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ORIGINAL ARTICLE

A new chiral derivatizing agent for the HPLC separation of α-amino acids on a standard reverse-phase column

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Abstract A new chiral derivatizing agent for α-amino acids is described which leads to diastereomers that can be separated by reverse-phase HPLC with direct detection by a diode array detector. The main advantage of the presented procedure is the fact that an excess of the derivatizing reagent can be employed as the product exhibits an absorption maximum at 360 nm, while the reagent has its absorption maximum at 260 nm. Therefore, it is possible to suppress the reagent signal by a detection wavelength of 400 nm leading to an easy and general method for the enantioseparation of a mixture of DL-amino acids and the determination of the enantiomeric purity of α-amino acid as exemplified by 16 different α-amino acids.

Keywords Sanger reagent · Marfey reagent · Chiral derivatization agent (CDA) · Amino acids · Enantiomeric purity determination

Introduction

Over the last years, a number of methods have been described for the detection of specific amino acids in complex mixtures and the determination of their chiral purity either by GC, HPLC, TLC, SFC or capillary electrophoresis. For the HPLC detection, indirect method has been well established that is preparing diastereomers by derivatizing the α-amino acid with an enantiomerically pure reagent, which at the same time provides better detection properties, so that the derivatives can be analyzed on a reverse-phase column. An ideal reagent should give good resolvable diastereomers in quantitative yield under mild conditions in a short reaction time without the formation of troublesome by-products and without an extra extraction step. It is also advantageous if the derivatives are better detectable than the substrate due to chromophoric or fluorophoric properties. Besides, the derivatizing reagent should be available in both enantiomeric forms to change the elution order eventually, and it should be easily available and stable (Ilisz et al. 2008; Görög and Gazdag 1994). A reagent which fulfils a lot of these requirements is the Marfey reagent **A** (Bhushan and Brückner 2004; Bhushan and Kumar 2009; Bhushan et al. 2009; Fisher et al. 2009; B'Hymer et al. 2003; Marfey 1984). A special advantage of this reagent is that the detection is done at 340 nm, so that the chromatogram is not interfered by impurities which are in the probe eventually. Often the Marfey reagent is called a chiral variant of the Sanger reagent **B** (Sanger 1945). However, the two compounds considerably differ in their absorption properties: compound **B** has a marker function as it is colorless having an absorption maximum at 232 nm (Simons 1979) while after reaction with an α-amino acid a yellow push–pull system is generated exhibiting an absorption maximum above 330 nm (Sanger 1949). The Marfey reagent **A** contains already a push–pull system by itself thus leading after reaction with an amino acid only to a small shift in wavelength. Therefore one can follow the reaction visually only in the case of the Sanger reagent and, besides, more importantly, the excess of derivatizing reagent is repressed and furthermore with different elution conditions or in complex mixtures, respectively, the product can be detected by diode array directly.

In this paper, the synthesis and application of a new derivatizing agent for α-amino acids is described which combines the advantages of the Sanger reagent (marker

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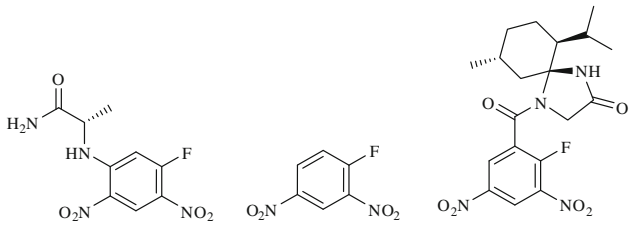
function) and the Marfey reagent (easy derivatization and good detection properties) and allowing the quick and easy analysis of D- and L-amino acids.



A **B** **1**

Marfey reagent **Sanger reagent**

Materials and methods

NMR spectra were recorded on a Bruker ARX 400 spectrometer. ^{1}H and ^{13}C chemical shifts are reported δ ppm relative to the used deutero solvent. Mass spectra were recorded on a Varian MAT 311A; IR spectra were recorded on a Nicolet FT-IR spectrometer Avatar 360. Elementary analyses were obtained with a Perkin-Elmer microelementary analyst 240B. Melting points were recorded on a Gallenkamp MFB-595 and are uncorrected. UV-absorbance spectra were recorded on a Shimadzu UV-160A. The HPLC system consisted of a Merck/Hitachi L 7100 pump, a DA L-7455 detector and a MZ Kromasil RP 18 column (250 × 4.6 mm, 5 µm). For TLC aluminum sheets silica gel 60 F_{254} from Merck were used. (−)-Menthone, glycinamide, trifluoracetic acid (TFA), *N*,*N*-diisopropylethylamine (DIPEA) and triethylamine (TEA) were purchased from Aldrich, Acros and Fluka. 2-Fluoro-3,5-dinitrobenzoic acid was synthesised according to the procedure of Petersen and Jensen (2001).

Synthesis of (5*S*,6*S*,9*R*)-6-isopropyl-9-methyl-1,4-diazaspiro[4.5]decan-2-one (**2**)

44.2 g of fine pulverized glycinamide hydrochloride was suspended in 700 ml of absolute methanol. After the addition of 61.6 g (400 mmol) (−)-menthone and 40.5 g (55.8 ml, 400 mmol) triethylamine, the reaction mixture was refluxed for 18 h. The solvent was removed under reduced pressure and the residue was dissolved in a mixture of water and diethyl ether. The water layer was extracted three times with diethyl ether. The collected organic layers were washed with sat. $NaHCO_3$ solution and brine and dried over Na_2SO_4. Evaporation under reduced pressure gave 84 g of a pale yellow oil that crystallized within 1 week. After addition of 30 ml pentane, the crystal slurry was transported on a suction filter and washed two times with a little pentane. Drying in vacuum afforded 13 g (18%) (2*S*,6*S*,9*R*)-6-isopropyl-9-methyl-1,4-diazaspiro[4.5]decan-2-one as a colorless solid (the purity is good enough for the next conversion. Recrystallisation from cyclohexane is possible and afforded fine needles). To get better yields, the mother liquor was evaporated and further product crystallized within a few weeks, which can be isolated as above. This procedure was repeated several times, whereby the yield increased over 50%.

TLC: R_f 0.27 (silica 60, 2-propanol/cyclohexane 25:75); mp 127°C (after recrystallisation). ^{1}H NMR (400 MHz, $CDCl_3$, 32°C): $\delta = 9.09$ (s, 1 H), 3.61 (d, $^{2}J_{H,H} = 16.2$ Hz, 1 H,), 3.50 (d, $^{2}J_{H,H} = 16.2$ Hz, 1 H), 2.10–2.01 (m, 1 H), 1.98 (s, 1 H, NH), 1.85–1.76 (m, 2 H), 1.67–1.58 (m, 2 H), 1.33–1.17 (m, 3 H), 0.92 (d, $^{3}J_{H,H} = 7$ Hz, 3 H), 0.89 (d, $^{3}J_{H,H} = 6.5$ Hz, 3 H), 0.86–0.82 (m, 1 H), 0.78 (d, $^{3}J_{H,H} = 6.9$ Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$, 32°C): $\delta = 176.5$ (C_2), 80.0 (C_5), 51.9 (C_6), 50.5 (C_{10}), 49.6 (C_3), 34.4 (C_8), 30.0 (C_9), 25.3 (C_{12}), 23.9 ($C_{13/14}$), 22.5 (C_7), 22.1 (C_{11}), 17.9 ($C_{13/14}$). IR (KBr): 3262, 3202, 3097, 2947, 2928, 2908, 2868, 2842, 1708, 1681, 1482, 1459, 1430, 1360, 1340, 1258, 1234, 1157, 991, 925, 884, 831, 723 cm^{-1}. Calcd for $C_{12}H_{22}N_2O$: C, 68.63; H, 10.54; N, 13.32. Found: C, 68.28; H, 10.71; N, 13.25. MS (EI, 70 eV): *m/z* (relative intensity) 210 (M^+, 36), 195 (29), 167 (14), 153 (33), 125 (100), 111 (22), 98 (61), 55 (27), 41 (47).

Synthesis of (5*R*,6*S*,9*R*)-4-(2-fluoro-3,5-dinitrobenzoyl)-6-isopropyl-9-methyl-1.4-diazaspiro[4.5]decan-2-one (**1**)

1.15 g (5 mmol) 2-fluoro-3,5-dinitrobenzoic acid was suspended in 10 ml dichloromethane. First 0.47 ml (5.5 mmol) oxalyl chloride was added followed by 40 µl DMF. The mixture was stirred for 1 h at ambient temperature while an emission of gas occurred. To the clear solution, 1.05 g (5 mmol) (5*S*,6*S*,9*R*)-6-isopropyl-9-methyl-1,4-diazaspiro[4.5]decan-2-one dissolved in 20 ml dichloromethane was added followed by 1.82 ml (11.0 mmol) DIPEA. After 1 h, the reaction mixture was washed two times with brine and dried over $NaSO_4$. The solvent was removed under reduced pressure and the crude product was isolated as red foam. Recrystallization from acetone/water 2:1 afforded the title compound as a pale yellow solid (yield 71%).

TLC: R_f 0.47 (silica, cyclohexane/ethyl acetate 1:2); mp. 158°C dec. ^{1}H NMR (400 MHz, $CDCl_3$, 32 °C): $\delta = 9.36$ (s, 1 H, H_4), 9.00 (dd, $^{4}J_{H,H} = 3.1$, $^{4}J_{H,F} = 6.1$ Hz, 1 H, H_{21}), 8.50 (dd, $^{4}J_{H,H} = 2.8$, $^{4}J_{H,F} = 4.3$ Hz, 1 H, H_{19}), 3.97 (AB, $^{2}J_{H,H} = 15.3$ Hz, 1 H, H_2), 3.91 (AB, $^{2}J_{H,H} = 14.8$ Hz, 1 H, H_2), 2.70 (dd, $^{3}J_{H,H}$ 13.0 and 2.3 Hz, 1 H, H_6), 2.53 (t, $^{2}J_{H,H} = 12.7$ Hz, 1 H, H_{10}), 1.89–1.75 (m, 4 H, $H_{7,8,10,12}$), 1.66 (m, 1 H, H_9), 1.30 (dq, 1 H, $^{2}J_{H,H} = {}^{3}J_{H,H} = {}^{3}J_{H,H} = 13.3$ Hz, $^{3}J_{H,H} = 2.9$ Hz, H_7), 1.12 (dq, 1 H,

$^2J_{H,H} = {}^3J_{H,H} = {}^3J_{H,H} = 12.6$ Hz, $^3J_{H,H} = 2.5$ Hz, H_8), 1.01 (d, 3 H, $^3J_{H,H} = 7.1$ Hz, $H_{13/14}$), 0.99 (d, $^3J_{H,H} = 6.1$ Hz, 3 H, H_{11}), 0.85 (d, $^3J_{H,H} = 6.6$ Hz, 3 H, $H_{13/14}$). ^{13}C NMR (100 MHz, $CDCl_3$, 32 °C): $\delta = 167.4$ (C_3), 159.0 (C_{15}), 154.0 (d, $^1J_{C,F} = 274.7$ Hz, C_{17}), 143.9 (d, $^4J_{C,F} = 4.1$ Hz, C_{20}), 137.8 (d, $^2J_{C,F} = 11.2$ Hz, C_{18}), 129.8 (d, $^2J_{C,F} = 21.4$ Hz, C_{16}), 128.2 (d, $^3J_{C,F} = 6.1$ Hz, C_{19}), 122.8 (C_{21}), 85.3 (C_5), 50.5 (C_2), 45.0 (C_6), 44.8 (C_{10}), 33.8 (C_8), 29.8 (C_9), 26.3 (C_{12}), 23.2 ($C_{13/14}$), 22.6 (C_7), 21.9 (C_{11}), 18.1 ($C_{13/14}$). ^{19}F NMR (376 MHz, $CDCl_3$, 32 °C): $\delta = -112.07$ (dd, $^4J_{F,H} = 4.8$ and 5.6 Hz, F_{17}), –112.16 (^{13}C satelite, $^1J_{F,C}$ 274.4 Hz, F_{17}). Calcd for $C_{19}H_{23}FN_4O_6$: C, 54.02; H, 5.49; N, 13.26. Found: C, 54.36; H, 5.19; N, 13.04. MS (EI, 70 eV): *m/z* (relative intensity) 422 (M^+, 36), 407 (16), 338 (33), 337 (100), 213 (41), 167 (25), 121 (21), 71 (15), 57 (29), 55 (21), 43 (18), 42 (23).

Typical procedure for derivatization of amino acids with **1**

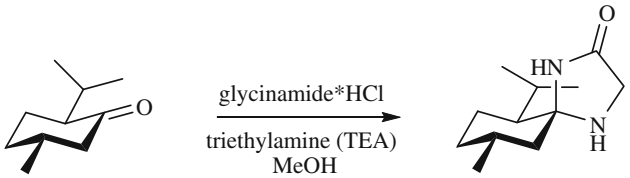
50 µl of a 20 mM aqueous amino acid solution containing 1% TEA was transferred to a 2 ml plastic tube and 100 µl of a 33 mM solution of **1** in acetonitrile were added. The mixture was diluted with acetonitrile up to a volume of 300 µl. The contents were mixed and heated to 60°C for 30 min. After cooling to room temperature, 10 µl of the mixture was injected on a HPLC column. Alternatively, the sample was acidified with 200 µl acetonitrile/1% TFA (aq.) 1:1 before injection.

Elution and detection

To separate diastereomers from amino acid mixtures, elution was done with a linear gradient of acetonitrile/1% TFA (aq.) from 50:50 to 80:20 during 1 h, flow rate 0.6 ml/min. To separate diastereomers of one amino acid, elution was done with acetonitrile/1% TFA (aq.) 70:30, 80:20 or 90:10 depending on the polarity of the amino acids, flow rate 0.8 ml/min. To observe the derivatizing reagent peak, a detection wavelength of 350 nm was used. To suppress the reagent signal, the detection was done at 400 nm.

Results and discussion

The chiral component of reagent **1** was developed originally as a chiral glycine equivalent **2**. Owing to the excellent diastereoselectivity using **2** as a chiral auxiliary to prepare amino acid derivatives (Altenbach et al. 1997, 2001), the *N,N*-acetal **2** seems to be an optimal compound to introduce chirality into a derivatizing reagent. **2** is easily synthesized from (−)-menthone and glycinamide (Scheme 1). The



50 %

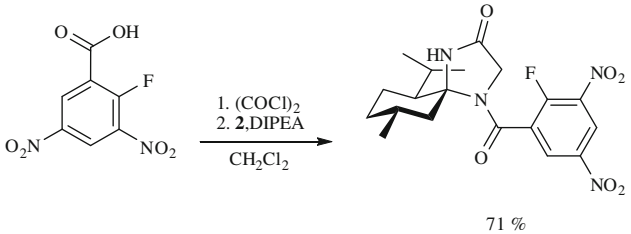
2

Scheme 1 *N,N*-acetalization of menthone with glycinamide

shown diastereomer **2** was isolated by crystallization, whereas the other diastereomer (with equatorial amide function) does not crystallize. The proposed structure of the isolated diastereomer **2** was evidenced by X-ray spectroscopy of a derivate (Kotthaus 2005). Owing to the low costs of all reagents (including (+)- or (−)-menthone), the moderate yield of 50% is acceptable.

To synthesize reagent **1**, 2-fluoro-3,5-dinitrobenzoyl chloride, prepared in situ from the acid **3** with oxalyl chloride, was stirred for 1 h with **2** and *N,N*-diisopropylethylamine (DIPEA) as base in dichloromethane (Scheme 2). The derived product can be isolated easily by crystallization (yield 71%). For derivatizing operations, we used a 33 mM solution of reagent **1** in acetonitrile. During our work it could be shown that this solution, stored at room temperature is stable for a minimum of 3 months. The UV spectrum from **1** (Fig. 1) exhibits a maximum at 260 nm, typically for such electron-deficient aromatics.

The marker function of the reagent could be seen after reaction with amino acids (Scheme 3). Immediately after the addition, the color of the reaction mixture changes from colorless to deep yellow, due to a change in the absorption maximum from 260 to 360 nm for the derived product (Fig. 1). The procedure to generate HPLC-compatible samples is very easy and needs no purification step. Complete conversion is reached after 30 min in acetonitrile/1% TEA (aq.) at 60°C. After cooling, the sample was injected directly onto the column. To separate derivatized amino acid mixtures, a gradient elution acetonitrile/TFA 0.1% (aq.) from 50:50 to 80:20 within 1 h was used. To



71 %

3 **1**

Scheme 2 Preparation of reagent **1**

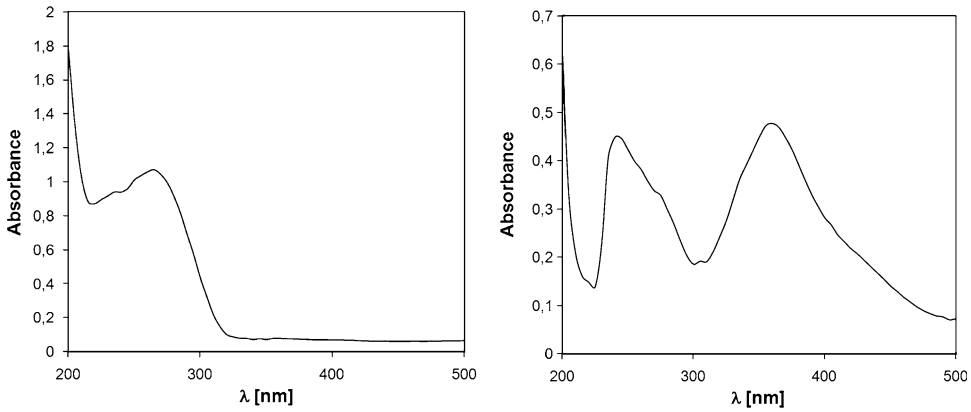
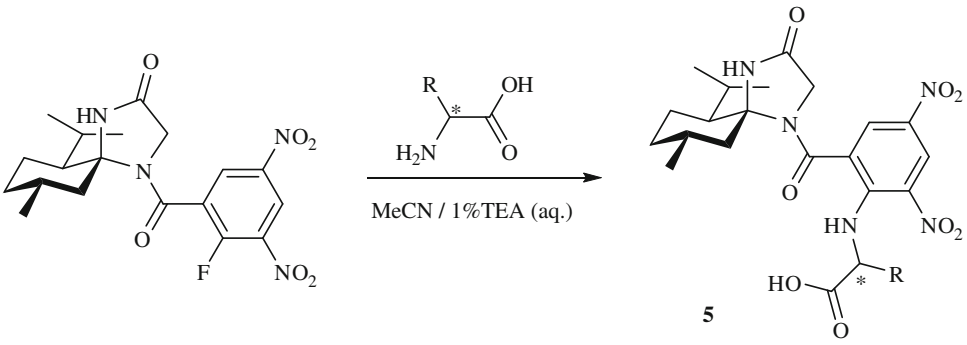


Fig. 1 *Left* UV spectrum of **1**; $c = 6.6 \times 10^{-5}$ mol/l in acetonitrile, $d = 0.5$ cm, *right* UV spectrum of **1** + valine, $c = 6.6 \times 10^{-5}$ mol/l in aqueous 1% TEA solution

Scheme 3 Derivatization of amino acids with reagent **1**



separate only two diastereomers, isocratic conditions from 70:30 to 90:10 were used.

Figure 2 demonstrates the advantage of the marker function exemplarily shown for derivatized DL-valine. The picture at the left shows an integrated chromatogram from 220 to 400 nm. The highest peak represents the excess of the derivatizing reagent **1**. The same sample detected at a fixed wavelength at 400 nm (illustrated at the right chromatogram in Fig. 2) shows only the two diastereomers of the derivatized amino acid. The area under the two peaks and of each measured amino acid racemate (Table 1) is equal (within the margin of error). An equal absorption of the racemates is necessary if one wants to calculate the enantiomeric excess of unknown samples.

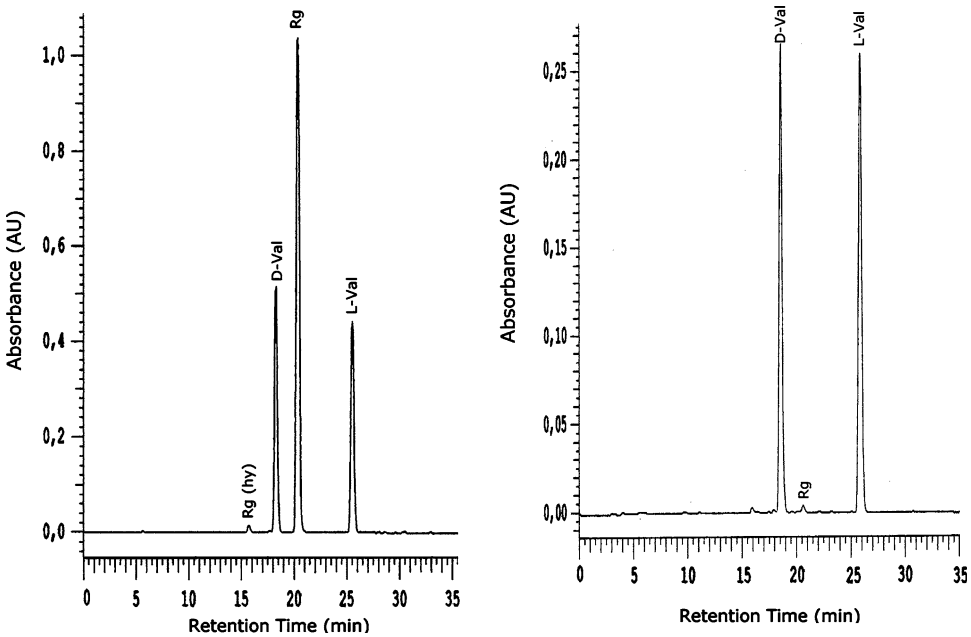


Fig. 2 HPLC chromatogram of valine derivatized with reagent **1**; *left* integrated chromatogram from $\lambda = 220$–400 nm, *right* the same sample at $\lambda = 400$ nm; MZ Kromasil RP 18, 0.6 ml/min; gradient MeCN/ 0.1% TFA 50:50 → 80:20 in 60 min

Table 1 Enantioseparation of amino acids after pre column derivatization with reagent **1** on an achiral phase (MZ Kromasil RP 18)

Number	DL-Amino acid	k^D	k^L	α
S1[a]	Tle	0.20	0.76	3.72
S2[a]	Phe	0.23	0.51	2.23
S3[b]	Lys	0.82	1.26	1.54
S4[c]	Val	0.99	2.20	2.23
S5[d]	Asn	1.16	0.95	1.23
S6[d]	Thr	1.78	2.91	1.64
S7[d]	Ser	1.87	1.32	1.42
S8[d]	Ala	2.72	4.13	1.51
S9[d]	Met	2.96	5.79	1.96
S10[d]	Abu	3.60	4.85	1.35
S11[d]	Trp	3.97	5.91	1.49
S12[d]	Val	4.12	6.81	1.65
S13[d]	Phg	4.16	5.69	1.37
S14[d]	Tle	4.19	8.77	2.09
S15[d]	Pro	4.20	4.36	1.04
S16[d]	Nor	4.45	6.97	1.56
S17[d]	Phe	4.54	7.48	1.65
S18[d]	Ile	5.34	8.12	1.52
S19[d]	Leu	6.07	9.24	1.52

Detection at $\lambda = 350$ nm

[a] MeCN/1%TFA 90:10, 0.8 ml/min

[b] MeCN/1%TFA 80:20, 0.8 ml/min

[c] MeCN/1%TFA 70:30, 0.8 ml/min

[d] Gradient MeCN/1%TFA 50:50 → 80:20 in 60 min, 0.6 ml/min; $k = t_r/t_0$; $\alpha = k''/k'$

In this way, one can easily determine the enantiomeric excess of amino acids, with the advantage of getting to see only the derivatized amino acids and not the excess of the derivatizing reagent or the impurities of a probe which do not react with the marker **1**.

Furthermore, the separation of the formed diastereomers is very good. Besides DL-valine, we tested 15 other amino acids which all could be baseline separated easily (Table 1, S1–S19). We observed that the elution order of DL-amino acids (derivatized with **1**) is normally first the D- than the L-isomer. The reverse elution order was only observed by amino acids with very polar side chains like Ser or Asn. Most separations shown in Table 1 were carried out by gradient elution MeCN/1% TFA 50:50 to 80:20 in 60 min. This method was primarily developed to separate amino acid mixtures. For the analysis of only one enantiomeric pair one can accelerate the separation time dramatically by the use of more polar conditions (compare Table 1, S1 with S14, S2 with S17 and S4 with S12).

Figure 3 shows the separation of an amino acid cocktail. In less than 45 min 10 different DL-amino acids were separated after derivatization with **1**.

Conclusion

In summary, the synthesis and application of a new chiral derivatizing agent for amino acids are reported that conjoins a lot of positive characteristics: the synthesis of the reagent is easy, the educts are cheap, both enantiomers are accessibly due to commercial availability of (+)- and (−)-menthone, the reagent and its solution are stable over a long time and derivatizations are fast and need no work up. The separations of our tested derivatized amino acids on reverse-phase HPLC were excellent and the derived diastereomers show the same absorption, so one is able to determine the enantiopurities of amino acids very easily.

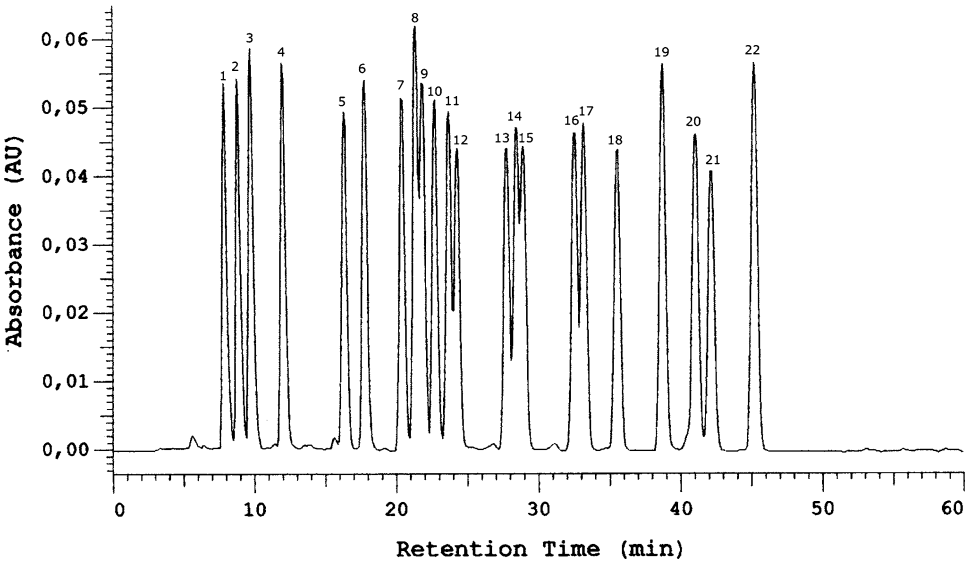


Fig. 3 Enantioseparation of 10 DL-amino acids after diastereomer formation with **1** on an achiral HPLC column. Conditions: phase MZ Kromasil RP 18; gradient MeCN/0.1% TFA 50:50 → 80:20 in 60 min, 0.6 ml/min; detection $\lambda = 300$–400 nm (integrated); peaks: *1* L-Asn, *2* D-Asn, *3* L-Ser, *4* D-Ser, *5* D-Met, *6* hydrol. reagent, *7* D-Trp, *8* D-Val, *9* D-Tle, *10* D-Nor, *11* D-Phe, *12* reagent, *13* L-Met; *14* L-Trp; *15* D-Leu; *16* L-Val; *17* L-Nor; *18* L-Phe; *19* D-Lys; *20* L-Tle; *21* L-Leu; *22* L-Lys

Conversion of **3** with other chiral auxiliaries for instance menthol or prolinamide etc. led to less satisfying results.

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