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RESEARCH PAPER

2-Acyl-dimedones as UV-active protective agents for chiral amino acids: enantiomer separations of the derivatives on chiral anion exchangers

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Abstract 2-Acetyldimedone and 12 related compounds were employed as UV-active pre-column derivatizing agents for amino acids. Direct enantioseparation of the products was achieved using chiral anion exchanger stationary phases in polar-organic mobile phase mode. Under basic conditions, the reagents´ cyclic β-tricarbonyl motifs can give rise to exo- and endocyclic enols through tautomerization. However, with primary amines (proteinogenic and unusual amino acids, aminosulfonic and aminophosphonic acids), we exclusively observed the formation of exocyclic enamine-type products. Reaction yields depended strongly on the 2-acyl modification of the reagent; in particular, we observed a significant decrease when electronegative or sterically demanding substituents were present in α-position to the exocyclic carbonyl group. In addition to improving UV detectability of the products, the introduction of this protective group facilitated successful enantiomer separations of the amino acid derivatives on *Cinchona*-based chiral anion exchangers. Particularly high enantiomer selectivity was observed in combination with stationary phases bearing a new variation of selectors with π-acidic (electron-poor) bis(trifluoromethyl)phenyl groups. No racemization of the analytes occurred at any stage of the analytical method including the deprotection, which was achieved with hydrazine.

Keywords Amino acids/peptides · Chiral analysis · HPLC · Ion chromatography/ion exchange · UV/VIS

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Introduction

State-of-the-art amino acid analysis requires efforts beyond the assessment of "just" the chemical composition of a sample (i.e. the constituting amino acids, their modifications and their respective quantities). Many physiological processes involve enantiomerically pure amino acids, peptides and proteins and are therefore highly enantiospecific. Thus, information on the stereochemical composition (the presence and percentage of D-amino acids) is of fundamental importance, especially in samples of pharmaceutical relevance.

The natural pool of proteinogenic amino acids, although relatively small, contains molecules of remarkable diversity. Aliphatic, aromatic, polar, acidic and basic functionalities must be addressed if one is to give a complete picture in terms of amino acid composition of a sample. The demand for stereoselective analysis further complicates this task; but thanks to dedicated efforts, numerous methods have been made available to analytical chemists by now. Popular approaches range from chiral derivatization of the analyte enantiomers—so-called "indirect methods" which involve the use of enantiomerically pure derivatizing agents for the formation of covalently bound diastereomeric entities and their resolution through conventional techniques—to enantioselective HPLC.

The latter represents the most widespread "direct approach" on enantioseparation. It is based on the formation of intermediate non-covalent complexes between a chiral auxiliary (selector, SO) and the analyte (selectand, SA) isomers. The enantiomers are resolved based on the differences in the stability of their respective complex with the chiral selector. The most prevalent manifestation of enantioselective chromatographic techniques is chiral stationary phase (CSP)-mode HPLC. It utilizes chiral selectors such as low-molecular weight compounds or natural polymers with defined stereochemistry which are covalently immobilized (often via a spacer) or coated onto solid support material. Stereoselective amino acid analysis is dominated by (glyco-)peptide and carbohydrate-based CSPs but numerous applications make use of (semi-)synthetic low-molecular weight selectors, too. Through well-considered combinations of functional groups, selectors can be "tailored" to specifically address a particular separation challenge.

The "brush-type" chiral stationary phases employed in this study (Fig. 1) have been developed to target *N*-protected amino acids and other chiral acidic compounds [1]. Chiral scaffolds originating from *Cinchona* alkaloids quinine (QN) and quinidine (QD) constitute the basis of chiral recognition in the selectors. In acidic environments, the protonated quinuclidine nitrogen acts as an anion exchanger (AX) and facilitates analyte retention through long-range electrostatic interaction. Additional functional groups, namely the quinoline system and the modified carbamate residue, give rise to enantiomer discrimination by their conformationally fixed positions in the selector molecule in analogy to the 3-point interaction model of chiral recognition [2]. It is important to understand that attractive (e.g. H-bonding, dipole–dipole, hydrophobic) as well as repulsive interactions (steric hindrance, charge repulsion) can contribute to enantiomer separation on CSPs.

Amino acids and peptides are frequently derivatized on their N and/or C termini prior to analysis to mask charged groups and to enhance UV detector response. Widely used achiral derivatizing reagents introduce 3,5-dinitrobenzoyl (DNB), 9-fluorenylmethoxycarbonyl (Fmoc) or *tert*-butyloxycarbonyl (Boc) groups. They have been demonstrated to permit chiral separations of amphoteric compounds on *Cinchona*-based

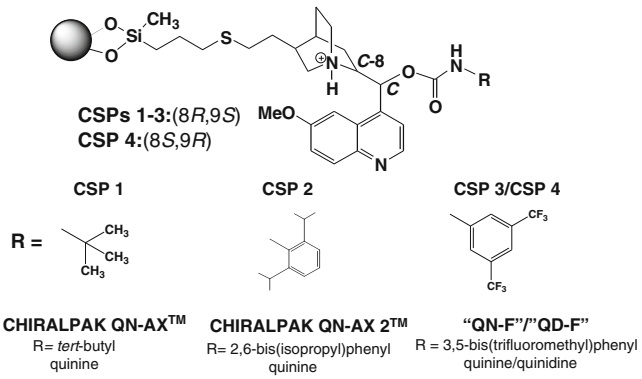


Fig. 1 Chiral anion exchange-type stationary phases used for the evaluation of enantioseparations of dimedone-protected amino acids. Column dimensions: 150×4 mm i.d., 5 μm material. Selector loadings: CSPs 1 and 2: commercial, CSP 3: 328 μmol SO/g silica, CSP 4: 295 μmol SO/g silica

anion exchangers by converting amino acids and peptides into negatively charged amide-type derivatives [1, 3].

In our study, we investigated less common reagents based on β-tricarbonyls [4]. This diverse group of natural compounds contains molecules which possess biological activities ranging from anti-bacterial to anti-inflammatory and anti-cancer [4–6]. Owing to favorable herbicidal properties, the main field of application of synthetic β-triketones is crop protection [7]. Recently, the aza- and oxazole heterocycles have seen growing interest in the exploitation of other aspects of their application potential [8, 9].

The high reactivity of β-triketone species towards amino groups, a well-known phenomenon in heterocyclic chemistry, has been utilized for the development of a new class of *N*-protective groups: 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) [10] and its homologues [11] have been employed in synthetic approaches to a wide variety of compounds, among them branched, cyclic and side-chain modified peptides [12–16], dendrimers [17], peptide nucleic acid (PNA) monomers [18] or tetra-orthogonally protected (2S,6R)-lanthionines [19].

β-Tricarbonyls are subject to tautomerization and their predominant enol forms react with primary amines to give enamino diketones, which in turn can isomerize to imines (see Figure S1, Electronic Supplementary Material) [4]. However, it was shown early on that 2-acetyldimedone and 2-acylcyclohexane-1,3-diones produce mostly enamine-type derivatives of amino acids [20]. Accordingly, the protection and deprotection procedures reported for enantiomerically pure amino acids were stereospecific and practically no racemization was observed [10, 11].

We studied 2-acetyl-dimedone and 12 structurally related compounds (Table 1 presents the reagents in their keto forms) for their ability to derivatize amphoteric molecules under conditions compatible with chiral anion exchange chromatography. In addition, they were expected to promote

both UV detector response and enantiomer selectivity on AX CSPs. The analyte set consisted of proteinogenic and unusual amino acids. Aminosulfonic and phosphonic acids were derivatized in order to assess the application range of the method (Electronic Supplementary Material, Table S2).

Furthermore, we investigated the removal of the protective group using hydrazines and monitored the stereochemical integrity of the analytes throughout the entire procedure.

Experimental

Materials

HPLC grade methanol, acetonitrile and acetone were from Merck (via VWR Austria, Vienna, Austria) and bi-distilled water was obtained from an in-house facility. NMR solvents were purchased from Deutero (Kastellaun, Germany).

Table 1 Di- and triketones (keto forms) employed as achiral derivatizing agents for amino acids and other amphoteric compounds

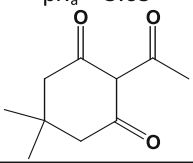
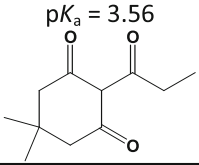
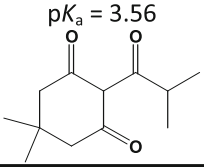
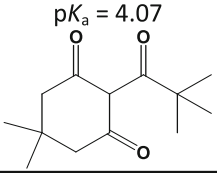
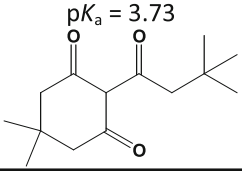
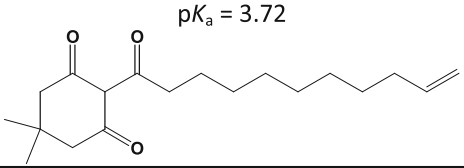
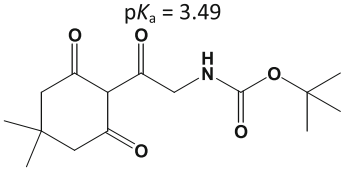
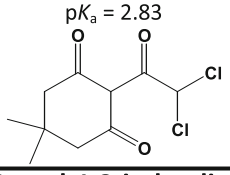
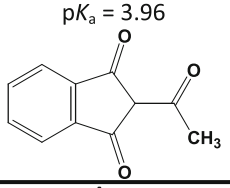
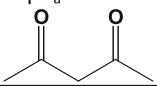
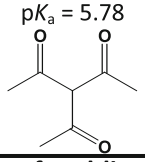
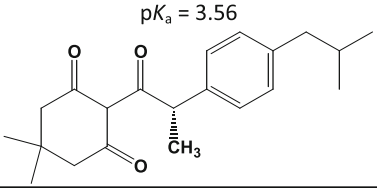
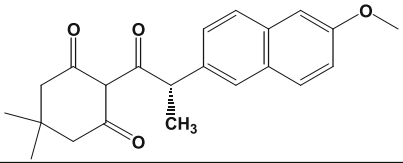
Reagent (Structure, pK_a)	**Reaction conditions** Default: reactant/reagent/base ratio 1 : 1.5 : 1.5, solvent: MeOH/H_2O 50:50 (v/v), base: TEA
2-Acetyldimedone pK_a = 3.65 	**a** 1:1.2, MeOH/H_2O 50:50 (v/v), 60 °C (10 hrs) (optimized conditions) Yields: > 95 % **b** L-leucine/Ac-DMD 1:1.5, iPrOH, TFA, 50 °C, overnight; yield: 50 %
2-Propionyldimedone pK_a = 3.56 	Reaction temperature: 60 °C Reaction time: 8 hrs
"2-Isobutyryldimedone" 2-(2´-methylpropanoyl)dimedone pK_a = 3.56 	Reaction temperature: 60 °C Reaction time: 8 hrs Reagent dissolved in 100% MeOH Yields: < 1 %
2-Pivaloyldimedone pK_a = 4.07 	Reaction temperature: 60 °C Reaction time: 8 hrs Yields: < 3 %
2-(3,3-dimethylbutanoyl)dimedone pK_a = 3.73 	Reagent dissolved in 100% MeOH Reaction temperature: **a** 40 °C **b** 60 °C Reaction time: **a** 8 hrs **b** 24 hrs Yields (**b**): < 8 %
"2-Undecenoyl-dimedone" 5,5-Dimethyl-2-(undec-10-enoyl)cyclohexane-1,3-dione pK_a = 3.72 	**a** yields: 40-80 % **b** amino acid/Ac-DMD 1:1.5, iPrOH, TFA, 50 °C, overnight; yield: 50 %

Table 1 (continued)

2-(*N*-Boc-glycyl)dimedone 5,5-Dimethyl-2-((*N*-*tert*-butyloxycarbonyl)glycyl)cyclohexene-1,3-dione pK_a = 3.49 	1:1.5, MeOH/H_2O 50:50 (v/v), 50 °C, 10 hrs
"2-(2´,2´-Dichloroacetyl)dimedone" 5,5-Dimethyl-2-(2,2-dichloroacetyl)cyclohexane-1,3-dione pK_a = 2.83 	Reaction temperature: 50 °C Reaction time: Overnight (12 hrs) 2-10 %
2-Acetyl-1,3-indandione pK_a = 3.96 	Reaction temperature: 50 °C Reaction time: Overnight (12 hrs) Yields: not determined
Acetylacetone pK_a = 8.94 	Reaction temperature: 40 °C Reaction time: 4 hrs Base: KOH
Triacetylmethane pK_a = 5.78 	Reaction temperature: 50 °C Reaction time: 18 hrs Yields: not determined
"2-Ibuprofenyldimedone" 5,5-Dimethyl-2-(2'-(4-(2-methylpropyl) phenyl)propanoyl)cyclohexene-1,3-dione pK_a = 3.56 	Reaction temperature: 50 °C Reaction time: Overnight (18 hrs) Yields: 3-8 % based on peak areas
"2-Naproxenyldimedone" 5,5-Dimethyl-2-(2'-(6-methoxynaphthyl) propanoyl)cyclohexene-1,3-dione pK_a = 3.54 	Reaction temperature: 50 °C Reaction time: Overnight (18 hrs) Yields: 3-8 % based on peak areas

pK^a values were calculated for the keto forms and refer to the abstraction of the acidic H atom [in position 2 of the ring]. "Reaction conditions" state deviations from the default conditions

Mobile phase additives diethylamine (DEA), formic acid (FA), acetic acid (AcOH) and ammonium acetate (NH_4OAc), all of analytical grade, were purchased from Fluka/Sigma-Aldrich (Vienna, Austria).

Bulk chemicals (of reagent grade or higher purity), technical grade solvents and standard amino(sulfonic) acids (analytical grade) were commercially available (Sigma-Aldrich, Vienna, Austria and Tokyo Chemical Industry, Tokyo, Japan). Chiral aminosulfonic acids were synthesized in-house in accordance with published procedures [21, 22]. *N*-protected amino acids were also produced in-house. Quinine (QN) and quinidine were obtained from Buchler (Braunschweig, Germany). All chemicals were used without further purification.

Triacetylmethane and acetylacetone are commercially available from Sigma-Aldrich. All other 2-acyldimedone reagents were synthesized and purified in-house based on published procedures [23, 24] (M. Kohout, *publication in preparation*).

HPLC experiments

Chromatographic experiments were carried out on an Agilent 1200 HPLC system equipped with on-line degasser, quaternary pump, autosampler, thermostated column compartment and diode array detector. Mobile phase flow was 1 mL/min. Separations were carried out at 25 °C (column thermostat). Samples were injected at concentrations of ca. 1 mg/mL and the injection volume was 10 μL. Acetone (1:20 *v*/*v* in methanol) was used to determine void times. The standard detection wavelengths were 254 and 310 nm depending on the absorbance maxima of the respective analytes. A Corona Charged Aerosol Detector (CAD; Dionex/Thermo Fisher Austria GmbH, Vienna, Austria) was employed for non-UV active solutes. Reaction yields were estimated based on peak areas of the reagent and the products and are stated in % of the total peak area.

CSPs

CSPs 1 and 2 were purchased from Chiral Technologies Europe (Illkirch, France). Novel anion exchanger CSPs 3 and 4 were prepared in analogy to published procedures by derivatizing native quinine (CSP 3) and quinidine (CSP 4) with 3,5-bis(trifluoromethyl)phenyl isocyanate (catalyst: dibutyltin dilaurate) and subsequent thiol-ene-click immobilization onto SH-modified and endcapped silica gel (Daisogel 120-5P from Daiso Co., Ltd., Düsseldorf, Germany. Particle size, 5 μm; pore diameter, 120 Å; surface area, 300 m^2/g; SH coverage, 680 μmol/g silica) [1, 25]. Selector coverage was calculated from the nitrogen content as determined by elemental analysis (EA 1108 CHNS-O Element Analyser, Carlo Erba/Thermo Scientific Austria GmbH, Vienna, Austria).

Mobile phases

Chiral anion exchangers were operated at a slightly acidic apparent pH (pH = 5–6) in polar-organic mobile phase mode.

Bulk solvents: methanol (MeOH), acetonitrile (ACN)

Mobile phase additives: formic acid (FA), acetic acid (HOAc), diethylamine (DEA), ammonium acetate (NH_4OAc).

Mobile phase 1	MeOH	50 mM FA, 25 mM DEA
Mobile phase 2	MeOH	25 mM FA, 12.5 mM DEA
Mobile phase 3	MeOH/ACN 90:10 (*v*/*v*)	25 mM FA, 12.5 mM DEA
Mobile phase 4	MeOH/H_2O 80:20 (*v*/*v*)	50 mM HOAc, 50 mM NH_4OAc
Mobile phase 5	MeOH	350 mM HOAc, 65 mM NH_4OAc

Methods

Choice of solutes

For most experiments reported in this contribution, the following proteinogenic amino acids containing a primary amino group were investigated: Phe, Trp, Ala, Asn, Asp, Val, Thr, Leu, Met, Ile, Tyr, Glu, Ser, Lys, Cys, Arg, His and Gln. Gly (achiral) and Pro (secondary) were included as "proof of concept". The first 8 amino acids, together with 2- and 3-aminobutyric acid (2-ABA and 3-ABA) constituted the "reduced analyte set". For straightforward readability and referencing, the respective derivatives are labeled with the name of the underlying amino acid in the tables and figures. Additional data can be found in the Electronic Supplementary Material.

Derivatization procedure

Equal volumes of stock solutions of amino acid (50 mM in MeOH/H_2O, 1:1 *v*/*v*), base (triethylamine, KOH, or quinine, 75 mM) and reagent (75 mM) were combined and incubated in a "Thermo-mixer compact" system from Eppendorf (Vienna, Austria). The vials were agitated at 1,250 rpm to ensure complete mixing. Variations of reactand/reagent ratio, reaction time and temperature are specified in Table 1. The amino acids were derivatized as racemates by default; for determination of elution orders single enantiomers were subjected to the same treatment if available.

The products were either dried using a Thermo Savant ISS110 vacuum centrifuge system (Thermo Fisher Scientific, Schwerte, Germany) and re-dissolved in methanol or diluted 1:1 with MeOH/H_2O (1:1, *v*/*v*) prior to HPLC-UV

analysis. Sample concentrations were adjusted to ca. 1 mg/mL for optimum UV detector response.

For the investigations on racemization and removal of the protective group, L-valine (12 mmol) was refluxed for 15 h with a 1.5-fold excess of acetyldimedone and triethylamine in methanol/water (50:50 *v*/*v*).

Purification of the product 2-acetyldimedone-L-valine was carried out on a semi-preparative HPLC system (Bischoff Chromatography, Leonberg, Germany) with two pumps, a manual injector (loop volume 1 mL; concentration of samples, 200–900 mg/mL) and a programmable Lambda 1010 UV/vis detector set to a detection wavelength of 254 nm. For purification, a 200×16-mm flash column was packed with in-house poly-imino-modified spherical silica gel 60 (particle size 40–63 μm). Elution was accomplished with a mobile phase composed of methanol, formic acid (20 mM) and ammonium formate (10 mM) at 10 mL/min (room temperature).

The derivatization under acidic conditions (see also Table 1) was carried out with L-leucine (7.4 mmol) and acetyldimedone (12.5 mmol) and the products were purified by flash chromatography (unmodified silica, CH_2Cl_2/MeOH 10:1 *v*/*v*).

The products were characterized by NMR experiments performed on a Bruker DRX 400 MHz NMR spectrometer. Spectra were recorded in MeOH-d_4 and $CHCl_3$, respectively, with tetramethylsilane as internal standard. Solvent signals were used as reference signals. Chemical shifts are stated in ppm (parts per million).

N-(1-(1-(4,4-dimethyl)-2,6-dioxocyclohexyldien)ethyl)-valine

Yield: quantitative; purity: 99.6 w% (determined with HPLC-CAD).

^{1}H-NMR (MeOH-d_4): δ=1.1 (s, 6H, CH_3), 1.06 (s, 6H, CH_3), 2.35 (m, 1H, CH), 2.4 (s, 4H, CH_2), 2.5 (s, 3H, CH_3), 4.2 (d, 1H, CH). MS (ESI, pos): 282.2 $[M+H]^+$.

N-(1-(1-(4,4-dimethyl-2,6-dioxocyclohexyldien)ethyl)-leucine

Yield: 50 %, white crystals (after flash chromatography).

^{1}H-NMR ($CHCl_3$-d_1): δ=0.98 (d, 3H, CH_3), 1.05 (d, 3H, CH_3), 1.1 (s, 6H, CH_3), 1.83 (m, 1H, CH), 1.90 (dd, 2H, CH_2), 2.52 (s, 3H, CH_3), 2.60 (s, 2H, CH_2), 2.62 (s, 2H, CH_2), 4.5 (t, 1H, CH), 10.3 (s, 1H, COOH).

Removal of the protective group

The 2-acetyldimedone derivative of L-valine (35 μmol) was dissolved in 1 mL of ethanol or acetonitrile and incubated with equimolar amounts of hydrazine monohydrate, methyl- or phenylhydrazine, respectively, at 40 °C [10, 26].

For the deprotection with immobilized hydrazine, spherical silica ("Kieselgel 60", Merck, Darmstadt, Germany) was derivatized with hydrazine monohydrate (Fluka) in-house (coverage according to elemental analysis, 750 μmol hydrazine/g silica).

The degree of deprotection and the stereochemical integrity of the product were assessed by chromatography on "taurine-QN" and "aminobutanesulfonic acid-QN" zwitterionic chiral stationary phases [22, 27] using a charged aerosol detector. Apart from the immobilized hydrazine, which had no effect on the protected amino acid, all reagents afforded L-valine in its free and enantiomerically pure form. The required incubation times for quantitative recovery of the free amino acid were 3 h with hydrazine and 24 h with methyl- and phenylhydrazine (reactant/reagent ratio 1:2), respectively.

Software

HPLC data were recorded with Agilent ChemStation software and evaluated using Microsoft Excel. ACD/Labs 7.0 was used for the calculation of pK_a values. NMR spectra were processed with SpinWorks 2.2.5 software.

Results and discussion

General

Derivatization procedure

On account of their amphoteric (zwitterionic) nature, amino acids are not ideally suitable for chiral enantiomer separations with single-charge ion exchangers. Zwitterionic chiral stationary phases bearing positively and negatively charged groups can overcome this limitation [22, 28]. In an alternative approach, condensing the amino acids with reagents featuring bi- or tricarbonyl motifs masks the positively charged amine and simultaneously introduces a strongly UV-active substituent. The resulting acidic entities are ideal target analytes for chiral anion exchangers because their newly gained structural complexity contributes significantly to the multi-modal chiral recognition mechanism.

A large variety of solute-reagent combinations had to be investigated in order to derive a relationship between the side-chain modifications of the 2-acyl-dimedones on the one hand and the reaction efficiency and enantioseparation results on the other. Therefore, after small-scale derivatization (mg amounts), the products were subjected to chromatographic evaluation (HPLC-UV) in a "dilute-and-shoot" approach. Systematic optimization of reaction conditions for the individual reagents was outside of the scope of this study.

The solutes and the respective reagent were incubated at elevated temperatures in a basic environment (pH=9–10) in

order to maximize conversion of the bi- and tricarbonyl motifs into the reactive enol(s). Control experiments proved the derivatization to be distinctly less efficient under acidic conditions on account of the requirement for the abstraction of an acidic proton in position 2 of the ring (Table 1, Experimental).

As illustrated in Figures S1a and S1b (Electronic Supplementary Material), most 2-acyl-1,3-cyclohexanediones predominantly form the endocyclic enol or, under basic conditions, the respective enolate [29]. Compared to the exocyclic enolate, the former is more efficiently stabilized by a hydrogen bond between the hydroxyl and a β-carbonyl group. The exocyclic enolate is consequently believed to be more reactive [30]. A reaction of the external enolate with amines, however, would require a nucleophilic attack on a C atom inside the ring, which, on the other hand, is sterically hindered (Electronic Supplementary Material, Figure S1b, bottom). These considerations may explain our observation that the reaction products were exclusively the ones originating from a regioselective reaction involving the endocyclic enol form. Unfortunately, literature reports on the reaction mechanism are as yet ambiguous [29, 31].

We were forced to acknowledge that the various reagents exhibit only limited specificity towards α-amino groups. With some reagents, we observed multiple *N*-derivatives of solutes with two primary amines such as lysine (ε-amino group).

In contrast to this, the derivatization of glutamic and aspartic acid was not effectively accomplished on account of their limited solubility in the employed reaction media. Yields for the respective derivatives were always inferior to those obtained for other amino acids and frequently no products were found at all.

In accordance with the proposed reaction mechanism and with previous studies [4], secondary amino acids such as proline and its six-membered homologue pipecolic acid (homoproline) did not react with enolates under the given conditions. The achiral amino acid glycine was usually derivatized successfully but is not discussed specifically as it obviously cannot be enantioseparated.

Chromatographic enantioseparation and CSP performance

2-Acyldimedones are weak acids [4]. Their calculated pK_a values are stated in Table 1.[1] Under the given mobile phase conditions (polar-organic, apparent pH=5–6), the reagents eluted from the anion exchangers close to the void time (retention factors ca. 0.1). We frequently observed low column efficiency reflected in comparatively broad peaks but with the exception of 2-acetyl-1,3-indandione, the reagent peaks never interfered with the analyte signals.

On the *Cinchona*-based AX CSPs, the enantiomers of acidic compounds are distinguished and separated due to a combination of simultaneous, non-covalent interactions with the basic selectors [1, 32]. In an acidic environment, the selector's protonated quinuclidine nitrogen represents the primary interaction site, retaining the negatively charged solute by means of a relatively strong, long-range electrostatic interaction. Hydrogen bonding between the carbamate group and suitable moieties of the analyte is one of the crucial increments to the chiral recognition mechanism. Dipole–dipole interactions, Van-der-Waals, solvophobic, aromatic π–π and steric interactions also contribute to chiral distinction on account of the transfixed arrangement of the respective interaction sites in the selector. Their combination eventually brings about the chromatographic separation of the analyte enantiomers.

All CSPs tested were capable of retaining the derivatized analytes, even though their selectivity was not always sufficient to achieve complete (baseline) enantiomer separation. At a first glance, all four chiral anion exchangers exhibited similar enantiorecognition performance but some aspects deserve special consideration.

When comparing the separation results of the derivatives of selected amino acids (Val and Phe), it is interesting that CSPs 3 and 4 give significantly higher enantiomer selectivity values for Phe derivatives than CSP 2. As this effect is not observed for the respective Val derivatives, it is reasonable to attribute it to the differences in the selectors´ aromatic carbamoyl residues: CSPs 3 and 4 feature a π-acidic bis(trifluoromethyl)phenyl group, while CSP 2 bears a π-basic diisopropylphenyl moiety. Obviously, ππ stacking plays a more significant role in the recognition mechanism of the enamine-type derivatives than what has been observed for amide-type ones.

While CSPs 1–3 are based on quinine, CSP 4 is derived from its pseudo-enantiomer quinidine. It is well-known that quinine and quinidine-based selectors usually give comparable enantioselectivity and resolution values but inverse elution sequences when applied in enantiomer separations. In some cases however, notably the enantiomer separations of propionyldimedone-valine or undecenoyldimedone-valine, CSPs 3 and 4 show marked differences in enantioselectivity which are reminiscent of their diastereomeric nature.

Effect of the 2-acyl substituent on derivatization and enantiomer separation

Taking into account reaction yields as well as enantiomer separation results, it is possible do deduce certain correlations between the 2-acyl substituents of the individual reagents and their suitability for this direct chiral amino acid separation.

[1] It should be noted that, strictly speaking, the concept of pH and pK_a values is limited to aqueous systems. It is not advisable to assume their equality in the polar-organic mobile phase employed for these chromatographic experiments but they may serve for rough estimations of the reagent´s affinities to the ion exchangers relative to the "more acidic" reaction products.

2-Acetyldimedone proved particularly successful in derivatizing a variety of solutes, among them both proteinogenic and unusual amino acids, aminosulfonic acids and other primary amines (see also Electronic Supplementary Material, Table S2). Enantiomer selectivity and resolution values obtained in the chiral separations were moderate but mostly sufficient for baseline separations. 2-Propionyldimedone provided for particularly high resolution but yields were inferior to those obtained with Dde.

The more sterically demanding the 2-acyl substituents, the lower were the respective product yields. In particular, yields were strongly affected by (methyl) groups in α-position to the exocyclic carbonyl group. The conversion of the original tricarbonyl into the enol is thought to be unaffected by the 2-acyl substituent [29], but steric hindrance is likely to impair the reaction with the amine. Consequently, isobutyryl- and pivaloyldimedone gave very low yields and those obtained with 2-(3,3-dimethylbutanoyl)dimedone were only marginally higher. In keeping with the chiral recognition mechanism of the CSPs depicted in Fig. 1, the respective products were nonetheless separated with high enantiomer selecitivity and chromatographic resolution. The acyclic reagents acetylacetone and triacetylmethane provided good yields but, due to a lack of steric interaction with the selectors, only mediocre separation results.

The combination of conformational flexibility provided by the methylene group in the α-position and the hydrophobic side chain of 2-undecenoyldimedone proved especially beneficial for amino acid derivatization and subsequent enantioseparation. Product yields were among the overall highest and chromatographic separations were characterized by strong retention and very good enantiomer selectivities. A discussion of selected aspects connected to the application of the individual reagents can be found below.

Deprotection procedure

According to the literature, hydrazine facilitates the deprotection of the analytes [10, 26]. A purified sample of 2-acetyldimedone-derivatized L-valine (Ac-DMD-L-Val) was incubated with aqueous hydrazine at elevated temperature and subsequently analysed on an AX CSP to demonstrate quantitative removal of the protective group (Fig. 2a).

Free hydrazine permitted straightforward quantitative cleavage of the protective group (no residual Ac-DMD-valine was found after 3 h of treatment), while methyl- and phenylhydrazine were less efficient (Fig. 2b). We believe that this finding can be explained by a) a steric hindrance of the reaction caused by the hydrazine substituent and b) its effects on the electron distribution which impair the reagent´s reactivity compared to free hydrazine.

Attempts to remove the protective group by incubating Ac-DMD-L-Val with hydrazine-modified silica particles left the acetyldimedone derivative intact. The hydrazine apparently loses its reactivity upon immobilization, possibly due to a stabilizing effect of silanol groups on the silica surface involving hydrogen bonds with the free amines.

Racemization of the analytes during protection–deprotection operations constitutes a potential source of errors in any enantioselective analytical method. All chromatograms obtained from enantiomerically pure educts were therefore carefully checked for the presence of isomers of the opposite configuration. In particular, we repeatedly investigated the Dde derivative of L-valine at different stages of processing. From a comparison of the peak areas of deprotected valine with those of the original amino acid sample, it was obvious that the stereochemical integrity of the amino acid was not affected by the protection–deprotection operations. This assessment was not easily feasible and required the application of chiral zwitterionic stationary phases [22, 27] in combination with a charged aerosol detector in order to facilitate a) enantiomer separation and b) detection of the structurally undemanding, non-UV active free amino acid. Both enantiomer separation and detection are accomplishable in a more straightforward way for *N*-protected amino acids such as the derivatization products investigated here.Discussion of individual reagents.

2-Acetyldimedone

2-Acetyldimedone (Ac-DMD, Dde) was chosen as the model reagent following promising results in preliminary tests. Reaction conditions (analyte/reagent ratio, solvent, temperature, reaction time) were optimized for Dde and subsequently applied to the other reagents, which may be partially responsible for the favorable yields reported for Dde.

As mentioned above, 2-acyl-5,5-dimethyl-1,3-cyclohexanediones generally prefer an endocyclic enolic bond [29] but under the reaction conditions employed in this study a significant portion of the reagent may be made up of exocyclic enol. Therefore, significantly less of the reagent may be available for reaction with the analyte. By employing a 1.5-fold excess of reagent we accounted for this shortcoming. Substituting the strong base KOH with triethylamine (TEA) or quinine (QN) had no effect on reaction yields, which justified our eventual choice of TEA. This base has been reported to accelerate tautomeric interconversion in β-tricarbonyl compounds [30] and is perfectly compatible with the chromatographic method.

In chromatographic runs, unreacted Ac-DMD (Dde) was always eluted close to the void time while the product capacity factors were between 0.7 (k_1, retention factor of first eluting enantiomer) and 10 (k_2, second eluting enantiomer, Table 2). The Ac-DMD peak was split, indicating the presence of two enol forms. However, we obtained

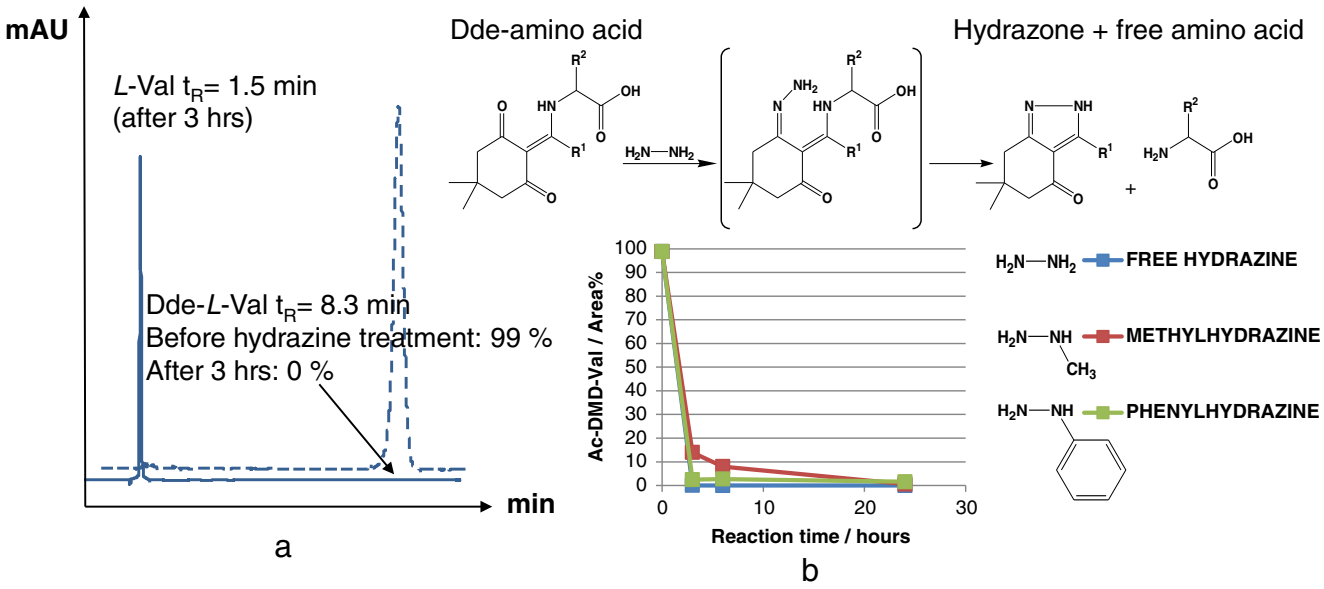


Fig. 2 Removal of the Dimedone (Dde) protective group with hydrazines. **a** The reaction afforded hydrazone and the free amino acid and was complete after 3 h. Chromatographic conditions: CSP 4, mobile phase: Methanol (50 mM formic acid, 25 mM diethylamine), 1 mL/min, 25 °C, detection: UV (254 nm), CAD. **b** Progress of deprotection reactions with different hydrazine reagents (reactand/reagent 1:1, 40 °C)

exclusively one type of product (i.e. one pair of peaks in the case of successful chiral separation) for most racemic amino acids. Unfortunately, the issue of double derivatization of amino acids with multiple potentially reactive groups (Lys, possibly also Ser—unequivocal results) could not be eliminated by adjustment of reaction times and/or temperature.

For lysine, mass spectrometric detection (ESI, negative mode) confirmed the presence of an N^{α},N^{ε}-bis-Dde derivative with an m/z ratio of 473.5 Da $[M\text{-}H]^-$ which was clearly separated from the more strongly retained N^{α} and N^{ε}Dde derivatives (m/z=309.5, $[M\text{-}H]^-$). The peaks belonging to the single derivatives were only partially resolved. Peak assignment could likely be achieved by fragmentation experiments.

On account of its low solubility, only a small percentage (<10 %) of glutamic acid could be converted into the enamine product. Utter failure of the derivatization reaction, noticeable in the absence of quantifiable peaks (>2 area per cent) in the relevant area of the chromatograms, was attributed to insufficient solubility (Asp) or dimerization (Cys).

Interestingly, the enantioselectivity towards the Dde-protected amino acids exhibited by in-house produced CSPs 3 and 4 was superior to that of the commercial anion exchangers CSPs 1 and 2 (Table 2). The π-acidic (electron-poor) bis(trifluoromethyl)phenyl motif is especially favorable for π–π interactions with the electron-rich aromatic indole and phenyl moieties of the respective amino acids (Phe, Trp) but can be regarded as generally beneficial for enamine-type products. In contrast to this, the enantiorecognition capabilities of CSPs 3 and 4 towards amide-type analytes are similar to the ones of CSPs 1 and 2. In the case of DNB-protected amino acids, CSP 1 is strikingly more selective than CSPs 3 and 4 (Electronic Supplementary Material, Table S1).

Amide-type *N*-protected amino acids tend to give the elution order *D before L* on quinine-based CSPs [33]. In contrast to this, the elution sequence for the majority of enamine-type 2-acetyldimedone derivatives was *L before D*, which corresponds to previously established recognition patterns of *Cinchona* alkaloids for amine-type 2,4-dinitrophenyl (DNP)derivatives [34]. The inversion reflects a change in the molecular recognition mechanism originating from an impaired capacity for hydrogen bond formation of the enamines with the carbamate moiety of the selectors.

Inconsistent elution orders for derivatives of glutamic acid (*L before D* on quinine-based CSPs) have also been observed before and were attributed to a dominating influence of the side chain carboxyl on the retention mechanism [34]. The results reported in Table 2 (CSP 2) may point in this direction. Quite unexpectedly, we also found reversed elution orders for Dde-Asn (*D before L* on CSP 1, L before D on CSP 4), but are presently incapable of providing a full explanation. As anticipated from the well-known pseudo-enantiomeric behavior of QN and QD, elution orders were reversed when switching from the quinine-based CSPs 1–3 to the quinidine-based CSP 4 (Fig. 3, [32]).

Palpable differences in selectivity and resolution between CSPs 3 and 4, whose selectors vary only in the configurations of the stereocenters at C-8 and C-9, serve as a reminder of the essentially diastereomeric nature of

Table 2 Chromatographic results for 2-acetyldimedone derivatives of proteinogenic amino acids

Analyte	CSP 1				CSP 2			
	k_2	α	R	EO	k_2	α	R	EO
Gly	3.29	n.a.	n.a.	n.a.	4.46	n.a.	n.a.	n.a.
Ala	2.48	1.05	0.93	L	3.41	1.11	1.21	L
Val	2.03	1.22	2.33	L	2.48	1.26	2.47	L
Met	2.93	1.10	1.36	L	4.10	1.17	1.99	L
Leu	2.04	1.11	1.26	L	2.39	1.18	1.77	L
Ile	1.97	1.20	2.18	L	2.28	1.26	2.68	L
Trp	5.39	1.21	2.82	L	8.00	1.21	2.29	L
Phe	3.30	1.13	1.78	L	4.32	1.13	1.51	L
Tyr	3.70	1.11	1.47	L	5.00	1.11	1.22	L
Thr	2.57	1.04	0.53	L	2.96	1.00	0.00	-
Asp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glu	11.17	1.10	1.43	n.d.	14.10	1.10	1.11	L
Asn	3.32	1.07	1.01	D	4.25	1.05	0.59	n.d.
Ser	3.04	1.05	0.60	L	3.85	1.07	0.66	L
Lys	2.82	1.13	1.35	L	4.20	1.19	1.89	L
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arg	3.30	1.07	0.99	n.d.	4.23	1.05	0.58	n.d.
His	1.45	1.00	0.00	n.a.	1.65	1.00	0.00	-
Gln	3.07	1.18	2.32	L	4.05	1.19	1.97	L
Pro	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Analyte	CSP 3				CSP 4			
	k2	α	R	EO	k2	α	R	EO
Gly	5.86	*	*	n.a.	5.88	*	*	n.a.
Ala	4.54	1.14	1.97	L	4.70	1.15	2.23	D
Val	3.42	1.23	2.07	L	3.74	1.22	2.44	D
Met	5.71	1.20	2.18	L	5.86	1.15	1.98	D
Leu	4.43	1.24	2.52	L	4.34	1.18	2.24	D
Ile	3.67	1.24	2.30	L	3.92	1.18	2.18	D
Trp	9.62	1.07	0.65	L	9.01	1.30	3.54	D
Phe	6.48	1.22	2.44	L	6.58	1.20	2.56	D
Tyr	6.46	1.19	1.98	L	6.48	1.20	2.40	D
Thr	4.19	1.08	0.75	L	4.03	1.09	1.08	D
Asp	n.d.	n.d	n.d.	n.d.	n.d.	n.d	n.d.	n.d.
Glu	5.40	1.15	1.69	n.d.	n.d.	n.d.	n.d.	n.d.
Asn	4.63	1.00	0.00	n.a.	4.60	1.06	0.70	L
Ser	5.05	1.08	0.75	L	4.62	1.08	0.92	D
Lys	6.46	1.28	2.83	L	6.85	1.08	0.98	D
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Arg	4.60	1.00	0.00	n.a.	4.60	1.07	0.68	n.d.
His	2.03	1.07	0.54	L	2.34	1.05	0.53	D
Gln	5.40	1.41	4.01	L	4.04	1.18	2.32	D
Pro	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

k_2: retention factor of second eluted enantiomer, α: selectivity, R_S: chromatographic resolution, EO: elution order (first eluted enantiomer), n.a.: not applicable, n.d.: not determined. Chromatograhic conditions: Mobile phase: MeOH, 50 mM formic acid, 25 mM diethylamine, 1 mL/min, 25 °C. Columns: 150×4 mm i.d., 5 µm material. Detection: UV (254 nm). For detailed discussion of the results, see text

QN and QD. If they were "true" enantiomers, chiral separations would lead to reversed elution orders but exactly the same selectivity for the product enantiomers but, as illustrated in Table 2, the QD-based CSP 4 performed better in the majority of cases.

The separation of isobaric amino acids demands special consideration in the context of mass spectrometric detection. We subjected the Ac-DMD (Dde) derivatives of (L)-*tert*-leucine, L-*allo*-leucine, and (L)-leucine to separation on CSP 2 in order to briefly estimate the suitability of our approach for

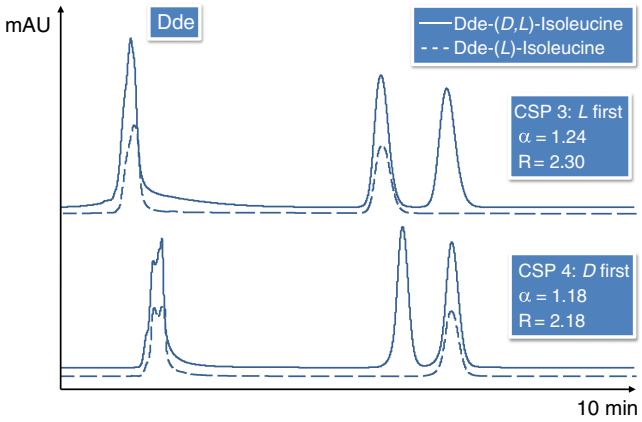


Fig. 3 Elution orders of 2-acetyldimedone (Ac-DMD) derivatives of Isoleucine on quinine-based CSP 3 and its pseudo-enantiomer, quinidine-based CSP 4. Conditions: Column dimensions: 150×4 mm, 5 μm material. Mobile phase: Methanol (50 mM formic acid, 25 mM diethylamine), 1 ml/min, 25 °C. Injection volume: 10 μL. Detection: UV (254 nm)

such a chemoselective (not enantio- or stereoselective) application. The CSP was capable of distinguishing the isomers, even though the separation of allo-Leu and Leu was not complete. Mobile phase optimization should eliminate this problem and facilitate baseline separation of all three isomers. The Ac-DMD/Dde modification also allowed for separation of the product isomers on a commercial achiral C-18 column. Dde derivatives of (D,L)-leucine, (D,L)-isoleucine, (D,L)-norleucine and (D,L)-*allo*-leucine could also be distinguished, albeit with only limited chemoselectivity (data not shown).

2-Propionyldimedone

2-Propionyldimedone succeeded in derivatizing highly diverse primary amines, among them *cis*-1,3-aminocyclohexanesulfonic acid, penicillamine and 2-aminobutyric acid as well as proteinogenic primary amino acids (Table 3).

The derivatives were separated with moderate enantioselectivity but the CSPs exhibited very high resolution power in many cases. Again, CSP 4 demonstrated particularly favorable separation characteristics, which points towards a higher specificity of QD-based selectors for the dimedone derivatives compared to QN-based ones. It is worth mentioning that this is not an effect of exclusion of the enantiomer with a less ideal fit but rather due to higher affinity for the more strongly retained one (k_1 values obtained with CSP 4 are not significantly smaller than those obtained with the other CSPs. k_2 values, however, are higher).

In contrast to all other analytes, the penicillamine product led to a peak pair in the chromatographic evaluation. Derivatization of both the NH_2 and the thiol groups of this multifunctional analyte seemed imaginable but could not be verified.

2-Isobutyryldimedone, 2-pivaloyldimedone

The reagents were not soluble in the water/methanol mixture that was employed for most other reagents, which prompted us to add the reagent in 100 % methanol to the other components. However, yields for the derivatization of proteinogenic amino acids were marginal (see Table 1). Chiral chromatography on CSP 2 did not produce any product peaks with more than 1 % of the total peak area for isobutyryldimedone derivatives. We attributed the failure of this procedure to the bulky substituent which prevents successful derivatization by sterically obstructing access of the nucleophile to the reaction center. Our hypothesis is corroborated by earlier findings of Rogers and Smith, who discovered that 2-isobutyryldimedone does not react with aniline and also suspected steric hindrance [4, 35]. On the other hand, some pivaloyldimedone products were separated with exceptionally high enantioselectivity and excellent resolution on CSPs 2 and 4 using mobile phase 1 (α>1.6, R>5 for aromatic amino acids, α>1.2, R>1.2 for aliphatic ones; data not shown). On account of its obvious recognition-enhancing properties, this reagent would be a worthy candidate for systematic optimization of the derivatization procedure.

2-(3,3-Dimethylbutanoyl)dimedone

In keeping with the statements made for isobutyryl- and pivaloyldimedone, neither changing the reaction medium to methanol nor increasing the temperature and reaction time could increase the yields for dimethylbutanoyldimedone derivatives above ca. 8 %. The fact that the dimethylbutanoyldimedone worked marginally better than the previously introduced reagents can be attributed to higher conformational flexibility permitted by the methylen group in α position to the electrophilic carbon atom.

The products could be enantioseparated with reasonable retention factors (< 5) on the chiral anion exchangers, especially on CSP 4 (Fig. 4). However, chiral recognition of dimethylbutanoyldimedone derivatives was inferior to the [few] separations observed for pivaloyldimedone products. Here, the additional methylene group separating the sterically demanding *tert*-butyl from the enamine nitrogen obviously poses a disadvantage in the sense that conformational flexibility of the interacting groups can compromise the efficiency of chiral recognition by low-molecular weight selectors.

2-(Undecenoyl)dimedone

With the exceptions outlined in the General section, most proteinogenic amino acids were converted into the respective enamine-type derivatives with reasonable yields by reaction with 2-undecenoyldimedone. The protective group

Table 3 Chromatographic results for 2-propionyldimedone derivatives of amino acids

Analyte	CSP 1			CSP 2			CSP 3			CSP 4		
	k_2	α	R_S	k_2	α	R_S	k_2	α	R_S	k_2	α	R_S
Phe	4.30	1.11	1.66	3.15	1.07	0.81	4.52	1.22	2.32	5.46	1.22	2.83
Trp	7.15	1.16	2.48	5.64	1.13	1.46	6.27	1.07	0.75	7.05	1.34	4.04
Ala	3.39	1.06	0.88	2.68	1.07	0.74	3.59	1.15	1.69	4.24	1.20	2.61
Asn	4.56	1.20	2.73	3.49	1.18	1.91	3.13	1.05	0.56	3.61	1.00	0.00
Asp	5.30	1.00	0.00	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.
Val	2.84	1.20	3.13	1.92	1.16	1.61	2.70	1.20	1.99	3.44	1.29	3.33
Thr	3.44	1.00	0.00	2.34	1.00	0.00	3.30	1.08	0.86	3.80	1.21	2.50
Leu	2.96	1.12	1.76	1.93	1.12	1.22	3.52	1.25	2.57	4.08	1.27	3.24
γ-ABA	1.39	1.11	1.17	0.81	1.09	0.53	1.63	1.22	1.87	1.23	1.25	2.18
β-ABA	3.16	1.14	2.04	2.32	1.12	1.32	3.20	1.18	1.90	3.80	1.21	2.57

k_2: retention factor of second eluted enantiomer, α: selectivity, R_S: chromatographic resolution. n.f.: not found, n.d.: not determined. Chromatograhic conditions: Mobile phase: MeOH, 50 mM formic acid, 25 mM diethylamine, 1 mL/min, 25 °C. Columns: 150×4 mm i.d., 5 µm material. Detection: UV (254 nm). CSPs 1–3: Quinine-based, CSP 4: Quinidine-based

proved highly beneficial in the chromatographic separations (Table 4).

Capacity factors were exceptionally high (*k* values typically above 5, in some cases above 15 with mobile phase 2), which is suggestive of a strong hydrophobic interaction increment binding the derivatives to the stationary phase. In keeping with this observation, CSPs 1–4 exhibited a high enantiomer recognition performance (Table 4, Fig. 5). With adjustments of the type and concentration of additives, more practical k values between 5 and 10 were feasible with only minor losses in enantiomer selectivity and resolution (Mobile Phase 5, Electronic Supplementary Material, Table S3).

Typically, the best enantioselectivity and resolution results were obtained for derivatives of amino acids with hydrophobic and aromatic side chains. This behavior can be explained on the basis of a hydrophobic cavity formation—the delivery of counter ions into the active center of the selector-solute interaction is slowed down, which in turn accounts for longer retention times. For lysine we found two peak pairs of unequal size due to double derivatization but we did not observe any product formation for histidine. Once again, the best overall results were obtained with quinidine-based CSP 4, even though it should be mentioned that this was the only CSP incapable of separating the Lys derivatives.

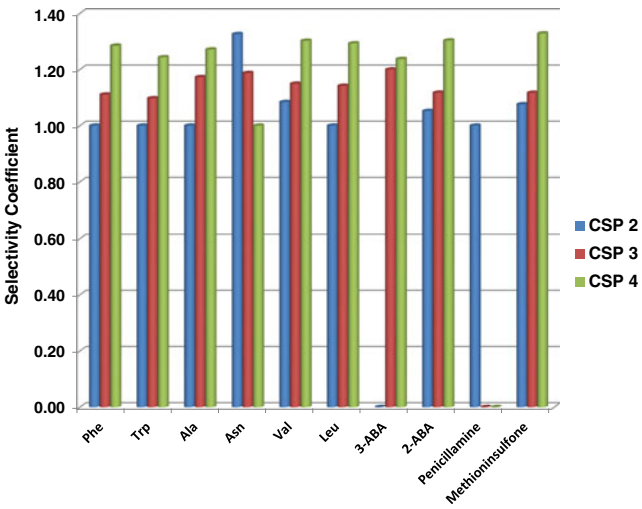


Fig. 4 Chiral recognition of neopentyldimedone-derivatized amino acid enantiomers on anion exchanger CSPs. Chromatographic conditions: CSPs 2–4 (150×4 mm i.d., 5 µm material), mobile phase: Methanol (50 mM formic acid, 25 mM diethylamine), 1 mL/min, 25 °C. Detection: UV (254 nm)

2-(N-Boc-glycyl)dimedone

Recounting the rather weak performance of reagents with sterically demanding 2-acyl-substituents, it was unexpected that 2-(*N*-Boc-glycyl)dimedone afforded satisfactory conversion rates for all ten analytes tested. The remarkably high reaction yields result from a favorable geometry brought about by hydrogen bonding between the carbonyl group at C-1 and the carbamate moiety of the 2-acyl substituent. Apparently, it supports the formation of a reactive endodyclic enol intermediate (Fig. 6, left). In addition, the protective group significantly contributed to the chiral recognition mechanism on CSPs 1, which was reflected in high enantioselectivity and excellent resolution ($\alpha > 1.2$, $R_S > 2.0$, Fig. 6, right). One disadvantage was that the reaction products of Gly, Ala and Glu could not be sufficiently separated from byproducts on CSP 2, which rendered their identification and quantification impossible. In addition, CSP 2 was not enantioselective towards Ser derivatives, which had been particularly well separated on CSP 1. Reduced selectivity of CSP 2 was also observed for most other amino acids except, interestingly, for valine.

Table 4 Chromatographic results obtained for 2-undecenoyldimedone derivatives of amino acids on chiral anion exchangers

Analyte	CSP 1			CSP 2			CSP 3			CSP 4		
	k_2	α	R_S	k_2	α	R_S	k_2	α	R_S	k_2	α	R_S
Ala	6.70	1.15	2.36	4.72	1.07	0.86	8.99	1.11	1.44	9.59	1.23	2.97
Gly	8.86	n.a.	n.a.	11.42	n.a.	n.a.	20.58	n.a.	n.a.	19.82	n.a.	n.a.
Glu	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.
Ile	5.60	1.31	4.48	3.29	1.19	2.24	7.33	1.21	2.61	8.70	1.40	4.89
Leu	5.77	1.19	2.75	3.50	1.10	1.29	8.95	1.22	2.75	9.50	1.33	4,36
Trp	12.52	1.18	2.89	9.52	1.12	1.60	14.96	1.06	0.68	14.79	1.30	3.74
Ser	9.08	1.24	3.77	5.57	1.09	1.12	11.31	1.14	1.82	10.28	1.27	3.56
Val	5.66	1.31	4.43	3.45	1.16	1.99	6.85	1.18	2.24	7.54	1.40	4.88
Phe	5.28	1.71	7.47	2.75	1.33	3.09	5.91	1.70	6.15	4.94	1.81	7.51
Tyr	9.68	1.17	2.68	6.83	1.11	1.38	11.80	1.20	2.35	12.65	1.32	4.04
Arg	8.16	1.07	1.23	5.90	1.13	1.67	8.56	1.00	0.00	8.64	1.00	0.00
Asn	8.18	1.07	1.18	5.89	1.13	1.67	9.12	1.07	0.73	8.62	1.00	0.00
Asp	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.
Cys	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.
Gln	7.76	1.19	2.94	5.40	1.09	1.11	7.30	1.11	1.31	7.90	1.21	2.78
His	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.
Lys[a]	7.44	1.24	3.24	5.30	1.13	1.51	16.94	1.33	3.62	15.19	1.00	0.00
Thr	7.68	1.22	3.31	4.33	1.00	0.00	10.72	1.27	3.19	9.95	1.40	5.07
Pro	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.	n.f.	n.d.	n.d.
Met	7.83	1.16	2.57	5.60	1.10	1.35	10.85	1.16	2.09	12.04	1.27	3.60

k_2: retention factor of second eluted enantiomer, α: selectivity, R_S: chromatographic resolution. n.f.: not found, n.d.: not determined, n.a.: not applicable. Chromatograhic conditions: Mobile phase: MeOH, 25 mM formic acid, 12.5 mM diethylamine, 1 mL/min, 25 °C. Columns: 150×4 mm i.d., 5 µm material. Detection: UV (254 nm). CSPs 1–3: Quinine-based, CSP 4: Quinidine-based

[a]Lysine: multiple derivatives, reported here are the results for the main product (>40 % based on peak areas)

2-(2',2'-Dichloroacetyl)dimedone

The derivatization of 10 representative amino acids with halogen-containing 2-acetyldimedone was severely impaired by the electron-withdrawing substituent. The reaction yields were between 2–10 % and thus considerably lower than those obtained with 2-acetyldimedone. We observed chromatographic separation of dichloroacetyldimedone into two peaks representing the two main enol forms with an area ratio of ca. 1:2 while for other reagents the ratio was roughly 1:1. The electron-withdrawing substituent obviously shifts the tautomeric equilibrium towards the exocyclic enol. Thus, it forces the nucleophilic amine to attack an endocyclic carbon, a pathway that is irrelevant for most other reagents due to steric hindrance. Accordingly, we found considerable amounts of byproducts in the relevant areas of the chromatograms. Overall yields including byproducts were between 7–13 %. The reasons are found in a combination of the electronic factors outlined above and significant steric hindrance exerted by the bulky dichloroacetyl residue. We concluded that a derivatization reaction which fails to provide unequivocal product distributions is unfeasible, especially in the challenging field of stereoselective analysis, and, thus, the reagent was deemed unsuitable.

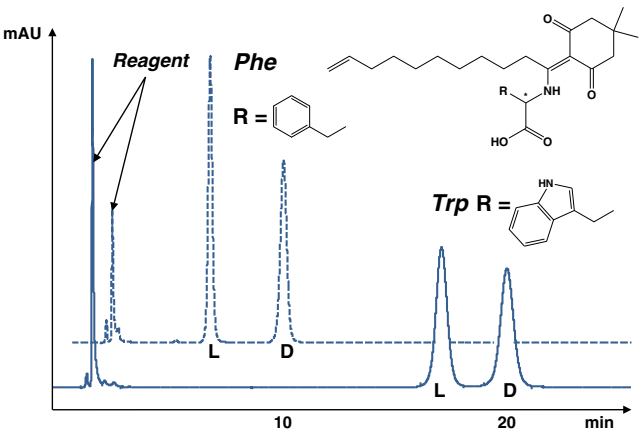


Fig. 5 Chromatographic enantioseparation of 2-undecenoyldimedone derivatives of racemic Phe and Trp. Chromatographic conditions: CSP 1 (150×4 mm i.d., 5 µm material), mobile phase: Methanol (50 mM formic acid, 25 mM diethylamine), 1 mL/min, 25 °C. Detection: UV (254 nm)

2-Acetyl-1,3-indandione

This structurally demanding, highly hydrophobic reagent was barely soluble in the reaction mixture but the reaction with 10 representative amino acids produced strongly UV-active derivatives. Unfortunately, acetylindandione proved impractical as a protective agent due to unfavorable chromatographic characteristics: While all other reagents were

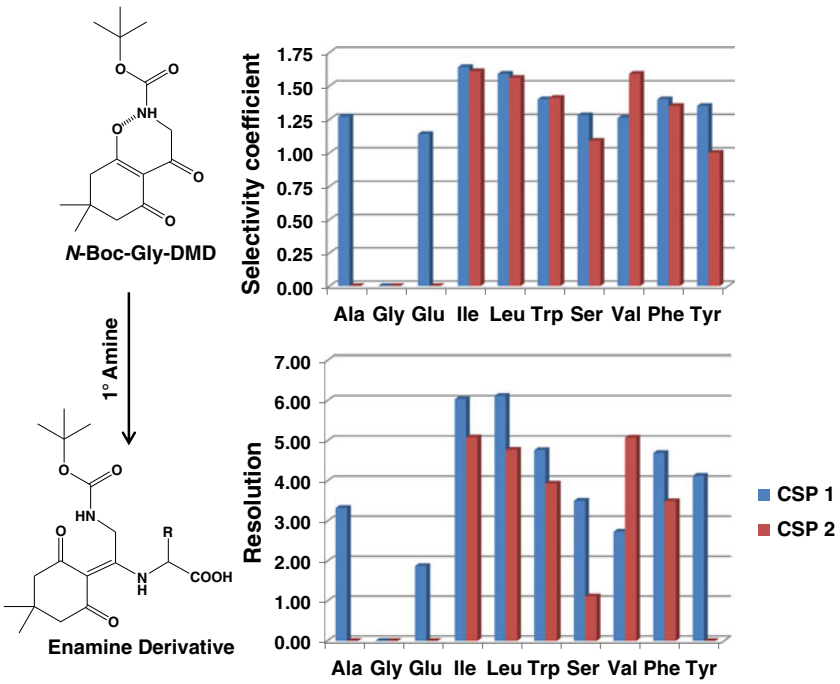


Fig. 6 Derivatization and enantioseparation of primary amino acids with *N*-Boc-glycyldimedone (*N*-Boc-Gly-DMD). Note that enantioselectivity and resolution are not defined for achiral glycine (Gly). Chromatographic conditions: Stationary phases: CSPs 1 and 2 (150×4 mm i.d., 5 µm material). Mobile phase: Methanol (50 mM FA, 25 mM DEA), 1 mL/min, 25 °C, detection: UV (254 nm)

well separated from the respective products on anion exchange CSPs 1–4 ($k_{reagent}$=ca. 0.1), acetylindandione was strongly retained ($k_{reagent}$=1–2) and produced two broad, heavily tailing peaks which frequently interfered with the analyte signals. This issue persisted on all four CSPs tested and could not be resolved through mobile phase modification with water, which is usually a reliable way to improve peak-shapes on ion-exchange stationary phases through moderation of the strong ionic interactions. Thus, we were forced to abandon this reagent as incompatible with our chromatographic application.

Acetylacetone and triacetylmethane

Compared to the cyclic tricarbonyl reagents, the highly symmetrical structures of acetylacetone (AcAc) and triacetylmethane (TAM) give rise to fewer tautomeric forms and only one enol form needs to be considered for the reaction with amines (see Table 1 for structures).

With KOH as a base we were able to obtain the AcAc derivatives of several proteinogenic amino acids (Leu, Ile, Val) as well as those of *trans*-1,2-aminocyclohexanesulfonic acid and *cis*-1,3-aminocyclohexanesulfonic acid. However, chromatographic analysis of the products (CSP 2, MP 4) revealed some shortcomings: While the reagent eluted very close to the void time and the product peak(s) were more strongly retained (retention factors between 0.4 and 5), enantiomer selectivities for the products were very weak (selectivity factors below 1.20).

In order to understand these findings, it is necessary to consider the increments to the chiral recognition mechanism of carbamoylated *Cinchona* alkaloids: The selectors are designed to exhibit high enantioselectivity for amide-type derivatives of amino acids, supported by hydrogen bond formation between the amide moieties and the carbamate residue of the selectors [34]. Repulsive steric interactions of the carbamoyl-bound residues (*tert*-butyl in CSP1, di(isopropyl)phenyl in CSP 2 and bis(trifluoromethyl)phenyl in CSPs 3 and 4) with amino acid side chains are another important contribution to chiral recognition. The derivatization with AcAc produces enamine-type products with reduced H bond acceptor properties. In addition, AcAc derivatization introduces a spatially undemanding substituent which is not sufficient for compensation of the loss of this important interaction site.

As opposed to the cyclic tricarbonyl compounds investigated in this study, TAM samples have been found to contain significant amounts of the keto form [36]. Nevertheless, TAM was capable of successfully converting 9 out of 10 amino acids (including achiral Gly; Glu conversion was incomplete due to solubility issues) into the respective enamines (Table 5). Under the conditions employed for this procedure, glutamic acid led to two (albeit barely detectable) sets of peaks, which hints at a side reaction. Unfortunately, we were not able to recover the peak pairs for further evaluation.

The TAM derivatization led to a shift in the absorbance maximum from 280 nm for the reagent to 310 nm for the derivatives. On the one hand, this thwarted efforts to estimate reaction yields based on peak areas but, on the other hand, it facilitated the straightforward evaluation of product retention factors and selectivities without interference from the reagent.

Table 5 Chromatographic results obtained for triacetylmethane (TAM) derivatives of amino acids on CSP 1

CSP 1				
Analyte	k_1	k_2	α	R_S
Ala	5.01	5.34	1.06	1.10
Ile	3.45	3.79	1.10	1.58
Leu	4.14	4.14	1.00	0.00
Trp	12.03	13.95	1.16	2.64
Ser	6.94	7.45	1.07	1.31
Val	3.58	3.94	1.10	1.59
Phe	7.74	8.57	1.11	2.06
Tyr	8.23	9.59	1.16	2.71

k_1, k_2: retention factors of first/second eluted enantiomer, α: selectivity, R_S: chromatographic resolution. CSP 1 (150×4 mm i.d., 5 μm material), mobile phase 2: MeOH, 25 mM formic acid, 12.5 mM diethylamine, 1 mL/min, 25 °C. Detection: UV (254 nm)

TAM derivatives of aromatic amino acids were well separated on CSP 1 with MP 2 (Table 5) but the products of aliphatic amino acids required MP 3 (10 % ACN, lower buffer concentration than MP 2) to achieve reasonable enantioselectivity and resolution (α values above 1.1, R_S>1.5, not shown). The latter conditions, on the other hand, were unsuitable for aromatic amino acid derivatives because acetonitrile interferes with π–π interactions, which are essential for the chiral recognition of this type of analytes.

"2-Ibuprofenyl-" and "2-Naproxenyldimedone"

The reagents were prepared from the enantiomerically pure drugs but their chiral 2-acyl substituents were found to have racemized during the synthetic procedure. Preliminary experiments investigating this phenomenon are given in the Electronic Supplementary Material. Further details and the feasibility of enantiomer separation of the reagents are the subject of currently ongoing studies (See also Electronic Supplementary Material section 5 and Figure S2).

Conclusion

In the course of this study, reagents incorporating a cyclic β-tricarbonyl motif as well as the acyclic triacetylmethane and the β-diketone acetylacetone were investigated for their suitability as derivatizing agents for primary amino acids. They were expected to enhance analyte enantiomer recognition on chiral anion exchangers and promote UV detectability.

In this context, 4 structurally related anion exchange-type chiral stationary phases were employed to chromatographically separate the product enantiomers and any excess reagent based on a multi-modal chiral recognition mechanism. The latter was significantly enhanced by the steric, hydrophobic and aromatic interaction sites introduced by the respective protective groups. In a study focusing on (enantiomer) selectivity and resolution values for the enamine-type derivatives, the products of 2-acetyldimedone- and 2-undecenoyldimedone were shown to possess favorable chromatographic characteristics.

Incompatibility of the solubilities of reagent and analytes was a commonly encountered issue, especially when amino acids with charged side chains and highly lipophilic reagents were combined. For solutes with more than one reactive amino group, we frequently observed multiple derivatization products, indicating limited selectivity of the reaction.

In accordance with a reaction mechanism involving a nucleophilic attack of the primary amine on the endocyclic enol form of the reagent, bulky 2-acyl substituents such as isobutyryl or pivaloyl were found to significantly impair product formation through steric hindrance. The overall highest reaction yields were consequently obtained with 2-acetyldimedone and 2-undecenoyldimedone. The enamine-type products were stable to degradation and racemization in acidic and basic environment but the analytes could easily be recovered through de-protection with hydrazine solution.

To sum up, the derivatization of amino acids with β-di- and tricarbonyl compounds was feasible as long as the 2-acyl substituent featured a methyl or a methylene group separating bulky moieties from the electrophilic carbonyl center. On the other hand, sterically demanding substituents were found to enhance chiral recognition on *Cinchona* carbamate anion exchangers. Despite the large number of well-established, straightforward achiral derivatization methods available, the method presented herein can certainly be regarded as an interesting concept for highly specific applications. More precisely, suitable target analytes for this methodology are primary amines and amino acids with only one reactive group (unequivocal derivatization results). If high chromatographic resolution is required (e.g. for preparative purposes), sterically demanding 2-acyl substituents such as pivaloyl are recommended but modification of the derivatization procedure might be required in order to facilitate quantitative product formation. Good product yields are obtained with 2-acetyl- or 2-undecenoyldimedone, both of which combined acceptable product yields with reasonable chromatographic separation performance on chiral anion exchangers. The application of the derivatization–separation–deprotection sequence for pharmaceutically relevant substances was outside the scope of this study. Nonetheless, it

appears particularly promising due to the preservation of the stereochemical integrity of the analyte, which was proven in the course of this study.

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