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DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON IMMOBILIZED PROTEIN STATIONARY PHASES

V. OPTICAL RESOLUTION OF N-(2,4-DINITROPHENYL)- AND DANSYL-D,L-AMINO ACIDS

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SUMMARY

Analytical-scale optical resolution of a series of N-(2,4-dinitrophenyl)- and dansyl-D,L-amino acids has been effected by the use of a bovine serum albumin silica column (Resolvosil). Decreasing retention was found for both types of amino acid with increasing pH or 1-propanol content of the mobile phase. In the dinitrophenyl series, the aspartic acid derivative showed very large enantiomeric separation factors compared with the glutamic acid homologue, and an analogous, but less pronounced, effect was found for the phenylglycine–phenylalanine pair. Fluorimetric studies of dansyl-alanine showed that by a simple post-column addition of 1-propanol the fluorescence yield can be increased by a factor of over 20, giving a very low detection limit. The usefulness of the analytical technique for the determination of the bacterial marker compounds, D-alanine and D-glutamic acid, present in cell wall hydrolysates, is emphasized.

INTRODUCTION

It has recently been suggested that the D-amino acids present in the peptidoglycan layer of bacteria, notably D-alanine and D-glutamic acid, could be useful as specific marker compounds for such microorganisms[1], and some exploratory investigations along these lines have already been performed by means of gas chromatography–mass spectrometry (GC–MS)[2]. These were based on separation of the enantiomers of alanine as N-heptafluorobutyryl isobutyl ester derivatives on a Chirasil-Val capillary column.

In connection with these efforts, we found it of interest to perform a general investigation of the possibilities of separating the enantiomers of some readily prepared derivatives of amino acids by chiral liquid chromatography (LC), *viz.* a series of N-(2,4-dinitrophenyl) (DNP) and N-dansyl derivatives, which permit detection with high sensitivity. The potential advantages over capillary GC would be the less

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complicated sample treatment and the substantially higher sample volume load capacity of the column.

EXPERIMENTAL

Amino acid derivatives

The DNP-amino acids were all synthesized according to the method described by Rao and Sober[3]. The purity of the compounds obtained was ascertained by m.p., ^{1}H NMR, IR and UV. The dansyl derivatives were obtained from Sigma (St. Louis, MO, U.S.A.), except for the derivatives of D-alanine and D-glutamic acid which were prepared by the procedure given by Taphui *et al.*[4], in yields of 48% and 32%, respectively.

Chromatography

The LC equipment was essentially the same as described previously[5]. The earlier part of the investigation was performed with a column different from the one used later, therefore the values given in Table I are not directly comparable with data given in the following Tables.

All chromatography was performed isocratically at a flow-rate of 1.0 or 1.5 ml/min. The DNP compounds were detected at 340 nm and the dansyl derivatives at 250 nm.

Peak assignment of all DNP-amino acids was made by chromatography of samples spiked with the appropriate L-enantiomer. Similarly, assignment of the elution order of dansyl-D,L-alanine and -D,L-glutamic acid was obtained by chromatography with the corresponding D-enantiomers. The L-enantiomer was eluted first in all DNP compounds, as well as for dansyl-alanine and -glutamic acid.

Fluorescence intensity measurements

The investigations of fluorescence intensity enhancement of dansyl-alanine by 1-propanol addition was performed with a Shimadzu Model RF-510 LC spectrofluorimeter. The excitation and emission wavelengths used were 347 and 396 nm, respectively. A 50 m*M* phosphate buffer (pH 6.9) containing 0–50% of 1-propanol was used as the solvent.

Amino acid analysis of bacterial cell wall hydrolysate

Cell walls (10.0 mg of dry weight) of *Bacillus megaterium* (ATCC 14581), prepared by a freeze-press technique[6], were hydrolysed with 3.0 ml of 6 *M* hydrochloric acid at 110°C for 24 h under nitrogen in a sealed tube[7]. The hydrolysis mixture was centrifuged on a bench-top centrifuge and the supernatant evaporated to dryness. The residue was dissolved in 4.5 ml of a 50 m*M* citrate buffer (pH 2.2). A 250-μl volume was taken for amino acid analysis.

RESULTS

As found earlier for N-aroyl-substituted amino acids[8], the retentions of the DNP derivatives differ widely depending on the chiral stationary phase used. From Table I it is apparent that the hydrophobic character of the amino acid side-chain

TABLE I

RETENTION DATA OBTAINED FOR THE SERIES OF DNP-AMINO ACIDS UNDER IDENTICAL CHROMATOGRAPHIC CONDITIONS

Mobile phase, phosphate buffer (50 m*M*)–2% 1-propanol (pH 6.84); flow-rate, 1.5 ml/min.

Amino acid function	k'_1	k'_2	α
Serine	2.99	3.46	1.16
Alanine	20.5	26.8	1.30
Methionine	19.8	80	4.0
Aspartic acid	10.5	75.5	7.21
Glutamic acid	11.1	13.5	1.21
Phenylglycine	36.2	93.6	2.59
Phenylalanine	44.8	64.3	1.44
Tyrosine	29.9	38.8	1.30

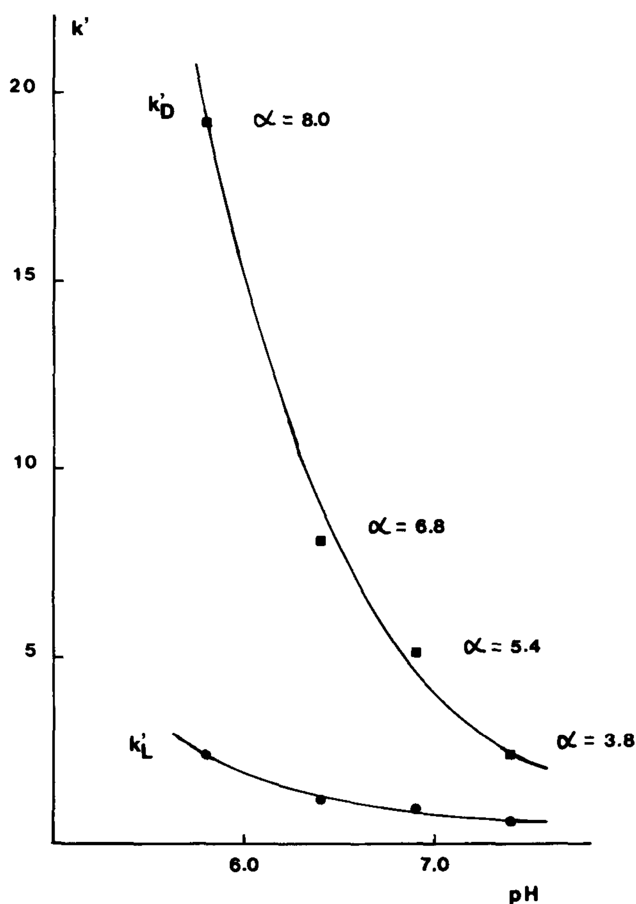

Fig. 1. Capacity ratios of L- and D-DNP-aspartic acid (k'_L and k'_D, respectively) as a function of mobile phase pH.

greatly influences the k'_1 value. Retention of the last-eluted enantiomer is probably highly dependent on rather subtle steric requirements; k'_2 is therefore even more variable and may give rise to high α-values. Of particular interest is the great difference in k'_2 (and α) between DNP-aspartic acid and its next higher homologue, DNP-glutamic acid. However, a more detailed study of the mobile phase dependence, performed with a different column, showed that this difference is highly pH-dependent as k'_2 of DNP-aspartic acid increases very rapidly with decreasing pH (Fig. 1).

The α-variation was much less pronounced for the dansyl derivatives, with values ranging between 1.0 and 2.3. Dansyl-tryptophan was an exception with a value of 4.0.

The results from studies of the influence of 1-propanol content, pH and buffer strength of the mobile phase system used, are given in Tables II–IV.

The general decrease in k' values with increasing 1-propanol content or pH is evident; however, there appears to be a more complex dependence on the buffer strength, in some cases giving minima in k'.

The amino acid composition of the cell wall material from *B. megaterium* is shown in Table V. Since the two dominating amino acids, alanine and glutamic acid, are well separated in the amino acid chromatogram; their respective isolation by ion-exchange chromatography prior to derivatization should therefore be feasible.

The performance of the chiral column used for the enantio-separations is illustrated by Fig. 2, which shows the resolution of the two derivatives of D,L-glutamic acid.

TABLE II

RETENTION DATA AS A FUNCTION OF 1-PROPANOL CONTENT OF THE MOBILE PHASE

Data are k'_1; k'_2; α values. Mobile phase, phosphate buffer (50 m*M*, pH 7.7); flow-rate 1.0 ml/min.

Amino acid derivative	*1-Propanol (%)*				
	0	*1*	*2*	*3*	*4*
DNP					
Serine	0.48; 0.70; 1.5	0.37; 0.47; 1.3	0.21; 0.25; 1.2	0.16; 0.16; 1.0	0.10; 0.10; 1.0
Tyrosine	4.3; 5.4; 1.2	2.9; 3.3; 1.1	2.2; 2.2; 1.0	1.7; 1.7; 1.0	1.2; 1.2; 1.0
Methionine		2.1; 8.6; 4.1	1.8; 5.6; 3.1	1.3; 4.1; 3.2	1.0; 2.5; 2.5
Glutamic acid		2.2; 9.5; 4.3	1.1; 4.2; 3.8	0.68; 1.7; 2.5	0.42; 1.0; 2.4
Aspartic acid		2.0; 5.4; 2.7	1.0; 2.4; 2.4	0.72; 1.7; 2.4	0.42; 0.84; 2.0
Alanine		1.4; 2.3; 1.6	1.0; 1.3; 1.3	1.0; 1.1; 1.1	0.68; 0.68; 1.0
Phenylalanine		7.5; 7.5; 1.0	4.7; 5.3; 1.1	4.2; 4.8; 1.1	2.8; 3.5; 1.2
Phenylglycine		6.6; 33.4; 5.1	5.1; 18.4; 3.6	4.6; 11.4; 2.5	3.7; 7.8; 2.1
Dansyl					
Serine		2.2; 2.2; 1.0	1.2; 1.4; 1.2	0.90; 1.2; 1.3	0.74; 1.0; 1.4
Threonine	2.2; 2.6; 1.2	1.6; 1.6; 1.0	1.3; 1.3; 1.0	1.0; 1.0; 1.0	0.55; 0.55; 1.0
Methionine	11.2; 11.2; 1.0	7.4; 7.4; 1.0	4.1; 4.1; 1.0		
Glutamic acid		0.85; 1.6; 1.9	0.68; 1.1; 1.6	0.53; 0.74; 1.4	0.35; 0.45; 1.3
Aspartic acid		2.0; 3.6; 1.8	1.5; 2.6; 1.7	1.2; 1.9; 1.6	0.95; 1.6; 1.7
Alanine		5.3; 7.8; 1.5	2.9; 3.9; 1.3	2.4; 3.2; 1.3	1.6; 2.0; 1.2
Phenylalanine		14.5; 18.8; 1.3	10.7; 14.4; 1.3	9.9; 13.8; 1.4	9.3; 12.8; 1.4

TABLE III

RETENTION DATA AS A FUNCTION OF pH OF THE MOBILE PHASE

Data are k'_1; k'_2; α values. Mobile phase, phosphate buffer (50 mM)–2% 1-propanol; flow-rate, 1.0 ml/min.

Amino acid derivative	*pH*			
	5.8	*6.4*	*6.9*	*7.4*
DNP				
Serine	0.47; 0.47; 1.0	0.32; 0.32; 1.0	0.25; 0.25; 1.0	0.21; 0.21; 1.0
Tyrosine	3.9; 5.2; 1.3	2.5; 3.3; 1.3	2.0; 2.4; 1.2	1.7; 1.9; 1.1
Methionine	3.2; 12.2; 3.8	1.8; 7.1; 3.9	1.6; 6.8; 4.2	1.4; 4.7; 3.4
Glutamic acid	2.5; 2.5; 1.0	1.2; 1.2; 1.0	0.80; 1.1; 1.4	0.58; 1.1; 1.9
Aspartic acid	2.4; 19.2; 8.0	1.2; 8.1; 6.8	0.95; 5.1; 5.4	0.63; 2.4; 3.8
Alanine	2.6; 2.9; 1.1	1.8; 2.1; 1.2	1.3; 1.6; 1.2	1.0; 1.2; 1.2
Phenylalanine	10.1; 12.8; 1.3	5.9; 7.7; 1.3	4.6; 5.8; 1.3	3.8; 4.1; 1.1
Phenylglycine	7.2; 14.9; 2.1	6.8; 14.5; 2.1	5.8; 13.2; 2.3	5.2; 11.3; 2.2
	5.7	*6.8*	*7.3*	*7.9*
Dansyl				
Serine	7.1; 9.4; 1.3	3.7; 4.6; 1.2	3.0; 3.7; 1.2	2.5; 2.5; 1.0
Threonine	4.9; 4.9; 1.0	3.2; 3.2; 1.0	2.2; 2.2; 1.0	1.9; 1.9; 1.0
Methionine	17.0; 17.0; 1.0	9.8; 9.8; 1.0	8.4; 8.4; 1.0	7.2; 7.2; 1.0
Glutamic acid	4.5; 5.1; 1.1	1.6; 1.9; 1.2	1.7; 2.3; 1.4	1.6; 3.4; 2.1
Aspartic acid	9.6; 14.9; 1.6	4.0; 6.4; 1.6	3.2; 5.4; 1.7	3.4; 6.9; 2.0
Alanine	21.5; 27.3; 1.3	8.6; 11.8; 1.4	7.5; 10.8; 1.4	6.4; 8.9; 1.4
Phenylalanine	38.2; 62.8; 1.6	28.6; 45.4; 1.6	22.2; 34.8; 1.6	26.8; 41.1; 1.5

TABLE IV

RETENTION DATA AS A FUNCTION OF BUFFER STRENGTH OF THE MOBILE PHASE

Data are k'_1; k'_2; α values. Mobile phase, phosphate buffer–2% 1-propanol (pH 7.7); flow-rate, 1.0 ml/min.

Amino acid derivative	*Buffer strength (mM)*			
	25	*50*	*75*	*100*
DNP				
Serine	0.21; 0.21; 1.0	0.21; 0.21; 1.0	0.15; 0.15; 1.0	0.15; 0.15; 1.0
Tyrosine	2.0; 2.4; 1.2	1.9; 1.9; 1.0	1.9; 1.9; 1.0	2.0; 2.2; 1.1
Methionine	1.6; 6.5; 4.1	1.6; 5.8; 3.6	1.5; 5.4; 3.6	1.6; 5.3; 3.3
Glutamic acid	2.0; 5.2; 2.6	0.79; 2.0; 2.5	0.64; 1.7; 2.5	0.50; 1.3; 2.6
Aspartic acid	1.2; 5.5; 4.6	0.84; 2.9; 3.4	0.68; 2.1; 3.1	0.50; 1.6; 3.2
Alanine	1.2; 1.4; 1.2	1.2; 1.4;1.2	1.0; 1.4; 1.4	0.90; 1.2; 1.3
Phenylalanine	6.5; 7.3; 1.1	5.6; 5.6; 1.0	5.3; 5.3; 1.0	4.9; 4.9; 1.0
Phenylglycine	5.2; 15.6; 3.0	4.3; 11.3; 2.6	5.3; 13.0; 2.4	6.8; 16.1; 2.4
	10	*50*	*75*	*100*
Dansyl				
Serine	1.8; 2.1; 1.2	1.8; 2.0; 1.1	1.6; 2.0; 1.2	1.6; 1.9; 1.2
Threonine	1.4; 1.4; 1.0	2.2; 2.2; 1.0	1.7; 1.7; 1.0	1.4; 1.4; 1.0
Methionine	5.1; 5.1; 1.0	4.8; 4.8; 1.0	4.5; 4.5; 1.0	4.2; 4.2; 1.0
Glutamic acid	1.3; 3.0; 2.3	0.79; 1.1; 1.4	0.68; 0.95; 1.4	0.67; 0.89; 1.3
Aspartic acid	2.8; 6.0; 2.2	1.8; 3.1; 1.7	1.3; 2.2; 1.7	1.3; 2.0; 1.5
Alanine	4.0; 5.8; 1.4	4.0; 5.4; 1.4	3.6; 5.0; 1.4	3.5; 4.7; 1.3
Phenylalanine	15.5; 25.2; 1.6	14.0; 20.4; 1.4	12.3; 16.9; 1.4	15.7; 21.5; 1.4

TABLE V

AMINO ACID COMPOSITION OF CELL WALL MATERIAL FROM *B. MEGATERIUM*

Amino acid	*Concentration in analyte (μM)*	*Based on dry weight (%)*
Aspartic acid	61	0.4
Threonine	42	0.2
Serine	47	0.2
Glutamic acid	1060	7.1
Glycine	77	0.3
Alanine	1460	5.9
Valine	54	0.3
Methionine	29	0.2
Diaminopimelic acid	850	7.3
Leucine	72	0.4
Tyrosine	25	0.2
Phenylalanine	28	0.2
Ethanolamine	63	0.2
Ornithine	3	0
Lysine	59	0.4
Histidine	14	0.1
Arginine	30	0.2

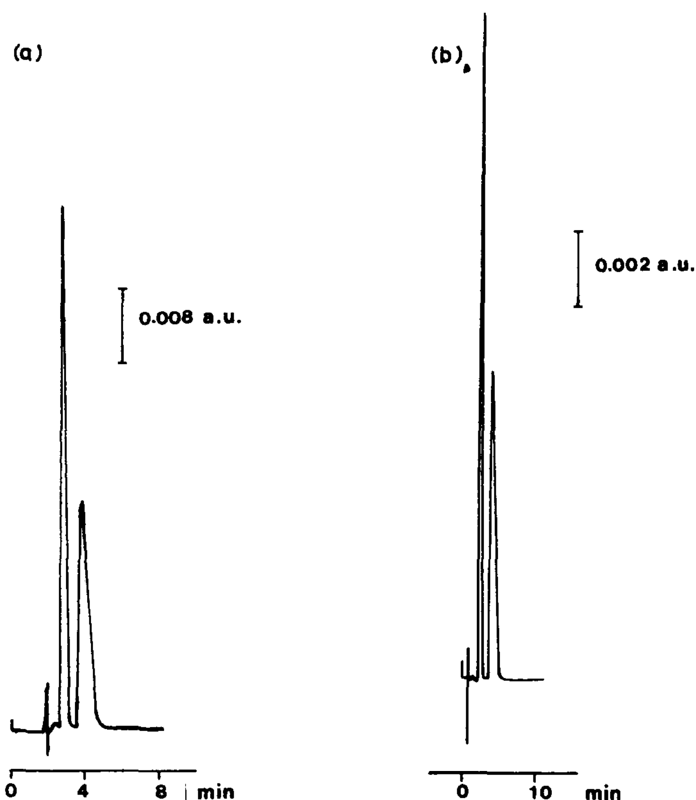

Fig. 2. Chromatograms of (a) DNP- and (b) dansyl-D,L-glutamic acid. Conditions used: 50 m*M* phosphate buffer; (a) 4% 1-propanol (pH 7.7), sample concentration 105 *μM*; (b) 2% 1-propanol (pH 7.9), sample concentration 138 *μM*.

Under the conditions used, the L-forms of all DNP-amino acids investigated were eluted prior to the corresponding D-enantiomers. The same elution order was found for dansyl-alanine and -glutamic acid; the elution order of the other dansyl amino acids was not determined.

DISCUSSION

The cell walls of Gram-positive bacteria are known to contain between 30 and 70% peptidoglycan[9]. Hydrolysates of peptidoglycan have been found to contain N-acetylglucosamine, diaminopimelic acid (L,L- or meso-form), D-alanine and D-glutamic acid as unique markers for this structure[10,11]. It has been shown that very little racemization of alanine (less than 0.5%) takes place during the acid hydrolysis[2], and the corresponding figure for glutamic acid should be virtually the same[12].

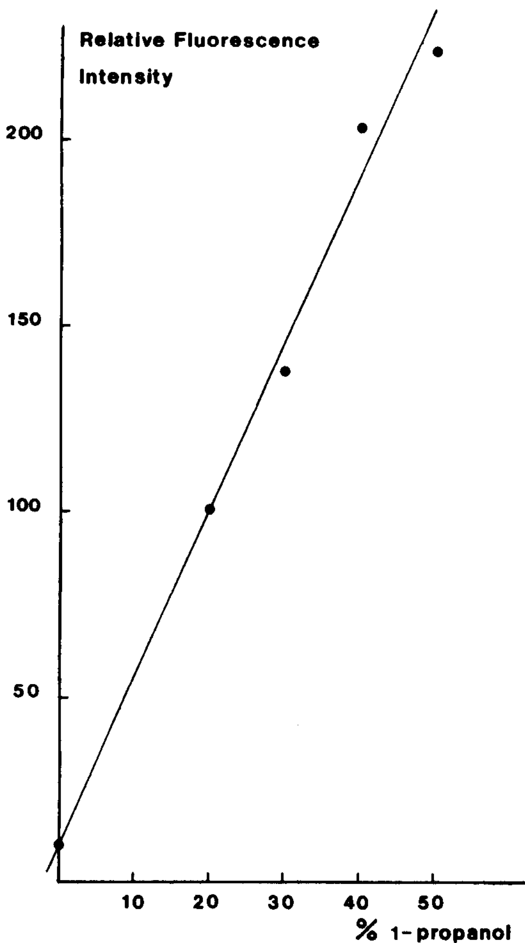

Fig. 3. Illustration of the increase in fluorescence yield from dansyl-D,L-glutamic acid with increasing 1-propanol content of the solvent.

The result from the amino acid analysis shows the high content of alanine and glutamic acid in the cell wall preparation used. Calculation gives roughly 6.6 and 4.8 μmol of alanine and glutamic acid, respectively, in the total hydrolysate, which corresponds to *ca.* 5.9 and 7.1%, respectively, based on dry weight. Therefore, preparative isolation of these amino acids by ion-exchange chromatography, followed by transformation to DNP or dansyl derivatives for an analysis of the D/L ratio by the chiral LC technique described above should present no particular problems. Since the glutamic acid in all cell wall peptidoglycans is thought to exist entirely as the D-enantiomer, our results should be useful for studies of this particular bacterial marker. The sensitivity obtained by the use of the dansyl derivative and the postcolumn addition of 1-propanol (Fig. 3) will permit determination of sub-nanomole amounts.

Further work in this field is in progress.

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