SYNTHESIS AND CHIROPTICAL PROPERTIES OF HETERODETIC CYCLIC HEXAPEPTIDES RELATED TO OXYTOCIN RING MOIETY*

Ivo FRIČ, Lyudmila I. LEONTEVA**, Petr MALOŇ, Karel JOŠT and Karel BLÁHA

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

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Cyclic disulfides of cysteinyl-tetraglycyl-cysteine (*Ia*), cysteinyl-tyrosyl-triglycyl-cysteine (*Ib*) and cysteinyl-tyrosyl-isoleucyl-diglycyl-cysteine (*Ic*) were synthesized by classical methods of peptide synthesis. The actions of solvent and of side chains in the positions 2 and 3 on the conformational arrangement of the peptide backbone and the disulfide group were investigated by means of CD spectroscopy. Some mechanisms which co-operate in stabilizing the oxytocin conformation were identified. Hence, it may be deduced, that the amino acid sequence in the positions 1—3 determines the spatial arrangement characteristic for oxytocin, at least in a protonating medium.

The present efforts[1-6] in utilizing the spectra of circular dichroism to obtain knowledge on conformation of neurohypophyseal hormones were largely of an extensive nature given above all by the use of numerous analogues prepared for the purpose of understanding the structure–biological activity relationships[7,8]. Although it was possible to draw some conformational conclusions agreeing in principle with the findings afforded by other methods[9-14], the results were rather of a diagnostic value, *i.e.* they enabled one to classify the particular types of analogues according to the characteristic structural modification[3,5]. To rationalize the approach to conformational studies of the natural compounds – oxytocin and vasopressin – it would be advantageous to investigate model compounds tailored directly for this purpose. Such models would facilitate the more detailed analysis of the complex CD curves of neurohypophyseal hormones as well as the recognition of the particular structural segment actions on the spatial arrangement of mother compounds.

In this report we describe the synthesis of three simple heterodetic cyclohexapeptides *Ia*–*Ic* retaining the following features of the parent oxytocin molecule: the size of the cyclohexapeptide fragment and the disulfide grouping.

In addition, compound *Ib* contains also the second amino-acid residue of the oxytocin sequence (tyrosine) and compound *Ic* the second and third residue (tyrosine

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** Visiting scientist, Leningrad State University, Leningrad, USSR.

and isoleucine). These compounds, together with the additional heterodetic cyclohexapeptide *VII* (prepared by Siemion and Klis[15]), were thoroughly investigated by CD spectroscopy in a series of solvents and in relation to temperature. We endeavoured to identify the factors of structure and environment capable of influencing the spatial arrangement of the peptide backbone and the disulfide group and to find relations between properties of the model compounds and those of the native hormone.

Synthesis of the hexapeptides* *Ia*–*Ic* was performed by fragment condensation. Protecting groups were chosen with respect to the need for their simultaneous removal in the last step by sodium in liquid ammonia (N-benzyloxycarbonyl, S-benzyl and benzyl ester). *o*-Nitrobenzenesulfenyl group was utilized for the temporary protection of the amino groups. A similar route was used[17–19] for the preparation of the compound *Ia*. In addition, we were able to present the full characterisation of this as well as the other two (*Ib* and *Ic*) hexapeptides. We have also proved the monomeric character of the compound *Ia* by partial substitution of the amino group[20,21].

H-Cys-Gly-Gly-Gly-Gly-Cys-OH

Ia

H-Cys-Tyr-Gly-Gly-Gly-Cys-OH

Ib

H-Cys-Tyr-Ile-Gly-Gly-Cys-OH

Ic

Z-Cys(Bzl)-Gly-Gly-R

IIa, R = OEt, *IIb*, R = N_2H_3

Nps-Gly-Gly-Cys(Bzl)-OBzl

III

Z-Cys(Bzl)-Gly-Gly-Gly-Gly-Cys(Bzl)-OBzl

IV

Nps-X-Gly-Gly-Cys(Bzl)-OBzl

Va, X = Gly, *Vb*, X = Ile

Z-Cys(Bzl)-Tyr-X-Gly-Gly-Cys(Bzl)-OBzl

VIa, X = Gly, *VIb*, X = Ile

H-Cys-Lys-Ala-Gly-Gly-Cys-OH

VII

* Amino acids used in this work were of L-configuration. Nomenclature and symbols follow the published proposals[16].

Synthesis of the compound *Ia* started with two tripeptide derivatives. The ester *IIa* was converted to hydrazide *IIb* and azide-condensed with the tripeptide ester obtained from the compound *III* by removal of the amino protecting group with HCl. The fully protected hexapeptide *IV* was reduced with sodium in liquid ammonia and oxidized in the aqueous solution with air oxygen. Preparation of the remaining two hexapeptides started again from the tripeptide *III*. After removal of the *o*-nitrobenzenesulfenyl group, the compound was acylated either with *o*-nitrobenzenesulfenylglycine and N,N'-dicyclohexylcarbodiimide (yielding the compound *Va*) or with N-hydroxysuccinimide ester of *o*-nitrobenzenesulfenylisoleucine, affording the tetradeptide *Vb*. In both cases the *o*-nitrobenzenesulfenyl group was split off by HCl and the free tetrapeptide esters were acylated with N-benzyloxycarbonyl-S-benzylcysteinyl-tyrosine azide prepared from the corresponding hydrazide[22]. Similarly to the case of the compound *IV*, the both protected hexapeptides *VIa* and *VIb* were reduced with sodium in liquid ammonia and oxidized with the air. Inorganic salts were removed using sulfonate cation exchanger and the products were purified by gel filtration on Bio-Gel P-4 in 1M acetic acid. The pure products were obtained in 30 – 60% yield.

EXPERIMENTAL

Melting points were determined on a Kofler block and are corrected. Samples for elemental analyses were dried 24—48 h at 130 Pa. Thin-layer chromatography was carried out on silica gel plates in the solvent systems 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), 1-butanol–pyridine–acetic acid–water (30 : 20 : 12 : 6) (S4). Electrophoresis was carried out on Whatman 3 MM paper in a moist chamber apparatus at 800 V for 1 h. The buffer solutions used were 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7). Spots were detected by ninhydrin or by chlorination. The presented R_F and E values relate to pure compounds. Samples for amino acid analysis were hydrolysed for 20 h in 6M-HCl (in ampoules sealed at 130 Pa). Analyses were performed on an automatic analyser (Developmental Workshops, Czechoslovak Academy of Sciences, type 6020). Reaction mixtures were evaporated on a rotatory evaporator at a bath temperature of 30—35°C under reduced pressure (water pump; with mixtures containing dimethylformamide an oil vacuum pump). Gel filtrations were performed in columns filled with Bio-Gel P—4 (Bio-Rad Laboratories, Richmond, Ca., USA).

o-Nitrobenzenesulfenylglycyl-glycine

To a solution of glycyl-glycine (13·2 g) in a mixture of 2M-NaOH (50 ml) and dioxan (125 ml) we added *o*-nitrobenzenesulfenyl chloride (20·6 g) and 2M-NaOH (60 ml) in a period of 10 min. The mixture was poured into cold water (1 l), the precipitate was filtered off and the solution was acidified with 1M-H_2SO_4. The precipitated product was filtered off and washed with water. Crystallization (ethanol) afforded 17·3 g (60%) of the product, m.p. 170 to 175°C. For $C_{10}H_{11}N_3O_5S$ (285·3) calculated: 42·10% C, 3·89% H, 14·73% N; found: 42·30% C, 3·94% H, 14·73% N.

o-Nitrobenzenesulfenylglycyl-glycyl-S-benzylcysteine Benzyl Ester (*III*)

S-Benzylcysteine benzyl ester *p*-toluenesulfonate was prepared according to the published procedure[23] in 88% yield. M.p. 158—161°C, $[\alpha]_D$ —19·2° (*c* 0·5, dimethylformamide); $E_{2.4}^{Gly}$ 1·2, $E_{5.7}^{His}$ 0·63. Reported[23] m.p. 159°C.

S-Benzylcysteine benzyl ester *p*-toluenesulfonate (40 g) dissolved in water was neutralized with sodium carbonate to pH 8—9. The solution was extracted with ethyl acetate, the extract was dried and taken down. The residue was taken up in dimethylformamide (70 ml), *o*-nitrobenzenesulfenylglycyl-glycine (23 g) and 1-hydroxybenzotriazole (12 g) were added. The solution was cooled to —20°C and N,N'-dicyclohexylcarbodiimide (18·3 g) was added. The mixture was stirred for 2 h at —20°C and overnight at 0°C. Dicyclohexylurea was filtered off, the solution was taken down and the residue was treated subsequently with light petroleum and ether. The crystalline portion was filtered and washed on the filter with 5% sodium carbonate solution, water, 1M-H_2SO_4 and water. Crystallization from 2-propanol yielded 25 g (54%) of the title compound, m.p. 125—127°C; $[\alpha]_D$ —25·4° (*c* 0·5, dimethylformamide). For $C_{27}H_{30}N_4O_5S_2$ 568·7) calculated: 57·02% C, 4·96% H, 9·85% N; found: 56·82% C, 4·93% H, 9·96% N.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycine Ethyl Ester (*IIa*)

Glycyl-glycine (1 g) was dissolved in 5·2M ethanolic HCl (5 ml) and after standing for 18 h at room temperature glycyl-glycine ethyl ester hydrochloride was precipitated with ether. Yield 1·5 g (93%), m.p. 184—186°C; $E_{2.4}^{Gly}$ 0·54, $E_{5.7}^{His}$ 0·88. Refs[17,18] give m.p. 185—186°C and 181 to 182°C.

N-Benzyloxycarbonyl-S-benzylcysteine (3·45 g), glycyl-glycine ethyl ester hydrochloride (2·75 g) and 1-hydroxybenzotriazole (1·35 g) were dissolved in dimethylformamide. The pH of the solution was adjusted to 7·5—8·0 (moist pH paper) by the addition of N-ethylpiperidine. The solution was cooled to —20°C and N,N'-dicyclohexylcarbodiimide (2·27 g) was added. The mixture was stirred for 2 h at —20°C, overnight at 0°C and 2 h at room temperature. After evaporation the residue was treated with ether, the crystalline portion was filtered and washed with 1M-HCl, water, 5% aqueous sodium hydrogen carbonate and water. Crystallization (2-propanol) afforded 4·4 g (86%) of *IIa*, m.p. 105—107°C, $[\alpha]_D$ —8·9° (*c* 0·6, ethanol). For $C_{24}H_{29}N_3O_6S$ (487·6) calculated: 59·12% C, 6·00% H, 8·62% N; found: 59·15% C, 6·01% H, 8·64% N. Ref.[23] gives m.p. 114°C, $[\alpha]_D$ —12·9° (*c* 3·2, ethanol).

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycine Hydrazide (*IIb*)

The compound was obtained in 80% yield by hydrazinolysis of the corresponding ethyl ester with hydrazine hydrate. M.p. 170—172°C, $[\alpha]_D$ —24·1° (*c* 0·5, dimethylformamide). Literature[23] gives m.p. 164°C.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycyl-glycyl-glycyl-S-benzylcysteine Benzyl Ester (*IV*)

To a solution of the protected tripeptide *III* (1·7 g) in dimethylformamide 3·6M-HCl in ether (1·75 ml) was added. After 4 min the mixture was evaporated and triturated with ether. To a cooled (—20°) solution of the protected hydrazide *IIb* (1·46 g) in dimethylformamide (5 ml) 6·5M-HCl in tetrahydrofuran (2·3 ml) and butyl nitrite (0·7 ml) were added. The pH of the mixture was adjusted to 8·5 and the dimethylformamide solution of the amino component was added. After standing for 24 h at 0°C, the mixture was taken down and the residue treated with 1M-HCl, water, 5%

aqueous sodium hydrogen carbonate and water. Crystallization from 2-propanol afforded 2·2 g (82%) of *IV*, m.p. 209—211°C, $[\alpha]_D$ —31·5° (*c* 1·1, pyridine). R_F 0·77 (S1), 0·65 (S2), 0·73 (S3), 0·71 (S4). Literature[23] gives m.p. 205—206°C, $[\alpha]_D$ —29·7° (*c* 1·1, pyridine).

Cyclic Disulfide of Cysteinyl-glycyl-glycyl-glycyl-glycyl-cysteine (*Ia*)

The protected hexapeptide *IV* (0·50 g) was reduced with sodium in liquid ammonia (0·5 l). After 2 min the blue solution was discoloured by addition of ammonium chloride and the ammonia was evaporated. The residue was dissolved in water, the pH was adjusted to 3—4 and the solution was extracted with ether. Then the pH value of the mixture was adjusted to 7 with 1M-NaOH, the solution was diluted with water to 0·5 l and air-oxidized for 2 h. The pH was adjusted to 3—5, the solution was concentrated to 100 ml and poured on a Dowex 50 (H^+-cycle) column. The column was washed with water and the peptidic material eluted with 10% pyridine (5°C). Freeze-drying of the eluate afforded 0·14 g (55%) of the product, which was further purified by gel filtration on a Bio-Gel P—4 column (140 × 2·5 cm) in 1M acetic acid (OD_{256} detection). One main peak of the peptidic material was localized; freeze-drying yielded 54% of *Ia* (total yield 30%); $E^{Gly}_{2.4}$ 0·77, $E^{Gly}_{5.7}$ 0·0; R_F 0·12 (S1), 0·02 (S2), 0·07 (S3), 0·25 (S4); $[\alpha]_D$ —24·0° (*c* 0·06, 1M acetic acid). Amino acid analysis: Gly 4·00, Cys 1·82. For $C_{14}H_{22}N_6O_7S_2.C_2H_4O_2.2{\cdot}5\ H_2O$ (555·6) calculated: 34·59% C, 5·62% H, 15·13% N; found: 34·68% C, 5·23% H, 15·09% N. The monomeric form of the peptide was proved by the determination of the number of amino groups[20,21].

o-Nitrobenzenesulfenylglycyl-glycyl-glycyl-S-benzylcysteine Benzyl Ester (*Va*)

The *o*-nitrobenzenesulfenyl group of the tripeptide *III* (1·7 g) was split off by the identical procedure as described for the compound *IV*. Hydrochloride of the tripeptide ester, together with the dicyclohexylammonium salt of *o*-nitrobenzenesulfenylglycine (1·23 g) and 1-hydroxybenzotriazole (0·40 g), were dissolved in dimethylformamide (20 ml), the mixture was cooled to —20°C and N,N'-dicyclohexylcarbodiimide (0·68 g) was added. After stirring for 2h at —20°C and for 12 h at 0°C the precipitated N,N'-dicyclohexylurea was filtered off, the filtrate was taken down and the residue triturated with light petroleum and ether. The crystalline portion was filtered and washed with 1M-H_2SO_4, water, 5% aqueous sodium hydrogen carbonate and water. Crystallization from 2-propanol afforded 1·35 g (75%) of product, m.p. 109—111°C; $[\alpha]_D$ —25·1° (*c* 0·5, dimethylformamide). For $C_{29}H_{31}N_5O_7S_2$ (625·7) calculated: 55·68% C, 4·99% H, 11·19% N; found: 55·90% C, 5·11% H, 11·49% N.

N-Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-glycyl-glycyl-glycyl-S-benzylcysteine Benzyl Ester (*VIa*)

To a solution of the protected tetrapeptide *Va* (1·25 g) in dimethylformamide (3 ml) 3·6M-HCl in ether (1·46 ml) was added and after standing for 2 min at room temperature the mixture was taken down and the residue triturated with ether. To a cooled (—20°C) dimethylformamide (10 ml) solution of N-benzyloxycarbonyl-S-benzylcysteinyl-tyrosine hydrazide (1·07 g) 4·0M-HCl in tetrahydrofuran (2·3 ml) and butyl nitrite (0·3 ml) were added. After stirring for 10 min at 0°C the solution was again cooled to —20°C and after adjusting the pH value to 8·5 with N-ethylpiperidine the dimethylformamide solution of the amino component was added. After standing for 24 h at 0°C and for 2 h at room temperature the mixture was evaporated and the residue was treated with 1M-HCl, water, 5% aqueous sodium hydrogen carbonate and water. The yield

of the product crystallized from 2-propanol was 1·70 g (88%). M.p. 172—176°C, $[\alpha]_D$ —32·8° (*c* 0·5, dimethylformamide); R_F 0·95 (S1), 0·80 (S2), 0·75 (S3), 0·85 (S4). For $C_{50}H_{54}N_6O_{10}S_2 . 0{\cdot}5\ H_2O$ (972·2) calculated: 61·76% C, 5·70% H, 8·65% N; found: 61·78% C, 5·88% H, 8·79% N.

Cyclic Disulfide of Cysteinyl-tyrosyl-glycyl-glycyl-glycyl-cysteine (*Ib*)

The protected hexapeptide *VIa* (0·50 g) was reduced with sodium in liquid ammonia and oxidized with the air at pH 7·0. Inorganic salts were removed with the aid of Dowex 50 (H^+-cycle; elution of the peptidic material by 10% aqueous pyridine). The procedure yielded 250 mg (83%) of the product exhibiting a single spot on electrophoresis (ninhydrin detected). The peptide was purified by gel filtration on a Bio-Gel P—4 column (140 × 2·5 cm) in 1M acetic acid (OD_{280} detection). The chromatographically and electrophoretically homogeneous product was obtained in 47% yield. $E^{Gly}_{2.4}$ 0·65, $E^{Gly}_{5.7}$ 0·0; R_F 0·30 (S1), 0·07 (S2), 0·20 (S3), 0·33 (S4). Amino acid analysis (hydrolysed in the presence of phenol): Gly 2·98, Cys 1·86, Tyr 1·03. $[\alpha]_D$ +14·7° (*c* 0·1, 1M acetic acid). For $C_{21}H_{28}N_6O_8S_2 . C_2H_4O_2 . 3\ H_2O$ (670·7) calculated: 41·18% C, 5·67% H, 12·53% N; found: 41·09% C, 5·17% H, 12·77% N.

o-Nitrobenzenesulfenylisoleucyl-glycyl-glycyl-S-benzylcysteine Benzyl Ester (*Vb*)

o-Nitrobenzenesulfenyl group of the protected tripeptide *III* (0·57 g) was split off as described for the compound *IV*. The tripeptide ester and *o*-nitrobenzenesulfenylisoleucine N-succinimidyl ester (0·57 g) were dissolved in dimethylformamide (2 ml). The pH value of the mixture was immediately adjusted to 8 by N-ethylpiperidine and the mixture was stirred for three days at room temperature. After evaporation the residue was taken up in ethyl acetate and the resulting solution was washed with 1M-H_2SO_4, water, 5% aqueous sodium hydrogen carbonate and water, dried with sodium sulfate and taken down. The residue was triturated successively with light petroleum and ether. Crystallization from 2-propanol afforded 0·55 g (80) of *Vb*, m.p. 155—157°C; R_F 0·88 (S1), 0·80 (S2), 0·83 (S3), 0·82 (S4). $[\alpha]_D$ —57·2° (*c* 0·3, dimethylformamide). For $C_{33}H_{49} . N_5O_7S_2$ (681·8) calculated: 56·63% C, 5·20% H, 10·01% N; found: 56·68% C, 5·68% H, 9·78% N.

N-Benzyloxycarbonyl-S-benzylcysteinyl-tyrosyl-isoleucyl-glycyl-glycyl-S-benzylcysteine Benzyl Ester (*VIb*)

To a solution of the protected tetrapeptide *Vb* (1·36 g) in dimethylformamide (5 ml) 3·6M-HCl in ether (1·46 ml) was added. After standing for 2 min at room temperature the mixture was evaporated, the residue was triturated with ether and the crystalline portion was filtered and washed with ether. To a solution of N-benzyloxycarbonyl-S-benzylcysteinyl-tyrosine hydrazide (1·07 g) in dimethylformamide (3 ml) 6·4M-HCl in tetrahydrofuran (1·56 ml) was added at —20°C. Butyl nitrite (0·3 ml) was added, the solution was stirred for 10 min at 0°C, cooled to —20°C and made alkaline with N-ethylpiperidine (pH 8·5). The dimethylformamide (3 ml) solution of the amino component was then added, the mixture was stirred for 48 h at 0°C, evaporated and the residue was triturated with water, 1M-HCl and water. Crystallization from the mixture 1-propanol–2-propanol afforded 1·63 g (80%) of the product with m.p. 173—175°C. $[\alpha]_D$ —32·5° (*c* 0·15, dimethylformamide); R_F 0·91 (S1), 0·84 (S2), 0·89 (S3), 0·89 (S4). For $C_{54}H_{62}N_6O_{10}S_2 . H_2O$ (1037) calculated: 62·53% C, 6·22% H, 8·10% N; found: 62·84% C, 6·16% H, 8·75% N.

Cyclic Disulfide of Cysteinyl-tyrosyl-isoleucyl-glycyl-glycyl-cysteine (*Ic*)

The protected hexapeptide *VIb* (0·50 g) was reduced with sodium in liquid ammonia. The oxidation was affectuated with the air (the total volume of 500 ml) during the period of 2 h at pH 7.

The solution was concentrated to 150 ml and applied on a Dowex 50 (H^+-cycle) column. The peptidic material was eluted with 10% aqueous pyridine, the eluate was concentrated and freeze-dried leading to 0·20 g (67%) of the electrophoretically homogeneous product, which was further purified by gel filtration on a Bio-Gel P—4 column (140 × 2·5 cm) in 3M acetic acid. The detected peptide (OD_{280}) was freezen-dried affording 57% overall yield of the product. $E^{Gly}_{2.4}$ 0·56, $E^{His}_{5.7}$ 0·00; R_F 0·50 (S1), 0·10 (S2), 0·37 (S3), 0·67 (S4). Amino acid analysis: Gly 2·09, Cys 1·71, Ile 1·07, Tyr 1·04. For $C_{25}H_{36}N_6O_8S_2.C_2H_4O_2.1{\cdot}5\,H_2O$ calculated: 46·34% C, 6·19% H, 12·01% N; found: 46·38% C, 6·02% H, 11·78% N.

Spectroscopic Measurements

The spectra of circular dichroism were recorded on a Roussel–Jouan Dichrographe CD 185 model II in quartz cells with an optical pathlength of 0·02—1·00 cm at 22—25°C (unless otherwise stated). The solutions were prepared by weighing the freeze-dried substances. Concentrations (about 0·3 mg ml^{-1}) were corrected for the respective content of water and acetic acid (see elemental analyses). The data are given as molar ellipticity values ([Θ], deg cm^2 $dmol^{-1}$) and are not corrected for the refractive index of solvent. The following solvents were used: 0·01M phosphate buffer (pH 7·5), 0·01M-HCl (pH 2), methanol, 2,2,2-trifluoroethanol, hexafluoroacetone trihydrate. Temperature dependences were measured in a mixture of solvents[24] (phosphate buffer, pH 7·5, water–ethanol–glycerol 1 : 1 : 1) using a cryostat cooled with liquid nitrogen.

RESULTS

Assignment of the dichroic bands to particular electronic transitions in the molecules of cyclohexapeptides under investigation is based on the previously reported interpretation of the CD spectra of oxytocin, vasopressin and their analogues[3–6]. In the compound *Ia* only amide and disulfide chromophores are present. The two CD bands in the long wavelength region (240 – 320 nm) may be assigned, in analogy with the spectra of model compounds[25,26], to $n-\sigma^*$ transitions of the disulfide chromophore: A negative band with the maximum at 270 – 277 nm and a shoulder in the region of 240 – 250 nm, indicating the presence of a positive band. The latter is, however, well developed only in aqueous solutions. The negative band is in turn stronger in non-aqueous solvents (particularly in methanol). Likewise, the hypsochromic shift of the crosspoint of the CD curves with the zero line (between 253 and 264 nm) indicates, that in non-aqueous solvents the dichroic intensity is transferred from the positive band to the negative disulfide band. Spectra measured in the acidified aqueous or methanolic solutions do not exhibit any significant changes in the region of the disulfide bands. Similarly, the acidic nature of hexafluoroacetone trihydrate compared with 2,2,2-trifluoroethanol does not apply. The negative disulfide band is markedly enhanced at low temperature in a mixture water–ethanol–glycerol (Table I).

Dichroic bands located in the short wavelength region of CD spectra of the cyclohexapeptide *Ia* may be assigned to electronic transitions within the amide groups. The bands are analogous to those of the homodetic cyclohexapeptides, *e.g.* cyclo-

(L-Leu-Gly_5)[27] and cyclo(Gly-L-Ala-L-Leu)$_2$[28], with respect to wavelength, intensity and sometimes even the sign. Thus, there is no reason to suppose any significant manifestation of the disulfide chromophore in this spectral region. The CD band

TABLE I
CD Data on NH_2-L-Cys-$(Gly)_4$-L-Cys-OH (*Ia*)

Solvent[a]	λ^b, nm ($[\Theta] \cdot 10^{-3}$, deg cm^2 dmol^{-1})					
	disulfide bands			amide bands		
				$n-\pi^*$	$\pi-\pi^*$	
Buffer, pH 7·5	276·5 (—0·31)	261 (0)	s 247 (+0·91)	215·5 (+6·1)	197 (—6·2)	185[c] (+10)
Methanol	273 (—1·29)	253 (0)	250[d] (+0·79)	222 (+16·1)	s 205 (+4·6)	194 (—8·1)
TFE	270 (—0·67)	253 (0)	250[d] (+0·29)	214 (+12·5)	199·5 (+20·9)	185[c] (—10)
Buffer, pH 4·9	276 (—0·33)	262 (0)	s 250 (+1·00)	219 (+17·1)	195·5 (—38·7)	
0·01M-HCl	277 (—0·23)	264 (0)	s 248 (+1·04)	221 (+16·1)	196 (—38·6)	
0·05M-HCl in methanol	273 (—1·38)	256 (0)	250[d] (+1·27)	222 (+26·3)	195 (—50·4)	
HFA	271 (—0·79)	252·5 (0)	250[d] (+0·36)	219 (+20·7)	188·5 (—37·9)	
B., pH 4·9 at +12·5°C	273 (—0·48)	260 (0)	s 250 (+1·07)	219 (+18·9)	194·5 (—41·6)	
B., pH 4·9 at +72°C	277 (—0·24)	261 (0)	s 250 (+0·65)	218·5 (+10·8)	195 (—17·8)	
Mixed at —80°C	268·5 (—2·69)	250·5 (0)	250[d] (+0·25)	226 (+6·9)	[e]	
Mixed at +40°C	268 (—0·82)	252 (0)	250[d] (+0·25)	223·5 (+4·9)	[e]	

[a] TFE 2,2,2-trifluoroethanol, HFA hexafluoroacetone trihydrate, mixed solvent is a mixture of ethanol, water and glycerol (1 : 1 : 1); [b] s denotes shoulder; [c] end value; [d] ellipticity value at 250 nm; [e] not measured.

of the amide $n-\pi^*$ transitions is located at 214–222 nm and exhibits a positive sign in all solvents. The bands in the 185–210 nm region belong to the components of the exciton splitted amide $\pi-\pi^*$ transition. We observe either a sole high intensity band or a couple of oppositely signed bands (the maximum of the short wavelength band being usually outside the region accessible to measurement). Parameters of the amide bands are markedly dependent on polarity and acidity of the solvent (Table I, Fig. 1). In the neutral aqueous solution intensities of the amide bands are low. In alcohols we observe a significant change in the $\pi-\pi^*$ transition region in addition to the intensity enhancement of the $n-\pi^*$ band. In methanol a shoulder of the positive band appears at 205 nm besides the low intensity negative band. The change is completed in 2,2,2-trifluoroethanol. The signs of the pair of the $\pi-\pi^*$ bands are opposite to those exhibited in the neutral buffer. Lowering the pH value of the aqueous solution to 4·9 results in almost threefold increase of the $n-\pi^*$ Cotton effect (combined with its bathochromic shift) and a sixfold increase of the negative Cotton effect due to the $\pi-\pi^*$ transition. No marked changes are observed when pH is further lowered to the value of 2. Acidification of the methanolic solution results in similar effects. In the proton donating hexafluoroacetone trihydrate the parameters of the amide bands are similar to those characteristic for the acidic aqueous solutions (Table I).

A slight increase in intensity of the positive $n-\pi^*$ band is observed when the temperature is lowered (in water–ethanol–glycerol). Similar but much more pronounced temperature dependence is exhibited by both the amide bands ($\Delta[\Theta]/\Delta t =$ $= 16$ and $136\,[\Theta]$ units per deg, respectively) in the acidic aqueous solution (pH 4·9).

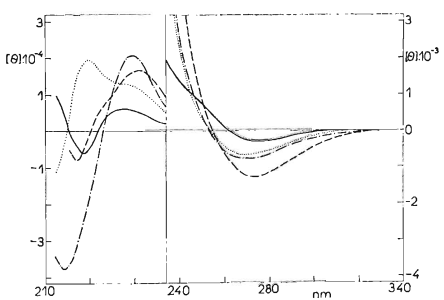

FIG. 1
Circular Dichroism Spectra of NH_2-L-Cys-$(Gly)_4$-L-Cys-OH (*Ia*) in Buffer, pH 7·5 (———), in Methanol (– – –), in 2,2,2-Trifluoroethanol (····), in Hexafluoroacetone Trihydrate (–·–·–·–)

CD spectra of the cyclohexapeptides *Ib* and *Ic* are more complex owing to the presence of the tyrosine aromatic side chain the dichroic bands of which are related

TABLE II
CD Data on NH_2-L-Cys-L-Tyr-$(Gly)_3$-L-Cys-OH (*Ib*) and N-Acetyl-L-tyrosine Methylamide

Solvent[a]	λ^b, nm ([Θ] . 10^{-3}, deg cm^2 dmol^{-1})					
	composite aromatic and disulfide band			composite amide and aromatic bands		
	NH_2-L-Cys-L-Tyr-$(Gly)_3$-L-Cys-OH (*Ib*)					
Buffer, pH 7·5	273 (—1·14)	252 (0)	226 (+19·9)	m 214·5 (+13·0)	199·5 (+43·1)	192[c] (—40)
Methanol	269 (—2·26)	249·5 (0)	230 (+17·5)	m 216·5 (+6·1)	200 (+65·3)	[d]
TFE	272·5 (—1·29)	249 (0)	225 (+12·8)	m 215 (+5·3)	200 (+43·1)	190[c] (—15)
0·01M-HCl	274·5 (—1·17)	254 (0)	225 (+40·0)	m 212·5 (+23·2)	202·5 (+41·8)	191·5[c] (—35)
HFA	272 (—1·40)	251 (0)	223 (+37·9)	m 211 (+22·4)	200 (+43·0)	
0·01M-NaOH	289 (—0·79)	269 (0)	240 (+16·6)	m 224 (+12·7)	205 (+43·5)	195[c] (+8)
Mixed at —80°C	264·5 (—4·19)	250 (0)	226·5 (+22·1)	m 215 (+10·2)	[d]	
Mixed at +40°C	267·5 (—2·59)	250 (0)	228·5 (+14·1)	m 217·5 (+4·8)	[d]	
	N-Acetyl-L-tyrosine Methylamide					
Buffer, pH 7·5	276 (—0·43)	—	224·5 (+18·9)	m 214 (+11·7)	199·5 (+43·9)	
Methanol	[d]		227·5 (+16·3)	m 215·5 (+6·0)	200 (+42·8)	
0·01M-NaOH	297·5 (+0·16)	—	240 (—9·8)	m 224 (+7·4)	206 (+29·5)	

[a] TFE denotes 2,2,2-trifluoroethanol, HFA hexafluoroacetone trihydrate, mixed solvent is a mixture of water, ethanol and glycerol (1 : 1 : 1); [b] m denotes minimum; [c] end value; [d] not measured.

to $^1B_{2u} \leftarrow {}^1A_{1g}$, $^1B_{1u} \leftarrow {}^1A_{1g}$, and $^1E_{1u} \leftarrow {}^1A_{1g}$ transitions of the benzene chromophore. These bands overlap with those of the amide and disulfide groups. The long

TABLE III
CD Data on NH_2-L-Cys-L-Tyr-L-Ile-$(Gly)_2$-L-Cys-OH (*Ic*) and Oxytocin

Solvent[a]	λ^b, nm ($[\Theta] \cdot 10^{-3}$, deg cm^2 dmol^{-1})							
	composite aromatic and disulfide band				composite amide and aromatic bands			
	NH_2-L-Cys-L-Tyr-L-Ile-$(Gly)_2$-L-Cys-OH (*Ic*)							
Buffer, pH 7·5		274 (−0·61)	s 255 (−0·37)	247 (0)	226 (+16·5)	m 214 (+8·6)	201 (+28·1)	189 (−43)
Methanol	s 282 (−1·10)		261·5 (−1·70)	247 (0)	228 (+24·8)	m 217 (+15·3)	203 (+60·2)	193[c] (−47)
TFE	s 281 (−0·65)		262 (−0·81)	243 (0)	225·5 (+7·9)	m 215 (+3·8)	201 (+22·8)	[d]
0·01M-HCl		273 (−0·77)	s 257 (−0·60)	245 (0)	225 (+21·6)	207[e] (+3·0)		190[c] (−50)
0·05M-HCl in methanol	s 281 (−0·52)		267 (−0·61)	247 (0)	226·5 (+24·2)	m 209 (+7·2)	203·5 (+10·1)	195[c] (−34)
HFA		273·5 (−1·72)	s 255	247 (0)	224 (+31·3)	s 213 (+12·9)	s 200 (−19·3)	192[c] (−39)
0·01M-NaOH		[d]			239 (+10·9)	m 220 (+4·7)	210 (+12·8)	[d]
Mixed at −80°C		262 (−1·58)		245·5 (0)	226 (+37·9)	m 215 (+23·2)	[d]	
Mixed at +40°C		261 (−1·22)		244 (0)	228·5 (+14·1)	m 218 (+5·4)	[d]	
	Oxytocin[f]							
Buffer, pH 3·0	282 (−0·45)	271 (0)	s 250 (+1·90)		226 (+22·3)	s 211 (−9·4)		196 (−70)
HFA	280 (−1·28)	263 (0)	s 251 (+2·30)		225 (+32·0)	s 213 (+16·8)		195 (−46)

[a] TFE 2,2,2-trifluoroethanol, HFA hexafluoroacetone trihydrate, mixed solvent is a mixture of ethanol, water and glycerol (1 : 1 : 1); [b] m denotes minimum, s shoulder; [c] end value; [d] not measured; [e] broad shoulder; [f] see ref.[3].

wavelength CD extremum lying within the limits of 261 and 280 nm represents now a composite band, the dichroic absorption of which is contributed by the $n-\sigma^*$ transition of the disulfide grouping as well as by the longest wavelength aromatic $\pi-\pi^*$ transition (B_{2u}). This composite band exhibits a negative sign for compounds *Ib* and *Ic*. In the case of compound *Ib* the related parameters are not much sensitive to a solvent change. It may be deduced from the course of the spectrum measured in the alkaline aqueous solution (Table II) that the intensity of the band is contributed more seriously by the aromatic transition. The intensity of the band appears approximately doubled only in methanolic solution probably due to the higher participation of the disulfide transition. The shoulder caused by the short wavelength disulfide transition, which is indicated in the spectra of *Ia* at 250 nm, is not detectable. The total dichroic absorption at 250 nm remains small positive or even a negative one (in methanol and 2,2,2-trifluoroethanol).

In general, the intensity of the long wavelength composite band of the cyclohexapeptide *Ic* (Table III, Fig. 4) is lower and distributed in a different way when compared with the compound *Ib*. The negative shoulder at about 255 nm (aqueous solutions, hexafluoroacetone) and the negative maximum at 261 nm (methanol, 2,2,2-trifluoroethanol) are related to the transitions within the disulfide group. The negative dichroism of the broad bands at 290 nm and higher wavelengths is of the same origin. The contribution of the aromatic transitions to the composite band intensity is significantly smaller than in the case of the compound *Ib*. The negative long wavelength

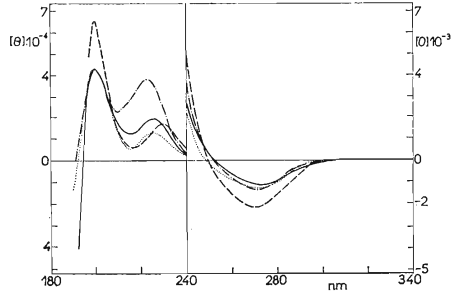

FIG. 2
Circular Dichroism Spectra of NH_2-L-Cys-L-Tyr-$(Gly)_3$-L-Cys-OH (*Ib*) in Buffer, pH 7·5 (———), in Methanol (– – –), in 2,2,2-Trifluoroethanol (· · · · · ·), in Hexafluoroacetone Trihydrate (–·–·–·–)

band of the compound *Ib* exhibits hypsochromic and hyperchromic effect on lowering the temperature (in water–ethanol–glycerol), while the analogous band of the compound *Ic* undergoes only insignificant changes.

The compounds *Ib* and *Ic* possess a single positive band at 223 – 230 nm (Tables II and III, Figs 2 and 4) which involves contributions of both the transition types located in this spectral region, *i.e.* $n-\pi^*$ transitions of the amide groups and the $^1B_{1u}$ transition of the aromatic chromophore. If positions of this maximum and of the $n-\pi^*$ maximum of *Ia* are compared and if the hypsochromic shift towards 240 nm in alkaline medium is considered it may be assumed, that for both the compounds, *Ib* and *Ic*, the positive ellipticity is marshalled by the contribution of the $^1B_{1u}$ transition located at slightly higher wavelength. Protonation of the primary α-amino group in acidic aqueous solution and in hexafluoroacetone results in an intensity increase of the composite positive band. This increase is, probably, enhanced by the $n-\pi^*$ component of the band (*cf.* spectra of the compound *Ia*) as may be deduced *e.g.* from the presence of a shoulder at about 215 nm in hexafluoroacetone solutions of the compounds *Ib* and *Ic* or from the difference spectra (Fig. 3).

The short wavelength region is also characterized by the overlap of closely neighbouring bands caused by $\pi-\pi^*$ amide transitions and $^1E_{1u}$ transition of the phenol chromophore. Assignment of bands located in this experimentally difficult region is uncertain even for simple model compounds[27,29]. Cyclopeptide *Ib* is characterized by a very strong positive band at 199 – 200 nm the dichroic absorption of which is only little sensitive to a change in solvent or in ionic state of the primary amino

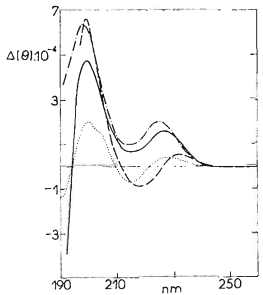

FIG. 3

Difference Circular Dichroism Spectra Obtained by Subtracting the Spectrum of NH_2-L-Cys-$(Gly)_4$-L-Cys-OH (*Ia*) from NH_2-L-Cys-L-Tyr-$(Gly)_3$-Cys-OH (*Ib*) in Buffer, pH 7·5 (———), in Methanol (– – –), in 2,2,2-Trifluoroethanol (· · · · ·), in Hexafluoroacetone Trihydrate (–·–·–)

group. In the alkaline aqueous solution, the band is shifted to 205 nm (*Ib*) or 210 nm (*Ic*) similarly to the behaviour of the aromatic bands at higher wavelengths (Tables II and III). In this context it is interesting to note that the simple model, N-acetyl-L-tyrosine N'-methylamide, displays spectra, which are almost identical with those of the cyclopeptide *Ib* including the shifts of dichroic bands in alkaline medium (Table II). Consequently, the short wavelength positive band of the compound *Ib* (and similarly the analogous band of *Ic*) is directly conditioned by the presence of tyrosine side chain and probably belongs to the aromatic $^1E_{1u}$ transition.

Contribution of the tyrosine side chain to the CD spectrum of the cyclopeptide *Ib* is well illustrated by the difference spectra obtained by subtracting the spectra of *Ia* from those of *Ib* (Fig. 3). The positive difference maximum found at about 225 – 228 nm indicates a dominant contribution of the B_{1u} transition to the composite positive band. This contribution is significantly lower for solutions in methanol and 2,2,2-trifluoroethanol in which the difference curve in the $n-\pi^*$ transition region (225 to 230 nm) becomes negative. These findings indicate that in the above-mentioned solvents introduction of the tyrosine residue into the molecule causes the amide $n-\pi^*$ band to decrease in intensity or even to change the sign.

TABLE IV
CD Data on NH_2-L-Cys-L-Lys-L-Ala-$(Gly)_2$-L-Cys-OH (*VII*)

Solvent[a]	λ^b, nm ($[\Theta]$. 10^{-3}, deg cm^2 dmol^{-1})						
	disulfide bands				amide bands		
					$n-\pi^*$	$\pi-\pi^*$	
Buffer, pH 7·0	273·5 (—0·45)	257 (0)	s 250 (+0·42)		217·5 (+15·0)	198·5 (—29)	
Methanol	272 (—1·35)	255 (0)	250[c] (+0·37)		220 (29·6)	194 (—25)	
TFE	270 (+0·21)	253·5 (0)	250[c] (—0·21)	238 (—1·5)	211 (+19·0)	201[d] (+15·5)	189·5 (+35)
0·01 M-HCl	272 (—0·46)	254 (0)	s 248 (+0·27)		220 (+10·1)	198 (—41)	
HFA	276 (—0·09)	263 (0)	256 (+0·07)	228·5 (—6·4)	211 (+2·6)	200·5 (—5·9)	191·5 (+21)

[a] TFA denotes 2,2,2-trifluoroethanol, HFA denotes hexafluoroacetone trihydrate; [b] s denotes shoulder; [c] ellipticity at 250 nm; [d] a negative band appearing as a positive minimum.

Contrary to the compound *Ib*, the dichroic absorption of the compound *Ic* in the short wavelength region is markedly solvent sensitive (Table III, Fig. 4). In methanolic solution, the above mentioned positive band exhibits about the same intensity as in the case of *Ib*, while in 2,2,2-trifluoroethanol and in buffer (pH 7·5) the intensity of the band (which could be again ascribed to the E_{1u} transition) is much lower. The band disappears in protonating solvents. In 0·01M-HCl we observe only

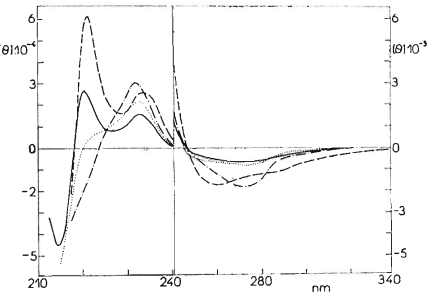

FIG. 4
Circular Dichroism Spectra of NH_2-L-Cys-L-Tyr-L-Ile-$(Gly)_2$-L-Cys-OH (*Ic*) in Buffer, pH 7·5 (———), in 0·01M-HCl (· · · · ·), in Methanol (— — —), in Hexafluoroacetone Trihydrate (-·-·-·-)

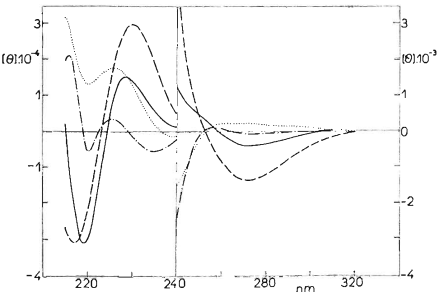

FIG. 5
Circular Dichroism Spectra of NH_2-L-Cys-L-Lys-L-Ala-$(Gly)_2$-L-Cys-OH (*VII*) in Buffer, pH 7·5 (———), in Methanol (— — —), in 2,2,2-Trifluoroethanol (· · · · · ·), in Hexafluoroacetone Trihydrate (-·-·-·-)

a broad shoulder at 207 nm on a curve sharply declining to negative ellipticity values. A shoulder at 200 nm in hexafluoroacetone indicates the presence of a negative band. There is one additional negative band in the spectra of compounds *Ib* and *Ic*. The maximum of this band is located outside the region accessible to measurement (except for *Ic* in neutral buffer). Temperature dependence of CD spectra of both compounds (measured in water–ethanol–glycerol) is qualitatively similar, *i.e.* intensity of the dichroic bands increases with decreasing temperature. With *Ic*, we observe a more pronounced temperature dependence of the B_{1u} maximum and of the minimum at 215 nm (which indicates changes of the amide $n-\pi^*$ band).

The cyclohexapeptide *VII* does not fall into the series of compounds *Ia*–*Ic* which simulate the stepwise build up of the oxytocin ring moiety. The compound does not contain aromatic chromophore, however, in the positions 2 and 3 amino acids with side chains are present.

Therefore, it will be interesting to compare its CD spectra with those of *Ia* and *Ic*. The spectra of *VII* in aqueous solutions and in methanol (Table IV, Fig. 5) are similar to those of *Ia*, as concerns number of bands and their respective signs. In the region of the disulfide bands, even a quantitative similarity exists, but for the compound *VII* the intensity is slightly changed in favour of the negative band. The same similarity is expressed also in the spectra measured in methanol: Intensity of the positive amide $n-\pi^*$ band increases while the negative ellipticity of the $\pi-\pi^*$ band does not change when compared with the neutral buffer. The bands related to compound *VII* are stronger. Protonation of the α-amino groups results with both compounds, *Ia* and *VII*, in comparably increased intensities of the negative $\pi-\pi^*$ band, however only with *VII* in a decrease of the $n-\pi^*$ band ellipticity. The compound *VII* exhibits markedly different behaviour in strongly polar organic solvents. In hexafluoroacetone, there are two bands in the region of the disulfide transitions: a weak negative band at long wavelength and a distinct positive band at 256 nm. Only one broad positive band at 270 nm is present in this region in 2,2,2-trifluoroethanol. The distinct negative band at 238 nm can be ascribed to the amide $n-\pi^*$ transition, with respect to the presence of a similar band in the hexafluoroacetone solution. Thus, contrary to the spectra of *Ia*, the compound *VII* exhibits a negative $n-\pi^*$ band at longer wavelength in addition to the positive one. The couple of $\pi-\pi^*$ bands is of opposite sense as compared with the spectra of *Ia* (2,2,2-trifluoroethanol solution), the long and short wavelength component being negative (positive minimum in 2,2,2-trifluoroethanol) and positive, respectively.

DISCUSSION

The CD spectra of cyclohexapeptides *Ia*–*Ic* and *VII* constitute an experimental basis facilitating conformational considerations. Molecular chirality of the compound *Ia* originates solely from the cystine moiety. The tetraglycine chain is supposed

to have appreciable conformational flexibility, due to the minimal steric restrictions. In solution, the compound probably exists as a mixture of conformers the dichroic contributions of which can efficiently compensate. This assumption is well met by CD spectra of the cyclohexapeptide *Ia* in neutral aqueous solution or in methanol. In these solvents the amide $\pi-\pi^*$ bands, the rotational strengths of which originate from the interactions among the amide groups and which, in consequence, reflect the peptide backbone conformation in the most sensitive way, exhibit very small intensities. The flat temperature dependence in water–ethanol–glycerol indicates small energy differences among conformers. The spectra in other solvents are characterized by a significant increase in the $\pi-\pi^*$ bands intensities which might be related to the predominance of a certain conformation of the peptide chain. The conformational type preferred in 2,2,2-trifluoroethanol, which is hydrogen-bonded to the carbonyl oxygen[30], is characterized by a couple of $\pi-\pi^*$ bands with signs opposite to those in the neutral buffer. Different conformational type seems to be preferred in aqueous and methanolic HCl or in hexafluoroacetone, where a strong negative temperature dependent $\pi-\pi^*$ band is observed. The enhancement of the $\pi-\pi^*$ bands intensities is accompanied by an increase in intensity of the amide $n-\pi^*$ band, the disulfide bands being unchanged. These spectral changes are caused primarily by protonation of the α-amino group and not by the properties of the solvent used. The corresponding conformational changes arise probably from the interaction of the positive charge with the dipoles of the adjacent amide groups. As far as the $\pi-\pi^*$ bands are concerned, the spectra of *VII* in aqueous solutions and in methanol exhibit similar properties to those of *Ia*. The higher band intensities of *VII* may be explained as an effect of the conformational flexibility limitations by the side chains, which results in the predominance of one conformational type. However, we may state that, under given conditions, both the compounds possess similar conformations and undergo similar conformational changes. The side chains in the positions 2 and 3 do not seriously influence the interactions of the compound *VII* with solvent. On the contrary, in 2,2,2-trifluoroethanol and in hexafluoroacetone the preferred conformation of *VII* is, according to the $\pi-\pi^*$ bands pattern, different from that characterizing the compound *Ia* under identical conditions. The difference could be explained by greater solvation ability and/or bulkiness of these solvents resulting with *VII* in steric interactions of the bound solvent molecules with side chains.

Intensity of the positive amide $n-\pi^*$ band of the compound *Ia* is changing in parallel with the intensity of the negative disulfide band in neutral as well as in protonating media. On the other hand, the amide $n-\pi^*$ band does not follow the changes of the $\pi-\pi^*$ band due to the solvent, excepting protonation of the α-amino group. This implies, that the properties of the positive amide $n-\pi^*$ band are not directly related to the conformation of the peptide backbone, but merely to the presence and manifestation of various conformations of the heterodetic joint of the cyclohexapeptide. (The positive amide $n-\pi^*$ band is not typical for homodetic cyclohexapeptides

possessing aliphatic side chains, *e.g.* cyclo(Gly-L-Ala-L-Leu)$_2$, cyclo(Gly-L-Val-L-Leu)$_2$[28], cyclo(Gly$_2$-L-Leu)$_2$[27].) We may deduce that most of the $n-\pi^*$ band rotational strength originates from the amide group adjacent to the cysteine residue in the position 1. A similar dependence of the $n-\pi^*$ component of composite positive band on the location of the sulfur atom as well as on the presence and ionic state of the α-amino group was previously observed with the series of oxytocin carba-analogues[3].

Circular dichroism of the amide $n-\pi^*$ transition is also influenced by the presence and nature of side chains. The origin of the positive band in the spectrum of *VII* is probably identical with that of *Ia*, while negative long wavelength band is likely to originate from other amide groups. The decrease in intensity of the positive $n-\pi^*$ band of *VII*, found in 0·01M-HCl in contrast to *Ia*, may be explained by the effect of the positively charged ε-amino group of the lysine residue. This charge compensates the effect of the charged α-amino group of cysteine 1 and/or causes an interaction resulting in a local conformational change.

CD bands related to the disulfide group represent a potential source of information concerning conformation of the heterodetic joint. ^{13}C-NMR spectra of oxytocin and its analogues[31] afford evidence on a significant conformational mobility around S—C bonds. As follows from the Raman spectra[32–34], several conformations of the disulfide group coexist in solutions of oxytocin and lysine-vasopressin. Hence, conformational mobility of the disulfide segment is to be expected also for the cyclohexapeptides under study. The observed changes of the disulfide bands may then be rationalized on the basis similar to that used by Maxfield and Scheraga[32] in their interpretation of published[1–5] CD spectra of neurohypophyseal hormones. The CD spectra of *Ia* and *VII* can be interpreted as a superposition of one positive band at 250 nm and a doublet (or several doublets) of bands which are symmetrically centered around the singlet band and have a short wavelength ($\lambda < 250$ nm) positive lobe and a long wavelength ($\lambda > 250$ nm) negative lobe. The positive lobes overlap with the stronger positive $n-\pi^*$ band and, hence, only the negative lobes can be identified in the spectra. The positive band at 250 nm, detected as a shoulder, indicates the presence of disulfide conformers with a torsion angle close to $\pm 90°$ (ref.[35,36]). Optical activity of these conformers arises solely from perturbations of the disulfide chromophore by the neighbouring chiral centres[26,37,38]. Their rotational strength may be comparable with that resulting from the inherent chirality of the disulfide group[26,37] and is given by the rotamer distribution around C_α—C_β bonds of the cystine residue[26]. Rotational strengths of the oppositely signed couple of bands originate then from the inherent chirality of the disulfide chromophore[35,36]. Hence, a negative band indicates the presence of conformers with the disulfide torsion angle significantly differring from $\pm 90°$.

We may suggest the following interpretation of the CD spectra of *Ia*: In aqueous solutions, the prevailing conformer with dihedral angle $\varphi = \pm 90°$ is significantly

accompanied by conformers with a different φ angle. From the small width of the negative band we may deduce that this group of conformers is more homogeneous (as to the φ values) than conformers population in methanolic solution. Location of the band maximum (271 – 277 nm) correlates best with a declination of 30° from ±90°. Due to the negative sign of the band, the dihedral angle values of +120° or −60° are expected[32,35]. (The position of the maximum is in agreement with that found for [2-hemicystine, 7-hemicystine]gramidicin S)[39]. In alcoholic solvents, the ratio of conformers is shifted in favour of the type involving dihedral angle values different from ±90°. This conformational type is probably prevailing in methanol. The greater width of the negative band implies the presence of a minor proportion of conformers exhibiting a dihedral angle declination greater than 30°. The effect of lowering the temperature, which is constrained to intensity changes of the negative band, is to be interpreted rather as a consequence of a restricted overall internal mobility of the molecule than as a change in the population of the particular conformational types.

Side chains of residues in positions 2 and 3 of *VII* have only a limited influence on the overall conformation of the disulfide moiety as far as aqueous and methanolic solutions are concerned (a certain increase in population of the conformational type with $\varphi \neq \pm 90°$). On the contrary, a manifestation of the structural differences between *Ia* and *VII* is found in strongly interacting polar solvents. The overall spectral change in hexafluoroacetone reflects a shift of the conformational equilibrium towards conformers with a rectangular arrangement of the disulfide group. (The observed intensity of the positive singlet at 257 nm which characterizes these conformers, may be low due to the superposition with the negative amide $n-\pi^*$ band.) The positive long wavelength band in 2,2,2-trifluoroethanol indicates the presence of conformers with the φ value differing from ±90° in the opposite sense (*e.g.* $\varphi = +60°$ or $-120°$). It is obvious, that the mentioned changes in conformation of the disulfide group of the compound *VII* must be connected with changes of the backbone conformation, which are indicated by the variations of amide $\pi-\pi^*$ bands.

CD spectra of *VII* in the region of the disulfide bands were previously investigated by Siemion and Klis[15]. According to our interpretation only the negative band at 270 – 273 nm is assigned to conformers with the dihedral angle φ differing from ±90° by about 30°. Assignment of a defined helicity to the disulfide group is a matter of choice between the two possible values of the φ angle (+120° or −60°). The value of +120° and, hence, the *P* helicity of the disulfide group seems to be preferable for the compound *VII* on the basis of molecular model studies[15]. The identical type of helicity ($\varphi = +90°$) may be assigned also to those conformers of *VII* which are well characterized by a positive singlet at 250 nm. Similar considerations may be applied to cyclopeptides *Ia–Ic* and even to oxytocin, the disulfide group of which has been suggested to possess the same *P*-helical arrangement (see *e.g.* ref.[10]). The

relationships between the disulfide bands of our model cyclopeptides and those exhibited by oxytocin indicate that disulfide groups in these compounds are arranged in a uniform manner, *i.e.* the torsion angle value may vary within certain limits, but does not change the sign.

There is no unequivocal preference for the energetically most favourable rectangular arrangement of the disulfide group, although the ring of this size represents a system with not too much strain. CD spectra similar to those of *Ia* are exhibited by cyclopeptides possessing similar structure Boc-L-Cys-Gly_x-L-Cys-OMe (ref.[40]), where $x = 3$, 4 and 5. Intensity of the long wavelength negative band of these compounds is not very dependent on the ring size. Therefore, the conformation or the distribution of conformers of the disulfide group seems to be given rather by interactions of substituents on the cystine α-carbon atoms than by the ring size.

Different situation applies to the compound *Ib*. Its CD spectra are dominated by bands related to transitions within the tyrosine side chain (at 275, 225 and 200 nm) and exhibit only a small solvent and temperature dependence. Rotational strengths of aromatic transitions in analogous peptides arise from their interactions with the $\pi-\pi^*$ transitions of the proximate amide groups (electric dipole coupling mechanism)[29,41,42]. In addition, it follows from the reported papers that the aromatic bands are dependent differently on the relevant dihedral angles. In general, the band due to the $^1B_{2u}$ transition, which is polarized perpendicularly to the direction of the C_β—C_γ bond, seems to be more sensitive to conformational changes[41]. A similar sensitivity should be expected for the short wavelength $^1E_{1u}$ transition, especially for its long wavelength component exhibiting the identical polarization[29]. The conformation of *Ib* is relatively stable and homogeneous, at least as far as the spatial relations of the aromatic nucleus to the neighbouring amide groups are concerned. Solvation interactions leave this spatial arrangement unchanged. Some ideas concerning the tyrosine conformation in *Ib* may be achieved on the basis of comparison with N-acetyl-L-tyrosine methylamide which possesses similar chiroptical properties including small sensitivity to solvent change. Detailed conformational energy calculations based on empirical energy functions[43] are available for this amide. The three most favourable conformations, which differ little in energy and possess the overall statistical weight of 70%, belong to the extended type of the peptide backbone conformation (C_5 conformation, $\Phi \sim -150°$, $\Psi \sim 140°$). As far as the side chain conformations are concerned, the largest population was calculated[43] for the staggered conformation ($\chi_1 = -180°$, $\chi_2 = \pm 90°$) in which the aromatic ring is close to the carbonyl group of tyrosine. The calculated[43] conformation conforms with spectral properties of N-acetyl-L-tyrosine methylamide. The solvent is not expected to influence seriously either the extended conformation of the peptide backbone or the side chain conformation. We assume that the tyrosine residue in *Ib* exists in a conformation close to that calculated[43] for the isolated dipeptide

unit and, hence, to the conformation suggested for the oxytocin ring moiety[9-11], *i.e.* a β-turn with tyrosine localized in the extended part. The tyrosine side chain seems to play an important role in stabilising β-turn conformation.

The effect of solvent on the spectra of *Ib* is more discernible on the intensity of the positive band located at 223–230 nm. In protonating solvents, this effect of charge on the α-amino group is mediated rather by the protonated α-amino group than by a conformational change of the aromatic side chain. In methanol, unusually high intensities are observed for the long wavelength negative band (similarly as found for *Ia* and *VII*) as well as for the short wavelength aromatic band. It is probable that in this solvent a more extensive reorganization operates, favouring conformations with the disulfide dihedral angle different from ±90°. This reorganization may result in a conformational change of the tyrosine side chain in *Ib* indicated by intensity change of the aromatic band at 200 nm.

The most important consequence of introducing the isoleucine residue into the position 3 of *Ic* consists in the fact that the dichroism of the band at 200 nm becomes strongly dependent on the solvent (contrary to *Ib*). Assuming the positive band at 200 nm to be of aromatic origin, we may interpret this spectral change as the result of a conformational change of the tyrosine side chain. It may be supposed that, in accord with calculations[43], the value of χ_2 related to tyrosine does not deviate too much from ±90°. Then the intensity changes of the positive band at 200 nm would correspond mainly to the deviations of χ_1 from the preferred value of 180°. These conformational changes could be caused by a steric interaction with the bulky side chain of isoleucine which represents the first corner residue of the β-turn conformation of *Ic*. The dependence on solvent may then be caused by the varying interactions of both the side chains in the particular solvent–solute complex.

Supposing that the tyrosine dipeptide unit is relatively rigid, a change in the Φ angle of the isoleucine residue would be critical in the first place. The conformational change of the peptide backbone may in turn take part in variations of the short wavelength region of CD spectra through contributions of the amide $\pi-\pi^*$ bands.

The smallest interaction of the tyrosine and isoleucine side chains is expected in methanol and in water–ethanol–glycerol at 40°C, as follows from the similarity of spectra exhibited by *Ib* and *Ic*. Under these conditions, both the compounds possess identical or similar conformations in the tyrosine containing segment. This conformation remains also preferred at low temperature where it is more pronounced for *Ic* than for *Ib*. A change in the tyrosine side chain conformation due to the interaction with solvent is indicated in 2,2,2-trifluoroethanol and in neutral buffer. The protonated α-amino group takes part in conformational changes in methanolic or aqueous HCl because of the interaction with the aromatic π-electron system. This interaction is more effective in *Ic* since the tyrosine side chain conformation is altered by the interaction with isoleucine. Both the above mentioned mechanisms are operating in strongly interacting hexafluoroacetone.

The long wavelength region of the spectra of *Ic* indicates that, compared with *Ib*, the stereochemistry of the disulfide group is further shifted in favour of conformations with the dihedral angle φ different from $\pm 90°$. The broad negative band at about 280 nm found in the methanolic solution suggests the presence of several conformers possessing various disulfide φ values. The present investigation of model cyclohexapeptides *Ia–Ic* and *VII* affords some information useful for a more detailed understanding of CD spectra of oxytocin and related natural or chemically modified neurohypophyseal hormones. The assignment of particular dichroic bands to the electronic transitions within the amide, disulfide and aromatic chromophores was firmly established. The tyrosine residue in position 2 most probably stabilizes the extended part of the oxytocin-ring conformation. Conformational space of the tyrosine side chain is seriously limited by the isoleucine side chain. Conformational effect caused by protonation of the α-amino group is inherent already to the non-substituted cyclohexapeptide ring. The compound *Ic*, the structure of which is most similar to that of oxytocin or tocinamide, possesses in protonating media CD spectra which are very similar below 240 nm to those of the full hormone. Thus, side chains in positions 2 and 3 are sufficient to determine the conformation which roughly corresponds to that of oxytocin. On the other hand, the model compound *Ic* differs significantly from oxytocin (more than *Ia*) as to the conformation of the disulfide group. Oxytocin and analogues assume[32] a dominant conformation with the dihedral angle φ close to $\pm 90°$. Conformation of the disulfide group is given rather by the character and interactions of substituents close to the heterodetic joint than by the ring size.

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