Acta 25, 575.
Harper, A. E., Benton, D. A., Winje, M. E., and Elvehjem, C. A. (1954), *Arch. Biochem. Biophys. 51*, 523.
Howell, R. K., and Lee, M. (1963), *Proc. Soc. Exp. Biol. Med. 113*, 660.
Johnson, W. A., and Connelly, J. L. (1972), *Biochemistry 11*, 1967.
Kanzaki, T., Hayakawa, T., Hamada, M., Fukoyoshi, Y., and Koike, M. (1969), *J. Biol. Chem. 244*, 1183.
Meister, A. (1957), Biochemistry of the Amino Acids, New York, N. Y., Academic Press, p 194.
Ness, G. (1971), Ph.D. Dissertation, University of North Dakota.
Nordlie, R. C., Hanson, T. L., Johns, P. T., and Lygre, D. G. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 593.
Silberman, J., Dancis, J., and Feigin, I. (1961), *Arch. Neurol. 5*, 351.
Snyderman, S. E. (1967), *in* Amino Acid Metabolism and Genetic Variation, Nyhan, W. L., Ed., New York, N. Y., McGraw-Hill Co.
Snyderman, S. E., Norton, P. M., Roitman, E., and Holt, L. E. (1964), *Pediatrics 34*, 454.
Tashian, R. E. (1961), *Metabolism 10*, 393.
Westall, R. G. (1963), *Arch. Dis. Childhood 38*, 485.
Wohlheuter, R. M., and Harper, A. E. (1970), *J. Biol. Chem. 245*, 2391.

Aromatic Side-Chain Cotton Effects in Cyclic Hexapeptides†

C. Allen Bush* and Don E. Gibbs‡

ABSTRACT: We report circular dichroism curves of the cyclic hexapeptides *cyclo*-(Gly$_2$-Tyr)$_2$, *cyclo*-Gly-His-Gly-Ala-Tyr-Gly, and *cyclo*-Gly-His-Gly-Tyr-Ala-Gly. Published nuclear magnetic resonance (nmr) data indicate that these compounds adopt conformations having all-trans peptide bonds and two β loops stabilized by trans-annular hydrogen bonds. The circular dichroism of the cyclic peptide in the 210–289-nm region is approximately separated into contributions of the peptide bonds and those of the side-chain chromophores. When a tyrosyl residue is the first corner residue of a β loop we estimate that it contributes a rotational strength of -0.242×10^{-40} cgs esu at 277 nm and 2.94×10^{-40} cgs esu at 227 nm. We further conclude that the circular dichroism of *cyclo*-Gly-His-Gly-Tyr-Ala-Gly contains contributions typical of a tyrosyl residue in an extended conformation. We also propose a simple coupled oscillator theory to calculate Cotton effects due to tyrosyl chromophores at 277 and 227 nm. We give calculated results for several backbone conformations which have been proposed on the basis of nmr data. We find satisfactory agreement with experiment for a tyrosyl residue as the first corner residue of a β loop ($\phi = -80°$, $\psi = 120°$) if we take the side-chain conformational angles $\chi_1 = 300°$ and $\chi_2 = 60°$. Our results on the circular dichroism of cyclic peptides may be generalized to the structure *cyclo*-(Gly-L-residue-D-residue)$_2$ and we use our method to reinterpret some circular dichroism data from other laboratories.

A cyclic hexapeptide is the smallest cyclic peptide which may have all-trans peptide bonds. A number of cyclic hexapeptides adopt a characteristic conformation having two β loops and two internal amide hydrogens in a conformation originally proposed by Schwyzer (1959). The internal amide protons may be detected by temperature dependence of nuclear magnetic resonance (nmr) (Kopple *et al.*, 1969a). In some, but not all cases, the internal amide proton may form trans-annular hydrogen bonds (Kopple *et al.*, 1972).

Further information on the conformation of cyclic hexapeptides can be gained from nmr by measuring the amide NH to CαH coupling constant. This coupling constant is related to the dihedral angle about the N–C bond (Ramachandran *et al.*, 1971; Bystrov *et al.*, 1969). Since this dihedral angle is closely related to the peptide conformation angle, ϕ_{CN}, such information is quite useful in building models of cyclic hexapeptides. Temperature dependence of chemical shift and coupling constants may be combined with conformational energy maps to propose complete backbone conformations of cyclic hexapeptides. Some generalizations about the influence of various side chains on the backbone conformation have been made recently (Kopple *et al.*, 1972).

The optical activity curves of cyclic peptides, although often similar in appearance to those of linear polypeptides, require an interpretation quite different from those of linear polypeptide structures such as α helix, β-pleated sheet, and random coil (Bush, 1971). An interesting illustration of this fact is offered by gramicidin S whose circular dichroism (CD) curve resembles that of an α helix (Urry *et al.*, 1969). In fact, a conformation, quite unlike an α helix, has been proposed based on nmr data (Stern *et al.*, 1968). This conformation, which is now widely accepted as correct, has two β loops. Yet optical activity calculations based on the model of Stern *et al.* (1968) yield an optical rotatory dispersion (ORD) curve which is consistent with experiment (Pysh, 1970). In the present study, we will approach the optical activity of cyclic peptides not from the standpoint of linear peptides but rather from the basis of cyclic peptide models whose conformation is known from nmr data.

In an earlier study, we showed differences in CD between

† From Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616. *Received February 14, 1972.* Research supported in part by The Petroleum Research Foundation of The American Chemical Society and by NSF Grant GP-20053.

‡ NSF College Teacher Research Participant. Present address: Department of Chemistry, University of Kansas, Lawrence, Kansas 66044.

TABLE I: Molar Extinction Coefficients and Ellipticities per Average Residue Molecular Weight for Some Cyclic Peptides.

Compound	Absorption		CD	
	λ_{max} (nm)	$\epsilon_{max} \times 10^{-3}$	λ_{max} (nm)	$[\theta]_\lambda$
cyclo-(Gly_2-L-Tyr)$_2$	192	18.7	198	−24,500
	222	2.8	207.5	−2,600
	275	0.42	219	−3,700
	(279 shoulder)		275	−300
cyclo-(Gly-L-His-Gly-L-Tyr-L-Ala-Gly)	192	11.2	198	+14,500
	(218 shoulder)		216	−2,800
	275	0.19	270	+90
	(280 shoulder)		286	−20
cyclo-(Gly-L-His-Gly-L-Ala-L-Tyr-Gly)	192	11.4	198	−10,000
	(218 shoulder)			
	275	0.19	275	−90
	(280 shoulder)			

the monosubstituted cyclic hexapeptide, *cyclo*-(Gly_5-Leu), and the 1,4-disubstituted compound, *cyclo*-(Gly_2-Leu)$_2$ (Ziegler and Bush, 1971). In that case, it has been proposed that the former compound has freedom to adopt a centrosymmetric-type backbone conformation, while the side chain of the latter constrains the backbone to a C_2 symmetry. This information has been incorporated into detailed model structures recently proposed for these compounds by Kopple *et al.* (1972). In the present study we relate the Cotton effect of the tyrosyl side chain to its position in the Schwyzer structure and extend the generalizations to some other cyclic peptides.

Experimental Section

The cyclic peptides were synthesized in the laboratory of Dr. K. D. Kopple of this department. The syntheses have been described by Kopple *et al.* (1972). Solutions were prepared in twice distilled water with agitation at 60°, passed through a membrane filter (Millipore RAWP 01300, 1.2 μ), and stored under refrigeration. Ultraviolet absorption spectra were determined with a Cary 15 spectrophotometer using fused silica cells (0.1, 0.2, and 1 cm) at ambient temperature (23–26°). Nitrogen purging was used throughout. Results were reproducible within 0.05 optical density unit and 0.2 nm.

Beer's law plots were obtained for each peptide at four to five concentrations in the range of 0.3×10^{-4} to 2×10^{-4} M. Molar extinction coefficients per residue were obtained from the slopes of these linear plots.

Circular dichroism was measured with the Cary 60 spectropolarimeter with the Model 6002 circular dichroism accessory. Results are reported as $[\theta]_\lambda$, ellipticity per average residue molecular weight

$$[\theta]_\lambda = \theta M/10c'l \quad (1)$$

where θ is the observed ellipticity in degrees for wavelength λ, M is the average residue molecular weight (in the present cases, molecular weight/6), c' is the concentration in grams per milliliter, and l is the pathlength in centimeters.

The conditions for CD measurements were the same as described above for the spectral determinations. Solutions with an optical density of 0.8–1.2 were employed. Measurements of θ above 200 nm were reproducible within 0.0004°, there was considerable loss of precision below this wavelength. As in obtaining the ultraviolet spectra, base lines with pure solvent were obtaied before and after each measurement involving a peptide. The work was repeated whenever significant variations occurred. Ionization of histidine and of tyrosine in our water solutions was found to be insignificant. Identical spectra and optical activities were obtained in water and in phosphate buffer (pH 7.85).

Results

The extinction coefficients per residue at the maxima for the three cyclic hexapeptides are given in Table I. The band at 275 nm is the long-wavelength absorption of neutral tyrosyl contained in all three of these compounds. In *cyclo*-(Gly_2-Tyr)$_2$ the band has approximately twice the intensity it has in *cyclo*-Gly-His-Gly-Ala-Tyr-Gly (c-GHGATG) and in *cyclo*-Gly-His-Gly-Tyr-Ala-Gly (c-GHGTAG) indicating no hypochromism in this band. The tyrosyl residue also has a band at 222 nm in the absorption spectrum of *cyclo*-(Gly_2-Tyr)$_2$. This band appears as a shoulder in the other two compounds as a result of histidyl absorption at 211 nm. The absorption maxima at 192 nm is due both to tyrosyl absorption and to the amide π-π^* bands which are split as a result of exciton interaction (Ziegler and Bush, 1971).

The CD peaks and troughs are also reported in Table I. The CD of c-GHGATG (Figure 1) in the short-wavelength range is seen to be quite similar to that of *cyclo*-Gly-His-Gly-Gly-Tyr-Gly (c-GHGGTG) (Ziegler and Bush, 1971). In spite of certain differences in the nmr spectra of these two compounds, we will interpret the CD results to indicate a close similarity in the conformation (C_2 type) of these two compounds (Kopple *et al.*, 1972).

In the region of the long-wavelength Cotton effect of tyrosyl (Figure 2) we notice similarity between *cyclo*-(Gly_2-Tyr)$_2$ and c-GHGATG, the former, which contains two tyrosyl residues, contributing a band approximately twice the intensity of the latter. The long-wavelength Cotton effects for these compounds are also similar to those observed for *cyclo*-(Gly_5-Tyr) and c-GHGGTG (Zeigler and Bush, 1971). In contrast, both the short-wavelength (Figure 1) and long-wavelength (Figure 2) regions of the CD curve for c-GHGTAG appear

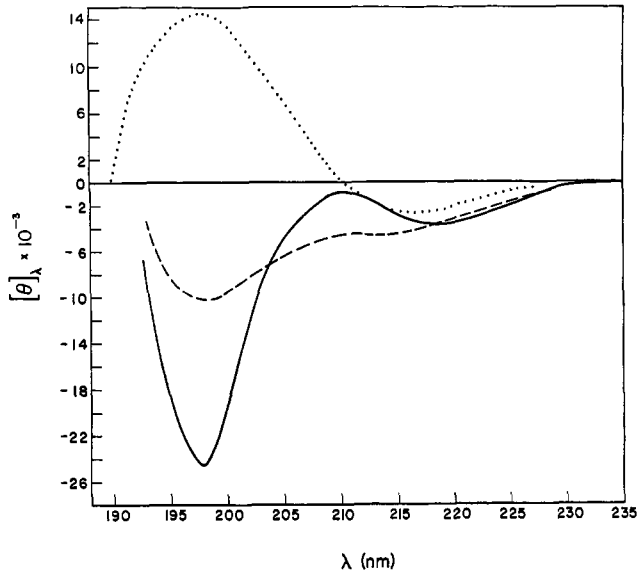

FIGURE 1: Circular dichroism in water solution of *cyclo*-(Gly_2-Tyr)$_2$, (——), *cyclo*-Gly-His-Gly-Ala-Tyr-Gly (– – – –), and of *cyclo*-Gly-His-Gly-Tyr-Ala-Gly (· · · · ·). [θ] is reported per average residue molecular weight. (See eq 1.)

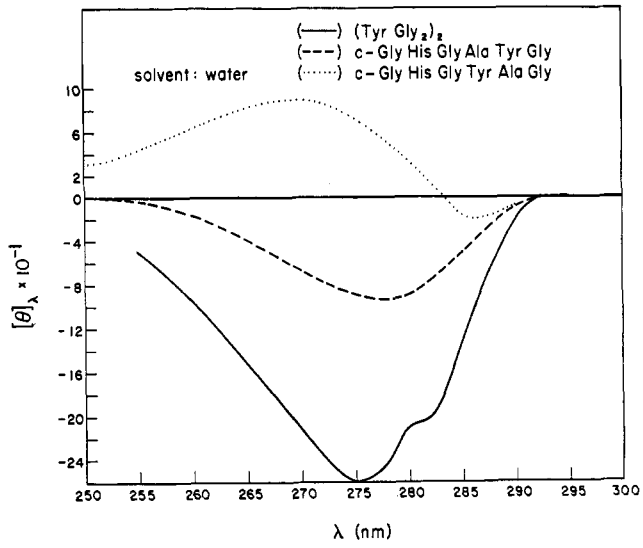

FIGURE 2: Circular dichroism in the aromatic region of water solutions of *cyclo*-(Gly_2-Tyr)$_2$ (——), *cyclo*-Gly-His-Gly-Ala-Tyr-Gly (– – – –), and of *cyclo*-Gly-His-Gly-Tyr-Ala-Gly (· · · · ·). [θ] is reported per average residue molecular weight. (See eq 1.)

quite different from those of the other two compounds we report here. We will show below how these differences may be interpreted as a result of differing position of the tyrosyl residue in the Schwyzer structure.

Following procedures used in a previous publication, we will separate the contribution to the CD curves into Cotton effects due to amide backbone and Cotton effects due to side chains (Ziegler and Bush, 1971). Although such a separation is not rigorously correct, we consider it approximately valid within certain limits. Clearly the Cotton effects in the 277-nm region are due mainly to the tyrosyl chromophore. Moreover, by subtracting the CD of a compound having the same conformation but no chromophoric side chains we may approximately obtain the tyrosyl Cotton effect at 227 nm. The CD bands in the 190–205-nm region arise from strong coupling of the amide π-π^* bands and the strong tyrosyl absorption at 195 nm. Our separation procedure is not valid in this range and we will not attempt to interpret these bands.

In order to extract the Cotton effects due to the side chains, we will subtract from the curves of Figure 1 the CD due to the peptide backbone in a C_2-type conformation as represented by the CD of *cyclo*-(Gly_2-Leu)$_2$ (Ziegler and Bush, 1971). We have previously shown that the tyrosyl residue enhances the n-π^* CD band of the amide making it necessary to multiply the *cyclo*-(Gly_2-Leu)$_2$ CD curve by a factor, *f*, in order for it to represent the amide contribution in tyrosyl-containing cyclic hexapeptide (see equation 2 of Ziegler and Bush, 1971). In estimating the contribution of the side chains to the CD of c-GHGGTG we subtracted 2.8 times the CD of *cyclo*-(Gly_2-Leu)$_2$. One can see that the same treatment of the CD of c-GHGATG (Figure 1) will lead to very similar results due to the similarity of the CD of c-GHGGTG and c-GHGATG. When we apply this same treatment to the CD of c-GHGTAG, the results (Figure 3) are quite different, and we will discuss below these differences in terms of conformation. The 275-nm band is positive in this case and also the 227-nm band is approximately two times stronger per tyrosyl residue than in the case of *cyclo*-(Gly_2-Tyr)$_2$ or in c-GHGATG (see Figure 3).

For the case of *cyclo*-(Gly_2-Tyr)$_2$ where there are two tyrosyl residues to enhance the amide n-π^* band, we find that the use of the factor, $f = 4$ in eq 2 of Ziegler and Bush (1971) leads to zero CD in the 210–215-nm region of the difference curve (Figure 3). We use this criterion for the choice of the factor since there is no absorption in the tyrosyl residue in this wavelength region. One might anticipate that the enhancement of the n-π^* by two tyrosyl residues would be just twice that of one tyrosyl group (*i.e.*, $f = 5.6$) but such does not seem to be the case.

Our experimental results may be summarized as follows. The CD of c-GHGATG is quite similar to that of c-GHGGTG. The CD of *cyclo*-(Gly_2-Tyr)$_2$ contains a contribution from the tyrosyl residues which is similar to the tyrosyl contribution to the CD curves of *cyclo*-Gly_5-Tyr, c-GHGGTG, and c-GHGATG. The contribution of the tyrosyl residue to the CD of c-GHGTAG differs from that of the former compounds.

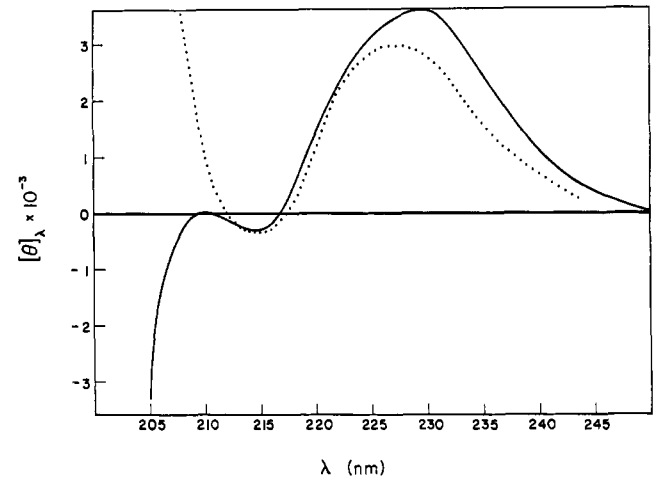

FIGURE 3: Difference curves representing Cotton effects due to side chains. Two tyrosyl residues of *cyclo*-(Gly_2-Tyr)$_2$ (——) and of the tyrosyl and histidyl residue of *cyclo*-Gly-His-Gly-Tyr-Ala-Gly (· · · · ·). [θ] is reported per average residue molecular weight. (See eq 1.)

TABLE II[a]

	R_{T1} (277 nm)	R_{T2} (227 nm)
First corner residue	−0.242	+2.94
Extended residue	0.248	9.82

[a] Rotational strengths of tyrosyl residues in β loops of cyclic hexapeptides. Units are 10^{-40} cgs esu per tyrosyl residue.

In the latter case the 277-nm band is mainly positive rather than negative (see Figure 2). Also the 227-nm band of c-GHGTAG is two to three times larger than that of *cyclo*-(Gly_2-Tyr)$_2$. (Note that in Figure 3 the ellipticity is reported per amide residue so that the CD per tyrosine of c-GHGTAG is about twice that of *cyclo*-(Gly_2-Tyr)$_2$ in the 227-nm region.) The exceptional behavior of c-GHGTAG is also evident in the short-wavelength region of Figure 1. Although we will not attempt to interpret these Cotton effects in the 190–200-nm region, they clearly indicate some differences between c-GHGTAG and the other tyrosine containing compounds we have discussed.

Discussion of Experimental Results

c-GHGATG has a CD curve (Figures 1 and 2) very similar to that of c-GHGGTG (Ziegler and Bush, 1971). These two compounds differ only by a single methyl group and the simplest interpretation of the CD curves would indicate that their conformations are quite similar. However, the temperature dependence of the nmr shows that c-GHGGATG has only one internal amide proton as compared to two internal amide protons for c-GHGGTG (Kopple *et al.*, 1972). We interpret this fact as an indication that the presence of the methyl group at Ala-4 bends that amide slightly out of the average plane of the cyclic peptide. Apparently this change is sufficiently subtle that it causes major differences in neither the tyrosyl CD bands (277 nm, 227 nm) nor in the amide backbone CD bands.

For two compounds whose CD is reported in this work, *cyclo*-(Gly_2-Tyr)$_2$ and c-GHGATG, the Cotton effects at 277 and 227 nm assigned to tyrosyl are quite similar to those bands in *cyclo*-Gly_5-Tyr and in c-GHGGTG (Ziegler and Bush, 1971). This similarity occurs in spite of the fact that the amide backbone of the monosubstituted compound (*cyclo*-Gly_5-Tyr) has a conformation different from the C_2 conformation of the 1,4-disubstituted peptides. We interpret this similarity to mean that the conformation of the amide backbone around the tyrosyl residue is identical in both the C_2 cyclic peptides and in the monosubstituted compound. This fact has recently been discussed in model building studies by Kopple *et al.* (1972).

In addition, the models proposed by Kopple *et al.* (1972) indicate that the tyrosyl residue in all these cases is the first corner residue of a β loop. Its conformation is approximately given by $\phi = -80°$, $\psi = 120°$ which places this corner residue in the broad minimum of the upper-left-hand corner of a Ramachandran energy plot.[1] We claim that the 277-nm Cotton effect of c-GHGATG (Figure 2) is typical for a tyrosyl residue in such a conformation. Moreover, within the accuracy of our approximate separation of amide and side-chain Cotton effects, the CD band at 227 nm for *cyclo*-(Gly_2-Tyr)$_2$ (Figure 3) is also typical for the tyrosyl residue in the first corner position of a β loop. The rotational strengths of the tyrosyl residue have been calculated from these data and are given in Table II.

The CD curve of c-GHGTAG differs substantially from that of its sequence isomer c-GHGATG (Figures 1 and 2). The 277-nm band of the former is positive and there are differences in the 200-nm region as well. The nmr results show some similarities between c-GHGATG and c-GHGTAG (Kopple *et al.*, 1972). Much of the steric constraint of the side chain on the backbone is due to the β-carbon atom. Therefore, we might imagine that the conformation of the backbone in these two isomers is identical and that the tyrosyl side chain belongs to the extended residue of the β loop in c-GHGTAG rather than to the corner residue as in the case of c-GHGATG. Thus the contribution of the amide CD bands is similar in the two compounds and the differences in the CD curves arise from different contribution of the tyrosyl residue.

This reasoning leads us to interpret the tyrosyl Cotton effects of the difference curve for c-GHGTAG (Figure 3) as characteristic of a tyrosyl residue in an extended chain conformation. We have calculated the rotational strengths for the 277- and 227-nm bands from the curve for c-GHGTAG (Figure 3) and recorded them in Table II. Our claim that these Cotton effects are characteristic of a tyrosyl residue in an extended conformation must be considered as tentative since we have only one example (c-GHGTAG) rather than four in the case of the first corner residue. The conformational angles of the residue in the extended position are $\phi = -70°$, $\psi = 180°$ in the model of Kopple *et al.* (1972).

The difference curves representing side-chain Cotton effects of c-GHGTAG and c-GHGATG (Figure 3) contain not only the contribution of the tyrosyl group, but also that of the histidyl side chain which has an absorption band at 211 nm. Although it is possible that CD bands due to histidyl occur at that wavelength, we suspect that these bands are very small compared to the contributions of tyrosyl residues. Some recent experiments on the diketopiperazines, *cyclo*-Gly-His, *cyclo*-Ser-His, *cyclo*-Asp-His, and *cyclo*-His-His, indicate that the contribution of the imidazole chromophore to the CD is quite small both at acidic and basic pH values (Ziaudin *et al.*, 1972). Although data on histidyl side-chain Cotton effects is meager, present indications are that its contributions to CD are quite small.

Theory

In this section we propose a simple theory for calculating the tyrosyl side-chain Cotton effects in order to test the plausibility of our interpretation. We also attempt to determine just how sensitive are the tyrosyl Cotton effects to the conformation of the amide backbone. Furthermore, since we expect the calculated rotational strengths to be sensitive to the side-chain orientation, we hope to deduce some information on the conformational angles, χ_1 and χ_2, in our cyclic peptides.

A complete theory of CD in cyclic peptides would contain three contributions. First one should include all interactions among the tyrosyl groups. A second contribution is the interaction among the amides, each having an n-π^* and a π-π^* transition. Third, one should include interactions between the tyrosyl absorption bands and those of the amides. Such a

[1] We follow the most recent convention on peptide conformation angles suggested by IUPAC–IUB (1970).

calculation, allowing for mixing of all these interactions, has been recently reported for poly(L-tyrosine) by Chen and Woody (1971).

In this study we propose a simplified theory which neglects certain of these interactions in an attempt to give a qualitative interpretation of the data. Since the tyrosyl residues are distant, at opposite ends of the *cyclo*-(Gly$_2$-Tyr)$_2$ structure, we may neglect interactions between them without sacrificing accuracy. The interactions among the amide n-π^* and π-π^* bands determine the Cotton effects which we assign to the amide backbone. In our treatment of the experimental data, we have separated these effects from the side-chain CD bands and our present theory is intended to calculate only the latter. Since amide Cotton effects have been very thoroughly treated by Schellman and his coworkers (Bailey *et al.*, 1969), we concentrate here on the side-chain Cotton effect.

It is the remaining interaction, that between the tyrosyl and the amides, which is calculated in this theory. These side-chain bands (see Figure 3) arise from interactions between the tyrosyl bands at 277 and 227 nm and the amide n-π^* and π-π^* transitions. In fact, we need consider only the interaction of the tyrosyl bands with the amide π-π^* transitions neglecting the n-π^* interaction for the following reasons.

The influence of the tyrosyl residue on the amide n-π^* band is evidenced by the enhancement of the n-π^* CD band in *cyclo*-Gly$_5$-Tyr over that in *cyclo*-Gly$_5$-Leu (Ziegler and Bush, 1971). This enhancement can be due either to static field effects or to coupling of the n-π^* magnetic transition moment (m_j) with the tyrosyl electric transition dipole moment (μ_i).[2] This latter coupling effect, ($m_j \cdot \mu_i$) coupling, will also influence the CD bands assigned to tyrosine. We neglect this coupling effect in calculating the tyrosyl CD bands assuming that most of the enhancement by the tyrosyl of the amide n-π^* CD band is due either to static field effects or to coupling with shorter wavelength (λ less than 210 nm) bands of tyrosine. The amide–tyrosyl interaction which remains is the electric dipole coupled oscillator effect involving the amide π-π^* transition with the tyrosyl bands at 277 and 227 nm. Recent optical activity calculations on tyrosine indicate that the electric dipole coupling mechanism is dominant for the Cotton effects of these two aromatic bands in the case of the free amino acid (Hooker and Schellman, 1970). Likewise, this effect is predominant in the case of poly(tyrosine) (Chen and Woody, 1971).

This coupled oscillator effect is then calculated in a dipole–dipole approximation following Tinoco (1963) and Bush and Tinoco (1967). For the electric transition dipole moments we use the values given by Chen and Woody (1971) which are summarized in Table III.

For the geometry of the cyclic peptide we use standard peptide bond angles and bond distances (Ramachandran and Sasisekharan, 1968). The dihedral angles ϕ and ψ are the standard peptide conformational angles and are taken from the backbone models proposed by Kopple *et al.* (1972). The conformational angles of the tyrosyl side chain, χ_1 and χ_2, are allowed to take on values corresponding to energy minima found in conformational calculations (Maigret *et al.*, 1971). For aromatic side chains, χ_1 takes on values of 60°, 180°, or 300° while χ_2 takes on values of 60° or 300°.

The calculated rotational strengths of the 277- and 227-nm tyrosyl transitions are given in Table IV for the six allowed side-chain conformations. We include results for three models in which the tyrosyl residue is the first corner residue of a β loop and for one model in which it is the extended residue. For the first three models in Table IV, all of which have tyrosyl in the first corner position, the results are similar. Consistent with our argument that the coupled oscillator mechanism dominates, the calculated rotational strengths are comparable to the measured values given in Table II. Keeping in mind the approximate nature of this optical activity theory, we conclude that the rotational strengths for all three of these models are in agreement with experiment in sign and order or magnitude for the side-chain orientation given by $\chi_1 = 300°$ and $\chi_2 = 60°$. This side-chain orientation is in agreement with the minimum energy calculations of Maigret *et al.* (1971). There is probably some conformational freedom of these side chains as indicated by the coupling constants measured by Kopple *et al.* (1972) and we conclude that the most favored angles are $\chi_1 = 300°$, $\chi_2 = 60°$.

The fourth model in Table IV represents a compound with the tyrosyl residue in an extended conformation and the results are somewhat different. No single value of χ_1 and χ_2 leads to agreement between theory and experiment for both the 277- and 227-nm CD bands. We interpret this result as an indication that conformational equilibrium is likely.

The fact that we observe a positive rotational strength for T1 (277 nm) in cGHGTAG (see Table II) implies that the conformation $\chi_1 = 180°$, $\chi_2 = 60°$ must contribute substantially. The conformations with $\chi_1 = 60°$ place the tyrosyl residue in contact with the backbone and we consider them to be unlikely. While our conclusions about the extended residue are less certain than those about the tyrosyl residue as the corner residue in the β loop, we suggest that $\chi_1 = 300°$ contributes and that $\chi_1 = 180°$ occurs in combination with $\chi_2 = 60°$. These conclusions are in approximate agreement with the results of Kopple (1971) on the cyclic heptapeptide evolidine in which phenylalanine appears as an extended residue adjacent to a β loop.

TABLE III: Electric Transition Dipole Moments.

	Wavelength (nm)	μ (Debye)	Polarization
Amide π-π^*	190	3.05	38°[a]
Tyrosyl-T1	277	1.14	Short axis
Tyrosyl-T2	227	2.45	Long axis

[a] Polarization in the amide plane, angle relative to C=O bond (Chen and Woody, 1971).

Application to Other Cyclic Peptides

The combination of nmr data, conformational calculation, and CD data now available for cyclic peptides makes it possible to apply some generalizations about conformation to compounds already described in the literature. Conformational calculations indicate that the corner residues of β loops may have either the same or opposite optical configuration leading to two different conformations of the β loop (Venkatachalam, 1968). Apparently the turn corresponding to different handedness, the L–D turn, is energetically favored when the sequence is L-Gly, as this is the conformation observed in the compounds discussed in this paper (Kopple

[2] We follow the notation of Tinoco (1962) for magnetic and electric transition moments of differing groups i and j.

TABLE IV: Calculated Rotational Strengths in 10^{-40} cgs esu.

	χ_1/χ_2	60/60	60/300	180/60	180/300	300/60	300/300
Corner residue	T1	2.9	−0.2	0.3	−0.7	−1.4	0.0
C_2 model[a]	T2	−6.4	−6.3	5.6	5.6	1.6	1.6
Monosubstituted model[b]	T1	2.3	−0.5	1.4	−0.7	−2.1	0.7
Centrosymmetric type	T2	−1.3	−1.2	−2.6	−2.6	3.8	3.9
c-GHGATG[c]	T1	2.2	−2.0	0.0	−0.8	−1.7	−0.4
	T2	−4.3	−4.3	4.8	4.8	10.3	10.3
Extended residue[d]	T1	−0.5	−1.6	0.6	−0.8	−1.7	−0.2
c-GHGTAG	T2	17.5	17.5	−3.2	−3.2	12.5	12.5

[a] Model of Table IV of Kopple *et al.* (1972). [b] Model of Table VB of Kopple *et al.* (1972). [c] Model of Table VIA of Kopple *et al.* (1972). [d] Model of Table VIA with Tyr and Ala position reversed.

et al., 1972). Moreover, the glycine seems to be the residue most easily accommodated in the extended residue of the structure, although this feature may be sacrificed in favor of the L–D turn as it is in the cases of c-GHGTAG and c-GHGATG.

One may use these principles to predict that any cyclic hexapeptide having the sequence *cyclo*-(L-D-Gly)$_2$ will adopt a conformation similar to the C_2 structure proposed by Kopple *et al.* (1972) having glycine as the extended residues. The CD curves of several cyclic hexapeptides meeting these requirements have been reported by Blaha *et al.* (1969, 1970). Their compound V [*cyclo*-(Gly-Leu-D-Phe)$_2$] and their compound VIIIB [*cyclo*-(Gly-S-benzylcysteine-D-Leu)$_2$] conform to our requirements, and their compound I [*cyclo*-(Gly-D-Phe-Leu)$_2$] has an enantiomeric relationship to our compounds.

Although the compounds of Blaha *et al.* (1969, 1970) contain benzyl side chains which have side-chain CD bands, we may compare their CD curves to that of *cyclo*-(Gly$_2$-Leu)$_2$ to look for similarities (Figure 2 of Ziegler and Bush, 1971). Allowing for a reversal in sign for compound I, all four of these compounds of Blaha *et al.* (1969, 1970) have negative CD bands in the 222–225-nm region which we assign to amide n-π^* Cotton effects from a backbone in the C_2 conformation. The band is larger by a factor of two to three in compounds I, V, and VIIIB than in *cyclo*-(Gly$_2$-Leu)$_2$. This enhancement may result from the increased rigidity of the backbone containing an L-D turn over an L-Gly turn or it could result from the perturbation of the amide n-π^* by the benzyl side chains as we have observed in the case of tyrosyl side chains.

Our interpretation differs from that of Blaha *et al.* (1969, 1970) who claim that the conformation of compounds V and VIIIB differ. Our interpretation is that the amide backbones are similar and that the chromophoric benzyl side chain has been moved from the second corner residue to the first corner residue in going from compound V [*cyclo*-(Gly-Leu-D-Phe)$_2$] to compound VIIIB [*cyclo*-(Gly-*S*-benzylcysteine-D-Leu)$_2$]. The benzyl chromophore has very strong bands in the 200-nm region, so changing its orientation could cause the substantial difference seen in the CD of these two compounds in that region.

We also differ with the claim of Blaha *et al.* (1969) that stacking of the phenylalanyl residues is important in compounds I and V. Stacking is usually strong only in heterocycles, not in benzyl groups, and the requirements of the amide backbone seem to predominate in keeping the aromatic residues at opposite ends of the C_2 structure.

The compounds of Blaha *et al.* (1969) could conceivably be used as models for the phenylalanyl chromophore in much the same way as we have used our compounds to represent tyrosyl side-chain Cotton effects. The contribution of amide CD could be approximately represented by the CD *cyclo*-(Gly$_2$-Leu)$_2$ or more desirably by that of *cyclo*-(Gly-D-Leu-L-Leu)$_2$ which has not been reported. CD bands due to phenylalanine appear at 213 nm and at 268 nm in the curves reported by Blaha *et al.* (1969, 1970).

Clearly it would be desirable to extend our analysis of aromatic side-chain Cotton effects to the study of some physiologically important peptides. β loops containing aromatic side chains are known to occur in several cases. Oxytocin has a tyrosyl residue occurring as an extended residue in the model of Urry *et al.* (1970). Unfortunately the negative Cotton effect near 280 nm seems to be more characteristic of the disulfide bond in oxytocin than of the tyrosyl residue (Beychok and Breslow, 1968).

Gramicidin S has a β loop, the turn of which is composed of –D-Phe-L-Pro– (Stern *et al.*, 1968). We might hope that the phenylalanyl Cotton effects for gramicidin S would be similar to those in compound I, *cyclo*-(Gly-D-Phe-L-Leu)$_2$, of Blaha *et al.* (1969) which has a positive CD band at 268 nm, but gramicidin S has a negative band at 268 nm (Urry *et al.*, 1969). We suspect that this discrepancy may arise from conformational differences due to the prolyl residue in gramicidin S. It may be that prolyl residues in corners of loops follow conformational rules different from those for other side chains. In support of this hypothesis, the CD of *cyclo*-(Gly$_2$-Pro)$_2$ which has been reported by Deber *et al.* (1970), differs substantially from that of *cyclo*-(Gly$_2$-Leu)$_2$ or *cyclo*-(Gly$_5$-Leu) (Ziegler and Bush, 1971). We interpret this difference as an indication that *cyclo*-(Gly$_2$-Pro)$_2$ may be in a different conformation perhaps containing cis peptide bonds.

Acknowledgment

It is a pleasure to acknowledge the help of Dr. K. D. Kopple who supplied all the peptides studied here as well as advice on their conformations.

References

Bailey, P. M., Neilsen, E. B., and Schellman, J. A. (1969), *J. Phys. Chem. 73*, 228.

Beychok, S., and Breslow, E. (1968), *J. Biol. Chem. 243*, 151.

Blaha, K., Frick, I., Bezpalova, Z., and Kaurov, A. (1970), *Collect. Czech. Chem. Commun. 35*, 3557.
Blaha, K., Frick, I., and Rudinger, J. (1969), *Collect. Czech. Chem. Commun. 34*, 3497.
Bush, C. A. (1971), *in* Physical Techniques in Biological Research, Vol. IA, Oster, G., Ed., New York, N. Y., Academic Press, p 439.
Bush, C. A., and Tinoco, I., Jr. (1967), *J. Mol. Biol. 23*, 601.
Bystrov, V. F., Portnova, S. L., Tsetlin, V. I., Ivanov, V. T., and Ovchinnikov, Yu. A. (1969), *Tetrahedron 25*, 493.
Chen, A. K., and Woody, R. W. (1971), *J. Amer. Chem. Soc. 93*, 29.
Deber, C. M., Scatturin, A., Vaidya, V. M., and Blout, E. R. (1970), *Peptides: Chem. Biochem., Proc. Amer. Peptide Symp., 1st 1968*, 163.
Hooker, T., and Schellman, J. A. (1970), *Biopolymers 9*, 1319.
IUPAC–IUB (1970), *Biochemistry 9*, 3471.
Kopple, K. D. (1971), *Biopolymers 10*, 1139.
Kopple, K. D., Go, A., Logan, R. H., and Savrda, J. (1972), *J. Amer. Chem. Soc. 94*, 973.
Kopple, K. D., Ohnishi, M., and Go, A. (1969a), *J. Amer. Chem. Soc. 91*, 4264.
Maigret, B., Perahia, D., and Pullman, B. (1971), *Biopolymers 10*, 107.
Pysh, E. S. (1970), *Science 167*, 290.
Ramachandran, G. N., Chandrasekaran, R., and Kopple, K. D. (1971), *Biopolymers 10*, 2113.
Ramachandran, G. N., and Sasisekharan, V. (1968), *Advan. Protein Chem. 23*, 2083.
Schywzer, R. (1959), *Rec. Chem. Progr. 20*, 147.
Stern, A., Gibbons, W. A., and Craig, L. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 734.
Tinoco, I., Jr. (1962), *Advan. Chem. Phys. 4*, 113.
Tinoco, I., Jr. (1963), *Rad. Res. 20*, 133.
Urry, D. W., Ohnishi, M., and Walter, R. (1970), *Proc. Nat. Acad. Sci. U. S. 6*, 111.
Urry, D. W., Ruiter, A. L., Starcher, B. C., and Hinners, T. A. (1969), *Antimicrob. Ag. Chemother. 1968*, 87.
Venkatachalam, C. M. (1968), *Biopolymers 6*, 1425.
Ziaudin, Kopple, K. D., and Bush, C. A. (1972), *Tetrahedron Lett.*, 483.
Ziegler, S. M., and Bush, C. A. (1971), *Biochemistry 10*, 1330.

Amino Acid Sequence of Thermolysin. Isolation and Characterization of the Fragments Obtained by Cleavage with Cyanogen Bromide†

Koiti Titani, Mark A. Hermodson, Lowell H. Ericsson, Kenneth A. Walsh, and Hans Neurath*

ABSTRACT: Commercial preparations of crystalline thermolysin were purified by gel filtration under conditions which minimized autolysis. Gel electrophoresis in the presence of sodium dodecyl sulfate and gel filtration in 6 M guanidine hydrochloride indicated the presence of a single polypeptide chain with a molecular weight of approximately 37,000–38,000. On the basis of the amino acid composition and metal content the minimum molecular weight was calculated to be 34,800. End-group analyses yielded amino-terminal isoleucine and carboxyl-terminal lysine. All attempts to detect carbohydrates gave negative results. As predicted from the presence of two methionine residues, three major fragments were obtained after treatment with cyanogen bromide. The molecular weights and amino acid compositions of these fragments were determined. End-group analyses of these fragments, together with characterization of a small amount of a larger overlap fragment, established the linear order within the polypeptide chain of thermolysin.

Thermolysin is an endopeptidase isolated in crystalline form from culture filtrates of the thermostable microorganism *Bacillus thermoproteolyticus* (Endo, 1962). The enzyme is representative of a group of neutral metalloproteases (Matsubara and Feder, 1971) which are largely bacterial in origin, operate at neutral pH, and are inhibited by chelating agents but not by inhibitors of "sulfhydryl" or "serine" proteases. The substrate specificity of neutral metalloproteases is preferentially directed toward internal peptide bonds wherein the imino nitrogen is contributed by hydrophobic residues (Matsubara and Feder, 1971). In this respect these enzymes resemble pancreatic carboxypeptidase A, although they are endopeptidases while the latter enzyme is an exopeptidase (Pétra, 1970).

Ohta *et al.* (1966) reported for thermolysin a preliminary molecular weight of 37,500 and an amino acid composition which revealed an abundance of hydrophobic residues. Latt *et al.* (1969) reported that the enzyme contains 1 g-atom of zinc per 37,500 g which is essential for the enzyme activity. In these features thermolysin resembles carboxypeptidase A (Neurath and Bradshaw, 1970). In contrast to carboxypeptidase A, thermolysin also contains 3–4 g-atoms of calcium per 37,500 g (Latt *et al.*, 1969) and maintains activity at higher temperatures than bovine carboxypeptidase A (Endo, 1962; Pétra, 1970). The presence of calcium ions appears to be

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received March 6, 1972.* This work has been supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (NP-18N).