Ultraviolet Spectra of Dehydropeptides by Double Beam Measurement

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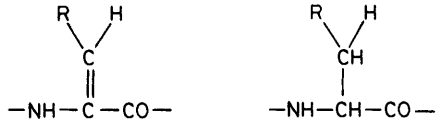
The double beam measurement (d.b.m.) method, in which the u.v. spectrum of an unsaturated peptide is scanned using the saturated analogue as a reference, allowed the isolation of the u.v. absorption peaks of the dehydroamino-acid moiety in unsaturated dehydropeptides (DHP). Three types of DHP were measured as models: ΔAla † peptides having no β-substituents, showed peaks at 200 and 240 nm; ΔLeu peptides with a β-alkyl substituent also showed two peaks at 200 and 220—230 nm; and ΔPhe peptides with a β-phenyl substituent had three peaks at 200, 220, and 280 nm. These distinct peaks were caused by steric effects about the double bond of the dehydroamino-acid moieties. The effect of configuration (*Z* or *E*) was also discussed.

MANY bioactive dehydropeptides (DHP), which contain αβ-unsaturated amino-acids (dehydroamino-acids), have been found in nature.[1,2] In recent years, studies on the synthesis of DHP gave been carred out in order to prepare potent analogues of peptide hormones and to develop further the structure–activity relationship in these compounds.[1-3] It is clear that the spectrometric determination of a dehydroamino-acid moiety in a peptide sequence is important for confirmation of the structure of a stereochemically pure synthetic DHP. ^1H and ^{13}C n.m.r. spectroscopy is often used for these purposes.[2]

We have synthesized several highly active dehydroenkephalins, for example [D-Ala[2], ΔPhe[4], Met[5]]enkephalin amide,[4] and during these syntheses,[5] we found that the u.v. spectrum could be used as a very useful monitor of the introduction of the dehydroamino-acid moiety and for the final characterization of the DHPs. Only a few reports of u.v. studies used for identification of synthetic and natural DHP have appeared.[6-9] However, in these investigations, the dehydroamino-acid moiety itself could not be clearly characterized because of the strong absorption by peptide bonds below 240 nm. Therefore, in this work, we intended to isolate the spectrum of the dehydroamino-acid moiety from the u.v. spectrum of the remainder of the peptide sequence.



(Ia) (Ib)

(1) R = H
(2) R = $CH(CH_3)_2$
(3) R = C_6H_5

Three types of DHP (Ia) were selected as models: (1a) a vinyl type, dehydroalanine (ΔAla); (2a) a β-alkylvinyl type, dehydroleucine (ΔLeu); (3a) a β-arylvinyl type, dehydrophenylalanine (ΔPhe). In order to extract the absorption of the dehydroamino-acid moiety, the saturated analogues (Ib) of these DHP were synthesized and used as references. A series of double beam measurements (d.b.m.) in which the u.v. spectrum of the unsaturated peptide is scanned using the saturated analogue as reference are described in this paper. The steric effect of the configuration, *Z* or *E*, on the u.v. spectra of DHP [types (2) and (3)] is also discussed.

† Abbreviations according to IUPAC–IUB Commission, *Biochemistry*, 1972, **11**, 1726, are used throughout. Additional abbreviations: Bu^tOCl, t-butyl hypochlorite; Δ, αβ-didehydro; DMF, *NN*-dimethylformamide; DBU, 1,5-diazabicyclo[5.4.0]-undec-5-ene; Boc, t-butoxycarbonyl; Bzl, benzyl.

EXPERIMENTAL

Spectra.—The u.v. spectra were measured on a Varian Cary 219 or a Beckman UV 5260 spectrophotometer. Acetonitrile, chloroform, and methanol were of spectroscopic quality and the remaining solvents were reagent grade. The measurements were performed such that the same concentrations of unsaturated and saturated peptides were used. ^1H N.m.r. spectra were recorded on a Varian EM-390 90 MHz spectrometer with tetramethylsilane as internal standard.

Peptides.—All peptides were synthesized by classical methods and finally purified by silica gel or Sephadex LH-20 column chromatography. Their purity was checked by t.l.c., ^1H n.m.r., and elemental analyses. The methods of DHP synthesis are described as follows without detailed description: the dehydroalanine residue was derived from an L-serine residue by tosylation followed by β-elimination using diethylamine;[10] dehydroleucine was prepared by *N*-chlorination of leucine esters with Bu^tOCl followed by dehydrochlorination and isomerization of the intermediate imine with DBU;[11] Boc-pGlu-ΔPhe-OBu^t (VIa) was made from the saturated peptide (VIb) by the same method as described for the ΔLeu-peptides,[12] but other ΔPhe peptides were prepared from *threo*-DL-β-phenylserine dipeptides by dehydroazlactonizations.[13] All ΔLeu and ΔPhe peptides except Bz-Δ^ELeu-OMe and Z-Δ^EPhe-OEt have the *Z*-configurations, as determined by ^1H n.m.r.[14,15]

RESULTS

The d.b.m. method allowed the isolation of the u.v. peaks below 250 nm as shown in Figures 1—7. The ΔAla peptides afforded a single sharp peak at 240 nm (Table 1, Figure 1). In the standard measurements, this peak (ε 4 000) is observed only when the number of amino-acid residues in the peptide is small as in (IIa) in Figure 1. In the case of large peptides or depsipeptides containing ΔAla, this peak is discernible only as a shoulder.[8,9] The regular u.v. absorption spectra of β-alkyl-substituted DHPs like dehydroaminobutyric

TABLE 1

Spectroscopic properties of dehydrodipeptides: chemical shift of vinyl proton in ^1H n.m.r. and u.v. absorption [a]

	Peptide	Chemical shift δ of vinyl proton ($CHCl_3$)	U.v. spectra MeOH λ/nm	ε	CH_3CN λ/nm	ε	95% EtOH λ/nm	ε	$CHCl_3$ λ/nm	ε
(IIa)	Boc-Phe-ΔAla-OMe	5.94, 6.66	242	7 000	244	5 300	(insoluble)			
(IIb)	Boc-Phe-Ala-OMe		<200		<200		<200			
(IIIa)	Boc-Phe-ΔLeu-OBzl	6.50 (d)	222	7 400	227	6 100	220	7 300		
(IIIb)	Boc-Phe-Leu-OBzl		<200		<200		<200			
(IVa)	Boc-pGlu-ΔPhe-OBu^t	7.42 (Ar envelope)	280	18 300	279	17 100	280	18 000	286	16 300
			221	11 700	221	11 000	221	10 900		
(IVb)	Boc-pGlu-Phe-OBu^t		<200		<200		<200			

[a] Series a were measured by the d.b.m. method while series b were measured by standard methods.

acid (ΔAbu), ΔVal, or ΔLeu peptides do not even show the shoulder,[7] because the peptide bond has a large absorption velow 230 nm. However, by the d.b.m. method, the ΔLeu peptides showed a distinct peak at 220—230 nm (ε 5 000—7 000), which was detected for the first time (Figures 2 and 4).

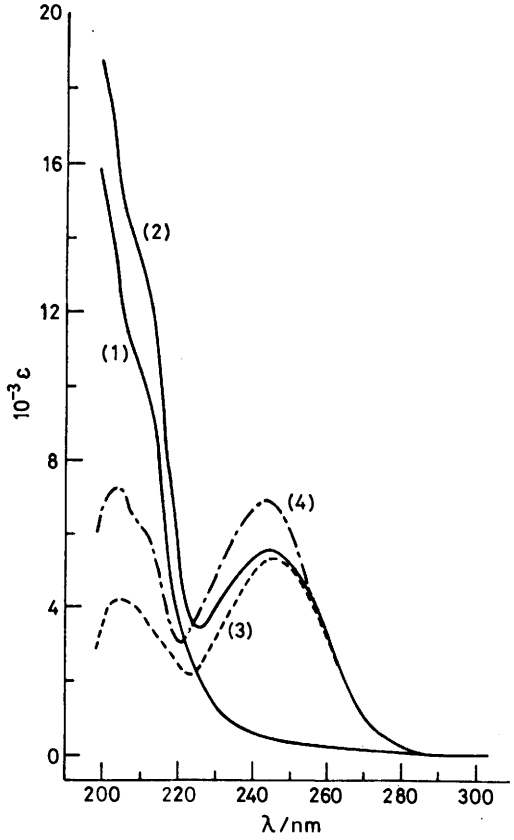


FIGURE 1 U.v. spectra of (1) Boc-Phe-Ala-OMe (IIb); (2) Boc-Phe-ΔAla-OMe (11a); (3) Boc-Phe-ΔAla-OMe (IIa) (d.b.m.) in CH_3CN (3.2×10^{-5}M), and (4) Boc-Phe-ΔAla-OMe (IIa) (d.b.m.) in MeOH (3.2×10^{-5}M)

The ΔPhe peptides showed a peak at 220 nm (ε 9 000—12 000) along with a strong peak at 280 nm (ε 16 000—20 000) (Figures 3 and 7).

Although all types of DHP showed another peak at *ca.* 200 nm in the d.b.m. spectra as shown in Figures 1—7, it was difficult to estimate the intensity of this peak exactly because measurements near 200 nm are quite limited by solvents and instrumental techniques. The solvents, CH_3CN, MeOH, and 95% EtOH, showed a very small peak below 205 nm, but the additional peak of all the DHPs near 200 nm was much more intense than that of solvents and should be recognized as a significant absorption.

The concentrations of both measured and referenced peptides must be identical and the purity of both peptides must be carefully checked by t.l.c., n.m.r., and elemental

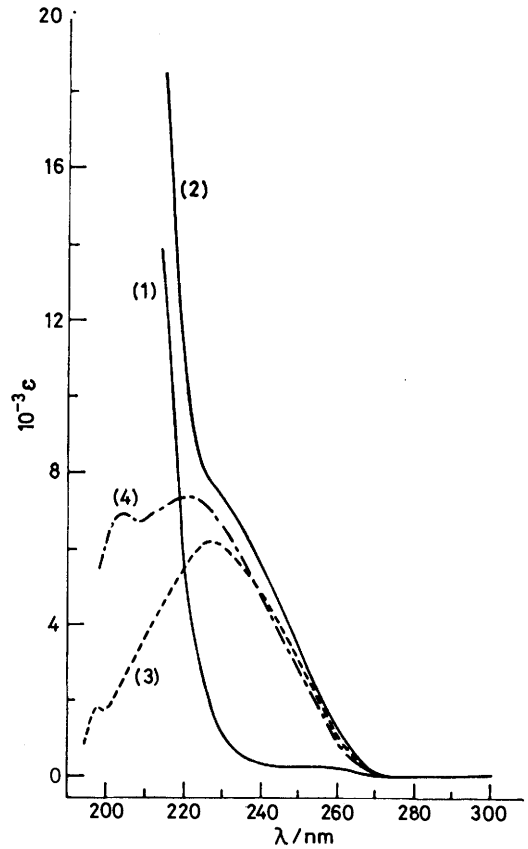


FIGURE 2 U.v. spectra of (1) Boc-Phe-Leu-OBzl (IIIb), (2) Boc-Phe-ΔLeu-OBzl (IIIa), (3) Boc-Phe-ΔLeu-OBzl (IIIa) (d.b.m.) in CH_3CN (3.2×10^{-5}M), and (4) Boc-Phe-ΔLeu-OBzl (IIIa) (d.b.m.) in MeOH (3.2×10^{-5}M)

analyses. Although the tetrapeptides (V) were measured in MeOH (Table 2, Figure 4), pentapeptides (VI) were dissolved in 0.1% DMF–MeOH because of their insolubility in neat MeOH (Figure 5). Since DMF interfered with measurements below 230 nm, the absorption of the ΔLeu peptide (VIa-2) could not be observed (Table 2). In $CHCl_3$ no peaks for ΔAla and ΔLeu were observed even though the d.b.m. method was used and there was no sig-

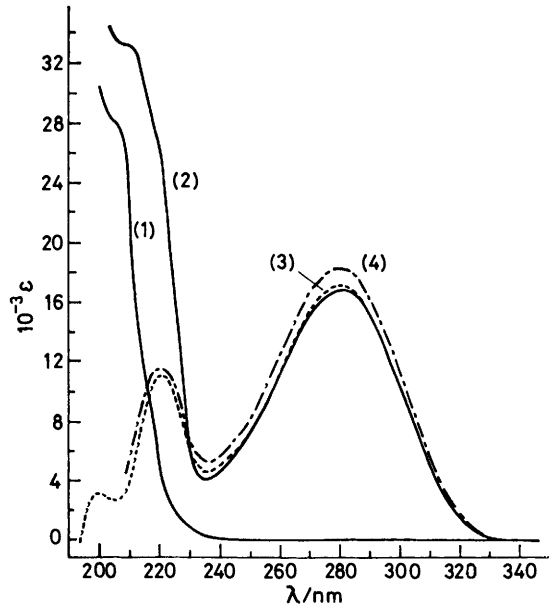


FIGURE 3 U.v. spectra of (1) Boc-pGlu-Phe-OBu^t (IVb), (2) Boc-pGlu-ΔPhe-OBu^t (IVa), (3) Boc-pGlu-ΔPhe-OBu^t (IVa) (d.b.m.) in CH_3CN (3.2×10^{-5}M), and (4) Boc-pGlu-ΔPhe-OBu^t (IVa) (d.b.m.) in MeOH (3.2×10^{-5}M)

nificant influence on the wavelength due to solvent changes as shown in Table 1. However, proton-donating solvents (MeOH and 95% EtOH) gave slightly more intense absorption, probably resulting from solvation of peptide bonds.

TABLE 2

Spectroscopic properties of dehydrotetra- and penta-peptides

	Peptide	Chemical shift δ of vinyl proton	U.v. spectra λ/nm	ε
(Va-2)	Boc-D-Ala-Gly-Phe-ΔLeu-OCH_2Ph	6.40 (d) [a]	221	7 400
(Va-3)	Boc-D-Ala-Gly-ΔPhe-Leu-OBzl	7.30 [a]	276	19 100
			222	9 200
(Vb)	Boc-D-Ala-Gly-Phe-Leu-OBzl		<200	
(VIa-1)	Z-Tyr(Cl_2Bzl)-ΔAla-Gly-Phe-Leu-OBzl	5.60, 6.20 [b]	241	4 100
(VIa-2)	Z-Tyr(Cl_2Bzl)-D-Ala-Gly-Phe-ΔLeu-OBzl	6.38 (d) [b]	<230	
(VIa-3)	Z-Tyr(Cl_2Bzl)-D-Ala-Gly-ΔPhe-Leu-OBzl	7.25 [b]	276	19 300
(VIb)	Z-Tyr(Cl_2Bzl)-D-Ala-Gly-Phe-Leu-OBzl		<200	

[a] In $CDCl_3$. [b] In [$^2H_6$]DMSO.

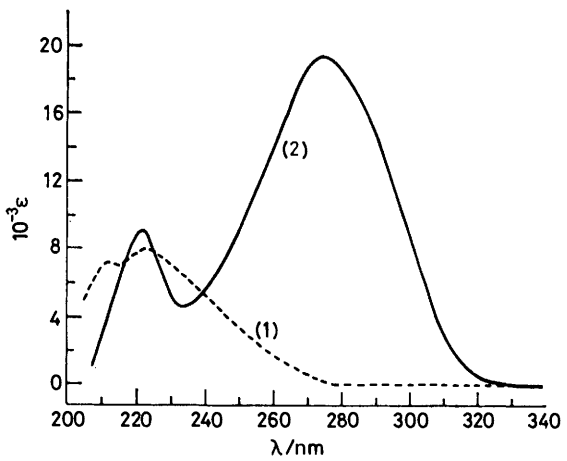


FIGURE 4 D.b.m. spectra of (1) Boc-D-Ala-Gly-Phe-ΔLeu-OBzl (Va-2) and (2) Boc-D-Ala-Gly-ΔPhe-Leu-OBzl (Va-3) in MeOH (8.4×10^{-5}M)

Acetonitrile seems to be the best solvent with respect to solubility, purity, and measurable region.

Z- and *E*-isomers of Bz-ΔLeu-OMe afforded the different

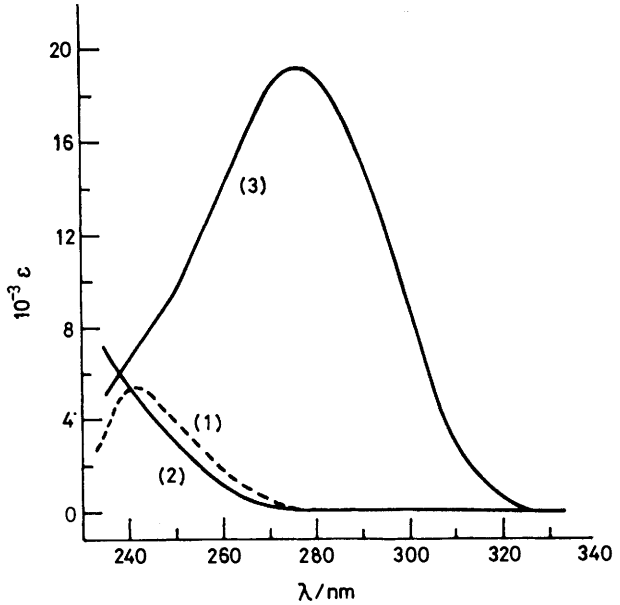


FIGURE 5 D.b.m. spectra of (1) *Z*-Tyr(Cl_2Bzl)-ΔAla-Gly-Phe-Leu-OBzl (VIa-1), (2) *X*-Tyr(Cl_2Bzl)-D-Ala-Gly-Phe-ΔLeu-OBzl) (VIa-2), and (3) *Z*-Tyr(Cl_2Bzl)-D-Ala Gly-ΔPhe-Leu-OBzl (VIa-3) in 0.1% DMF–MeOH (2.6×10^{-5}M)

TABLE 3

Stereoscopic properties of *Z*- and *E*-isomers of dehydroamino-acid derivatives

	Amino-acid derivative	Chemical shift δ of vinyl proton $CDCl_3$	CF_3CO_2H	Δδ (p.p.m.)	λ/nm	ε
(VIIa-2*Z*)	Bz-Δ^{Z}Leu-OMe	6.65 (d)	7.15 (d)	+0.50	245	5 200
(VIIa-2*E*)	Bz-Δ^{E}Leu-OMe	7.09 (d)	6.60 (d)	−0.49	252	9 100
(VIIb)	Bz-Leu-OMe				224	10 600
(VIIIa-3*Z*)	*Z*-Δ^{Z}Phe-OEt	(7.20—7.40) [a]			280	17 000
					221	9 300
(VIIIa-3*E*)	*Z*-Δ^{E}Phe-OEt	7.57	~7.30 [a]	−0.27	278	15 700
					221	9 800
(VIIb)	*Z*-Phe-OEt				<200	

[a] Contained within the aromatic envelope.

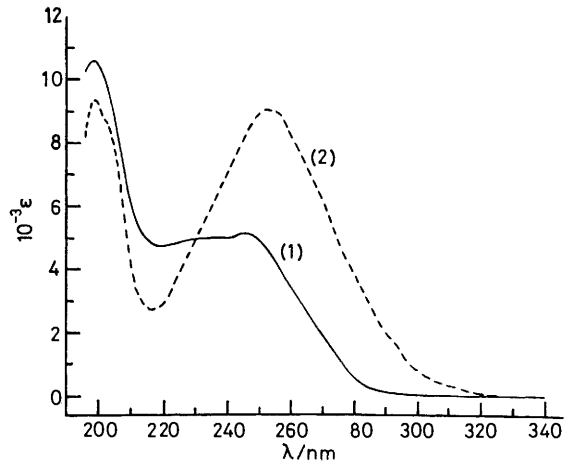


FIGURE 6 D.b.m. spectra of (1) PhCO-Δ^{Z}Leu-OMe (VIIIa-2*Z*) and (2) PhCO-Δ^{E}Leu-OMe (VIIa-2*E*) in CH_3CN (8.0×10^{-5}M)

peaks and intensities (Table 3, Figure 6), but on the other hand, the *Z*- and *E*-isomers of *Z*-ΔPhe-OEt showed almost the same pattern of peaks and intensities, appearing at 200, 220, and 280 nm (Table 3, Figure 7).

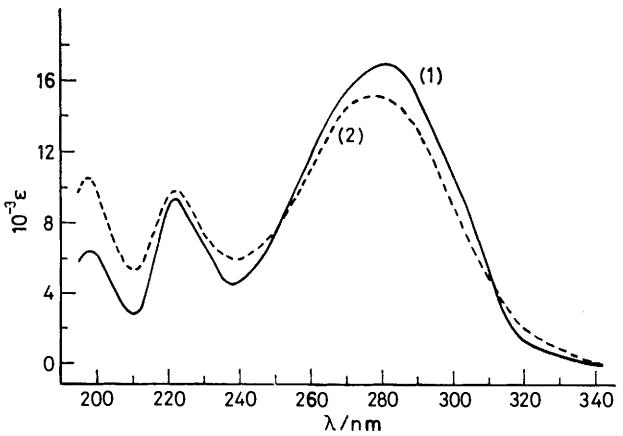


FIGURE 7 D.b.m. spectra of (1) *Z*-Δ^{Z}Phe-OEt (VIIIa-3*Z*) and (2) *Z*-Δ^{E} Phe-OEt (VIIIa-3*E*) in CH_3CN (6.1×10^{-5}M)

DISCUSSION

The absorption of an ethylene chromophore is due to a $\pi \rightarrow \pi^*$ transition, which shows intense absorption bands (K bands) in the 200—300 nm region in the u.v. spectrum. Since DHPs have a dehydroamino-acid moiety, this K band absorption should be shown distinctly by the d.b.m. method. Fieser [16] summarized the extensive rules of enone absorption as enunciated by Woodward. According to Woodward's rules, the base value of an αβ-unsaturated carboxylic acid or ester is 195 nm. Since the ΔAla peptides (no β-substituents) have $\lambda_{max.}$ at 240 nm (Tables 1 and 3) and the ΔAla derivatives like *Z*-ΔAla-OH have the same $\lambda_{max.}$ at 240 nm,[11] the increment can be estimated as +45 nm for an α-amino and/or α-peptidyl (-NHCO-R) substituent, which is well known to have partial double bond character.[17] The β-phenyl substitution of ΔPhe peptides gets a +40 nm additional increment in its $\lambda_{max.}$ (280 nm), if the 240 nm absorption peak of the ΔAla peptides is used as the base value for DHP. On the other hand, an alkyl substituent like an isopropyl group showed a 10—20 nm decrement, since the ΔLeu peptides showed the $\lambda_{max.}$ at 220—230 nm. In addition to this, all of the DHPs had an additional peak at *ca.* 200 nm. These results suggested some other effects on the u.v. absorption of DHPs.

Forbes [18] has delineated three types of steric effect on a conjugated system: type 1, steric interactions which cause intensity changes without causing any change in wavelength; type 2, steric interactions which cause a change in both wavelength and absorption intensity; and type 3, steric interactions which cause separation of the absorption into two distinct entities because of inhibition of conjugation. Crotonic acid showed only one peak at 202 nm by the d.b.m. method, while the ΔAla and ΔLeu peptides had two distinct peaks: ΔAla (202 and 240 nm in CH_3CN); ΔLeu (195 and 227 nm in CH_3CN) (Figures 1 and 2). The steric interactions which would occur in the ΔAla and ΔLeu peptides by the α-peptidyl (-NHCO-R) substitution might cause both type 2 and 3 steric effects, although there could be no exact determination of the intensity of peaks near 200 nm because of instrumental limitations. On the other hand, the ΔPhe peptides showed three distinct peaks at 203, 221, and 279 nm (Figure 3). It is certain that the β-phenyl substitution caused both type 2 and 3 steric effects because the absorption at 221 nm was much more intense than those of the ΔAla and ΔLeu peptides. The strong absorption of the ΔPhe peptides at 280 nm is attributed to the new conjugation of phenyl ring, double bond, and peptide bonds. Such effects as observed for DHP should be independent of the size of peptides (Tables 1—3, Figures 1—5).

In order to explain the wavelength change of 10—20 nm between the ΔAla and ΔLeu or ΔPhe peptides in the region of 220—240 nm, we considered that it might be attributed to another steric effect. The ΔLeu and ΔPhe peptides exist as *Z*- and *E*-isomers as regard with β-substitution and all the ΔLeu and ΔPhe peptides herein measured have the *Z*-configuration. Unfortunately, the synthesis of the *E*-isomers of the ΔLeu and ΔPhe peptides is very difficult and has not yet been accomplished. Rich and Mathiaparanam [6] reported the u.v. absorption peaks of both isomers of Boc-NMeAla-Leu-ΔPhe-Gly-OMe: *Z*-isomer; $\lambda_{max.}$ 276 nm, (ε 18 400), *E*-isomer; $\lambda_{max.}$ 282 nm (ε 9 080). However, Rao and Filler [19] observed almost the same intensities for the *Z*-and *E*-isomers of PhCO-ΔPhe-OH and PhCO-ΔPhe-OEt. In this study, the *Z*- and *E*-isomers of *Z*-ΔPhe-OEt (VIIIa-3*Z* and 3*E*) showed almost the same patterns and intensities in the 200—300 nm region (Table 3, Figure 7). However, the *Z*- and *E*-isomers of PhCO-ΔLeu-OMe (VIIa-2*Z* and 2*E*) showed different patterns and intensities (Table 3, Figure 6). The intensity of the *E*-isomer, $\lambda_{max.}$ 252 nm (ε 9 100), was about twice that of the *Z*-isomer, $\lambda_{max.}$ 245 nm (ε 5 200). Although the reasons for these differences are not clear, it seems that an amino-protecting group like benzoyl might be included in the con-

jugation and thus show some effect (Figure 6). We think it is important to deal with DHPs, not dehydroamino-acid derivatives, in order to elucidate the steric effects on u.v. spectra.

In general, the syntheses of saturated peptides are much easier than those of unsaturated peptides so that the d.p.m. method can be used to determine the presence of a dehydroamino-acid moiety in a natural compound. Further data compilation, like those of Rich *et al.*,[6] and Caccamese *et al.*,[20] may even allow the assignment of configuration to the dehydro-moiety.

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REFERENCES

[1] Y. Shimohigashi and N. Izumiya, *Yuki Gosei Kagaku Kyokai Shi*, 1978, **36**, 1023.
[2] U. Schmidt, J. Häusler, E. Ohler, and H. Poisel, *Prog. Chem. Nat. Prod.*, 1979, **37**, 252.
[3] C. Shin, *Yuki Gosei Kagaku Kyokai Shi*, 1979, **37**, 830.
[4] M. L. English and C. H. Stammer, *Biochem. Biophys. Res. Commun.*, 1978, **85**, 780.
[5] Y. Shimohigashi and C. H. Stammer, *Biochem. Biophys. Res. Commun.*, submitted for publication.
[6] D. H. Rich and P. Mathiaparanam, *Tetrahedron Lett.*, 1974, 4037.
[7] J. P. Greenstein and M. Winitz, ' Chemistry of the Amino Acids,' Wiley, New York, 1961, vol. 1, p. 856.
[8] Y. Shimohigashi, S. Lee, H. Aoyagi, T. Kato, and N. Izumiya, *Int. J. Pept. Protein Res.*, 1977, **10**, 197.
[9] Y. Shimohigashi and N. Izumiya, *Int. J. Pept. Protein Res.*, 1978, **12**, 7.
[10] I. Photaki, *J. Am. Chem. Soc.*, 1963, **85**, 1123.
[11] H. Poisel and U. Schmidt, *Angew. Chem. Int. Ed. Engl.*, 1976, **15**, 294.
[12] H. Poisel, *Chem. Ber.*, 1977, **110**, 948.
[13] M. Bergmann, V. Schmidt, and A. Miekeley, *Z. Physiolog. Chem.*, 1930, **187**, 264.
[14] D. Rich and J. Tam, *J. Org. Chem.*, 1977, **42**, 3815.
[15] A. Srinivasan and R. Olsen, *Tetrahedron Lett.*, 1976, 891.
[16] L. M. Fieser and M. Fieser, ' Steroids,' Reinhold, New York, 1959; ' Natural Products Related to Phenanthrene,' Reinhold, New York, 1949, p. 184.
[17] L. Pauling, R. B. Corey, and H. R. Branson, *Proc. Natl. Acad. Sci. USA*, 1951, **37**, 205.
[18] W. F. Forbes, ' Steric Effects in Conjugated Systems,' ed. G. W. Gray, Butterworths, London, 1958, p. 62.
[19] Y. S. Rao and R. Filler, *Synthesis*, 1975, 749.
[20] S. Caccamese, P. Azzolina, and M. Davino, *Chromatographia*, 1979, **12**, 545.