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DETERMINATION OF D-AMINO ACIDS. II. USE OF A BIFUNCTIONAL REAGENT, 1,5-DIFLUORO-2,4-DINITROBENZENE

by

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1-fluoro-2,4-dinitrophenyl-5-L-alanine amide has been synthesized in high yield (76%) from 1,5-difluoro-2,4-dinitrobenzene and L-Ala-NH_2. This compound contains a reactive fluorine atom which can be used for the reaction with a mixture of L- and D-amino acids. The resulting diastereomers which are obtained in quantitative yield can be separated and estimated by HPLC. With the five amino acids studied (Ala, Asp, Glu, Met and Phe), L-diastereomers were eluted from the reverse-phase column before D-diastereomers. This behavior can be explained by a stronger intramolecular hydrogen bonding in the latter diastereomer. When artificial mixtures of the five amino acids containing known proportions of L- and D-isomers were derivatized with the reagent and the reaction products analyzed by HPLC, it was possible to determine the relative content of each isomer in a nanomole range.

1. INTRODUCTION

A bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene, FFDNB, has previously been used as a cross-linking reagent in protein chemistry (2, 3, 4, 6). It reacts mainly with uncharged amino groups in a protein and with phenolic hydroxyl groups and sulfhydryl groups. The derivatives are stable, so that cross-linked amino acids can be isolated after protein hydrolysis (2, 3). A useful feature of the bridge chromophore (-NH-DNP-NH-) is its high light absorption at 340 nm with an $\varepsilon_M \cong 3 \times 10^4$ (2).

We have considered that this reagent can also be used to prepare diastereomers of amino acids and, thus, can be very useful in quantitative determination of D-amino acids in protein hydrolysates. We have first synthesized FDNP-L-alanine amide (FDNP-L-Ala-NH_2) and used this as a reagent for derivatization of a D- and L-amino acid in a mixture. The resulting diastereomers were separated and quantitated by HPLC.

Abbreviations: DMSO = dimethylsulfoxide; DNDEAP-a.a. = N-(2,4-dinitro-5-diethylaminophenyl)-amino acid; DNP = 2,4-dinitrophenyl; FDAA = F-DNP-L-Ala-NH_2 = 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; FFDNB = 1,5 difluoro-2,4-dinitrobenzene; HO-DAA = 1-hydroxy-2,4-dinitrophenyl-5-L-alanine amide; HPLC = high performance liquid chromatography; L-Ala-$NH_2 \cdot HCl$ = L-Alanine amide hydrochloride; TEAP = triethylammonium phosphate; TLC = thin-layer chromatography. Other abbreviations are according to the guideline of the IUPAC-IUB, Commission of Biochemical Nomenclature.

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2. MATERIALS AND METHODS

2.1. Materials

FFDNB was purchased from Fluka AG, Switzerland. It had m.p. 74-75 °C and showed only a single spot on a silica TLC sheet with an Rf value of 0.7 (benzene). L-Alanine amide hydrochloride was from Bachem Feinchemikalien AG, Switzerland. L- and D-amino acids were purchased from Sigma, USA. Polygram, Sil G/UV 254 precoated sheets were purchased from Macherey-Nagel Co., West Germany. All other chemicals and solvents were of analytical grade and were obtained from Merck, W. Germany.

2.2. Methods

2.2.1. Synthesis of FDNP-L-Ala-NH_2 (FDAA)

This synthesis was similar to that described for 5-fluoro-2,4-dinitrodiethylaniline (5). It was essential to maintain conditions exactly as indicated, otherwise, the yield of the desired compound was low and, instead, a hydrolyzed product was obtained. A sample of L-Ala-$NH_2 \cdot HCl$ (472 mg, 3.81 millimoles) was dissolved in 3.9 ml 1 N-NaOH and immediately 60 ml acetone was added. About 10 g of anhydrous $MgSO_4$ was added and the contents stirred at room temperature for about 3 hours. $MgSO_4$ was removed by filtration and washed twice with little acetone. FFDNB (668 mg, 3.27 millimoles) was dissolved in 15 ml acetone. To this solution was added dropwise under magnetic stirring the acetone solution of L-Ala-NH_2. After addition, the contents were stirred for an additional 0.5 hour. Equal volume of water was added resulting in formation of the golden-yellow scales which were filtered, washed first with little 2:1 water-acetone mixture, then with water and finally dried in the air and in the dark. The yield was 0.5 g (56% of theory, mol. wt. 272), m.p. 224-226 °C. From the mother liquor, upon removal of more acetone under water pump, another fraction of crystals was obtained in the same way as above. The yield was 180 mg (20% of theory), m.p. 222-223 °C. Both fractions had the same Rf value (0.4) upon TCL on silica with ethylacetate as solvent. When subjected to HPLC using TEAP/acetonitrile systems (section *2.2.3*) both fractions had the same elution peak (and represented 95% of the total area). The ultraviolet spectrum of the product obtained in 25 mM-TEAP, pH 3.0, and 50% acetonitrile had maxima at 264 nm, 338 nm ($\varepsilon_M \cong 1.5 \times 10^4$) and a shoulder at 380nm (a spectrum similar to the hydrolyzed sample, see Figure 1).

2.2.2. Synthesis and HPLC of L- and D-diastereomers of amino acids

These syntheses were closely patterned after those used for the synthesis of DNDEAP-amino acids (5). Aqueous solutions (50 mM) of ten amino acids (D- and L-isomers of Ala, Asp, Glu, Met and Phe) were used as starting materials for synthesis. 50 µl (2.5 micromoles) of each solution was placed in separate 2 ml plastic micro centrifuge tubes. To each was added 100 µl of 1% acetone solution of FDAA (1 mg, 3.6 micromoles), the molar ratio of FDAA to amino acid 1.4:1, followed by 20 µl of 1 M-$NaHCO_3$ (20 micromoles). The contents were mixed and heated over a hot plate at 30-40 °C for 1 hour with frequent mixing. After cooling to room temperature, 10 µl (20 micromoles) of 2 M-HCl was added to each reaction mixture. After mixing, the contents were dried in a vaccuum desicator over NaOH pellets. The residues were dissolved in 0.5 ml DMSO affording ten solutions each 5.0 mM (based on amino acid). Analysis of these solutions by HPLC under conditions given below indicated that the derivatization reaction was quantitative (see below). A 1:1 dilution of these solutions was made (2.5 mM) and 5 µl samples of each (12.5 nanomoles) were pooled together and injected for HPLC.

2.2.3. Chromatography

HPLC was done with equipment described in the preceding paper. Elution was done with a linear gradient of acetonitrile in 50 mM-TEAP buffer, pH 3.0, from 10% to 50% acetonitrile during 1 hour, flow rate 2 ml/min, analysis of the effluent at 340 nm. This wave length was chosen because it is close to λ_{max} of most of the compounds investigated. All the solvents were filtered and degassed before use.

TLC was done with pre-coated plastic sheets containing 0.25 mm layers of silica gel impregnated with fluorescent indicator (Polygram

Sil G/UV 254). Ethyl acetate, p-dioxane and p-dioxane-benzene (3:1) mixture were used as solvents.

2.2.4. Derivatization and quantitation of D-isomers in mixtures of known proportions of L- and D-isomers

Four amino acid mixtures and a reagent blank were prepared in separate plastic 2 ml micro centrifuge tubes. Mixture 1 contained 60% of L- and 40% of D-isomers of Ala, Asp, Glu, Met and Phe (12 µl of each 50 mM solution of L-isomer and 8 µl of each 50 mM solution of D-isomer). The total volume of the mixture was 100 µl (5 micromoles of all amino acids). Mixture 2 was similarly prepared but contained 80% L-isomer and 20% D-isomer and mixture 3 contained 90% L-isomer and 10% D-isomer. Mixture 4 contained only L-isomers and the reagent blank 100 µl water. Each mixture and the reagent blank was treated with 200 µl of 1% acetone solution of FDAA (2 mg, 7.2 micromoles, the molar ratio of FDAA to amino acid 1.4:1). 40 µl of 1 M-$NaHCO_3$ (40 micromoles) was added, the contents mixed and then heated for 1 hour over a hot plate at 30 °C-40 °C with frequent mixing. After cooling to room temperature, 20 µl of 2 M-HCl (40 micromoles) were added, and the contents dried in a vacuum desiccator over NaOH pellets.

The reaction residues, each containing 5 micromoles of total amino acids (except blank), were dissolved in 0.5 ml DMSO affording 10 mM solutions. Aliquots (10 µl, 100 nanomoles) of each mixture and 10 µl of the 1:10 diluted reagent blank were injected for HPLC under conditions described in section *2.2.3*.

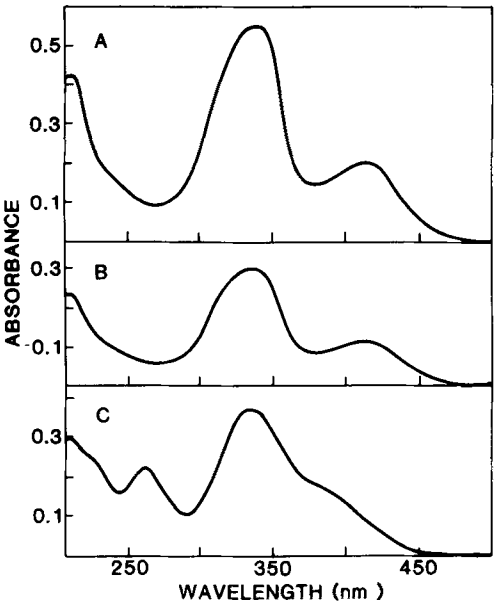

Figure 1. Ultraviolet spectra of L- and D-diastereomers of Ala in 25 mM-TEAP buffer, pH 3.0, and 50% acetonitrile. A, D-Ala-DNP-L-Ala-NH_2 (20 µM solution); B, L-Ala-DNP-L-Ala-NH_2 (10 µM solution); C, hydrolyzed reagent (HO-DNP-L-Ala-NH_2), 30 µM solution).

3. RESULTS

3.1. Spectral characteristics of diastereomers

All ten diastereomers studied have very similar ultraviolet absorption spectra. A typical spectrum is shown for D- and L-diastereomers of Ala in Figure 1. The spectra of both diastereomers are very similar and are characterized by a λ_{max} at 338 nm($\varepsilon_M \cong 3.0 \times 10^4$) and 414 nm ($\varepsilon_M \cong 1.1 \times 10^4$). The ratio of absorption at these two wavelengths is 2.7 which is characteristic of a -NH-DNP-NH- chromophore (2,3). In contrast, the spectrum of HO-DNP-L-Ala-NH_2 (the hydrolyzed reagent) has λ_{max} at 264 nm 338 nm ($\varepsilon_M \cong 1.5 \times 10^4$) and a shoulder at 380 mm. The λ_{max} and the ε_M values are slightly different for diastereomers of different amino acids and vary slightly with the nature of the solvent used. The spectra are stable if the solutions are kept in the dark, otherwise, a gradual change occurs as a result of a photochemical decomposition of the absorbing chromophore.

3.2. Chromatographic characteristics of diastereomers of Ala, Asp, Glu, Met and Phe

The HPLC elution pattern of the ten diastereomers of Ala, Asp, Glu, Met and Phe showed good separation. The separation of D- and L-diastereomers is best for Met followed by that of Phe, Ala, Glu and Asp. The hydrolyzed reagent appears as a sharp peak and is separated from all the diastereomers.

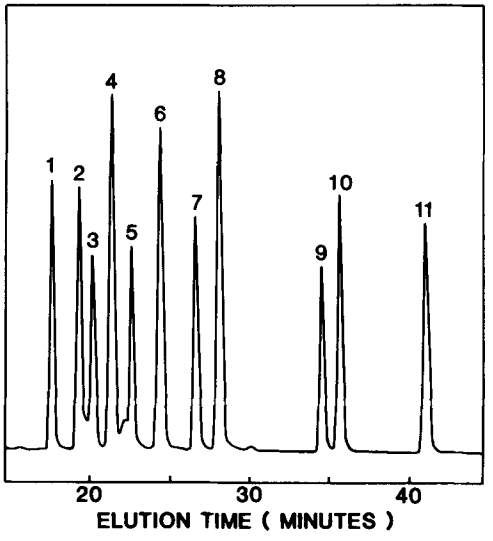

Figure 2. HPLC of mixture 1 containing L- and D-diastereomers of Ala, Asp, Glu, Met, Phe in 60% L- and 40% D-proportion. Peak 1, L-Asp-DNP-L-Ala-NH_2, 17.68 min; Peak 2, L-Glu-DNP-L-Ala-NH_2, 19.40 min; peak 3, D-Asp-DNP-L-AlaNH_2, 20.28 min; peak 4, L-Ala-DNP-L-Ala-NH_2, 21.40 min; peak 5, D-Glu-DNP-L-Ala-NH_2, 22.71 min; peak 6, HO-DNP-L-Ala-NH_2 (hydrolyzed reagent) 24.48 min; peak 7, D-Ala-DNP-L-Ala-NH_2, 26.72 min; peak 8, L-Met-DNP-L-Ala-NH_2, 28.21 min; peak 9, D-Met-DNP-L-Ala-NH_2, 34.66 min; peak 10, L-Phe-DNP-L-Ala-NH_2, 35.82 min; peak 11, D-Phe-DNP-L-Ala-NH_2, 41.22 min. 10 µl sample (100 nanomoles) of 10 mM solution of all amino acid derivatives, containing 8 nanomoles of each D-isomer and 12 nanomoles of each L-isomer, was used for HPLC under conditions described in section *2.2.3*.

This is exemplified in Figure 2 which shows the HPLC pattern of the amino acid mixture containing 40% D-isomer after derivatization with the FDAA reagent as described in section *2.2.4*. The hydrolyzed reagent is peak 6 and the peaks corresponding to five D-isomers are: peak 3, D-Asp-DNP-L-Ala-NH_2; peak 5, D-Glu-DNP-L-Ala-NH_2; peak 7, D-Ala-DNP-L-Ala-NH_2; peak 9, D-Met-DNP-L-Ala-NH_2 and peak 11, D-Phe-DNP-L-Ala-NH_2. The chromatograms of mixtures with 20%, 10% and 0% D-isomers, respectively, are not shown but they are very similar to the one shown in Figure 2, except that peaks corresponding to D-isomers get progressively smaller for the 20% D- and the 10% D-mixtures and the peaks for the L-isomers get progressively larger. The chromatogram of the reagent blank had only one peak (peak 6). The quantitative evaluation of hydrolyzed reagent peak gave a value of 1.4×10^4 HPLC area units per nanomole. Using this value in evaluating peak 6 in the chromatograms of the four amino acid mixtures indicated that the amount of hydrolyzed reagent found (27%) corresponded to the excess reagent (28%) used for the derivatization reaction. This result confirms that no unexpected side reactions occur.

The amount of D-isomer in the three mixtures was calculated from the HPLC area corresponding to that particular isomer and there was a linear relationship between % D-area and % D-isomer in the three mixtures (Figure 3). The slopes are not completely identical because ε_M values at 340 nm vary slightly for different diastereomers of the same amino acid.

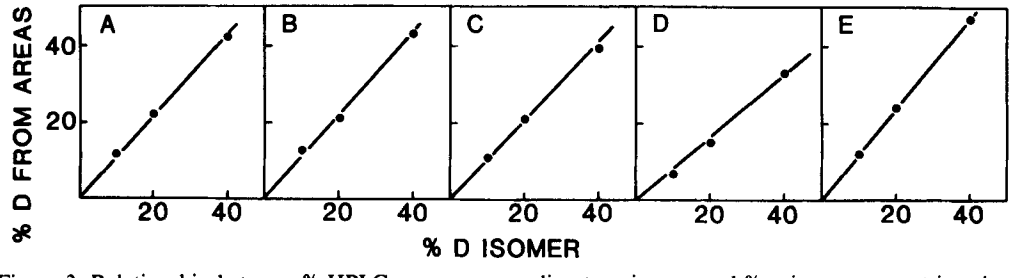

Figure 3. Relationship between % HPLC area corresponding to D-isomer and % D-isomer present in mixture 1. A, D-Asp-DNP-L-Ala-NH_2 (slope 1.06); B, D-Glu-DNP-L-Ala-NH_2 (slope 1.10); C, D-Ala-DNP-L-Ala-NH_2 (slope 1.03); D, D-Met-DNP-L-Ala-NH_2 (slope 0.81); E, D-Phe-DNP-L-Ala-NH_2 (slope 1.19). The % D-area was calculated from: % D = [D-area/ (D-area + L-area)] × 100.

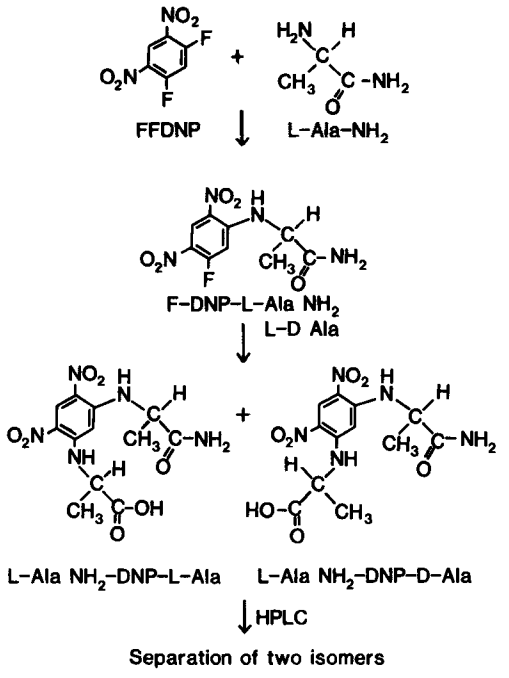

Figure 4. An outline of the reaction sequence used for the synthesis of FDAA reagent and for the derivatization of L- and D-isomers of amino acids. Conditions for the synthesis and for the HPLC are given in the text.

4. DISCUSSION

The reactions which were used to prepare diastereomers of the five amino acids discussed in this paper are outlined in Figure 4. The initial reaction is between FFDNB and L-Ala-NH_2 to form F-DNP-L-Ala-NH_2. Amide was chosen because it is quite stable and apparently is not easily racemized. The next step involves the reaction with a mixture of L- and D-isomers of any amino acid. The product is a mixture of diastereomers which can easily be separated and quantitated by HPLC. This method of resolution and quantitation of optical isomers is quite flexible because in principle one can prepare a reagent containing any optically active amino acid in place of L-Ala-NH_2 and use it for the second reaction. It has the advantage over the method of MANNING and MOORE (1) in that it does not produce oligomeric products which can be formed when an L-amino acid N-carboxyanhydride reacts with a mixture of L- and D-amino acids. Another advantage is the availability of a stable, highly absorbing chromophore, which permits determination of diastereomers in the nanomole range and the rapidity of determination inherent in the HPLC method.

Analysis of the chromatograms of the four amino acid mixtures and of the experimental reagent blank showed that the derivatization reaction was quantitative. The expected amount of the reagent was bound to the amino acids (see Figure 3) and the excess recovered as a single peak in quantitative yield.

It was of interest to find that L-diastereomers are eluted from the column before D-diastereomers. The reason for this behavior is probably due to a stronger intramolecular H-bonding in D- than in L-diastereomer. One can expect that the carboxyl group can hydrogen bond either to an ortho-situated nitro group producing a 9-membered ring or, more likely, to the carbonyl oxygen of the meta-situated L-Ala-NH_2 forming a 12-membered ring. Stronger H-bonding in a D-diasteromer would produce a more hydrophobic molecule which would be expected to interact more strongly with the reverse-phase column and thus have a longer retention time than an L-diastereomer.

When one compares the differences in elution times of the five pairs of diastereomers (see Figure 2) one obtains the following order: Met (7.1 min.) > Phe (5.4 min.) > Ala (5.2 min.) > Glu (3.2 min.) > Asp (2.5 min.). It is clear that the nature of the amino acid side-chain is responsible for this behavior. The ionizable side-chains of Asp and Glu decrease the separation whereas neutral and hydrophobic side-chains increase it.

The method of quantitation of D-isomers in a mixture is quite simple. It compares HPLC peak areas of the two diastereomers of the same amino acid in the same chromatogram. In this way, the experimental conditions are the same for both isomers.

The present method has so far been applied to determination of D-isomers of five amino acids. In principle, it can be applied to other amino acids, but the separation of the diastereomers may require different conditions for HPLC.

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