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Synthesis and FT-IR spectral elucidation of dipeptide with aromatic amino acid

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Small peptides can provide useful models for studying the interaction forces responsible for the structure and activity of larger proteins. One approach is to study small peptide by "protecting" the ends with groups that eliminate the functionality of the N and C terminus. The synthesis of dipeptide containing aromatic amino acid was carried out by well know procedures - method of mixed anhydrides was applied, where N-protected Phe and C-protected Ala react in the presence of Piv-Cl. The FT-IR- spectral analysis in solid state was used for characterization and elucidation of typical bands of the investigated compound. The obtained results for the IR-bands of amide C=O-NH fragments and other vibrations of the aromatic residues can be provids a structural information about the configuration of amide O=C-NH group in the peptide molecules and corresponding amide planes in the peptide molecules.

Key words: amino acids**,** dipeptide H-Phe-Ala-OH, IR - spectral analysis

INTRODUCTION

Phenylalanine is one of the twenty biologically naturally occurring amino acids that can be found in protein. It contains an amino group, a phenyl ring and a carboxylic group. It is an essential amino acid.

Small, biologically active peptides were first described about 40 years ago. Bioactive peptides are specific protein fragments, that have a positive impact on body functions or conditions and may ultimately influence on the health [1]. Upon oral administration, they may affect the major body systems - namely, the cardiovascular, digestive, immune and nervous systems, depending on their amino acid sequence. Essentially, these molecules play a hormonal role: they act at specific receptor sites at different locations in the organism. Mostly the peptides are transported from the site of release to the site of biological activity through the blood or lymphatic fluid.

Many studies of receptor binding have been carried out to determine the affinity and specificity of the peptides for the target cells. Different kinds of analysis have been undertaken to understand better the mechanisms of recognition, binding and signal triggering when the peptide interacts with the cellular surface. It became clear, that peptides might have tremendous potential for medical and pharmaceutical applications.

The short peptides are potential drug candidates for pharmaceutical and biotech industries. Synthetic short peptides are also extensively developed as agonistic or antagonistic ligands, that function in a similar manner to antibodies, soluble receptors and protein ligands. Characterization of the peptides in solution is often performed in the presence of organic solvents, which can presumably generate the structure bound to the target surface and also enhance the solubility of the peptides [2].

There have been several spectroscopic studies on the behavior of many amino acids and peptides including phenylalanine and on complexes involving amino acids, organic molecules and metal ions [3–8]. Infrared spectroscopy is often used for obtaining both structural and conformational information from biological samples, especially proteins [9-14] and amino acids [15–18].

On the other hand, the possibilities of the IR- and especially IR-LD spectroscopy have been demonstrated in series of papers, dealing with the IR-characteristic band assignment and structural elucidation of small peptides [19-22]. As a part of these systematic study our aim is to be presented the synthesis of the dipeptide *L*-phenylalanyl-*L*-alanine (*H-Phe-Ala-OH*) and examination of the correlation IR-spectroscopic characteristics-structure of the dipeptide depicted in Fig. 1.

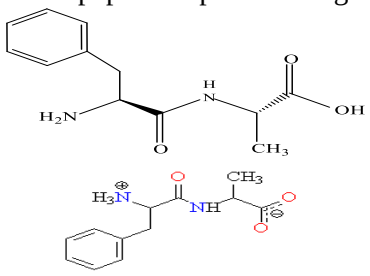

Fig. 1. Chemical diagram of the Phe-Ala-OH and zwitterionic structure.

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EXPERIMENTAL

Synthesis

Boc-Phe-OH (1): Phenylalanine (1.7 g, 10 mM) was added to the solution of $NaHCO_3$ (0.9 g, 10.5 mM) in 25 ml water and was cooled to 0°C. A solution of Boc_2O (2.3 g, 10.5 mM) in 25 ml *i*-PrOH was added to this mixture. The reaction mixture was stirred for 24 hours at room temperature. The process was monitored by TLC CT_1 ($CHCl_3$: MeOH : H_2O, 80 : 30: 5). At the end of the process *i*-PrOH was evaporated, aqueous solution was acidified with dry $NaHSO_4$ to pH 3 and three extractions with EtOAc were made (3 x 30 ml). Combined organic layers were washed with saturated solution of NaCl, dried over Na_2SO_4, filtered and EtOAc was evaporated. Pure Boc-Phe-OH crystalized on air and 2.5 g (98 %) was obtained.

HCl.Ala-OMe (2): 10 ml Methanol were cool to -10°C and SO_2Cl (2.9 ml, 40 mM) was added dropwise. After 5 min alanine (1.8 g, 20 mM) was added. The reaction was completed after 2 hours, methanol was evaporated and white crystal product (2.3 g (76 %) was obtained.

Boc-Phe-Ala-OMe (3): Boc-Phe-OH (1.5 g, 6 mM) and NMM (0.66 ml, 6 mM) were dissolved in THF (10 ml) and were cooled to -10°C. Piv-Cl (0.74 ml, 6 mM) was added carefully at this temperature. After 10 min a solution of HCl.Ala-OMe (0.9 g, 6 mM) in THF (10 ml) with Et_3N (0.9 ml, 6 mM) was added. The reaction completed after 24 hours. THF was evaporated under reduced pressure, residue was dissolved in EtOAc (30 ml) and was washed consequently with $NaHCO_3$ (3 x 30 ml), $NaHSO_4$ (3 x 30 ml), and water to pH 7. Organic layer was dried over Na_2SO_4 and EtOAc was evaporated. 1.1 g (53 %) was obtained.

Boc-Phe-Ala-OH (4): Fully protected dipeptide 3 (1.1 g, 3.1 mM) was dissolved in mixture of dioxane : water 1:1 (20 ml). A drop of methanol solution of thymolphthaleine was added and after that 1N NaOH was added dropwise until color of the solution (blue) remained constant. Dioxane was evaporated and aqueous solution was acidified with dry $NaHSO_4$ to pH 3. The dipeptide was obtained from aqueous solution with EtOAc extractions (3 x 20 ml). Combined organic layers were washed with water to pH 7, dried over Na_2SO_4 and EtOAc was evaporated. 0.8 g (73%) product was obtained.

Phe-Ala (5): Boc-Phe-Ala-OH (0.8 g, 2.5 mM) was dissolved in CH_2Cl_2 (2 ml) and TFA (1 ml) was added. The reaction completed in 1 hour at room temperature. The solvent was evaporated and the crude product was purified by column purification.

Methods

The IR-spectra of the compound were recorded using a Thermo Scientific Nicolet iS10 FT-IR spectrometer (4000 – 400 cm^{-1}) with ATR accessory. A spectral resolution of ± 4 cm^{-1} was used and 64 scans were accumulated. The solid state IR spectra were recorded using ATR accessory and technique.

RESULTS AND DISCUSSION

Peptides and proteins play an important role in modern biology. A key step in peptide production is the formation of the peptide bond, which involves amide bond formation [23]. The process usually requires activation of a carboxylic acid moiety in the presence of coupling reagents. Activation consists of the replacement of the hydroxyl group of the carboxylic acid with a leaving group as the acid would otherwise simply form salts with the amine. The reaction of the activated intermediate and the amine is known as the coupling reaction and the activators are coupling reagents [24].

The dipeptide, Phe-Ala was synthesized using synthetic scheme shown in Fig. 2. Method of mixed anhydrides was applied, where N-protected Pro and C-protected Ala reacted in the presence of Piv-Cl. In order to obtain free dipeptide, protecting groups (Me ester and Boc-group) were removed subsequently. Alkaline hydrolysis was used for ester removal and acid hydrolysis – for Boc-group deprotection. Crude product was purified by gel filtration.

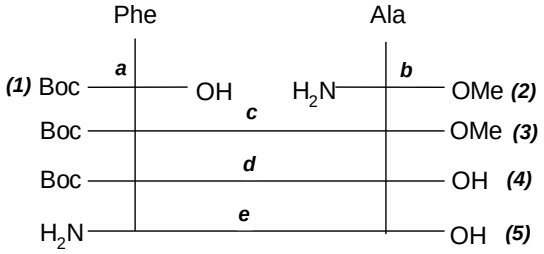

Fig. 2. Synthesis of L-phenylalanyl-L-alanine: a) Boc_2O, $NaHCO_3$, *i*-PrOH, H_2O; b) SO_2Cl, MeOH; c) Piv-Cl, NMM, Et_3N, THF; d) 1N NaOH, dioxin:H_2O (1:1); TFA, CH_2Cl_2.

IR-spectral analysis

Like in our previous investigations on peptide systems [25, 26], the vibrational analysis support well the experimental IR-characteristic bands only assigned to molecular motions of

functional groups which does not participate in intermolecular interactions in solid-state.

The obtained results about the investigated compound are assigned on the basis of known IR-data about similar systems. The characteristic IR-bands of the dipeptide *H-Phe-Ala-OH* are listed and assigned in Table (1). The comparison and assignment of the solid-state IR-spectra of the studied compound is done, using the statement that pure dipeptide stabilize H_3N^+, R-COO^- (*Phe-Ala-OH*) as a zwitterion with characteristic IR-spectral bands of -NH_3^+ and -COO^-groups.

The IR-characteristic bands assignment shown in Table 1 is done by preliminary deconvolution and curve-fitting on the IR-spectroscopic patterns. The spectral analysis shows the presence of bands assigned of the protonated amino group -NH_3^+ in the 3300 – 2400 cm^{-1} region. The broad maximum for studied compound in this region corresponds to symmetric and asymmetric stretching vibrations of protonated NH_3^+-group.

The IR-spectrum of *H-Phe-Ala-OH* is characterized with an intensive band of ν_{NH} at 3250 cm^{-1}. The obtained low frequency shifting is a result of the participation of NH amide group in intermolecular interactions. The discussed band is low-frequency shifted too in corresponding hydrochloride of *H-Phe-Ala-OH* with ~70 cm^{-1} supposing the participation of the NH group in strong hydrogen bonding (Tabl.1).

The $\delta^{as}{}_{NH3}{}^+$, $\delta'^{as}{}_{NH3}{}^+$, and $\delta^{s}{}_{NH3}$, bending maxima are at about 1690 - 1620 cm^{-1} region. The observed two bands at 1692, 1684 cm^{-1} characterized asymmetric banding vibrations ($\delta^{as}{}_{NH3}{}^+$). The band at 1546 cm^{-1} is assigned to the symmetric banding vibrations ($\delta^{s}{}_{NH3}{}^+$). The *amide I* bands of the peptide is observed about 1679 cm^{-1}, while the *amide II* (1525 cm^{-1}) is strongly depends of the type of intermolecular interactions. The typical COO- maxima are in the 1600 – 1400 cm^{-1} spectral range. The character of the band at 1610 cm^{-1} and 1403 cm^{-1} are assigned and belonging to the asymmetric and symmetric stretching modes of COO-fragment ($\nu^{as}{}_{COO}{}^-$ and $\nu^{s}{}_{COO}{}^-$) in the molecule.

The protonation of the zwitterionic dipeptide leads to disappearance of the characteristic IR-bands of COO^-, *i.e.* $\nu^{as}{}_{COO}{}^-$, $\nu^{s}{}_{COO}{}^-$ and an observation of the bands of COOH group. The protonation leads to disappearance of the typical -COO^- maxima in 1600-1400 cm^{-1} spectral range. The character of the 1610 cm^{-1} and 1403 cm^{-1} bands as $\nu^{as}{}_{COO}{}^-$ and $\nu^{s}{}_{COO}{}^-$ in *H-Phe-Ala-OH* is confirmed in addition by the obtained IR-spectrum of protonated form as hydrochloride salt. It is characterized with the disappearance of the maxima above and a new peak at 1735 cm^{-1} is appeared, which correspond to $\nu_{C=O}$ stretching vibration of the restored COOH group in the salt.

Table 1. IR-characteristic bands of *Phe-Ala-OH and Phe-Ala-OH.HCl*

Assignment ν (cm^1)	H-Phe-Ala-OH.	H-Phe-Ala-OH.HCl
$\nu^{as}{}_{NH3}{}^+$, $\nu'^{as}{}_{NH3}{}^+$, $\nu^{s}{}_{NH3}{}^+$	3200 – 2700	3200 – 2700
ν_{OH}	3365	3400
ν_{NH}	3275	3200
$\delta^{as}{}_{NH3}{}^+$, $\delta'^{as}{}_{NH3}{}^+$	1692, 1684	1654, 1650
$\delta^{s}{}_{NH3}{}^+$	1546	1511
$\nu_{C=O}$ (COOH group)	-	1735
$\nu_{C=O}$ (Amide I)	1679	1670
δ_{NH} (Amide II)	1525	1571
$\nu^{as}{}_{COO}{}^-$	1610	-
$\nu^{s}{}_{COO}{}^-$	1403	-

CONCLUSSION

The dipeptide *H-Phe-Ala-OH* was synthesized by well know method of mixed anhydrides in the presence of Piv-Cl. The spectral investigation, inclucuds IR-characteristic bands determination of the studied dipeptide *H-Phe-Ala-OH* were carried out.

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СИНТЕЗ И ИЧ-ФТ СПЕКТРАЛНО ИЗЯСНЯВАНЕ НА ДИПЕПТИДИ С АРОМАТНА АМИНОКИСЕЛИНА

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(Резюме)

Малките пептиди могат да представляват полезни модели за изучаване силите на взаимодействие, отговорни за структурата и активността на по-големи протеини. Синтезът на дипептид, съдържащ ароматна аминокиселина е извършен по добре известна методика - приложен е метода на смесените анхидриди, където аминокиселините фенилаланин е N-защитена, а аланин С-защитена реагират в присъствие на пивалоил хлорид. Спектралният ИЧ-ФТ анализ в твърдо състояние може да се използва за допълнително охарактеризиране и изясняване на типични ивици от изследваното съединение. Получените резултати за ивиците на амид фрагментите (C=O, NH), както и на други ивици от ароматния остатък могат да дадат полезна структурна информация относно конфигурацията на амидната група и съответните амидни равнини в пептидните молекули.