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Application of (1*S*,2*S*)- and (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propylisothiocyanate to the indirect enantioseparation of racemic proteinogenic amino acids

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Abstract

The application of (1*S*,2*S*)- or (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propylisothiocyanate as a new chiral derivatizing agent for the resolution of compounds possessing an amino group is described. The reagent is easily accessible in both enantiomeric forms after a simple two-step synthesis. Its applicability was demonstrated on the example of the resolution of a series of α-amino acids. The diastereomeric thiourea derivatives produced were separated by reversed-phase high-performance liquid chromatography. The effects of pH, temperature and reagent excess on the derivatization kinetics were investigated, as were the effects of pH and organic modifier on the separation.

Keywords: Enantiomer separation; Derivatization, LC; Diacetoxynitrophenylpropylisothiocyanate; Amino acids

1. Introduction

Separation of the enantiomers of a racemate by applying chiral derivatizing agents (CDAs) is widely used in high-performance liquid chromatography (HPLC). This indirect resolution involves pre-column derivatization of the enantiomers with a CDA, and subsequent separation of the diastereomers formed on achiral HPLC columns. Its popularity does not seem to have decreased, despite the appearance of highly effective direct methods [1,2]. The CDAs developed for this purpose [3–7] include many isothiocyanate-based compounds [8–11]. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC or TAGIT) was introduced almost 20 years ago [10], but is still one of the most widely applied CDAs. *N*-[(2-Isothiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide (CDITC) [11] and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS) [12] are highly effective fluorescence-active, chiral tagging reagents.

The isothiocyanate group is selective towards primary and secondary amines under mild conditions, no other free functional groups need to be protected, and the thiourea derivatives produced allow very sensitive UV detection. However, the commercially available reagents are sometimes rather expensive, or available in only one enantiomeric form.

The present paper describes a new isothiocyanate-containing CDA, (1*S*,2*S*)- or (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propylisothiocyanate [(*S*,*S*)- or (*R*,*R*)-DANI], and demonstrates its applicability on

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the example of the separation of α-amino acid enantiomers (for preliminary results, see Ref. [13]). The diastereomers formed were analyzed on a reversed-phased (C_{18}) column, mixtures of 0.1% aqueous trifluoroacetic acid (TFA) and methanol (MeOH) or acetonitrile (MeCN) being used for elution. The conditions of the derivatization (pH, reagent excess and temperature) were optimized. The effects of the pH of the eluent and the organic modifier on the separation, and the elution sequence of the thioureas, were investigated in detail.

2. Experimental

2.1. Chemicals and reagents

TFA, acetic acid and triethylamine (TEA) of analytical reagent grade, and MeOH and MeCN of HPLC grade were obtained from Merck. 0.1% aqueous TFA and 0.1 *M* sodium acetate buffers were prepared with triply distilled water and purified further by filtration through a 0.45-μm Millipore filter, type HV (Molsheim, France). Racemic and enantiomerically pure amino acids were commercial products of Sigma (St. Louis, MO, USA). Glacial acetic acid, acetyl chloride, sodium hydrogencarbonate and thiophosgene of analytical reagent grade were from Aldrich (Steinheim, Germany). (1*S*,2*S*)- and (1*R*,2*R*)-2-amino-1-(4-nitrophenyl)-1,3-propanediol were generous gifts of Egis Pharmaceuticals (Budapest, Hungary).

2.2. Apparatus

The HPLC system consisted of an M-600 low-pressure gradient pump equipped with an M-486 tunable absorbance detector and an M-996 photodiode array detector, and Millenium software version 2.1 (Waters Chromatography, Milford, MA, USA). The injector with a 20-μl loop was from Rheodyne (Cotati, CA, USA). Reversed-phase analyses were performed on a Nova-Pak C_{18} column, 150×3.9 mm I.D., 4 μm particle size (Waters Chromatography).

2.3. Other experimental methods

IR spectra were recorded with a Perkin-Elmer Paragon 1000 Fourier transform (FT) IR spectrometer (Perkin-Elmer, Beaconsfield, UK). Optical rotations were measured with a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Überlingen, Germany). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DRX 400 spectrometer (Bruker Analitische Messtechnik, Rheinstetten-Forchheim, Germany).

2.4. Synthesis and features of (S,S)- and (R,R)-DANI

The reagent is readily available in both enantiomeric forms after a straightforward two-step synthesis (Fig. 1). The (*S*,*S*) form of 2-amino-1-(4-nitrophenyl)-1,3-propanediol (**1**, Fig. 1) is a side-product of chloramphenicol synthesis, while the (*R*,*R*) form is the effective isomer. The L-base [(*S*,*S*) form] was earlier successfully employed for the resolution of racemic chiral phosphoric acids and amino acids [14]. As chloramphenicol synthesis is carried out on a large scale, both enantiomers are available in chirally pure form in large quantities.

Starting from the trifunctional (1*S*,2*S*)-**1**, the first reaction step was performed in glacial acetic acid with acetyl chloride as acylating reagent. The resulting hydrochloride of the 1,3-diacetoxy analogue of the starting compound was treated with thiophosgene in a biphasic system in the presence of sodium hydrogencarbonate, to furnish the corresponding DANI (**2**). The crude product was purified by distillation or on a silica gel column (dichloromethane–methanol, 10:0.1, v/v). The overall yield for the enantiomer (1*S*,2*S*)-**2** was 60% [13]. Its physicochemical data: a pale-yellow oil; R_F=0.72 (toluene–methanol, 4:1); $[\alpha]_D^{23}$=+72.4 (c=1, MeOH); IR (KBr) 2039 cm^{-1} very intense, broad absorption band ($\nu_{N=C=S}$); ^{1}H NMR δ (ppm) in C^2HCl_3: 2.13 (3H, s, 3-$OCOCH_3$), 2.23 (3H, s, 1-$OCOCH_3$), 4.01–4.06 (1H, dd, J=11.2 Hz, 6.3 Hz, 3-H), 4.21–4.24 (1H, ddd, J=6.3 Hz, 5.0 Hz, 5.0 Hz, 2-H), 4.29–4.33 (1H, dd, J=11.2 Hz, 5.0 Hz, 3-H), 5.98–6.00 (1H, d, J=5.0 Hz, 1-H), 7.55–7.57 and 8.27–8.30 (2H, d, J=8.7 Hz, Ar-H); ^{13}C NMR δ (ppm) in C^2HCl_3: 20.02 (1C), 21.17 (1C), 60.68 (1C), 62.85 (1C), 73.12 (1C), 124.54 (2C), 128.05 (2C), 139.69 (1C), 143.23 (1C), 148.64 (1C), 169.69 (1C), 170.52 (1C).

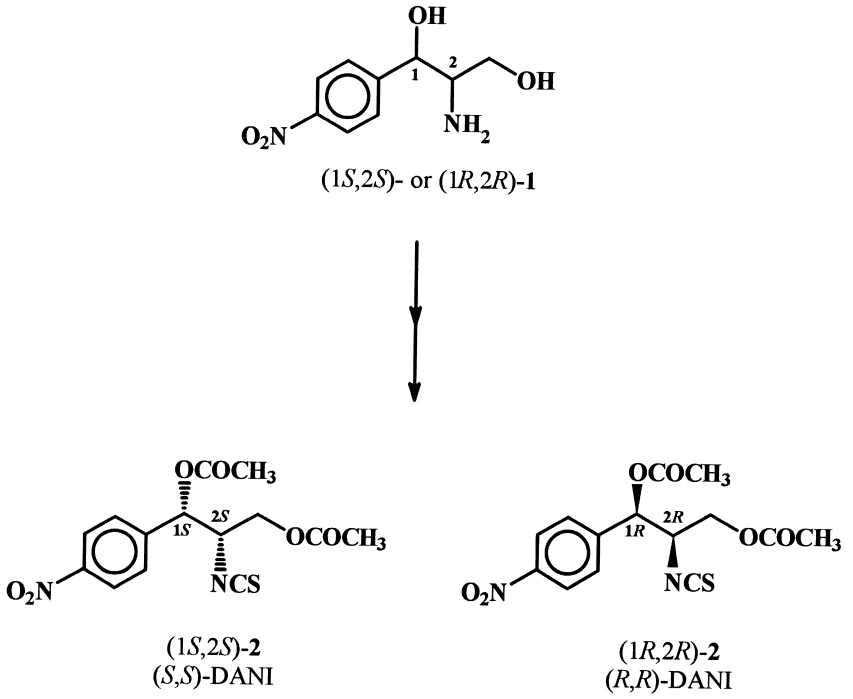

Fig. 1. Synthesis and structure of (*S*,*S*)- or (*R*,*R*)-DANI.

(*R*,*R*)-DANI [(1*R*,2*R*)-**2**] was obtained from the appropriate (1*R*,2*R*)-**1**. Yield 59%; $[\alpha]_D^{23} = -73.2$ ($c = 1$, MeOH); other data are the same as for (1*S*,2*S*)-**2**.

An advantage of the reagent is that it is stable under a nitrogen atmosphere in a refrigerator without decomposition for several months. Its solution in MeCN is likewise stable for at least five months, even at room temperature.

2.5. Chiral purity of the reagent

(*S*,*S*)- and (*R*,*R*)-DANI were derivatized with (*S*)-Val of known enantiomeric purity (Aldrich 99%). The enantiomeric purity of both reagent enantiomers was found to be 99%. (Detection limit ca. 0.1% *R* enantiomer in an excess of *S*.)

2.6. Derivatization procedure

A 30 m*M* aqueous solution of the amino acid was diluted to 5 m*M* final concentration with 0.4% TEA (in MeCN–water, 1:1, pH~11). To 100 μl of this solution, 100 μl of the reagent (10 m*M* in MeCN) was added; the ratio of the reagent to the amino acid was 2:1. The mixture was thermostated at 60°C for 2 h. The derivatives produced were injected onto a reversed-phase HPLC column after a five-fold dilution with the eluent, the pH of the samples thus being weakly acidic (pH~5.5).

If necessary, the excess of the reagent can be removed with glycine. To a 20-μl aliquot of the reaction mixture, was added 50 μl of glycine (50 m*M* in water) and the mixture was thermostated for a further 30 min at 60°C.

The derivatized amino acids were detected at 245 nm. The UV spectra of (*S*,*S*)-DANI and a corresponding thiourea derivative are depicted in Fig. 2.

3. Results and discussion

3.1. Derivatization kinetics

The derivatized samples can be stored in a refrigerator without significant decomposition for one

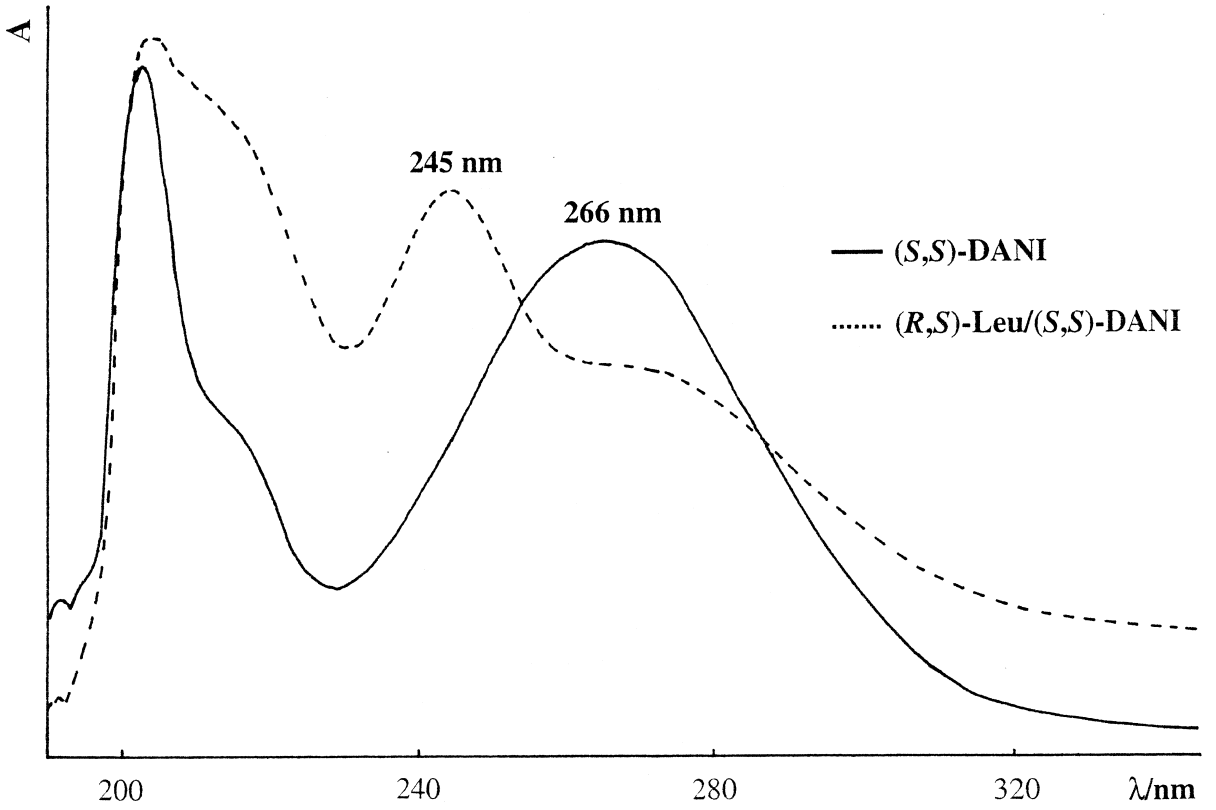

Fig. 2. UV spectra of (*S*,*S*)-DANI and (*R*,*S*)-Leu/(*S*,*S*)-DANI. Column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, photodiode array; eluent, TFA–MeOH (45:55, v/v).

week. No racemization of either the CDA or the amino acids was observed under any of the reaction conditions tested.

3.1.1. Effect of pH

Derivatizations were carried out in an MeCN–aqueous TEA (1:1) mixture with (*R*,*S*)- and separately with (*R*)- and (*S*)-Val, using (*S*,*S*)-DANI as CDA. The pH of the aqueous component was varied via change of the concentration of TEA. Reaction yields were compared at different pH values. Fig. 3 clearly shows that there is practically no derivatization of (*R*,*S*)-Val with (*S*,*S*)-DANI at pH<9. The same results were obtained when (*R*)- or (*S*)-Val was derivatized with (*S*,*S*)- or (*R*,*R*)-DANI. For further reactions, therefore, 0.4% aqueous TEA (pH~11) was used.

3.1.2. Effect of reagent excess

In order to prevent any kinetic resolution, it is advisable to use an excess of CDA. It is also crucial for the derivatization to proceed quantitatively. The reactions were followed on thin-layer chromatography (TLC) by visualizing the spots with ninhydrin. When the integrated peak area reached a maximum, the TLC experiments did not demonstrate any unreacted amino acid, thereby indicating that derivatization was quantitative.

The effect of the reagent excess on the derivatization was studied at 60°C with representative racemic amino acids: Ala (neutral), Val (neutral, sterically hindered), Phe (aromatic), Asp (acidic) and Arg (basic). (*S*,*S*)-DANI was used for derivatization. For illustration, data on (*R*,*S*)-Val are shown in Fig. 4. At CDA:amino acid=2:1, the reaction was com-

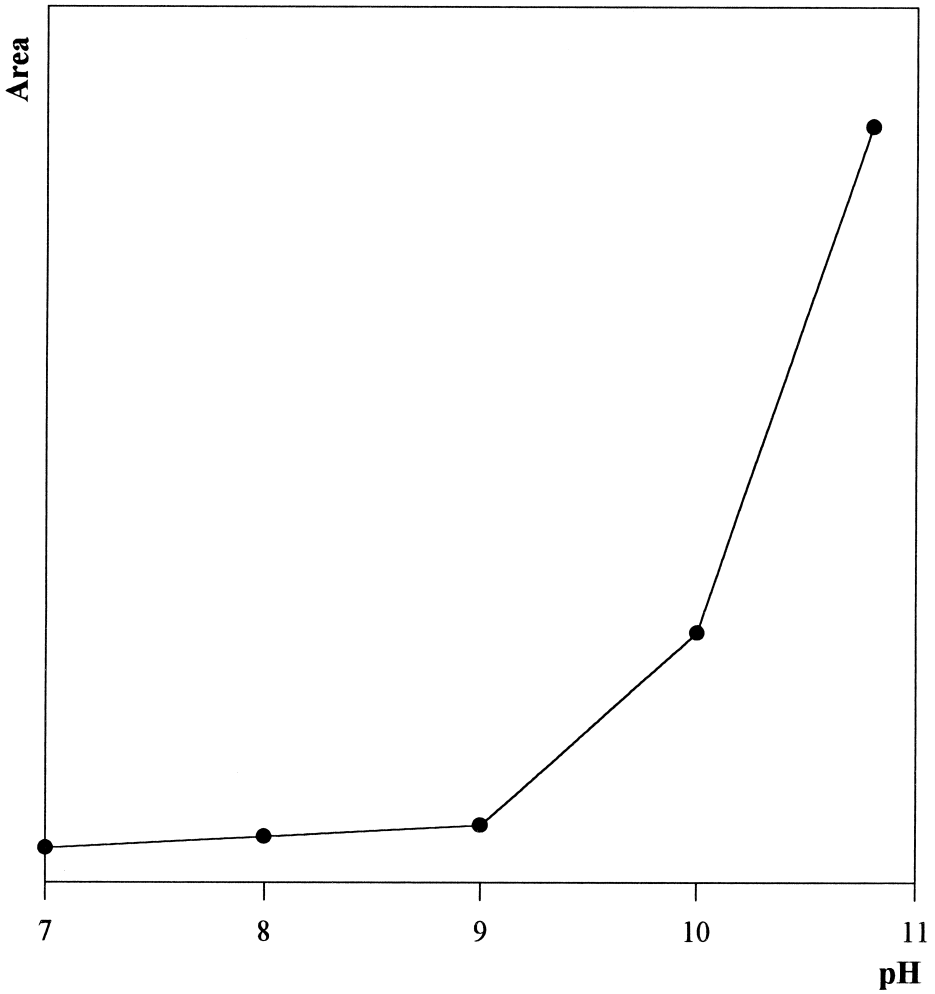

Fig. 3. pH dependence of reaction yield of (*R*,*S*)-Val/(*S*,*S*)-DANI derivatives [only the results for (*R*)-Val/(*S*,*S*)-DANI are shown]. Conditions of derivatization: temperature, ambient; time, 3 h; molar ratio of CDA:amino acid=2:1. Chromatographic conditions: column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, 245 nm.

plete within 2 h. At a lower ratio (CDA:amino acid=1:1 or 1.5:1), a very slight kinetic resolution, caused by the different reaction rates of the enantiomers, was detected. At a higher reagent excess (CDA:amino acid=5:1), the derivatization goes to completion within a shorter time, but it is recommended to use an optimum excess of CDA, especially if elimination of the reagent peak is necessary. As concerns the other amino acids, the same conclusions can be drawn. The use of a twofold excess of CDA is therefore proposed.

3.1.3. Effect of temperature

The effect of temperature on the reaction yield with the above-mentioned amino acids was studied at CDA:amino acid=2:1. The results were very similar for both enantiomers and for different analytes. Temperature rise favorably affected the reaction times (Fig. 5). At room temperature, 7 h was necessary for complete derivatization; at 60°C, the reaction time was shortened to 2 h and the higher temperature did not decrease the stability of the diastereomers.

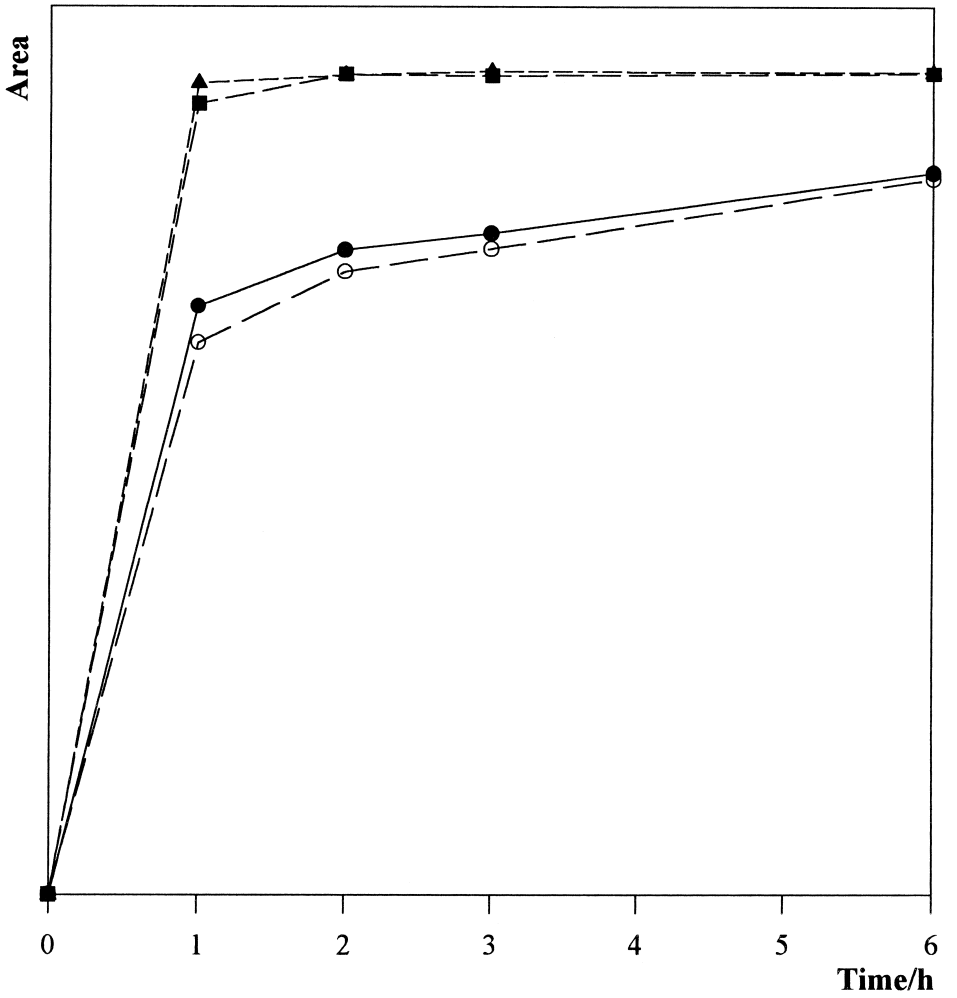

Fig. 4. Effect of reagent excess on yield of derivatization of (*R*,*S*)-Val/(*S*,*S*)-DANI. Conditions of derivatization: pH, ~11; time, 0–6 h; temperature, 60°C. Chromatographic conditions: column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, 245 nm. ●, CDA:amino acid=1:1, (*R*)-Val; ○, CDA:amino acid=1:1, (*S*)-Val; ■, CDA:amino acid=2:1, (*R*)- and (*S*)-Val; ▲, CDA:amino acid=5:1, (*R*)- and (*S*)-Val.

Derivatizations carried out under the optimized conditions resulted in diastereomer peaks with similar peak areas for the different amino acids examined. The only exception was Lys, which formed bis derivatives; the relative peak area of such a derivative was about twice that in the other cases. The derivatization kinetics was practically the same when (*R*,*R*)-DANI was used as CDA.

In consequence, the optimized conditions were well applicable for all the amino acids tested in this study. However, the reaction times and yields may be affected by other functionalities present besides the amino group in the analyte, and also by steric hindrance. A check on the quantitativeness of derivatizations is therefore proposed for new compounds, as also recommended by other authors [9].

3.2. HPLC analyses

3.2.1. Effect of pH of mobile phase on separation

To perform analyses on a reversed-phase column and to ensure reproducibility, the degree of ionization of the carboxylate group should be kept constant. Acidic eluents are therefore required. The

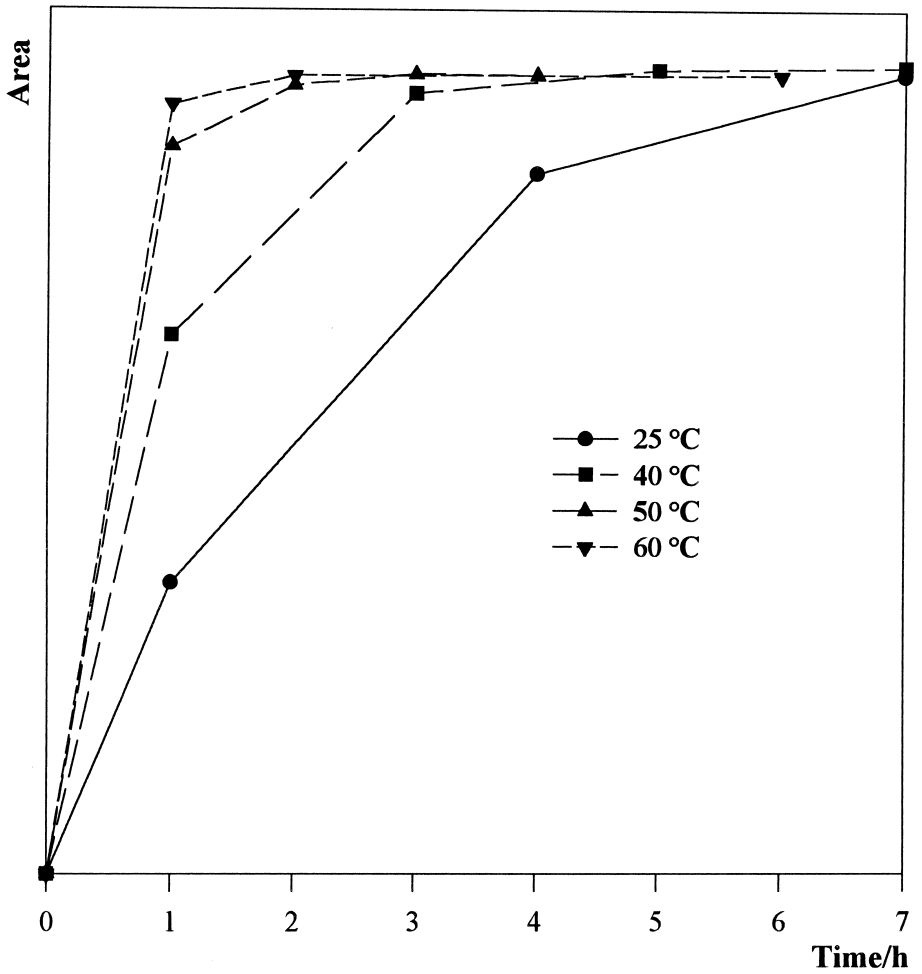

Fig. 5. Effect of temperature on yield of derivatization of (*R*,*S*)-Val/(*S*,*S*)-DANI. Conditions of derivatization: pH, ~11; time, 0–7 h; temperature, 25–60°C; molar ratio of CDA:amino acid=2:1. Chromatographic conditions: column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, 245 nm. Only the results for (*R*)-Val/(*S*,*S*)-DANI are shown.

effect of pH on the separation was investigated in the pH range 2–4.6. In order to ensure the constant ionic strength of the mobile phase, acetate buffers of different pH were prepared from 0.1 *M* aqueous sodium acetate with acetic acid. The results obtained for Val derivatives with (*S*,*S*)-DANI are listed in Table 1. Besides the retention factors of the diastereomers, the *k* value of the excess of CDA (k_3) is indicated. The eluent with pH 3 displayed the best selectivity. The α value achieved with the mobile phase with pH 2 was also very good and the resolution was satisfactory. With regard to the shorter retention times of the diastereomers and also the

Table 1
Effect of pH on separation of (*S*,*S*)-DANI-derivatized Val diastereomers[a]

pH	k_1	k_2	α	R_S	k_3
2.0	1.90	2.60	1.37	1.52	5.62
3.0	4.79	6.81	1.42	2.13	17.42
4.0	2.36	3.02	1.28	1.00	17.54
4.6	1.51	1.94	1.28	0.69	17.04

[a] Column, Nova-Pak C_{18} 150×3.9 mm I.D. (4 μm); flow-rate, 0.8 ml min^{-1}; detection, 245 nm; mobile phase composition, 0.1 *M* sodium acetate (pH as indicated)–MeOH (50:50, v/v). k_1: Retention factor of first-eluting diastereomer; k_2: retention factor of second-eluting diastereomer; k_3: retention factor of reagent peak.

essentially shorter overall analysis time, the use of eluents with pH 2 is proposed. (It was also found for several other amino acids that a retention time longer about 30–40% is needed to obtain a similar resolution with the mobile phase with pH 3 than for pH 2.) For this, 0.1% aqueous TFA (pH~2) was applied.

3.2.2. Effect of organic modifier

Chromatographic parameters of the (*S*,*S*)-DANI-derivatized amino acids are listed in Tables 2 and 3. All of the examined amino acids were separated with at least one organic modifier (MeOH or MeCN). As concerns the selectivity of the organic modifiers, significant differences were observed. As regards the number of diastereomers that can be separated, MeOH seems to be more effective. In the cases when both MeOH and MeCN could be applied successfully, MeOH usually resulted in shorter retention times and similar or better R_s values than those for MeCN. However, in the cases of Ser and Trp, no separation occurred with MeOH-containing mobile phases, whereas MeCN-containing eluents did allow appropriate resolution of the derivatives. Chromatograms of the derivatized amino acids may be seen in Figs. 6 and 7.

Table 2
Chromatographic data on (*S*,*S*)-DANI-derivatized amino acids with MeOH as organic modifier[a]

Compound	Eluent	k_1	k_2	α	R_s	e.s.[b]
Gly	F	1.42	–	–	–	–
Asp	B	25.18	28.66	1.14	1.50	*R*<*S*
Glu	A	35.55	38.88	1.09	1.15	*R*<*S*
Arg	B	10.39	13.17	1.27	1.76	*S*<*R*
His	B	10.66	14.26	1.34	2.28	*S*<*R*
Lys[c]	E	35.66	40.31	1.13	1.45	*R*<*S*
Asn	A	20.58	22.76	1.11	1.13	*S*<*R*
Gln	A	19.83	22.04	1.11	1.17	*S*<*R*
Trp	E	22.00	22.00	1.00	0.00	–
Met	E	8.03	10.66	1.33	2.27	*R*<*S*
Ser	B	15.33	15.33	1.00	0.00	–
Thr	D	6.85	7.66	1.12	1.83	*R*<*S*
Pro	C	7.64	9.55	1.25	1.78	*R*<*S*
Ala	E	4.02	5.47	1.36	1.57	*R*<*S*
Val	F	3.39	4.86	1.43	1.75	*R*<*S*
Leu	F	5.91	8.44	1.43	2.83	*R*<*S*
Ile	F	5.60	8.65	1.54	3.28	*R*<*S*
Phe	F	8.20	11.71	1.43	2.67	*R*<*S*
Tyr	D	15.88	17.86	1.12	1.26	*R*<*S*

[a] Column, Nova-Pak C_{18} 150×3.9 mm I.D. (4 μm); flow-rate, 0.8 ml min^{-1}; detection, 245 nm; mobile phase compositions, TFA–MeOH (v/v), (A) 70:30, (B) 65:35, (C) 60:40, (D) 55:45, (E) 50:50, (F) 45:55.
[b] Elution sequence.
[c] Bis derivative.

Table 3
Chromatographic data on (*S*,*S*)-DANI-derivatized amino acids with MeCN as organic modifier[a]

Compound	Eluent	k_1	k_2	α	R_s	e.s.[b]
Gly	C	5.10	–	–	–	–
Asp	A	30.72	32.41	1.06	0.54	*R*<*S*
Glu	A	25.70	25.70	1.00	0.00	–
Arg	A	16.51	19.82	1.20	1.56	*S*<*R*
His	B	4.58	5.61	1.22	1.26	*S*<*R*
Lys[c]	E	11.36	12.95	1.14	1.47	*S*<*R*
Asn	A	16.27	16.27	1.00	0.00	–
Gln	A	14.31	14.31	1.00	0.00	–
Trp	C	33.20	37.86	1.14	1.31	*S*<*R*
Met	C	15.73	17.46	1.11	1.24	*R*<*S*
Ser	A	18.87	21.11	1.12	1.30	*R*<*S*
Thr	B	9.33	11.61	1.24	2.12	*R*<*S*
Pro	B	13.95	16.57	1.19	1.93	*R*<*S*
Ala	C	6.35	7.49	1.18	1.41	*R*<*S*
Val	C	16.81	19.76	1.18	1.98	*R*<*S*
Leu	C	25.34	27.88	1.10	1.30	*R*<*S*
Ile	C	26.67	30.52	1.14	1.60	*R*<*S*
Phe	C	6.37	6.77	1.06	0.50	*S*<*R*
	D	14.66	14.66	1.00	0.00	–
	E	35.26	36.93	1.05	0.53	*R*<*S*
Tyr	C	11.31	12.12	1.07	0.94	*S*<*R*

[a] Column, Nova-Pak C_{18} 150×3.9 mm I.D. (4 μm); flow-rate, 0.8 ml min^{-1}; detection, 245 nm; mobile phase compositions, TFA–MeCN (v/v), (A) 80:20, (B) 75:25, (C) 70:30, (D) 65:35, (E) 60:40.
[b] Elution sequence.
[c] Bis derivative.

3.2.3. Sequence of elution

The sequence of elution of the diastereomers was studied when (*S*,*S*)-DANI was used for derivatization. Besides other important factors, hydrogen bonding may play a significant role in establishing the conformational differences between the diastereomers, but no general rule could be found for the elution sequence of the thiourea derivatives.

For acidic amino acids (Asp and Glu), the (*R**, *S*,*S*) diastereomer eluted before the (*S**, *S*,*S*) derivative when they were separable (the asterisk refers to the configuration of the asymmetric center in the analyte).

Of the basic amino acids, Lys formed bis deriva-

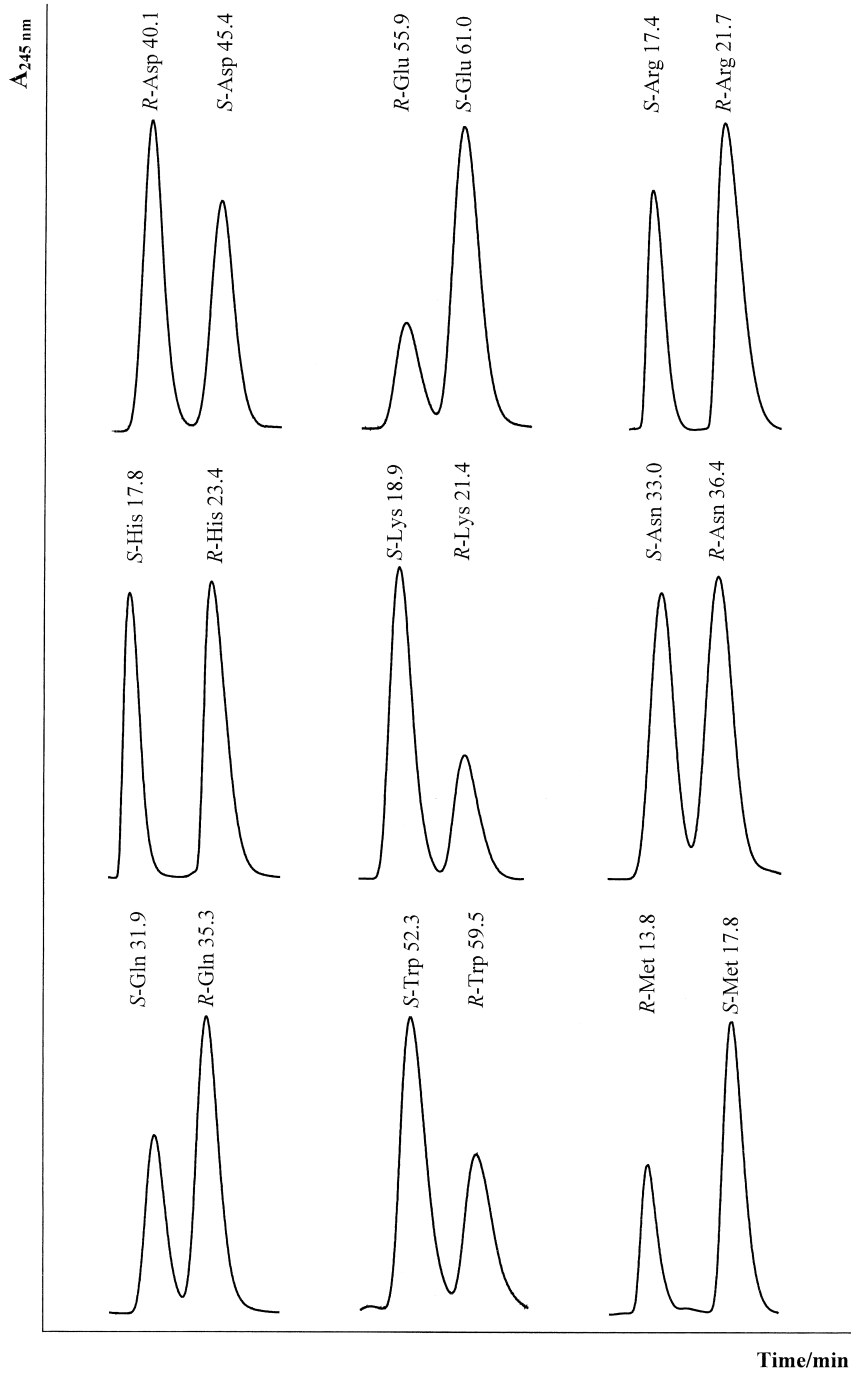

Fig. 6. Chromatograms of amino acid diastereomers formed with (*S*,*S*)-DANI. Column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, 245 nm; eluent (v/v), Asp TFA–MeOH (65:35) (*R*:*S*=5:4), Glu TFA–MeOH (70:30) (*R*:*S*=1:3), Arg TFA–MeOH (65:35) (*R*:*S*=2:1), His TFA–MeOH (65:35) (*R*:*S*=2:1), Lys TFA–MeCN (60:40) (*R*:*S*=1:2), Asn TFA–MeOH (70:30) (*R*:*S*=5:4), Gln TFA–MeOH (70:30) (*R*:*S*=3:2), Trp TFA–MeCN (70:30) (*R*:*S*=1:2), Met TFA–MeOH (50:50) (*R*:*S*=1:2). *R*:*S* means the molar ratio of the diastereomers to be analyzed.

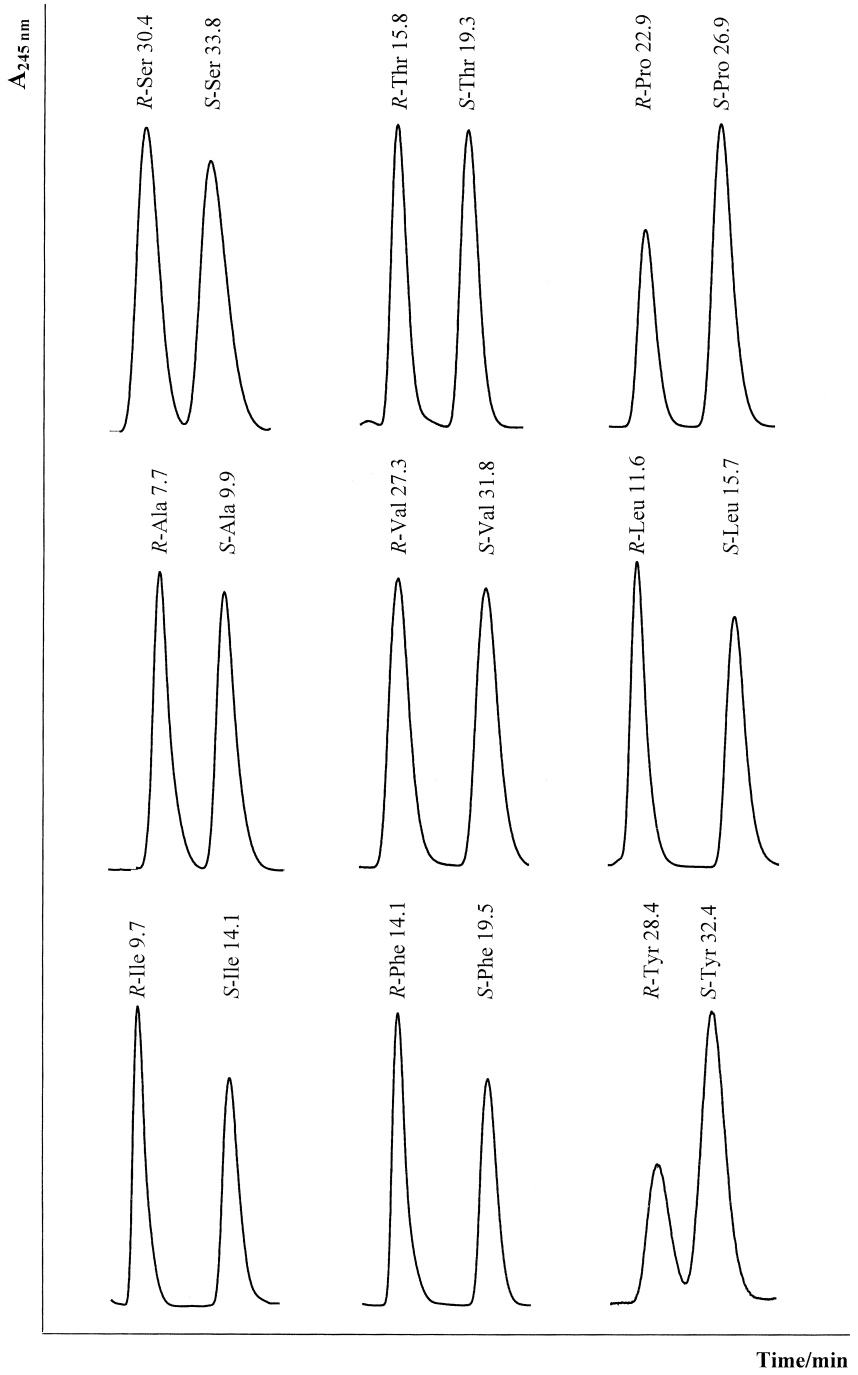

Fig. 7. Chromatograms of amino acid diastereomers formed with (*S*,*S*)-DANI (continued). Column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, 245 nm; eluent (v/v), Ser TFA–MeCN (80:20) (*R*:*S*=1:1), Thr TFA–MeCN (75:25) (*R*:*S*=1:1), Pro TFA–MeCN (75:25) (*R*:*S*=1:2), Ala TFA–MeOH (50:50) (*R*:*S*=1:1), Val TFA–MeCN (70:30) (*R*:*S*=1:1), Leu TFA–MeOH (45:55) (*R*:*S*=1:1), Ile TFA–MeOH (45:55) (*R*:*S*=1:1), Phe TFA–MeOH (45:55) (*R*:*S*=1:1), Tyr TFA–MeOH (55:45) (*R*:*S*=1:2). *R*:*S* means the molar ratio of the diastereomers to be analyzed.

tives; with the MeOH-containing eluent, $R<S$, while with the MeCN-containing eluent, the sequence was $S<R$. In the other two cases, i.e., for Arg and for His, where the additional NH or NH_2 remains free, the sequence was $S<R$ with both organic modifiers.

In most cases, the sequence of elution for neutral amino acids was found to be $R<S$ with both MeOH- and MeCN-containing mobile phases. However, derivatives where the amino acid contained another NH or NH_2 besides the α-NH_2 (Asn, Gln or Trp) or an aromatic side-chain (Phe and Tyr) were exceptions. Due to the presence of the other NH in the cases of Asn, Gln, Trp and also the basic Arg and His, the amino acid probably can form an additional hydrogen bond, which may contribute to the $S<R$ elution sequence.

As concerns the aromatic amino acids, (*R,S*)-Phe exhibited unusual chromatographic behavior. In MeCN-containing eluents, not only was the R_s value very low (in contrast with the excellent resolution obtained with the MeOH-containing mobile phase, $R_s=2.67$), but the elution sequence of the diastereomers was also changed by variation of the amount of MeCN in the eluent. At TFA–MeCN (70:30, v/v), the elution sequence was $R<S$ ($R_s=0.50$). At TFA–MeCN (65:35, v/v), complete coelution of the peaks was observed, while at (60:40, v/v) the sequence was $S>R$ ($R_s=0.53$). For the other aromatic amino acid, i.e., Tyr, change of the organic modifier from MeOH to MeCN also resulted in a reversal of the elution sequence, but the resolution did not alter dramatically (from $R_s=1.26$ to $R_s=0.96$).

3.2.4. Possibility for appropriate selection of elution sequence

In the determination of enantiomeric impurity, it may be important to ensure that the impurity elutes before the main peak [2,15]. This can easily be done if the CDA is available in both enantiomeric forms.

(*R,S*)-Val was derivatized with both (*S,S*)- and (*R,R*)-DANI. As expected, the sequence of elution of the diastereomers was different when the opposite reagent enantiomer was applied (Fig. 8). It is also clear from Fig. 8 that the amount of the impurity can be determined much more precisely when the small peak elutes first; and the difference in the accuracy of determination in the two cases increases with decreasing impurity.

The limit of detection was determined for Val to be 0.16 nmol ml^{-1} at 245 nm.

4. Conclusions

The results demonstrate that the new reagent is capable of the resolution of a series of amino acids.

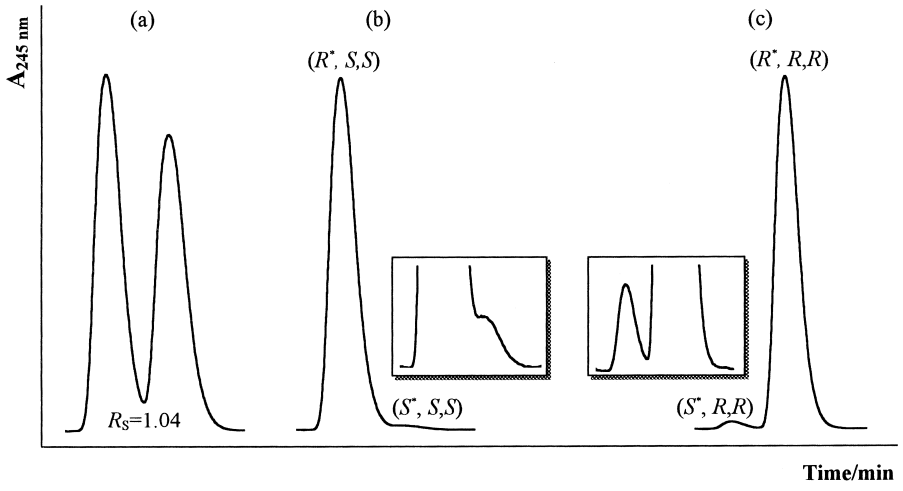

Fig. 8. Selection of the elution sequence. Column, Nova-Pak C_{18}; flow-rate, 0.8 ml min^{-1}; detection, 245 nm; eluent, TFA–MeCN (68:32, v/v); analyte, Val; $k_1=13.4$, $k_2=14.6$. (a) (*R,S*)-Val/(*S,S*)-DANI or (*R,S*)-Val/(*R,R*)-DANI; (b) (*R*)-Val/(*S,S*)-DANI:(*S*)-Val/(*S,S*)-DANI=100:1.2; (c) (*R*)-Val/(*R,R*)-DANI:(*S*)-Val/(*R,R*)-DANI=100:1.9.

Since the isothiocyanate group in DANI is attached to a chiral center, the optimum distance between the asymmetric centers in the reagent and in the analyte (as an essential requirement for the production of separable diastereomeric derivatives) is ensured. A further important structural feature, the conformational rigidity of the derivatives, is also provided by the two *O*-acetyl groups in the 1,3 position. Besides its high stability and simple synthesis, a further major advantage of the new reagent is the possibility of appropriate selection of the elution sequence since it is available in both enantiomeric forms. This can be important in the detection and determination of enantiomeric purity.

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