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Simple and efficient preparation of (R)- and (S)-enantiomers of α-carbon deuterium-labelled α-amino acids

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SUMMARY

A procedure for the synthesis of (R)- and (S)-enantiomers of α-carbon deuterium-labelled α-amino acids, exemplified for (R)- and (S)-[2-$^{2}H_{1}$]-Leu is described. Starting from the respective (S)- or (R)-enantiomer or from the racemic mixture of an α-amino acid the selective proton exchange at the α-carbon is carried out by racemization via a Schiff base in monodeuterated acetic acid as solvent which serves as deuterium source.

After N-protection the racemic mixture is liquid chromatographically separated into the individual (R)- and (S)-enantiomers on preparative scale employing a chiral anion exchanger based on carbamoylated quinine as chiral selector. After deprotection the enantiomerically pure products can be obtained in good yields.

Key words: α-carbon deuterated α-amino acids, synthesis, racemization, enantiomeric separation, (R)- and (S)-[2-$^{2}H_{1}$]-Leu.

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INTRODUCTION

Regio- and stereospecific deuterium-labelled α-amino acids are important building blocks in organic chemistry and find application as probes for the elucidation of biosynthetic pathways or reaction mechanisms by NMR and MS either as substrate or incorporated into peptides or proteins (1).

Besides this application deuterated compounds are also widely used as reference compounds in mass-spectrometry, e.g. as internal standards.

Previously described syntheses of (R)- and (S)-enantiomers of C_α-deuterated amino acids (2-8) have high costs or the reactions have to be done under protective atmosphere and with sensitive and expensive catalysts. Other authors use enzymatic synthesis for stereoselectively labelling of α-amino acids (9-12). These methods require exactly controlled reaction conditions and the enzyme used has to be selective for the α-amino acid to be labelled.

In this contribution a simple and inexpensive method for the preparation of (R)- and (S)-enantiomers of α-carbon deuterium-labelled α-amino acids is described. Starting from the cheap natural (S)-α-amino acid (or alternatively from an (R)-enantiomer or from a racemic mixture) the C_α-hydrogen is selectively exchanged by deuterium by racemization of this stereogenic centre in monodeuterated acetic acid via the Schiff base protocol (see Scheme 1). Subsequently, the racemic mixture of the N-protected amino acid is liquid chromatographically separated into the (R)- and (S)-enantiomers on preparative scale employing a weak anion exchange type chiral stationary phase (CSP) based on quinine carbamate (9,10,11) (See Fig 1). The collected enantiomerically pure fractions get deprotected yielding the resulting amino acids without loss of deuteration.

For HPLC enantioseparation the deuterated amino acids have to be converted to N-protected derivatives (as Boc, FMOC, Z, DNZ amino acids), which can easily be enantioseparated and deprotected. The practicability of this method is demonstrated by the preparation of the (R)- and (S)- enantiomers of α-carbon deuterated leucine, (R) and (S)-[2-$^2H_1$]-Leu.

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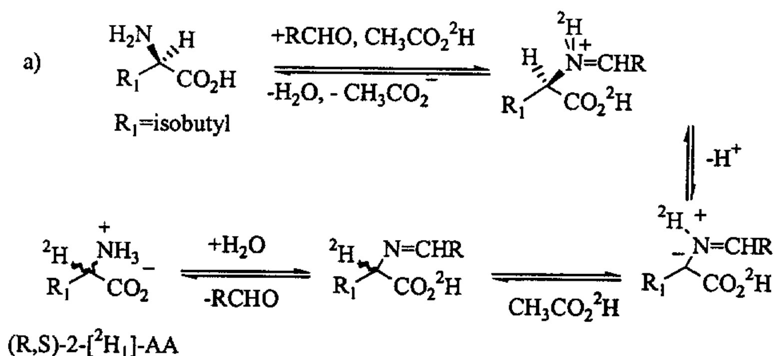
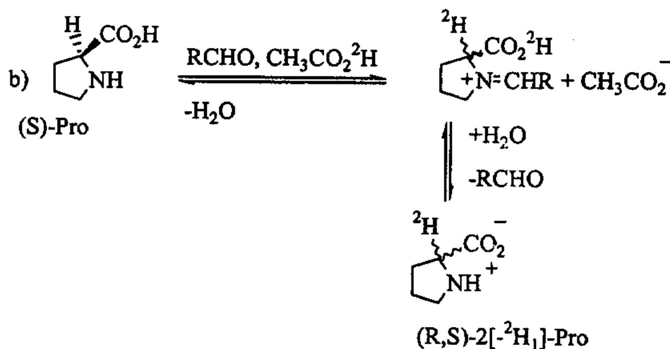
RESULTS AND DISCUSSION

C_α-DEUTERIUM LABELLING BY RACEMIZATION REACTION

Racemization methods in the contest of enantioseparation processes of racemic mixtures are of high interest for the chemical and pharmaceutical industry, since via this protocol the otherwise wasted enantiomer may be transformed and recycled into the production process for the wanted enantiomer, thus improving the overall productivity. For α-amino acids a convenient racemization method via Schiff base formation in acetic acid has been proposed by S. Yamada et al. (13). If this simple and inexpensive reaction is carried out in an excess of





Proposed reaction scheme of selective deuterium exchange accompanied by racemization of primary and secondary amino acids in monodeuterated acetic acid: the intermediate Schiff base formation is accompanied by its cleavage due to the water cleavage and addition during reaction. Due to the large excess of $CH_3CO_2{}^2H$ the deutero exchange is dominant

Scheme 1

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deuterated solvent (monodeutero acetic acid instead of acetic acid) the corresponding racemic C_{α}-deuterium labelled amino acids are easily accessible in high chemical purity (extent of deuterium exchange usually >99%) as outlined in reaction scheme 1.

The extent of deuterium exchange was proved by ^{1}H-NMR and mass spectrometry, respectively, and the results are summarised in Tab. 1.

Salicylaldehyde (SA) as Schiff base forming catalyst gave the best results, but other aromatic aldehydes, such as 2-nitrobenzaldehyde, can also be used and only catalytic amounts of aldehydes in the range of 1-10 mol% are necessary (13). We applied however 10-20 mol%.

The molar extent of monodeuterated acetic acid used as reagent and solvent was about 100% fold (related to acidic H)

Table 1

Reaction conditions and yield for the racemization and deuteration reaction

Product	**Starting material**	**Aldehyde**	**Acid**	**Yield**[a]	**Atom % ^{2}H**[b]	**React. time**
(R,S)-2-[$^{2}H_{1}$]-Leu	(S)-Leu 200 mg	SA 0.03 ml	$AcO^{2}H$ 9.4 ml	97%	>99%	1 h
(R,S)-2-[$^{2}H_{1}$]-Ala	(S)-Ala 150 mg	SA 0.03 ml	$AcO^{2}H$ 9.4 ml	74%	>97%	1 h
(R,S)-2-[$^{2}H_{1}$]-Pro	(S)-Pro 500 mg	SA 0.04 ml	$AcO^{2}H$ 9.4 ml	96%	>95%	4 h
(R,S)-2-[$^{2}H_{1}$]-Phe	(S)-Phe 100 mg	SA 0.02 ml	$AcO^{2}H$ 9.4 ml	68%	>98%	1 h
(R,S)-2-[$^{2}H_{1}$]-Phg	(S)-Phg 100 mg	SA 0.02 ml	$AcO^{2}H$ 9.4 ml	60%	>98%	1 h
2,2-[$^{2}H_{1}$]-Gly	Gly 100 mg	SA 0.02 ml	$AcO^{2}H$ 9.4 ml	98%	100%	1 h

[a] refers to chemical yield after workup of amino acid before N-protection and enantioseparation process

[b] determined by mass spectrometry

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It was found that the optimal temperature for this reaction is 100 °C and 60-70 minutes reaction time. Compared to Leu the reaction time had to be increased for Phe to complete the racemization and hydrogen-deuterium exchange.
The method can be also applied to secondary α-amino acids like Pro.
However, in these cases the reaction time was 4 hours due to reduced reactivity.

CHROMATOGRAPHIC SEPARATION OF (R)- AND (S)-ENANTIOMERS OF C_α-DEUTERIUM LABELLED α-AMINO ACIDS

Thus prepared racemic mixtures of C_α-deuterium labelled α-amino acids can be separated into the individual (R)- and (S)-enantiomers on a chiral weak anion exchange (WAX) type stationary phase (see Fig. 1) (16). This WAX type CSP derived from quinine carbamate as chiral selector (SO) separates the enantiomers of chiral acidic compounds (SAs) under buffered aqueous-organic or strictly organic mobile phases with high enantioselectivity. This WAX type CSP exhibits optimal exchange capacity and enantioselectivity at apparent mobile phase pH below 6.0. However, this is near the isoelectric point of amino acids, e.g. pI of Leu is 5.98. This means that they are not negatively charged and therefore not retained on the WAX type CSP under mobile phase conditions at which the stationary phase exhibits exchange capacity and enantioselectivity. Therefore, the amino group is protected. All of the N-protection groups commonly employed in amino acid and peptide chemistry can be applied, i.e. Boc, FMOC, Z, DNZ, DNP protection groups, etc. Table 2 lists the enantioseparation results of some selected nondeuterated and C_α-deuterated amino acids with different protection groups on the quinine carbamate type CSP. As expected, the retention (k′), the enantioselectivity (α) as well as elution order (e.o.) of C_α-deuterated α-amino acids are equal to the corresponding hydrogen analogues. This chromatographic method was used analytically to control the progress and extent of racemization and to determine the enantiomeric excess (ee) of the final products, e.g. (R)- and (S)-[2-$^2H_1$]-Leu, which can be easily obtained by simple up-scaling the analytical chromatographic procedure. Thus, 100 mg of the racemate of Z-protected [2-$^2H_1$]-Leu have been injected onto a

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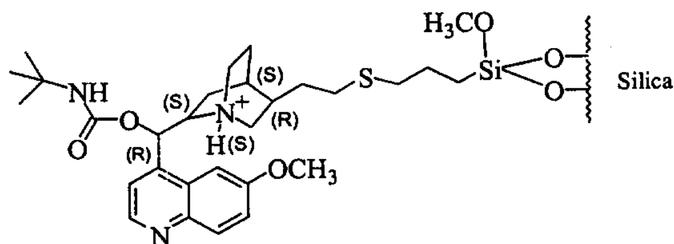
preparative column of the WAX type CSP based on the quinine carbamate (see Experimental). The first eluting (R)-enantiomer could be collected with very high enantiomeric excess (ee>99%). In order to ensure a similar high enantiomeric purity for the second eluting (S)-enantiomer a middle fraction, which can be rechromatographed, had to be cut before collecting the second enantiomer. Thus, the enantiomeric excess of (S)-[2-$^{2}H_{1}$]-Leu exceeded 97% ee. The quality and ee-value of the (S)-enantiomer could easily be increased if a larger volume of mixed fraction would be cut-out, or if the separation is performed with the corresponding quinidine carbamate type CSP that exhibits, owing to its "pseudo-enantiomeric" behaviour, reversed elution order, i.e. Z-(S)-2-[$^{2}H_{1}$]-Leu before Z-(R)-2-[$^{2}H_{1}$]-Leu. From the eluates containing the product, the Z-amino acid derivative could be recovered in 95% chemical yield. Finally the Z-group was removed by hydrogenation with Pd-C as a catalyst. The analytical control of the enantiomeric purity or (R)- and (S)-2-[$^{2}H_{1}$]-Leu clearly showed that no racemization by which the C_{α}-deuterium could have been exchanged by hydrogen occurred during separation and work-up (see Fig. 2). This was confirmed also by the mass spectra of the (S)- and (R)-products which clearly demonstrated no change of deuterium labelling initially performed.

As examined on analytical scale for a broad spectrum of natural and synthetic primary and secondary α-amino acids, this general method can be easily adapted for these compounds on a preparative scale.



The quinine carbamate selector (SO)

Fig. 1

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Table 2

Chromatographic data for the analytical enantiomer separation of N-protected derivatives of some α-amino acids on a *tert.*butyl carbamoyl quinine type CSP.

Amino Acid (AA)	Protection Group								
	DNP[a]			DNZ[a]			Z[a]		
	k'_1[b]	α[b]	e.o.[c]	k'_1[b]	α[b]	e.o.[c]	k'_1[b]	α[b]	e.o.[c]
(R,S)-Ala	15.93	1.18	(R)	8.38	1.84	(S)	6.43	1.15	(S)
(R,S)-2-[$^2H_1$]-Ala	15.93	1.18	(R)	8.35	1.84	(S)	6.41	1.14	(S)
(R,S)-Leu	14.60	1.25	(R)	8.84	2.56	(S)	8.86	1.28	(S)
(R,S)-2-[$^2H_1$]-Leu	14.61	1.25	(R)	8.87	2.56	(S)	8.85	1.25	(S)
(R,S)-Pro	13.82	1.34	(R)	9.40	1.18	(S)	6.00	1.00	(S)
(R,S)-2-[$^2H_1$]-Pro	13.79	1.33	(R)	9.41	1.18	(S)	5.99	1.00	(S)
(R,S)-Phe	27.64	1.29	(R)	11.10	1.95	(S)	8.95	1.22	(S)
(R,S)-2-[$^2H_1$]-Phe	27.60	1.27	(R)	11.08	1.93	(S)	8.95	1.22	(S)

a) DNP: 2,4-dinitrophenyl; DNZ: 3,5-dinitrobenzyloxycarbonyl; Z: benzyloxycarbonyl

b) $k'=(t_r-t_0)/t_0$; $\alpha=k_2'/k_1'$

c) e.o.: elution order, configuration of the enantiomer with higher affinity to the chiral selector thus being longer retained

EXPERIMENTAL

Materials, Instrumentation and Chromatographic Methods

(S)- Leucine (Leu), (S)-phenylalanine (Phe), (S)-proline (Pro), (S)-alanine (Ala), (S)-phenylglycine (Phg), monodeuterated acetic acid ($CH_3CO_2{}^2H$, 98% atom ^2H), reagent grade glacial acetic acid for HPLC (CH_3CO_2H), methanol p.a. (MeOH), ^2H_2O p.a., palladium on activated charcoal, sodium sulphate dry, magnesium sulphate dry, glacial acetic acid, HCl p.a. and triethylamine were purchased from Fluka (Buchs, Switzerland). Salicylaldehyde p.a. (SA), nitrosalicylaldehyde p.a., methanol LiChrosolve grade for HPLC (MeOH),

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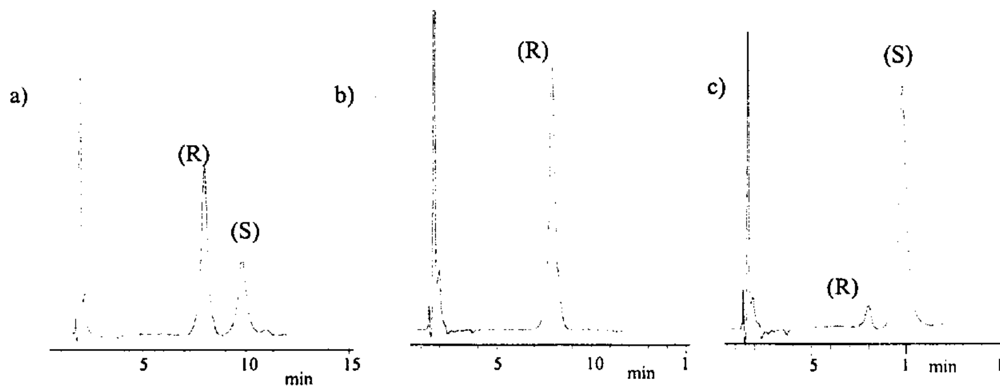

Determination of the enantiomeric purity of (R)- and (S)- [$^2H_1$]-Leu as Z-derivatives by enantioselective chromatography on quinine carbamate type CSP

a) mixture of (R) and (S) enantiomers
b) (R)-enantiomer
c) (S)-enantiomer

Fig. 2

ammonium acetate p.a., were obtained from Merck (Darmstadt, Germany). Methylene chloride p.a., *n*-hexane p.a. and diethylether p.a. were obtained from Loba Feinchemie (Fischamend, Austria). All reagents and solvents were used without further purification.

Water, HPLC grade, was used to prepare the buffer solution for the mobile phase. The mobile phases were degassed before use by ultrasonication.

3,5-dinitrobenzylchloroformate (DNZ-Cl) was prepared from 3,5-dinitrobenzylalcohol (Aldrich, Steinheim, Germany) and phosgene (0.34 g phosgene/ml dioxane) (Fluka, Buchs, Switzerland). 2,4-Dinitrofluorobenzene (DNFB, Sanger's reagent) was obtained from Aldrich (Steinheim, Germany).

NMR spectra were measured with a 250 MHz spectrometer, Bruker DPX 250 (Bremen, Germany). All spectra were measured in ^2H_2O as a solvent.

MS spectra were recorded on a PE Sciex API 365-electrospray mass spectrometer (PE-Sciex, Toronto, Canada) and VG Quattro MS (Micromass, Manchester, UK).

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Preparation of racemic mixtures of C_α-deuterium labelled α-amino acids

C_α-deuterated α-amino acids were prepared following a racemization procedure developed by Yamada et al. (13) but carried out in deuterated acetic acid. The procedure is exemplified by the synthesis of racemic 2-[$^2H_1$]-Leu: 200 mg (1.52 mM) of (S)-Leu were dissolved in 9.4 mL (0.16 M) $CH_3CO_2{}^2H$ and 0.03 mL (0.3 mM) salicylaldehyde were added. The reaction mixture was stirred at 100° C for one hour. After reaction, the solution was concentrated under reduced pressure to dryness. The residue was treated with 2 mL of ^2H_2O for 15 min then diluted with water, treated with charcoal and filtered. The filtrate was then concentrated under reduced pressure to dryness. 15 mL of methanol were added to the residue.

The crystalline precipitate was collected to yield 194 mg (97%) of the C_α-deuterated (R,S)-2-[$^2H_1$]-Leu. m.p. 270-279°C, NMR: 0,95 ppm (s, 6H), 1,65 ppm (m, 3H), MS: 132 m/z (MH^+, 98%) , deuteration level >98 % (MS), ee (HPLC) 0%

Analytical control of racemization process by enantioselective liquid chromatography

The success and extent of racemization of the treated α-amino acids and thus of deuterium exchange was monitored by enantioselective chromatography employing an anion exchange type chiral stationary phase (CSP) with a quinine carbamate type chiral selector (see Fig. 2). Pre-column derivatization of the deuterium labelled amino acids with broadly used N-protection reagents (DNFB, DNZ-Cl, Z-Cl, etc.) was necessary. The following protocols were used for the preparation of the respective DNP, DNZ or Z derivatives.

1 mg of the racemic deuterium-labelled amino acid was dissolved in 200 μL of carbonate buffer (0.1 M sodium bicarbonate adjusted to pH 9.5 with 0.1 M

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sodium carbonate). A 50µL volume of a solution containing either DNFB, DNZ-Cl or Z-Cl (each 5% in acetonitrile) was added and the solution was vortex-mixed. After heating at 50°C for 1 hour the reaction mixture was diluted with 800 µL of the mobile phase (see below) and an aliquot of 20 µL was injected onto the CSP.

Analytical chromatography was performed on a HP 1050 liquid chromatograph from Hewlett Packard, consisting of a quaternary gradient pump, an autosampler, variable wavelength detector and a HP Chemstation for data evaluation. The stationary phase (See. Fig. 2) was prepared by immobilisation of *tert*.butyl carbamoylated quinine onto 3-mercaptopropyl modified silica (prepared from Kromasil 100-5) (16). The column dimensions were 150 x 4.6 mm I.D.

A mixture of methanol and 0.1 M ammonium acetate (80/20; v/v), adjusted to $pH_{app.}$= 6.0 by adding glacial acetic acid, was used as mobile phase. The analytical separations were carried out with a flow-rate of 1 mL/min.

Preparative liquid chromatographic separation of racemic 2-[$^2H_1$]-Leu as Z-derivative

The racemic mixture of the C_α-deuterated leucine was preparatively separated as Z-derivative on a milligram to gram scale to obtain, after cleavage of the Z-group, both enantiomers of 2-[$^2H_1$]-Leu in high enantiomeric excess.

Thus, 50 mg of the racemic (R,S)-2-[$^2H_1$]-Leu, were dissolved in 1 mL carbonate buffer (0.1 M sodium bicarbonate adjusted to pH 9.5 with 0.1 M sodium carbonate). To this mixture 1mL of a solution of Z-chloride (8% w/v in dioxane) was added. The solution was stirred at 50°C for 12 hours. After this time the reaction mixture was extracted 3 times with diethylether. The remaining aqueous phase was acidified with $HCl_{conc.}$ and stirred for 15 min. The solution was extracted 4 times with methylene chloride and the collected organic phases were dried over sodium sulfate. The solvent was evaporated and the residue was treated with *n*-hexane.

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Yield: 72 mg (71.5%) of (R,S) product
Mass: 267 m/z (MH^+, 98%), % ^2H: >98% (MS)

This product was subjected to preparative liquid chromatography (see below). The fractions of the eluate containing the enantiomers were collected separately, while a small mixed fraction was discarded.

The HPLC instrument for the preparative liquid chromatography consisted of a pump L-6000 and a UV detector L-4000 (both from Merck), a Rheodyne injector (model 7125) with a 3 mL loop and an integrator from Hewlett Packard (Model HP 3396A). The same type of chiral stationary phase as described for analytical scale chromatographic separations was used for the preparative separation, however, the particle diameter of the preparative chiral sorbent was 15 μm. The column dimensions were 150 x 16 mm I.D. As a mobile phase methanol with 20 mM triethylammonium acetate (adjusted with glacial acetic acid to an apparent pH of 6.0) was used. The injection volume was 2 mL (50 mg/mL sample concentration). The flow-rate was 9 mL/min and the detection wavelength was 254 nm.

All fractions containing product to be recovered were combined and acidified with 0.5 M HCl to pH = 2. After extraction with ethyl acetate (3 x 25 mL) the combined organic phases were washed (3 x 25 mL brine), dried with sodium sulfate and after filtration the solvent was evaporated.

(S)-Z-[2-$^2H_1$]-Leu: Yield: 32.3 mg (89.7%), ee = 97% (S),
267 m/z (MH^+, 98%)
(R)-Z-[2-$^2H_1$]-Leu: Yield: 27.4 mg (76.1%), ee= > 98% (R),
267 m/z (MH^+, 98%),

Hydrogenolytic cleavage of the Z-group

32 mg of the (S)- and 27 mg of the (R)-deuterium-labelled and Z-protected amino acids were dissolved in 20 mL of dry ethanol. 20 mg of Pd-C as a catalyst

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were added to the solution. The solution was then shaken under hydrogen for 2 hours (100-120 psi). After 2 hours the catalyst was filtered off and the filtrate was evaporated to dryness. Crystallisation from ethanol/water (50/50, v/v) yielded the respective free (R)- and (S)-Leu products.

(R)-[2-$^2H_1$]-Leu: 12.1 mg (89%), m.p. 230°-234° C, 133 m/z (MH^+, 99%), ee=97%.

(S)-[2-$^2H_1$]-Leu: 14.3 mg (89%), m.p. 231°-235° C, 133 m/z (MH^+, 99%), ee=>98%.

(R)-[2-$^2H_1$]-Leu + (S)-[$^2H_1$]-Leu = 26.4 mg (52.8% of starting racemic material).

Conclusion

The reported and widely applicable synthesis in combination with liquid chromatographic enantiomer separation of N-protected C_α-deuterated α-amino acids, exemplified on leucine, is a powerful process to obtain this material and the parent α-amino acids also on large scale. The productivity rate of the batch chromatography method may be easily further increased implementing the continuously working simulated moving bed (SMB) chromatography approach. The enantiomeric purity of the recovered compounds is very high and the production costs are low compared to other labelling methods. There is also no toxic waste and no special materials are needed for the synthesis, for the separation and for the deprotection.

REFERENCES

1. Ramalingam, K.; Nanjappan, P.; Kalvin, D. M.; Woodard, R. W.; *Tetrahedron*, **1992**, *44*, 5597.
2. Kajiwara, M; Lee, S. F.; Scott, I. A.; Akhtar, M.; Jones, C. R.; Jordan, P. M; *J. Chem. Soc. Chem. Commun.*, **1978**, 967-968.
3. Tilley, K.; Akhtar, M.; Gani, D; *J. Chem. Soc. Perkin Trans I*, **1994**, 3079-3087.

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4. Hammon, D.P.; Massy-Westropp, R.P.; Razzino, P.; *Tetrahedron*, **1993**, *49*, 6419-4928.
5. Elemes, Y.; Ragnarsson, U.; *Chem. Commun.*, **1996**, 935-936.
6. Thanassi, J. W; *J. Org. Chem.*, **1971**, *36*, 3019-3020.
7. Belokon, Y. N.; Melikyan, A.S.; Saleleva, T. F.; Bakhmutov, V. I.; Vitt S. V.; Belikov, V. M.; *Tetrahedron*, **1980**, *36*, 2327-2335.
8. Seebach, D.; Dziadulewicz, E.; Behrendt, L.; Cantoreggi, S.; Fitzi, R.; *Liebigs Ann. Chem.*, **1989**, 1215-1232.
9. Milne, J.J.; Malthouse, J. P.; *J. Biochem.*, **1996**, *314*, 787-791.
10. Milne, J.J.; Malthouse, J. P.; *Biochem. Soc. Trans.*, **1996**, *24*, 133.
11. Williams, J. S.; Rosevear, P.R.; *Biochemistry*, **1991**, *30*, 6412-6416.
12. Fitzpatrick, T.B.; Malthouse, J.P.; *Eur. J. Biochem.*, **1998**, *252(1)*, 113-117.
13. Yamada, S.; Hongo, C.; Yoshioka, R.; Chibata, I.; *J. Org. Chem.*, **1983**, *48*, 843-846.
14. Piette, V.; Lämmerhofer, M.; Bischoff, K.; Lindner, W.; *Chirality*, **1997**, *9*, 157-161.
15. Kleidernigg, O.P.; Lämmerhofer, M.; Lindner, W.; *Enantiomer*, **1996**, *1*, 387-402.
16. Lämmerhofer, M.; Lindner, W.; *J. Chromatogr. A*, **1996**, *741*, 341-348.
17. Houben-Weyl, *Methoden der Organischen Chemie*, **1972**, Vol. 15, 4. Aufl. Synthese von Peptide I, E. Wünsch, Ed.

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