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Determination of the Enantiomeric Excess of α-Hydroxy Acids

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Abstract: This work describes a convenient and accurate method for optical purity determination of α-hydroxy acids. Their derivatization with commercially available valine methyl ester affords diastereoisomers easily separable by HPLC using achiral C_{18} columns.

Hydroxy acids are important as constituents of biologically active molecules and of great interest for the pharmaceutical industry. The synthesis of depsipeptides or cyclodepsipeptides and analogs requires the multistep preparation of "exotic" α-hydroxy acids. As the optical purity influences biological properties, it is important to be able to verify the optical purity of synthetic α-hydroxy acids. Among various methods for the determination of optical purity $[\alpha]_D$, the oldest optical rotation measurement still remains widely used[1,2] due to its simplicity and speed despite its low accuracy and the possibility of large errors due to the presence of chiral impurities. Moreover, reference values are needed to calculate the optical purity. The NMR technique is a more accurate method (sensitivity about 1%). However, as only diastereoisomers are distinguished, it is necessary to modify the α–hydroxy acids by complexation with a chiral compound (chiral lanthanide shift reagent or chiral solvent) or by derivatization by reaction with an enantiomerically pure reagent[2]. In the latter case, the enantiomeric excess determination is carried out by ^{1}H, ^{13}C, ^{19}F or ^{31}P NMR[3-11], depending on the reagent used. The HPLC method is widely employed and extremely accurate, showing a sensitivity of about 0.1%. Chiral supports allow a direct analysis of compounds possessing only one stereogenic center, but they are expensive and often very specific. The enantiomeric separation is due to interactions between the substrate and the chiral entity linked to the solid phase of the column. These interactions are due to hydrogen bonds[12,13] or π-donor - π-acceptor interactions[14-16]. Copper salts are sometimes added for complexing the substrate with the stereogenic centers of the support[17-19]. The column chirality is obtained either by simple adsorption of a chiral substance (as a bovin serum albumin[20]) or by covalent fixation of chiral compounds (such as cyclodextrins[21], amino acids[14], etc.). Achiral columns are not so expensive but can separate only diastereoisomers, thus a derivatization of compounds bearing only one stereogenic center is needed. This approach is used particularly for determination of the enantiomeric excess of amino acids. The reagents generally employed are sugars[17,22-24] or amino acids[25-27] bearing a reactive function (isocyanate, fluoronitrophenyl, etc.) able to form a covalent bond with the compound to be analysed. Marfey's reagent[26] (1-fluoro-2,4-dinitro phenyl-5-(L)alanine amide) is of particular interest because it is able to effect a complete analysis of amino acids with enantiomeric excess determination.

RESULTS AND DISCUSSION

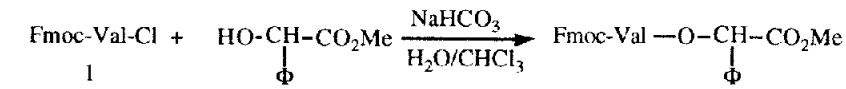
We describe in this paper a general analytical method for determination of the enantiomeric excess of α-hydroxy acids. Mandelic acid, whose two enantiomers are commercially available was chosen as a test compound for several derivatization approaches. Bonner[28] has shown that this hydroxy acid racemizes easily in the course of derivatization reactions.Hence it is an excellent model for checking for the absence of racemization in our derivatization reactions.

NMR determination : Our inspiration came from previous work in our laboratory[29,30] using the chiral shift reagent tris[3(heptafluoropropylhydroxymethylene)-d-camphorato] EuropiumIII, ($Eu(hfc)_3$). In this method, the compound to be studied was derivatized by groups able to undergo complexation with europium and whose NMR signals were preferably singlets for an easy analysis. We first used the methyl ester of mandelic acid. Progressive addition of the europium salt leads to a splitting of signals but also broadens them, making analysis in the solvents used ($CDCl_3$ or C_6D_6) impossible. For the corresponding O-acetyl derivative, the methoxy and acetyl signals are split, but only weakly broadened so that the integration is easy ($CDCl_3$, 90 MHz, 0.6 equivalents of Eu salt). However, the necessity of a double derivatization and an accuracy not exceeding 2-3% minimise the interest for this method.

HPLC determination : Considering the numerous HPLC advantages : high accuracy, very good sensitivity, and the requirement for only very small quantities of crude products, we chose this technique for the enantomeric excess determination of α-hydroxy acids. Taking into account the specificity and the high cost of chiral columns, we decided to employ a non-chiral column and to transform α-hydroxy acids into diastereoisomers by derivatization with a homochiral reagent.

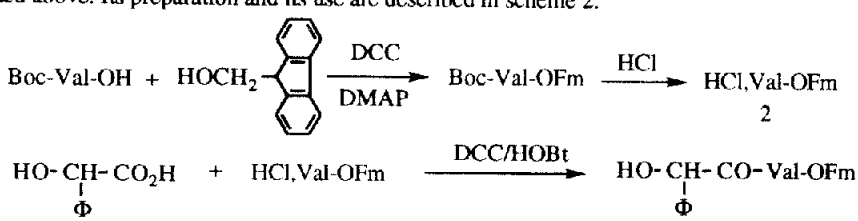
We have to select a chiral derivatizating reagent possessing some particular properties : (i) a good reactivity able to undergo complete and easy derivatization of hydroxy acids and so avoiding any kinetic enrichment of the two diastereoisomers, (ii) a bulky group so that the diastereoisomers formed are easily separable by HPLC, (iii) a chromophore which absorbs at a specific wave-length in which only diastereoisomers formed and reagent excess are detected thus allowing a direct analysis of the reaction medium and avoiding any enrichment of one of the diastereoisomers during the purification steps.

On the basis of these considerations, we chose as the derivatizing reagent Fmoc-(L) valine chloride **1**, a highly activated form of this bulky amino acid in which N-protection by the Fmoc group constitutes an excellent chromophore possessing a maximum absorbance at 262 nm. As the two functions of hydroxy acids can react with an acid chloride, mandelic acid was converted into its methyl ester and then derivatizated with Fmoc-(L) valine chloride following Carpino's method[32] (Scheme 1).



Scheme 1

However, all attempts to separate the two diastereoisomers in a C_{18} reverse phase column were unsuccessful. For this reason we tried to use (L) valine fluorenylmethyl ester **2** as a reagent having all the criteria cited above. Its preparation and its use are described in scheme 2.

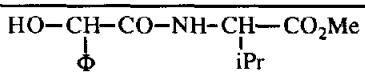
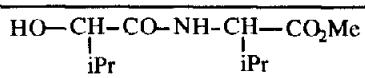
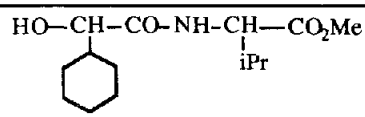
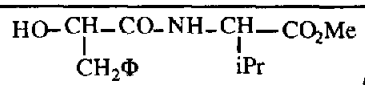
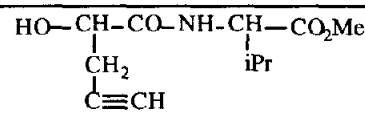
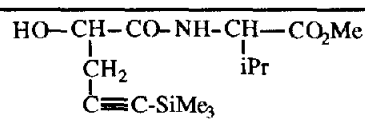
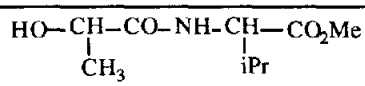


Scheme 2

The use of the DCC/HOBt coupling system allows amide bond formation without any ester being formed, thus avoiding the protection of the mandelic acid alcohol function. In this case, the two diastereoisomers obtained with racemic mandelic acid were separated with an excellent resolution (R_S=2.2) on a C_{18} column.

However, the inconvenience of this method is due to the poor yields in the multistep preparation of **2**. Thus, it was decided to test methyl (L) valinate **3**, a commercially available product, stable as its hydrochloride form. In the absence of a chromophore, the wavelength required for the detection of the derivatized hydroxy acids was 214 nm. The coupling reaction between valine methyl ester and racemic mandelic acid led to a mixture of two diastereoisomers for which separation on a C_{18} column is very good (R_S=3.4). The reaction mixture analysis showed that coupling reagents and starting materials are eluted faster than the derivatized hydroxy acids. So, it is not necessary to purify derivatized products before their HPLC analysis. The absence of racemization was checked by derivatization of the (D) enantiomer of mandelic acid. The (L,L) diastereoisomer arising from a possible racemization is not observed by HPLC (with a detection threshold of about 0.1%). With a view to generalisation of this method, other α-hydroxy acids were tested (Table 1). Derivatization conditions are identical to those described for mandelic acid.

Table 1: HPLC analysis of derivatized α-hydroxy acids

Compound	t'_1	t'_2	α	R_S
 (a)	12.16 (D,L)	18.56 (L,L)	1.5	3.4
 (b)	5.33 (D,L)	7.83 (L,L)	1.5	3.1
 (c)	12.08 (D,L)	18.33 (D,D)	1.5	2.2
 (d)	13.50 (D,L)	21.17 (L,L)	1.6	3.6
 (e)	14.25 (D,L)	18.83 (L,L)	1.3	2.9
 (c)	21.67 (D,L)	27.67 (L,L)	1.3	3.3
 (d)	39.17 (D,L)	50.50 (D,D)	1.3	1.3

Eluents: **(a)** 45% MeOH/55% H_2O; **(b)** 53% MeOH/47% H_2O; **(c)** 57% MeOH/43% H_2O; **(d)** 50% MeOH/50% H_2O; **(e)** 30% MeOH/70% H_2O.

t'_1 et t'_2 are corrected retention times in minutes (measured retention time reduced by the elution time of non-retained products),corresponding respectively to diastereoisomers D,L (or L,D) and L,L (or D,D).

$\alpha = t'_2/t'_1$ is a selectivity and $R_S = t'_2 - t'_1 / W_1+W_2$ the resolution, W_1 and W_2 being the pic width at half-high.

These results show that even in the most unfavourable cases (lactic acid or propargyl glycolic acid), the HPLC separation is sufficient to obtain a good analysis. We also note that in all cases studied, diastereoisomer L,D (or D,L) has a shorter retention time than the corresponding L,L (or D,D) diastereoisomer.

CONCLUSION

The absence of racemization in the coupling reaction of mandelic acid with (L) valine methyl ester and the possibility to effect HPLC analysis directly on the reaction mixture are guarantees for the reliability of results obtained by this method. The successful generalization to the analysis of other α-hydroxy acids shows that derivatization by (L) valine methyl ester followed by HPLC analysis with non-chiral columns is a simple and general method for enantomeric excess determination of α-hydroxy acids.

EXPERIMENTAL

All melting points are uncorrected. ^1H NMR spectra were obtained on a Brüker WP 80/CW spectrometer with TMS as the internal standard. HPLC was carried out with a Waters equipment (two pumps model 510, a variable UV detector model 484 and a 820 Maxima workstation) using a column Spherisorb 5 ODS (30cm). Fmoc valine and valine methyl ester are commercially available products.

Synthesis of (L) methyl mandelate

To a stirred anhydrous methanolic solution (50ml) of (L) mandelic acid (13 mmol, 2g) were added dropwise 26 mmol (3.3 ml) of trimethylsilyl chloride. After stirring for 24h at room temperature, methanol was removed and the residue was taken up in ether (100ml). The organic solution was washed with a saturated solution of $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$. After removal of the solvent, the crude product was recrystallized from hexane giving 1.7g of the product as white solid. 77% yield ; Rf 0.6 (ether) ; m.p. 54-56°C (56-58, Fluka product); $[\alpha]_D$ 144° (c=2, MeOH) ; ^1H NMR ($CDCl_3$) : δ (ppm) : 7.3 (m,5H,C_6H_5) ; 5.2 (s,1H,C_6H_5-C<u>H</u>) ; 3.4 (s,3H,CO_2<u>CH_3</u>).

Synthesis of O-acetyl methyl mandelate

A mixture of 1g (6mmol) of methyl mandelate, 0.61g (6mmol) of acetic anhydride and 0.07g (0.6mmol) of DMAP in 30ml of dichloromethane was stirred at room temperature for 18h. The solution was then successively washed with 1N citric acid solution, saturated sodium hydrogen carbonate and water until neutral pH. The organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure leading to 1.25g of an oily product. 100% yield ; R_f 0.75 (hexane/ethyl acetate 50/50) ; ^1H NMR ($CDCl_3$), δ (ppm): 7.3 (m, 5H, Ph) ; 6 (s, 1H, Ph-C<u>H</u>) ; 3.6 (s, 3H, CO_2CH_3) ; 2.2 (s, 3H, CH_3 of O-Ac).

NMR measurement of the enantiomeric excess

A solution containing about 50μmol of the mixture of enantiomers in 0.5ml of deuterated solvent ($CDCl_3$, C_6D_6) was prepared and its NMR spectrum recorded. To this solution was added progressively (by 0.1eq. portions) the europium salt ($Eu(hfc)_3$) until at least one of spectrum signals split into two signals which could be integrated separately. The ratio between the integration of the two signals corresponds to the ratio between the two enantiomers, allowing the calculation of the enantiomeric excess.

Synthesis of Fmoc-Val-Cl

A solution of Fmoc-Val-OH (2.7g, 8.3mmol) and thionyl chloride (5.9ml, 81mmol) in dichloromethane (50ml) was heated at reflux for 1h. After cooling at room temperature, 50ml of hexane was added. The crystalline product that formed was filtered off, dried under reduced pressure and stored under nitrogen. 80% yield ; m.p. 110°C (Lit.[32] 111-112°C).

Derivatization of (D,L) methyl mandelate by Fmoc-Val-Cl

A solution of 30mg (0.2mmol) of (D,L) methyl mandelate in chloroform (3ml) was added to a mixture containing 3ml of $CHCl_3$ and 3ml of a 10% solution of $NaHCO_3$. After vigorous stirring for 10min., the organic layer was washed with water and dried over $MgSO_4$. Solvent evaporation gave 0.1g of the product as

an oil. 100% yield ; R_f 0.2 (acetone/hexane 10/90) ; ^1H NMR ($CDCl_3$), δ (ppm) : 7.4 (m, 13H, Ph and Aromat. H of Fmoc) ; 5.3 (m, 1H, NH) ; 5.1 (s, 1H, Ph-CH) ; 4.3 (m, 4H, non Aromat. H of Fmoc and CHα) ; 3.7 (s, 3H, CO_2CH_3) ; 2.1 (m,1H, iPr) ; 0.9 (d, 8 Hz, 6H, iPr) ; HPLC (214 nm, eluent : MeOH / H_2O 60 % / 40 %) t_R = 29,6 mn.

Synthesis of Boc-Val-OFm

To a cool (-30°C) solution of 2.17g (10mmol) of Boc-Val-OH and 1.96g (10mmol) of 9-fluorenylmethanol in dichloromethane (5ml) was added dropwise with stirring 2.27g (11mmol) of DCC in 5ml of dichloromethane and 0.12g (1mmol) of DMAP in 2ml of dichloromethane. The resulting solution was stirred at the same temperature for 30 minutes and at room temperature for another 4 hours. DCU that formed was filtered off and the filtrate washed successively with 1N citric acid solution, saturated sodium hydrogen carbonate solution and water until neutral pH. The organic layer was dried over $MgSO_4$ and gave after solvent evaporation an oil which was purified by column chromatography on silica gel, eluent hexane/ether 50/50 leading to 2.15g of a white solid. 54% yield ; m.p. 60°C ; R_f 0.6 (hexane/ether 50/50) ; ^1H RMN ($CDCl_3$) δ (ppm) : 7.4 (m, 8H, Aromat.) ; 5 (d, 1H;, NH) ; 4.4 (m, 3H, non Aromat. of Fm) ; 4.2 (m, 1H, CHα) ; 1.8 (m, 1H, iPr) ; 1.4 (s, 9H, tBu) ; 0.8 (d, 7 Hz, 6H, iPr).

Deprotection of Boc-Val-OFm

2.15g (5.4mmol) of Boc-Val-OFm in 30ml of dioxan saturated with HCl was stirred at room temperature for 3 hours. To the oily residue obtained after solvent evaporation was added a mixture of ether/petroleum ether 50/50 until precipitation of the hydrochloride. The solid was filtered of and dried under reduced pressure. 55% yield ; m.p. > 180°C ; ^1H RMN (DMSO) δ (ppm) : 7.4 (m, 8H, Aromat.) ; 4.5 (m, 3H, non Aromat. of Fm) ; 4.2 (m, 1H, CHα) ; 1.8 (m, 1H, iPr) ; 0.8 (d, 8 Hz, 6H, iPr).

Derivatization of mandelic acid by Val-OFm

Neutralization of HCl, Val-OFm. A mixture of 0.9g (2.7mmol) of HCl, Val-OFm in 50ml of ethyl acetate and 20ml of $NaHCO_3$ 5% solution was vigorously stirred during 15 minutes. The organic layer was washed with water until neutral pH, dried over $MgSO_4$ and the solvent was evaporated.

Derivatization : Val-OFm obtained above (0.8g, 2.7mmol) was added to 5ml of CH_2Cl_2 containing 0.41g (2.7mmol) of mandelic acid and 0.4 (2.9mmol) of HOBt. To this mixture cooled at -30°C was slowly added 0.67g (3.2mmol) of DCC dissolved in 2ml of CH_2Cl_2. After completion of the addition, the reaction mixture was stirred for 30 minutes at -30°C and then for 12 hours at room temperature. The precipitate of DCU was filtered off and 20ml of CH_2Cl_2 was added to the filtrate. The solution was successively washed with 1N citric acid (2X10ml), saturated $HNaCO_3$ (2X10ml), water (10ml) and dried over $MgSO_4$. The oily product obtained after solvent evaporation was taken up in a minimum of acetone and stored in a refrigerator bringing about the precipitation of remaining DCU. After filtration and acetone evaporation, 0.8g of a brown oil was obtained. 75% yield ; ^1H RMN ($CDCl_3$) δ (ppm) : 7.6 (s, 5H, Ph) ; 7.3 (m, 8H, Aromat. of Fm) ; 5 (s, 1H, Ph-CH) ; 4.4 (m, 3H, non Aromat. of Fm) ; 4.1 (m, 1H, CHα) ; 1.9 (m, 1H, iPr) ; 0.7 (d, 8 Hz, 6H, iPr) ; HPLC (264 nm, MeOH / H_2O 68 % / 32 %) t_{R1} = 34.7 mn ; t_{R2} = 39.8 mn ; α = 1.2; R_S = 2.2.

Derivatization of mandelic acid by Val-OMe

A suspension of HCl, Val-OMe in anhydrous ether cooled at -30°C was neutralized by a gaseous flow of NH_3 for 30 minutes. Insoluble materials were filtered off and the ethereal solution evaporated to dryness to give Val-OMe as an oil. 1g (7.8mmol) of Val-OMe was added to a stirred solution containing 1g (6.5mmol) of mandelic acid and 0.96g (7.2mmol) of HOBt in 10ml of CH_2Cl_2. To this solution cooled at -30°C was added 1.6g (7.8mmol) of DCC dissolved in 5ml of CH_2Cl_2. Stirring was maintained for 30 minutes at -30°C and then

for 12 hours at room temperature. The precipitate of DCU was filtered off and 50ml of CH_2Cl_2 added to the filtrate. The solution was successively washed with 1N citric acid (2X15ml), saturated $HNaCO_3$ (2X15ml), water (15ml) and dried over $MgSO_4$. Solvent evaporation gave an oil which was taken up in a minimum of acetone and stored at 0°C. After filtration of the remaining DCU and solvent evaporation, 1.5g of an brown oil was obtained. 88% yield ; R_f 0.57 (EtOAc) ; ^1H RMN ($CDCl_3$) δ (ppm) : 7,3 (s, 5H, Aromat.) ; 6.1 (m, 1H, NH) ; 5.2 (s, 1H, Ph-CH) ; 4.4 (m, 1H, CHα) ; 3.4 (s, 3H, CO_2CH_3) ; 1.8 (m, 1H, iPr) ; HPLC (214 nm, éluent : MeOH / H_2O 45 % / 55 %) t_{R1} = 12.16 mn , t_{R2} = 18.56 mn; α = 1.5; R_S =3.4.

Derivatization of the other hydroxyacids by Val-OMe

The derivatization of hydroxy acids summarized in table 1 was conducted following the same procedure that used for mandelic acid. HPLC analysis of crude reaction products do not shown the presence of important impurities allowing so a direct enantiomeric excess analysis.

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