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Peptides as bioorganic models

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Abstract. This article develops the possibility of using conformationally rigid peptides as bioorganic model systems. Stereochemical constraints on peptide backbone folding may be introduced by the judicious use of sequences containing α-aminoisobutyric acid and proline. The design of synthetic peptide models of 3_{10}-helical and β-turn conformations is reviewed. Attempts at generating model antiparallel β-sheet peptides are discussed. The use of disulphide crosslinks is illustrated in the application of cystine peptides to generate models for β-turn and antiparallel β-sheet conformations. Using a conformationally well-defined backbone as a skeleton attempts to generate models for protein binding sites are examined. Helical retinylidene-lysine peptides are introduced as models for the bacteriorhodopsin chromophore. Lysine containing peptides and chiral diamines are explored as model-binding sites for bilirubin and gossypol. An attempt to model the active site disulphide loop of the redox protein, thioredoxin, is described.

Keywords. Peptide conformation; bioorganic models; alpha-aminoisobutyryl peptides; bacteriorhodopsin; bilirubin; gossypol; thioredoxin; peptide models.

1. Introduction

Biological recognition processes involve extremely specific interactions between relatively complex molecules. Peptides and proteins often participate in important molecular recognition events in biology of which, receptor-effector, antigen-antibody and enzyme substrate interactions are a few well-known examples. The precise folding patterns of peptide backbones can serve to precisely orient functional sidechains in three-dimensional space. Proteins thus have the ability to generate remarkable molecular cavities, which provide a means of recognizing complementary structures. Studies in the area of biomimetic chemistry have resulted in the design of a variety of extraordinary structures which in turn have allowed the development of a wide range of 'host-guest' chemistry (Kellog 1982). Crown ethers, macrobicyclic cryptands, cyclophanes and cyclodextrins (Cram and Trueblood 1981; Weber and Vögtle 1981; Cram 1983; Breslow 1982; Lehn 1978; Cram and Katz 1983) provide illustrative examples of molecular surfaces, with specific binding abilities. The structural flexibility of peptide backbones and the multiplicity of sidechain conformations have severely limited attempts to design peptide structures, which will mimic the binding and functional characteristics of natural protein systems (Chakravarty *et al* 1973; Gutte *et al* 1979; Fukushima *et al* 1979; Schultz *et al* 1982; Moser *et al* 1983). It is clear that an understanding of the factors determining peptide chain folding must precede any attempt to rationally design and engineer the construction of a synthetic peptide with specific functional abilities. This article describes studies being carried out in the author's laboratory, which attempt to develop synthetic peptides as bioorganic model systems. Our interest in this area is based on studies of stereochemically constrained peptides, carried out during work aimed at understanding the conformation and

function of alamethicin and related membrane channel forming polypeptides (Nagaraj and Balaram 1981a; Mathew and Balaram 1983; Prasad and Balaram 1984). These systems contain high proportions of the unusual α,α-dialkylated amino acid, α-aminoisobutyric acid (Aib). The imino acids proline (Pro) and hydroxyproline (Hyp) also occur in these natural products. While examining the structural chemistry of peptides containing these residues it became clear that the stabilization of specific backbone conformations can be achieved using a judicious choice of sequences with Aib and Pro residues. Further restrictions on chain folding can be imposed by cyclization involving disulphide bridging between cysteine (Cys) residues in a given sequence. In this report a brief account of the design of peptides with defined backbone conformations is first presented, followed by a survey of a few examples of the use of peptides as model systems in bioorganic chemistry.

2. Design of conformationally defined peptides

2.1 *Helical conformations*

The peptide backbone has two main degrees of freedom about the N–C^α (ϕ) and C^α–CO(ψ) bonds, with the peptide units being largely restricted to a *trans* geometry ($\omega \sim 180°$). For residues having only a single C^α substituent, relatively large regions of ϕ, ψ space are stereochemically allowed and these are generally delineated on a two-dimensional ϕ-ψ (Ramachandran) map (Ramachandran and Sasisekharan 1968). All the major elements of protein secondary structures like the α-helix, β-sheet and reverse turn (β and γ) conformations lie within the allowed regions of the ϕ–ψ map. Upon incorporation of a second alkyl substituent at C^α, as in Aib, the stereochemically allowed regions are severely limited, with the theoretically computed energy minima lying in the left and right handed $3_{10}/\alpha$-helical regions of the conformational map ($\phi \sim \pm 60° \pm 20°$, $\psi \sim \pm 30° \pm 20°$) (figure 1). The expectation that Aib residues largely favour helical conformations has been borne out by the results of a large number of crystal structure investigations, (summarized partially in figure 1) which have been reviewed elsewhere (Prasad and Balaram 1984; Toniolo *et al* 1983). Extensive spectroscopic studies of Aib containing oligopeptides suggest that sequences of the type –$(Aib)_n$- and –$(Aib\text{-}X)_n$-adopt largely 3_{10}-helical conformations stabilized by intramolecular $4 \rightarrow 1$ hydrogen bonds (Nagaraj *et al* 1979; Venkatachalapathi and Balaram 1981; Nagaraj and Balaram 1981b; Iqbal and Balaram 1981a, b, c 1982a, b; Vijayakumar and Balaram 1983a, b). Evidence for the formation of α-helical structures by Aib containing peptides, which contain other L-amino acids interspersed in the sequence, has come from single crystal x-ray diffraction studies of the 11-residue peptide, Boc-$(Ala\text{-}Aib)_2$-Ala-Glu(OBzl)-$(Ala\text{-}Aib)_2$-Ala-OMe (Butters *et al* 1981) (figure 2) and the 20-residue natural peptide, alamethicin (Fox and Richards 1982). However, 3_{10} helix formation has been noted in the crystal structure of the decapeptide, Boc-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-OMe (Francis *et al* 1983) (figure 2). While the tendency of Aib containing sequences to favour helical folding has been established beyond doubt, the role of sequence and environmental effects in determining the precise nature of the helical structure (3_{10} or α) remains to be elucidated. Thus, sequences rich in Aib can be used to generate helical oligopeptide conformations. It should however be noted that sidechain distributions differ in the 3_{10} and α-helical conformations, with the former being characterized by 3 residues per turn

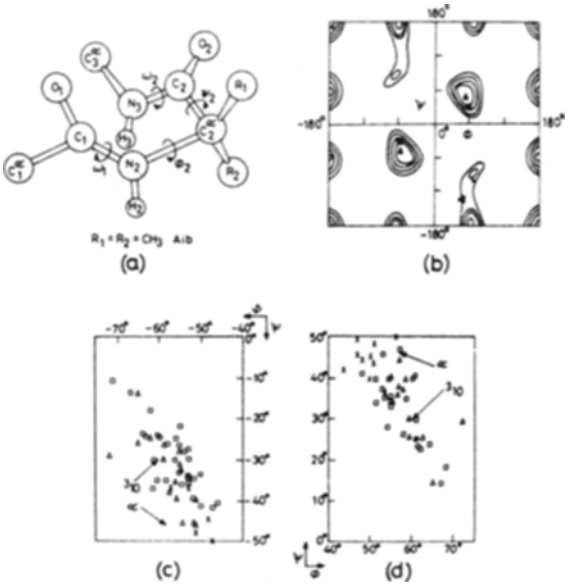


Figure 1. (**a**) Definition of conformational angles for Acetyl-Aib-N-methylamide. (**b**) Potential energy map for the Aib residue. Contours are drawn at 1 kcal mol^{-1} intervals with respect to the innermost contour enclosing the minimum. The ideal 3_{10} (■) and α- (▲) helical conformations are marked. ● represent crystal structure observations of Aib in non-helical conformations. (**c**, **d**) Crystallographic observations for Aib residues in peptides in the right (**c**) and left (**d**) handed helical regions. Tips of arrows indicate ideal 3_{10} and α-helices. △ N-terminal residue in protected peptides. □ Non-terminal residue X C-terminal Aib present as ester or acid.

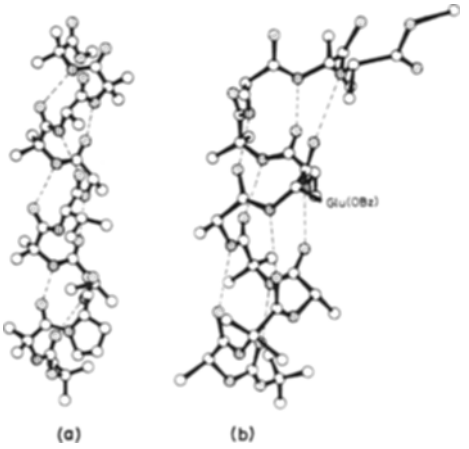
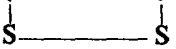


Figure 2. 3_{10} and α-helical structures observed in Aib peptide crystal structures. (**a**) Boc-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-OMe, 3_{10}-helix (Francis *et al* 1983) (**b**) Boc-Ala-Aib-Ala-Aib-Ala-Glu(OBzl)-Ala-Aib-Ala-Aib-Ala-OMe, α-helix (redrawn from Butters *et al* 1981).

of the helix, while the latter has 3·6 residues per helical turn (Ramachandran and Sasisekharan 1968).

2.2 *β-turn conformations*

Aib and Pro residues have a marked tendency to stabilize β-turn structures, in which the direction of peptide chain propagation is reversed, with two residues occupying the pivotal corner positions. β-turns have been classified into several types, differing in the ϕ, ψ values for the corner residues (Venkatachalam 1968). The ϕ value for a residue in the corner position of types I, II and III β-turns is constrained to $\sim -60°$. This is the preferred region for Aib, while in L-Pro the constraints of cyclization imposed by the pyrrolidine ring, necessarily limit ϕ_{Pro} to values of $\sim -60° \pm 15°$. The formation of specific β-turn conformations by Aib and Pro containing peptides has been exemplified in several crystal structures (Prasad and Balaram 1983, 1984). ^{1}H NMR, CD and IR studies in solution suggest that these folded conformations are, in fact, retained in solution. For the -Pro-Aib-sequence, which is an almost obligatory β-turn sequence, it has been shown that both types II and III β-turns are energetically accessible. The type II structure ($\phi_{Pro} = -58°$, $\psi_{Pro} = 139°$, $\phi_{Aib} = 61°$, $\psi_{Aib} = 25°$) has been detected in the solid state for Piv-Pro-Aib-NHMe (Prasad *et al* 1982), while the type III structure ($\phi_{Pro} = -59°$, $\psi_{Pro} = -34°$, $\phi_{Aib} = -62°$, $\psi_{Aib} = -18°$) is observed in the cyclic disulphide, Boc-Cys-Pro-Aib-Cys-NHMe (Ravi *et al* 1983). Circular dichroism and



nuclear Overhauser effect studies suggest, that in solution both structures can be populated depending upon the solvent conditions (Rao *et al* 1983; Crisma *et al* 1983). The body of available results suggests that Aib-Pro and Pro-Aib sequences can be used to generate reverse turns in acyclic peptides with a considerable degree of certainty.

2.3 *β-sheet conformations*

Both parallel and antiparallel β-sheet structures are observed in proteins (Richardson 1981). However, there have been few attempts to generate models for these structures using synthetic peptides. The associated β-sheet structures have been exclusively studied using linear homooligopeptide models (Toniolo 1977). However, the precise nature of the associated species remains to be established, in these cases. It would therefore, be desirable to establish criteria for the design of peptides, folded to generate an antiparallel β-sheet conformation as illustrated in figure 3. In choosing a synthetic target it would be desirable to incorporate a sequence, which nucleates a β-turn (hairpin bend) or γ-turn in the centre of the peptide and to incorporate residues, which favour extended β-sheet conformations. With this end in view a series of peptides of the type Boc-$(Val)_n$-Aib-Pro-$(Val)_m$-OMe ($n = 2,3$ and $m = 3$), Boc-$(Val)_n$-X-$(Val)_m$-OMe (X = Aib, Pro; $n = 2,3$ and $m = 3$), have been synthesized. The Aib-Pro sequence was chosen to ensure chain reversal by β-turn formation, while the introduction of a single Aib or Pro residue in an oligo-Val sequence might favour γ-turn formation (figure 3). Val containing sequences were chosen in view of the propensity of this residue to occur in β-sheet conformations (Chou and Fasman 1978). Figure 4 shows the CD spectra of Boc-$(Val)_2$-Aib-Pro-$(Val)_3$-OMe in different solvents. The appearance of a negative CD band at 224 nm in dioxane is suggestive of a β-sheet conformation (H Balaram, unpublished results). However, the observed solvent dependence suggests a degree of

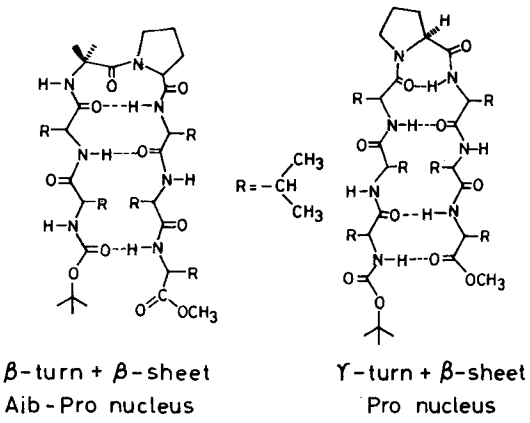


Figure 3. Possible nucleation of β-sheet conformations. Speculative conformations for Boc-$(Val)_2$-Aib-Pro-$(Val)_3$-OMe and Boc-$(Val)_3$-Pro-$(Val)_3$-OMe.

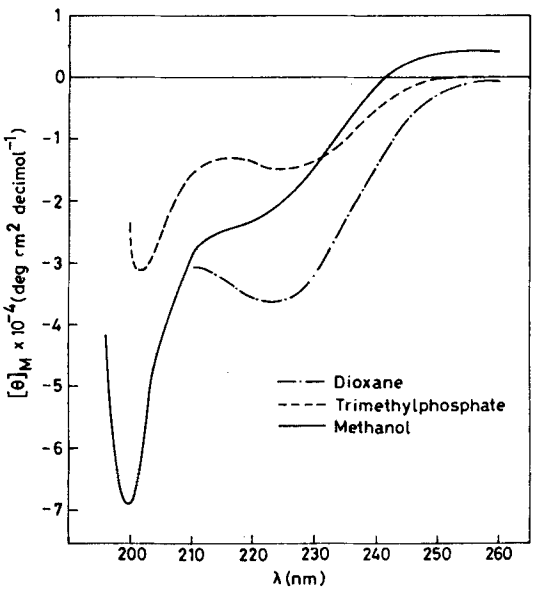


Figure 4. CD spectra of Boc-$(Val)_2$-Aib-Pro-$(Val)_3$-OMe in different solvents.

conformational flexibility. Further spectral studies, presently in progress, should allow the delineation of the conformational features of these putative β-sheet model peptides.

2.4 *Covalent constraints introduced by disulphide bridging*

Disulphide crosslinks can be introduced into peptides to stabilize specific chain conformations. In developing this approach cyclic and acyclic cystine peptides have been synthesized. Peptides of the type Boc-Cys-Pro-X-Cys-NHMe (X = Gly, L-Ala,

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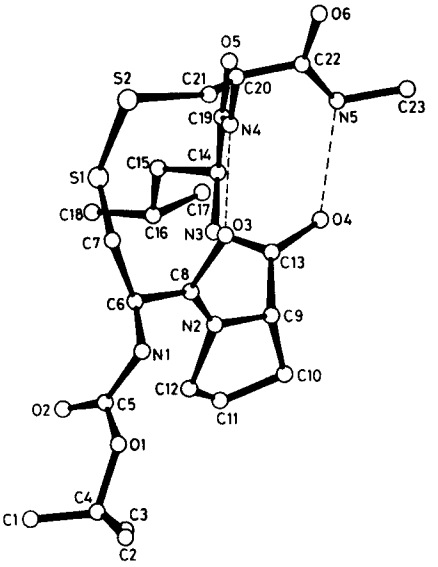

Figure 5. Solid state conformation of Boc–Cys–Pro–Leu–Cys–NHMe.
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D-Ala, Aib, Leu, Val, Phe) provide rigid models for β-turn structures. Detailed spectroscopic (^{1}H, ^{13}C, NMR, IR, CD, Raman) studies have unambiguously established these peptides as β-turn structures (Ravi *et al* 1983; Ravi and Balaram 1984; Rao *et al* 1983; Ishizaki *et al* 1981; Venkatachalapathi *et al* 1982). Crystallographic studies on the X = Leu (Prasad, unpublished) and Aib peptides (Ravi *et al* 1983) demonstrate that a consecutive β-turn or an incipient, disulphide bridged, 3_{10}-helix occurs in both peptides in the solid state. Figure 5 illustrates the solid state conformation of the Pro-Leu cyclic disulphide.

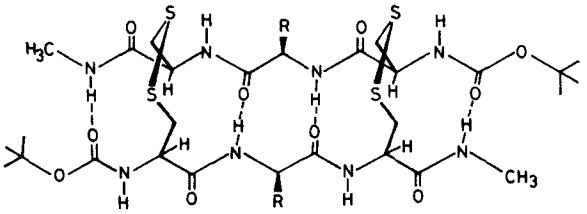
Bis-cystine peptides of the type Boc–Cys–X–Cys–NHMe
MeHN–Cys–X–Cys–Boc

(X = L-Ala, D-Ala, Gly, L-Leu) have been shown to adopt anti-parallel β-sheet conformations stabilized by two S–S crosslinks, located on the same face of the sheet (figure 6a). These peptides are characterized by unusually low field shifted Cys C$^\alpha$H resonances in ^{1}H NMR spectra of $CDCl_3$ solutions, high $J_{HNC^\alpha H}$ values ($\sim$ 9 Hz) and extremely solvent shielded X–NH and $-NHCH_3$ resonances (Ravi 1983; Kishore unpublished results). From the conformation depicted in figure 6a, it is seen that the *bis*-cystine cyclic systems could serve as a starting point for designing peptide cavities, lined by sulphur atoms. Cystine can also be used to stabilize the antiparallel β-sheet conformation in acyclic sequences. In this connection, symmetrical peptides of the type Boc–Ala–Gly–Cys–Leu–Phe–OMe

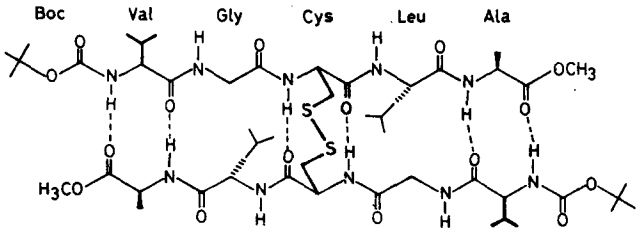
Boc–Ala–Gly–Cys–Leu–Phe–OMe and

Boc–Val–Gly–Cys–Leu–Ala–OMe

Boc–Val–Gly–Cys–Leu–Ala–OMe



(a)



(b)

Figure 6. (**a**) Proposed conformation for the cyclic bis-cystine peptides. (**b**) Probable antiparallel β-sheet conformation in the peptide Boc–Val–Gly–Cys–Leu–Ala–OMe

Boc–Val–Gly–Cys–Leu–Ala–OMe

have been synthesized. Preliminary ^{1}H NMR measurements indicate extensive aggregation, as evidenced by line broadening. The conformation likely to be preferred is shown in figure 6b (Raj, unpublished results).

3. Design of bioorganic models

3.1 *Retinylidene-lysine peptides as models for the bacteriorhodopsin chromophore*

The protein bacteriorhodopsin constitutes the major protein of the purple membrane of *Halobacterium halobium* and is involved in the conversion of photon energy into a transmembrane electro-chemical H^+ gradient (Stoeckenius and Bogomolni 1982; Stoeckenius 1980). The retinal chromophore is attached *via* a Schiff base linkage to Lys-216, which lies in a membrane spanning helical segment of the protein (Stoeckenius and Bogomolni 1982). The absorption spectrum of the protein has a band at $\sim$ 560 nm, which has been ascribed to a protonated Schiff base (PRSB) chromophore. However, this band is considerably redshifted as compared to model PRSB's obtained from retinal and model amines, leading to the suggestion that the spectral shift (opsin shift), is a result of the environmental effects due to proximate protein residues (Nakanishi *et al* 1980).

In an attempt to evaluate neighbouring group effects the peptides Boc-Aib-Asp-Aib-Aib-Lys-Aib-OMe and Boc-Aib-Asp-Aib-Ala-Aib-Lys-Aib-OMe have been synthesized. The choice of $(\text{Aib-X})_n$ sequences compells 3_{10} helical folding, thereby bringing the Asp and Lys residues within a reasonable distance for interaction. Figure 7 illustrates the disposition of residues assuming an ideal 3_{10} helical geometry for the peptide backbone. Table 1 summarizes the λ_{max} values obtained for the retinylidene peptides in

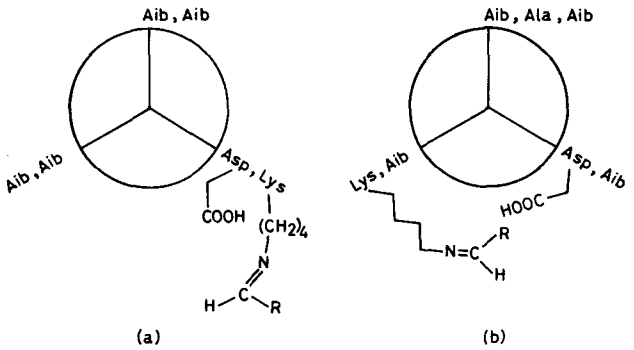


Figure 7. Helical wheel representations of peptides Boc-Aib-Asp-Aib-Aib-Lys-Aib-OMe (**a**) and Boc-Aib-Asp-Aib-Ala-Aib-Lys-Aib-OMe (**b**), in an ideal 3_{10} helical conformation. In (**a**) the Lys and Asp residues lie adjacent on the same face of the helix. In (**b**) the Asp and Lys residues lie on different faces with the Lys residue being translated by one turn of the helix.

protonated and unprotonated forms in different solvents. The results in solvents like $CHCl_3$ and CH_2Cl_2 suggest that the proximal Asp residue may stabilize the PRSB ground state by hydrogen bonding, in poorly solvating media thereby reducing the magnitude of the protonation red shift (Vijayakumar and Balaram 1984). These results suggest that a detailed examination of the effect of proximate residues is indeed possible, using the Aib-X sequences to provide a rigid helical scaffolding.

3.2 *Models for binding sites on proteins: Bilirubin and gossypol*

Proteins often interact with small molecules by specific multipoint interactions involving electrostatic, hydrogen bonding and hydrophobic effects. We have chosen to design peptide binding sites for the acyclic tetrapyrrole pigment, bilirubin and the phenolic triterpene gossypol (figure 8). Bilirubin, a degradation product of heme, is an extremely important metabolite involved in a variety of disease states (Berthelot *et al* 1982). The pigment has a strong affinity for serum albumins and binds noncovalently to a high affinity site on the protein (Brodersen 1982). The hydrophobic nature of this site and the involvement of charged residues like Lys and Arg have been inferred from several biochemical studies (Jacobsen 1975, 1977). Gossypol is acquiring increasing importance as a male antifertility agent (Quian *et al* 1980), in addition to possible uses against herpes virus (Wichmann *et al* 1982) and in the treatment of malaria (Vander Jagt *et al* 1982). This binaphthyl derivative has been isolated in the racemic (±) form from cottonseed oil (Abou Donia 1976) and in optically active (+) form from the bark of the tree *Thespesia populnea* (Datta *et al* 1968; King and De Silva 1968). A resolution of optical isomers (atropoisomers) has not been effected and (−) gossypol is, as yet, unavailable for biological studies. Gossypol inhibits a variety of nucleotide binding enzymes, of which its specific inhibition of the lactate dehydrogenase of sperm (LDH-X) is of special interest (Olgiati and Toscano 1983). It has also been shown to bind to serum albumins and glutathione-S-transferases, at the same site as bilirubin (Royer and Vander Jagt 1983; Vander Jagt *et al* 1983). The presence of two aldehyde groups on gossypol suggests that bifunctional attachment to a protein *via* Schiff base formation, involving two proximal Lys residues is an attractive possibility.

Table 1. Absorption band positions for retinylidene peptides[a] in various solvents.

Solvent	Peptide 1			Peptide 2			Peptide 3		
	SB λ_{max} (nm)	SBH^{+}[b] λ_{max} (nm)	$\Delta\nu$ (cm^{-1})	SB λ_{max} (nm)	SBH^{+} λ_{max} (nm)	$\Delta\nu$ (cm^{-1})	SB λ_{max} (nm)	SBH^{+} λ_{max} (nm)	$\Delta\nu$ (cm^{-1})
Dimethylsulphoxide	357	435	5023	356	435	5101	355	439	5390
Methanol	358	446	5511	357	443	5438	357	444	5489
n-Octanol	361	443	5127	360	432	4501	357	439	5232
Ethylacetate	339	434	6457	347	433	5724	346	439	6123
Chloroform	337	410	5283	336	408	5252	336	460	8023
Dichloromethane	—	—	—	—	—	—	356	498	8010

[a] Peptide 1 = Boc-Aib-Asp-Aib-Aib-Lys-Aib-OMe; Peptide 2 = Boc-Aib-Asp-Aib-Ala-Aib-Lys-Aib-OMe; Peptide 3 = Boc-Aib-Lys-Aib-OMe.
[b] Protonation was effected by adding excess trichloroacetic acid (0·028 M).

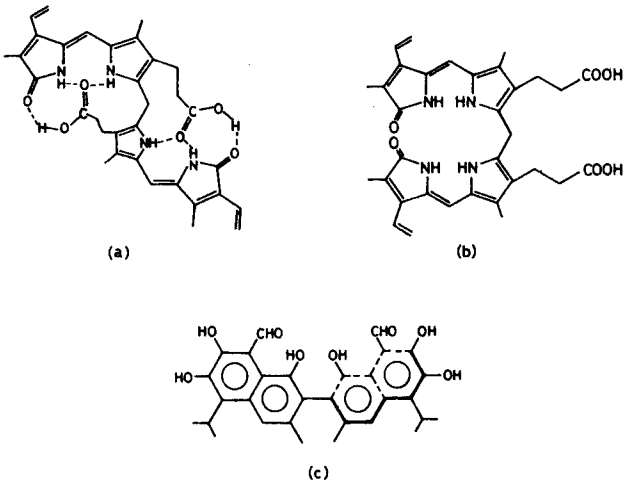


Figure 8. Extended (**a**) and folded (**b**) conformations of bilirubin IX-α. (**c**) Gossypol.

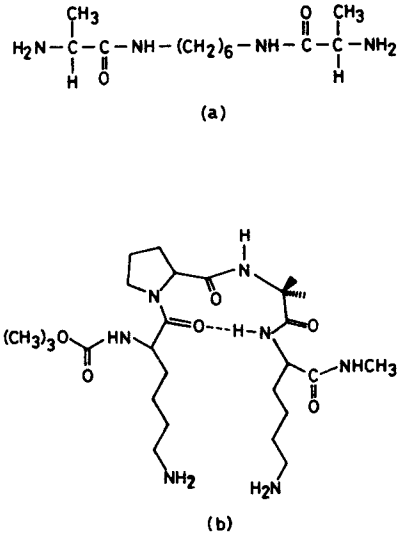


Figure 9. Structures of N,N'-bis-L-alanyl-hexamethylenediamine (**a**) and Boc-Lys-Pro-Aib-Lys-NHMe in a probable β-turn conformation (**b**).

We have therefore explored the possibility of developing diamines and di-Lys peptides as model receptors for both bilirubin and gossypol. Figure 9 illustrates the structures of two molecules that have been synthesized as a first step in this direction. Di-L-alanyl hexamethylenediamine is a chiral diamine and it was hoped that specific interactions with bilirubin could be monitored by induction of optical activity in the pigment (Blauer and Wagniere 1975). The CD spectra of bilirubin in the presence of HSA at pH 8·7 and with the chiral diamine in dioxane, are compared in figure 10. The

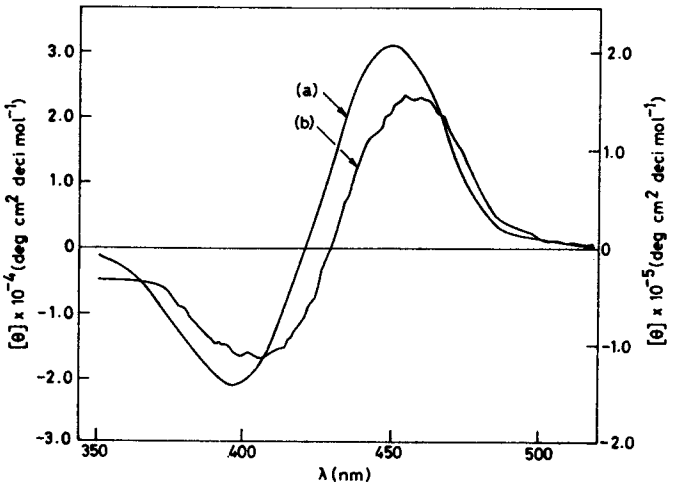


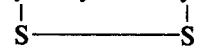
Figure 10. CD spectra of bilirubin in the presence of human serum albumin (HSA) and di-L-alanylhexamethylene diamine. (a) HSA $3{\cdot}46 \times 10^{-5}$ M, bilirubin $3{\cdot}46 \times 10^{-5}$ M, pH 8·7, phosphate buffer. (b) Diamine $2{\cdot}1 \times 10^{-3}$ M, bilirubin $2{\cdot}06 \times 10^{-5}$ M, dioxane. Right scale is for trace (a). Left scale is for trace (b).

similarity of the signs of the CD bands at ~ 400 nm (negative) and ~ 450 nm (positive) suggests that the same helical twist of the dipyrrylmethene chromophores, is maintained in both cases. In contrast, entirely different CD spectra are generated in the presence of α-helical and β-sheet forms of poly-L-lysine in aqueous solution (Marr-Leisy and Balaram 1984).

Figure 11 shows the CD spectrum of (+) gossypol in dioxane and the influence of the addition of the peptide Boc-Lys-Pro-Aib-Lys-NHMe. This peptide was chosen to permit β-turn formation at the Pro-Aib segment, thereby allowing the Lys residues the possibility of binding simultaneously to the two aldehyde moieties on gossypol. The dramatic changes in CD spectra suggest that this expectation has been realized. Further support for the bifunctional interaction comes from the lack of significant changes in (+) gossypol CD spectra in the presence of Ac-Lys-NHMe (Whaley *et al*, unpublished).

3.3 *Models of protein active sites*

Attempts to model the active sites of enzymes like proteases have thus far met with limited success (Schultz *et al* 1982). We have, therefore, chosen to develop models for redox sites, which are composed of small disulphide loops. Thioredoxin, an ubiquitous protein, is of great importance in a variety of cellular redox processes (Holmgren 1981a). The active site of the protein consists of the fourteen membered loop formed between Cys residues 32 and 35 (figure 12). Structural changes on reduction of the disulphide have been monitored by following the enhancement of Trp fluorescence (Holmgren 1981b). The peptides Boc–Cys–Gly–Pro–Cys–NHMe



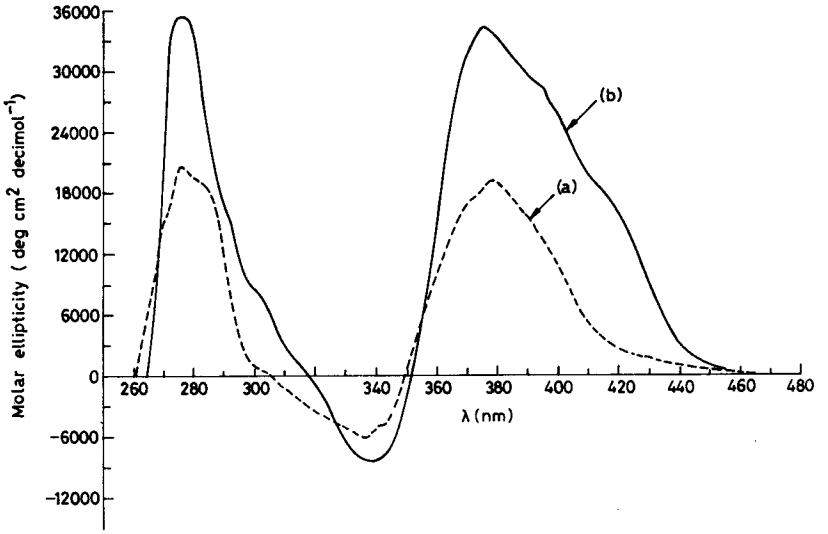


Figure 11. CD spectra of (+) gossypol and a peptide complex. (a) (+) Gossypol 6×10^{-6} M in dioxane. (b) (+) Gossypol 6×10^{-6} M and 5×10^{-5} M Boc-Lys-Pro-Aib-Lys-NHMe in dioxane.

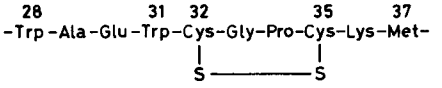


Figure 12. Partial sequence of *E. coli* thioredoxin in the vicinity of the active site.

and Boc–Trp–Cys–Gly–Pro–Cys–NHMe have been synthesized as models for the
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thioredoxin active site. ^{1}H NMR and CD studies favour a consecutive β-turn conformation in the former (Ravi and Balaram 1983). Figure 13 shows the effect of disulphide reduction on the Trp containing peptide model. It is observed that the Trp fluorescence enhancement parallels that observed for the native protein (Kishore *et al* 1983). The active site peptide of the protein glutaredoxin (Holmgren 1981a), –Cys–Pro–Tyr–Cys
S——S
has also been synthesized and studies on the synthesis of the thioredoxin segment shown in figure 12 are underway (Kishore, unpublished). A comparison of the structural, spectroscopic and redox properties of these systems will be of value in elucidating further details of the molecular mechanisms involved at the redox active site in thioredoxin and related proteins.

4. Conclusions

An attempt has been made in this article to emphasize the utility of designing peptides with defined backbone conformations. A few examples of their application in

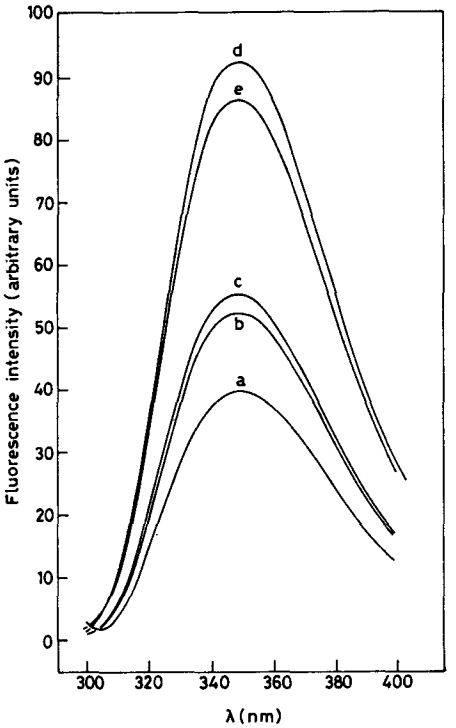

Figure 13. Fluorescence spectra of Boc–Trp–Cys–Gly–Pro–Cys–NHMe excited at

S————S

290 nm, in water. (a) Peptide (b) Peptide + dithiothreitol (DTT) 20 minutes after addition of reducing agent (c) 50 minutes after DTT addition. (d) L-Trp (e) L-Trp + DTT. Curves (d) and (e) represent controls.

bioorganic chemistry have been briefly illustrated. Of necessity, these have been limited to examples emanating from the author's laboratory. Significant obstacles remain in the synthesis of peptides containing more than one conformational domain and in the rational control of sidechain flexibility. There is little doubt that intensified activity in this area should rightfully position peptide chemistry as a central part of bioorganic chemistry.

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