 mesh) and *N*-Fmoc-*N*-trityl-L-histidine were purchased from Bachem chemical Company (Budenforf, Switzerland). Boc-β-alanine-OH was obtained from Aldrich Company (St. Louis, MO, USA). Coupling reagents: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) was purchased from Fluka Chemical Company (Buchs, Switzerland). Scavengers: anisole and phenol; solvents: trifluoroacetic acid (TFA), piperazine, *N*,*N*-di-isopropylethylamine (DIPEA), diethyl ether, dichloromethane (DCM), *N*,*N*-dimethyl formamide (DMF) and methanol (MeOH); metals: $AlCl_3$, $CuCl_2$, $HgCl_2$ and $Pb(NO_3)_2$ were obtained from Merck (Darmstadt, Germany).

Methods

PG Instruments T80 double beam ultraviolet-visible (UV-Vis) spectrophotometer (Leicestershire, UK) was used for UV-Vis measurements. The Fourier transform infrared spectra (FTIR) were recorded on a Shimadzu FTIR-8400S spectrophotometer (Kyoto, Japan) in the range of 400-4000 cm^{-1}. Proton (^1H) nuclear magnetic ressonance (NMR) spectra were recorded on a Bruker DRX 250 (300 MHz) spectrometer (Billerica, MA, USA) in water. Mass-spectra was recorded on an Agilent 6410 Triple Quadrupole liquid chromatography-mass spectrometry (LC-MS) using Agilent 1200 series HPLC system (Santa Clara, CA, USA) column C18 (250 × 4.6 mm, 5 µm); mobile phase A, H_2O and B, methanol; flow rate of 1 mL min^{-1}, 20 µL, total run time of 40 min.

Loading of trityl chloride resin (Scheme 1)

Peptide synthesis was carried out using trityl chloride resin. Trityl chloride resin (2 g) (**1**) was swelled in dichloromethane (1 h). *N*-Fmoc-*N*-trityl-*L*-histidine (2.47 g, 2 equivalents) (**2**), DIPEA (1 mL), DCM (2.5 mL) and DMF (5 mL) were added to the reaction vessel. The mixture was shaked for 2 h. After 2 h, the resin was filtered and washed thoroughly under nitrogen atmosphere with DMF (5 mL × 3) and after that with DCM (5 mL × 3).



Scheme 1. Attachment of the first amino acid *N*-Fmoc-*N*-trityl-*L*-histidine to the resin. DIPEA: *N*,*N*-Diisopropylethylamine.

Capping

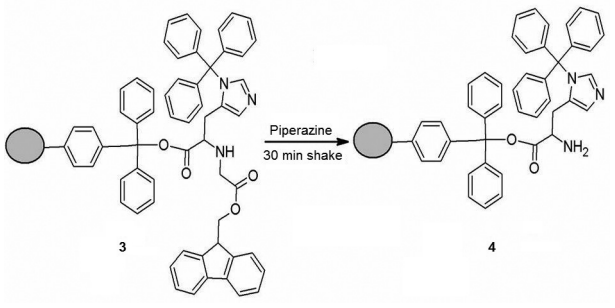
After the first amino acid be loaded to the resin, the un-reacted sites must be end-capped with methanol (MeOH) to ensure that future reactions do not react at those unloaded sites. The process of methanol capping is fairly simple and involves a mixture of DCM, MeOH, and DIPEA in a 80:15:5 ratio added to the resin and shaked for 15 min. The resin must be washed under nitrogen atmosphere with DMF (5 mL × 3) and then with DCM (5 mL × 3) to remove any excess of MeOH and DIPEA before being re-swollen and coupled with the next amino acid.[12]

Deprotection of Fmoc group (Scheme 2)

Removal of the Fmoc group (**3**) was obtained by the addition of 10% piperazine, shaked for 30 min under nitrogen atmosphere and after 30 min, washed with DMF (5 mL × 3). The monitoring of the complete cleavage of the Fmoc was performed with color detector (acetaldehyde/chloranil), for detection of free terminal amino groups (**4**). This detector will do the transition from white to green if an amino group is present.[13] Solution **1** is composed by 2% acetaldehyde in DMF, and solution **2** by 2% chloranil in DMF. A few beads of resin are placed in a small test tube and 2-5 drops of each solution are added. After a short mixing, the mixture is left at room temperature for 5 min. The result dark blue to green beads is positive for Fmoc cleavage, and colorless to yellowish beads is negative for Fmoc cleavage.

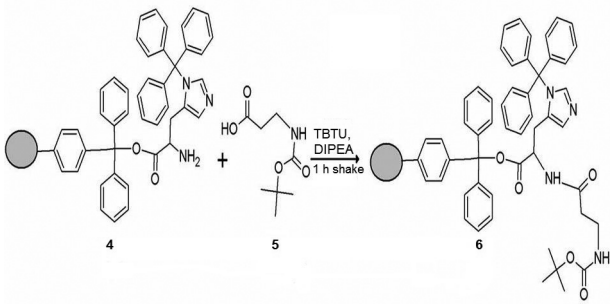
Coupling of second amino acid Boc-β-alanine-OH to the resin (Scheme 3)

After loading the first amino acid on trityl chloride resin (**4**), the second amino acid Boc-β-alanine-OH (0.75 g,



Scheme 2. Deprotection of 9-fluorenylmethyloxycarbonyl (Fmoc) group from the resin.

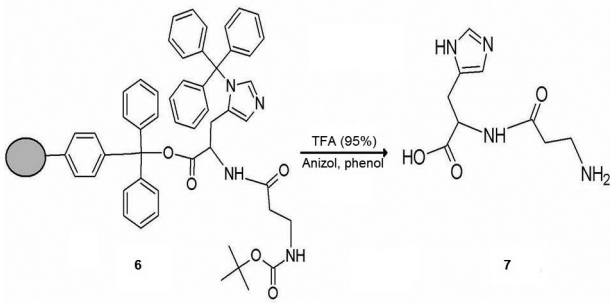
2 equivalents) (**5**) was treated with coupling reagents (TBTU 0.32 g, 2 equivalents in DIPEA 1 mL), DCM (10 mL) to form a solution, which then was added to the resin and shaked for 1 h at room temperature. The reaction was terminated by performing the chloranil test, colorless to yellowish beads positive for coupling the second amino acid (**6**). After coupling, resin was filtered and washed with DMF (5 mL × 3), after that with DCM (5 mL × 3) and then with MeOH (5 mL × 3), respectively, and dried *in vacuo*.



Scheme 3. Coupling of second amino acid Boc-β-alanine-OH to the resin. DIPEA: *N*,*N*-Diisopropylethylamine; TBTU: *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate.

Deprotection of Boc group and cleavage from the resin (Scheme 4)

In the last step of the synthesis, peptide was cleaved from the resin with a mixture of TFA (1 mL), anizol (0.3 mL), phenol (0.3 g) and was shaken mechanically at room temperature for 2 h. One important consideration when selecting the cleavage cocktail are scavengers, such as anizol, phenol, triisopropylsilane (TIPS), 1, 2-ethanedithiol (EDT), thioanisole or water. The reason the scavengers are often added is because during the course of cleavage, highly reactive cationic species can be generated, which can cause damage to the structure. The purpose of a scavenger is to extinguish any reactive species that may be generated during revelation from TFA cleavage.[14] At the end of 2 h, the resin was filtered and washed with DMF (5 mL × 3) and after that with DCM (5 mL × 3). The filtrate was evaporated under reduced pressure and the resulting mixture precipitated by adding diethylether (chemical formula = $C_9H_{14}N_4O_3$, molar mass = 226.23 g mol^{-1}, melting point = 258 °C, yield = 90%; Scheme 4). Boc is acid labile and sensitive to acid cleavage. A common process used to cleave the Boc off the amine while on a solid phase resin is to add a solution of 1:1 TFA in DCM (10 mL) at room temperature for 1 h. The amine will form a TFA salt and must be washed with 3 successive steps of 1:1 DCM and DIPEA (10 mL g^{-1} resin) to prepare the deprotected amine product for the next addition step. If using 2-chlorotritylchloride resin, this will also cleave it, forming the resin as well as the amine. It is recommended to use a base labile resin if one needs to remove the Boc for further alkylation prior to deprotection from a resin.[15] (Yield = 90%, melting point = 258 °C). Elemental analysis: calculated C (47.78%), H (6.24%), N (24.77%), O (21.22%). Molecular formula: $C_9H_{14}N_4O_3$; formula weight: 226.2324.



Scheme 4. Deprotection of *tert*-butyloxycarbonyl (Boc) group and cleavage from the resin. TFA: Trifluoroacetic acid.

Results and Discussion

Characterization of synthesised dipeptide (histidine-β-alanine)

Dipeptide was successfully synthesized via the standard BOC method. The synthesis of dipeptide (histidine-β-alanine) was approved by using UV-Vis, FTIR, ^{1}H NMR and LC-MS analysis. The UV-Vis absorbance spectra of histidine-β-alanine obtained in water at 25 °C is presented in Figure 1. The results show that the maximum peaks were appeared at 214 and 264 nm, which can be assigned to π→π* and n→π*, respectively.

FTIR (KBr) ν / cm^{-1} 3226 (NH_2), 1641 (amide), 1563 (imidazol); ^{1}H NMR (300 MHz, D_2O) δ 2.60 (m, 2 Hs, 12H), 2.92 (dd, 1 Hs, 6H), 3.08 (dd, 1 Hs, 6H), 3.16 (m, 2 Hs, 11H), 4.40 (dd, 1 Hs, 7H), 6.89 (s, 1 Hs, 4H), 7.66 (dd, 1 Hs, 2H), 7.89 (imidazole ring). The LC-MS analysis showed a single mass peak in $[M + H]^+$ and $[M]^-$, which

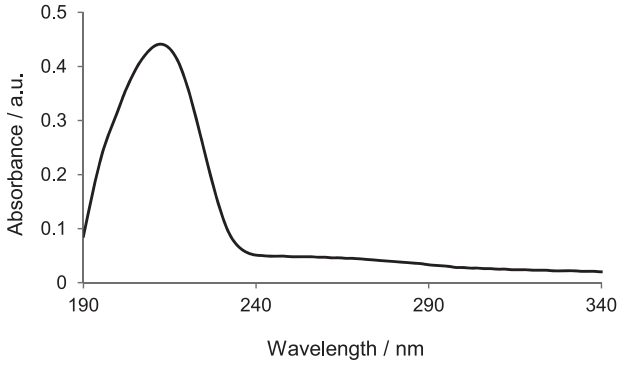


Figure 1. Ultraviolet visible (UV-Vis) absorption spectrum of dipeptide (histidine-β-alanine).

correspond to molecular weight for dipeptide calculated for $C_9H_{14}N_4O_3$: 226.23; found *m/z* $[M + H]^+$: 227.000 and *m/z* $[M]^-$: 224.800 (Figure 2).

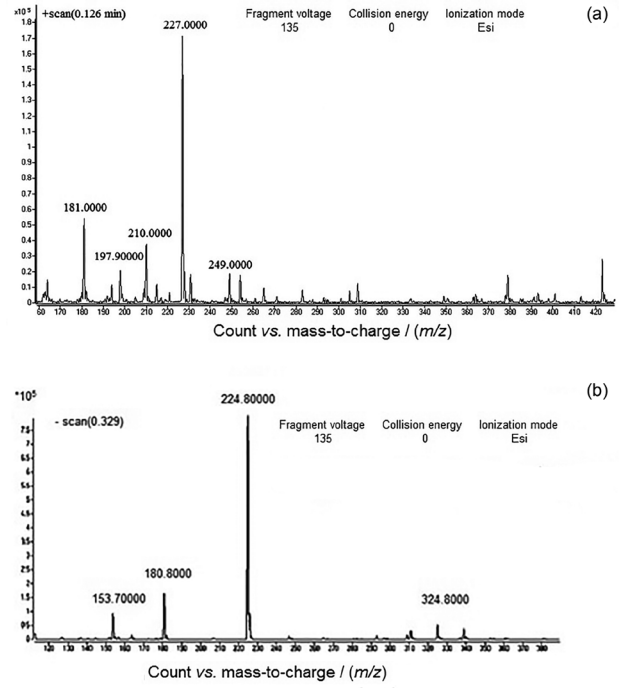


Figure 2. Liquid chromatograph-mass spectrometry (LC-MS) chromatogram of dipeptide (histidine-β-alanine): (a) +scan; and (b) –scan.

Study of chelating property of dipeptide (histidine-β-alanine) *in vitro*

The bioactivities behaviors of histidine-β-alanine depend on its complexation of metal cations. This peptide is a multidentate ligand with five potential metal-coordinating sites (two N of imidazole ring, one carboxylate group, an amide linkage and a terminal amino group). Both tetrahedral and octahedral types of complexes can be formed, but the accurate configuration and chelating ability of dipeptide can depend on size of the metal cation, ligand-to-metal ratios, and the ionic strength of the supporting solution.[11]

To study of kinetic reactions between dipeptide (histidine-β-alanine) and various metal ions, and changes of absorption in wavelength of 214 nm (maximum wavelength of dipeptide) were investigated by UV-Vis spectrophotometer when concentration of dipeptide was kept constant at 10^{-4} mol L^{-1} *vs.* metal ions with different concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} mol L^{-1}). After interaction of dipeptide with various metal ions, the maximum absorption peak of dipeptide reduced differently. For example, at concentration of 10^{-4} mol L^{-1} of metals, Pb^{2+} ion was shown the most efficient absorbing by chelating reaction (Figure 3).

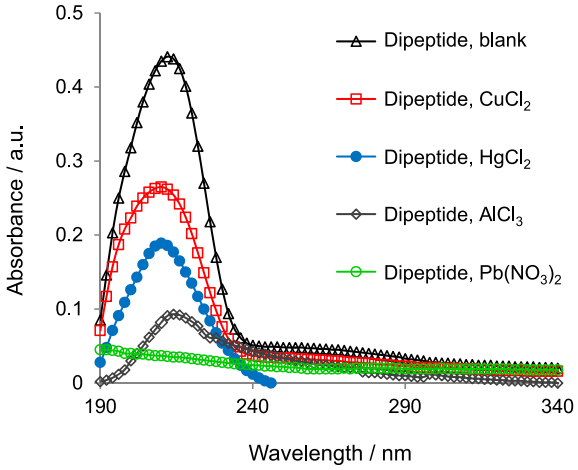


Figure 3. Ultraviolet visible (UV-Vis) absorption spectra of dipeptide (10^{-4} mol L^{-1}) with $AlCl_3$, $CuCl_2$, $HgCl_2$ and $Pb(NO_3)_2$.

The results listed in Table 1 show that in initial times (about 10-15 min), chelating reactions of dipeptide (concentration of 10^{-4} mol L^{-1}) were faster in presence of metals which have same concentration (10^{-4} mol L^{-1}) and lower concentration (10^{-5} mol L^{-1}), but in higher concentration these reactions carried out slower (Figures 4-6). As seen in these figures, the order of the observed strength chelating adsorption in different concentrations is: (*i*) 10^{-5} mol L^{-1}: $Pb^{2+} > Cu^{2+} > Hg^{2+} = Al^{3+}$; (*ii*) 10^{-4} mol L^{-1}: $Pb^{2+} > Al^{3+} > Hg^{2+} > Cu^{2+}$; and (*iii*) 10^{-3} mol L^{-1}: $Al^{3+} > Cu^{2+} > Hg^{2+} > Pb^{2+}$.

At low concentration of metal ions, Pb^{2+} is more adsorbed from the solution. It can occur because Pb^{2+} has a larger radius rather than other cations; therefore, it can be easily coordinated to dipeptide. At high concentration of metal ions (10^{-3} mol L^{-1}), the adsorption of Al^{3+} is the best. Al^{3+} has the smallest radius and more charge than the other cations, which lead to more ability of it for accepting pair electron from N or O atoms of peptide. However, the coordination of dipeptide with Al^{3+} is poor but the higher ability for accepting electron pairs increase its adsorption.

Table 1. Kinetic analysis of different concentration of metals with fixed concentration of dipeptide, at 10^{-4} mol L^{-1} after 30 min

Metal concentration / (mol L^{-1})	Al	Cu	Pb	Hg
1×10^{-1}	6×10^{-6}	-6×10^{-5}	1×10^{-8}	1×10^{-6}
1×10^{-2}	-1×10^{-5}	4×10^{-7}	6×10^{-6}	2×10^{-6}
1×10^{-3}	-5×10^{-5}	-1×10^{-6}	3×10^{-6}	1×10^{-5}
1×10^{-4}	-2×10^{-5}	-2×10^{-6}	-7×10^{-5}	-3×10^{-7}
1×10^{-5}	-2×10^{-5}	6×10^{-6}	-7×10^{-5}	-4×10^{-5}
2×10^{-5}	-4×10^{-5}	4×10^{-7}	-2×10^{-5}	-3×10^{-5}
3×10^{-5}	-6×10^{-5}	-1×10^{-6}	-2×10^{-5}	9×10^{-7}
4×10^{-5}	-4×10^{-4}	-2×10^{-6}	-3×10^{-5}	-5×10^{-6}
5×10^{-5}	-1×10^{-4}	6×10^{-6}	-2×10^{-6}	-6×10^{-6}

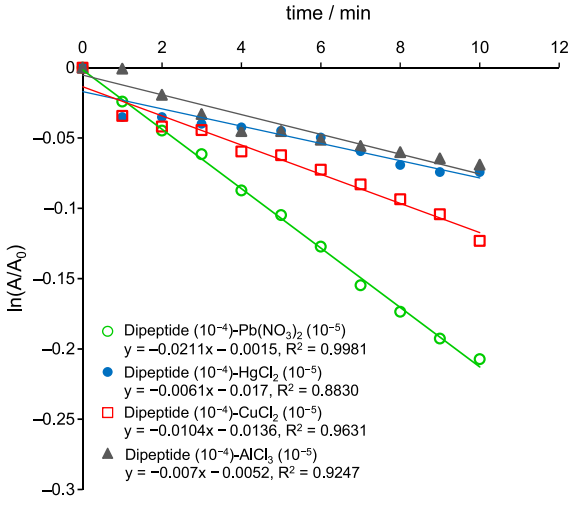


Figure 4. Kinetic study of dipeptide (histidine-β-alanine, 10^{-4} mol L^{-1}) with $AlCl_3$, $CuCl_2$, $HgCl_2$ and $Pb(NO_3)_2$ (10^{-5} mol L^{-1}) in wavelength of 214 nm.

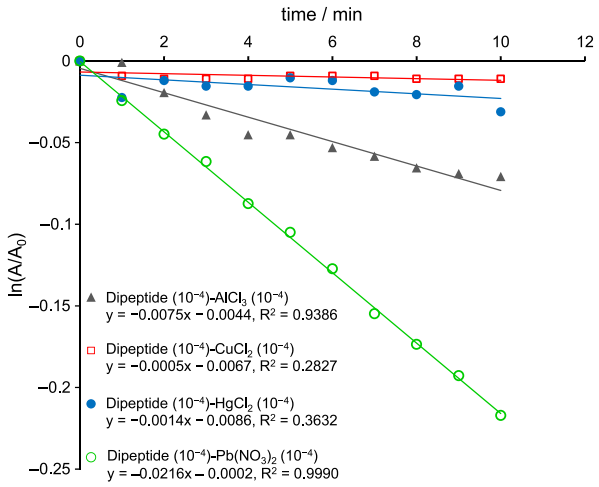


Figure 5. Kinetic study of dipeptide (histidine-β-alanine, 10^{-4} mol L^{-1}) with $AlCl_3$, $CuCl_2$, $HgCl_2$ and $Pb(NO_3)_2$ (10^{-4} mol L^{-1}) in wavelength of 214 nm.

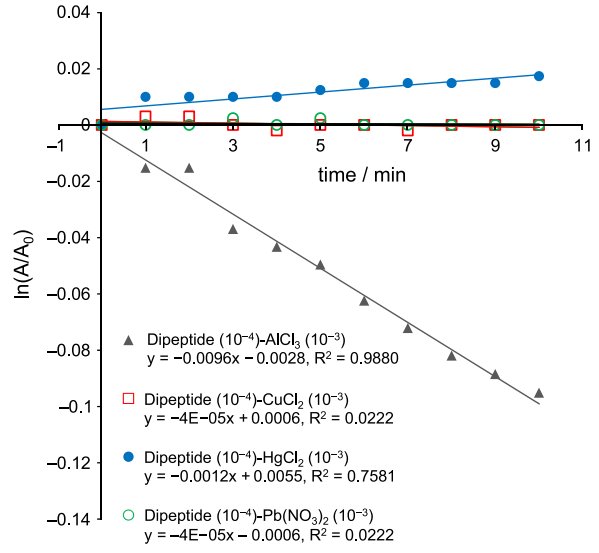


Figure 6. Kinetic study of dipeptide (histidine-β-alanine, (10^{-4} mol L^{-1}) with $AlCl_3$, $CuCl_2$, $HgCl_2$ and $Pb(NO_3)_2$ (10^{-3} mol L^{-1}) in wavelength of 214 nm.

Theoretical

Theoretical methods

The evaluation of computer system for PASS on the set of dipeptide using multilevel neighborhoods of atoms (MNA) descriptor was studied. PASS results for a compound are presented as a list of activity names and probability activity (Pa) values in Table 2.

Theoretical study of dipeptide (histidine-β-alanine)

Lipotropic compounds are those that help catalyse the breakdown of fat during metabolism in the body. It has been predicted that dipeptide (histidine-β-alanine) produces modest short-term weight loss in some people, along with the advantages that are the lowest side effects (1.3 to 6.9%) and good pharmacological activity. As an illustration, this compound can be introduced as a suitable in obesity with 56.7% lipotropic effect. There are many conditions, diseases and disorders, including sleep apnea, teeth grinding, allergies and colds, snoring, frequent urination, fibromyalgia, nightmares, etc. that can cause sleep disturbances, which the treatment depends on the causes, but dipeptide with 54.2% of pharmacological effect can be a treatment for sleep disorders. Aminoacylhistidine dipeptidases (EC 3.4.13.3, also Xaa-His dipeptidase, X-His dipeptidase and PepD) are zinc-containing metallopeptidase, which catalyse the cleaving and release of a *N*-terminal amino acid, usually neutral or hydrophobic residue, from Xaa-His dipeptides or polypeptides.[16-19] Dipeptide has the role of inhibition by 88.7% PASS activity. Histidine ammonia lyase (HAL) catalyses the elimination of ammonia from the substrate to form (*E*)-urocanate, which the dipeptide here is the inhibitor through the molecular mechanisms with 78.2% of biological activity.

Table 2. Portion of the predicted probability activity (P_a) spectra for dipeptide (histidine-β-alanine)

Pharmacological effects	Pa / %	Molecular mechanisms	Pa / %	Side effects and toxicity	Pa / %
Lipotropic	56.7	X-his dipeptidase inhibitor	88.7	ulcerogenic	6.9
Sleep disorders treatment	54.2	histidine ammonia-lyase inhibitor	78.2	embryotoxic	5.1
Bone formation stimulant	45.4	histidine-tRNA ligase inhibitor	77.7	vytotoxic	4.9
Antihypertensive	42.0	phytepsin inhibitor	66.1	eye irritation	1.8
Hematopoietic	40.4	acrosin inhibitor	61.1	mutagenic	1.6
Myelodysplastic syndrome treatment	32.2	lysozyme inhibitor	53.5	skin irritation	1.4
Restenosis treatment	31.4	chelator	18.4	carcinogenic	1.3

Also, dipeptide is the histidine-tRNA ligase, phytepsin, acrosin and lysozyme inhibitor by 77.7, 66.1, 61.1 and 53.5%, respectively. According to Table 2, it is observed that controversial point as a dietary supplement seems to have all the same chelating properties, and it offers a possibility for an inexpensive oral chelation therapy but it has 18.4% PASS activity as a chelator, which shows the chelation chemistry of dipeptide through mixed complexes. In fact, this dipeptide with drug-likeness of 0.916 and new biological properties presented in this study can be utilized to identify numerous medicine applications.

Conclusions

In this work, dipeptide (histidine-β-alanine) as a chelating agent was synthesized via chemical method, SPPS. This method allows performing synthesis much more quickly compared to solution phase chemistry, which requires long purification methods after each intermediate step. The study of chelating property of dipeptide (*in vitro*) by UV-Vis absorbance spectra revealed that chelating activity depends on concentration of metals, molar ratio and kind of metals. In addition, the pharmacological and biological activities of this dipeptide were examined by PASS program. It offers a possibility for an oral chelation therapy and it has 18.4% PASS activity. In fact, dipeptide (histidine-β-alanine) with novel biological properties presented in this study can be utilized to identify numerous medicine applications.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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