CHIROPTICAL PROPERTIES OF FLUORESCAMINE CONDENSATION COMPOUNDS WITH DIPEPTIDES *IN SITU*

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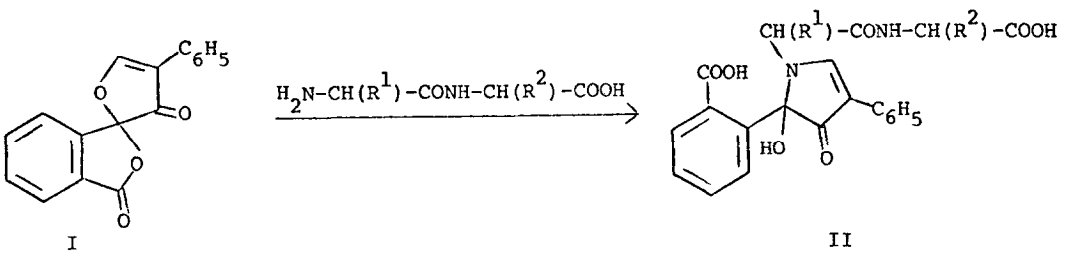
SUMMARY

The free amino group of a dipeptide reacts efficiently with fluorescamine (FLURAM®) to form pyrrolinone-type chromophores with long wavelength absorption maxima in the 380 nm region. A simple test tube procedure is described which allows *in situ* determination of the absolute configuration of the NH_2-terminal amino acids of the dipeptides based on the chiroptical properties of their chromophoric derivatives.

INTRODUCTION

We have recently reported on the application of fluorescamine, 4-phenylspiro(furan-2(3H), 1'-phthalan)-3,3'-dione I as a chromophoric reagent for the determination of the absolute configuration of primary (1) and secondary (2) amino acids *in situ*. Fluorescamine also reacts efficiently with the amino group of a dipeptide (3) to form pyrrolinone-type chromophores II:



I II

This reaction is simple and fast and can be performed in test tubes under mild conditions. Chromophore II is chiroptically active and CD spectra can be obtained from the reaction mixtures without isolation of the product.

EXPERIMENTAL

A. Reagents

The dipeptides were purchased from Vega-Fox Biochemicals and ICN Life Sciences Group and were used without further purification. Fluorescamine (Fluram®) was obtained from Hoffmann-La Roche Inc. and spectral grade dioxane and methanol from the Fischer Scientific Co. The phosphate buffer (pH 8.0, 0.05 M) was prepared according to Clark and Lubs (4) using AR-grade chemicals from Mallinckrodt Chemical Works.

B. Method

Two milliliters of a 0.004M solution of fluorescamine in dioxane is rapidly added to 2 ml of a 0.002M (concentration may range between 10^{-2} and 0.5×10^{-6}M) solution of a peptide in 0.05M phosphate buffer pH 8.0 in a test tube. The reaction mixture is stirred for ca 15 sec on a Vortex mixer, transferred to a 0.1 cm cell (or into a cell of different length, depending on the dipeptide concentration), and the CD spectra are recorded on a JASCO Spectropolarimeter, Model 20 between 450 and 240 nm. If necessary, the reaction mixtures are diluted with phosphate buffer pH 8/dioxane 1:1 v/v.

For some peptides the general procedure is modified: 4 ml of phosphate buffer pH 8 is mixed in a test tube with 2 ml of a 0.002M solution of a dipeptide in phosphate buffer pH 8/methanol 1:1 v/v and to this mixture is rapidly added 2 ml of a 0.004M solution of fluorescamine in dioxane. The reaction mixture is stirred for 15 sec and the CD spectra are measured as described above.

In two cases (L-lysyl-L-tryptophan and L-phenylalanyl-L-isoleucine) the reaction was also carried out in organic solvents: 2 ml of a 0.004M solution of fluorescamine in dioxane was mixed with 2 ml of a 0.002M solution of a dipeptide in methanol. After 4 hrs the CD spectra were recorded.

The spectra are difficult to obtain below 240 nm because of the high absorption of the reagent, especially if its concentration is higher than 0.004M or if cells longer than 0.1 cm are used.

RESULTS AND DISCUSSION

For the reasons previously discussed (5), the dipeptides are dissolved in phosphate buffer pH 8-8.5 and reacted with fluorescamine in dioxane. When the peptide concentration is in the range of 0.01-0.001M, a twofold excess of fluorescamine is sufficient for analytical purposes, but lower dipeptide concentrations ($\leq 10^{-4}$M) require a 20-40 fold excess of the reagent (6). Under standard conditions the reactions are complete within one minute and the chromophore is stable at least for 1-2 hrs.

The UV absorption spectra of the pyrrolinone-type chromophores II, arising from the reaction of fluorescamine with dipeptides, (3,6) show maxima

TABLE I

Cotton Effects in CD Spectra of Reaction Products of Dipeptides with Fluorescamine in Situ

			1st		2nd		3rd	Cotton Effect
	Dipeptide	Solvent[a]	nm	$(\theta)x10^{-3}$	nm	$(\theta)x10^{-3}$	nm	$(\theta)x10^{-3}$
1.	D-Alanyl-D-alanine	B	384	+15.58	324	+ 8.75	268	-36.00
2.	D-Alanyl-L-alanine	B	382	+ 6.25	325	+11.20	276	-26.81
3.	L-Alanyl-D-alanine	B	381	- 6.39	326	-11.02	276	+26.85
4.	L-Alanyl-D-phenylalanine	B	380	- 3.65	323	-14.82	275	+18.78
5.	D-Alanyl-D-phenylalanine	B	376	+ 5.15	333	+ 7.20	276	-27.60
6.	L-Arginyl-L-isoleucine[b]	B	381	-29.40	308	- 5.80	264	+66.15
7.	L-Arginyl-L-isoleucine	B/M	382	-16.05	315	- 6.16	264	+34.42
8.	α-D-Glutamyl-D-glutamic acid	B	385	+ 8.88	322	+ 8.22	264	-28.11
9.	Glycyl-L-leucine	B	383	- 8.06	327	- 4.65	265	+ 8.47
10.	Glycyl-D-leucine	B	385	+ 7.14	321	+ 5.03	270	- 8.58
11.	Glycyl-L-phenylalanine	B	388	- 2.27	336	- 6.55	278	+ 5.15
12.	Glycyl-D-phenylalanine	B	386	+ 3.10	336	+ 7.28	279	- 5.06
13.	L-Leucyl-L-alanine	B	390	- 5.11	318	- 8.15	269	+28.41
14.	L-Leucyl-L-proline	B	386	- 7.61	318	-20.58	270	+37.19
15.	L-Lysyl-L-tryptophan	B	403	+ 9.20	325	-10.21	272	+29.62
16.	L-Lysyl-L-tryptophan	M	380	- 5.63	316	- 7.85	264	+36.00
17.	L-Phenylalanyl-L-isoleucine	B	380	+ 4.51	324	-15.65	285	+ 1.05
18.	L-Phenylalanyl-L-isoleucine	M	390	-12.20	315	-30.19	281	+14.05
19.	L-Valyl-L-valine	B	378	+ 4.11	315	- 7.19	270	+45.03
20.	L-Valyl-L-valine	B/M	385	- 5.15	317	- 6.67	268	+33.19
21.	D-Valyl-D-valine	B	378	- 3.90	316	+ 6.45	270	-43.65
22.	D-Valyl-D-valine	B/M	387	+ 4.78	315	+ 6.39	268	-32.80
23.	D-Valyl-L-valine	B	385	- 4.70	319	+ 8.27	270	-43.15
24.	D-Valyl-L-valine	B/M	387	+ 5.80	319	+ 7.35	272	-31.63

(a) B = phosphate buffer pH 8/dioxane 1:1 v/v; B/M = phosphate buffer pH 8/dioxane/methanol 62.5:25:12.5 v/v.; M = dioxane/methanol 1:1 v/v., reaction time 4 hrs.

(b) Under standard conditions an additonal Cotton effect is observed at 336 nm, $(\theta)\ x10^{-3}$ = + 4.80

at 280-285 nm (ε = 16000-18000) and at 380-390 nm (ε = 6000-7000). As expected, these electronic transitions recognize the chirality of the α-C atom of the NH_2-terminal amino acid of a dipeptide and consequently the chromophoric derivatives afford CD spectra with Cotton effects located in the region of the UV maxima. An additional Cotton effect is observed at 315-325 nm (here the UV spectra show minima), presumably due to a coupled oscillator mechanism (7).

A number of dipeptides were reacted with fluorescamine and the CD spectra were recorded in the above-mentioned spectral range. The position, sign, and intensity of the Cotton effects are summarized in Table I. In Figures 1 and 2 the spectra of D-alanyl-L-alanine, L-alanyl- D-alanine, L-leucyl-L-proline, L-valyl-L-valine and D-valyl- D-valine are shown.

The pyrrolinone-type chromophores have three characteristic Cotton

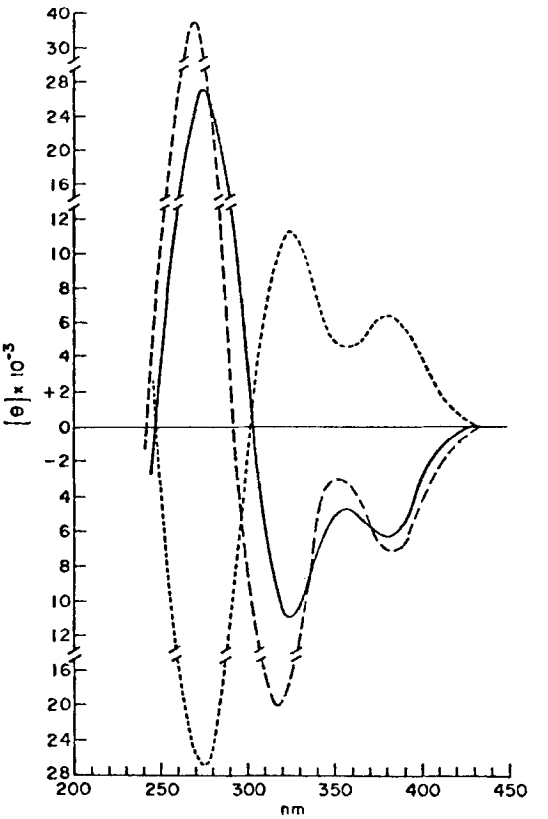


Fig. 1. CD spectra of the in situ reaction mixtures of D-alanyl-L-alanine (----), L-alanyl-D-alanine (——) and L-leucyl-L-proline (— —) with fluorescamine in 0.05 M phosphate buffer pH 8/dioxane, 1:1, v/v.

effects between 400 and 260 nm. In most cases, when the NH_2-terminal amino acid of the dipeptide derivative has an L-configuration, the first Cotton effect (at 403-375 nm) and the second one (at 336-308 nm) are negative, whereas the sign of the third Cotton effect (at 285-264 nm) is positive. Within the experimental error, the CD curves of the chromophores derived from NH_2-terminal amino acids of D-configuration are mirror images of those derived from NH_2-terminal amino acids of L-configuration. As can be seen from the experimental results, the sign of the Cotton effects of the pyrrolinone-type chromophore depends only on the configuration of the NH_2-terminal amino acid of the corresponding dipeptide. Evidently the configuration of the COOH-terminal amino acid has only a minor influence on the amplitude of the Cotton effects. Furthermore, it is interesting that the configurational characteristics are transmitted through the glycine moiety, although the intensities of the Cotton effects are reduced (Table I).

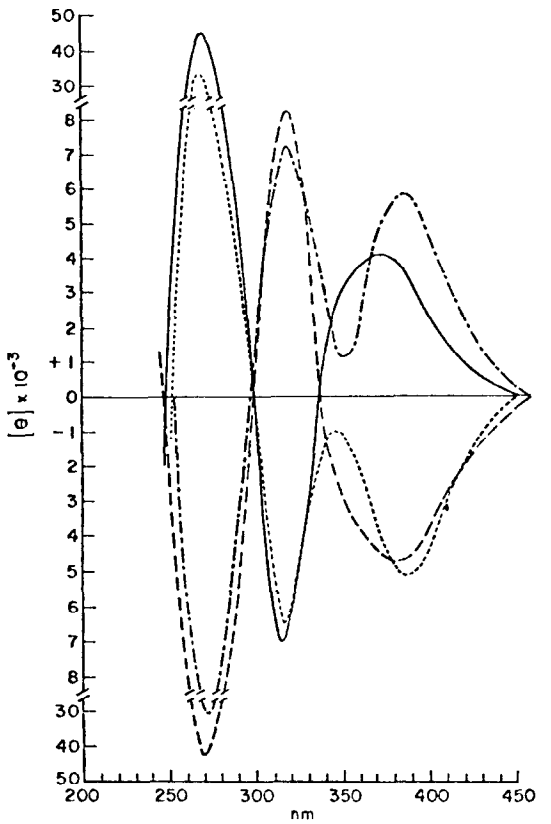


Fig. 2. CD spectra of the in situ reaction mixtures of L-valyl-L-valine (——) and D-valyl-L-valine (— —) with fluorescamine in 0.05 M phosphate buffer pH 8.0/dioxane 1:1 v/v. The spectra of the reaction mixtures of L-valyl-L-valine (----) and D-valyl-L valine (-.-) with fluorescamine in phosphate buffer/dioxane/methanol 62.5:25:12.5 v/v.

A few exceptions were encountered regarding the sign of the first Cotton effect (see entries 15, 17, 19, 21 and 23 in Table I). In these cases the Cottor effect in the 403-375 nm area is positive for the derivative of a dipeptide with an NH_2-terminal L-amino acid and negative for those with an NH_2-terminal D-amino acid, as demonstrated in Fig. 2. The same sequence of first Cotton effects was observed with chromophoric fluorescamine derivatives of α-amino acids (1). The sign of these first Cotton effects is sensitive to the polarity of the solvent (Fig. 2) and the exception to the general rule may be caused by the differences in hydration or by differences of the conformation of the dipeptide moiety (8). But in any case, the sign of the second and third Cotton effect can

be safely used for the determination of the absolute configuration of the NH_2-terminal amino acid of a dipeptide using the following empirical rule:

Configuration	Sign of the 2nd and 3rd Cotton Effects
L	⊖ ⊕
D	⊕ ⊖

As in the case of primary (1) and secondary (2) amino acids, the main advantage of this fluorescamine method is its simplicity. Under standard conditions, as little as 0.1-1.0 μg/ml of dipeptides has been routinely reacted with fluorescamine and useful CD spectra of the reaction mixtures were obtained.

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8. Temperature and pH studies are planned for the clarification of the observed exceptions to the general rule.