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APPLICATION OF MDPF AND FLUORESCAMINE XV CHIROPTICAL PROPERTIES OF MDPF CONDENSATION COMPOUNDS WITH DIPEPTIDES IN SITU[1]

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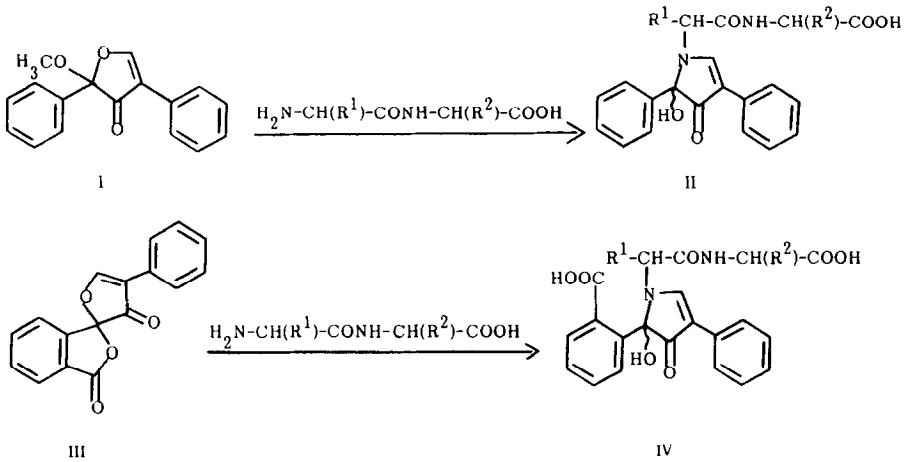
Summary: 2-Methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) reacts readily with the free amino group of a dipeptide to form a pyrrolinone-type chromophore with absorption maxima at 275-285 and 370-390 nm. A simple test tube procedure is described which allows in situ correlation of the absolute configuration of the NH_2-terminal amino acid of a dipeptide with the chiroptical properties of its chromophoric derivative. In several cases, unexpected deviation of the chiroptical characteristics from previously established empirical rules is observed.

We have recently described the application of 2-methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) I as a new chromophoric reagent for the correlation of the absolute configuration of primary α-amino acids with their chiroptical properties (2,3).



The UV spectra of the pyrrolinone-type chromophore II (4), arising from the reaction of MDPF with amino groups, show maxima at 280-285 nm (ε = 16000-18000) and at 380-390 nm (ε = 6000-7000). Derivatives containing an aromatic moiety, such as a phenol (tyrosine) or indole (tryptophan) in the side chain, show additional UV absorption stemming from these substituents (at 260-290 nm). As expected, the pyrrolinone electronic transitions recognize the chirality of the α-carbon atom, and

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consequently, the chromophoric derivatives II 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 (5).

First, twenty-four amino acids were derivatized with MDPF and their chiroptical properties were measured in methanol (2). It was found that without exception, the first Cotton effect in the CD spectra of the L-amino acid derivatives (at 403-375 nm) is always positive, and the second one (at 336-308 nm) is negative; whereas, the third Cotton effect (at 285-264 nm) is again positive. Within the experimental error, the CD curves of the D-amino acid-derived chromophores are mirror images of those of the L-amino acids. Subsequently, it was demonstrated that the chromophoric reaction between MDPF and an amino acid (3), and fluorescamine and an amino acid (6), can be carried out in a test tube, and that the CD spectra can be obtained from the resulting reaction mixtures without the isolation of the chromophoric derivatives. Again, it was demonstrated that the first and third Cotton effects of the chromophores derived from L-amino acids and MDPF are positive, and the second one is negative. The only exception to this rule was the alanine reaction product, where the sign of the first Cotton effect is reversed, but the sign of the second and third ones remained unchanged. In the presence of excess reagent (UV maximum in methanol at 241 nm, ϵ = 18800 and at 308 nm, ϵ = 3500), the third Cotton effect is sometimes difficult to measure.

During the investigation of chiroptical properties of fluorescamine III with α-amino acid esters (7) and dipeptides (8), it was found that the sign of the first Cotton effect (at 403-370 nm) varies and is not a function of configuration alone, but remains dependent upon the nature of the substitute at the α-carbon as well.

Because of these irregularities, it was of interest to investigate the chiroptical properties of chromophore II *in situ*, obtained from the reaction of dipeptides with MDPF (4). The reaction is simple, 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. MDPF was obtained from Hoffmann-La Roche Inc., and spectral grade methanol and dimethylformamide (DMF) from Fisher Scientific Company. The phosphate buffer pH 8.0, (0.05 M) was prepared according to Clark and Lubs (9) using AR-grade chemicals from Mallinckrodt Chemical Works. Triethylamine was obtained from Eastman Kodak Company.

B. Method

a. General Procedure: 2 ml of a 0.02 M solution of a dipeptide in 0.05 M phosphate buffer pH 8.0 is mixed in a test tube with 0.1 ml of triethylamine (18 equivalents) and with 1.9 ml of a 0.0527 M (2.5 equivalents) solution of MDPF in

methanol (10) under stirring for 10 seconds on a Vortex type mixer. The reaction mixture is kept for 60 minutes and the CD spectra are recorded on a JASCO Spectropolarimeter, Model ORD/CD-J-20 in a 0.01 cm cell between 450 and 240 nm. When necessary, the reaction mixture is diluted with methanol/phosphate buffer pH 8.0, 1:1, v./v.

b. Micro Procedure: Useful CD spectra (signal to noise ratio higher than 10) are regularly obtained with 0.005 - 0.01 mg of dipeptides. In order to avoid working with dilute solutions, where the reaction is slow or incomplete, 0.01 mg of a dipeptide is dissolved in 0.01 ml phosphate buffer pH 8.0 and reacted with 2 equivalents of MDPF in methanol containing 18 equivalents of triethylamine. After standing at room temperature for one hour, the volume of the reaction mixture is brought up to a total of 3 ml with methanol/phosphate buffer pH 8.0, 1:1, v./v., and the CD spectra are recorded in a 5 cm cell between 450 and 240 nm.

c. Non-aqueous Procedure: 2 ml of a 0.02 M solution of a dipeptide in dimethylformamide (DMF) is neutralized with 2 equivalents of triethylamine in a test tube and is reacted with 2 ml of a 0.04 M solution of MDPF in methanol. After standing for 16 hrs at room temperature, the CD spectra of the reaction mixtures are recorded between 450 and 240 nm in a 0.01 cm cell.

Under standard conditions, the reactions are completed within 1 hr, and the chromophore is stable at least for several hours.

Results and Discussion

A number of dipeptides were reacted with MDPF I, and the CD spectra of the reaction mixture containing N-substituted-3,5-diphenyl-5-hydroxy-2-pyrrolin-4-one II were recorded between 450 and 250 nm. The positions, signs [predicted and observed (2,3)] and intensities of the Cotton effects are summarized in Table I. In Figure 1, the spectra of the in situ reaction mixtures of I with L-isoleucyl-L-phenylalanine and D-valyl-L-valine are shown.

The pyrrolinone-type chromophore II has three characteristic Cotton effects between 410 and 260 nm. As described before (2,3), the signs of the Cotton effects of the chromophoric derivatives obtained from the reaction of MDPF and α-amino acids (isolated and in situ) appeared to be solely a function of the absolute configuration at the α-carbon atom of the parent amino acid, regardless of the nature of the substituents: in the L-series, the first Cotton effects at 403-370 nm were positive (the only exception was that the in situ reaction product between L-alanine and MDPF), the second Cotton effect at 336-308 nm negative and the third ones at 285-260 nm again positive.

As seen in Table I and Figure 1, for the dipeptides the above empirical rule holds without exception only for the third Cotton effects (in the presence of an aromatic side chain and an excess of the reagent, the third Cotton effect is difficult to measure). The third Cotton effect is positive for a NH_2-terminal L-amino acid of a dipeptide and negative for a NH_2-terminal D-amino acid. The sign of the first and second Cotton effects varies. It is not a function of the configuration alone, but are dependent upon the nature of the amino acid side chain as well. Similar variations in the sign of the Cotton effects of several other chromophoric derivatives of α-amino acids or α-amino acid ester has been described in the literature (11). In the case of

TABLE I

Cotton Effects in CD Spectra of Reaction Products of Dipeptides with MDPF in situ (in Phosphate Buffer pH 8/Methanol 1:1 v./v., unless otherwise stated; value of [θ] based on the dipeptide concentration)

	Peptide	1st		2nd		3rd Cotton Effect	
		nm	[θ] x 10^3	nm	[θ] x 10^{-3}	nm	[θ] x 10^{-3}
1.	D-Ala-D-Ala	383	(-)[a] + [b]11.50	320	(+) c	267	(-) - 14.8
2.	D-Ala-L-Ala	380	(-) + 12.0	317	(+) + 10.6	277	(-) - 28.0
3.	L-Ala-D-Ala[d]	379	(+) - 11.0	318	(-) - 9.6	275	(+) + 24.0
4.	L-Ala-D-Ala[d]	390	(+) + 1.8	329	(-) - 11.5	281	(+) + 18.6
5.	D-Val-D-Val[d]	386	(-) + 6.4	320	(+) - 4.0	263	(-) - 31.2
6.	D-Val-D-Val[d]	383	(-) - 1.6	333	(+) + 1.0	270	(-) - 17.6
7.	D-Val-L-Val[d]	380	(-) + 3.2	320	(+) + 8.5	263	(-) - 27.6
8.	D-Val-L-Val[d]	383	(-) - 21.0	328	(+) + 29.5	275	(-) - 27.6
9.	L-Leu-L-Ala[d]	380	(+) - 8.1	314	(-) - 1.9	262	(+) + 20.8
10.	L-Leu-L-Ala[d]	382	(+) + 3.5	333	(-) - 6.8	280	(+) + 16.0
11.	D-Ser-D-Ala	383	(-) + 12.6	328	(+) - 2.2	278	(-) - 19.2
12.	L-Arg-L-Ile	381	(+) - 10.3	325	(-) + 7.7	260	(+) + 22.4
13.	D-Glu-D-Glu	385	(-) + 8.3	309	(+) + 1.3	255	(-) - 21.2
14.	L-Ala-L-Phe	377	(+) - 5.3	318	(-) - 5.8	262	(+) + 13.6
15.	L-Val-L-Trp	382	(+) - 6.6	318	(-) + 2.3	261	(+) + 38.0
16.	L-Ile-L-Phe[d]	379	(+) - 2.1	316	(-) + 1.8	262	(+) + 32.0
17.	L-Ile-L-Phe	385	(+) + 13.8	329	(-) - 8.4	277	(+) + 30.0
18.	D-Leu-L-Tyr	390	(-) - 4.8	319	(+) + 18.4	278	(-) - 14.8
19.	L-Leu-L-Tyr	396	(+) + 2.3	324	(-) - 3.50	262	(+) + 33.2
20.	L-Lys-L-Trp	397	(+) + 7.9	323	(-) - 7.1	274	(+) + 21.2
21.	L-His-L-Leu	370	(+) + 0.9	310	(-) - 5.2	265	(+) + 6.8
22.	L-Phe-L-Val	382	(+) + 6.1	320	(-) - 14.6	275	(+) + 1.6
23.	L-Tyr-L-Ala	370	(+) + 1.3	315	(-) - 17.6		e
24.	L-Trp-L-Val	380	(+) + 10.2	305	(-) - 10.0		e
25.	L-Trp-L-Leu	381	(+) + 5.2	295	(-) - 12.0		e
26.	L-Phe-L-Trp	384	(+) + 10.8	324	(-) - 16.8	270	(+) + 4.8

a = Predicted and b = Observed signs of the Cotton effects (2,3).
c = No pronounced Cotton effect observed.
d = In dimethylformamide/methanol 1:1 v./v.
e = Not measured because of too high absorption.

the CD spectra of the reaction products between α-amino acid esters and dipeptides with fluorescamine, only the sign of the first Cotton effect was found to be sensitive to the nature of the substituent at the α-carbon but the sign of the second and third Cotton effect would be safely used for the determination of the absolute configuration of the α-amino acid esters and the NH_2-terminal amino acid of a dipeptide using the following empirical rule (7,8).

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

It is seen from Table I that when the NH_2-terminal amino acid of a dipeptide has an aromatic side chain (entries 21-26), the sign of the three Cotton effects correlates with the previously described empirical rule derived for the chiroptical properties of the pyrrolinone type chromophores II obtained from the reaction of α-amino acids and MDPF.

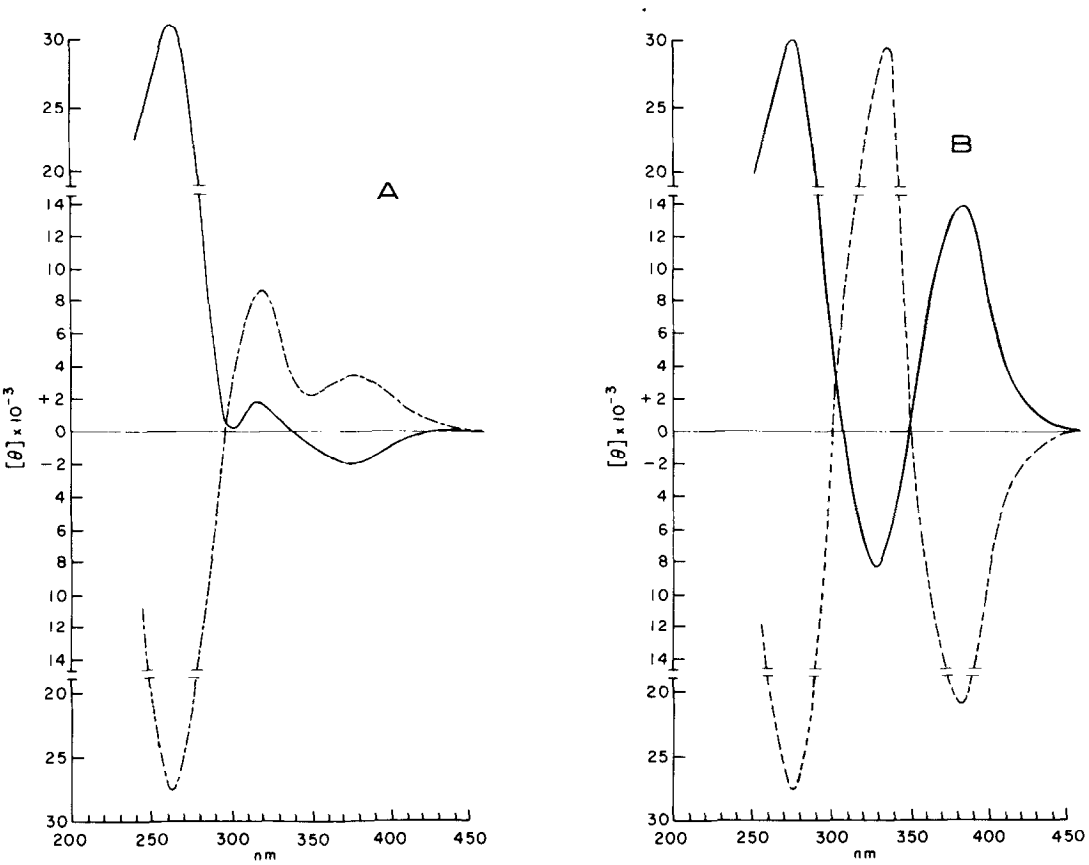

Fig. 1 CD spectra of the in situ reaction mixture of L-isoleucyl-L-phenylalanine (——) and D-valyl-L-valine (— —) with MDPF in 0.05 M phosphate buffer pH 8.00/methanol, 1:1, v./v. (A) and in dimethylformamide/methanol, 1:1, v./v. (B).

Several reactions were also carried in a methanol/DMF, solvent system and the CD spectra of the reaction mixtures were recorded in situ (entries 4, 6, 8, 10 and 17). Under these conditions, the signs of the first and/or second (but not the third) Cotton effects were reversed, and they now obey the empirical rule which applies to the amino acids.

The CD spectra of the synthesized pyrrolinone derivative of L-isoleucyl-L-phenylalanine (12) were recorded in phosphate buffer pH 8/methanol, 1:1, v./v., and in methanol/dimethylformamide, 1:1, v./v., and compared with the reaction products obtained in situ (entries 16 and 17). These results are demonstrated in Figure 2. The behavior of the isolated compound parallels that of the in situ mixture. The sign of the third Cottom effect is independent of solvent, but those of the first and second Cotton effects are not.

The sensitivity of the sign of the first and the second Cotton effects to the polarity of the solvent and the nature of the substituent at the α -carbon may be caused by the differences in hydration or by differences of the conformation of the dipeptide moiety (13). The additional COOH group at the phenyl ring of the

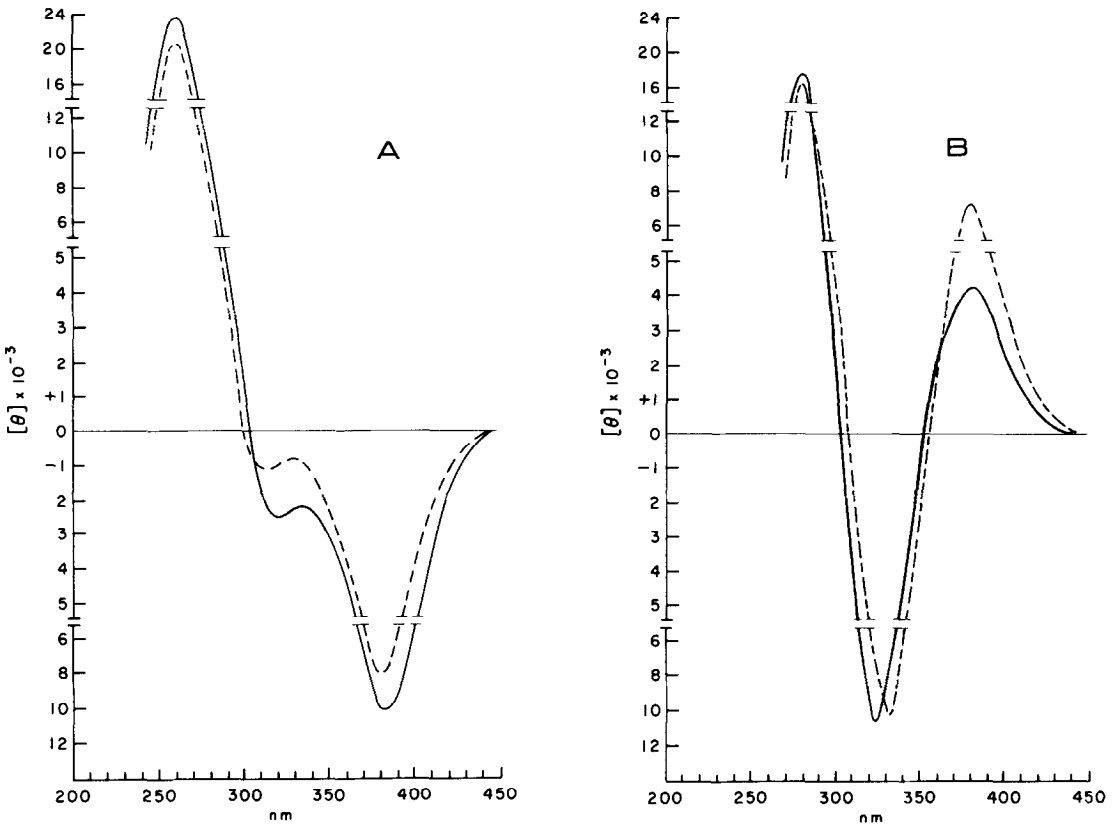

Fig. 2 CD spectra of synthesized (isolated) chromophoric derivative of L-leucyl-L-alanine (——) and at the in situ reaction mixture of L-leucyl-L-alanine with MDPF (— —) in 0.05 M phosphate buffer/methanol, 1:1, v./v. (A) and in methanol/dimethylformamide, 1:1, v./v. (B).

pyrrolinone-type chromophore IV might be responsible for the difference in chiroptical properties of the reaction products of dipeptides with MDPF and fluorescamine.

In summary, the sign of the third Cotton effect can be safely used for the determination of the absolute configuration of the NH_2-terminal amino acid of dipeptides. It is positive for the L- and negative for the D-configuration. When the NH_2-terminal amino acid carries an aromatic side chain, the previously mentioned empirical rule (2,3) for all three Cotton effects of amino acid derivatives applies. In the case of the aliphatic NH_2-terminal amino acids, it is advisable to react proper model dipeptides with known configuration with MDPF (and compare the CD spectra) if the third Cotton effect cannot be measured properly. Furthermore, for the determination of the absolute configuration of an amino acid in a more complex peptide, it may be essential to isolate and characterize the chromophoric derivative. In such cases, MDPF will be the reagent of choice when compared with the fluorescamine compounds of type IV because they tend to undergo hydrolysis upon isolation.

The main advantage of this MDPF method is its simplicity. Under standard conditions, as little as 0.01-0.005 mg/ml of dipeptides has been routinely reacted with MDPF and useful CD spectra of the reaction mixtures were obtained.

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13. Additional investigation of solvent, temperature and pH effects are planned.