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A fast and simple *in situ* method for the determination of the absolute configuration of amino acid esters using the chiroptical properties of their Eu(FOD)$_3$ complexes*

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Summary. The NMR shift reagent, Europium(III)-tris-(1,1,1,2,2,3,3)-heptafluoro-7,7-dimethyl-4-6-octanedione [Eu(fod)$_3$], complexes efficiently with α-amino acid esters in chloroform. These complexes exhibit characteristic circular dichroism (CD) spectral patterns in the 350–250 nm region. A fast and simple procedure (also in microscale) has been worked out which utilizes the signs of these CD bands for the determination of the absolute configuration at the α-carbon atom *in situ*. In the L-series, a positive CD band is observed at around 310 nm and a negative one in the 290–280 nm region. The CD spectra of the Eu complexes of the D-isomers are mirror images of those of the L-configurations. An empirical rule is proposed.

Keywords: Amino acids – α-Amino acid esters – Absolute configuration – CD – Eu(fod)$_3$

Introduction

Most of the several hundred amino acids (in addition to the twenty common building elements of proteins) known to occur in the nature (Fowden, 1973; Scannell and Preuss, 1974) possess the L-configuration[1] at the asymmetric α-carbon atom, but D-amino acids, isolated mainly from microbial products, are being encountered with increasing frequency. Therefore, the determination of the absolute configurations of naturally occurring amino acids is an important recurring task. Chiroptical methods, such as Optical Rotatory Dispersion

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[1] The L- or Ls- configuration at C-2 in α-amino acids as their esters are usually, but not always, (S) in the Cahn-Ingold-Prelog convention (Cahn et al., 1966).

(ORD) and Circular Dichroism (CD), have been utilized (among other techniques, such as chromatographic methods, single crystal X-ray analysis, etc.) in various ways for configurational assignments of amino acids and their analogues (Fowden et al., 1971; Polonski, 1975; Listowsky et al., 1979; Korver and Liefkens, 1980; Toome and Weigele, 1981; Toniolo, 1985). All aliphatic L-α-amino acids show, in their CD spectra at pH 1, a positive Cotton effect near 209 nm, which is generally assigned to the $n \rightarrow \pi^*$ transition of the carbonyl chromophore. The CD spectra of D-α-amino acids exhibit a negative Cotton effect in the same area. The chiroptical properties of the α-amino acid esters are similar to those of the free acids (Polonski, 1975). Their spectra are relatively simple and can be measured directly with the state-of-the-art ORD/CD spectropolarimeters which extend to the vacuum *UV* range.

A number of sector rules have been described in the literature and have been recently reviewed (Toome and Weigele, 1981; Toniolo, 1985). But it seems that no single rule has yet achieved an universal applicability. For all practical purposes, one is left with empirical comparisons.

However, if unusual conformational constraints and/or additional interfering chromophoric substituents, such as in the case of aromatic amino acids, are present, a more complex chiroptical pattern occurs. In addition, the substitution pattern of this aromatic moiety, as described, in the case of o-, m-, and p-tyrosine, influences the sign of the long wavelength CD bands (^1L_b transition) in the 280–250 nm region (Hooker, Jr. and Schellman, 1970). Therefore, in practice, it is advantageous for safe configurational assignment to form new chromophoric derivatives whose chiroptical properties reflect the absolute configuration at the α-carbon atom. These chromophores should absorb above 300 nm where the aromatic compounds and other chromophoric substituents (and possible impurities) are transparent. Also, the new chromophoric derivatives should have a rather high Kuhn's dissymmetry factor g, which is defined as $\Delta\varepsilon/\varepsilon$, to afford CD spectra with favorable signal-to-noise ratios.

A number of reagents and methods for the preparation of chromophoric derivatives (Toome and Weigele, 1981; Toniolo, 1985) and transition metal complexes (Frelek et al., 1985) of α-amino acids, α-amino acid esters and analogues have been reviewed in the literature and their chiroptical properties have been discussed. Some of their adherent disadvantages have also been noted: derivatization is sometimes accompanied by partial racemization; reaction mixtures have to be heated over a period of time or stand at ambient temperature for several hours. In several instances, the signs of the Cotton effects of the chromophoric derivatives are not a function of the configuration alone, but are also dependent upon the nature of the substituents at the α-carbon as well. In addition, some of the reagents may also react with hydroxy and sulfhydryl groups. In the case of the CD spectra of transition metal complexes (Mo, Rh, Ru, etc.) of amino acids and their derivatives, up to seven Cotton effects of variable intensity are present between 750 and 300 nm which make the assignment of these bands tedious in the presence of other optically active chromophores. A hexadecant rule has been proposed for the correlation of the sign of the Cotton effect with the configuration of the optically active ligands, but it has been stated that it is preferable to rely on empirical correlations (Frelek et al.,

1985). Furthermore, an excess of the ligand can change the sign of the main Cotton effect in the 400–300 nm region (Nakanishi et al., 1971; Frelek et al., 1985).

The NMR shift reagent $Pr(dpm)_3$ has been used for the determination of the absolute configuration of vicinal diols (Nakanishi et al., 1971; Dillon and Nakanishi, 1975). Subsequently, using the same reagent under anhydrous conditions, we noticed that the intensity and the duration of the CD spectra of these complexes varied widely. On the other hand, by employing the stronger chelating reagent, $Eu(fod)_3$, it has been possible to obtain CD spectra exhibiting very strong induced split Cotton effects, centered at about 300 nm, which were essentially stable over a period of 30 days in reagent grade chloroform or carbontetrachloride (Partridge et al., 1976). Recently, we have demonstrated (Wegrzynski and Toome, 1991) that α- and β-hydroxy-carboxylic acids, esters, and ethers also easily form complexes with $Eu(fod)_3$ in chloroform at room temperature and their chiroptical properties can be used for the determination of their absolute configuration *in situ*. An empirical rule has been proposed.

Based on these experiences, we have now extended this technique to α-amino acid esters. These compounds also react efficiently with $Eu(fod)_3$ and it is assumed that they form 1 : 1 complexes[2] in chloroform similar to α-hydroxy-carboxylic acid esters (Nakanishi et al., 1971; Andersen et al., 1974; Dillon and Nakanishi, 1975):

$$R_1\text{—}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}COOR_2 + Eu(fod)_3 \leftrightarrow R_1\text{—}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}COOR_2 \cdot Eu(fod)_3$$

where R_1 represents characteristic substituents of α-amino acid esters at the α-carbon and R_2 describes the nature of the ester moieties (allyl, benzyl, ethyl, methyl, *p*-nitrobenzyl or *t*-butyl groups).

This reaction is simple and fast and can be performed in test tubes at room temperature, and the CD spectra are obtained from the resulting reaction mixtures *in situ*.

Experimental procedure

A. Materials

Amino acids, amino acid esters and N-alkyl amino acids were purchased from Aldrich, Fluka, ICN and Sigma Chemical Companies and were used without further purification. Spectral grade chloroform (without ethanol as preservative) was obtained from American Burdick and Jackson Chemical Company and Europium (III)-tris-(1,1,1,2,2,3,3)-heptafluoro-7,7-dimethyl-4,6-octadiene [Eu(fod)3] was purchased from Norell, Inc.

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[2] Although we have not carried out kinetic or stoichiometric measurements (they are planned), it is assumed in the literature (Nakanishi et al., 1971; Andersen et al., 1974) that $Eu(fod)_3$ forms with bifunctional electron donating ligands such as α-amino acid esters an octa-co-ordinated complex.

B. Methods

1. Standard method

Two milliliters of a 4×10^{-4} M solution of $Eu(fod)_3$ in chloroform is added to 2 ml of a 4×10^{-4} M solution of an amino acid ester in chloroform in a test tube. The mixture is stirred on a Vortex type mixer and the CD spectrum of the *in situ* reaction mixture is recorded in a 0.01 dm stoppered quartz cell after standing 10 minutes (if not stated otherwise) at ambient temperature.

2. Micromethod (modified standard procedure)

Two milliliters of a 4×10^{-4} M solution of $Eu(fod)_3$ in chloroform is added to 2 ml of a 4×10^{-5} solution of an amino acid ester in chloroform and it is continued as described under the standard method. Alternately, 0.2 milliliters of a 4×10^{-4} M solution of $Eu(fod)_3$ solution in chloroform is added to a 0.2 ml of a 4×10^{-4} M solution of an amino acid ester in chloroform. The mixture is stirred on a Vortex type mixer and after standing about 10 minutes at ambient temperature, it is diluted with chloroform to the required cell volume.

C. Measurements

CD spectra were recorded on a JASCO spectropolarimeter, Model J-500A between 450 and 250 nm at ambient temperature. The spectropolarimeter was calibrated with D-10-camphorsulfonic acid in water. The CD intensity calculations are based on the concentrations of the amino acid esters and are expressed in molecular ellipticities $[\theta]$, $(deg \cdot cm^2 \cdot dmole^{-1})$.

Results and discussion

As in the case of α-hydroxy acids and their derivatives (16), the reaction between the α-amino acid esters and $Eu(fod)_3$ in 1 : 1 molar ratio in chloroform is also almost instantaneous, provided they are soluble in chloroform. Esters were used either as hydrochlorides or as *p*-toluenesulfonates as indicated in Table 1. For practical purposes and for the uniformity of the analytical results, the reaction mixtures were left to stand for 10 minutes before the CD spectra were recorded. The complex is stable for at least 30 days. Since some of the amino acid esters salts are only sparingly soluble in chloroform, it has been observed that in those cases, the intensity of the CD maxima slightly increases over a period of a few days[3,4]. The $Eu(fod)_3$ solution is stable in chloroform for at least 30 days at ambient temperature, without any precautions.

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[3] No complex formation was observed with most of the free amino acids within a reasonable time period due to their insolubility in chloroform. Only proline is quite soluble and reacts readily with $Eu(fod)_3$; where as valine for instance reacts very slowly. The CD spectra of their $Eu(fod)_3$ complexes are also included in Figure 3. In addition, alanine methyl ester hydrochloride and histidine methyl ester dihydrochloride did not react with $Eu(fod)_3$. The solubility problem is under investigation.

[4] Some differences in the intensities have been observed, but these could be caused by the variation of their solubilities in chloroform: increased solubility in the case of the larger, more lipophilic ester moiety. On the other hand, it is possible that for instance a tertiary butyl ester is sterically forcing an amino acid to assume a higher degree of a preferred conformation in solution than in the case of the methyl ester. Further experiments are planned to investigate these differences.

Table 1. CD Spectra data of $Eu(fod)_3$ complex with α-amino acid esters in chloroform *in situ*

Compound	1st Cotton Effect		2nd Cotton Effect	
	λ nm	$[\theta] \times 10^{-3}$	λ nm	$[\theta] \times 10^{+3}$
1. L-Alanine benzyl ester · HCl	312	+22.25	286	−19.75
2. D-Alanine benzyl ester p-toluene-sulfonate salt	312	−24.10	285	+22.52
3. L-Alanine t-butyl ester · HCl	309	+23.75	285	−21.65
4. L-Aspartic acid 4-benzyl ester	316	+4.25	283	−6.00
5. L-Aspartic acid diallyl ester p-toluene-sulfonate salt	312	+30.52	285	−29.51
6. L-Aspartic acid dibenzyl ester p-toluene-sulfonate salt	313	+30.50	285	−29.12
7. L-Aspartic acid dimethyl ester · HCl	310	+22.25	285	−21.56
8. D-Aspartic acid dimethyl ester · HCl	312	−21.75	283	+19.77
9. L-Cysteine ethyl ester · HCl	310	+21.73	284	−20.75
10. L-Cysteine methyl ester · HCl	310	+7.75	285	−7.72
11. L-Glutamic acid dimethyl ester · HCl	310	+12.15	284	−12.75
12. L-Histidine benzyl ester di-p-toluene-sulfonate salt	311	+11.82	285	−11.23
13. L-Isoleucine methyl ester · HCl	310	+16.75	284	−19.00
14. L-Isoleucine t-butyl ester · HCl	308	+44.00	284	−41.49
15. L-Leucine ethyl ester · HCl	309	+21.15	285	−20.26
16. L-Leucine methyl ester · HCl	310	+26.00	285	−25.00
17. D-Leucine methyl ester · HCl	311	−24.52	285	+22.85
18. L-Methionine methyl ester · HCl	309	+7.75	284	−8.70
19. L-Phenylalanine benzyl ester p-toluene-sulfonate salt	308	+12.75	283	−14.00
20. L-Phenylalanine methyl ester · HCl	303	+6.50	283	−7.22
21. D-Phenylalanine methyl ester · HCl	301	−8.90	284	+8.24
22. L-Phenylalanine t-butyl ester · HCl	304	+12.25	282	−11.25
23. L-Proline methyl ester · HCl	312	+61.25	284	−50.00
24. L-Serine benzyl ester · HCl	313	+5.52	286	−4.26
25. L-Tryptophan benzyl ester · HCl	306	+10.55	281	−7.23
26. D-Tryptophan methyl ester · HCl	303	−6.41	280	+3.12
27. L-Tyrosine benzyl ester p-toluene-sulfonate salt	307	+6.75	281	−12.18
28. L-Tyrosine methyl ester	306	+10.85	281	−12.45
29. L-Tyrosine t-butyl ester	303	+23.25	282	−17.00
30. L-Valine methyl ester · HCl	307	+23.32	283	−23.25
31. L-Valine p-nitrobenzyl ester · HBr	311	+55.00	284	−39.32

It should be noted here that the chloroform (without ethanol), purchased from Burdick and Jackson, was the most suitable commercially available chloroform for this reaction and was used without purification. Analytical grade chloroforms from other suppliers, even after treatment with molecular sieves were not at all (no measurable complexation observed), or only sparingly suitable as a solvent for this reaction.

A number of optically active α-amino acid esters of known absolute configuration were reacted with $Eu(fod)_3$ in chloroform and the CD spectra of the reaction mixtures were recorded *in situ* between 400 and 250 nm. The positions,

signs, and intensities of the Cotton effects are summarized in Table 1. Fig. 1 exhibits the CD spectra of D- and L-leucine methyl ester hydrochloride reaction products with $Eu(fod)_3$ in chloroform *in situ*. For comparison (in order to demonstrate the drastic difference in the position and intensity of the CD maxima) the CD spectrum of the L-leucine ethyl ester hydrochloride in ethanol is also shown. An additional example is demonstrated in Fig. 2, where the CD spectrum of the L-tyrosine methyl ester hydrochloride in ethanol is compared with the CD curve of the tyrosine-t-butyl ester hydrochloride reaction product with $Eu(fod)_3$ in chloroform *in situ*.

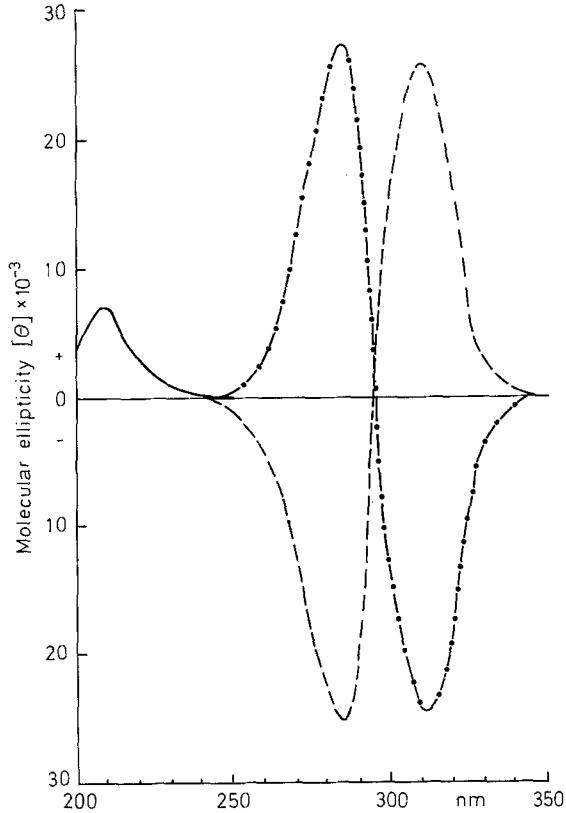


Fig. 1. CD spectra of L-leucine ethyl ester hydrochloride in ethanol (—) and D- and L-leucine methyl ester hydrochloride reaction products with $Eu(fod)_3$ in chloroform (–●– and – – respectively) *in situ*

As seen in Table 1 and demonstrated in Figs. 1 and 2, the CD spectra of the 1 : 1 mixtures of the solutes and $Eu(fod)_3$ in chloroform exhibit induced split CD curves centered at about 300–290 nm. It has been stated (Nakanishi et al., 1971) that it is not clear whether the two CD extrema of opposite signs are related to exciton splitting encountered in the aromatic chirality method. In the L-series, the first Cotton effect at around 320–305 nm is positive and the second at about 290–280 nm is negative. In the D-series, the CD spectra are mirror images to those of L-enantiomers within the experimental error. The signs of the Cotton

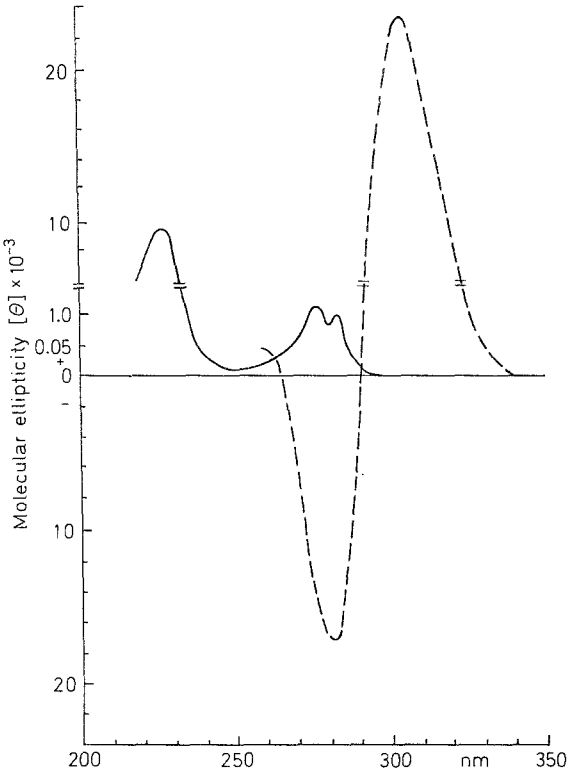


Fig. 2. CD spectra of L-tyrosine methyl ester hydrochloride in ethanol (—) and L-tyrosine-t-butyl ester reaction product with $Eu(fod)_3$ in chloroform (– –) *in situ*

effects are, so far without exception, not affected by the nature of the α-substituent (aliphatic, with or without other functional groups and aromatic amino acid esters) or by the nature, including steric effects, of the ester moiety[5] as summarized in Fig. 3. In addition, the secondary amino acids, L- and D-prolines and the primary amino acid, L-valine (the latter is only sparingly soluble in chloroform)[4,5] complexed with $Eu(fod)_3$ show a similar CD spectral pattern as their esters.

Since the CD bands are on the average of high intensity, this method is also useful in microscale where as little as 4 μg/ml or less of an amino acid ester can be reacted with $Eu(fod)_3$ to produce analytically useful CD spectra (signal-to-noise ratio better than 5 : 1).

Although there are still uncertainties about the conformational equilibria of α-amino acids in solution (Polonski, 1975; Listowsky et al., 1979), it is assumed that the predominant conformation (at least in the solution in the presence of a lanthanide complexing reagent such as $Eu(fod)_3$) is the one which has the α-amino group eclipsed with the carbonyl group (Polonski, 1975). They are shown below in the Newman projection, drawn with the carboxyl or ester group

[5] CD studies of the $Eu(fod)_3$ complexes with cyclic amino acid analogues such as L-(2)-amino-4-butyro-lactone, D-homocysteine thiolactone and L-(—)3-amino-ε-caprolactam are planned.

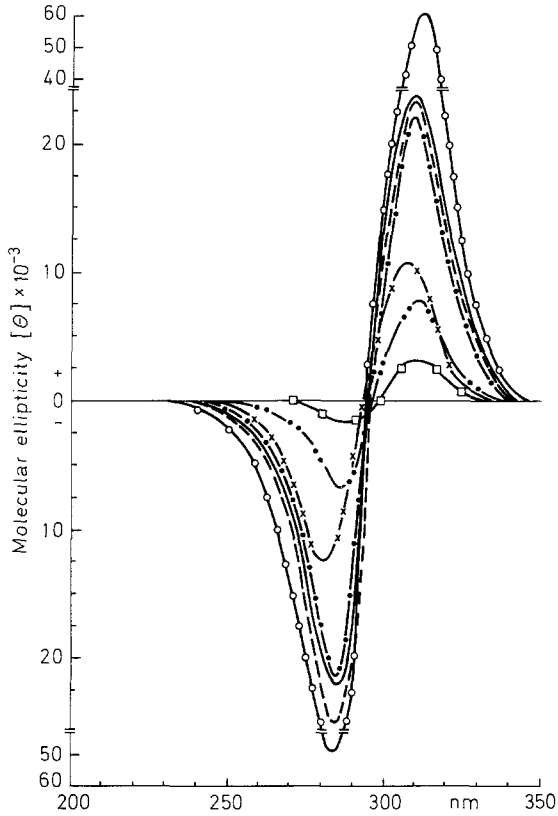
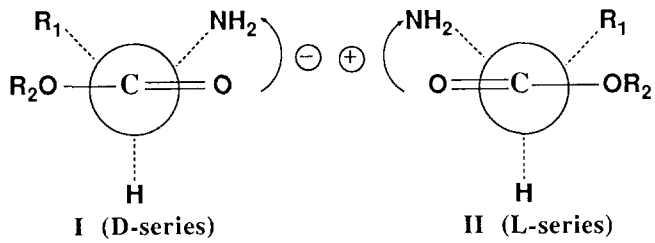


Fig. 3. CD spectra of L-alanine *t*-butyl ester hydrochloride (—), L-leucine methyl ester hydrochloride (– –), L-aspartic acid dimethyl ester hydrochloride (–●–), L-cysteine methyl ester hydrochloride (–●●–), L-tyrosine methyl ester hydrochloride (– × –), L-proline (–○–), and L-valine (–□–) reaction products with $Eu(fod)_3$ in chloroform *in situ*

in front and the amino substituted C-2 carbon in the back (I for D-series and II for L-series):



I (D-series) II (L-series)

The sign of the Cotton effect at around 310 nm is indicated by the arrow drawn from the carbonyl to the amino group at the C-2 carbon. It is defined arbitrarily as negative if the arrow is drawn counterclockwise, and positive if it is drawn clockwise. As an empirical rule, the negative arrow is associated with the first negative Cotton effect in the 315–305 nm area and the D-configuration and the positive arrow with the first positive Cotton effect and L-configuration of an

α-amino acid ester (or a free acid when soluble in chloroform), structures I and II respectively.

Conclusion

Our empirical rule holds in all cases investigated (including proline and proline esters), and it is not dependent on the nature of the α-substituent. This procedure is a simple, fast, and reliable analytical method. It allows the determination of the absolute configuration of α-amino acid esters based on the chiroptical properties of their Europium complexes *in situ* (also in microscale).

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