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THE SYNTHESIS OF L (-) AND D (+) LUPINIC ACID

B. Shadid and H.C. van der Plas*,
Laboratory of Organic Chemistry, Agricultural University Wageningen,
Dreijenplein 8, 6703 HB Wageningen, The Netherlands
and
W.H.J. Boesten, J. Kamphuis, E.M. Meijer and H.E. Schoemaker,
DSM Research, Bio-organic chemistry section,
P.O. Box 18, 6160 MD Geleen, The Netherlands

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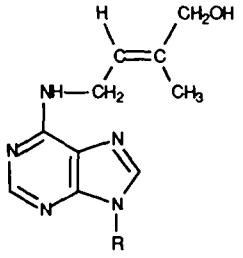
Abstract: Optically active L-lupinic acid (e.e. > 95%) and D-Lupinic acid amide were obtained by enantioselective hydrolysis of a racemic mixture of the amide of D,L-lupinic acid with aminopeptidase from *Pseudomonas putida*. Hydrolysis of D-Lupinic acid amide gave D-Lupinic acid.

INTRODUCTION

L-amino acids, containing a heterocyclic ring on the β-position of alanine, occur widely in nature and are produced in plants. In these non-protein L-amino acids six-membered heterocyclic rings (trigonelline, mimosine, willardine, and isowillardine), five-membered rings (quisqualic acid, β-(pyrazol-1-yl)alanine, β-(3-isoxazolin-5-on-2-yl)alanine, β-(5-methylisoxazolin-3-on-2-yl)alanine, β-(3-amino-1,2,4-triazol-1-yl)alanine, β-(2-furoyl)alanine, as well as bicyclic heterocycles (lupinic acid, β-(6-benzoylaminopurin-9-yl)alanine, histidinoalanine are identified as β-heterocyclic moieties. There is continuing interest in the synthesis of these naturally-occuring heterocyclic β-substituted alanines[1]. Plant extracts have been isolated, which contain enzyme systems (β-substituted alanine syntheses), which catalyze the syntheses of β-substituted heterocyclic alanines from O-acetyl L-serine and the appropriate heterocycle. It was found that neither serine, nor its O-phospho and its O-sulpho derivative could serve as donor of the alanyl moiety. Attempts are reported to prepare above-mentioned β-substituted alanines biomimetically using a pyridoxal-5'-phosphate (PLP) catalyzed chemical reaction between O-acetyl L-serine and the heterocycle in the presence of metal ions, especially gallium ions. The yields are usually low-to-very low; 30% yields or more are mentioned for β-(pyrazol-1-yl)-alanine (40-45%), β-(5-methylisoxazolin-3-on-2-yl)alanine (0.15%) and β-(3-amino-1,2,4-triazol-1-yl)alanine (30-35%)[1].

Due to our interest in the use of enzymes in synthetic procedures we turned our attention to the preparation of the β-purinyl-L-alanine derivative, called L-lupinic acid **1a**, which is isolated from *lupinus angustifolins* [2] and which is a principal metabolite of the phytohormone *trans*-zeatine (**E-1b**), being one of the most effective natural stimulants of plant cell division.



1a: R = $CH_2CH(NH_2)CO_2H$

1b: R = H

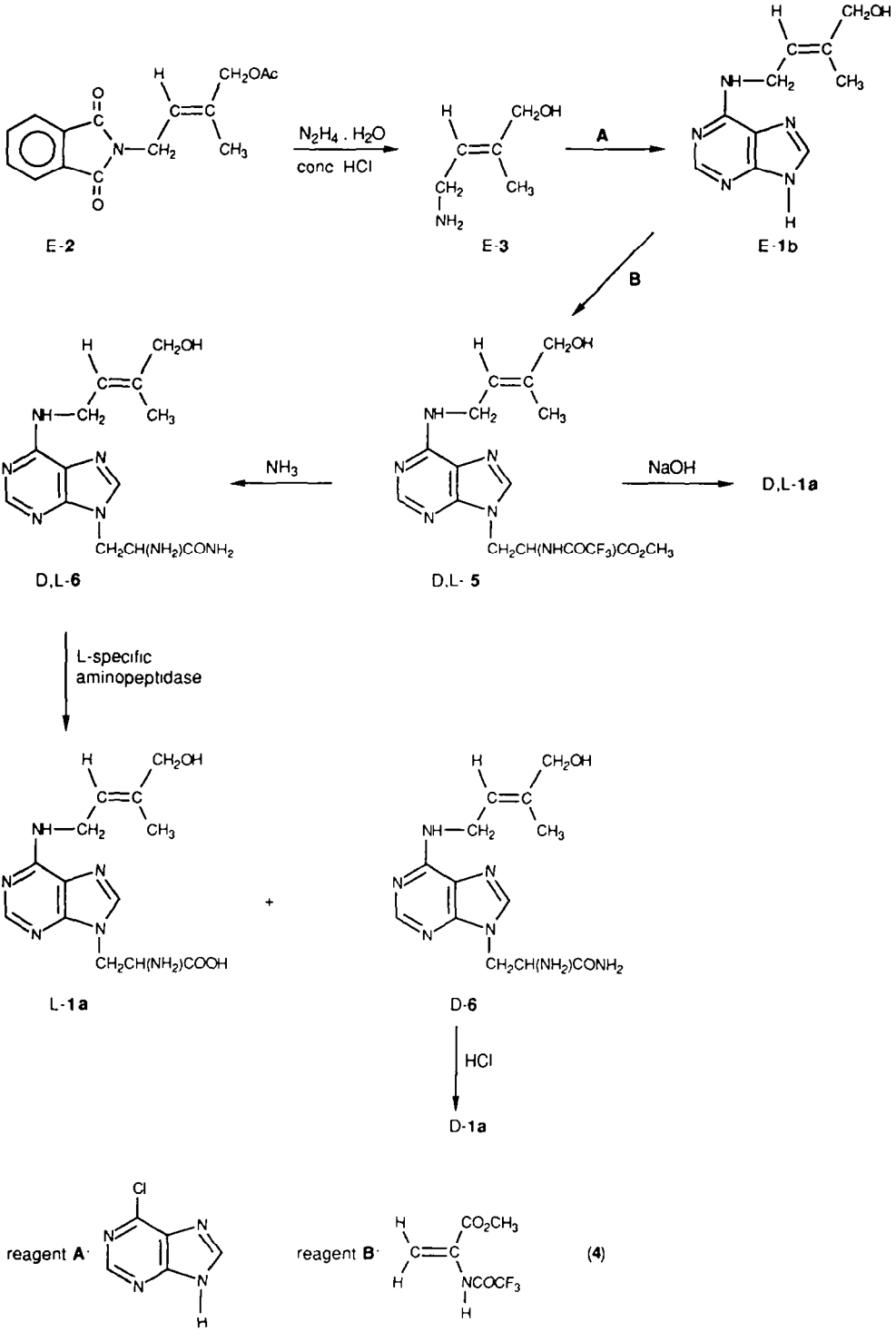
The chemical synthesis of a racemic mixture of D,L-**1a** has been described[2]. However, pure L-lupinic acid (L-**1a**) and D-lupinic acid (D-**1a**) have never been prepared. Only the enzymatic formation of L-**1a** from *trans*-zeatin (E-**1b**) and O-acetyl L-serine, using as enzyme source extracts of Lupinus seedlings has been reported. Attempts to prepare L-**1a** by a PLP-catalyzed reaction between E-**1b** and O-acetyl-L-serine met with little succes (yield about 1,5%)[3].

For the production of optically pure D- and L-amino acids enzymatic resolution methods have proven to be powerful tools. The enzymatic resolution process developed by DSM (Dutch State Mines), based on the stereospecific hydrolysis of amino acid amides by using the L-specific aminopeptidase from *Pseudomonas putida* has found wide application[4]. We wanted to study if this enzyme would also be useful for the preparation of L-lupinic acid (together with D-lupinic acid amide) from DL-lupinic acid amide; from D-lupinic acid amide D-lupinic acid could be obtained. This work is part of a program of cooperation between our laboratory and the Center of Agricultural and Biological Research at Wageningen directed to study the metabolism and physiological effects of cytokinins in plants.

RESULTS AND DISCUSSION

The strategy we have adopted for the synthesis of L-lupinic acid (L-**1a**) and D-lupinic acid (D-**1a**) consists of the following steps i) the synthesis of *trans*-zeatine (E-**1b**) ii) the synthesis of trifluoroacetylaminoacrylate **4**, iii) coupling of **4** with E-**1b** at position 9 of the purine ring into D,L-**5**, iv) removal of the protecting trifluoroacetyl group and conversion of the ester group into the amide function affording D,L-lupinic acid amide (D,L-**6**), v) enzymatic stereospecific hydrolysis of DL-**6** with L-specific aminopeptidase into L-lupinic acid (L-**1a**) and D-lupinic acid amide (D-**6**), vi) the chemical conversion of D-Lupinic acid amide (D-**6**) under mild acid condition into D-Lupinic acid (D-**1a**).

Our synthesis of *trans*-zeatin (E-**1b**) is a modification of two syntheses, being already reported[5,6]. In our work N-(4-acetoxy-3-methyl-E-but-2-enyl) phthalimide (E-**2**), being prepared by a procedure described before, was converted in a one-pot reaction into 4-hydroxy-3-methyl-E-


E-2
N2H4 . H2O
conc HCl
E-3
A
E-1b
B
D,L-6
NH3
D,L- 5
NaOH
D,L-1a
L-specific
aminopeptidase
L-1a
+
D-6
HCl
D-1a
reagent A:
reagent B:
(4)

but-2-enylamine (E-**3**) by treatment with 85% aqueous hydrazine to remove the phthaloyl group and subsequent heating with hydrochloric acid to hydrolyse the acetate group (yield 85%). Coupling of E-**3** with 6-chloropurine (reagent **A**) in refluxing butanol in the presence of triethylamine gave *trans*-zeatine (E-**1b**). The coupling of E-**1b** with methyl trifluoroacetylamino acrylate (reagent **B**: **4**) into β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]-N-trifluoroacetylalanine methyl ester (D,L-**5**) requires difficult conditions[7]. Optimum yields (about 80%) were obtained when a solution of an equimolar amount of **4** and E-**1b** in dimethyl sulphoxide, containing potassium bicarbonate was stirred for three days at 25°C. Using the stronger base potassium carbonate instead of potassium bicarbonate resulted in lower yields of D,L-**5**. Removal of the protecting N-trifluoroacetylgroup in D,L-**5** and conversion of the ester group into a carboxylic amide function was realized by heating a solution of D,L-**5** in butanol, being saturated with ammonia, for three days at 35°C in a sealed tube. The yield of the racemic mixture of D,L-lupinic acid amide (D,L-**6**) amounted to 85%.

The resolution was succesfully achieved by using L-aminopeptidase from *Pseudomonas putida*, which converts L-carbonamide **6** into the corresponding L-lupinic acid L-**1a**, but does not hydrolyse D-carbonamide **6**. By use of a strong basic ion-exchange column the L-lupinic acid (L-**1a**) could be easily separated from the D-lupinic acid amide (D-**6**). D-Lupinic acid amide (D-**6**) was treated with 6N hydrochloric acid at room temperature to obtain D-Lupinic acid (D-**1a**).

The optical purities of L-**1a** and D-**6** were established by reacting each of these compounds as well as the racemic mixture of D,L-**1a** (obtained by treatment of a solution of D,L-**5** in dioxane-water with aqueous sodium hydroxide at room temperature) with pure S-2-chloropropionyl chloride[8]. This reagent reacts with D,L-α-amino acids into the corresponding diastereoisomeric N-(S-2-chloropropionyl) derivatives. In the ^{1}H-NMR spectra of these D,L derivatives the chemical shift for the methyl doublet of the CH_3-CHCl moiety is found to be different for both diastereoisomers. When this technique is applied to D,L-**1a** we observed two methyl doublets of the CH_3-CHCl part at 1,53 and 1,56 ppm. However, the ^{1}H-NMR spectrum of the N-(S-2-chloropropionyl) derivative of L-**1a** exhibits only a CH_3-CHCl methyl doublet at 1,53 ppm; the N(S-2-chloropropionyl) derivative of D-**6** only gave the methyl doublet at 1,56 ppm. From these results it can be unequivocally concluded that the enzymatic separation was succesful and that L-lupinic acid has been obtained with high optical purity (e.e > 95%). Acid hydrolysis of D-**6** gave optically pure D-lupinic acid (D-**1a**).

EXPERIMENTAL PART

General methods and materials

Scheicher and Schül DC Fertigfolien F1500 LS 254 were used for TLC, the following solvent systems were used: System A (chloroform/methanol, 90:10, v/v), system B (chloroform/methanol, 86:14, v/v), system C (chloroform/methanol, 80:20, v/v), system D (chloroform/methanol/concentrated ammonium hydroxide, 60:40:20, v/v). ^{1}H-NMR-spectra were measured

using a Hitachi Prekin-Elmer R-248 or a Varian EM 390 spectrometer. ^{13}C-NMR-spectra were measured at 75.460 MHz using a Bruker CXP 300 spectrometer; proton noise decoupling was used. Mass-spectra were recorded on an AEI MS 902 instrument, equiped with a VG/ZAB console. Ultraviolet spectra were determined using a Beckmann Du-7 or an Amico Dw-2A-spectrometer. Melting points were determined on a Kofler hot stage equipped with a microscope and a polarizer. They are uncorrected. Optical rotations were measured at 20° C with a Perkin Elmer 141 polarimeter.

Dimethylsulphoxide was dried by stirring with CaH_2 for 16 h, then distilled under reduced pressure and stored over molecular sieves 4A. Triethylamine was dried by refluxing with CaH_2 for 16 h, then distilled and stored over molecular sieves 4A. n-Butanol (analyzed grade, Baker) was used without further purification. n-Butanolic ammonia was prepared by passing dry (KOH) ammonia gas through butanol at -20°C until saturation.

Synthesis of 4-hydroxy-3-methyl-E-but-2-enylamine (E-**3**)

A mixture of N-(4-acetoxy-3-methyl-E-but-2-enyl)phthalimide (E-**2**) (9.46, 40.7 mmol), 85% aqueous hydrazine hydrate solution (2.44 ml, 40.8 mmol) and methanol (100 ml) was refluxed for 1 h with stirring. TLC-analysis (system A) indicated that under these conditions complete conversion of the starting material took place into a product having Rf=0. After cooling of the solution, water (25 ml) and concentrated hydrochloric acid (25 ml) were added and the reaction mixture was heated under reflux for 1 h. After cooling to 0°C phthalylhydrazide precipitated, which was removed by filtration; the filtrate was evaporated at 30°C in a rotary evaporator. The residue was dissolved in water (20 ml) and the insoluble material was removed by filtration; the filtrate was made alkaline untill pH of 10 with 4N aqueous sodium hydroxide solution. This solution was continuously extracted with chloroform. The organic extract was, after drying on $MgSO_4$ evaporated to give the aminoalcohol E-**3**. Yield 3.5 g (85%), oil. ^{1}H-NMR-spectral data are identical with those reported in the literature[5].

Synthesis of *trans*-zeatin (E-**1b**)

A mixture of aminoalcohol E-3 (3.5 g, 34.7 mmol), 6-chloropurine (5.02 gram, 32.7 mmol), anhydrous triethylamine (4.7 ml) and n-butanol (150 ml) was boiled under reflux for 3 h. TLC-analysis (system B) indicated that the conversion of the starting material was complete. The solvent was removed and the residue recrystallized twice, first from water, then from ethanol, to give *trans*-zeatin as white crystals. Yield 5.73 g (80%), m.p. 208-209°C, m.p. lit. 209-210°C, ^{1}H-NMR and ^{13}C-NMR spectral data were identical as described[9,10].

Synthesis of D,L-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]-N-trifluoroacetylalanine methylester (D,L-**5**).

A mixture of *trans*-zeatin, (E-**1b**) (5.7 g, 26,2 mmol), methyl 2-trifluoro-acetylaminoacrylate[2] (5.2 g, 26,2 mmol) dry dimethylsulphoxide (25 ml) and $KHCO_3$ (150 mg) was stirred for 3 days at 20°C, TLC-analysis (system C) indicated that then the reaction was complete. The reaction mixture was poured into water (50 ml), extracted with chloroform (3x100 ml) and the combined

organic extracts were dried with $MgSO_4$. The organic layer was concentrated to an oil, which was chromatographed on a column of silica gel (230-400 mesh ASTM). Elution with chloroform/methanol (100:0 → 98:2, v/v) gave after evaporation pure D,L-**5** as an oil. Yield 8.16 g (75%), Rf=0,8 system C.

^{1}H-NMR ($CDCl_3$): δ 1.70, s, 3H, CH_3; 3.92, s, 3H, $COOCH_3$; 4.10, s, 2H, CH_2O; 4.30, t, J=3.8 Hz, 2H, CH_2N; 4.8-5.2, m, 3H, C_α-H, 2x C_β-H; 5.57, t, J=6.2 Hz, 1H, CH=C; 6.75, t, J=3,8 Hz, 1H, NHCH$_2$; 7.81, s, 1H, H-2; 8.42, s, 1H, H-8; 10.20, m, 1H, $NHCOCF_3$; ^{13}C-NMR ($CDCl_3$): δ 167.73, $COOCH_3$); 157.57, q, J_{C-F}=39 Hz, $COCF_3$; 154.48, C_6; 152.70, C_2; 148.68, C_4; 139.96, C_8; 138.90, CH_3C=C; 119.82, CH=C; 119.15, C_5; 115.40, q, J_{C-F}=287 Hz, CF_3; 66.90, CH_2OH; 52.94, OCH_3; 52.80, C_α; 44.23, $NHCH_2$; 38.3, C_β, 13.52, CH_3; FD/MS: m/z: 416, [M$^+$]; 396, [(M-HF)$^+$]; 385, [(M-CH_3O)$^+$]; 357, [(M-$C_2H_3O_2$)$^+$].

Synthesis of D,L-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]alanine carbonamide (D,L-**6**)

The racemic mixture D,L-**5** (6.74 g, 16.2 mmol) was dissolved in dry n-butanol (150 ml), which was saturated with NH_3; the solution was heated at 30°C for 3 days in a sealed tube. TLC-analysis (system C) showed the complete absence of D,L-**5** and formation of a new compound with Rf=0.23. The solution was evaporated to an oil, which was crystallized from methanol to give D,L-**6** as white crystals. Yield 4.2 g (85%). mp. 190-192°C, [Rf=0.23 (system C, Rf=0.86 (system D).

^{1}H-NMR (DMSO-d6): δ 1.73, s, 3H, CH_3; 3.66, dd, J=8.1 Hz and 5.2 Hz, 1H, C_α-H; 3.84, d,J =5.7 Hz, CH_2O; 4.17, m, 3H, NH-CH_2 and C_β-H; 4.45, dd, J=13.9 Hz and 5.2 Hz, 1H, C_β-H; 4.79, t, J=5.7 Hz, 1H, OH; 5.59, t, J=6.1 Hz, 1H, CH=C; 7.22 and 7.55, br s, 2H, $CONH_2$; 7.85, br s, 1H, NHCH$_2$; 8.07, s, 1H, H-2; 8.27, s, 1H, H-8; ^{13}C-NMR (D_2O): δ 176.55, C=O; 154.46, C_6; 152.75, C_2; 148.39, C_4; 141.99, C_8; 138.75, CH_3C=C; 121.11, CH=C; 118.70, C_5; 66.89, CH_2O, 54.19, C_α; 47.18, NH CH_2, 38.63, C_β, 13.28, CH_3; FD/MS: m/z 306, [(M+H)$^+$]; 305 [M$^+$]; 219 [(M-$C_3H_7N_2O$)$^+$].

UV-spectra showed λ_{max} at the pH of 3, of 6 and of 11 at 266.3, 269 and 270 nm, respectively, being characteristic of (N^6,N^9)-disubstituted adenines[11].

Synthesis of D,L-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]alanine. (D,L-**1a**)

A stirred solution of the ester D,L-**5** (140 mg, 0.34 mmol) in dioxane (10 ml) and H_2O (5 ml) was treated with 1 N NaOH for 4h. The pH of the solution changed from 6 to 10, after 4h at 20°C. TLC-analysis (system D) indicated complete conversion of the starting material. The solution was percolated through a column (50 ml) of the anion exchange resin Dowex AC-1-X8 (OH^--form); the column was washed with water (150 ml) and then eluted with 0.1 N HCl. The eluate was passed directly through a column (60 ml) of cation exchange resin Dowex AC-50W-X8 (H^+-form). Then the column was washed with water and eluted with ammonia (1N). This eluate was evaporated to dryness and the residue was crystallized from ethanol/water.

Yield: (±)-Lupinic acid (D,L-**1a**): 128 mg (70%), m.p. 215-216°C; lit.[1]: 216-217°C, [Rf=0 (system C), Rf=0.74 (system D)].

^{1}H-NMR (DMSO-d6): δ 1.72, s, 3H, CH_3; 3.83, s, 2H, CH_2O; 3.88, dd, J=4.3 Hz and 8.2 Hz, 1H, C_α-H; 4.17, br s, 2H, NHCH_2; 4.56-4.70, m, 2H, 2x C_β-H; 4.88, br s, 1H, CH_2-OH; 5.57, t, J=5.9 Hz, 1H, CH=C; 7.92, br s, 1H NH-CH_2; 8.17, s, 1H, H-2; 8.27, s, 1H, H-8; ^{13}C-NMR (DMSO-d6): δ 171.08,

C=O; 155.69, C_6; 154.10, C_2; 150.16, C_4; 143,19, C_8, 139.99, $CH_3\underline{C}$=C; 121.83, $\underline{C}$H=C; 120.02, C_5; 67.72, CH_2O; 55.89, C_α; 45.91, $NHCH_2$; 14.84, CH_3; FD/MS: m/z: 306 [M^+]; 262 [$(M-CO_2)^+$]; 218[$(M-C_3H_6O_2N)^+$].

Synthesis of L-β-[6-(4-hydroxy-3-methyl-E-but-2-enyl amino)purine-9-yl]alanine (L-lupinic acid, L-**1a**)

The amide of D,L-lupinic acid (D,L-**6**) (2.0 gram, 4.93 mmol) was dissolved in 40 ml water and treated for 20 h at 40°C at pH=10[12] with aminopeptidase from *Pseudomonas putida* (4) (0.4 g of crude cells containing about 1% of active enzyme). The L-amino acid (L-**1a**) was separated from the D-amide (D-**6**) by the use of a strong basic ion-exchange column (see previous sections). Elution with 2N acetic acid gives L-lupinic acid (L-**1a**) 0.85 g, Rf=0.74 (system D), $[\alpha]_D^{20}$ = -24.5 (c=1, H_2O) and the unreacted D-lupinic acid amide (D-6) 0.78 g, Rf=0.86 (system D),$[\alpha]_D^{20}$ = -99 (c=1, H_2O). ^{1}H-NMR and ^{13}C-NMR and mass spectrometric data of compound L-**1a** and D-**6** are identical with those of D,L-**1a** and D.L-**6**, respectively.

Synthesis of D-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purine-9-yl]alanine (D-Lupinic acid D-**1a**)

A solution of the unreacted D-Lupinic acid amide (D-6) (120 mg, 0.40 mmol) in H_2O (5 ml) was treated with 6N HCl (2 ml). After one week at 20°C, TLC analysis (system D) showed complete conversion op the starting material with Rf=0 86 into the product D-**1a**, Rf=0.74. The reaction solution was neutralized with NaOH (4N) to pH=7.5. The reaction solution was concentrated to a small volume (1 ml) and applied to silanized silica gel RP18. The column was eluted with water applying a methanol gradient (0→60%), which gave after evaporation D-**1a** as a white solid. Yield 95 mg (77%), Rf=0.78, $[\alpha]_D^{20}$ = +26 (c=1, H_2O). ^{1}H-NMR, ^{13}C-NMR and mass spectrometric data of D-**1a** are identical with those of D,L-**1a**.

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12. At this pH a very small amount of D,L-carbonamide is hydrolyzed into the D,L-acid.