Electronic and Vibrational Optical Activity of Several Peptides Related to Neurohypophyseal Hormones: Disulfide Group Conformation

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ABSTRACT:

Electronic and vibrational optical activity of the set of neurohypophyseal hormones and their analogs was investigated to clarify the S—S bond solution conformation. The selected compounds include oxytocin (I), lysine vasopressin (II), arginine vasopressin (III), and their analogs (IV–IX), differing widely in their pharmacological properties. We have extended the already known electronic circular dichroism data by new information provided by vibrational circular dichroism (VCD) and Raman optical activity (ROA). The use of VCD brought additional details on three-dimensional structure of the chain reversal in the ring moiety and on its left handedness. Furthermore, Raman scattering and ROA allowed us to deduce the sense of the disulfide bond torsion. © *2012 Wiley Periodicals, Inc. Biopolymers 97: 923–932, 2012.*

Keywords: neurohypophyseal hormones; disulfide bridge; chiroptical spectroscopy; circular dichroism; Raman optical activity; vibrational circular dichroism; oxytocin; vasopressin; analogs; conformation

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INTRODUCTION

Neurohypophyseal hormones (NHHs) are among the longest recognized biologically active peptides. Oxytocin (OT) and arginine vasopressin (AVP—human hormone) are structurally related nonapeptides, synthesized in hypothalamic neurons, stored in the posterior pituitary gland (neurohypophysis, NH), and released into the circulation as NHHs with very different physiological roles (Gimpl and Fahrenholz[1]). The primary physiological role of OT is milk ejection and contraction of the uterus, whereas AVP is involved in regulation of cardiovascular functions and acts as a hormone with antidiuretic

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Table I List of Samples

Compound	Accepted Title	Structure[a]	Basic Pharmacological Effects (Peripheral)
Neurohypophyseal hormones			
I	Oxytocin	**C** Y I Q N **C** P L G-NH_2	Uterotonic (UTT), milk ejecting (ME)
II	Arginine vasopressin Natural human hormone	**C** Y F Q N **C** P R G-NH_2	Antidiuretic (AD), pressoric (PR)
III	Lysine vasopressin Hog hormone	**C** Y F Q N **C** P K G-NH_2	Antidiuretic (AD), pressoric (PR)
Analogs produced as bulk pharmaceutical chemicals for remedies			
IV	Methyloxytocin[b]	**C** Y(OMe) I Q N **C** P L G-NH_2	UTT (lower activity and protracted effect)
V	Atosiban[c]	**Mpr** y(OEt) I T N **C** P Orn G-NH_2	UTT, inhibitor OTR, V1 a V2 receptors
VI	Desmopressin[b]	**Mpr** Y F Q N **C** P r G-NH_2	AD (DDAVP) (V2 agonist in diabetes insipidus)
VII	Terlipressin[c]	G G G **C** Y F Q N **C** P K G-NH_2	PR low and very protracted effect—used for esophageal bleeding
Analogs			
VIII	NHH inhibitor	cpm**C** y I T N **C** P Orn-NH_2	UTT, strong OTR inhibitor
IX	Ring model	**C** G G G N **C**-NH_2	Not known, for conformational studies only

[a] In all structures, the Cys (or Mpr) residues are connected via the disulfide bridge and form a heterodetic ring closure (indicated in Bold type); Mpr, mercaptopropionic acid; cmpC, β-cyclopentamethylene-cysteine. Lower case letters are used to denote D-amino acid residues.

[b] Methyloxytocin was used as the safe uterotonic stimulator (later replaced by even safer Carbetocin (deamino-carba[1]-Tyr(OMe)[2]-oxytocin)).

[c] Atosiban is able to antagonize uterotonic effect of oxytocin and is used for the suppression of the development of unwanted uterotonic activity.

[d] Desmopressin (also known as DDAVP) is still considered as one of the most successful very powerful antidiuretic drugs.

[e] Terlipressin (also known as glypressin) is hormonogen-like peptide with very protracted pressoric activity which is used in the various bleedings disorders and during vascular surgery.

function. New experimental approaches disclosed the role of OT and AVP as important regulatory factors in the brain (maternal and social behavior, learning and memory, stress, etc.). At present, it is well recognized that NHHs act in central nervous system and this role seems to be perhaps equally important in comparison with the peripheral effects already described in detail.[2] Central activities of NHHs are currently studied from various aspects. Both hormones act through G-protein-coupled receptors, OT through OT receptor, whereas AVP by activation receptors V1a, V1b (or V3), and V2.[1] However, the receptor selectivity is not absolute and there exists some crosstalk between vasopressin (VP) and OT receptors. Amino acid sequence of OT including its disulfide bridge was elucidated in 1953 by Du Vigneaud et al.[3] OT was the first peptidic hormone that was successfully synthesized, shortly after its discovery.[3,4] Syntheses of OT and AVP analogs with agonistic and antagonistic properties, based on the modulation of their receptor activity, followed soon; some of these drugs have important therapeutic use (Table I). V2 agonists are used for the treatment of diabetes insipidus and its antagonists may be used in the case of congestive heart failure; terlipressin is used against esophageal bleeding. OT antagonist atosiban was approved for the treatment of preterm labor. Nowadays, many (i.e. hundreds of) synthetic analogs and structural descendants of NHHs exist. Analogs of NHHs were utilized as drugs in both human and veterinary medicine and their investigation significantly contributed to knowledge of general methodics of QSAR studies, inhibitor, and receptor investigation.[2,5]

Structural studies of NHHs are numerous as well. The molecules display considerable conformational constraints and limited conformational mobility. Their spatial arrangement has been thoroughly investigated. There are numerous nuclear magnetic resonance (NMR) (^{1}H, ^{13}C, relaxation times, and Overhauser effect)[6–22] (for a review, see Hruby and Lebl[23]) and molecular spectroscopy (CD,[24–28] Raman,[27–31] and fluorescence[32–34]) studies. Several analogs were crystallized and the X-ray studies of them[35,36] or of the complex with neurophysin[37,38] and trypsin[39] provide a conception of their three-dimensional structure. Structural studies in solution are mainly based on NMR and electronic CD spectroscopies. The results indicate that NHH molecules—at least the ring parts—are remarkably consistent in their spatial arrangements. It is difficult to relate quite variant biological properties, which respond sensitively to even minor changes in structure of NHH analogs, with the fact that three-dimensional structures of these molecules vary little. NMR studies have provided considerable information about NHH structural arrangement in solution, but it has not been useful for investigating conformation around the disulfide bridge. Disulfide group is a characteristic and important structural element of NHH molecules because it closes the 20-membered ring which is essential for

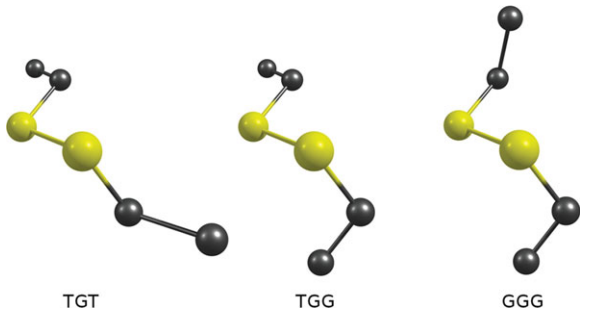

FIGURE 1 Frequent conformations of the disulfide bridge, C—C—S—S—C—C. Left: TGT; middle: TGG; right: GGG (G, *gauche*; T, *trans*). Sulfur atoms are shown in yellow.

the manifestation of pharmacological activities. However, the ring can be modified, leading often to analogs more stable toward cleaving enzymes. In addition, some studies indicate that NHH conformation might react sensitively to the interaction of disulfide group with the aromatic side chain at position 2.[40] This interaction was also confirmed by data from high-performance liquid chromatography (HPLC)[41,42] and NMR and fluorescence[43] studies of OT analogs modified with replacement of sulfur atoms by methylene groups or S—O groups.

Many spectroscopic studies of disulfide conformation in NHHs and their analogs have been already made.[24–31,44–50] Generally, disulfide bridge in peptides is quite flexible and can adopt various geometries. However, statistical treatment of known protein structures reveals preferences for several conformational types. There are three general conformations of the disulfide group (Figure 1), all with the central dihedral angle (C—S—S—C, i.e. around the S—S bond) nearly $\pm 90^{\circ}$ denoted as GGG, GGT, and TGT (in this three-letter symbolics the initial and final G or T designates *gauche*- or *trans*-conformation of the C—C—S—S or S—S—C—C termini; the central G is a standard designation of the S—S conformation with χ_{S-S} nearly $\pm 90^{\circ}$). These conformations can be distinguished by standard Raman spectroscopy which allows detecting individual C—S and S—S stretching modes and identifying corresponding disulfide conformations. It is generally accepted that GGG, TGG, and TGT conformations give rise to the respective S—S stretching signals nearly 510, 525, and 540 cm^{-1} (Refs. [51–53]). Conformations of the disulfide bond with prevailing GGG arrangement were found in the spectra of most agonistic OT and VP analogs.[27–31] On the other hand, different disulfide bond conformations can be found in some OT antagonists, for example molecules having penicillamine in position 1−.[45] Although the information obtained mainly by Raman spectroscopy is valuable, it cannot distinguish between right- and left-handed arrangements of the system. However, nonplanar disulfide group is chiral and this makes its handedness approachable by chiroptical methods involving electronic and vibrational circular dichroism (VCD) and Raman optical activity (ROA). There are some difficulties with investigation of disulfide groups by electronic circular dichroism (ECD) owing to group symmetry, orbital degeneracy, and strong overlap of disulfide bands with the amide transitions at 190–250 nm and with π–π^* transitions of aromatic residues at 250–300 nm (see, e.g. Refs. [27,54]). We have shown earlier that useful information about the disulfide conformation might be obtained using methods of vibrational optical activity (VOA), particularly ROA which is a chiral variant of Raman spectroscopy. Our earlier experimental and theoretical results indicate that ROA might represent a long sought-after solution of disulfide group conformation and might be capable of distinguishing between its right- and left-handed chirality.[55,56]

For the present study, we take advantage of the fact that NHHs are used as drugs in various indications for the treatment of disorders in both human and veterinary medicine and for this purpose they are manufactured under the rules of current good manufacturing practice (cGMP) in large quantities and in high purity as described in the Pharmacopeia. We selected a set of such compounds (Table I), including hormones themselves (OT I, arginine, and lysine vasopressins II, III), their pharmaceutically used analogs (methyloxytocin IV, atosiban V, desmopressin VI, and terlipressin VII) and related models (VIII, IX). Although the selected compounds differ widely in their pharmacological properties, these are achieved just by little changes of their primary and perhaps secondary structures. Our list forms a suitable basis for a more detailed structural scrutiny which could provide us the still missing view. We intend to investigate these compounds with a combination of several optical and chiroptical spectroscopies including ECD, VCD, Raman spectroscopy, and ROA. The use of vibrational chiroptical methods (VCD and ROA) might provide additional information because neither amide (VCD and ROA) nor disulfide (ROA) signals overlap with the π–π^* bands of aromatic side chains as they unfortunately do in ECD spectra. In addition, these methods, in particular the ROA, provide insight even into small-scale structural changes. Comparison of new chiroptical data with already known results of conformational studies should further elucidate the role of disulfides in NHHs structure and function.

MATERIALS AND METHODS

The hormones I–III and their analogs IV–VII were bulk-synthesized pharmaceutical chemicals prepared under cGMP (Polypeptide Group). They were fully analytically characterized and used without further purification. The sample of inhibitor VIII was provided by Prof. Manning (Medical College of Ohio) and used without further purification. The simplified ring model IX was prepared using

standard procedures of solid-phase peptide synthesis employing Fmoc/*tert*-butyl strategy, TFA reagent cleavage from the resin, oxidation, and standard HPLC purification. The synthesized peptide was at least 95% pure (based on HPLC).

Electronic absorption and ECD spectra were measured in solutions on Jasco J-815 spectrometer equipped with a Peltier-based temperature control module. The solvents included 0.01*M* phosphate buffer (pH = 7.5), 0.01*M* HCl (pH = 2) and, for compound II, 2,2,2-trifluoroethanol. Concentrations were in the approximate range of 5–20 $\times \cdot 10^{-4}$ mol L^{-1}. We used quartz cells with the optical path length of 1 cm. The spectra were recorded in the spectral region 255–360 nm mostly at room temperature but we also measured the dependence on temperature in the range of 5–75°C. Each spectrum was obtained as an average of three subsequent computer-controlled scans taken at the scanning speed of 50 nm/min and the time constant of 4 s. The final ECD and absorption spectra are expressed as the respective values of $\Delta\varepsilon$ and ε (L mol^{-1} cm^{-1}) (Figure. 2). ECD and absorption of compound VIII in low pH was not measured because of too low quantity which was available.

VCD was measured on Bruker FTIR spectrometer equipped with the VCD/IRRAS module. The setup used for amide I and mid IR measurements (1800–1200 cm^{-1}) was based on IFS-66/S FTIR and included: VCD/IRRAS PMA 37 attachment, BaF_2 polarizer, ZnSe photoelastic modulator (Hinds), MCT detector (InfraRed Associates), and a lock-in amplifier (SR830 Stanford Instruments). The spectra were recorded at the resolution of 8 cm^{-1} in D_2O at the concentration range of 4–9 $\cdot 10^{-2}$ mol L^{-1}. We used a demountable sample cell (path length, 0.025 mm) with CaF_2 windows. Our standard measurement protocol involved averaging of four 30 min blocks of interferograms. Corresponding solvent scans were subtracted as background. The compounds II, VIII, and IX showed the presence of TFA as an absorption signal at 1671 cm^{-1} which has been subtracted. Final data were plotted as the respective ε((absorption) and $\Delta(\varepsilon$((VCD) values normalized to number of amino acid residues. Second derivatives of absorption spectra were calculated using GRAMS/AI software (Thermo Electron Corporation) to enable decomposition and detailed interpretation of overlapping amide I band components according to a procedure described elsewhere.[57]

Raman scattering and ROA spectra were measured on the commercial scattered circular polarization (SCP) ROA spectrometer (Chiral Raman, Bio Tools, USA)[58] and on the incident circular polarization (ICP) Raman/ROA instrument built at the Institute of Physics, Charles University in Prague working in backscattering.[59] The latter spectrometer is based on a fast stigmatic spectrograph HoloSpec HS-f/1.4 (Kaiser Optical Systems) with an interchangeable holographic transmission grating and a back-illuminated CCD detection system (Roper Scientific, 1340 × 100 pixels). The compounds I–VII and IX for Raman and ROA measurements were dissolved in distilled water at ambient temperature (20°C) and filtered through a 0.22-μm Millipore filter into quartz ROA micro-cells (~60 μL, 4 × 3 mm, Starna Scientific) with antireflectively coated windows. The pH of aqueous solutions was adjusted with 0.2*M* HCl to a value of ~3. For the compound VIII, we were not able to obtain Raman and ROA spectra because of its low quantity which allowed only the measurement of ECD (in neutral buffer) and VCD. The conditions for ROA experiments were set on the two instruments as follows: Chiral Raman: excitation wavelength 532 nm, laser power at the sample 250–300 mW, spectral resolution 7 cm^{-1}; noncommercial spectrometer: excitation wavelength 514.5 nm, laser power at the sample 550–650 mW, spectral resolution 6.5 cm^{-1}. We used concentrations in the range of 6–10 $\times \cdot 10^{-2}$ mol L^{-1}. Acquisition time was optimized to get reasonable *S*/*N* ratio in ROA spectra (3–4 days). Spectra were processed by subtracting the solvent signal and correcting the baseline by polynomial fitting (5th order polynomial). The final ROA spectra are presented as ($I^R - I^L$) and the Raman spectra as ($I^R + I^L$) where I^R and I^L are Raman intensities in right- and left- circularly polarized ICP or SCP laser light. Numerical data treatment was done using GRAMS/AI software (Thermo Electron). Decomposition of Raman spectra into single bands was made using second derivatives.

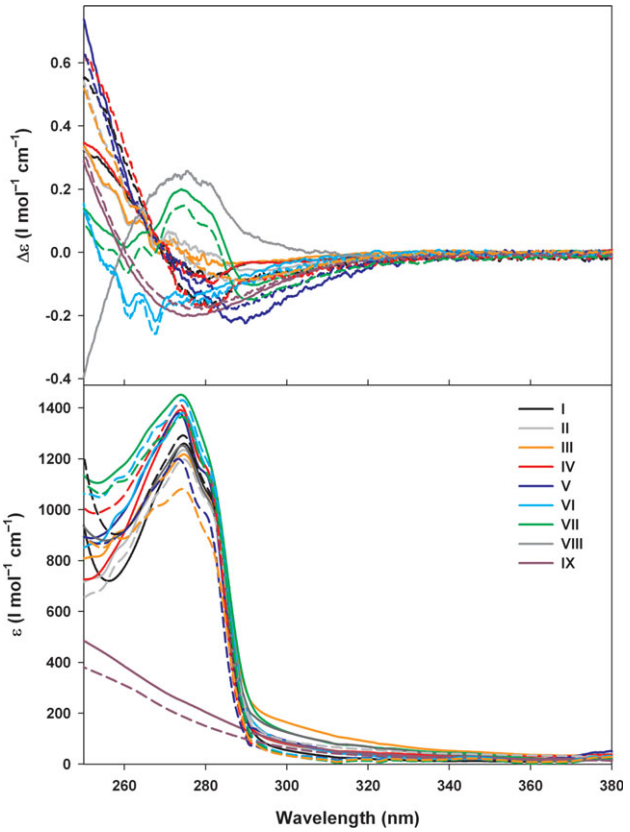

FIGURE 2 ECD (top) and UV absorption (bottom) of compound I (OT), II (AVP), III (lysine vasopressin), IV (methyloxytocin), V (atosiban), VI (desmopressin), VII (terlipressin), VIII (NHH inhibitor), and IX (ring model) measured in phosphate buffer (solid) and 0.01*M* HCl (dashed).

RESULTS AND DISCUSSION

We have measured ECD and electronic absorption of the whole series of compounds I–IX at neutral (pH 7.5, phosphate buffer) and acidic (pH 2, 0.01*M* HCl) conditions in the disulfide transition range (250–380 nm). Even in the case of compounds I–III, VI, IX, which have been previously measured,[25,26,28,48] we have collected the new data to have all spectra measured under comparable conditions. The results are shown in Figure 2. Below 280 nm, the disulfide bands

unfortunately overlap with the long-wavelength π–π^* bands of aromatic chromophores (Tyr in OT analogs, Tyr and Phe in VP analogs) and consequently pure disulfide signal can be observed only in the ECD spectra of IX which possesses no aromatic side chains. The interfering π–π^* bands show typical vibronic structure (tyrosine in absorption, phenylalanine in ECD—at 255, 261, and 268 nm). This phenomenon complicates spectra and makes isolation of disulfide contributions more difficult. In the presence of aromatic residues, we can thus safely ascribe disulfide origin to just the long wavelength tail in the 300–340 nm region as has been also recommended earlier.[60] On the basis of this long-wavelength disulfide CD band, we can classify our compounds I–IX into three categories: The first category (a) includes compounds with a negative band of low intensity; this is by far the most numerous category including all mainstream agonists I–IV, desmopressin VI and terlipressin VII. Although differences between their spectra are definitely over the error bars, the general course of these curves remains similar. A significant difference is observed only with OT I and methyloxytocin IV in low pH. In these latter cases, intensity of the negative disulfide band markedly increases on acidification. The second category (b) includes atosiban V with the disulfide band of clearly higher intensity. The spectra of the ring model IX form the transition between the first two categories. Although the differences between spectra belonging to classes (a) and (b) are quantitative and rather subtle, the category (c) shows disulfide band of the opposite sign. There is only one compound in this category—the inhibitor VIII. At shorter wavelengths, it is sometimes possible to observe a second disulfide band as predicted by the theory. With our compounds, it is observable only in the ECD spectrum of IX having no aromatic residues. This compound represents a simplified model of the OT ring with Gly residues replacing tyrosine and isoleucine. In neutral buffer, it exhibits a negative ECD band at 275 nm which can be considered a pure disulfide manifestation. In the early studies,[24] there were attempts to correlate the analogous 280 nm negative ECD band in I (also in IV, see above) and its pH dependence with the effect of possible protonation of the neighboring α-NH_2 group on the disulfide chromophore. However, no effect of protonation on disulfide conformation can be seen in ECD spectra of the ring model IX with Gly at position 2. This might indicate that the disulfide chromophore is influenced by neighboring Tyr (or Ile—Ref. [24]) residue. Much smaller changes can be observed in the spectra of NHH analogs lacking the α-NH_2 group (V, VI). The long-wavelength spectral tail which we used above for the classification of our compounds/spectra into (a, b, c) classes can be observed in spectra of all compounds I–IX. Its sign does not correlate with either the presence or the absence of α-NH_2 group (V, VI) and the correlation with configuration of the amino acid at the position 2 (V, VIII) is uncertain. However, it is interesting but hardly causally significant that this band somehow correlates with pharmacological properties: the agonists all belong to class (a) with small and similar disulfide bands, whereas the inactive IX and antagonists V, VIII display spectral differences and fall into categories (b, c). Consequences and importance of the D-configuration of aromatic amino acid for biological activities of OT analogs and their spectral properties were studied earlier in detail with both conformationally relaxed and constrained amino acids.[61] Analogs with D-amino acid clearly belong to different structural classes.

There have been many attempts to correlate experimental signs and magnitudes of disulfide bands with sense and magnitude of the disulfide twist on the basis of theoretical considerations and calculations.[54,62,63] Although nowadays it is not difficult to execute such a calculation at a very good theoretical level[55,64] (TD DFT calculation using B3LYP functional and at least the 6-31G* basis set may be considered a reasonable level of calculation), the results of such a calculation suffer numerous problems. The low-energy conformation of the C—S—S—C grouping is a perpendicular conformation with $\chi_{S-S} = \pm 90^\circ$. It would seem advantageous that this conformation represents simultaneously the arrangement having topologically maximal possible chirality. However, it is most unfortunate that the two lowest energy electronic transitions of the disulfide group are exactly degenerate in this conformation and consequently the sign of predicted CD sharply changes at $\chi_{S-S} = \pm 90^\circ$, where the two bands cancel and no CD is predicted. This situation has been known for some time[54,62,63] and it is usually generalized in a requirement that for the theoretical interpretation not only the sign but also some measure of the magnitude of the χ_{S-S} angle is needed (with values of χ_{S-S} close to $\pm 90^\circ$ this information needs to be rather precise). Applied to our case of NHH analogs, we may only say that similar course of disulfide-related ECD as shown in Figure 2 may mean similar S—S bridge conformation, but such a finding is not certain and there is a need for additional unambiguous information.

Vibrational and particularly chiral vibrational spectra should provide some still-missing structural details of NHHs, which are inaccessible by other spectroscopies. In addition, VOA promises to enrich significantly the current understanding of NHH's structure–function relationship. VCD and IR absorption spectra in the mid IR region (1300–1800 cm^{-1}) include signals owing to amide I (in D_2O amide I′) and amide II (II′) vibrations. In its current state of development, VCD cannot provide direct information on disulfide arrangement as vibrational bands owing to C—S—S—C

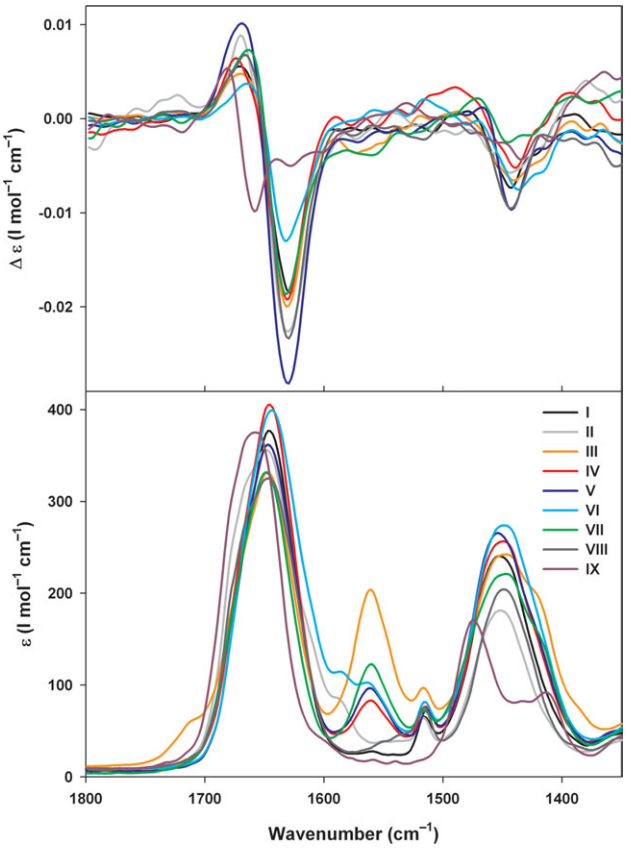

FIGURE 3 VCD (top) and IR absorption (bottom) of compound I (OT), II (AVP), III (lysine vasopressin), IV (methyloxytocin), V (atosiban), VI (desmopressin), VII (terlipressin), VIII (NHH inhibitor), and IX (ring model) measured in D_2O (spectra are normalized to the number of amino acid residues). The less compressed spectra are shown in Supporting Information material.

grouping lie outside the experimentally accessible region. The relevant region (~250–800 cm^{-1}) can be accessed by IR absorption spectroscopy, but the bands related to C—S and S—S bonds are usually invisible or of very low intensity. Vibrational transitions related to disulfide group are more conveniently accessed by Raman/ROA spectroscopy. On the other hand, VCD combined with IR spectroscopy can provide detailed information on peptide backbone and strengthen the knowledge given by other techniques. In our case, this means resolving information provided by ECD and NMR into detailed conformationally defined fragments. The spectra of compounds I–IX in D_2O are shown in Figure 3. The bands observed in absorption in amide I spectral region indicate the presence of the random coil/PPII structure (these two structures cannot be distinguished solely by IR spectroscopy) or of an α-helix (at ~1645 cm^{-1}, however α-helix can be excluded owing to VCD and Raman spectra—see below) together with the β-turn structure (shoulder at ~1665 cm^{-1}). VCD pattern owing to these signals has been described earlier[65] and its sensitive response to changes and types of secondary structures in peptides/proteins is well understood.[66] Our NHHs and their derivatives exhibit remarkably constant VCD spectral pattern consisting of a rather intense negative couplet corresponding to amide I′ at ~1650 cm^{-1} (a positive VCD band at ~1670 cm^{-1} together with a negative band at ~1630 cm^{-1}) and a smaller negative band at ~1440 cm^{-1} (amide II′). The positions of particular bands in I–VIII do not appear to be dependent on particular structures, only in the analog IX the position of amide I′ VCD bands is shifted to higher wavenumbers by about 25 cm^{-1}. The observed VCD patterns resemble very closely polyproline II (PPII) type secondary structures with a positive/negative couplet in the amide I (I′) region and a negative band in the amide II (II′) region. This type of conformation and associated VCD spectra has been extensively studied by Dukor and Keiderling.[67] Although it is generally difficult to distinguish between the left-handed helical PPII-like conformation and the truly unordered state of a peptide/protein, we can rather safely derive that the general three-dimensional ring pattern of NHHs includes a PPII-type left helical turn. For the extent of PPII turn, we can get an indication from the corresponding VCD intensities. If we calculate $\Delta A/A$ or alternately $\Delta\varepsilon/\varepsilon$ values for our compounds and compare these with intensities measured for real polyprolines,[67] we find our spectra approximately three times less intense. This indicates that a direction reversal of NHH ring probably involves just two to three amino acid residues. VCD intensities in the amide I′ and amide II′ region allow also some differentiation between compounds I and IX. The lowest intensity is shown by the compound IX in accordance with its assumed bigger flexibility.

Raman and ROA spectra provide a different point of view. Although there is also a significant manifestation of backbone conformation (it is shown in Raman/ROA mainly via extended amide III at 1240–1310 cm^{-1} [Ref. 68] and amide I bands), the most useful information follows from the low-wavenumber region (<800 cm^{-1}) with the prominent bands owing to disulfide vibrations. The spectra of compounds I–VII and IX in H_2O are shown in Figure 4 and the extracted Raman frequencies (amide I, amide III, ν_{C-S} and ν_{S-S}) obtained from the spectral decomposition using second derivatives are listed in Table II. Conformational analysis and band assignment was proposed with respect to the previous Raman investigations of OT (I), AVP (II), and their analogs.[27–31,45]

Raman bands of compounds I–VII in amide I and amide III region agree with the already expected presence of both random coil and β-sheet/β-turn structures[53,68] (Table II). Amide I and amide III bands in compound IX (the simplified

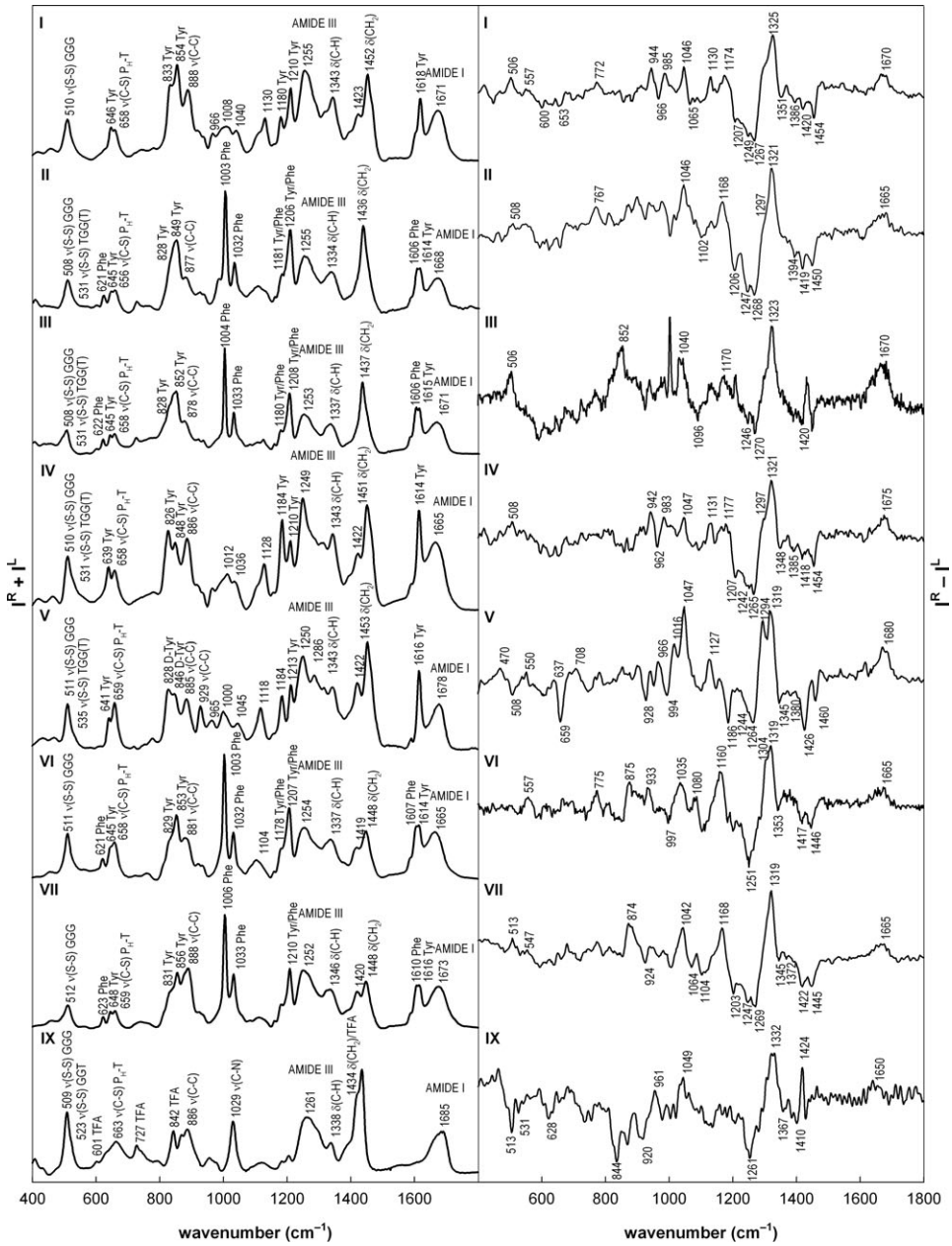

FIGURE 4 Raman scattering (left) and ROA (right) spectra of compounds I (OT), II (AVP), III (lysine vasopressin), IV (methyloxytocin), V (atosiban), VI (desmopressin), VII (terlipressin), and IX (ring model) measured at acidic pH. These spectra are shown at higher resolution in Supporting Information material.

model of the OT ring moiety) are shifted to higher wavenumbers and indicate the presence of β-sheet/β-turn structure. Raman bands at 850 and 830 cm^{-1} correspond to tyrosine side chain and their relative intensities are related to its environment.[69] The I_{850}/I_{830} intensity ratio exceeds 1 for compounds I–III, VI, VII, and IX, indicating that the Tyr residue is probably exposed to solvent. On the other hand, the ratio lower than 1 is observed for compounds IV and V where Tyr (2) OH group is alkylated and therefore no suitable partner for hydrogen bonding to the solvent is available. ROA spectra in this region show for compounds I–VII a sharp positive band at ~1320 cm^{-1} (extended amide III) and a weak positive band with the maximum at ~1665–1680 cm^{-1} (amide I). These findings further strengthen arguments in favor of the already indicated presence of PPII type secondary structure.[70–72] The additional negative band between ~1242–1251 cm^{-1} in compounds I–VII has been previously assigned to an antiparallel β-strand.[70] The presence of a short segment of

Table II C—S, S—S, and Amide-Related Frequencies in the Raman Spectra of Compounds I–VII, IX[a]

	Frequency (cm^{-1})							
Band assignment	I	II	III	IV	V	VI	VII	IX
Amide I	1686	1691	1690	1683	1687	1684	1685	1692
	1678	1683	1681				1682	
	1668	1666	1671	1667	1667	1670	1668	1672
	1659	1658		1657		1657	1653	
Amide III	1292	1285	1291	1288	1286	1286		1274
	1267	1263	1268	1274			1268	1257
	1250	1249	1247	1247	1250		1243	1247
		1238			1235			
ν(C—S) P_H	658	656	658	658	659	658	659	663
ν(S—S) TGG(T)		531sh	533sh	533sh	531sh			
ν(S—S) TGG								523sh
ν(S—S) GGG	510	508	508	509	511	511	512	508

[a] TGG(T)—Weak bands do not allow reliable distinguishing between G and T conformation on S—C dihedral angle.
sh, Shoulder.

this type of structure is quite probable. A positive band at ~1295 cm^{-1} and negative bands at ~1350 and ~1385 cm^{-1} in ROA spectra of I, IV, V, and VI might in addition indicate the presence of a β-turn-like structure.[72,73] VCD/IR/Raman and ROA data on NHHs (OT I, AVP II, LVP III, desmopressin VI) agree with the backbone arrangement found by NMR spectroscopy in combination with molecular dynamics (MD) simulations[19,21,22,74,75] although the latter data were often obtained in a different solvent (dimethyl sulfoxide) (Table I in Ref. [76]). NMR results favor β-turn (over the residues 2,3 or 3,4) or γ-turn conformations (desmopressin). However, NMR spectroscopy is not sensitive to disulfide group conformation.

Unlike in ECD spectra where Tyr and disulfide-related bands overlap and obscure each other, signals owing to the disulfide bridge (ν_{S-S} and ν_{C-S} vibrations) can be distinguished clearly in Raman and ROA spectra. Although a broad water band at 400–520 cm^{-1} overlaps just slightly with the S—S stretching band, it usually does not obscure the ν_{S-S} signal. The strong S—S stretching band in Raman spectra of all the measured compounds (I–VII and IX) at ~510 cm^{-1} is an indicator of prevailing GGG conformation of C—C—S—S—C—C grouping.[52] Second derivative spectra reveal a shoulder at ~523 cm^{-1} (IX) or ~530 cm^{-1} (I, II, IV), and ~535 cm^{-1} (V) which indicates a small fraction of the respective GGT and TGT conformation.[52,53] Raman signal of C—S stretching vibrations can be found in the region of 655–663 cm^{-1} for all studied compounds, indicating prevailing P_H conformation of the X—CH—CH_2—CH moiety (X=H, C, or N) with H_α atom in trans-position to the sulfur atom.[77] Theoretical analysis of ROA in S—S stretching region (490–550 cm^{-1}) indicates that the corresponding signals reflect rather sensitively differences in the absolute configuration/conformation of the disulfide group and that there is an unequivocal relationship of ROA sign and handedness of the C—S—S—C group.[55,56] A positive ROA signal of S—S stretching in I, III (~506 cm^{-1}), II, IV (~508 cm^{-1}), and VII (~513 cm^{-1}) indicates right-handed chirality of the disulfide group in all these compounds. On the contrary, a negative signal in ROA spectra of V (sharp negative band at 508 cm^{-1}) and IX (sharp negative band at 513 cm^{-1}) might indicate left-handed chirality of disulfide group. No ROA signal of the S—S stretching vibration can be clearly distinguished in VI. This may be owing to higher flexibility of the disulfide bridge in this compound (no N-terminus in this compound) with both negative and positive disulfide conformations possible or by the presence of D-arginine in position 8. The compound VI exhibits positive ROA signal at ~550 cm^{-1} which can also be found in ROA spectra of I, II, IV, V, and VII; however, its origin is probably not related to disulfide vibrations. As already noted, the above ROA data of our set of NHH agonists I–IV, VII indicate right-handed disulfide chirality. This finding conforms to ECD at least in the sense that these compounds all consistently exhibit very similar ECD. On the other hand, the inhibitor V and the inactive model IX behave differently. Sometimes, inhibitor-like properties relate to modifications in the position 1. The incorporation of β-mercaptopropionic acid leads to compounds lacking α-amino group (1-deamino analogs V, VI, VIII). Such modification may quite logically change disulfide conformation as documented most clearly by ROA. At the same time, it brings a different behavior toward a pH change. This

has been already noted in the past.[49] It is further noteworthy that a right-handed disulfide arrangement has been detected in most existing X-ray studies of agonistic NHH analogs[35,36] and of their complexes with carrier proteins.[37–39] HPLC[41,42] and NMR and fluorescence[43] studies of OT analogs support the existence of an interaction of sulfur in position 6 with the aromatic ring of tyrosine. The fact that right-handed helicity of the disulfide bridge in the crystal structure of deamino OT shows the possible proximity of aromatic side chain and the sulfur in position 6 as well, speaks in favor of the conclusion that the conformation in solution is close to the conformation found in the crystal, including the helicity of the disulfide bridge. We have shown that VOA can provide similar knowledge also for samples in solution.

CONCLUSIONS

We have collected the basic series of vibrational optical activity data related to NHHs and some of their analogs. The data show remarkable conformational consistency within most compounds. VCD and particularly ROA agree with the previously found and/or suggested structural data based mainly on NMR and MD simulations together with X-ray studies of NHH–neurophysin complexes. VOA extends the already existing knowledge in several significant details. The main-chain reversal of the ring moiety is undoubtedly left-handed. This follows from VCD pattern in amide I region, which quite closely resembles polyproline II type helix or maybe a certain type of β-turn. This finding is further supported by ROA because NHH spectra contain a prominent marker band of the PPII structure at 1320 cm^{-1}. Furthermore, ROA spectra contain information about arrangement of the disulfide group, which is otherwise difficult to obtain. Interestingly, the investigated NHH agonists seem to possess identical right handedness of the disulfide, whereas the inactive or inhibitory compounds show a tendency to opposite arrangement. This will be a matter of further investigation. Vibrational optical activity proved its ability to provide useful data on peptide conformation even in the presence of aromatic side chains, unlike ECD.

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