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Enantioselective HPLC combined with spectroscopic methods: A valid strategy to determine the absolute configuration of potential β-secretase inhibitors

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ABSTRACT

A direct HPLC enantioseparation of three representative compounds of a new family of potential non-peptide β-secretase inhibitors was performed on the immobilized Chiralpak IA chiral stationary phase. Semipreparative amounts of enantiopure forms were collected and submitted to stereochemical characterization. The absolute configuration was assigned by a multi-step methodology based on the combination of Mosher's method with circular dichroism (CD) spectroscopy. The results from the NMR/CD study fully correlated the configurational assignment obtained by a second approach involving single crystal X-ray diffraction.

1. Introduction

Interest in the field of the enantioseparation of chiral drugs has increased greatly in the last two decades. The observation that, in some cases, two enantiomers of biologically active compounds exhibit different pharmacological activities and/or toxicity makes the production of enantiopure forms a key step into the development of safer and more active drugs. In this context, direct chiral separation using chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC) has become the most practical way for both resolving racemic samples on analytical and preparative scale, and determining enantiomeric purity [1]. The advantages of enantioselective HPLC over the other tools of enantioseparation are several. The wide availability of commercial CSPs with complementary resolving power allows to rapidly develop HPLC systems capable of resolving heterogeneous classes of chiral compounds. In addition, the analytical enantioseparations usually require short analysis times and can be easily scaled-up for multi mg applications. Taking into account the importance of the three-dimensional structure in the discrimination process occurring at enzymatic and receptorial level, the determination of absolute configuration (AC) of chiral candidate drug molecules is an inevitable and complementary step to enantioseparation in the drug development process.

Single crystal X-ray diffraction is commonly viewed as the most valuable tool for the stereochemical assignment of chiral compounds. However, it has some limitations related to the highly specific equipment that needs special expertise, and to the sample which requires good-quality single crystals and the presence of heavy atoms in the structure. In recent years, other spectroscopic methods such as circular dichroism (CD), vibrational circular dichroism (VCD) and optical rotatory dispersion (ORD) [2–7] have been successfully used for establishing the AC of many classes of molecules. Additionally, classical NMR approaches such as Pirkle and Mosher's methods [8–10] continue to have a remarkable success in this area due to their simplicity and reliability. The NMR techniques are particularly attractive because they can be applied to non-crystalline compounds using equipments familiar to most research laboratories.

One way to assign AC by NMR entails derivatization of the substrate with chiral auxiliary agents to form two diastereomeric derivatives that can be differentiated by NMR spectroscopy. The sense of chemical shift nonequivalence can then be correlated to AC through configurational models. The NMR methodology has been improved by introduction of Riguera's modifications [11] to the pioneer Mosher's technique and by use of new chiral derivatizing

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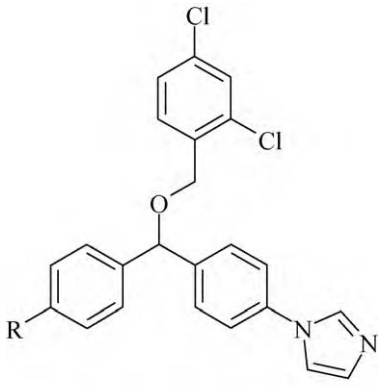

Compound	R
1	H
2	4-CH_3
3	4-Cl

Fig. 1. Structures of the chiral analytes **1**–**3**.

agents [12]. Its reliability has been demonstrated mainly on chiral alcohols, amines and carboxylic acids [11].

Whatever technique used, empirical or theoretical, to avoid false assignment it is suggested confirming the AC by a second methodology.

Here we report on the direct HPLC resolution of a small series of potential non-peptide β-secretase inhibitors (compounds **1**–**3**, Fig. 1) [13]. The β-secretase enzyme plays a critical role in the amyloid cascade and it is as an attractive therapeutic target for the treatment of Alzheimer's disease [14]. The diarylmethanol intermediate (Fig. 2) of the compounds **1**–**3** is structurally related to the key scaffold of several important drugs such as H1-antihistaminic (doxylamine, *p*-methyldiphenhydramine) and antifungal agents (miconazole, econazole) [15].

In order to increase our knowledge about the influence of chirality on the efficacy and biological interactions of this novel and interesting class of compounds, we isolated multi mg amounts of enantiomerically pure forms using the immobilized Chiralpak IA as CSP and developed a multi-step strategy to determine their AC. The direct stereochemical characterization of **1**–**3** by X-ray crystallography was not possible because they are oils at room temperature. This drawback was overcome via: (i) assignment of the AC of the alcoholic precursor of **2** (compound **2i**, Fig. 2) using the Mosher's method; (ii) assignment of the AC of the parent ether **2** by the chemical correlation method; (iii) comparison of the circular dichroism spectra of the enantiomers of **1**–**3** collected on semipreparative scale. As a further confirmation of this stereochemical assignment an independent approach was applied.

2. Experimental

2.1. Chiral HPLC

HPLC enantioseparations and diastereoseparations were performed by using the stainless-steel Chiralpak IA (250 mm × 4.6 mm I.D. and 250 × 10 mm I.D.) and Chiralpak AD (250 mm × 4.6 mm I.D. and 250 × 10 mm I.D.) (Chiral Technologies Europe, Illkirch, France) columns, respectively. All chemical solvents for HPLC, synthesis and spectral grade solvents were purchased from Aldrich (Italy) and used without further purification.

The analytical HPLC apparatus consisted of a PerkinElmer (Norwalk, CT, USA) 200 lc pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 20-μL sample loop, a HPLC Dionex CC-100 oven (Sunnyvale, CA, USA) and a Jasco (Jasco, Tokyo, Japan) Model CD 2095 Plus UV/CD detector. For semipreparative separations a PerkinElmer 200 LC pump equipped with a Rheodyne injector, a 500 μL sample loop, a PerkinElmer LC 101 oven and Waters 484 detector (Waters Corporation, Milford, MA, USA) were used. The signal was acquired and processed by Clarity software (DataApex, Prague, The Czech Republic).

2.2. Polarimetry

Specific rotations were measured at five wavelenghts (589, 578, 546, 436 and 365 nm) by a PerkinElmer polarimeter model 241 equipped with Na/Hg lamps. The volume of the cell was 1 mL and the optical path was 10 cm. The system was set at a temperature of 20 °C.

2.3. Circular dichroism

The circular dichroism spectra were measured by using a Jasco Model J-700 spectropolarimeter. The optical path and temperature were set at 0.1 mm and 25 °C, respectively. All CD spectra were recorded using a scan speed of 50 nm/min and spectral bandwidth of 1 nm. The spectra are average computed over three instrumental scans and the intensities are presented in terms of ellipticity values (mdeg).

2.4. Synthesis

Compounds **1**–**3** were sinthesized by a chemical pathway reported elsewhere [16].

2.4.1. *(R)-((S)-(4-(1H-Imidazol-1-yl)phenyl)(p-tolyl)methyl) 2-methoxy-2-phenylacetate (diastereomer **2m-1**) and (R)-((R)-(4-(1H-imidazol-1-yl)phenyl)(p-tolyl)methyl) 2-methoxy-2-phenylacetate (diastereomer **2m-2**)*

4-Dimethylaminopyridine (DMAP) (4.52 mg, 0.037 mmol) was added all at once to a mixture of (4-(1*H*-imidazol-1-yl)phenyl)(*p*-tolyl)methanol (100 mg, 0.38 mmol),

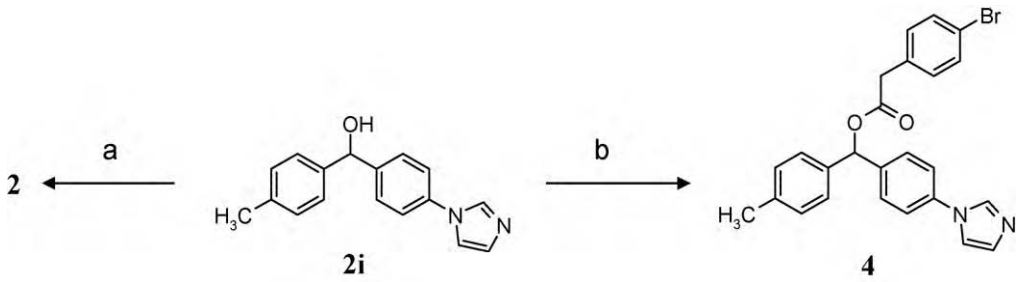

Fig. 2. Synthesis of compounds **2** and **4**. (a) 2,4-Dichloro-1-chloromethylbenzene, NaH, THF-DMSO, reflux, 30 °C. (b) 2-(4-Bromophenyl)acetic acid, DCC, DMAP, anhydrous DCM, 25 °C, overnight, Ar stream.

(*R*)-methoxyphenylacetic acid (MPA) (56.92 mg, 0.34 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (77.45 mg, 0.37 mmol) in anhydrous dichloromethane (DCM) (5 mL) under Ar stream. The reaction mixture was stirred at 25 °C overnight, cooled at 0 °C and filtered. The filtrate was evaporated and purified by column chromatography (silica gel, ethyl acetate as mobile phase) to furnish (*R*)-((*S*)-(4-(1*H*-Imidazol-1-yl)phenyl)(*p*-tolyl)methyl) 2-methoxy-2-phenylacetate (**2-m1**) and (*R*)-((*R*)-(4-(1*H*-imidazol-1-yl)phenyl)(*p*-tolyl)methyl) 2-methoxy-2-phenylacetate (**2-m2**).

2m-1. ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H), 3.43 (s, 3H), 4.90 (s, 1H), 6.89 (s, 1H), 6.94 (d, J = 8.9 Hz, 1H), 7.04 (d, J = 7.9 Hz, 1H), 7.22 (m, 1H), 7.28 (m, 1H), 7.32–7.34 (m, 2H), 7.37–7.39 (m, 7 H), 7.44–7.48 (m, 2H), 7.84 ppm (m, 1H). IR: ν 1730 cm^{-1}.

2m-2. ^{1}H NMR ($CDCl_3$): δ 2.51 (s, 3H), 3.43 (s, 3H), 4.90 (s, 1H), 6.90 (s, 1H), 7.04–7.07 (m, 2H), 7.16–7.21 (m, 8H), 7.34–7.41 (m, 3H), 7.44–7.49 (m, 2H), 7.79 ppm (m, 1H). IR: ν 1730 cm^{-1}.

2.4.2. (R,S)-(4-(1H-Imidazol-1-yl)phenyl)(p-tolyl)methyl 2-(4-bromophenyl)acetate (4)

DMAP (16 mg, 0.13 mmol) was added all at once to a mixture of (4-(1*H*-imidazol-1-yl)phenyl)(*p*-tolyl)methanol (330 mg, 1.25 mmol), 2-(4-bromophenyl)acetic acid (270 mg, 1.24 mmol) and DCC (260 mg, 1.25 mmol) in anhydrous DCM (30 mL) under Ar stream. The reaction mixture was stirred at 25 °C overnight, cooled at 0 °C and filtered. The filtrate was evaporated and purified by column chromatography (silica gel, ethyl acetate as mobile phase) to furnish (*R*,*S*)-(4-(1*H*-imidazol-1-yl)phenyl)(*p*-tolyl)methyl 2-(4-bromophenyl)acetate (**4**) as white solid (500 mg, 87%), mp 100–105 °C (from ethanol). ^{1}H NMR ($CDCl_3$): δ 2.34 (s, 3H), 3.69 (s, 2H), 6.85 (s, 1H), 7.14–7.17 (m, 6H), 7.20 (m, 1H), 7.25 (m, 1H), 7.30–7.36 (m, 4H), 7.43–7.47 (m, 2H), 7.82 ppm (m, 1H). IR: ν 1736 cm^{-1}. Melting points (mp) were determined on a SMP1 apparatus (Stuart Scientific) and are uncorrected. Infrared spectra (IR) were run on a SpectrumOne FT-ATR spectrophotometer (PerkinElmer). Band position and absorption ranges are given in cm^{-1}. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker 400 MHz FT spectrometer in the indicated solvent. Chemical shifts are expressed in δ units (ppm) from tetramethylsilane. Chromatography purifications were performed on silica gel from Macherey–Nagel (70–230 mesh). Silica gel TLC cards from Macherey–Nagel (silica gel precoated aluminum cards with fluorescent indicator visualizable at 254 nm) were used for thin layer chromatography (TLC). Developed plates were visualized by a Spectroline ENF 260C/FE UV apparatus. Organic solutions were dried over anhydrous sodium sulfate. Evaporation of the solvents was carried out on a Buchi Rotavapor R-210 equipped with a Buchi V-850 vacuum controller and Buchi V-700 (~5 mbar) and V-710 (~2 mbar) vacuum pumps.

2.5. X-ray diffraction analysis

(*R*)-(−)-**4**: $C_{25}H_{21}BrN_2O_2$, M = 461.35, orthorhombic, space group *P* 21 21 21, a = 6.147(1), b = 12.115(1), c = 28.168(1) å, V = 2097.7(4) å^3, Z = 4, D_c = 1.461, μ = 2.863 mm^{-1}, F(0 0 0) = 944.

8594 reflections were collected with a 4.81 < θ < 70.60 range with a completeness to theta 95.7%; 3528 were independent, the parameters were 271 and the final R index was 0.0349 for reflections having $I > 2\sigma I$, and 0.0479 for all data. Flack parameter is 0.028(19) for the exact configuration. Colourless prismatic shaped crystals suitable for collection were obtained and X-ray analysis was carried out with a Goniometer Oxford Diffraction KM4 Xcalibur2 at room temperature. Cu/Kα radiation (40 mA/−40 kV), monochromated by an Oxford Diffraction Enhance ULTRA assembly, and an Oxford Diffraction Excalibur PX Ultra CCD were used for cell parameters determination and data collection. The integrated intensities, measured using the ω scan mode, were corrected for Lorentz and polarization effects [17]. Direct methods of SIR2004 [18] were used in solving the structure and it was refined using the full-matrix least squares on F^2 provided by SHELXL97 [19].

Multi-scan symmetry-related measurement was used as experimental absorption correction type. The non-hydrogen atoms were refined anisotropically while the hydrogen atoms were refined as isotropic and all of them were assigned in calculated positions.

3. Results and discussion

3.1. Semipreparative enantioselective HPLC and polarimetric analysis

Among many commercially available CSPs for HPLC, amylose tris(3,5-dimethylphenylcarbamate) immobilized onto a silica support (Chiralpak IA) [20] is one of the most widely used. The main advantages of this CSP are the high chiral recognition ability towards many classes of racemates [21–30] and the full compatibility with any type of solvents [31–34]. For these reasons it was interesting for us to evaluate the performance of the Chiralpak IA CSP on the enantioselective separation of **1**–**3**. As a result of a screening of various eluents, we were able to obtain a baseline HPLC separation of the enantiomers of **1** and **2** using ethyl acetate–DEA 100–0.1 (v/v) as mobile phase. The enantioseparation of **3** was almost null in the same conditions as well as using other elution modes. Only with the mixture methyl *tert*-butylether (M*t*BE)–acetonitrile–DEA 40–60–0.1 (v/v/v) an appreciable enantioseparation was observed. In Table 1 are resumed the best chromatographic results obtained. The lower enantioselectivity noted for the compound **3** was probably due to a change in enantiorecognition process caused by the presence of the chlorine atom at the *para* position of the phenyl ring. This hypothesis is corroborated by the inversion of elution order of elution of the enantiomers of **3** with respect to **1** and **2** (the AC assignment is reported below). The chromatographic analytical methods were scaled-up with a 250 mm × 10 mm I.D. Chiralpak IA column. Single semipreparative runs of **1**–**3** afforded mg amounts of each enantiomer with ee ≥ 99% in less of 15 min (Table 2, Fig. 3).

Specific rotations of **1**–**3** measured at 589 nm and shown in Table 2 deserve a brief comment. Usually, the polarimetric data are used for confirming the enantiomeric relationship of chiral samples or determining their enantiomeric purity. More sophisticated applications entail the prediction of the absolute configuration by

Table 1
Chromatographic data for the resolution of **1**–**4**.

Compound	Mobile phase	k_1 (AC)[a]	a	Rs
1	Ethyl acetate-DEA 100-0.1 (v/v)	1.10 (*R*)	1.46	4.21
2	Ethyl acetate-DEA 100-0.1 (v/v)	1.20 (*R*)	1.44	4.39
3	MtBE-acetonitrile-DEA 40-60-0.1 (v/v/v)	2.04 (*S*)	1.12	1.72
4	Ethyl acetate-DEA 100-0.1 (v/v)	1.07 (*S*)	1.57	4.20

[a] : absolute configuration for the first eluting enantiomer.
Column, Chiralpak IA (250 mm × 4.6 mm I.D.); flow rate, 1.0 mL min^{-1}; temperature, 25 °C; detection, UV at 280 nm.

Table 2
Enantiomeric excess (ee) and specific rotation of the enantiomers of **1**–**4.**

Compound	V[a] (ml)	A[b] (mg)	F1[c]		F2[c]	
			e.e. (%)	$[\alpha]_D^{20}$	e.e. (%)	$[\alpha]_D^{20}$
1	0.2	16	>99	2.3	>99	−2.5
2	0.5	16	>99	12.7	>99	−12.0
3	0.2	2	>99	−4.1	99	3.9
4	0.3	25	>99.0	1.6	>99.0	−1.5

Column, Chiralpak IA (250 mm × 10 mm I.D.); flow rate, 3.5 mL min^{-1}; temperature, 25 °C; detection, UV at 280 nm.
[a,b]: Volume and amount of racemic sample injected in a single semipreparative run.
[c]: F1 and F2 means first and second eluting enantiomer, respectively.

theoretical specific optical rotation calculations. In the case of the compounds investigated in this work a special caution should be dedicated in the processing of polarimetric data. The optical rotation measured at 589 nm and in ethanol solution was very small particularly for the compounds **1** and **3**. For example, the enantiomers of **1** showed specific rotations of +2.3 and −2.5 which are values close to the cryptochirality condition. In order to find higher values we measured the specific rotation at five shorter wavelengths (578, 546, 436, 365 and 302 nm). As shown in Fig. 4 the ORD curve for **2** was monotonic while compounds **1** and **3** showed a change of sign and a maximum value at 302 nm. The wavelength dependence was particularly strong for the compound **3**, which showed an increase in specific rotation of about two orders of magnitude from 589 to 302 nm.

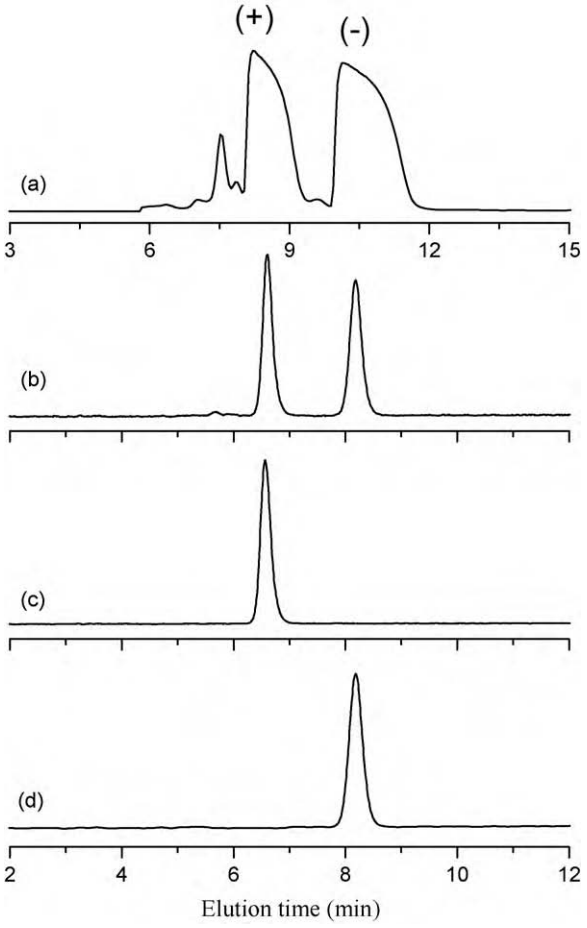

Fig. 3. Trace (a): semipreparative separation of 16 mg of **2**. Column: Chiralpak IA 250 mm × 10 mm I.D.; eluent: ethyl acetate–DEA 100:0.1 (v/v); flow rate: 3.5 mL min^{-1}; temperature: 25 °C; detection: UV at 280 nm. Trace (b): analytical separation of **2**. Traces (c) and (d): purity control of the single fractions collected at semipreparative scale. Column: Chiralpak IA (250 mm × 4.6 mm I.D.); eluent: ethyl acetate–DEA 100:0.1 (v/v); flow rate: 1.0 mL min^{-1}; temperature: 25 °C; detection: UV at 280 nm.

3.2. Determination of the absolute configuration

The presence of a secondary alcohol as intermediate in the synthesis of **1**–**3** (Fig. 2) prompted us to develop a multi-step strategy for the AC assignment based on ^{1}H NMR anisotropy method.

A stepwise summary of the Mosher's procedure employed to assign the AC of the selected alcohol **2i** is presented in Fig. 5. Racemic **2i** was esterified with (*R*)-MPA to give a diastereomeric mixture of MPA esters (**2m**). The diastereomeric pair was separated by HPLC on the Chiralpak AD CSP, which consists of amylose tris(3,5-dimethylphenylcarbamate) the same selector as in the IA CSP but coated onto silica gel particles. The eluent was the mixture *n*-hexane-2-propanol 50:50 (v/v). In these conditions, separation and resolution factor values of 1.83 and 8.09, respectively, were

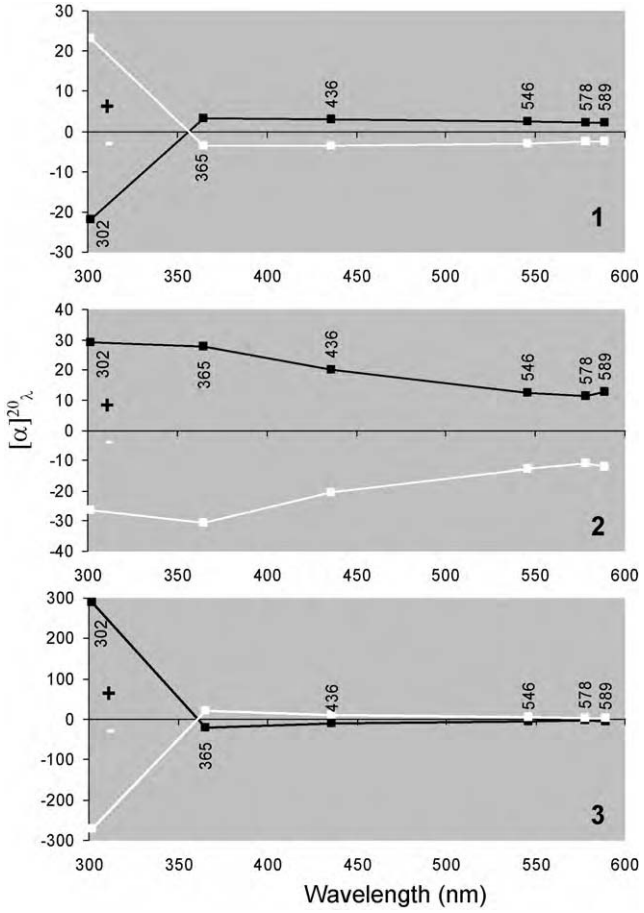

Fig. 4. ORD curves of the first eluted enantiomer (black) and second-eluted enantiomer (white) of the compounds **1**–**3** in ethanol.

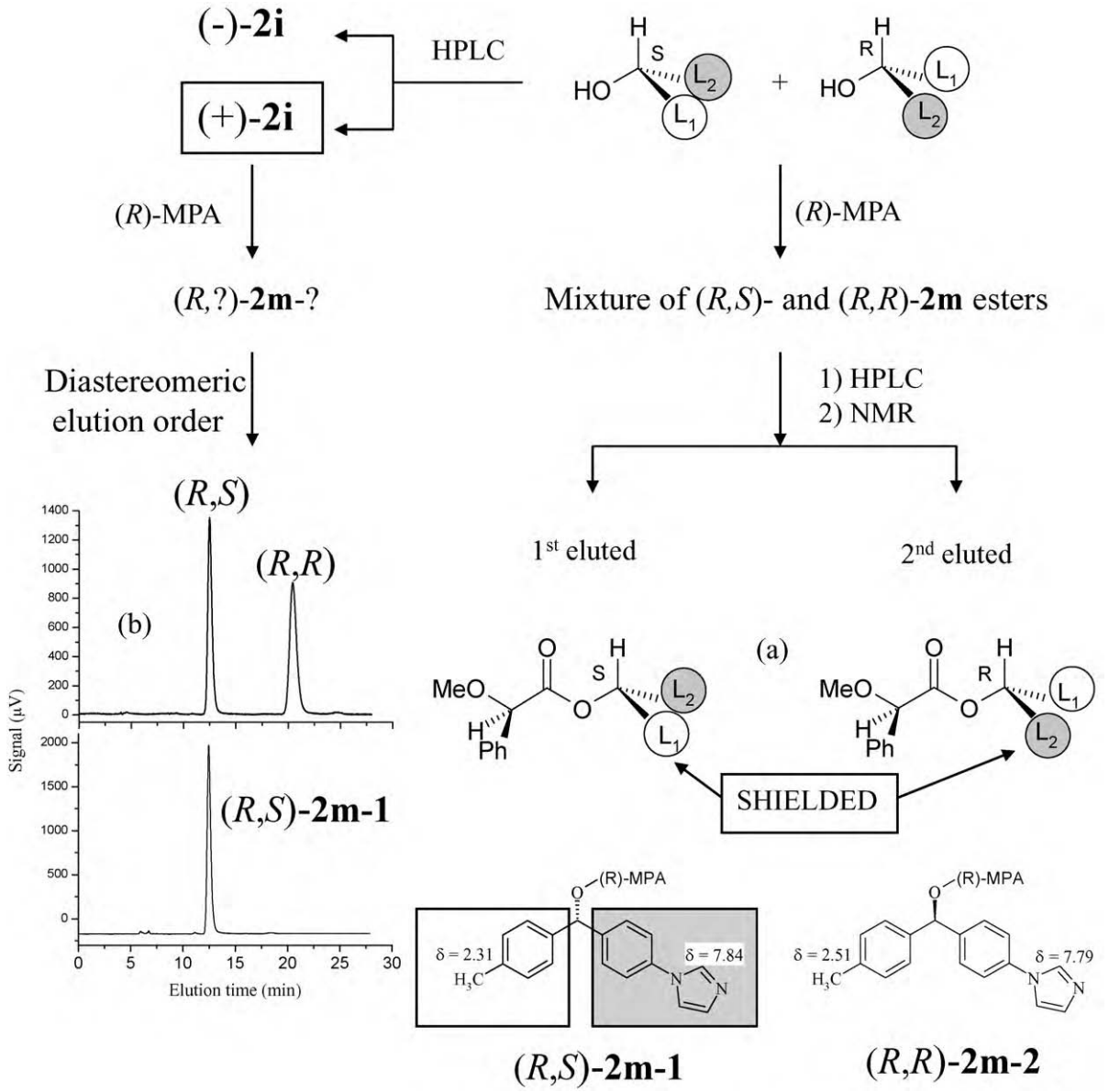

Fig. 5. General strategy for the assignment of the absolute configuration of **2i**. (a) sp conformation and selected ^{1}H NMR chemical shifts for **2m-1** and **2m-2** MPA esters; (b) a comparison between the HPLC chromatograms of the diastereomeric mixture (*R*,*S*)+(*R*,*R*) and the (*R*,?)-**2m**-? ester coming from the enantiomer (+)-**2i**. Column: Chiralpak AD (250 × 4.6 mm I.D.); eluent: *n*-hexane-2-propanol 50:50 (v/v); flow rate: 1.0 mL min^{-1}; temperature: 25 °C; detector: UV at 254 nm.

obtained. The high diastereoselectivity of amylose-based AD CSP combined with the good sample solubility in the mobile phase permitted a productive scaling-up at semipreparative level. By using a 1-cm I.D. AD column, an amount of 30 mg of sample was injected and the two esters were isolated with a diastereomeric excess >99% and yield >90%. Polarimetric analysis indicated that both diastereomers were levorotatory in ethanol solution {first eluted diastereomer, **2m-1**: $[\alpha]_D^{20}$-13 (*c* 0.3, ethanol), de>99%; second-eluted diastereomer, **2m-2**: $[\alpha]_D^{20}$-31 (*c* 0.3, ethanol), de>99%}.

Afterwards, the ^{1}H NMR spectra of **2m-1** and **2m-2** MPA esters were recorded and the signals of L_1 (the *p*-tolyl group which is linked to the stereogenic center of unknown configuration) and L_2 (the portion containing the imidazolyl group) were assigned.

It is well known that, in the most cases, an energetically favorite synperiplanar (sp) arrangement of the Mosher ester is expected with the MPA methoxy and carbonyl oxygen arranged on the same plane [11] (Fig. 5a).

On that basis, negative values of $\Delta\delta^{1,2}$ (which is defined as the difference in the chemical shift of the signals in the first eluting ester and the chemical shift of the same signal in the second eluting ester) imply a close arrangement between a fragment of the parent chiral alcohol and the shielding MPA phenyl ring and this in turn is related, *via* the sp model, to the AC of the parent alcohol. Positive values of $\Delta\delta^{1,2}$ are indicative of a remote relative disposition between fragments of the alcohol portion and the MPA phenyl ring.

In the first eluting ester, we observed negative $\Delta\delta^{1,2}$ values for the hydrogens of the substituent L_1 indicating their location on the same half-space of the shielding phenyl ring of the (*R*)-MPA auxiliary moiety. Positive $\Delta\delta^{1,2}$ values were observed for the hydrogens of substituent L_2, indicating their location on the other half-space where the shielding effect of the (*R*)-MPA phenyl ring is negligible (Fig. 5a).

The comparison of the ^{1}H NMR spectra of **2m-1** and **2m-2** clearly indicated that the protons of the methyl group linked at the 4-position of the phenyl ring of **2m-1** were largely shifted up-field (δ = 2.31 ppm, $\Delta\delta^{1,2}$ = −0.20) and the H2 (δ = 7.84 ppm, $\Delta\delta^{1,2}$ = +0.05 ppm) proton of imidazole group shifted down-field.

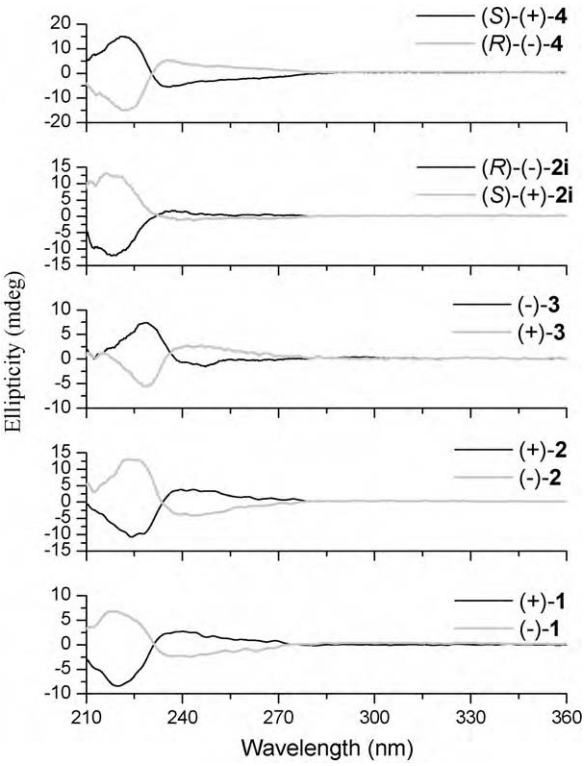

Fig. 6. CD spectra of the first eluted enantiomer (black) and second-eluted enantiomer (grey) on the IA CSP of the compounds **1**–**4**, and **2i** in ethanol at 25 °C.

These findings, applied to the sp models of the MPA esters, indicated that the first eluted diastereomer **2m**-**1** had the (*R*,*S*) AC, and, consequently, the second-eluted diastereomer **2m**-**2** the (*R*,*R*) AC.

Once determined the AC of the two MPA esters, we synthesized the (*R*,?)-MPA ester from the (+)-enantiomer of **2i** of unknown configuration. A comparison of the chromatograms of (*R*,?)-**2m**-? MPA ester and the mixture (*R*,*S*) + (*R*,*R*) (Fig 5b) clearly indicated the diastereomeric elution order: the (*R*,*S*)-**2m**-**1** ester coming from the alcohol (+)-**2i** was eluted before than (*R*,*R*)-**2m**-**2**. Therefore, to the second-eluted enantiomer on the AD CSP (+)-**2i** the (*S*) configuration was assigned.

In order to extend the stereochemical assignment to the ethers **1**–**3**, the enantiopure forms of **2i** of known stereochemistry were converted to the corresponding ether **2** using the same procedure adopted for the racemic sample [16]. The stereochemical course of reaction was monitored by enantioselective HPLC on the Chiralpak IA CSP using the chromatographic conditions described in Table 1. The esterification of the enantiomers of the key intermediate **2i** occurred in stereoconservative way as demonstrated by the high ee values (>99%) observed. The AC assignment of **2** by chemical correlation led to the following results: the (*R*)-configuration was attributed to the first eluted enantiomer on the IA CSP) [(*R*)-(+)-**2**] and the (*S*)-configuration to the second-eluted enantiomer [(*S*)-(−)-**2**].

After establishing the AC of the enantiomers of **2i** and **2**, we were able to assign the AC of the other two ethers **1** and **3** by CD technique. The CD spectra of **1**–**3** and **2i** are showed in Fig. 6. As expected, the enantiomeric forms exhibited specular pattern. By comparing the CD spectra of the compounds **1** and **3** to those of the references alcohol **2i** and ester **2** it appears that the nature of the substituent at the *para* position of the phenyl ring or at alcoholic oxygen did not substantially influence the CD behaviour. Only compound **3** showed a slight red-shift of the representative CD bands due to the effect of the chlorine atom. The results obtained by NMR and CD measurements allowed us to define a believable

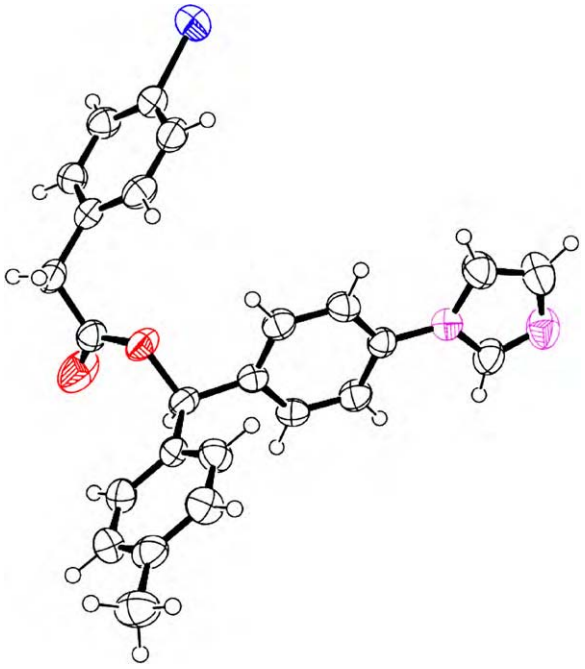

Fig. 7. An ORTEP view of the molecular structure of (*R*)-(−)-**4**.

parallelism between AC and CD band position: to the enantiomers showing an ellipticity minimum located around 240 nm and a maximum near 220 nm may be assigned the (*S*)-configuration, and, naturally, a reversed sign of the ellitipticity is expected from the (*R*)-enantiomers.

We wanted to confirm the stereochemical results based on the ^{1}H NMR anisotropy method using an alternative procedure. As already mentioned, single-crystal X-ray crystallography can not be used for the direct stereochemical assignment of ethers **1**–**3** because they are liquids at room temperature. Hence, starting from the alcohol **2i**, we synthesized the ester **4** bearing a heavy atom such as bromine essential for three-dimensional structure studies by crystallographic technique. After the synthetic step, a semipreparative enantiomeric separation of racemic ester **4** was accomplished on the Chiralpak IA CSP. Once again, the CSP based on amylose tris(3,5-dimethylphenylcarbamate) revealed high enantioselectivity and efficiency permitting a multi mg and fast resolution of the racemic sample (Table 2).

The second-eluted ester (−)-**4** was crystallized from ethanol/acetone. A single crystal was subjected to X-ray analysis affording the ORTEP drawing as shown in Fig. 7, from which the AC was clearly determined as (*R*). Consequently, to the dextrorotatory enantiomer of **4** was assigned the (*S*) configuration [(*S*)-(+)-**4**].

Subsequent comparison of the CD spectra of the enantiomers of **4** with those of the reference compounds **2** and **2i** (Fig. 6) fully confirmed the assignment obtained by NMR analysis. As reported in Table 1 the (*S*)-enantiomer of the ester **4** was eluted prior to the (*R*)-enantiomer, an enantiomer elution order reversed with respect to the compounds **1** and **2**. Inversion of the elution order was noted also for the enantiomers of **3**. These results confirm the risk of stereochemical predictions of structurally related compounds only based on the enantiomer elution order on polysaccharide CSPs and the difficulty to establish the mechanism of the recognition process occurring on these type of selectors [35].

4. Conclusions

Enantioselective HPLC proved here again an invaluable tool for supporting the AC determination of new biologically active

molecules. The easy access to mg amounts of enantiopure forms by HPLC on Chirapak IA CSP allowed us to develop an easy strategy to determine the AC of three ethers **1**–**3** endowed with potential β-secretase inhibitor activity. The stereochemical characterization was carried out via AC assignment of the alcohol intermediate **2i**. The elucidation of AC consisted of a straightforward protocol, the first step of which was the resolution on semipreparative scale of the chiral diarylmethanol intermediate **2i** combined to the NMR analysis of the corresponding Mosher esters. In the next step, the AC of the ether **2** was assigned by the chemical correlation method. As a final step, the AC of the two ethers **1** and **3** was indirectly assigned by CD analysis. These results suggest a clear agreement between the AC and the sign of the CD bands and can serve as a reference for studies on stereochemistry of similar scaffolds.

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