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A GENERAL AND ACCURATE NMR DETERMINATION OF THE ENANTIOMERIC PURITY OF α-AMINOACIDS AND α-AMINOACID DERIVATIVES

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Abstract - Derivatization of α-aminoacids, α-aminoesters and α-aminolactones as N-acetyl derivatives allow the accurate NMR determination of the enantiomeric purity. In these conditions the major coordination site with a chiral shift reagent will correspond to the NMR observation site. Experimental factors leading to the highest ΔΔδ values are ascertained. No straightforward correlation with absolute configurations can be established.

INTRODUCTION

Several methods have been used to determine the enantiomeric purity of aminoacids either directly or after derivatization : measurement of the rotatory power (cf. ref. 1 for criticisms), biochemical technics[2], isotopic dilution[1,3], liquid chromatography[4], vapour chromatography[5] and NMR.

NMR methods imply either the use of a chiral solvent[6] or the beforehand addition of a chiral auxiliary in order to convert the aminoacid into a mixture of two diastereoisomers whose respective NMR signals (^1H, ^{13}C or ^{19}F) are most of the time distinct[7]. The difference in chemical shift may still be increased by complexing the diastereoisomers with an achiral shift reagent[8].

An important simplification of the latter technic consists in the direct addition of a chiral lanthanide salt to the enantiomeric mixture; as a result, the two formed diastereoisomeric complexes can be easily distinguished by NMR. In that case the aminoacids are first transformed into their methyl esters, in order to allow their solubilisation in the usual NMR solvents (furthermore, compounds possessing acidic groups cause partial decomposition of the lanthanide chelates[9]) and to obtain two distinct thin peaks, the integration of which will measure the enantiomeric purity[10-16]. In less frequent cases, the methyl signal of a t-butyl ester[17] was used and even the Hα signal of an ethyl ester[18], but the magnitude of the shift between the two non-equivalent signals, and therefore the accuracy, were not specified. This classical derivatization into aminoacid methyl esters lacks generality; other esters (benzyl, trimethylsilyl...) and α-aminolactones cannot be directly studied. We also observed that urethane protected methyl aminoesters cannot be used. For example, Z-Val-OCH_3 and Boc-Val-OCH_3 with 0.3 molar equivalent of Eu(hfc)$_3$* in $CDCl_3$ or C_6D_6 solution, give small chemical shift variations of the methyl signal ($\Delta\delta$ = 0.1-0.2 ppm); a rapid broadening of the peaks (even with 0.05 molar equivalent of shift reagent) occurs and the splitting of the OCH_3 signal cannot be observed at 90 MHz.

Furthermore, methyl esters do not appear to be the best type of derivatives for allowing the largest separation of the signals corresponding to the two

*Eu(tfc)$_3$: Tris(3-(trifluoromethylhydroxymethylene)-d-camphorato) europium III
Eu(hfc)$_3$: Tris(3-(heptafluoropropylhydroxymethylene)-d-camphorato) europium III
Eu(dcm)$_3$: Tris(d,d-dicampholylmethanato) europium III
Eu(fod)$_3$: Tris(6,6,7,7,8,8,8-heptafluoro-2,2-dimethyl-3,5-octanedionato) europium III

diastereoisomeric complexes. The commonly used europium ß-diketonates are Lewis acids which strongly complex amines and weakly complex esters[9,19]. The preferential complexation site with methyl aminoesters (the amine function) is thus relatively distant from the NMR observation site (the ester group); this does not correspond to the best analytical conditions. Rackham[20] observed a far better separation of the methoxy signals of the two diastereoisomers by converting aminoacid methyl esters into their N-trifluoroacetyl derivatives. According to this author, the amino group loses its donor function as a result of the inductive effect of the CF_3 group and the carbonyl group of the ester function becomes the unique coordination site.

Due to the better coordination of lanthanides with amides than with esters[9,21,22], we propose in this work to transform aminoacids into their N-acetyl derivatives. In this manner, we will benefit from an interesting methodology using the lanthanide coordination site as NMR observation site. Moreover, this method appears to be much more general, for as we will see, it can be applied not only to α-aminoacids but also to all types of α-aminoesters and α-aminolactones.

Recently it has been claimed[14,15] that for some heterocyclic N-acetyl aminoacid methyl esters, there is no separation of the acetyl signals, and that the chemical shifts between the methoxy signals are very small. These results may be ascribed to unsuitable experimental conditions. Since extremely varied experimental conditions used in the different quoted publications[10-15,20,21] (nature of the lanthanide salt, of the solvent, proportion of reagents, substrate concentrations), it is not possible to make proper comparisons of the results of the literature. We thus intended to define standard conditions leading to the best shift between the acetyl signals.

RESULTS

First of all, we recorded the NMR spectra of 0.1 molar $CDCl_3$ solutions of N-acetyl methyl ß-alaninate after addition of increasing amounts of $Eu(hfc)_3$ (the choice of this reagent will be sustained further). We note (fig 1) that for the methylene** and methyl groups on each side of the amide carbonyle, NMR signals undergo a variation of chemical shift ($\Delta\delta$) much stronger compared with those corresponding to the same groups adjacent to the ester carbonyle. This result confirms the preferential coordination of the amide function.

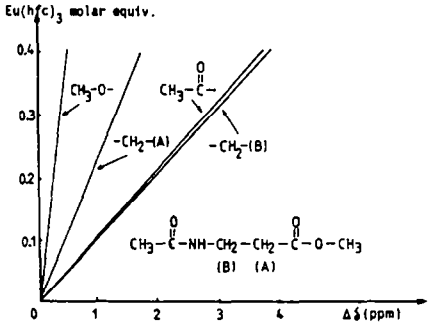

Figure 1

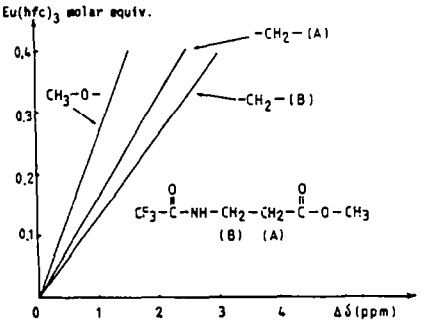

Figure 2

** This CH_2 appears as a multiplet due to the coupling with the neighbouring NH and is consequently easily differenciated from the other CH_2 which appears as a triplet.

Furthermore when we replace the acetamido by the trifluoroacetamido group, we note that the most important variation of δ corresponds to the methylene adjacent to the amide function (Fig 2). This result shows that, in opposition to Rackham's interpretation[20], the presence of a strong electron withdrawing trifluoacetyl group is insufficient to totally suppress the coordination with the amide group. This remark confirms the great difference existing between amide and ester functions as far as coodination with lanthanides is concerned.

Among chiral lanthanide salts, three europium salts* : $Eu(tfc)_3$, $Eu(hfc)_3$, and $Eu(dcm)_3$, are most commonly quoted in the literature. $Eu(tfc)_3$ was the first used to measure enantiomeric shift differences of aminoacid methyl esters[10]. But poor results were observed with N-acetyl ß-hetaryl methyl alaninates[14,15]. As far as we know, no experience involving $Eu(dcm)_3$ was ever realised with aminoacid derivatives; on the other hand, $Eu(hfc)_3$ was successfully used by some authors[16,20,21,23,24]. In order to choose the most efficient reagent, we have measured chemical shift variations ($\Delta\delta$) and enantiomeric shift separations ($\Delta\Delta\delta$) of the methyl signals of a reference product, D,L N-acetyl methyl leucinate, after addition of equivalent amounts of $Eu(hfc)_3$ and $Eu(dcm)_3$.The reported values (table 1) show without ambiguity that $Eu(hfc)_3$ provokes a far better effect.

		$Eu(dcm)_3$	$Eu(hfc)_3$
CH_3-CONH-	Δδ	1.13	2.63
	ΔΔδ	0.06	0.26
$-CO_2CH_3$	Δδ	0.86	0.90
	ΔΔδ	0.00	0.12

Table 1- Δδ and ΔΔδ values in ppm of the methyl signals of racemic 0.1 molar N-acetyl methyl leucinate in presence of 0.3 molar equivalent of $Eu(dcm)_3$ or $Eu(hfc)_3$ (solvent $CDCl_3$).

The nature of the solvent is also an important factor; in order to select the most suitable one, we recorded NMR spectra of the same reference product: D,L N-acetyl methyl leucinate (0.1 molar solution) in presence of an identical amount (0.3 molar equivalent) of $Eu(hfc)_3$ in six different solvents: CCl_4, $CDCl_3$, C_6D_6, CD_3CN, furan and 1,1,2,2-tetrachloroethane. The last one was rejected because addition of even a small amount of $Eu(hfc)_3$ provokes a broadening of the two methyl signals. Among the five other solvents, $CDCl_3$ and C_6D_6 lead to the best results. Nevertheless, further studies have shown that in some cases (such as N-acetyl methyl phenylalaninate) the system $CDCl_3$-$Eu(hfc)_3$ did not allow any signal separation. So we kept C_6D_6 as standard solvent for all the subsequent measurements.

We next looked for the best concentration of the shift reagent. Increasing quantities of $Eu(hfc)_3$ (from 0 to 1 molar equivalent) were added to 0.1 mole/l solution of D,L N-acetyl methyl valinate and D,L N-acetyl methyl alaninate in C_6D_6. We observed in both cases a larger shift of the acetyl signal, as compared to the methoxy signal, which confirms the preferential complexation of the amide group. Moreover, the splitting of the acetyl signal always occurs first. Finally, the variation of the ΔΔδ values for the acetyl group depends on the nature of the aminoacid. With valine, ΔΔδ values increase regularly with the added quantities of shift reagent (Fig 3). On the other hand, with alanine we observed a maximum corresponding to 0.2-0.4 molar equivalent of $Eu(hfc)_3$ (Fig 4). A similar result was already pointed out in the literature[25].

Taking these results into account, we chose in our following experiments to use 0.3 equivalent of $Eu(hfc)_3$ per equivalent of the aminoacid substrate.To illustrate the general application of these standard conditions, the spectra of eleven racemic N-acetyl aminoacid methyl esters have been recorded; we have gathered in Table 2 the variations of

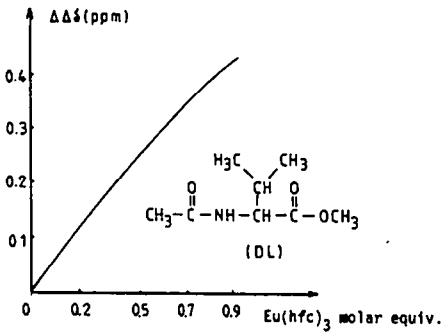

Figure 3

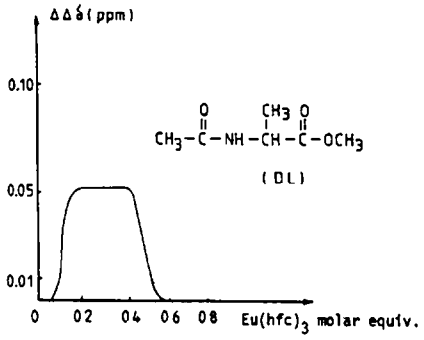

Figure 4

N-Ac amino esters	CH_3-CONH-		$-CO_2CH_3$	
	Δδ	ΔΔδ	Δδ	ΔΔδ
Ala	3.51	0.06	0.54	0.02
Val	3.24	0.16	0.50	0.02
Leu	3.36	0.40	0.54	0.09
Pro	1.90	0.14	0.52	0.06
Phenylglycine	2.85	0.16	0.32	0.03
Phe	3.05	0.06	0.37	0.04
Met	3.22	0.17	0.47	0.06
Ser(OBzl)	4.20	0.14	0.50	0.00
Bromohomoserine	2.65	0.11	0.65	0.10
Glu(OCH_3)	3.00	0.10	0.50	0.04
			0.55	0.00
Asp(OCH_3)	3.42	0.13	0.47	0.03
			0.45	0.00

Table 2- Δδ and ΔΔδ values in ppm of the methyl signals of racemic 0.1 molar N-acetyl aminoacid methyl esters with 0.3 molar equivalent of Eu(hfc)3 (solvent C_6D_6)

chemical shifts (Δδ), as well as the enantiomeric shift separation (ΔΔδ) of the amide and ester methyl signals in C_6D_6 solution in presence of 0.3 molar equivalent of Eu(hfc)3.

In the case of D,L N-acetyl tryptophane methyl ester, it is necessary to add one or two drops of pyridine to the C_6D_6 solution in order to assure complete solubilisation; pyridine being a consuming reagent, it therefore becomes necessary to increase the quantity of Eu(hfc)3 in order to get acceptable values (Table 3).

Eu(hfc)3	0.8 equiv.		1.5 equiv.	
	Δδ	ΔΔδ	Δδ	ΔΔδ
CH_3-CONH-	0.85	0.03	1.53	0.10
$-CO_2CH_3$	0.12	0.00	0.21	0.00

Table 3- Δδ and ΔΔδ values in ppm of the methyl signals of racemic N-acetyl tryptophan methyl ester (0.1 molar solution, solvent C_6D_6+2 drops of pyridine).

The strong chemical shift variation Δδ of the acetyl signal results in the occurrence of this signal in a zone generally free from other signals; therefore a good detection of the minor enantiomer is possible and the integration of the two signals is possible with a good precision. We have checked that it is easy to detect 1% of the D-enantiomer in a D,L mixture of N-acetyl methyl leucinate (prepared by weighing) with a routine spectrometer working at 90 MHz; 0.5% of the minor enantiomer can be detected with a 360 MHz machine.

The presence of a methyl ester function is not essential for the of determination of optical purity; our method can also be applied to various esters of N-acetyl aminoacids, especially those used in peptide synthesis such as benzyl, t-butyl and trimethylsilyl esters.

As an example, we recorded the spectra of D,L N-acetyl benzyl alaninate and D,L N-acetyl t-butyl valinate under the same standard conditions (Table 4); we note that in both cases the separation between the enantiomeric acetyl signals ($\Delta\Delta\delta$) is even better than for the corresponding methyl esters, probably as a result of the increased steric hindrance to coordination of the benzyl and t-butyl esters.

Amino esters	CH_3-CONH-		$-CO_2CH_3$		$-CO_2tBu$	
	$\Delta\delta$	$\Delta\Delta\delta$	$\Delta\delta$	$\Delta\Delta\delta$	$\Delta\delta$	$\Delta\Delta\delta$
Ac-Ala-OMe	3.51	0.06				
Ac-Ala-OBzl	3.32	0.12				
Val-OMe			1.70	0.08		
Ac-Val-OMe	3.24	0.16	0.50	0.02		
Val-OtBu					0.85	0.10
Ac-Val-OtBu	3.25	0.30			0.35	0.01
Ac-Leu-$OSiMe_3$	0.48	0.12				
Ac-α-Amino-butyrolactone	2.86	0.24				

Table 4- $\Delta\delta$ and $\Delta\Delta\delta$ values in ppm of some racemic 0.1 molar N-acetyl aminoacid esters with 0.3 molar equiv. of $Eu(hfc)_3$ (solvent C_6D_6).

We will now question the possibility of directly using aminoacid benzyl and t-butyl esters without derivatization. Actually in the case of alanine benzyl ester, the methylene protons become enantiotopic with the first addition of europium salt which is noxious to a precise measurement. In the case of valine t-butyl ester, the chemical shift variation $\Delta\delta$ of the thin methyl peak is small and the $\Delta\Delta\delta$ value is smaller than for the corresponding N-acetyl derivative. Results given in Table 4 clearly show the marked advantage of the N-acetyl derivatization.Two other examples, D,L N-acetyl trimethylsilyl leucinate and D,L N-acetyl α-aminobutyrolactone, illustrate the general applicability of our method (Table 4).

It would also be interesting to apply the same method to the N-acetyl aminoacids themselves. However it is generally claimed that acid substrates provoke partial decomposition of the lanthanide chelates[9]; nevertheless $Eu(fod)_3$* have been used with phenols and carboxylic acids[25]. On the other hand, an important difficulty lies in the insolubility of most of the N-acetyl aminoacids in C_6D_6. We first tried to use a cosolvent to C_6D_6; CD_3OD seemed to be the best, as one or two drops added to C_6D_6 were enough to solubilise the substrate. But CD_3OD is likely to coordinate with the europium salt; we have effectively checked that by addition of one or two drops of CD_3OD to the C_6D_6 solutions of the N-acetyl aminoacid methyl esters studied above, a decrease of both $\Delta\delta$ and $\Delta\Delta\delta$ values occurs, however with conservation of distinct methyl signals. NMR spectra of N-acetyl leucine and N-acetyl phenylalanine, registered with the same conditions of solvent (5ml of C_6D_6 + one drop of CD_3OD) and concentration (0.1 mole/l) and in presence of 0.4 equivalent of $Eu(hfc)_3$, show $\Delta\Delta\delta$ values of 0.18 and 0.14 ppm respectively, which are largely sufficient for a correct evaluation of the enantiomeric purity.

Nevertheless we observed a slight broadening of the peaks that may be damageable for the precision of the measurements; in this case the method of Parfitt et al[26] can be used in order to calculate the optical purity with a convenient accuracy. But it is also possible to avoid broadening the signal by adding a stoechiometric quantity of an organic base to the solution . Although the formed carboxylate anion and the amide group can competitively coordinate with the europium salt, the $\Delta\Delta\delta$ values for the enantiomeric acetyl signals remain large enough. We have gathered (Table 5) the $\Delta\delta$ and $\Delta\Delta\delta$ values observed with

N-acetyl leucine in the presence of four different bases. The best results are obtained with N-methylmorpholine.

Bases	$\Delta\delta$	$\Delta\Delta\delta$
Imidazole	0.36	0.00
N-Methylimidazole	0.33	0.07
Morpholine	0.49	0.11
N-Methylmorpholine	0.51	0.21

Table 5- $\Delta\delta$ and $\Delta\Delta\delta$ values in ppm of the acetyl signal of racemic 0.1 molar N-Ac leucine in presence of 0.4 molar equiv. of $Eu(hfc)_3$ and some organic bases (solvent: C_6D_6 + 1 drop of CD_3OD).

We have applied our method to determine the optical purity of aminoacids resulting from asymmetric synthesis. Actually many of the asymmetric synthesis lead first to the corresponding methyl esters[4g,4h,27] whose optical purity is a measure of the asymmetric induction efficiency. But after acid hydrolysis into the corresponding aminoacids, optical purities are rarely questioned, although drastic acid conditions can provoke partial racemisation[28]. Two pure aminoacid methyl esters (methyl L-leucinate and methyl L-phenylalaninate) have been hydrolysed in the standard conditions: 3 hours boiling with 2N HCl[24]. After N-acetylation, NMR spectra have been registered in the standard conditions (cf. table 5) and in the presence of N-methylmorpholine. The D-enantiomer could not be detected with leucine; but on the other hand, formation of 7% of D-enantiomer (as determined by integration of the signals) has been observed with the phenylalanine derivative.

In conclusion, the N-acetyl derivatization is much more general than other methods to determine the optical purity of aminoacid derivatives. Under our experimental standard conditions, the observed $\Delta\Delta\delta$ values of the acetyl signals easily allow the detection of less than 1% of the minor enantiomer, whatever the nature of the ester (methyl, benzyl, t-butyl, trimethylsilyl...); this method can also be applied to α-aminolactones as well as to aminoacids themselves.

Some authors[6,8,10,14,15,20] have tried to correlate enantiomeric shift differences with absolute configuration of α-aminoacids. Conflicting results were obtained, which is not surprising; it has also been found with other substrates (alcohols, amines...) that the experimental conditions has an important bearing on the sign of $\Delta\Delta\delta$ [15,25,29-31]. Our own results (Table 6) confirm that even taking into consideration the N-acetyl group, it is not possible to achieve an inambiguous correlation.

	CH_3-CONH-		-CO_2CH_3	
	D	L	D	L
Ac-Ala-OMe	+	-	+	-
Ac-Leu-OMe	+	-	+	-
Ac-Val-OMe	+	-	+	-
Ac-Glu(OMe)OMe	+	-	-	+
			-	+
Ac-Met-OMe	+	-	+	-
Ac-Asp(OMe)OMe	+	-	-	+
			-	+
Ac-Ser(OBzl)OMe	+	-	**	**
Ac-Phe-OMe	-	+	+	-
Ac-Trp-OMe *	-	+	+	-
Ac-Pro-OMe	+	-	+	-
Ac-Phenylglycine-OMe	+	-	-	+

Table 6- Respective positions of methyl signals
+ : methyl signal to upper fields
- : methyl signal to lower fields
solvent C_6D_6, except (*) C_6D_6 with one drop of C_5D_5N
(**) no signal separation.

EXPERIMENTAL

All NMR spectra were recorded with a Varian EM 390 (90 MHz) or a Varian WM 360 WB (360 MHz) spectrometer. L and D N-acetyl aminoacids were purchased from SIGMA Chemical Company or BACHEM AG; they can also be prepared as described below. N-Acetyl α-aminobutyrolactone was synthesized according to Fillmann and Alberson[32] and N-acetyl methyl β-alaninate according to Fusier[33].

N-Acetyl aminoacids

Sodium bicarbonate (30 mmoles) is added to a slurry of aminoacid (15 mmoles) in 50 ml of a 1-1 dioxane-water mixture; the mixture is stirred at room temperature or at 60°C until complete solubilisation, then acetic anhydride (15 mmoles) is slowly added and the stirring continued until completion of the reaction (overnight) (thin layer chromatography); after concentration under vacuum, the aqueous solution is acidified to pH 1.5 with a 1N HCl solution then extracted three times with ethyl acetate. The organic phase is finally distilled under vacuum (yield 75%-85%).

N-Acetyl aminoacid methyl esters

An etheral solution of diazomethane (1 equiv.) is added to a methanolic solution of N-acetyl aminoacid at 10°C. After evaporation of the solvent the N-acetyl aminoacid methyl ester is obtained in nearly quantitative yield.

N-Acetyl t-butyl valinate and N-acetyl benzyl alaninate

Imidazole (5 mmoles) and 1-acetyl imidazole (5 mmoles) are added to a solution of t-butyl valinate hydrochloride (purchased from BACHEM AG) or benzyl alaninate hydrochloride (purchased from SIGMA Chemical Company) (5 mmoles) in CH_2Cl_2 (15 ml). After evaporation of the organic phase the residue is purified by column chromatography on silica gel (eluent:ethyl acetate).

N-Ac-Val-OtBu, m.p.78°C, (80%), (Found: C,61.14; H,10.02. Calc. for $C_{11}H_{21}NO_3$: C 61.36, H 9.83).

N-Ac-Ala-OBzl, oil (77%), (Found: C, 65.29; H, 6.70. Calc. for $C_{12}H_{15}NO_3$: C 65.14, H 6.83).

The same method can be used to acetylate aminoacid methyl esters.

N-Acetyl trimethylsilyl leucinate

A stirred slurry of N-acetyl leucine (5 mmoles) in hexamethyldisilazane (3 ml) is heated under N_2 on a steam bath; after 15 minutes the medium becomes homogeneous and heating is continued for one hour; the excess of hexamethyldisilazane is distilled under vacuum and the water sensitive remaining oil (1.2g) is used without further purification. NMR (C_6D_6), δ(ppm): 0.25 ($SiMe_3$), 0.85 (d,$2CH_3$), 1.65 (m,3H), 1.75 (CH_3CO), 4.75 (m,1H).

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