ANALYSIS OF ENANTIOMERIC EXCESS AND DETERMINATION OF ABSOLUTE CONFIGURATION OF CHIRAL COMPOUNDS AT ENAMINE

1. Introduction

In the last decade, development of new drugs increasingly requires the use of chiral compounds as single stereoisomers.[1] The main fundamental reason for this lies in the fact that almost all the biological targets are chiral, and the drug-receptor interaction requires strict match of chirality. The formal reason results from strengthening the regulatory guidance for submitting new drug applications in Europe and USA which concern chirality issues. The priority is given to chiral drugs as pure stereoisomers, and pharmacological properties of all the stereoisomeric forms should be studied thoroughly where possible.[2] In turn, this tendency caused increased attention to chiral analytical techniques, in particular, analysis of optical purity of enantiomers. In this article, we outline the general strategy of the optical purity analysis (determination of enantiomeric excess, *ee*) of chiral building blocks and screening compounds at Enamine company. Approaches used for establishment the absolute configuration will also be briefly discussed.

2. Analysis of enantiomeric excess – a general strategy

In rare cases when the analyzed compounds are properly described in the literature and their chiroptical properties were reported, determination of their *ee* at Enamine follows the literature procedures. The specific rotation α_D is used most often; however, knowing the low reliability of the specific rotation measurements, we usually confirm the results by chromatographic or other techniques described below.

In all cases the analysis commences from finding the optimal conditions for the reliable distinction and quantitative detection of enantiomers using the corresponding racemates. The racemates are prepared either by independent synthetic routes, or by mixing *R*- and *S*-enantiomers, if available. The workflow of finding the optimal conditions is shown in the scheme 1.

First, the racemates are carefully checked for chemical purity by NMR spectroscopy and LC-MS/GC-MS. Only the samples having > 95% of chemical purity are then subjected for further analysis. Liquid chromatography on chiral stationary phases is then applied. The chromatography conditions (stationary phase, eluent, temperature, flow rate etc.) are usually varied to achieve, ideally, the base-line separation of the chromatography peaks corresponding to each enantiomer. Enamine has a large database of previously performed analyses which helps our experienced analytical chemists to find the optimal conditions quickly. In the cases where the selectivity of the method is not sufficient and the peaks are substantially overlapped (less than 5% of the samples analyzed currently) other techniques are applied. ^{1}H-NMR in the presence of chiral lanthanide shift reagents (LSR) or other

additives, derivatization by chiral derivatizing agents followed by NMR or HPLC are the methods of choice; they allow for the *ee* determination in virtually all the cases.

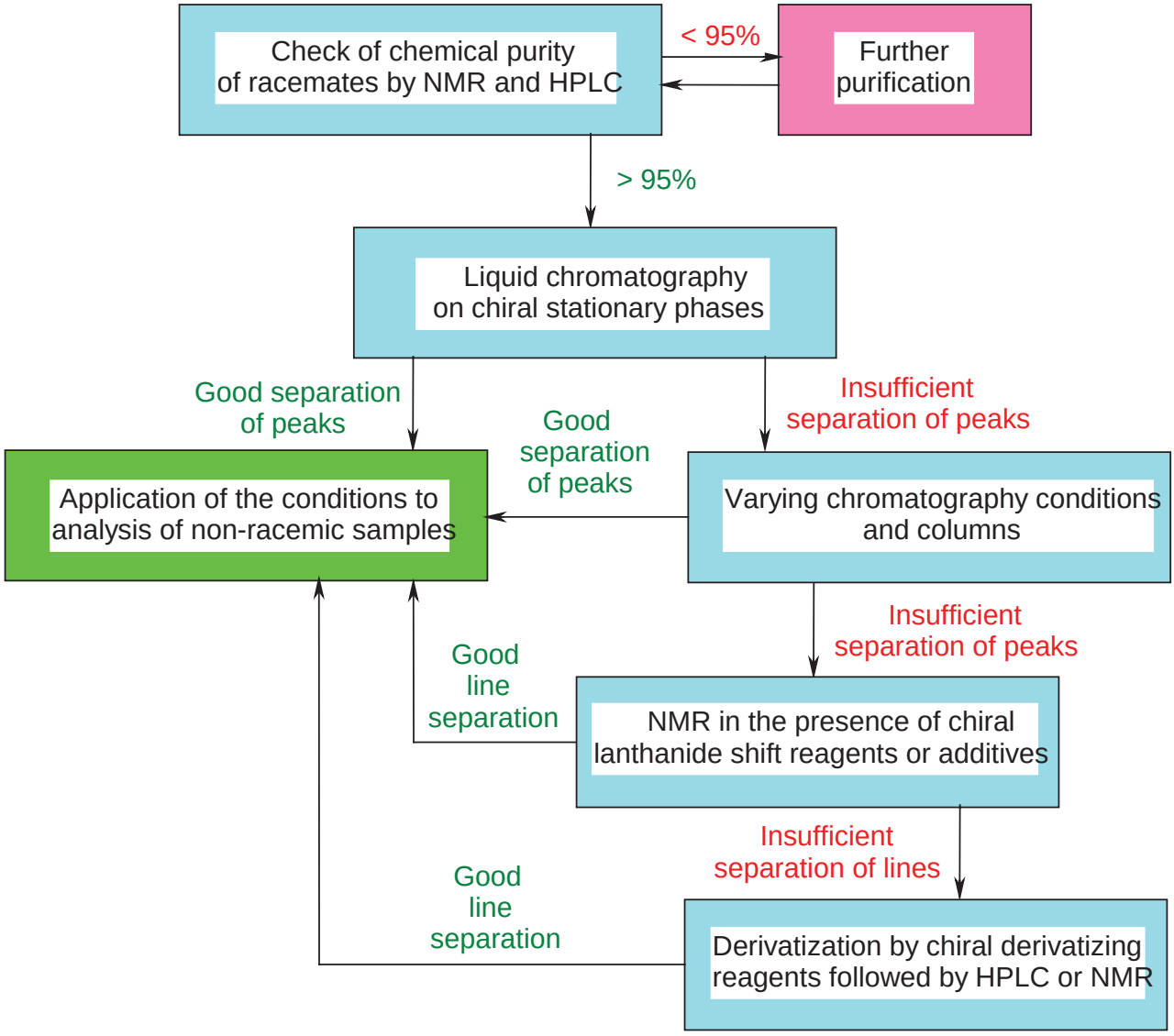


Figure 1. Finding the optimal conditions for the *ee* determination of a non-racemic compound using corresponding racemates.

In the following sections we outline the techniques for the *ee* determination in more details and illustrate them by several examples.

3. Liquid chromatography on chiral stationary phases

Chromatographic analysis of enantiomers is successful if the retention times of both isomers are significantly different, leading to separation of the chromatography peaks to the baseline (good selectivity). The choice of the chiral stationary phase is most critical for the success. Different chiral HPLC columns are available commercially; their offer is very dynamic as a

result of intensive scientific research in this area. The columns currently used at Enamine are listed in Table 1, together with their important functional characteristics. The columns are connected to the serial Agilent 1100 chromatographic system, equipped with automatic sampler and diode array detector. The chromatograph will be updated soon with a polarimetric detector for the simultaneous measurements of the optical rotation.

Table 1. Chiral HPLC columns used at Enamine.

Trade name/ company	Stationary phase	Column dimension	Particle size
CHIRALCEL ® OJ-H Daicel Chemical Industries Ltd.	Cellulose tris(4-methylbenzoate) on silica support	250x4,6 mm	5 μm
CHIRALPAK ® IB Daicel Chemical Industries Ltd.	Cellulose tris(3,5-dimethylphenyl carbamate) on silica support	250x4,6 mm	5 μm
CHIRALPAK ® IA Daicel Chemical Industries Ltd.	Amylose tris(3,5-dimethylphenyl carbamate) on silica support	250x4,6 mm	5 μm
CHIROBIOTIC ™ V2 SUPELCO ®	Vancomycin on silica support	250x4,6 mm	5 μm
CHIROBIOTIC ™ R SUPELCO ®	Ristocetin A on silica support	250x4,6 mm	5 μm
CHIROBIOTIC ™ TAG SUPELCO ®	Teicoplanin aglycone on silica support	250x4,6 mm	5 μm
CHIROBIOTIC ™ T SUPELCO ®	Teicoplanin on silica support	250x4,6 mm	5 μm
ChiraDex ® Merck	Silica particles with covalently bonded beta-cyclodextrin	250x4 mm	5 μm

The columns listed in the Table 1 allow, in our experience, successful analysis of more than 95% chiral non-racemic compounds produced at Enamine. Chiral alcohols, amines, aminoalcohols, alpha- and beta- amino acids and their derivatives (including N-Boc- and N-Fmoc-protected), heterocyclic compounds, sulfur-containing compounds (sulfoxides) were analyzed with excellent or satisfactory selectivity, using normal or reverse phase regime. Finding the appropriate column and optimal conditions for achieving good selectivity is by no means a trivial process, however. When the conditions are found, they are applied to the analysis of the non-racemic samples. Comparison with the racemate and integration gives the data for the *ee* calculation (which is, by a simple definition, 100% minus percentage of the racemate). This is illustrated by the HPLC traces obtained for 8-fluoro-chroman-4-ol (Figures 2,3), racemate and a sample with >99% *ee*.

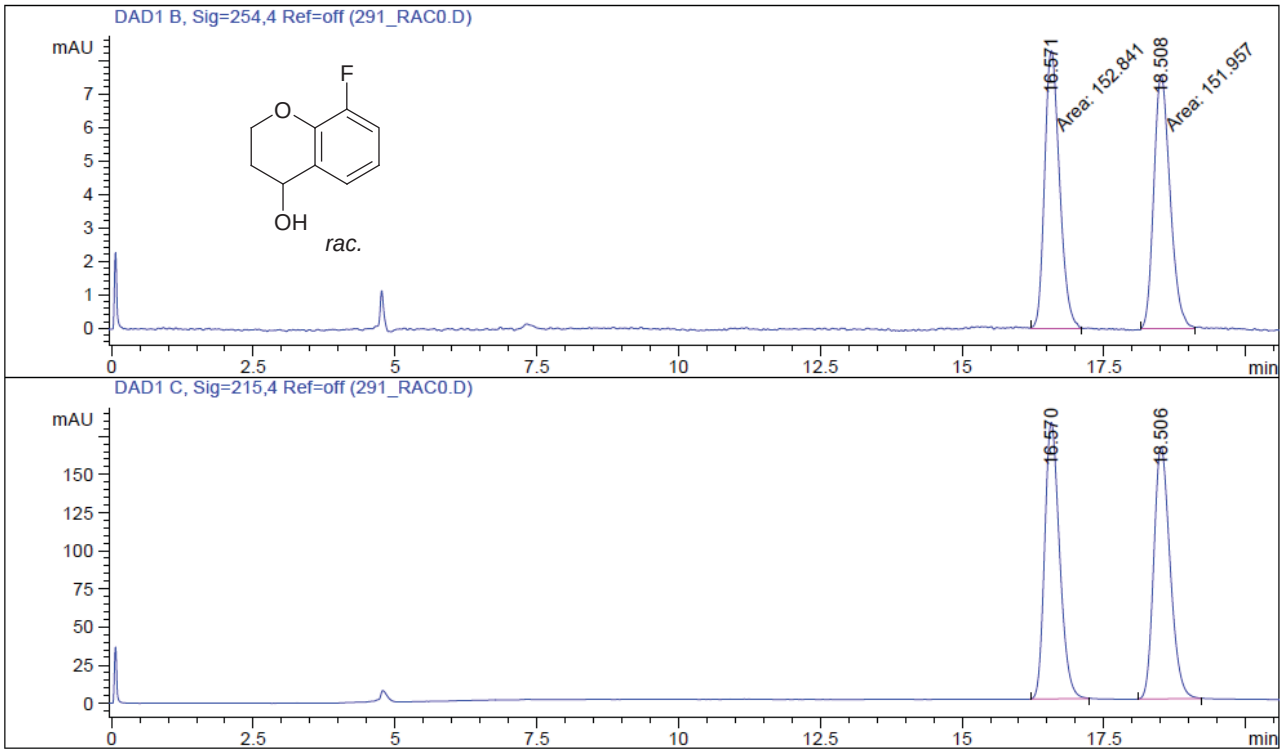


Figure 2. HPLC traces obtained for a racemic mixture of 8-fluoro-chroman-4-ol (CHIRALPAK ® IB column, hexane-isopropanol (95:5) mixture as an eluent, flow rate 0.7 mL/min, 23 °C)

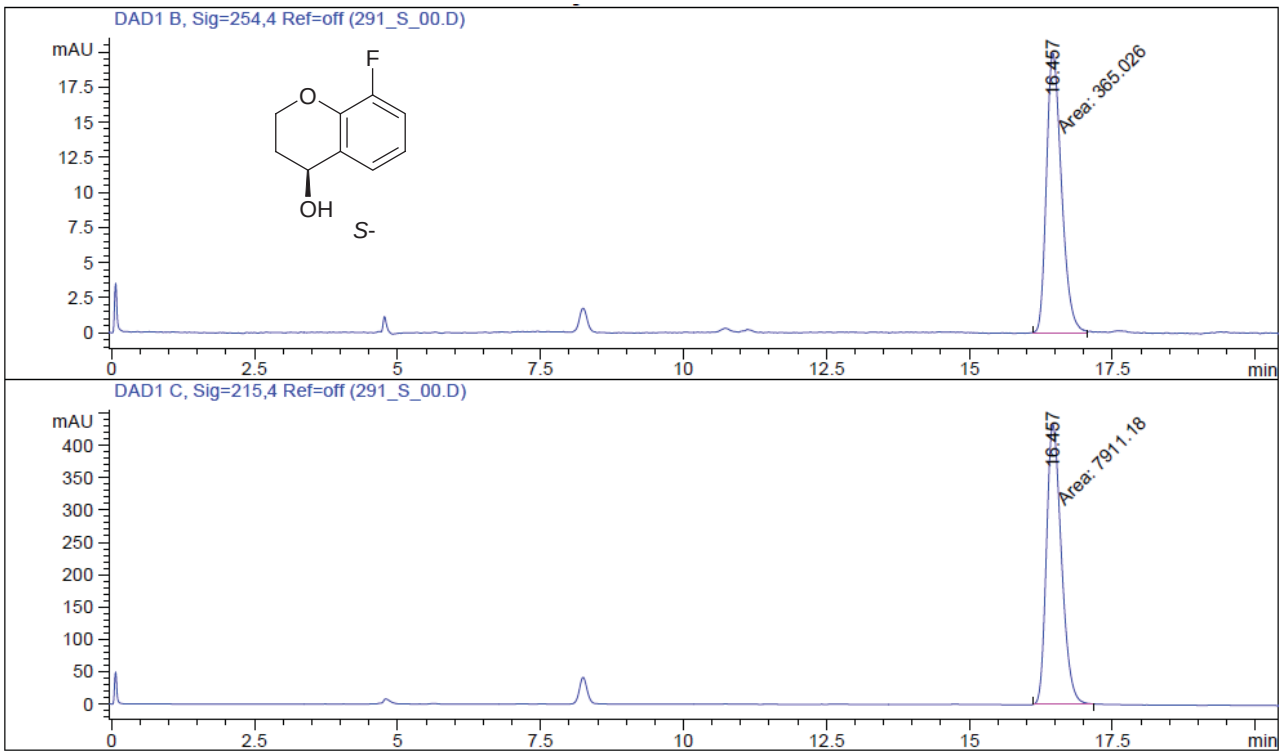


Figure 3. HPLC traces obtained for (4-*S*)-8-fluoro-chroman-4-ol (CHIRALPAK ® IB column, hexane-isopropanol (95:5) mixture as an eluent, flow rate 0.7 mL/min, 23 °C)

In some cases, however, sufficient peak separation for both enantiomers could not be achieved, despite extensive experimentation. For example, 1-benzofuran-2-yl-ethanol racemate analysis revealed overlapping chromatography peaks (like shown in the Figure 4), on all our columns tested under broad range of conditions. Alternative techniques are used in such cases, and first of all, ^{1}H-NMR in the presence of chiral lanthanide shift reagents or other chiral additives.[3]

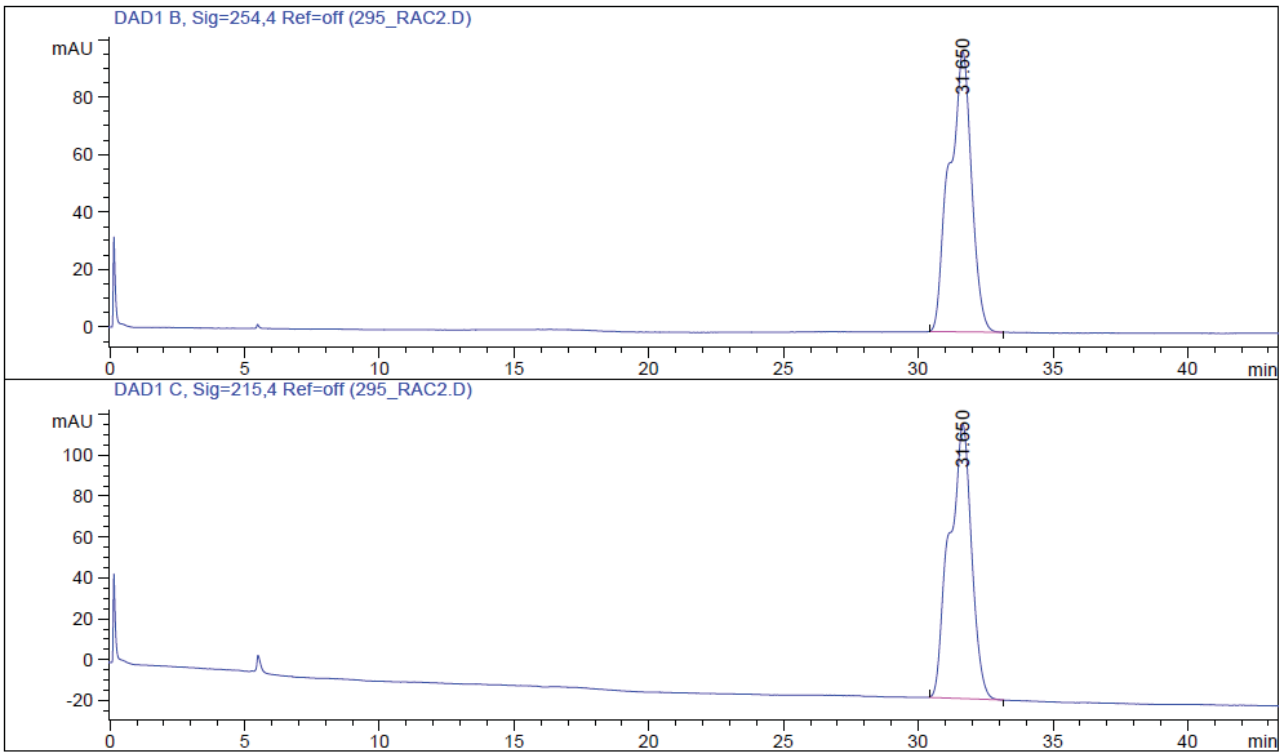
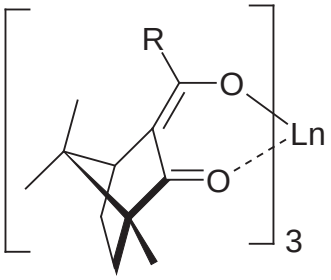


Figure 4. HPLC traces obtained for 1-benzofuran-2-yl-ethanol (CHIRALCEL ® OJ-H column, hexane-isopropanol (93:7) mixture as an eluent, flow rate 0.6 mL/min, 20 °C)

4. Determination of ee by NMR in the presence of chiral Lanthanide Shift Reagents (LSR) or other chiral additives

The commonly used chiral LSR are neutral complexes of general formula Ln(β-dik*)$_3$, where Ln is a trivalent paramagnetic lanthanide ion (any except of La, Ce, Gd and Lu; Eu and Pr are most often used), β-dik* is a chelating chiral beta-diketonate ligand.[4,5] The complexes with camphor-derived ligands Eu(TFC)$_3$ and Eu(HFBC)$_3$ which are used at Enamine are shown in Figure 5. The metal ions in the complexes are coordinatively unsaturated; they may form 1:1 and 1:2 adducts with different organic compounds (substrates) possessing hard Lewis-base groups (hydroxy-, amino-, carbonyl groups, different nitrogen-containing aromatic heterocycles etc.) in low-polar solvents (dichloromethane, chloroform, benzene). The paramagnetic lanthanide ions in the adducts cause significant (1-100 ppm) shifts of the signals in the NMR spectra of the substrates. LSR and adducts are in fast (in the NMR time scale) equilibrium in solutions, therefore, addition of small portions of an LSR to a substrate

solution results in incremental induced shifts of the substrate signals, the position of which is averaged by the fast LSR-adduct equilibrium. At first approximation, the induced shifts linearly depend on the amount of the reagent if the molar ratio of the LSR to the substrate is small (less than approximately 0.1).



Ln = Eu, Pr;
R = CF_3 ($Ln(TFC)_3$);
R = CF_2CF_3 ($Ln(HFBC)_3$);

Figure 5. Most commonly used chiral lanthanide shift reagents

Like in the case of the *ee* determination by HPLC, analysis of the non-racemic sample is preceded by finding the optimal LSR concentration using the corresponding racemates. Chiral LSR form diastereomeric adducts with both enantiomers of racemic chiral substrates. The diastereomeric adducts may have different induced shifts, therefore, at some LSR/substrate ratio one might observe separation of the NMR peaks from the same groups in both enantiomers of the racemate. Usually, ^{1}H-NMR spectroscopy is used for the measurements; in this case, europium reagents shift most of the signals downfield, praseodymium – upfield. In the usual run, the racemate is dissolved in a dry solvent ($CDCl_3$ or CD_2Cl_2, 15-20 mg in 0.5 cm^3), then small portions (1-3 mg) of $Eu(TFC)_3$ or $Eu(HFBC)_3$ are added, recording the ^{1}H-NMR spectra after each addition. The spectra reveal the induced shifts of the NMR signals; they are clearly visible in a stacking plot. Figure 6 (lower four traces) shows the results of the measurements for 1-benzofuran-2-yl-ethanol, for which the chromatographic analysis of the ee failed in our hands (see the preceding section). In favorable cases like shown in the Figure 6, such “LSR-titration” leads to duplicated signals from some groups of protons in the racemate at certain molar LSR/substrate ratio (CH(OH) proton signal in Figure 6c,d). They correspond to the diastereomeric adducts formed by two enantiomers with the LSR. In the next step, spectra of the non-racemic samples are measured in the presence of LSR at the concentration which led to clear separation of the signals in the racemate (see upper two traces in Figure 6). Integration of the signals allows sufficiently accurate determination of the *ee*, provided no overlap with other NMR peaks occurred. In our experience, good chances for successful analysis have secondary alcohols, primary, secondary and tertiary amines, and α-amino acid methyl esters. In the latter case, the ^{1}H-NMR signal of the OMe group is indicative, as shown in Figure 7.

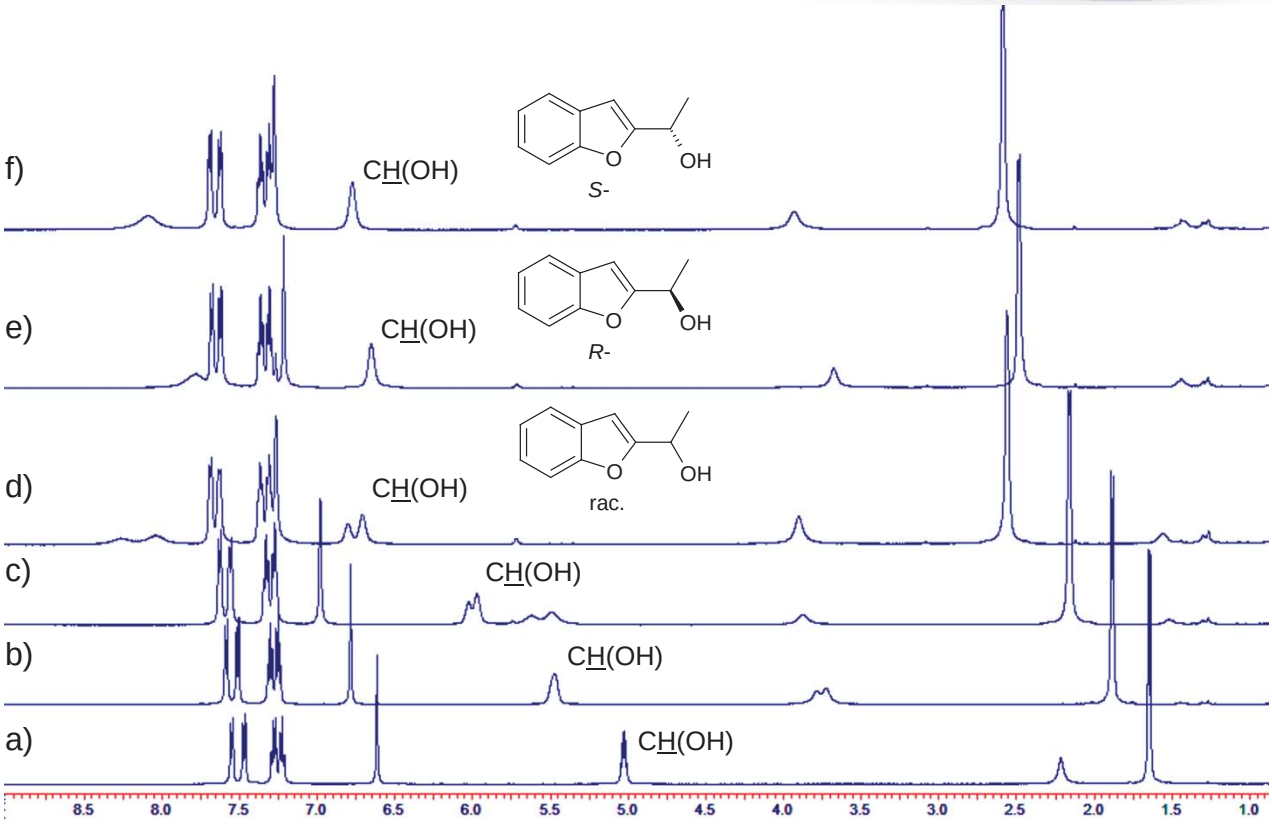


Figure 6. "LSR titration" of the racemate (a-d) and analysis of the ee of the non-racemic samples (e, f) of 1-benzofuran-2-yl-ethanol. a) ^{1}H-NMR spectrum of racemic 1-benzofuran-2-yl-ethanol (15.5 mg in 0.5 mL of $CDCl_3$); b-d) ^{1}H-NMR spectra of the racemate solution in the presence of chiral LSR Eu(HFBC)$_3$ (2.6, 2.8, 3.9 mg correspondingly); e) ^{1}H-NMR spectrum of (1*R*)-1-benzofuran-2-yl-ethanol (16.2 mg in 0.5 mL of $CDCl_3$) in the presence of Eu(HFBC)$_3$ (9.5 mg); f) ^{1}H-NMR spectrum of (1*S*)-1-benzofuran-2-yl-ethanol (15.1 mg in 0.5 mL of $CDCl_3$) in the presence of Eu(HFBC)$_3$ (8.9 mg).

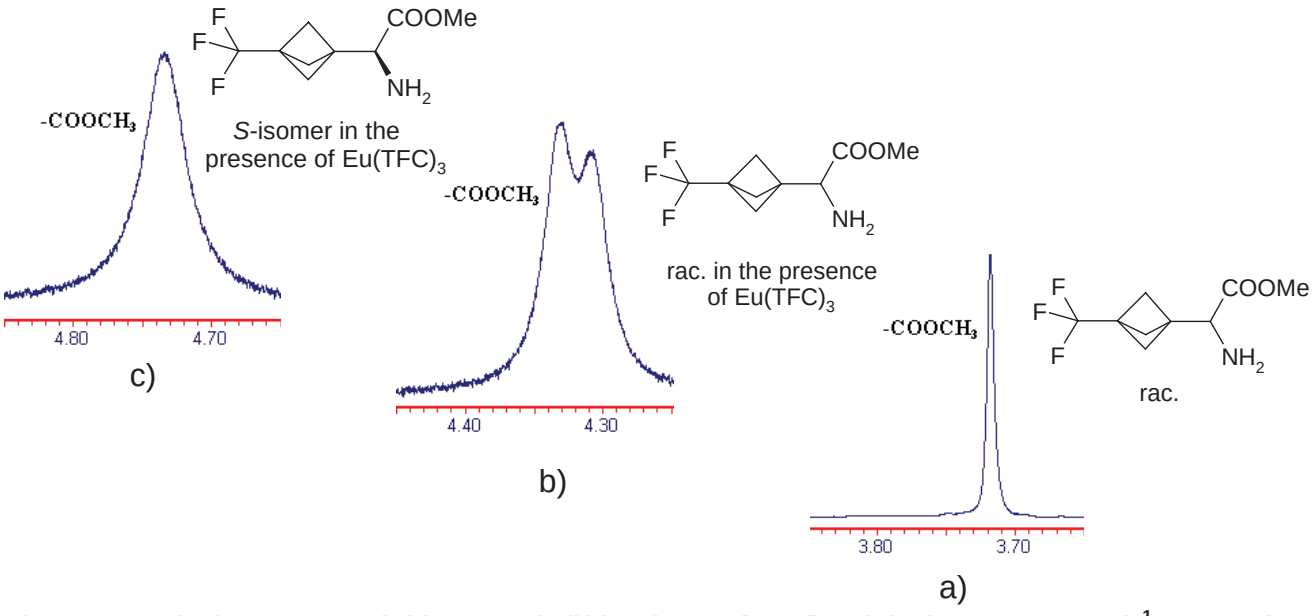


Figure 7. Analysis of ee of 3-(trifluoromethyl)bicyclopent-[1.1.1]-1-ylglycine, CF3-Bpg. a) ^{1}H-NMR signal of the OMe group of the racemate; b) ^{1}H-NMR spectrum fragment of the racemate solution in the presence of chiral LSR; c) ^{1}H-NMR spectrum fragment of the *S*-isomer in the presence of chiral LSR.

Apart from LSR, other chiral additives may be used, which form stable adducts ("solvates") with the analyzed compounds and cause induced shifts of the NMR signals. Many "solvating" agents suitable for *ee* determination were reported in the literature,[3] some of them are shown in Figure 8.

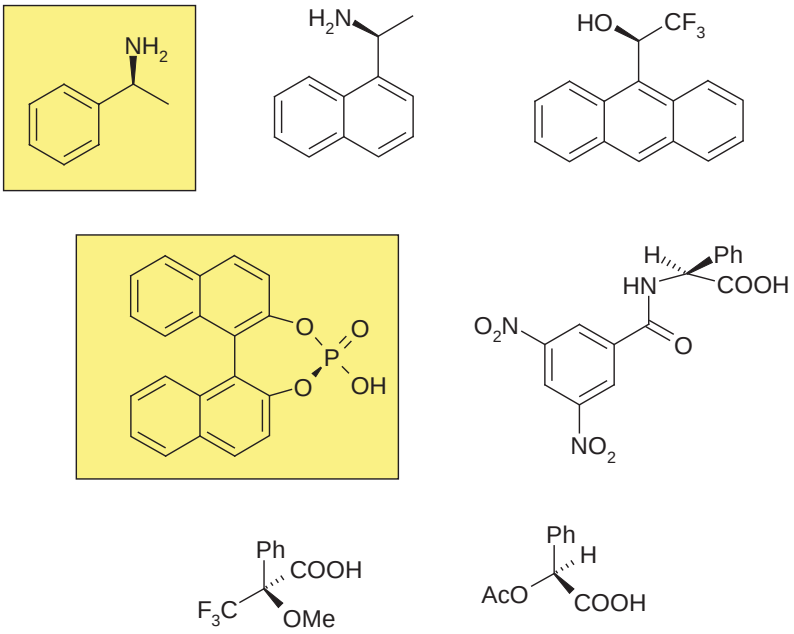


Figure 8. Common solvating agents used for chiral analysis by NMR (one enantiomer is shown in each case, the use of the other is also possible).

Like in the case of the LSR use described above, enantiomers might have different NMR spectra in the presence of an equivalent or more of the additives. Deuteriochloroform or benzene-D_6 are used as the solvents. The enantiomeric composition may be determined from the corresponding peak areas, provided the shift difference is large enough. Two types of additives are most often used at Enamine, as they are commercially available and relatively cheap: ((1*R*-) or (1*S*)-1-phenylethylamine and both enantiomers of 4-oxo-3,5-dioxa-4lambda*5*-phospha-cyclohepta[2,1-a;3,4-a'] dinaphthalen-4-ol (high-lighted in yellow in Figure 8). The former are used to analyze chiral carboxylic acids or N-protected amino acids; the latter are applicable as the additive in analyses of chiral amines. Many other functional groups in the substrates are tolerated.

5. Derivatization by chiral derivatizing agents followed by HPLC or NMR analysis

Chiral derivatizing reagents convert the analyzed compounds into diastereomeric derivatives. They give nonequivalent NMR spectra or can be separated and analyzed by HPLC on non-chiral stationary phases.[6,7] Derivatization is an additional laboratory step; a care must be taken to ensure that there will be no diastereomeric enrichment during the synthesis of the derivatives (for example, the use of crystallization or chromatography for the isolation of the derivatives should be avoided). There is also a risk of racemization for some substrates. Derivatization of the corresponding racemates prior to the analysis of the non-racemic

samples is obligatory, as in all the cases described above. NMR spectra or the HPLC analysis of the derivatized racemates show if the analysis is sufficiently selective, and the diastereomers formed by both enantiomers give clearly different measurable characteristics.

A number of chiral derivatizing reagents is known, they were developed for analysis of specific organic compound classes. Most of them are available commercially, and can be used at Enamine. One of the most universal reagents is the so-called Mosher's acid chloride (Figure 9), which is used here to analyze chiral primary and secondary amines and alcohols which can be easily converted to the corresponding amides and esters with the Mosher's reagent.

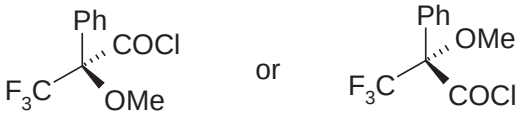
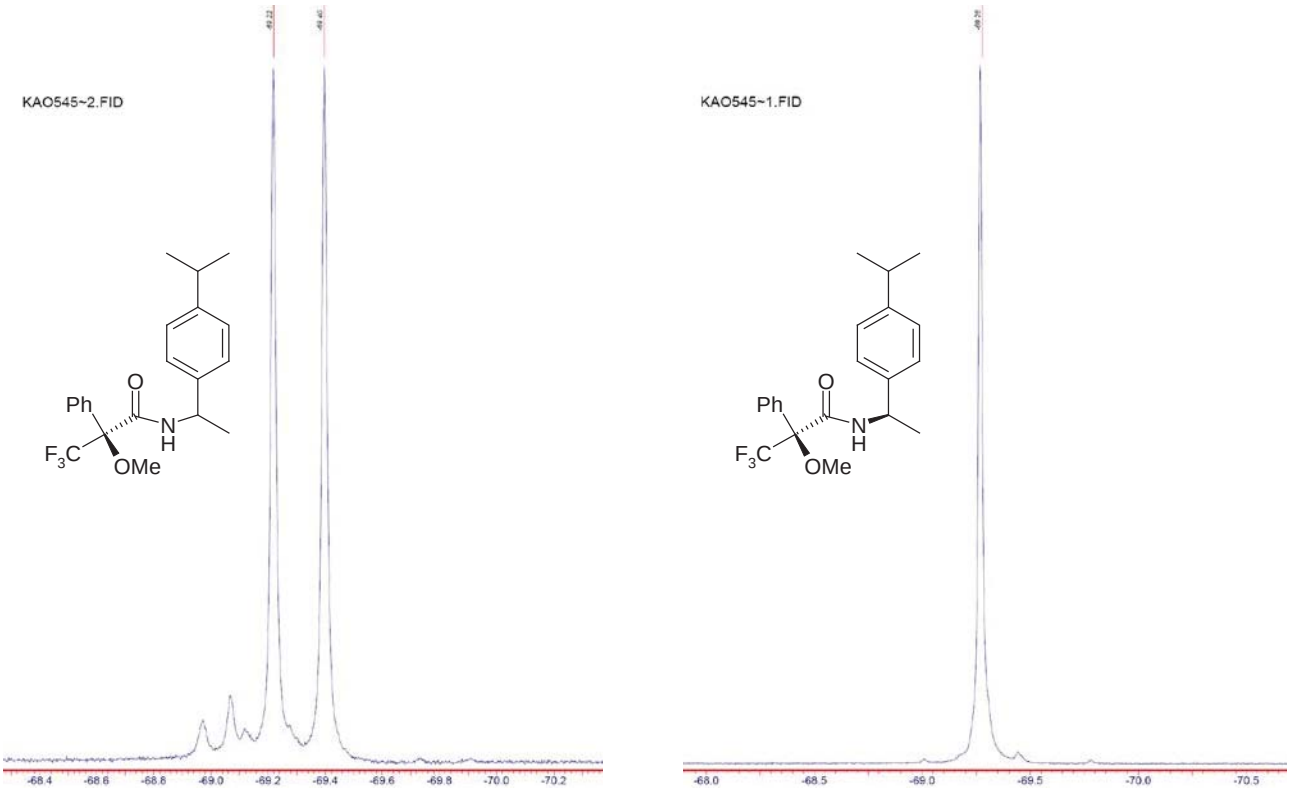


Figure 9. Mosher's reagents ((*S*-) and (*R*)-3,3,3-trifluoro-2-methoxy-2-phenylpropionyl chlorides)

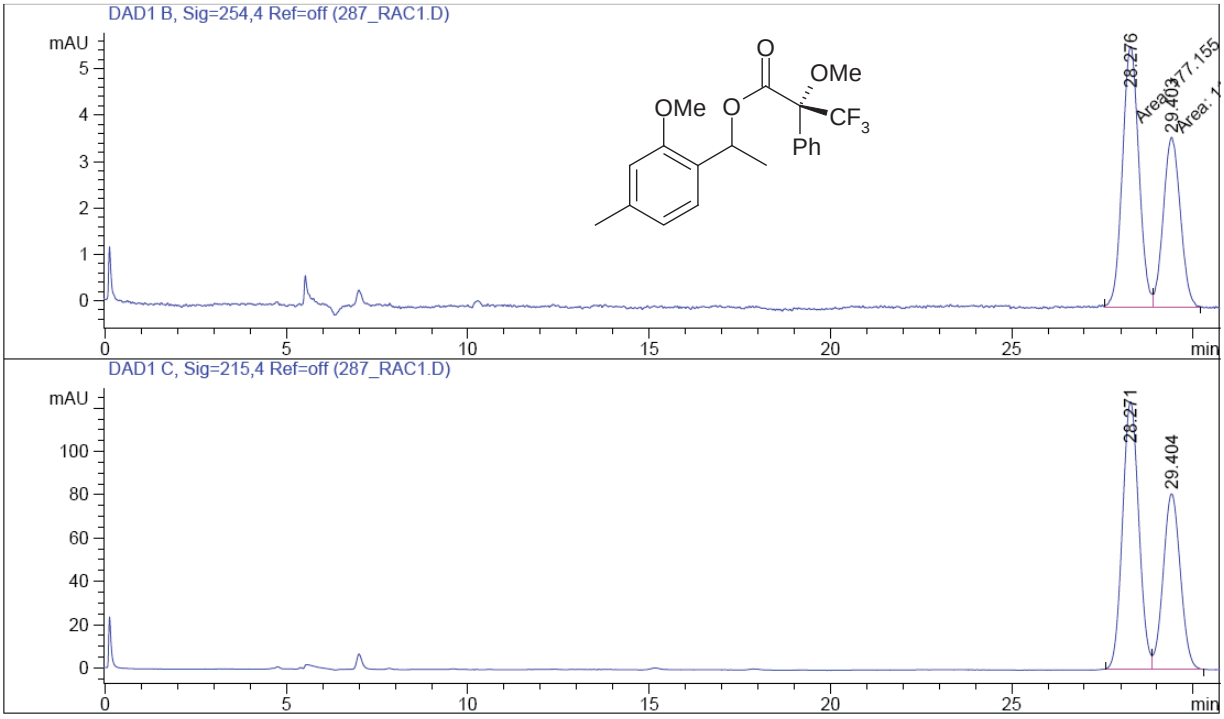
The diastereomeric amides and esters are usually analyzed by ^{1}H- or/and ^{19}F-NMR. They are usually separable by normal-phase HPLC on non-chiral analytical chromatography columns. Examples of the analysis are shown in figures 10 and 11.



a) **b)**

Figure 10. ^{19}F-NMR spectra of the Mosher's derivatives of 1-(4-isopropyl-phenyl)-ethylamine a) of a racemic sample; b) of the (*R*)-isomer, ee > 97%

a)



b)

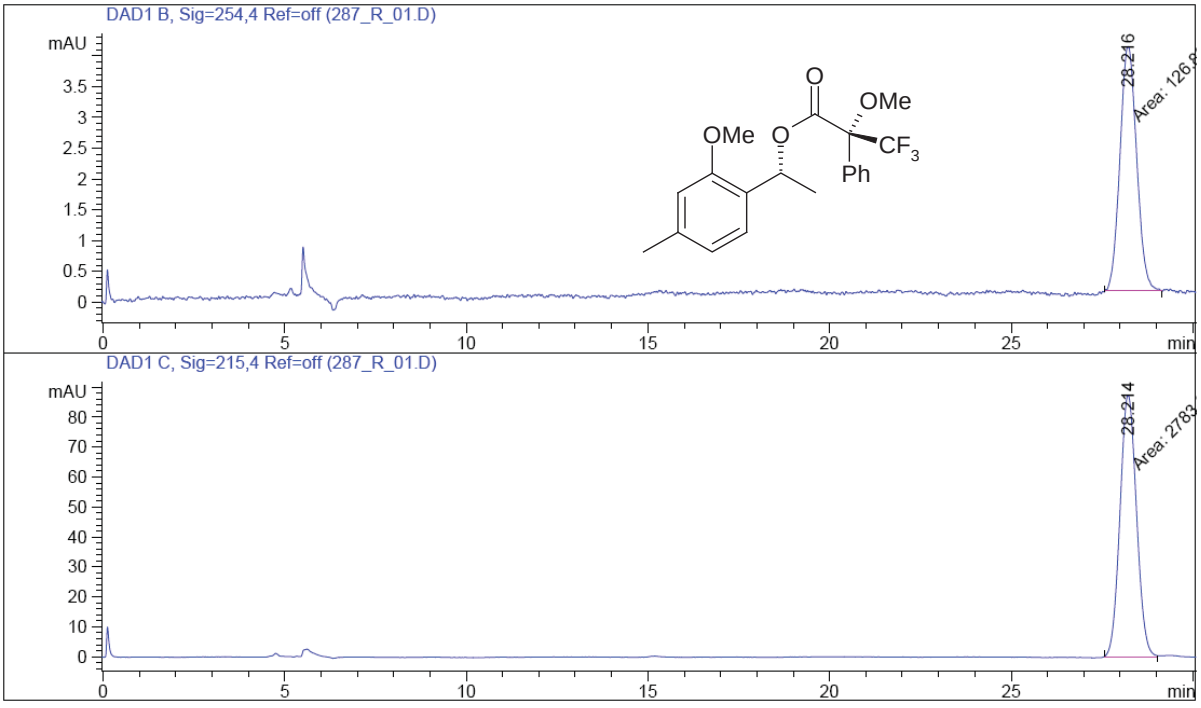


Figure 10. HPLC traces (of the Mosher's derivatives of 1-(2-Methoxy-4-methyl-phenyl)-ethanol a) of a mixture of the (*R*)- and (*S*)-isomers; b) of the pure (*R*)-isomer, ee > 99%

6. Determination of absolute configuration of pure enantiomers

Reliable establishment of the absolute configuration is a tedious task. In most cases assignments of the absolute stereochemistry is done preliminary by the general knowledge on the stereoselectivity of the process used to prepare the compounds. For example, if a compound was prepared by an enzymatic kinetic resolution, literature data on the configuration of the consumed/produced compound are taken into account.[8] The same arguments are applied when an established catalytic asymmetric transformation was used as the key stereoselective step. Empirical knowledge on the catalyst performance allowed formulation of simple rules capable to predict the stereochemical outcome of the reaction. For some powerful asymmetric catalytic systems (Sharpless asymmetric hydroxylation, CBS C=O group reduction) the rules are valid even in the case of chiral substrates, where asymmetric induction might interfere. There are also approaches based on the use of chiral LSR[9] or NMR of diastereomeric derivatives with an auxiliary optically pure reagents[6] to assign stereochemistry with some confidence.

Confirmation of the absolute configuration of the known compounds with the established configuration is made by comparison of the experimental and published chiroptical properties of the compounds or intermediates.

However, when unambiguous determination of the absolute configuration of an unknown chiral compounds is required, we use X-Ray crystallography of diastereomeric derivatives or X-Ray of single crystals with the analysis of anomalous scattering (Flack parameter).[10,11]

The choice of the derivatization reagent which brings the internal chirality reference to the derivatives depends on each individual substrate, but the main requirement to the reagents is the ability to produce crystallizable material, for the crystals to be of good quality. In an example (Figure 11), the absolute configuration of an aminonitrile (6-(1-Phenylethyl)-6-aza-bicyclo[3.2.1]octane-5-carbonitrile) was established by an X-Ray study of its chiral N-phenylethyl derivative.

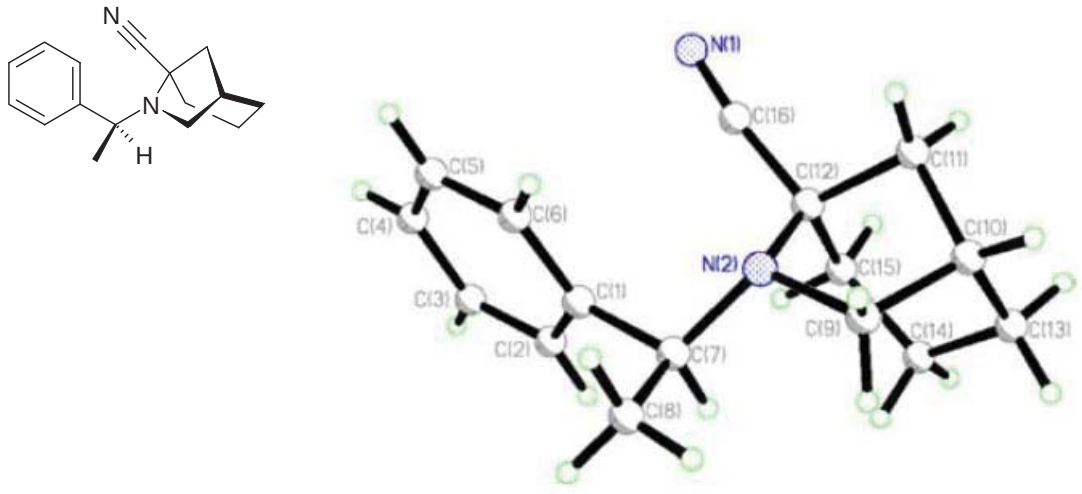


Figure 11. Molecular structure of (1*S*,5*R*)-6-((1*S*)-1-Phenylethyl)-6-aza-bicyclo[3.2.1]octane-5-carbonitrile as determined by X-Ray crystallography.

In some cases, stereochemistry of the diastereomeric derivatives can be assigned by NOE studies. 2D H,H-NOESY experiments are most informative, even in very complicated case, like shown in Figure 12 for a compound possessing axial chirality.

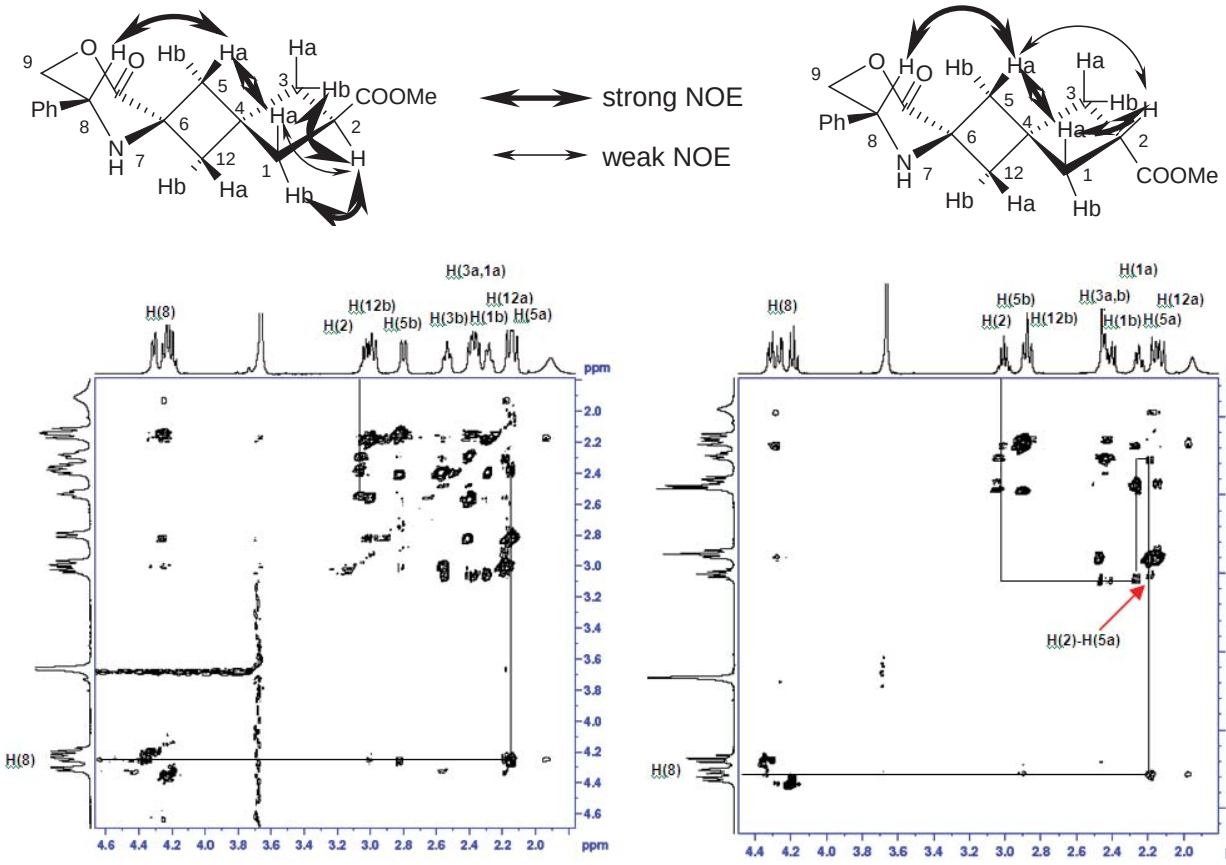


Figure 12. Axially chiral (*R*)- and (*S*)-2-amino-spiro[3.3]heptane-2,6-dicarboxylic acids were transformed in the tricyclic derivatives with optically pure (*R*)-α-phenylglycinol. For one of the diastereomers, shown right, there is a continuous path of strong NOE correlations from H(8) to H(2) signals, which proves the configuration shown. Another convincing proof of the stereochemistry of this isomer is the NOE correlation peak H(2)–H(5a) (marked by a red arrow), possible only for this diastereomer. For the other compound, shown left, the strong NOE correlation paths from H(8) and from H(2) are not convergent.

X-Ray diffraction with the use of the Flack parameter for the absolute configuration determination is done in rare cases (Figure 13). Good quality crystals are required.

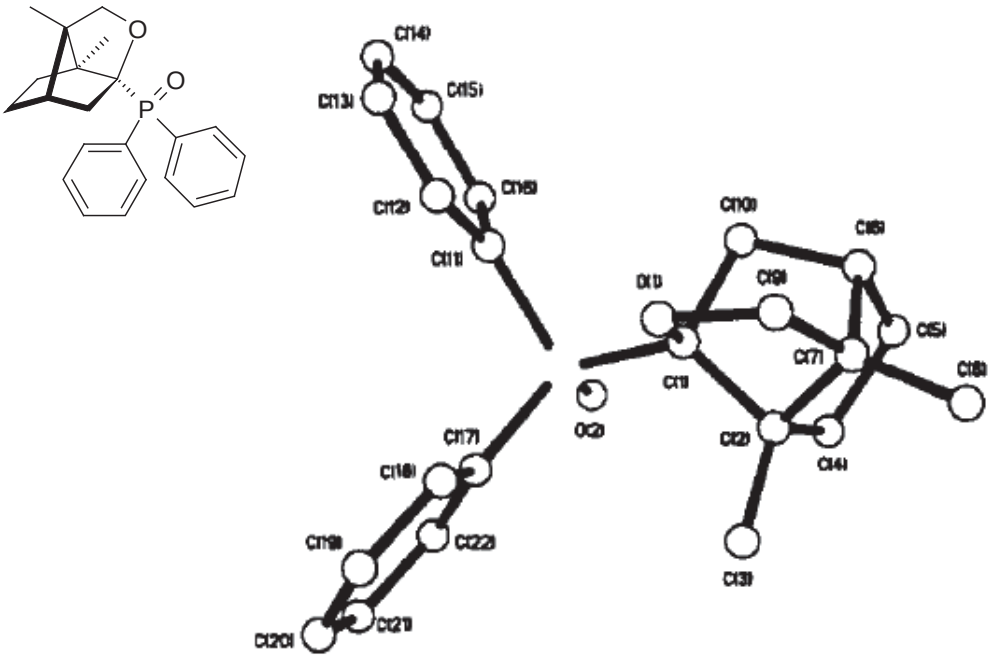


Figure 13. Molecular structure of (1S,3R,6S,7R)-3-(Diphenyl-phosphinoyl)-6,7-dimethyl-4-oxa-tricyclo-[4.3.0.0*3,7*]nonane. Absolute configuration was determined by single-crystal X-Ray diffraction.

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