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Note

Separation of the enantiomers of N-protected α-amino acids as anilide and 3,5-dimethylanilide derivatives

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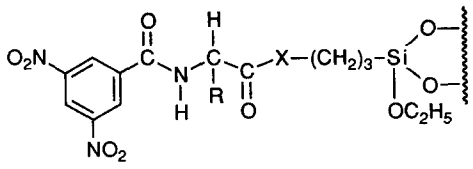
Accelerated interest in synthetic peptides has led to an increased demand for a method to detect and quantitate minor amounts of enantiomeric impurity in the N-protected amino acids used to prepare these peptides[1–3]. Especially important is the question of the enantiomeric purity of these N-protected amino acids *after* activation of the C-terminal carboxyl group with peptide coupling reagents. Coupling of the carboxyl activated N-protected amino acid with a chiral reagent may lead to chromatographically separable diastereomers. However, it may be difficult to obtain (and verify the purity of) chiral reagents of 100% enantiomeric purity. Since, in these situations, one is normally looking for rather low levels of enantiomeric impurities, the determination of enantiomeric purity by the direct separation of enantiomers is inherently less prone to error than is the determination of the ratio of diastereomeric derivatives.

The enantiomers of chiral acids are frequently separated by high-performance liquid chromatography as amide derivatives on chiral stationary phases (CSPs). The enantiomers of anilide-type derivatives are consistently separable upon one or more of the π-acidic CSPs developed in these laboratories[4,5]. The separation of the enantiomers of anilide and 3,5-dimethylanilide derivatives of representative N-protected α-amino acids upon several commercially available π-acidic CSPs is discussed herein. The analytes were chromatographed on both covalently and ionically bonded CSPs (Fig. 1), derived from (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine, (CSPs **1** and **2**) and (*S*)-N-(3,5-dinitrobenzoyl)leucine (CSPs **3** and **4**), using a mobile phase of 2-propanol in hexane, and, in the case of the covalent CSPs, also with methanol–water. While this paper focuses on the separation of enantiomers, such separations will



CSP	R	X
1	C_6H_5	NH
2	C_6H_5	$O^{-+}NH_3$
3	iso-C_4H_9	NH
4	iso-C_4H_9	$O^{-+}NH_3$

Fig. 1. CSPs derived from (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine and (*S*)-N-(3,5-dinitrobenzoyl)leucine.

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clearly facilitate the study of the extent of racemization of N-protected amino acids activated with peptide coupling reagents.

EXPERIMENTAL

Chromatography was performed with an Anspec-Bischoff model 2200 isocratic HPLC pump, a Rheodyne Model 7125 injector with a 20-μl sample loop and a Milton Roy-LDC UV Monitor D® fixed-wavelength detector (254 nm). The chromatographic columns, 250 × 4.6 mm I.D. packed with modified 5-μm silica were obtained from Regis. The analog output from the detector was amplified by an external amplifier and converted to a digital signal by a MetraByte Crom-1 A/D® board, controlled by Labtech Acquire® software, installed in an IBM XT® personal computer. The data were analyzed by custom software written and compiled in Microsoft QuickBASIC®.

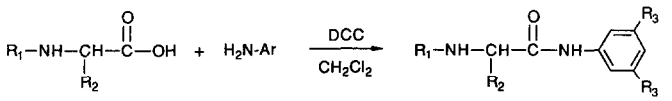
Generalized derivatization

The N-protected α-amino acids were formed by standard peptide synthesis methods[6].

Anilide derivatives were formed by allowing equal molar quantities of N-protected α-amino acid, aniline (or 3,5-dimethylaniline) and dicyclohexyl carbodiimide (DCC) to react in dichloromethane for 1 h followed by removal (filtration) of insoluble dicyclohexylurea (DCU). Residual DCU is present in the samples; however, DCU has little absorbance at 254 nm and does not interfere with the chromatographic analysis. In most cases the residual DCU prevented accurate determination of the melting points of the analytes. However ^{1}H NMR and mass spectral data are in accord with the assigned structures of the derivatives.

RESULTS AND DISCUSSION

Several α-amino acids (alanine, valine, leucine and phenylalanine) were N-protected with the benzyloxycarbonyl (CBZ), *tert.*-butyloxycarbonyl (BOC) and 9-fluorenylmethyloxycarbonyl (FMOC) protecting groups. These N-protected α-amino acids were subsequently derivatized with aniline and 3,5-dimethylaniline through the agency of DCC to give the corresponding anilides.

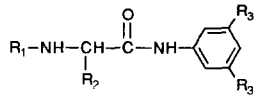


1 2 3

Chromatographic data for the normal-phase separation of the enantiomers of these α-amino acid derivatives on CSPs **1** and **3** are presented in Table I and for CSPs **2** and **4** in Table II. Reversed-phase chromatographic data on CSPs **1** and **3** are shown in Table III. All of the α-amino acid anilides chromatographed are separable on each CSP using 2-propanol in hexane as the mobile phase. The chromatographic separation factor, α, for the 3,5-dimethylanilides is larger than for the anilides in all cases. CSP **3** affords resolution suitable for accurate integration of the peaks of the

TABLE I

NORMAL-PHASE SEPARATION OF THE ANILIDE AND 3,5-DIMETHYLANILIDE DERIVATIVES OF N-PROTECTED α-AMINO ACIDS ON (*R*)-CSP 1 AND (*S*)-CSP 3



α = Chromatographic separation factor; R_s = r = resolution; k'_1 = capacity factor for the first eluted enantiomer using 2-propanol–hexane (5:95, v/v) as the mobile phase; flow-rate 2 ml/min.

Compound	R_1	R_2	R_3	*CSP 1*			*CSP 3*		
				α	R_s	k'_1	α	R_s	k'_1
3a	FMOC	CH_3	CH_3	1.49	2.78	18.53	2.35	4.36	13.85
3b	FMOC	CH_3	H	1.44	2.44	17.98	2.12	3.72	14.97
3c	FMOC	iso-C_3H_7	CH_3	1.66[a]	3.55	8.50	2.45[b]	3.97	6.97
3d	FMOC	iso-C_3H_7	H	1.50[a]	2.86	8.47	2.03[b]	3.57	7.35
3e	FMOC	iso-C_4H_9	CH_3	1.70	3.97	8.60	3.19	5.44	6.55
3f	FMOC	iso-C_4H_9	H	1.57	3.42	8.59	2.80	4.94	6.94
3g	FMOC	C_6H_5CO	CH_3	1.83	3.95	19.35	2.23	3.69	15.84
3h	FMOC	C_6H_5CO	H	1.68	3.64	18.12	1.99	3.40	16.38
3i	BOC	CH_3	CH_3	1.34	2.23	3.64	1.61	2.70	3.74
3j	BOC	CH_3	H	1.28	1.87	3.25	1.47	2.32	3.46
3k	BOC	iso-C_3H_7	CH_3	1.48	2.88	1.59	1.75	2.84	1.74
3l	BOC	iso-C_3H_7	H	1.38	2.27	1.55	1.59	2.37	1.77
3m	BOC	iso-C_4H_9	CH_3	1.52[a]	3.11	1.75	2.18[b]	3.90	1.87
3n	BOC	iso-C_4H_9	H	1.43	2.82	1.67	1.98	3.90	1.85
3o	BOC	C_6H_5CO	CH_3	1.56	3.51	3.86	1.62	2.58	4.40
3p	BOC	C_6H_5CO	H	1.48	3.30	3.62	1.55	2.54	4.27
3q	CBZ	CH_3	CH_3	1.37	2.53	14.25	2.14	4.49	14.73
3r	CBZ	CH_3	H	1.33	2.27	12.52	1.94	3.86	13.42
3s	CBZ	iso-C_3H_7	CH_3	1.55	3.46	6.26	2.13	4.22	6.51
3t	CBZ	iso-C_3H_7	H	1.44	3.00	5.96	1.88	3.82	7.02
3u	CBZ	iso-C_4H_9	CH_3	1.62	3.81	6.60	2.81	5.71	6.66
3v	CBZ	iso-C_4H_9	H	1.52	3.51	6.19	2.52	5.52	6.56
3w	CBZ	C_6H_5CO	CH_3	1.57	3.45	14.17	1.83	3.12	17.38
3x	CBZ	C_6H_5CO	H	1.53	3.21	13.65	1.66	2.82	17.79

[a] The (*R*)-enantiomer is most retained.
[b] The (*S*)-enantiomer is most retained.

enantiomers for all derivatives using 2-propanol in hexane and for all but the BOC derivatives using the reverse mobile phase. In all cases surveyed, the homochiral diasteromeric adsorbate (*R,R* or *S,S*) is the more stable. That is, the absolute configuration of the most retained enantiomer is the same as that of the CSP. While an explicit statement of the mechanism of chiral recognition must await further study, preliminary data suggests that a face to face approach of the analyte and CSP occurs as a consequence of π–π and dipole stacking interactions. The more stable of the diastereomeric adsorbates is the one having the bulky substituents on the stereogenic centers of the CSP and analyte external to the stack[7].

TABLE II

NORMAL-PHASE SEPARATION OF THE ANILIDE AND 3,5-DIMETHYLANILIDE DERIVATIVES OF N-PROTECTED α-AMINO ACIDS ON (*R*)-CSP **2** AND (*S*)-CSP **4**

For structural formula and symbol definitions, see Table I

Compound	*CSP 2*			*CSP 4*		
	α	R	k'_1	α	R_s	k'_1
3a	1.64	2.79	3.85	2.52	4.87	2.43
3b	1.49	2.25	4.39	2.26	4.61	2.77
3c	1.51[a]	2.49	1.44	2.10[b]	3.87	0.90
3d	1.32[a]	1.84	1.74	1.81[b]	3.33	1.13
3e	1.41	2.29	1.41	2.26	4.75	0.92
3f	1.26	1.60	1.75	2.01	4.31	1.14
3g	1.61	2.98	3.00	2.23	4.30	1.77
3h	1.45	2.46	3.52	1.99	3.92	2.20
3i	1.38	1.87	0.97	2.11	3.91	0.75
3j	1.29	1.69	1.09	1.91	3.61	0.84
3k	1.35	1.47	0.34	1.87	2.56	0.26
3l	1.21	1.01	0.43	1.66	2.23	0.34
3m	1.25[a]	1.05	0.35	1.94[b]	2.86	0.28
3n	1.18	1.20	0.42	1.78	2.73	0.35
3o	1.38	1.96	0.79	1.91	3.19	0.59
3p	1.29	1.66	0.93	1.77	3.36	0.73
3q	1.59	2.81	3.40	2.55	5.19	2.51
3r	1.46	2.39	3.72	2.35	4.74	2.80
3s	1.51	2.37	1.18	2.26	4.43	0.89
3t	1.33	1.82	1.40	1.97	3.80	1.06
3u	1.37	2.08	1.15	2.47	4.73	0.91
3v	1.26	1.59	1.38	2.22	4.36	1.12
3w	1.51	2.66	2.58	2.39	4.05	1.87
3x	1.39	2.32	2.94	2.17	4.05	2.28

[a] The (*R*)-enantiomer is most retained.
[b] The (*S*)-enantiomer is most retained.

CONCLUSION

The enantiomers of the 3,5-dimethylanilide and anilide derivatives of N-protected α-amino acids are readily separated on both covalently and ionically bonded α-acidic CSPs derived from phenylglycine and leucine. Proper selection of CSP and mobile phase will afford resolution sufficient for quantitation of enantiomeric excesses by integration for all analytes discussed. While the data presented herein is by no means an exhaustive survey of all α-amino acids of potential interest, the methods discussed should be readily extended to other N-protected α-amino acids. The wide spread availibility, chromatographic efficiency and the ability to use both normal and reversed phase eluents make the CSPs discussed especially attractive for these analyses.

TABLE III

REVERSED-PHASE SEPARATION OF THE ANILIDE AND 3,5-DIMETHYLANILIDE DERIVATIVES OF N-PROTECTED α-AMINO ACIDS ON (*R*)-CSP **1** AND (*S*)-CSP **3**

For structural formula and symbol definitions, see Table I, except k'_1 = capacity factor for the first eluted enantiomer using water–methanol (10:90) as the mobile phase; flow-rate 2 ml/min.

Compound	*CSP 1*			*CSP 3*		
	α	*R*	k'_1	α	R_s	k'_1
3a	1.35	3.89	3.01	1.69	5.67	1.67
3b	1.19	2.25	2.14	1.42	3.43	1.15
3c	1.40[a]	4.26	2.72	1.70[b]	5.74	1.52
3d	1.22[a]	2.42	2.05	1.39[b]	3.13	1.14
3e	1.41	4.29	2.82	1.90	7.01	1.66
3f	1.23	2.61	2.12	1.55	4.35	1.18
3g	1.53	5.90	3.94	1.70	6.18	2.15
3h	1.31	3.51	2.92	1.43	3.70	1.57
3i	1.13	1.07	0.81	1.30	1.62	0.49
3j	1.00	0.00	0.68	1.19	≈0.00	0.33
3k	1.17	1.35	0.76	1.34	1.77	0.46
3l	1.08	≈0.00	0.62	1.18	0.72	0.34
3m	1.19[a]	1.42	0.80	1.44[b]	2.42	0.50
3n	1.09	≈0.00	0.64	1.26	1.14	0.36
3o	1.27	2.34	1.08	1.36	2.43	0.66
3p	1.14	1.16	0.84	1.21	1.16	0.47
3q	1.22	2.26	1.40	1.57	4.11	0.80
3r	1.12	1.13	1.01	1.34	2.15	0.55
3s	1.28	2.63	1.26	1.61	4.06	0.75
3t	1.16	1.35	0.97	1.34	2.15	0.55
3u	1.30	2.82	1.32	1.80	5.20	0.82
3v	1.16	1.48	1.00	1.51	3.10	0.57
3w	1.39	3.86	1.86	1.59	4.34	1.08
3x	1.22	2.24	1.38	1.35	2.67	0.78

[a] The (*R*)-enantiomer is most retained.
[b] The (*S*)-enantiomer is most retained.

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REFERENCES

1 J. Maibaum and D. H. Rich, *J. Org. Chem.*, 53 (1988) 869.
2 Y. Okamoto, R. Aburatnani, Y. Kaida and K. Hatada, *Chem. Lett.*, (1988) 1125.
3 S. Görög, B. Herényi and M. Löw, *J. Chromatogr.*, 353 (1986) 417.
4 W. H. Pirkle and J. E. McCune, *J. Chromatogr.*, 469 (1989) 67.
5 W. H. Pirkle and J. E. McCune, *J. Chromatogr.*, 471 (1989) 271.
6 M. Bodansky and A. Bodansky, *The Practice of Peptide Synthesis*, Springer, New York, 1984.
7 D. S. Reno, *Ph.D. Thesis*, University of Illinois, Urbana, IL, 1987.