# THREE CYCLOHEXAPEPTIDES MODELLING THE OXYTOCIN RING MOIETY: SYNTHESIS AND CIRCULAR DICHROISM*

Jan HLAVÁČEK, Ivo FRIČ, 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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A series of cyclic disulfides cysteinyl-triglycyl-asparaginyl-cysteine (*Ib*), cysteinyl-diglycyl-glutaminyl-asparaginyl-cysteine (*Ic*), and cysteinyl-glycyl-isoleucyl-glutaminyl-asparaginyl-cysteine (*Id*) has been prepared. The effect of gradual attachment of side chains to the ring on the peptide backbone and the disulfide group conformation has been studied using circular dichroism. Introduction of side chains reduces substantially the conformational mobility of the backbone, but not enough to let any conformational β-turn type predominate (in polar solvents). Conformation of the disulfide group is essentially independent of the peptide moiety of the molecule and is influenced by specific solvation.

In our previous paper[1] we have described the synthesis of three heterodetic cyclohexapeptides derived from the ring part of oxytocin: The parent model compound H-Cys-$(Gly)_4$-Cys-OH (*Ia*)**, its derivative with tyrosine instead of glycine in position 2, and the still more substituted analogue having, in addition to the tyrosine in position 2, an isoleucine residue in position 3. CD studies revealed that incorporation of side chains effectively reduces conformational mobility of the unsubstituted parent cyclohexapeptide. The tyrosine side chain probably stabilizes an extended conformation of the corresponding dipeptide unit. Introduction of isoleucine into position 3 affects the tyrosine conformation to such an extent that in protic solvents the CD spectra of the cyclohexapeptide with these two side chains are essentially the same as those of oxytocin and the compound has a similar conformation in the region of positions 1 to 3. The two disulfide bands were ascribed to two conformational types of this grouping: Besides the rectangular arrangement there are present – and usually rather predominate – conformers with torsion angle significantly different from 90°.

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** The nomenclature and symbols of the amino acids, peptides and protecting groups obey the IUPAC-IUB recommendations. The chiral amino acids used in this work are of the L-configuration.

In the present study we prepared a complementary series of model heterodetic cyclohexapeptides *Ib*–*Id* without tyrosine residue. They were prepared by stepwise synthesis of the oxytocin ring starting from the carboxyl terminus of the peptide chain. The effect of non-aromatic side chains on the peptide backbone conformation and the disulfide grouping was studied by CD spectroscopy.*

The cyclic hexapeptides *Ib*–*Id* were obtained by clasical methods of peptide synthesis in solution; most of the protecting groups were selected so as they could be removed by reduction with sodium in liquid ammonia. Tert-butyl esters of the earlier described[3] peptides with carboxy-terminal S-benzylcysteine were used as intermediates in the synthesis of the desired hexapeptides.

H-Cys-Gly-Gly-Gly-Gly-Cys-OH
*Ia*

H-Cys-Gly-Gly-Gly-Asn-Cys-OH
*Ib*

H-Cys-Gly-Gly-Gln-Asn-Cys-OH
*Ic*

H-Cys-Gly-Ile-Gln-Asn-Cys-OH
*Id*

Trt-Gly-Asn-Cys(Bzl)-OBu$^t$
*II*

Trt-Gly-X-Asn-Cys(Bzl)-OBu$^t$
*IIIa*, X = Gly *IIIb*, X = Gln

Z-Cys(Bzl)-Gly-X-Y-Asn-Cys(Bzl)-OR

*IVa*, X = Gly; Y = Gly; R = Bu$^t$
*IVb*, X = Gly; Y = Gly; R = H
*IVc*, X = Gly; Y = Gln; R = Bu$^t$
*IVd*, X = Gly; Y = Gln; R = H
*IVe*, X = Ile; Y = Gln; R = Bu$^t$
*IVf*, X = Ile; Y = Gln; R = H

Tripeptide *II* was prepared by acylation of asparaginyl-S-benzylcysteine tert-butyl ester[3] with N-(triphenylmethyl)glycine *p*-nitrophenyl ester. After removal of the amino-protecting group, the acylation was repeated to give the tetrapeptide *IIIa*. The triphenylmethyl group was cleaved off and the peptide was converted to the protected hexapeptide *IVa* by condensation with N-benzyloxycarbonyl-S-benzyl-cysteinyl-glycine promoted with dicyclohexylcarbodiimide–pentafluorophenol. In a similar way we obtained also the protected hexapeptides *IVc* (*via* tetrapeptide *IIIb*) and *IVe*. Treatment with trifluoroacetic acid removed tert-butyl group from the hexapeptides *IVa*, *IVc*, and *IVe* under formation of compounds *IVb*, *IVd*, and *IVf* which on reduction with sodium in liquid ammonia, oxidative cyclization by air oxygen, desalting on an ion-exchange resin and gel filtration afforded the cyclo-hexapeptides *Ib*–*Id*.

* Some of the experimental data in this communication have been already published[2].

## EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Analytical samples were dried over phosphorus pentoxide for 24—48 h at room temperature at 150 Pa. Analytical electrophoresis was carried out on a Whatman 3 MM paper at 800 V (45—60 min) in 1M acetic acid (pH 2·4) or a pyridine–acetate buffer (pH 5·7). Thin-layer chromatography was performed on silica gel-coated plates (Kieselgel, Merck) in the systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5; S1), 2-butanol–25% ammonia–water (85 : 7·5 : 7·5; S2), 1-butanol –acetic acid–water (4 : 1 : 1; S3), 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6; S4). Spots were detected with ninhydrine or the chlorination method. Samples for amino acid analyses were hydrolyzed with 6M-HCl at 110°C for 20 h in ampoules sealed at 150 Pa. The analyses were performed on an automatic analyzer type 6020 (Developmental Workshops, Czechoslovak Academy of Sciences). Optical rotations were measured on a Perkin–Elmer 141 MCA instrument. Reaction mixtures were taken down under diminished pressure (aspirator) on a rotatory evaporator at bath temperature 30—35°C; samples containing dimethylformamide were evaporated at about 150 Pa. Gel filtration was done on 100 × 1 cm columns packed with Bio-Gel P-4 in 1M-$CH_3COOH$. The peptide material was detected at 254 nm.

### Triphenylmethylglycyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*II*)

N-(Triphenylmethyl)glycine *p*-nitrophenyl ester (1·3 g) was added portionwise to a solution of asparaginyl-S-benzylcysteine tert-butyl ester hydrochloride[3] (prepared from 1·1 g of the *o*-nitrobenzenesulfenyl derivative) in dimethylformamide (9 ml) which had been adjusted to pH 8 with N-ethylpiperidine. After stirring for 30 h at room temperature, dimethylformamide was evaporated and the residue washed successively with light petroleum, ether, 5% sodium hydrogen carbonate, water, and ether. The obtained material (0·8 g), m.p. 184—195°C, was crystallized from ethanol–dimethylformamide–light petroleum affording 0·75 g (55%) of product, m.p. 195—197°C, $[\alpha]_D$ —16·9° (*c* 0·2, dimethylformamide); $R_F$ 0·80 (S1), 0·75 (S2), 0·84 (S3), 0·82 (S4). For $C_{39}H_{44}N_4O_5S$ (680·9) calculated: 68·80% C, 6·51% H, 8·23% N, 4·71% S; found: 69·15% C, 6·62% H, 8·30% N, 4·75% S. Amino acid analysis: Gly 1·01; Asp 1·00; Cys(Bzl) 0·92.

### Triphenylmethylglycyl-glycyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*IIIa*)

Tripeptide *II* (0·75 g) was dissolved in 80% acetic acid. After standing at 30°C for 45 min the solution was evaporated and the residue dried by azeotropic distillation with ethanol–benzene mixture; $E_{2,4}^{Gly}$ 0·85, $E_{5,7}^{His}$ 0·60. A solution of the residue in dimethylformamide (5 ml) was adjusted to pH 8 with N-ethylpiperidine and N-(triphenylmethyl)glycine *p*-nitrophenyl ester (1·07 g) was added. After stirring at room temperature for 20 h, the mixture was worked up in the same manner as described for *II*. Yield 0·58 g, m.p. 182—186°C. Crystallization from dimethylformamide–ether afforded 0·47 g (64%) of the title compound, m.p. 186—187°C, $[\alpha]_D$ —31·9° (*c* 0·2, dimethylformamide). $R_F$ 0·67 (S1), 0·66 (S2), 0·75 (S3), 0·78 (S4). For $C_{41}H_{47}N_5O_6S$ (737·9) calculated: 66·73% C, 6·42% H, 9·49% N, 4·35% S; found: 66·76% C, 6·40% H, 9·76% N, 4·15% S. Amino acid analysis: Gly 1·87; Asp 1·00; Cys(Bzl) 0·93.

### N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycyl-glycyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*IVa*)

Peptide *IIIa* (0·40 g) was stripped of the triphenylmethyl group in the same manner as described above; $E_{2,4}^{Gly}$ 0·96, $E_{5,7}^{His}$ 0·53. The residue was dissolved in dimethylformamide (5 ml) and N-benzyloxycarbonyl-S-benzylcysteinyl-glycine[4,5] (0·24 g) and N-ethylpiperidine (0·14 ml) were

added. The solution was cooled to 0°C and dicyclohexylcarbodiimide–pentafluorophenol[6,7] complex (0·52 g) was added. After standing at 0°C for 20 h, the precipitated dicyclohexylurea was filtered, dimethylformamide evaporated and the residue triturated successively with light petroleum, ether, 5% sodium hydrogen carbonate solution, 1M-HCl, water and ether (0·35 g, m.p. 172—177°C). Crystallization from aqueous dimethylformamide gave 0·32 g (60%) of the product m.p. 176—178°C, $[\alpha]_D$ −41·0° (*c* 0·2, dimethylformamide); $R_F$ 0·62 (S1), 0·70 (S2), 0·75 (S3), 0·75 (S4). For $C_{42}H_{53}N_7O_{10}S_2.0{\cdot}5\,H_2O$ (889·1) calculated: 56·74% C, 6·12% H, 11·02% N, 7·21% S; found: 56·75% C, 5·91% H 11·04% N, 7·02% S. Amino acid analysis: Gly 2·74; Asp 1·00; Cys(Bzl) 1·82.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycyl-
-glycyl-asparaginyl-S-benzylcysteine (*IVb*)

Compound *IVa* (0·18 g) was dissolved in trifluoroacetic acid (1·7 ml). After standing at room temperature for 2 h, trifluoroacetic acid was evaporated and the residue azeotropically dried (benzene–ether). The solid residue suspension in benzene–ether was collected on filter, washed with ether (0·14 g; m.p. 215—217°C) and crystallized from aqueous dimethylformamide to give 0·12 g (71%) of product, m.p. 216—217°C, $[\alpha]_D$ −31·7° (*c* 0·2, dimethylformamide); $R_F$ 0·47 (S1), 0·71 (S2), 0·48 (S3), 0·57 (S4). For $C_{38}H_{45}N_7O_{10}S_2.H_2O$ (842·0) calculated: 54·21% C, 5·61% H, 11·65% N, 7·62% S; found: 54·11% C, 5·78% H, 11·41% N, 7·39% S.

Triphenylmethylglycyl-glutaminyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*IIIb*)

N-(Triphenylmethyl)glycine *p*-nitrophenyl ester (1·0 g) was added in portions to a solution of glutaminyl-asparaginyl-S-benzylcysteine tert-butyl ester hydrochloride[3] (prepared from 0·6 g of the corresponding *o*-nitrobenzenesulfenyl derivative) in dimethylformamide (5 ml) which had been adjusted to pH 8 with N-ethylpiperidine. The mixture was stirred at room temperature for 20 h and processed in the same manner as described for compound *II* (0·59 g; m.p. 179 to 188°C). Crystallization from dimethylformamide–ether afforded 0·55 g (74%) of product m.p. 187—189°C, $[\alpha]_D$ −28·2° (*c* 0·1, dimethylformamide); $R_F$ 0·65 (S1), 0·60 (S2), 0·68 (S3), 0·78 (S4). For $C_{47}H_{52}N_6O_7S.H_2O$ (827·0) calculated: 63·90% C, 6·58% H, 10·16% N, 3·87% S; found: 63·93% C, 6·21% H, 9·84% N, 3·67% S. Amino acid analysis: Gly 1·01; Asp 0·92; Glu 1·00; Cys(Bzl) 0·89.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycyl-glutaminyl-
-asparaginyl-S-benzylcysteine Tert-butyl Ester (*IVc*)

The triphenylmethyl group was removed from compound *IIIb* (0·50 g) in the same manner as described for *IIIa*; $E^{Gly}_{2,4}$ 1·0, $E^{His}_{5,7}$ 0·53. The condensation with N-benzyloxycarbonyl-S-benzylcysteinyl-glycine (0·28 g) was performed in dimethylformamide (4 ml) in the presence of dicyclohexylcarbodiimide–pentafluorophenol complex (0·59 g), affording 0·45 g of product m.p. 195—203°C which was crystallized from aqueous dimethylformamide; yield 0·41 g (63%), m.p. 205—207°C, $[\alpha]_D$ −36·9° (*c* 0·2, dimethylformamide); $R_F$ 0·87 (S1), 0·75 (S2), 0·86 (S3), 0·73 (S4). For $C_{45}H_{58}N_8O_{11}S_2$ (951·1) calculated: 56·83% C, 6·15% H, 11·78% N; found: 56·79% C, 6·05% H, 11·63% N. Amino acid analysis: Gly 1·83; Asp 1·03; Glu 1·00; Cys(Bzl) 1·82.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-glycyl-
-glutaminyl-asparaginyl-S-benzylcysteine (*IVd*)

The tert-butyl protecting group in compound *IVc* (0·26 g) was removed as described for *IVb* (0·18 g; m.p. 206—211°C). Crystallization from aqueous dimethylformamide afforded 0·16 g

(65%) of product m.p. 210—211°C, $[\alpha]_D^{22}$ −32·5° (*c* 0·2, dimethylformamide); $R_F$ 0·64 (S1), 0·67 (S3), 0·73 (S4). For $C_{41}H_{50}N_8O_{11}S_2.H_2O$ (913·0) calculated: 53·94% C, 5·74% H, 12·27% N, 7·02% S; found: 53·86% C, 5·66% H, 11·89% N, 7·24% S.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-isoleucyl-
-glutaminyl-asparaginyl-S-benzylcysteine Tert-butyl Ester (*IVe*)

Isoleucyl-glutaminyl-asparaginyl-S-benzylcysteine tert-butyl ester hydrochloride[3] (from 0·47 g of the corresponding *o*-nitrobenzenesulfenyl derivative) was treated with N-benzyloxycarbonyl-S--benzylcysteinyl-glycine (0·23 g) and dicyclohexylcarbodiimide–pentafluorophenol complex (0·55 g) in dimethylformamide (7 ml) to give 0·59 g of product m.p. 217—223°C. Crystallization from aqueous dimethylformamide afforded 0·56 g (93%) of the title compound, m.p. 222—224°C, $[\alpha]_D$ −52·2° (*c* 0·1, dimethylformamide); $R_F$ 0·70 (S1), 0·53 (S3), 0·72 (S4). For $C_{49}H_{66}N_8O_{11}S_2$ (1 007) calculated: 58·43% C, 6·60% H, 11·12% N, 6·37% S; found: 58·61% C, 6·55% H, 10·95% N, 6·51% S. Amino acid analysis: Gly 1·04; Asp 1·01; Glu 1·00; Ile 0·97, Cys(Bzl) 1·82.

N-Benzyloxycarbonyl-S-benzylcysteinyl-glycyl-isoleucyl-
-glutaminyl-asparaginyl-S-benzylcysteine (*IVf*)

The tert-butyl ester protecting group was removed from compound *IVe* (0·56 g) in the same manner as described for *IVb* (0·46 g; m.p. 220—226°C). Crystallization from aqueous dimethylformamide gave 0·42 g (79%) of product, m.p. 225—227°C, $[\alpha]_D$ −29·4° (*c* 0·3, dimethylformamide); $R_F$ 0·65 (S1), 0·66 (S2), 0·67 (S3), 0·69 (S4). For $C_{45}H_{58}N_8O_{11}S_2.H_2O$ (965·2) calculated: 55·77% C, 6·24% H, 11·56% N, 6·62% S; found: 55·89% C, 6·14% H, 11·69% N, 6·38% S.

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

Compound *IVb* (100 mg) was reduced with sodium in liquid ammonia[8] (100 ml) to blue coloration persisting for 1 min. After decoloration with ammonium chloride and removal of ammonia, the residue was dissolved in 2% HCl (100 ml, final pH 2). The solution was washed with ethyl acetate and ether, adjusted to pH 7 with 0·01M-NaOH (total volume 200 ml) and stirred slowly at room temperaure for 2 h. The pH value was adjusted to 3·5, the solution freeze-dried, and the residue dissolved in water (100 ml) and applied on a column of Dowex 50 ($H^+$ form). The column was washed with water and the peptide material eluted with 10% aqueous pyridine. The eluates were freeze-dried and the residue was purified by gel filtration on a column of Bio-Gel P-4 (in 1M-$CH_3COOH$). The product was obtained as lyophilizate (18 mg); $R_F$ 0·15 (S1), 0·07 (S3), 0·24 (S4); $E_{2,4}^{Gly}$ 0·70, $E_{5,7}^{His}$ 0·40. Amino acid analysis: Gly 2·90, Asp 1·00, 1/2 Cys 2·02. For $C_{16}H_{25}N_7O_8S_2.CH_3COOH.2{\cdot}5\,H_2O$ (612·6) calculated: 35·29% C, 5·52% H, 16·00% N; found: 35·50% C, 5·92% H, 15·84% N.

Cyclic Disulfide of Cysteinyl-glycyl-glycyl-glutaminyl-asparaginyl-cysteine (*Ic*)

Peptide *IVd* (150 mg) was reduced with sodium in liquid ammonia, oxidized with air oxygen and the product was purified by gel filtration as described for *Ia* to give 43 mg of *Ic*; $R_F$ 0·09 (S1), 0·04 (S3), 0·19 (S4); $E_{2,4}^{Gly}$ 0·68, $E_{5,7}^{His}$ 0·38. Amino acid analysis: Gly 1·97, Asp 1·00, Glu 0·99, 1/2 Cys 2·04. For $C_{19}H_{37}N_8O_9S_2.CH_3COOH.1{\cdot}5\,H_2O$ (665·7) calculated: 37·88% C, 5·60% H, 16·83% N; found: 37·90% C, 5·37% H, 16·60% N.

Cyclic Disulfide of Cysteinyl-glycyl-isoleucyl-
-glutaminyl-asparaginyl-cysteine (*Id*)

The protected peptide *IVf* (200 mg) was converted to the title compound (31 mg) by the procedure described for *Ia*. $R_F$ 0·33 (S1), 0·15 (S3), 0·35 (S4), $E^{Gly}_{2,4}$ 0·64, $E^{His}_{5,7}$ 0·35. Amino acid analysis: Gly 1·04, Asp 1·00, Glu 0·96, Ile 0·98, 1/2 Cys 2·04. For $C_{23}H_{38}N_8O_9S_2.CH_3COOH.2{\cdot}5\ H_2O$ (721·7) calculated: 41·61% C, 6·56% H, 15·53% N; found: 41·38% C, 6·71% H, 15·48% N.

Spectroscopic Measurements

The CD spectra were recorded on a Roussel-Jouan Dichrograph CD 185 model II in cells of 0·02–1 cm optical path-length 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 content of water and acetic acid using the elemental analyses given in the synthetic part. Spectral data in figures and tables are presented in molar ellipticity values ($[\Theta]$, deg $cm^2$ . . $dmol^{-1}$). The following solvents were used: phosphate buffer (*c* 0·01 mol $l^{-1}$, pH 7·5), 0·01M-HCl, methanol, 2,2,2-trifluoroethanol, and hexafluoroacetone trihydrate. Temperature experiments were carried out in a Jobin-Yvon cryostat cooled with liquid nitrogen using a mixed solvent (neutral buffer–ethanol–glycerol 1 : 1 : 1).

## RESULTS AND DISCUSSION

Compounds *Ib*–*Id* contain only the disulfide and amide chromophores. We ascribe dichroic bands at less than 210 nm to the $\pi$–$\pi^*$ transition, and bands between 210 and 240 nm to the $n$–$\pi^*$ transition of the amide groups. The well-discernible pair of bands due to the $n$-$\sigma^*$ transitions of the disulfide group is located at 240–300 nm. Spectra of the studied cyclopeptides did not contain other disulfide bands. The assignment of CD bands is based on our previous studies[1,2,9].

*Peptide Backbone*

The CD data for cyclohexapeptides *Ib*–*Id* are summarized in Tables I–III. All the spectra exhibit a characteristic strong band due to the amide $\pi$–$\pi^*$ transition at 195–200 nm (Fig. 1; see also Fig. 1 in ref.[2]). The intensity of the amide $\pi$–$\pi^*$ band depends on the structure of the cyclohexapeptide and on the solvent as shown by the diagram in Fig. 2 (Figs 2–6 include also data for the parent cyclopeptide *Ia*, taken from ref.[1]).

The most effective mechanism giving rise to the rotational strength of amide $\pi$–$\pi^*$ transitions is the dynamic coupling of these transitions in neighbouring amide groups[10]. Since this coupling is a function of the torsion angles $\Phi$ and $\Psi$ in the individual diamide units, amide $\pi$–$\pi^*$ bands reflect the peptide backbone conformation. Our interpretation of CD spectra of the cyclohexapeptide *Ia* is based on the assumption[1] that its peptide chain is considerably flexible and in solution exists as an ensemble of energetically little differing conformers. This assumption is confirmed also by conformational calculations[11]. In the CD spectra, this conformational

TABLE I

CD data of H-L-Cys-(Gly)$_3$-L-Asn-L-Cys-OH (*Ib*)

| Solvent[a] | $\lambda^b$, nm ([Θ] . $10^{-3}$, deg cm$^2$ dmol$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | disulfide bands | | | | amide bands | | |
| Buffer, pH 7·5 | 277 (−0·36) | 262 (0) | 247 (+0·78) | 218 (−11·2) | m214 (−10·9) | 196 (−29) | — — |
| 0·01M-HCl | 273 (−0·62) | 257 (0) | s242 (+1·05) | — — | 227 (+2·4) | 197 (−63) | — — |
| TFE | s267 (+0·59) | | s250 (+1·11) | — — | 218 (+12·8) | 198 (−50) | 186[c] (+10) |
| HFA | 271 (−0·77) | 255 (0) | s244 (+1·14) | — — | 222·5 (+6·4) | 200[c] (−46) | — — |
| Methanol | 286 (−0·24) | 270·5 (0) | s255 (+0·66) | — — | 219 (+17·6) | 198 (−26) | — — |

[a] TFE 2,2,2-trifluoroethanol, HFA hexafluoroacetone trihydrate; [b] s shoulder, m minimum; [c] end value, ellipticity value at the shortest attainable wavelength.

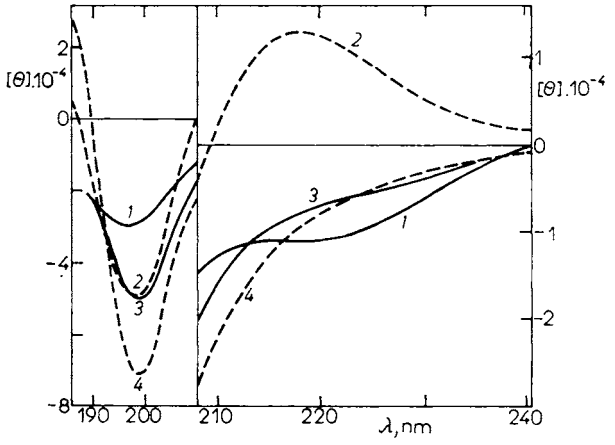


FIG. 1

Circular dichroism spectra of H-Cys-(Gly)$_3$-Asn-Cys-OH (*Ib*) *1* and *2* and of H-Cys-Gly-Ile-Gln-Asn-Cys-OH (*Id*) *3* and *4* in (———) phosphate buffer pH 7·5 and (– – – –) trifluoroethanol

flexibility is manifested by low intensities of the $\pi$–$\pi^*$ bands and their considerable solvent-sensitivity leading even to change of the sign (Fig. 2).

Attachment of asparagine side chain in position 5 (compound *Ib*) leads to a substantial simplification of the solvent dependence of the amide $\pi$–$\pi^*$ bands and to their general shift toward negative values (Fig. 2). Introduction of another side chain into position 4 (compound *Ic*) increases further this effect. The spectral changes can be explained by decrease in conformational freedom of the peptide chain (*i.e.* limitation of the accessible region of torsion angles $\Phi$ and $\Psi$) due to the spatial demands of the side chains and the new chiral centers introduced. Predominantly, the homoconjugated amide groups in the substituted cyclohexaptide are oriented in one of the two possible enantiomeric arrangements.

On the contrary, attachment of the third, isoleucine, side chain (compound *Id*)

TABLE II

CD data of H-L-Cys-(Gly)$_2$-L-Gln-L-Asn-L-Cys-OH (*Ic*)

| Solvent[a] | $\lambda^b$, nm ([$\Theta$] . $10^{-3}$, deg cm$^2$ dmol$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | disulfide bands | | | amide bands | | |
| Buffer, pH 7·5 | 269·5 (−0·79) | | m248·5 (−0·39) | s216 (−15·0) | 195 (−66) | — — |
| 0·01M-HCl | 265 (−0·50) | | m248 (−0·28) | — — | 197·5 (−97) | — — |
| TFE | 278 (−0·30) | 266 (0) | s244 (+1·45) | 220 (+8·6) | 197 (−72) | — — |
| HFA | 274 (−0·52) | 259 (0) | 242 (+1·10) | — — | 200[c] (−95) | — — |
| Methanol | 278 (−0·71) | 261 (0) | s245 (+1·60) | 224·5 (+5·6) | 200 (−78) | — — |
| Mixed solvent at +40°C | not measured | | | s222·5 (−3·7) | not measured | |
| Mixed solvent at −80°C | not measured | | | 217·5 (+24·1) | not measured | |

[a] TFE 2,2,2-trifluoroethanol, HFA hexafluoroacetone trihydrate, mixed solvent: ethanol–water–glycerol (1 : 1 : 1); [b] s shoulder, m minimum; [c] end value, ellipticity value at shortest attainable wavelength.

reduces (except in trifluoroethanol) the intensity of the negative $\pi$–$\pi^*$ band (Table III, Fig. 2). Such change can be due to a certain conformational rearrangement of the peptide chain (e.g. a transition from an extended to a β-turn conformation).

The dependence of the $\pi$–$\pi^*$ band intensity on the cyclopeptide structure is roughly parallel for all the solvents (Fig. 2) indicating that neither solvation interactions nor intramolecular hydrogen bonds play decisive role in determining the peptide backbone conformation. In solvents ionizing primary α-amino groups (0·01M-HCl and hexafluoroacetone trihydrate), the negative $\pi$–$\pi^*$ band is stronger than in neutral solvents for all the peptide studied. Evidently, the effect of charged α-amino group on dipoles of the amide groups near the heterodetic junction leads to a greater rigidity of the peptide. A long-range conformational effect of charged α-amino group in oxytocin has also been observed by NMR spectroscopy[12].

In the region of $n$–$\pi^*$ amide transitions, the CD spectra of cyclopeptides *Ia*–*Id* display two bands: a positive one at shorter wavelengths and a negative one at longer wavelengths. Since the energetic differences between the transitions are only small, the bands strongly overlap and compensate, sometimes together form a broad

TABLE III

CD data of H-L-Cys-Gly-L-Ile-L-Gln-L-Asn-L-Cys-OH (*Id*)

| Solvent[a] | $\lambda$[b], nm ($[\Theta] \cdot 10^{-3}$, deg cm$^2$ dmol$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | disulfide bands | | | amide bands | | |
| Buffer, pH 7·5 | 285 (−0·10) | 270 (0) | 248 (+0·37) | s225 (−3·3) | 199·5 (−43) | — — |
| 0·01M-HCl | 281 (−0·20) | 267 (0) | s243 (+0·91) | 225·5 (+2·4) | 199·5 (−65) | — — |
| TFE | 267 (−0·72) | — — | m248·5 (−0·36) | — — | 198 (−79) | — — |
| HFA | 276·5 (−0·34) | 260 (0) | 241·5 (+1·35) | — — | 200 (−61) | — — |
| Mixed solvent at +40°C | 291 (−0·18) | 269·5 (0) | 252 (+0·59) | s225 (−1·7) | not measured | |
| Mixed solvent at −80°C | 277 (−0·64) | 262 (0) | s244 (+3·00) | 223·5 (+9·5) | not measured | |

[a] TFE 2,2,2-trifluoroethanol, HFA hexafluoroacetone trihydrate, mixed solvent: ethanol–water–glycerol (1 : 1 : 1); [b] s shoulder, m minimum.

shoulder or, when weak, merge with the stronger $\pi$–$\pi^*$ band and escape identification (Fig. 1). The existence of two $n$–$\pi^*$ bands of opposite sign is well seen from the temperature dependence of spectra of compounds *Ic* and *Id* (Tables II and III, see also Fig. 2 in ref.[2]).

The dependence of the amide $n$–$\pi^*$ bands on the structure of cyclopeptides is somewhat more influenced by the solvent than in the case of the $\pi$–$\pi^*$ bands (Fig. 3; on the $y$-axis are plotted ellipticities at the positive $n$–$\pi^*$ maximum wavelength for *Ia* in the given solvent). In aqueous solvents and hexafluoroacetone, substitution with the first side chain (peptide *Ib*) is manifested by a steep decrease in intensity of the positive $n$–$\pi^*$ band whereas in alcohols no intensity change was observed. There is a reciprocal relationship between the amide $n$–$\pi^*$ and disulfide bands: with substitution, the latter undergo only small change, if any, in aqueous solvents whereas a marked change was observed in alcohols (Figs 4 and 5). We can assume

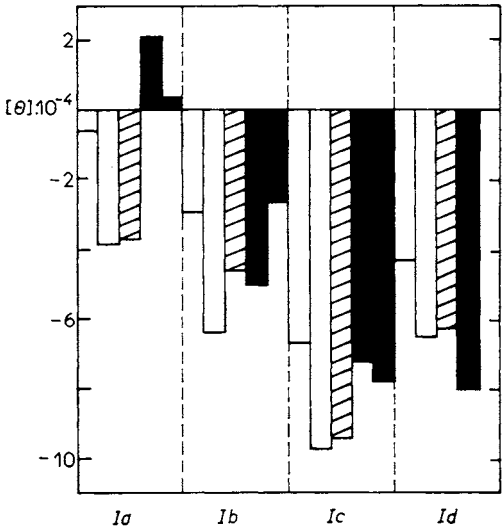


FIG. 2

Column graph showing the intensities of the $\pi$–$\pi^*$ amide circular dichroism bands in cyclohexapeptides *Ia*—*Id* as a function of structure and solvent. Solvents from left to right: phosphate buffer pH 7·5, 0·01M-HCl (open bars), hexafluoroacetone trihydrate (dashed bar), trifluoroethanol and methanol (filled bars)

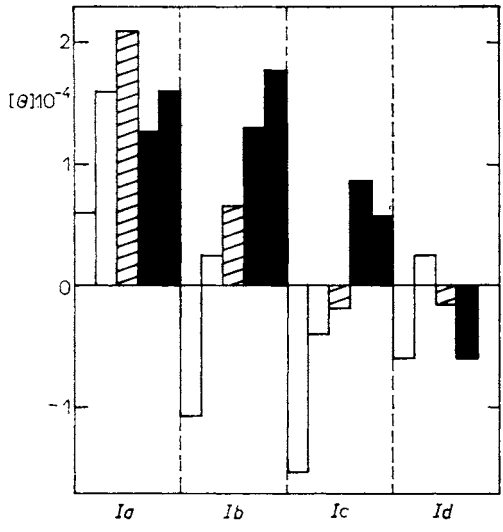


FIG. 3

Column graph showing the intensities of the $n$–$\pi^*$ amide circular dichroism bands in cyclohexapeptides *Ia*—*Id* as a function of structure and solvent. Solvents from left to right: phosphate buffer pH 7·5, 0·01M-HCl (open bars), hexafluoroacetone trihydrate (dashed bar), trifluoroethanol and methanol (filled bars)

that the corresponding conformational change takes place in the vicinity of the disulfide group.

The steric demands of the asparagine side chain can be apparently satisfied by conformational change of either the disulfide group or of its neighbourhood, depending on the nature and direction of the solvation interactions. This observation also shows that solvation should be taken as a factor influencing the disulfide group conformation.

With increasing substitution of the peptide ring in the series *Ia*–*Id*, the originally predominating positive $n$–$\pi^*$ band tends to give way to the negative one (Fig. 3). The positive $n$–$\pi^*$ band can be ascribed to the fragment consisting of the disulfide group and the adjacent amide groups, which in the incompletely substituted cyclopeptide is conformationally and chirally better defined than the rest of the molecule. This agrees also with the fact that ionization of the α-amino group in 0·01M-HCl and in hexafluoroacetone trihydrate increases the positive $n$–$\pi^*$ band in the spectra of peptides *Ia*–*Id* (Fig. 3, Tables I–III). Since the rotational strength of $n$–$\pi^*$ transitions is determined predominantly by short-range interactions, the band is, apparently, due to the first amide group in the peptide chain (*cf.* ref.[13]). On the other hand, the negative $n$–$\pi^*$ band represents a contribution of diamide units (in some of the extended conformations) in other positions of the peptide chain. The relative increase of the positive $n$–$\pi^*$ band was observed also upon cooling of peptide *Ic* or *Id* in the mixed solvent (Tables II and III). (Temperature dependence of the amide $\pi$–$\pi^*$ bands has not been studied.) This effect can be explained by

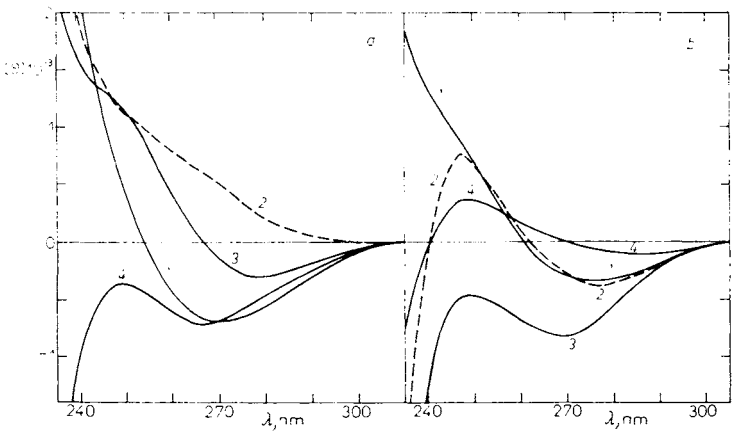


FIG. 4

Circular dichroism spectra in trifluoroethanol *a* and phosphate buffer pH 7·5 *b* of cyclohexapeptides *1 Ia*, *2 Ib*, *3 Ic*, and *4 Id*

greater rigidity of the molecular fragment, consisting of the disulfide group and the adjacent amide groups, caused by low temperature and enhanced solvation.

Both types of amide bands reflect thus essentially the same conformational changes accompanying the stepwise introduction of side chains into the cyclopeptide, *i.e.* the increasing relative rigidity of the peptide chain and possible conformational rearrangement caused by substitution with isoleucine side chain. As expected, the CD amide bands of *Id* are considerably similar to those of oxytocin analogues devoid of tyrosine residue in position 2 (refs[2,14]). The typical spectrum is characterized by a negative $\pi$–$\pi^*$ band at 200 nm and a shoulder of a negative $n$–$\pi^*$ band at 225 nm (mean ellipticity values *per* amide bond in neutral aqueous medium amount to about 5 400 deg cm$^2$ dmol$^{-1}$ for the $\pi$–$\pi^*$ and 700 deg cm$^2$ dmol$^{-1}$ for the $n$–$\pi^*$ band). When interpreting this spectrum in terms of the overall conformation of the peptide, we have to take into account the possible presence of a β-turn conformation in the cyclic peptide. However, since the CD spectra of the peptide *Id*,

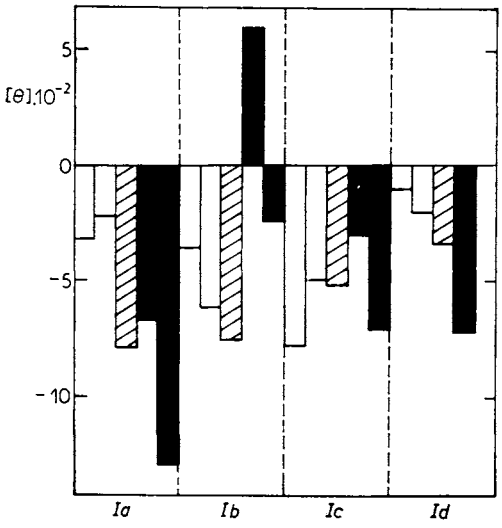


FIG. 5

Column graph showing the intensities of the long-wavelength disulfide circular dichroism bands in cyclohexapeptides *Ia*—*Id* as a function of structure and solvent. Solvents from left to right: phosphate buffer pH 7·5, 0·01M-HCl (open bars), hexafluoroacetone trihydrate (dashed bar), trifluoroethanol and methanol (filled bars)

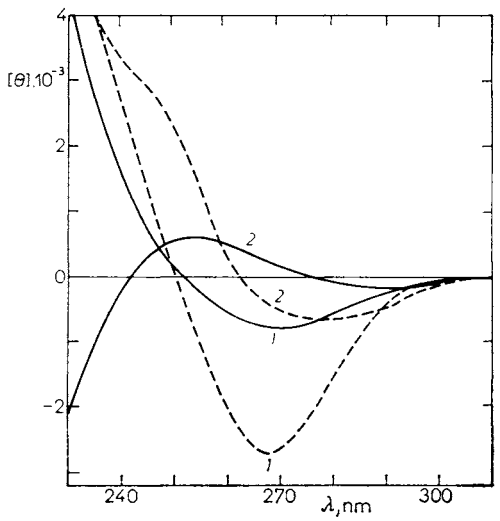


FIG. 6

The effect of temperature on the circular dichroism spectra of *1* H-Cys-(Gly)$_4$-Cys-OH (*Ia*) and *2* H-Cys-Gly-Ile-Gln-Asn-Cys-OH (*Id*) in a mixed solvent (neutral buffer–ethanol–glycerol 1 : 1 : 1). (———) +40°C, (– – – –) −80°C

as well as of simpler members of the studied series, do not resemble any theoretical or experimental spectra of known β-turn types[2,14], β-turn as a predominating conformation in studied solutions can be excluded. Also according to Hollósi and coworkers[15], the spectral type exhibited by *Id* indicates absence of β-turns.

The spectral parameters of *Id* most resemble those of random coil polypeptides, denaturated proteins, and unordered linear oligopeptides[10]. It seems thus that the peptide chain in compound *Id* in the studied solutions exists also in an unordered state as an ensemble of conformers[10] with a limited range of torsion angles $\Phi$ and $\Psi$. Our measurements indicate that the population of individual conformers in this set can be influenced by intermolecular interactions of the solvent–solute type whereas intramolecular hydrogen bonding interactions contribute only little to conformational stabilization. The considerable flexibility of the peptide chain in *Id* is consistent with the assumed unordered state.

*Disulfide Group*

CD spectra of the disulfide chromophore in oxytocin hormones or in model heterodetic cyclohexapeptides have been studied and interpreted in terms of conformation in several papers[1,12,16–19]. Spectra of compounds *Ia*–*Id* display two disulfide bands. One, mostly negative, occurs at 270 nm or higher wavelengths, the second, positive, is located at about 250 nm (Fig. 4, Table I–III). The apparent shape and intensity of the bands are affected by their mutual overlap and particularly by the overlap with the neighbouring $n$–$\pi^*$ amide band. This band can be of either sign and its intensity can change within wide range (Fig. 3). Therefore, the short-wavelength disulfide band may appear as a maximum, a more or less discernible shoulder or may merge with the amide band, depending on the situation. Thus, for a quantitative evaluation, the longer-wavelength band is more suitable. The Tables I–III include also the characteristic position of the cross-point of the CD curve with the zero line between the two disulfide bands.

Structure of peptides *Ia*–*Id* influences the parameters of both disulfide bands which in this context appear as inversely proportional: With increasing intensity of the negative band its maximum and the cross-point are shifted to shorter wavelengths and the intensity of the positive band decreases. When $\lambda_{max}$ of the negative band is 270 nm or lower, we often observe only a negative minimum instead of the positive band (*e.g.* curve *4* in Fig. 4*a*). These two disulfide bands can be therefore ascribed to two conformational types of the disulfide group whose relative population in solution depends on the cyclopeptide structure. However, the disulfide bands are affected not only by conformational changes of the disulfide chromophore itself but also by changes in its neighbourhood since the rotational strength of disulfide transitions arises at least by two mechanisms. This also explains numerous irregularities

in the mutual relationships between the disulfide bands. We ascribe the positive band at 250 nm to the rectangular conformation[1,2,14] whose optical activity arises through perturbation of the chromophore by neighbouring chirally disposed groups (one-electron mechanism), whereas the long-wavelength negative band is due to deformed transoid conformations with torsion angle significantly larger than 90° (refs[1,2,14]). The inherent chirality of these conformers is an important source of their optical activity. In both conformational types the disulfide group has probably the same, right-handed, helicity $P(\varphi > 0°)$, or, if it exists as a mixture of both enantiomers, the right-handed one predominates; (*cf.* X-ray diffraction analysis of oxytocin[20]). Consequently, the positive long-wavelength band observed for *Ib* in trifluoroethanol (Figs 4 and 5) belongs to a cisoid conformer of the disulfide group with $\varphi < +90°$.

The CD disulfide bands of cyclohexapeptides *Ia*–*Id* in two typical solvents – phosphate neutral buffer and trifluoroethanol – are shown in Fig. 4. The response of the disulfide bands to structural changes in the cyclopeptides depends considerably on the solvent. Thus, *e.g.* the disulfide bands of peptides *Ia* and *Ib* are almost identical in the phosphate buffer whereas in trifluoroethanol they become different. The spectrum of *Ia* is shifted into the negative, that of *Ib* into the positive ellipticity region. The dependence of intensity of the long-wavelength disulfide band on the structure of *Ia*–*Id* (Fig. 5) differs in aqueous solvent and in alcohols (in trifluoroethanol and methanol, whereas the acidic geminal diol hexafluoroacetone trihydrate acts rather as an aqueous solvent). The long-wavelength disulfide band achieves extremely high intensities in alcohol solutions: The strongest negative band has been found for *Ia* in methanol (Fig. 5) and the only case of a positive band was recorded for *Ib* in trifluoroethanol (Fig. 4 and 5). The observations, together with the fact that the disulfide and amide $\pi$–$\pi^*$ CD bands cannot be successfully correlated, indicate that the disulfide conformation is influenced directly by solvation interactions (and not merely by solvent-induced conformational changes of the peptide backbone). These interactions seem to be particularly strong in alcoholic solvents.The relations between structure and solvation interactions of the disulfide group have been already discussed above in connection with the amide $n$–$\pi^*$ bands.

Evidently, conformation of the disulfide group is relatively independent of conformation of the peptide backbone. The disulfide long-wavelength band is also much less affected by the ionization state of the primary α-amino group than are both the amide bands (Fig. 5). On the other hand, there is a certain analogy in the response of the disulfide and amide bands to introduction of the isoleucine side chain (*i.e.* to the change from *Ic* to *Id*). In both solvents in Fig. 4 the disulfide bands of *Id* differ substantially from those of *Ic* but resemble more closely the spectrum of the structurally most distant unsubstituted peptide *Ia*. This behaviour is probably connected with arrangement of the side chains along the peptide backbone. Relative to the disulfide group, the arrangement of the side chains in peptide *Ic* is the least

symmetrical whereas in *Id* the symmetry is partly restored and is formally closer to that of *Ia*.

In terms of conformation, the CD spectra can be interpreted so that in aqueous solvents the most populated form is the rectangular conformation of the disulfide group in peptide *Id*, for which the band parameters are near those for oxytocin. On the contrary, in trifluoroethanol the disulfide group of *Id* exists almost entirely in a non-rectangular strained conformation and the spectrum has no resemblance to that of oxytocin.

Temperature dependence of the disulfide bands is illustrated by the spectra of cyclopeptides *Ia* and *Id* (Fig. 6). At low temperature the molecule has lower flexibility which results in higher intensity of the disulfide bands. In the spectrum of the unsubstituted cyclohexapeptide *Ia* there is no change in the band position or width, indicating no dependence of conformation on temperature. In the case of peptide *Id* the negative maximum and the point of intersection with the zero line shift towards shorter wavelengths with decreasing temperature, indicating a shift of conformational equilibrium in favour of a non-rectangular form. From the similarity of the disulfide bands of *Id* in alcohols and in the polar mixed solvent (neutral buffer–ethanol–glycerol 1 : 1 : 1) it follows that in the latter the effect of the alcohol components predominates. The equilibrium shift to the side of strained conformers of the disulfide group should be explained by its higher solvation at lower temperature.

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