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ENANTIOMERIC RESOLUTION OF AMINO ACID DERIVATIVES ON CHIRAL STATIONARY PHASES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Chiral stationary phases (CSPs) containing urethane-blocked α-arylglycines as chiral groups were synthesized and found to have good enantioselectivity for several N^{α}-acylamino acid esters. Depending on the derivatization pattern of the amino acids, two different mechanisms of adsorption, generating opposite elution orders, could be observed. The best results were obtained utilizing amino acids as their N^{α}-3,5-dinitrobenzoylamino acid esters. The high resolution makes this method suitable for racemization studies in peptide and protein chemistry, as was demonstrated in some examples.

The mechanism of enantiomeric adsorption of N^{α}-3,5-dinitrobenzoylamino acid esters on such CSPs could be elucidated by a new method based on energy calculations for the reversible diastereomeric complexes. The calculated structures of the adsorption complexes agreed well with the observed elution orders of the amino acid derivatives.

INTRODUCTION

Differential enantiomeric adsorption of N^{α}-3,5-dinitrobenzoylamino acid esters on chiral stationary phases (CSPs) has been observed by several workers[1–6]. In almost every instance, CSPs carrying aromatic residues, such as phenyl or naphthyl, as a complementary binding site for the 3,5-dinitrobenzoyl group were used. This is analogous to the phenylglycine-containing stationary phase we have described earlier[7]. Aromatic interactions between the 3,5-dinitrobenzoyl group and the aromatic residue, linked to the α-C atom of the chiral group, seemed to be important for chiral recognition.

Analogous to aromatic charge-transfer complexes, such interactions can be described as electron donor–acceptor or π–π interactions by the valence bond method[8]. The extent of binding is mainly influenced by the tendency of the electron

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acceptor to receive an electron (characterized by the electron affinity) and the tendency of the electron donor to liberate an electron (characterized by the first ionization potential). If such interactions play an important role in enantioselective adsorption, variations in donor and/or acceptor strength should clearly influence the separations based on this effect.

In general, enantioselective adsorption can be described by a simple model, introduced by Dalgliesh[9], which reduces chiral recognition to the reversible formation of diastereomeric complexes between the chiral group of the CSP and the derivative that is adsorbed. For resolution, at least three simultaneous interactions must occur. Thus, in addition to the π–π interactions, two further binding sites must exist. In order to shed more light on the adsorption mechanism of amino acid derivatives on chiral phases, this paper deals with the chromatography of some amino acid derivatives with varying binding sites on CSP Type I and II (see Fig. 1).

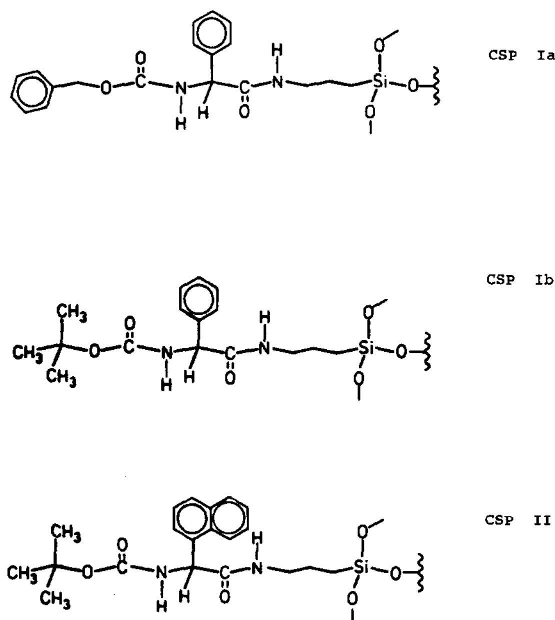


Fig. 1. Structures of chiral stationary phases Ia, Ib and II.

EXPERIMENTAL

Materials and methods

D-Phenylglycine was purchased from E. Merck (Darmstadt, F.R.G.) (1-Naphthyl)glycine was synthesized as a racemic mixture in about 50% yield from 1-naphthaldehyde by the Bucherer and Steiner variation of the Strecker synthesis[10].

N^{α}-*tert.*-Butyloxycarbonyl (Boc)-amino acids were obtained by the method of Moroder *et al.*[11], using di-*tert.*-butyl dicarbonate. N^{α}-benzyloxycarbonyl (*Z*)-amino acids were obtained by acylation of amino acids with benzyloxycarbonyl chloride[12]. Racemic Boc-(1-naphthyl)glycine was converted into pure Boc-L-(1-naphthyl)glycine

with high enantiomeric excess by esterification of the racemate with diazomethane and subsequent enzymatic hydrolysis with chymotrypsin at pH 7.9. Linking of chiral groups to the polymer matrix followed the N,N'-dicyclohexylcarboiimide–1-hydroxybenzotriazole route[13]. γ-Aminopropyl-functionalized silica was prepared from LiChrosorb Si 100, 5 μm (E. Merck), and 3-triethoxysilylpropylamine[13]. To reduce the polarity from remaining SiOH groups, all modified silica gels were end-capped with $(CH_3)_3SiCl$.

Chromatographic columns (250 × 4.5 mm I.D.) were slurry-packed by conventional techniques to yield CSPs Ia, Ib and II. CSP Ia contained 0.49 mmol of (*Z*)-D-Phg–$NH(CH_2)_3Si(OC_2H_5)_2$– per gram of gel, CSP Ib contained 0.48 mmol of Boc–D-Phg–$NH(CH_2)_3Si(OC_2H_5)_2$– per gram of gel and CSP II contained 0.39 mmol of Boc–L-(1-naphthyl)Gly–$Si(OC_2H_5)_2$– per gram of gel, where Phg represents phenylglycine.

N^α-3,5-Dinitrobenzoylamino acid esters were obtained from the pure amino acids (or amino acid mixtures of peptide hydrolysates) by esterification with 2-propanol–hydrochloric acid and subsequent acylation with 3,5-dinitrobenzoyl chloride. Other N^α-acylated amino acid derivatives were obtained in a similar manner. Detailed instructions for preparation are given in ref. 13. All new compounds showed the expected analytical and spectroscopic data (elemental analysis, amino acid analysis, ^1H NMR, ^{13}C NMR).

Chromatography

Experiments were carried out with a Perkin-Elmer (Überlingen, F.R.G.) Series 3B liquid chromatograph, equipped with a Perkin-Elmer LC-75 variable-wavelength detector. Solvents were glass-distilled before use. Solutes were injected in concentrations of 0.2–0.5 mg/ml, using a 10-μl sample loop.

RESULTS AND DISCUSSION

In every instance (except with proline), the enantiomeric N^α-3,5-dinitrobenzoylamino acid 2-propyl esters were resolved by chromatography on CSPs Ia and II. The racemization test on a synthetic Leu^5-enkephalin derivative, synthesized by our group[14], gives evidence for the analytical applicability of this chromatographic method in peptide chemistry. For this purpose, 5 mg of the peptide were cleaved by acid hydrolysis (6 *M* hydrochloric acid, 24 h, 110°C). The hydrolysate was subjected to the described derivatization procedure[13] and resolved on CSP Ia.

Fig. 2 shows the chromatogram of a test mixture (A) and of the hydrolysate (B). As can be seen, only slight racemization occurred with tyrosine. Evaluation of the peak size gave 2.9 ± 0.2% of D-Tyr in the hydrolysate of the peptide. As other investigations[15] showed, this content of D-enantiomer is due to racemization during hydrolysis, so that the synthesized Leu^5-enkephalin may be considered to be free from D-amino acids.

As Tables I–III show, chromatography of N^α-3,5-dinitrobenzoylamino acid esters on CSPs I and II always produces the same elution orders of the enantiomers. With the D-phenylglycine-containing CSP I, the L-enantiomer is more strongly retained than the D-enantiomer. On L-(1-naphthyl)glycine-containing CSP II the elution orders are the opposite, owing to the configurational change from CSP I to CSP

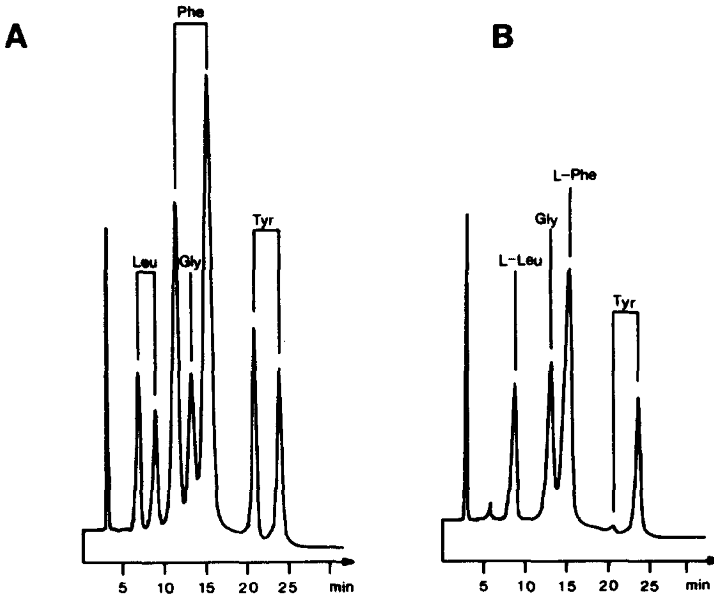


Fig. 2. Chromatogram of N^{α}-3,5-dinitrobenzoylamino acid 2-propyl esters on CSP Ia. Column, 250 × 4.5 mm I.D.; stepwise elution, 12 min 11% 2-propanol in hexane then 25 min 25% 2-propanol in hexane, flow-rate 1 ml/min. (A) Sample, test mixture of D/L-Leu, Gly, D/L-Phe and D/L-Tyr. (B) Sample, hydrolysate of a synthetic Leu^5-enkephalin.

TABLE I

ENANTIOMER SEPARATION OF N^{α}-3,5-DINITROBENZOYLAMINO ACID 2-PROPYL ESTERS ON CSP Ia

k'_D = Capacity factor for the D-isomer; k'_L = capacity factor the L-isomer.

Amino acid	k'_D	k'_L	α	*Mobile phase (%, v/v, 2-propanol in hexane)*
Alanine	3.27	4.31	1.31	7.5
Valine	2.14	3.48	1.63	7.5
Leucine	1.91	2.88	1.51	7.5
Isoleucine	1.84	3.15	1.71	7.5
Proline	2.67	2.67	1.00	7.5
Aspartic acid	2.51	3.00	1.19	7.5
Glutamic acid	2.52	3.39	1.35	7.5
Serine	7.09	7.50	1.06	7.5
Threonine	4.68	5.58	1.19	7.5
Phenylglycine	3.64	4.93	1.36	7.5
Phenylalanine	3.76	5.45	1.45	7.5
Tyrosine	3.53	5.11	1.45	15.0
Lysine	5.42	6.94	1.28	30.0
Methionine	4.52	6.51	1.44	7.5

TABLE II

ENANTIOMER SEPARATION OF N^{α}-3,5-DINITROBENZOYLAMINO ACID 2-PROPYL ESTERS ON CSP Ib

Amino acid	k'_D	k'_L	α	*Mobile phase (%, v/v, 2-propanol in hexane)*
Alanine	1.77	2.16	1.22	7.5
Valine	0.90	1.42	1.57	7.5
Leucine	0.85	1.36	1.60	7.5
Isoleucine	0.79	1.26	1.59	7.5
Proline	2.60	2.60	1.00	7.5
Aspartic acid	1.05	1.24	1.18	7.5
Glutamic acid	1.15	1.51	1.31	7.5
Serine	3.00	4.14	1.38	7.5
Threonine	2.76	3.15	1.14	7.5
Phenylglycine	1.32	1.75	1.32	7.5
Phenylalanine	1.42	1.92	1.35	7.5
Tyrosine	3.76	5.10	1.36	10.0
Lysine	3.63	4.61	1.27	15.0
Methionine	1.83	2.48	1.35	7.5

II. This observation suggests that in both instances the same adsorption mechanism (mechanism A) is operative.

Further, comparison of Tables II and III shows that on CSP II all derivatives are adsorbed with enhanced capacity factors under the same chromatographic conditions, although CSP II has the lowest content of chiral groups. We suggest that this stronger adsorption is due to the lower first ionization potential (8.12 eV) of the

TABLE III

ENANTIOMER SEPARATION OF N^{α}-3,5-DINITROBENZOYLAMINO ACID 2-PROPYL ESTERS ON CSP II

Amino acid	k'_D	k'_L	α	*Mobile phase (%, v/v, 2-propanol in hexane)*
Alanine	7.56	6.56	1.15	7.5
Valine	4.96	3.05	1.63	7.5
Leucine	4.87	2.82	1.75	7.5
Isoleucine	4.49	2.65	1.69	7.5
Proline	7.12	7.12	1.00	7.5
Aspartic acid	4.14	3.49	1.19	7.5
Glutamic acid	4.99	3.42	1.46	7.5
Serine	8.81	6.61	1.33	15.0
Threonine	4.30	3.79	1.14	15.0
Phenylglycine	6.36	5.23	1.22	7.5
Phenylalanine	6.85	5.46	1.25	7.5
Tyrosine	5.54	4.47	1.24	25.0
Lysine	7.11	5.88	1.21	40.0
Methionine	4.12	3.38	1.22	7.5

naphthyl groups in comparison with the first ionization potential of 9.24 eV for the phenyl groups. This makes the former a stronger donor and, therefore, a preferred binding site in interactions with the 3,5-dinitrobenzoyl group of the derivatives.

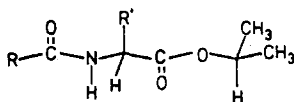
Further evidence for the presence of π–π interactions was obtained from chromatographic results with the N^{α}-acylated amino acid 2-propyl esters listed in Table IV. While N^{α}-3,5-dinitrobenzoylamino acid 2-propyl esters are clearly resolved on both CSPs I and II by the same adsorption mechanism (indicated by the same elution orders), there is a sharp decrease in capacity and separation factors on changing to N^{α}-pentafluorobenzoylamino acid 2-propyl esters. However, the elution orders remain the same.

Replacement of the N^{α}-pentafluorobenzoyl group with the N^{α}-benzoyl group afforded different effects on both types of CSP. Enantiomers of N^{α}-pentafluorobenzoylated and of N^{α}-benzoylated amino acid derivatives are not resolved on CSP I. On CSP II, however, these derivatives are resolved, but with a change in elution order. This indicates that a new mechanism of adsorption (mechanism B) is now operative. When the N^{α}-benzoyl group is replaced with the N^{α}-acetyl group, enantioselective adsorption again takes place on both types of CSP. The elution orders indicate that mechanism B now dominates the separations on CSPs I and II.

In general, the capacity factors, k', and separation factors, α, decrease in parallel to the π-acceptor activities (influenced by the electron-withdrawing effect of the substituents). The N^{α}-3,5-dinitrobenzoyl system, which is believed to be the strongest π-acceptor, shows the largest k' and α values.

TABLE IV

ENANTIOMER SEPARATIONS OF VARIOUS N^{α}-ACYLAMINO ACID 2-PROPYL ESTERS



CSP	*R*	*R'*	k'_D	k'_L	α	*Mobile phase (%, v/v, 2-propanol in hexane)*	*Flow-rate (ml/min)*
Ib		$-CH(CH_3)_2$	0.61	0.75	1.22	2.0	0.5
II			1.29	1.15	1.12	1.0	1.0
Ib		$-CH_2-C_6H_5$	1.03	1.27	1.23	2.0	0.5
II			2.13	1.81	1.18	1.0	1.0
Ib		$-C_6H_5$	3.08	3.08	1.00	2.0	0.5
II			1.57	1.67	1.06	2.0	1.0
Ib		$-CH_2-C_6H_5$	3.76	3.76	1.00	2.0	1.0
II			1.77	1.90	1.07	2.0	1.0
Ib	CH_3-	$-C_6H_5$	2.91	2.77	1.05	2.0	0.5
II			4.16	4.40	1.06	2.0	1.0
Ib		$-CH_2-C_6H_5$	3.07	2.88	1.07	2.0	0.5
II			5.13	5.56	1.08	2.0	1.0

Simultaneously with the decrease in π-acceptor activity, another competing mechanism (mechanism B) occurs, which increasingly dominates the separations. The fact that even N^{α}-acetylamino acid 2-propyl esters are resolved by this competing mechanism shows that it is not based on π–π interactions, as is mechanism A. Mechanism B is believed to depend much more on a combination of hydrogen bonds and steric repulsion. This would explain the fact that mechanism B is more active on CSP II, which carries the large 1-naphthyl group. The actual nature of mechanism B is still unknown, but the observed elution orders indicate that it might be the same mechanism that is operative on two different CSPs, also capable of resolving N^{α}-acetylated amino acid esters, as reported by Dobashi *et al.*[16] and Ôi and Kitahara[17].

However, the nature of adsorption mechanism A is now clear. As could be seen from the chromatography of proline and other N-methylated amino acids[13] which are not resolved on CSPs I and II, the amide NH group (as a potential hydrogen donor) of the amino acid derivatives also seems to be essential as a binding site in addition to the π–π interactions of the two aromatic groups. The fact that N^{α}-3,5-dinitrobenzoyl-1-phenylethylamine[13], which for this discussion may be considered to be a decarboxylated amino acid, is likewise unresolved on our CSP makes it likely that the carbonyl oxygen of the ester group (as a potential hydrogen acceptor) acts as the third binding site, as required by theory.

Binding sites of N^{α}-3,5-dinitrobenzoylamino acid 2-propyl esters on CSPs I and II are shown by arrows in Fig. 3A and complementary binding sites, belonging to the chiral groups of CSPs I and II, are indicated in Fig. 3B.

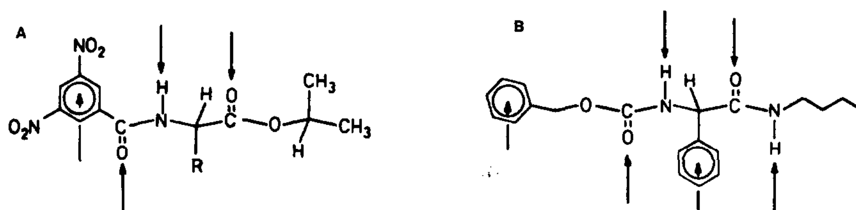


Fig. 3. (A) Binding sites of N^{α}-3,5-dinitrobenzoylamino acid 2-propyl esters on CSPs I and II. (B) Complementary binding sites belonging to the chiral groups of CSPs I and II.

At this stage of our investigation we used a computer program to determine the actual geometry of the adsorption complex. This program (Chem.X, developed and distributed by Chemical Design, Oxford, IK) is based on energy calculations of the adsorption complexes. The total potential energy (V) is calculated as the sum $V = V_b + V_\theta + V_\Phi + V_{nb} + V_e + V_{HB}$, where V_b = bond-stretching and compression terms, V_θ = valence angle bending terms, V_Φ = internal rotational or torsional terms, V_{nb} = non-bonding interactions, V_e = electrostatic or Coulomb interactions and V_{HB} = hydrogen bonds. Total potential energies calculated in this manner were then minimized as a function of molecular coordinates by an iteration procedure to give the most stable complex conformation. After successive exchange of all possible binding sites, at least one physically significant complex geometry remains, which agrees very well with the observed elution orders.

Fig. 4A shows this energy-minimized complex conformation for the adsorption

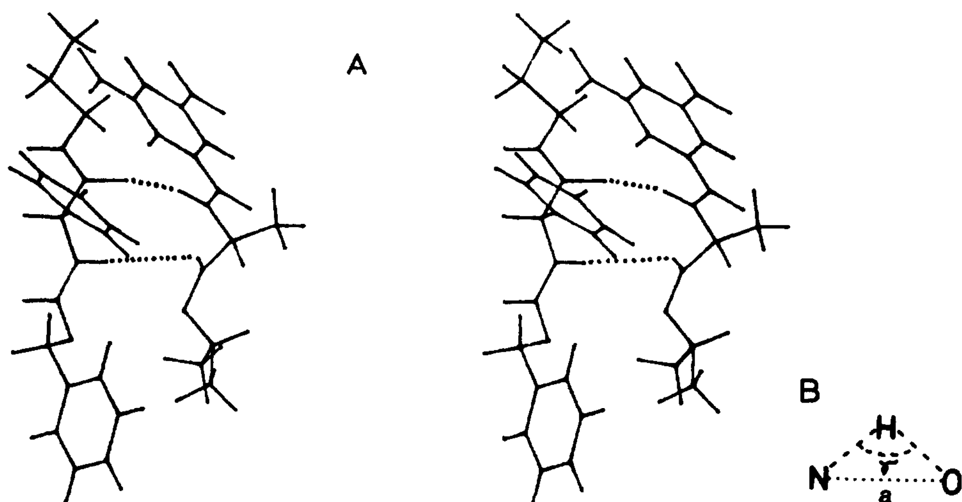


Fig. 4. (A) Stereo pair of the diastereomeric adsorption complex N^{α}-3,5-dinitrobenzoylamino-L-alanine 2-propyl ester with CSP Ia. (B) Intermolecular distances and angles in hydrogen bonds.

of the N^{α}-3,5-dinitrobenzoyl-L-alanine 2-propyl ester on CSP Ia, which is due to the larger capacity factor of the more stable diastereomeric complex. Chiral recognition consequently results from two hydrogen bonds (dotted lines) between the amino acid derivative and the chiral group of the CSP. The geometry is determined by an intermolecular distance a = 3.3 Å and angle γ = 12.8° (Fig. 4B). These data are in good agreement with other calculated or measured data for strong hydrogen bonds in peptides and their derivatives[18]. Further, Fig. 4A shows that the aromatic systems of the 3,5-dinitrobenzoyl group and the phenyl group, attached to the α-C atom of the CSP, are almost parallel to each other, with a dihedral angle of 12.0° and a distance of 3.7 Å. In this arrangement, the two aromatic systems are able to build up a third binding site by efficient π–π interactions, as required by theory. Similar data have been cited in the literature for many stable organic electron donor–acceptor complexes[19].

Application of the same procedure to the adsorption of the D-enantiomeric alanine derivative yields the complex structure shown in Fig. 5. In this instance, adsorption also results from the action of two hydrogen bonds with an intermolecular distance a = 3.1 Å and angle γ = 6.7°. However, in contrast to the adsorption of the L-enantiomer, the aromatic system of the 3,5-dinitrobenzoyl group and the phenyl group are widely separated from each other, enclosing a dihedral angle of over 90°. This arrangement no longer allows appreciable π–π interactions, resulting in lower adsorption of the D-enantiomer.

According to this model, chiral recognition therefore depends on two hydrogen bonds and on π–π interactions. Only the L-enantiomer is strongly attached to the CSP by the full number of these three interactions, thus yielding long retention times, whereas the D-enantiomer is held by only two of these interactions, which results in a weaker adsorption and therefore a shorter retention time.

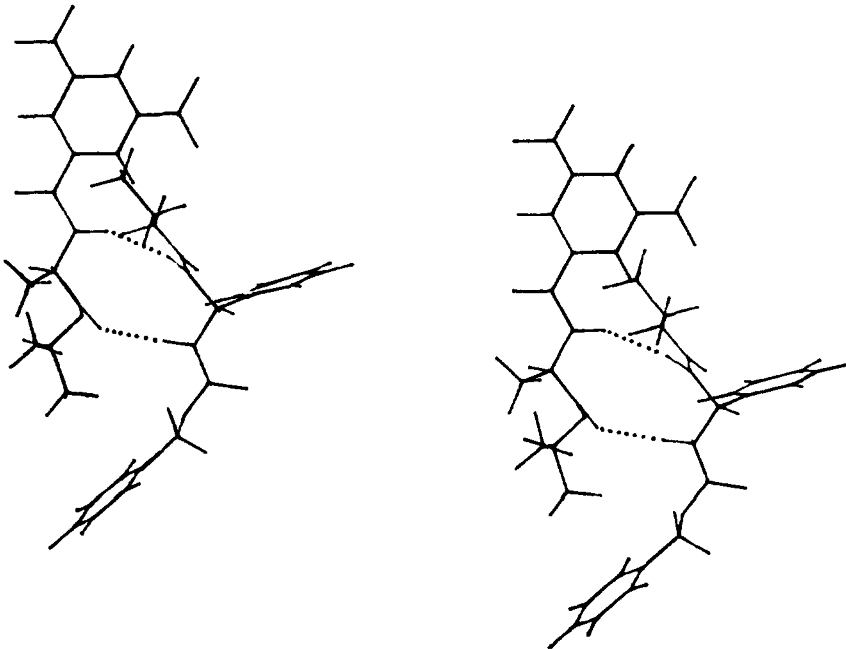


Fig. 5. Stereo pair of the diastereomeric adsorption complex of N$^{\alpha}$-3,5-dinitrobenzoylamino-D-alanine 2-propyl ester with CSP Ia.

As calculations show, chiral recognition of 3,5-dinitrobenzoylamino acid esters on CSP II follows the same mechanism. However, according to steric requirements, chiral separations on CSP II are believed to be much more affected by the competing mechanism B, which produces opposite elution orders. Therefore, competition between the two mechanisms would cause a decrease in separation factors, which might explain why the separation factors on CSP II are not significantly larger than those on CSP I, as would be expected from the theory of π–π interactions.

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