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Spectroscopic, theoretical and structural characterization of hydrogensquarates of L-threonyl-L-serine and L-serine

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Summary. Hydrogensquarates of dipeptide L-threonyl-L-serine (*H-Thr-Ser-OH*) and L-serine (*HSq* × *Ser*) have been synthesized, isolated and spectroscopic characterized by solid-state linear-polarized IR-spectroscopy, ^{1}H- and ^{13}C-NMR, ESI-MS and HPLC with tandem masspectrometry (MS-MS) methods. The structures of the salts and neutral dipeptide have been predicted theoretically by ab initio calculations. In the case of *H-Thr-Ser-OH* the theoretical data are supported by IR-LD ones. The hydrogensquarates consist in positive charged dipeptide or amino acid moiety and negative hydrogensquarate anion (*HSq*) stabilizing by strong intermolecular hydrogen bonds. The data about the L-serine hydrogensquarate are compared with known crystallographic data thus indicating a good correlation between the theoretical predicted structures and experimentally obtained by single crystal X-ray diffraction.

Keywords: H-Thr-Ser-OH – Serine – Hydrogensquarates – Solid-state IR-LD – ab initio calculations – ^{1}H- and ^{13}C-NMR – ESI-MS – HPLC-MS-MS

Introduction

The protonated forms of amino acids and peptides are of interest for a range of preparative and pharmaceutical applications. Furthermore, the squaric acid and its optically active amino acid amides crystallized noncentrosymmetrically and are of great interest for non-linear optical and electro optical application (Chemla and Zyss, 1987; Nalwa et al., 1997). In last years the bioactivity of squaric acid and some derivatives as inhibitors and VLA-4 antagonists (Xie et al., 2004) or potassium channel openers (Gilbert et al., 2000) or some derivatives of squaric acid based peptides inhibits matrix metalloprotease-1 (Onaran et al., 2005) have been also reported.

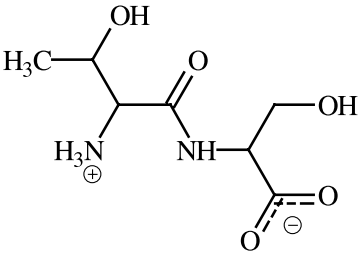
Therefore, in this paper we reported the results of synthesis, isolation and spectroscopic characterization of hydrogensquarates of dipeptide *H-Thr-Ser-OH* ($H_2Sq \times$ *H-Thr-Ser-OH*) and pure amino acid L-serine ($H_2Sq \times Ser$) shown in Scheme 1.

Experimental

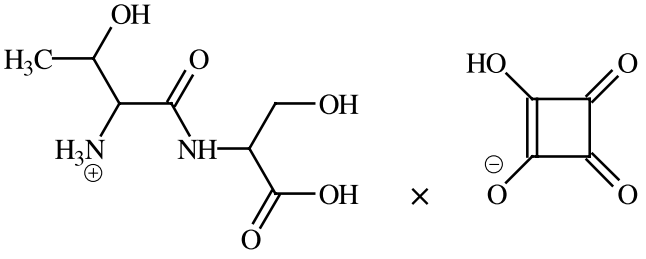
Materials and methods

The dipeptide H-Thr-Ser-OH (≥97.00%) and L-Serine were purchased from Bachem Organics (Switzerland) and were used without further purification.

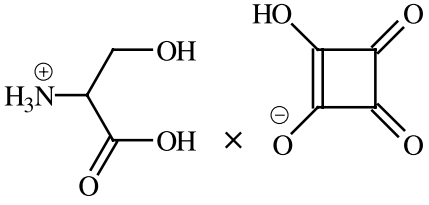
10. The 4000–400 cm^{-1} solid-state IR-spectra were recorded on a Bomem Michelson 100 FT-IR Spectrometer (resolution 2 cm^{-1}, 150 scans) equipped with a Perkin Elmer wire-grid polarizer. The oriented solid samples were obtained as a suspension in a nematic liquid crystal of the 4′-cyano-4′-alkylbicyclohexyl type (ZLI 1695, Merck), mesomorphic at room temperature. Its poor IR-spectrum allows the recording of the guest-compound bands in the whole 4000–400 cm^{-1} ranges. The presence of an isolated nitrile stretching IR-band at 2236 cm^{-1} serves additionally as an orientation indicator. The effective orientation of the samples was achieved through the following procedure: 5 mg of the compound studied was mixed with the liquid crystal substance until a slightly viscous suspension was obtained. The phase thus prepared was pressed between two KBr-plates for which in advance one direction had been rubbed out by means of fine sandpaper. The grinding of the mull in the rubbing direction promotes an additional orientation of the sample (Ivanova et al., 2006a–c; Kolev, 2006). IR-LD spectroscopy and the interpretation of the linear-polarized IR-spectra are described in (Michl and Thulstrup, 1986). The method consists of subtraction of the perpendicular spectrum, (IR_s, resulting from a 90° angle between the polarized light beam electric vector and the orientation of the sample) from the parallel one (IR_p) obtained with a co-linear mutual orientation. The recorded *difference* ($IR_p - IR_s$) spectrum divides the corresponding parallel (A_p) and perpendicular (A_s) integrated absorbencies of each band into positive values originating from transition moments which form average angles with the orientation direction (**n**) between 0° and 54.7° (magic angle), and negative ones corresponding to transition moments between 54.7° and 90°. In the *reducing-difference*



H-Thr-Ser-OH



$H_2Sq \times H$-Thr-Ser-OH



$H_2Sq \times$ Ser

Scheme 1. Chemical diagram of studied compounds

procedure, the perpendicular spectrum multiplied by the parameter $\boldsymbol{c}$, is subtracted from the parallel one and $\boldsymbol{c}$ allowed to vary until at least a band or sets of bands are eliminated. The simultaneous disappearance of these bands in the obtained *reduced* IR-LD spectrum ($IR_p - \boldsymbol{c}IR_s$) indicates a co-linearity of the corresponding transition moments, thus giving rise to information regarding the mutual disposition of the molecular fragments. This elimination method is graphically carried out using a subtracting procedure attached to the program for processing of IR spectra.

HPLC MS-MS

Measurements are made using TSQ Quantum AM ULTRA (Thermo Electron Corporation) instrument under the conditions presented in Table 1. Two flows were used: (i) A – H_2O and 0.1% HCOOH and (ii) B – CH_3CN and 0.1% HCOOH.

ESI mass spectrometry

A triple quadruple mass spectrometer (TSQ 7000 Thermo Electron, Dreieich, Germany) equipped with an ESI 2 source was used and operated with the following conditions: capillary temperature 190 °C; sheath gas 45 psi and spray voltage 4.0 kV. The sample was injected in the ion source by an autosampler (Surveyor) with a flow rate of 0.2 ml/min a pure acetonitrile. 1 mg/ml of the sample was dissolved in acetonitrile. The data obtained was processed using an Excalibur 1.4 software.

Table 1. HPLC/MS-MS conditions

N	*t* [min]	A [%]	B [%]	Rate [$\mu l \cdot min^{-1}$]
0	0.00	100	0	200
1	3.00	100	0	200
2	8.00	65	35	200
3	9.00	0	100	200
4	14.00	0	100	200
5	14.50	100	0	200
6	20.00	100	0	200

^1H- and ^{13}C-NMR measurements

Measurements, referenced to sodium 3-(trimethylsylyl)-tetradeuteriopropionate, were made at 298 K with a Bruker DRX-400 spectrometer using 5 mm tubes and D_2O as solvent.

11. Optimisation of the structures of protonated *H-Thr-Ser-OH* and Ser were carried out by ab initio calculations (RHF and UHF) at 6–31++G** basis set, using Dalton 2.0 program package.

Synthesis

The hydrogensquarate of L-Serine, [$C_7H_9NO_7$], was obtained according the procedure described (Kolev et al., 1998). Found: C, 35.77; H, 3.87; N, 5.94; [$C_7H_9NO_7$] calcd.: C, 35.75; H, 3.86; N, 5.96%. The ESI-MS spectrum of $H_2Sq \times$ Ser shows a peak at m/z 124.1, corresponding to NH_4^+ adduct with positive charged $[C_3H_8O_3N]^+$ (molecule weight of 106.2).

The hydrogensquarate of *H-Thr-Ser-OH* (*HSq* × *H-Thr-Ser-OH*), [$C_{11}H_{15}N_2O_9$], was obtained according the procedure: an aqueous solution of dipeptide *H-Thr-Ser-O*H (5 ml, 0.2060 g) was mixed with 5 ml 0.1140 g squaric acid in same solvent at equimolar ratio 1:1. The white precipitate is formed after 5 days and were filtered, washed with H_2O and dried at 298 K in air. (Found: C, 41.37; H, 4.77; N, 8.78; [$C_{11}H_{15}N_2O_9$] calcd.: C, 41.39; H, 4.74; N, 8.77%. Yield 66%). The HPLC MS/MS data of dipeptide salt shown a peak in curve relative abundance *vs.* *t* (Fig. 1A) at $t = 1.86$ min corresponding to m/z 435 (Fig. 1B). Last peak should be corresponds to *H-Thr-Ser-OH*. . .2HSq dimmer with molecule weight of 435.5.

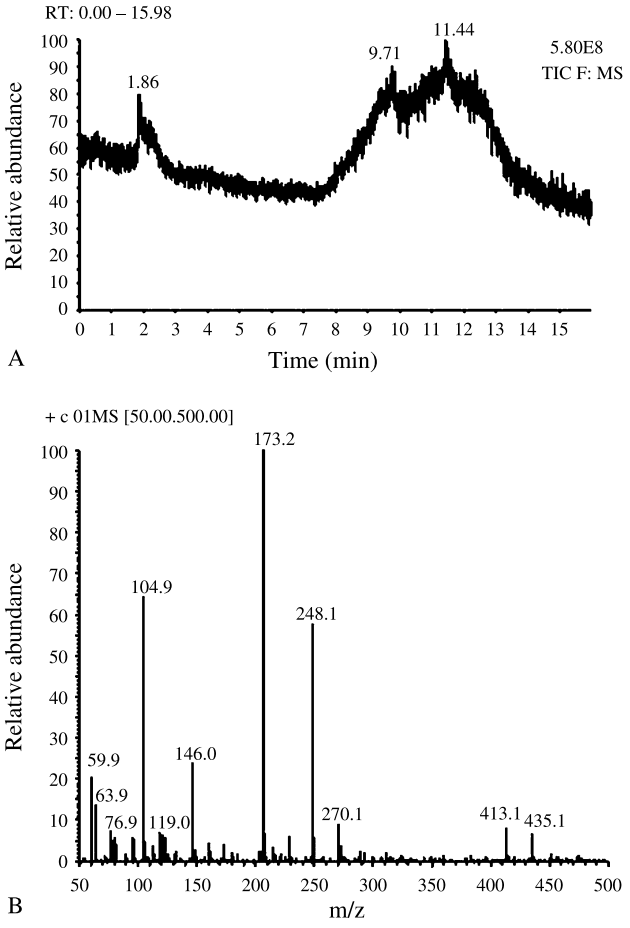
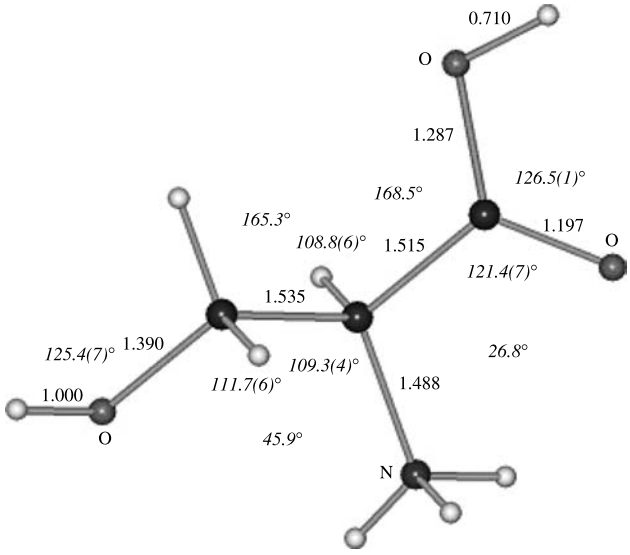


Fig. 1. HPLC MS/MS data of *H_2Sq × H-Thr-Ser-OH*. **A** HPLC, **B** MS-MS



Scheme 2. Optimized structure of protonated amino acid

Results and discussion

Theoretical calculations

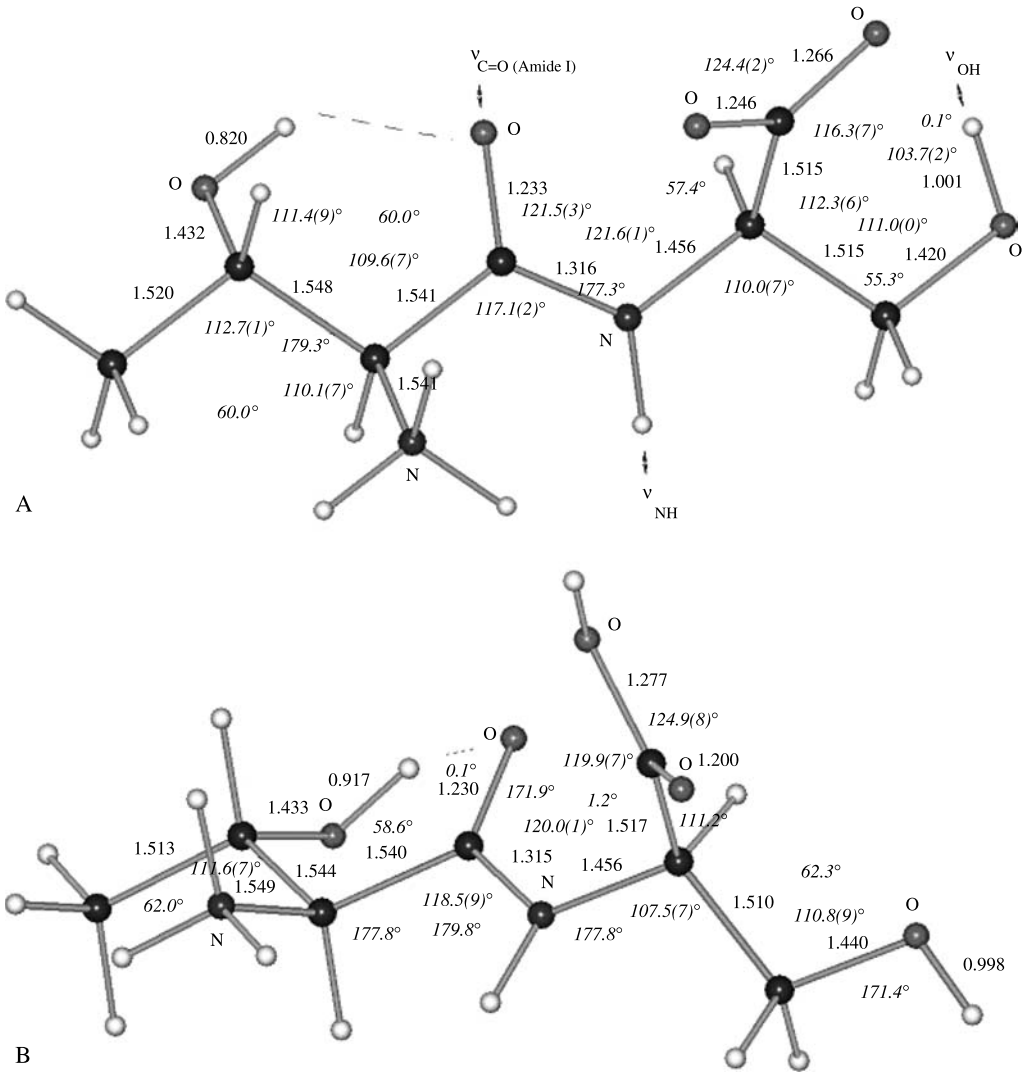
The validity of the used theoretical approach for structural prediction of both neutral and protonated forms of dipeptide *H-Thr-Ser-OH* is made using a correlation between the experimental single crystal X-ray data of protonated form obtained of serine in hydrpohensquarate (Kolev et al., 1998). The calculated geometry parameters are listed in Scheme 2 and their comparison with (Kolev et al., 1998) indicated a differences less then 0.034 Å and 3.5°.

The conformational analysis was generated by energy minimisation through an optimization of torsion angles. The geometry parameters of both *H-Thr-Ser-OH* (Scheme 3A) and its protonated form (Scheme 3B) are shown, where the listed data correspond to the most stable conformers with E_{rel} equal to 0.6 and 0.4 kJ/mol, respectively.

12. These results assumed a near *trans*-configuration of amide fragments in neutral form of *H-Thr-Ser-OH*, leading to a near co-linear orientation of the stretching C=O ($\nu_{C=O}$, amide I) and NH (ν_{NH}) vibrations (torsion angle of 177.3(6)°). The transition moment of stretching vibration of OH group in *seryl*-side chain is also co-linear disposed of amide I mode in zwiterion peptide in contrast to protonated form, where the *seryl*-chain ν_{OH} is near to perpendicular oriented. Independently, that the protonation declines the geometry of the neutral form discussed, both the compounds are characterized with a disposition of *Thr*-side chain OH-group towards the C=O amide group leading to a presumption for stabilization of intramolecular hydrogen C=O. . .HO bond, which could be explained the low frequency shift of amide I peak at 1640 cm^{-1} (see below). The protonation of dipeptide leads of a *transoide* configuration of amide fragment with a torsion angle of 171.8(5)°. The optimised structural parameters in both forms of bond lengths and angles for both systems studied (Scheme 2) and the values obtained are in good agreement with X-ray diffraction data for other peptides with *Thr*- and *Ser*-side chains. In alanylthreonine and glycylthreonine the –N(H)–C(=O)– angles are 173.9° and 174.3° and in seryl-leucine – 177.8° (Yadava et al., 1973; Netland et al., 2004; Slowikowska and Lipkowski, 2001; Moen et al., 2004). The protonation of the COO^--group leads to the expected inequality in the C–O bond lengths with 1.200 Å (C=O) and 1.277 Å (C–OH), respectively.

Conventional and linear-polarized IR-spectra of H-Thr-Ser-OH and H_2Sq × H-Thr-Ser-OH

IR-characteristic bands assignments of dipeptide *H-Thr-Ser-OH* are (Fig. 2.1): (i) the broad maximum for all



Scheme 3. Optimized structure of neutral (**A**) and protonated (**B**) peptide molecule

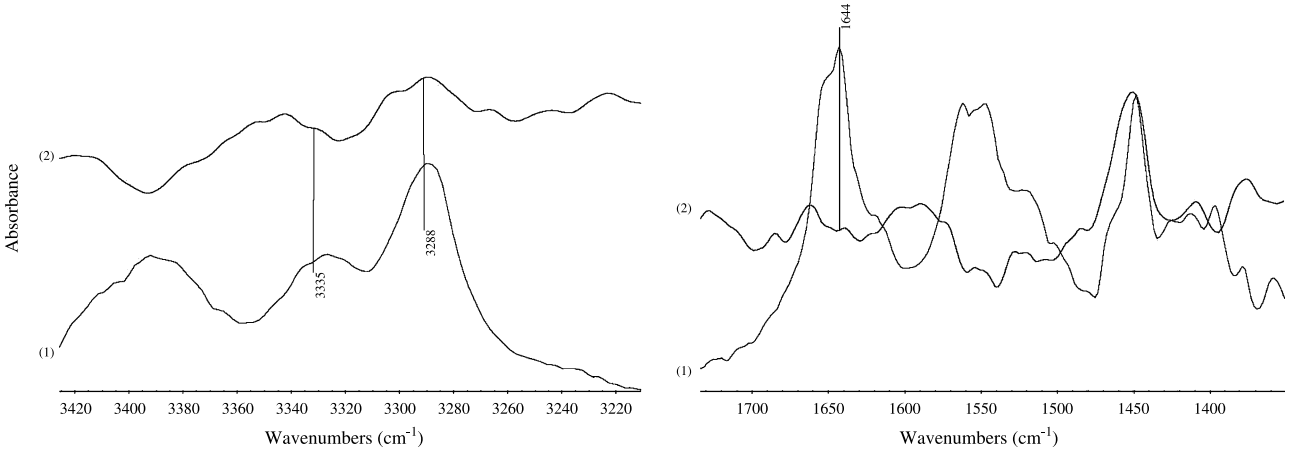


Fig. 2. Conventional IR-(1) and reduced IR-LD (2) spectrum of *H-Thr-Ser-OH* after elimination of the peak at 1644 cm^{-1}

compounds at 3200–2600 cm^{-1} corresponds to symmetric and asymmetric stretching vibrations of $-NH_3^+$-group. The corresponding band for *Ser* is in 3000–2600 cm^{-1} region. The $\delta^{as}_{NH_3^+}$, $\delta'^{as}_{NH_3^+}$ and $\delta^{s}_{NH_3^+}$ bending maxima in *H-Thr-Ser-OH* are at about 1614 and 1515 cm^{-1} and a high frequency shift with 23 cm^{-1} for *Ser* is obtained, (ii) the highest frequencies at 3390, 3335 and 3288 cm^{-1} belong to ν_{OH} of both amino acids side chains and of ν_{NH} stretching mode in dipeptide. *Ser* ν_{OH} peak in pure amino acid is observed at 3357 cm^{-1}; (iii) the maximum at 1644 cm^{-1} can be assigned to amide I ($\nu_{C=O}$) and the 1558 cm^{-1} – to amide II (δ_{NH}); (iv) to asymmetric and symmetric stretching modes of COO^- group (ν^{as}_{COO-} and ν^{s}_{COO-}) are attributed the maxima at 1560 and 1400 cm^{-1}, which disappeared in the protonated form of dipeptide. In the IR-spectrum of L-serine the discussed peaks are at 1622 and 1416 cm^{-1}, respectively.

The application of the reducing-difference procedure of the polarized IR-LD spectra of *H-Thr-Ser-OH* leads to following results: (i) Elimination of amide I and ν_{NH} maxima at 1644 and 3288 cm^{-1} (Fig. 2.2) assumes a co-linearity of their transition moments, which is realized in the predicted theoretical structure of the dipeptide (Scheme 2A). The procedure only leads to a strong reduction of ν_{OH} peak at 3335 cm^{-1}. Taking into account the calculated geometry of *H-Thr-Ser-OH*, the ν_{OH} transition moment of *Ser*-side chain is collinear to amide I, supposing an attribution of last peak to ν_{OH} of this amino acid.

The protonation in *H_2Sq × H-Thr-Ser-OH and H_2Sq × Ser* leads to disappearance of ν^{as}_{COO-} and ν^{s}_{COO-} bands, typical for a zwiterion dipeptide and amino acid and the observation of a new ones at 1700 and 1745 cm^{-1} belonging to $\nu_{C=O}$ stretching vibrations (Figs. 3.1 and 3.2). The amorphous character *of H_2Sq × H-Thr-Ser-OH* resulted to broad maxima in the IR-spectroscopic pattern, requiring a deconvolution and curve-fitting procedure for obtaining of number and position of IR-absorption peaks. The low frequency shift of amide I peak with 10 cm^{-1} in protonated form of *H-Thr-Ser-OH* compared to the zwiterion could be explained by the C=O...X intermolecular interaction. The other maxima in *H_2Sq × H-Thr-Ser-OH* are at 2933 cm^{-1} (ν_{NH}), 1637 cm^{-1} (amide I), 1575 cm^{-1} ($\delta^{as}_{NH_3^+}$) and 1566 cm^{-1} (amide II). The IR-spectra of both hydrogensquarates studied show series of IR-bands, characteristic for HSq^- ion about 1820, 1600 and 1500 cm^{-1} assigned to combination of ν_{C-O}, ν_{C-C} and $\nu_{C=C}$ modes. The peak positions are typical for the other obtained hydrogensquarates of peptides and amino-acid amides (Kolev et al., 1998). The reducing-difference procedure applied to polarized IR-LD spectra of both hydrogensquarates leads to observation of the elimination of last three maxima in different dichroic ratios, confirming their assignment as combination of stretching modes. On the other hand in the case of *HSq × Ser*, the crystallographic data indicated the presence of two hydrogensquarate anions with planes, mutual oriented at an angle of 86.3° (Scheme 4), which also leads to elimination

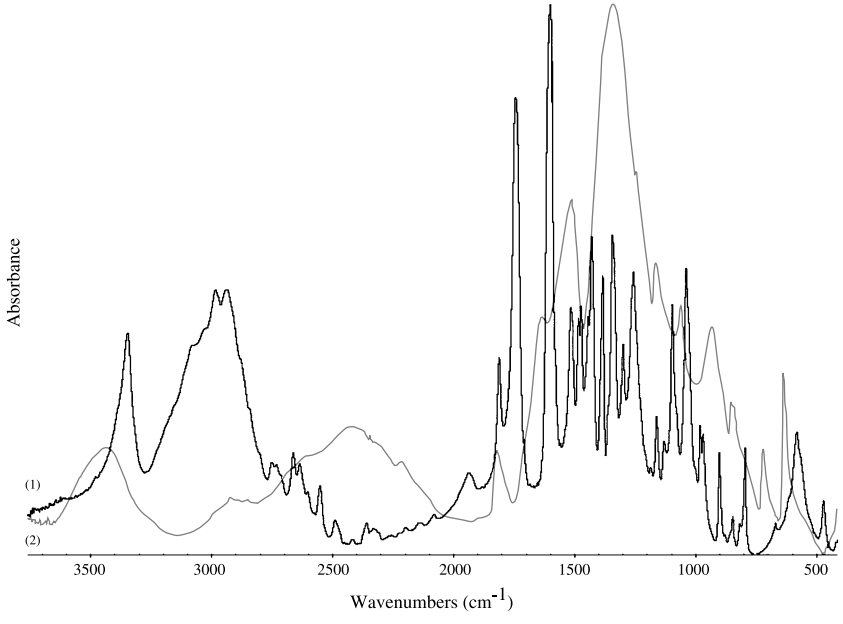
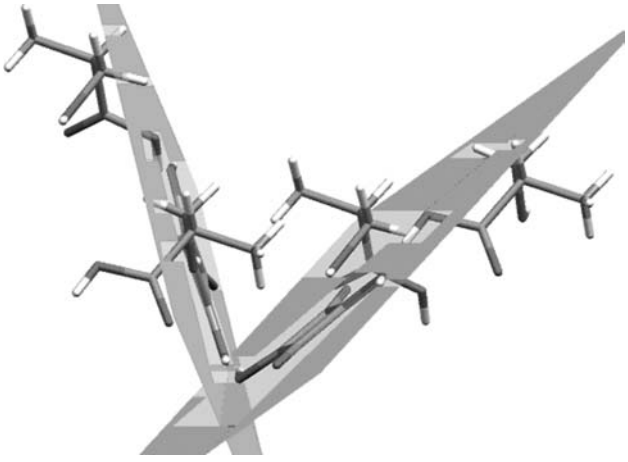


Fig. 3. Solid-state IR-spectra of *H_2Sq × Ser* and *H_2Sq × H-Thr-Ser-OH*



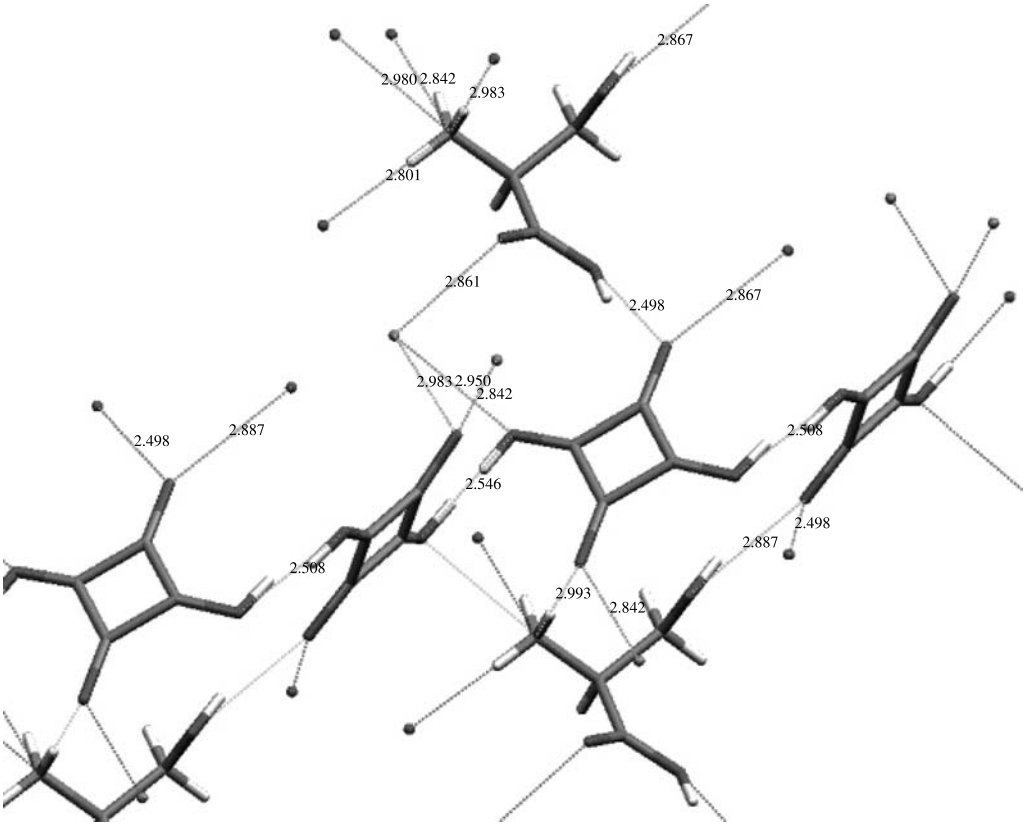
Scheme 4. Unit cell

of the corresponding stretching *HSq* maxima in different dichroic ratio.

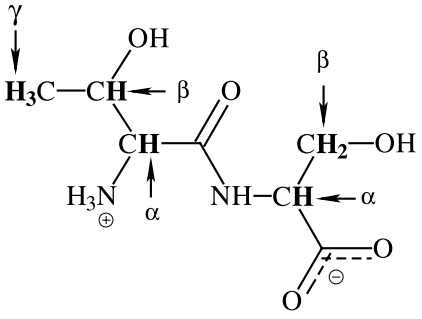
The broad maximum in whole 3200–2000 cm^{-1} IR-spectral region of *HSq* × *Ser* indicates a strong intermolecular H-bonds (Scheme 5), which are established crystallographically (Kolev et al., 1998) and forming between (*HSq*$^-$)O$^-$. . .H–O(*HSq*$^-$) at 1,3-position of HSq$^-$ moieties with bond length of 2.546 A. In addition the following other intermolecular H-bonds with corresponding lengths are formed (Scheme 5) (Kolev et al., 1998) (*HSq*) C=O. . .HO(C=O)(*Ser*) (2.498 Å), (*Ser*)OH. . .O=C(*HSq*-) (2.867 Å), (*Ser*)NH. . .O=C(*HSq*-) (2.950, 2.983, 2.842 Å) and (*Ser*)NH. . .O=C(OH)(*Ser*) (2.861 Å), respectively.

^{1}H- and ^{13}C-NMR data

The nuclear magnetic resonance spectra of hydrogensquarates of serine and *H-Thr-Ser-OH* are compared with the pure amino acids (Nord et al., 2004) and zwiterion form of dipeptide. The chemical shifts assignment is carried out using the numbering illustrated in Scheme 4. Both in simple amino acid and in dipeptide the ^{1}H NMR spectra are low informative as far as the protonation of –COO$^-$-groups during the formation of corresponding hydrogensquarates leads to a downfield shifting of α-C**H** protons in *seryl*- and *theonyl*-side chains less than 0.5–0.7 ppm. The corresponding data are 4.05 ppm (*Ser*), 4.29 ppm (*seryl*-side chain) and 3.88 ppm (*threonyl*-side chain). The β-**CH$_2$** signals in L-serine (pure amino acid) and *seryl*-side chain (in the dipeptide) as well as the β-C**H** and γ-C**H$_3$**



Scheme 5. Hydrogen bonding



Scheme 6. Proton

signals of *threonyl*-fragment are affected in low degree and are observed at 5.50 ppm (in *Ser*), 5.47 ppm (in dipeptide *seryl*-side chains), 4.30 and 1.40 ppm (in dipeptide *threonyl*-side chains), respectively (Scheme 6).

In the ^{13}C-NMR data both hydrogensquarates are characterized with four new signals at 186.8, 180.3, 174.9, 169.2 ppm (*$H_2Sq \times Ser$*) and at 189.0, 182.7, 177.1, 172.2 ppm (*$H_2Sq \times H$-Thr-Ser-OH*), belonging to HSq ion (Onaran et al., 2005).

Conclusions

By means of solid-state IR-LD spectroscopy, ^{1}H- and ^{13}C-NMR, ESI-MS and HPLC-MS-MS methods and by ab initio calculations at RHF and UHF level of theory and 6–31++ G** basis set the hydrogensquarates of L-serine and *H-Thr-Ser-OH* are characterized spectroscopic and structurally. On basis of these data, the following essential conclusions can be drawn: (i) the *H-Thr-Ser-OH* is characterized with a *trans*-configuration of amide fragments and the protonation leads to a *transoide* one of the –N(H)–C(=O)-group; (ii) In the zwiterion and protonated form of dipeptide a geometry with suitable disposed *threony*l-side chain OH group for stabilizing of intramolecular H-bond with C=O group is predicted; (iii) hydrogensquarate of *H-Thr-Ser-OH* consists in positive charged dipeptide moiety and negative one hydrogensquarate anion (HSq^-) stabilizing by strong intermolecular hydrogen bonds; (iv) vibrational assignment of both system studied has been obtained by means of solid-state IR-LD spectroscopy of oriented samples in nematic liquid crystal suspension.

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