# Syntheses of Derivatives of $N^2$-Acetyl-$N^3$-glycyl-L-2,3-diaminopropionic Acid and Their Hydrolyses by Trypsin

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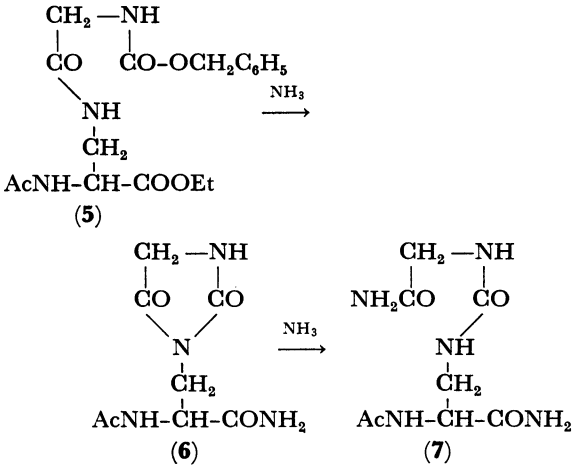
**Synopsis.** Amide and ethyl ester of $N^2$-acetyl-$N^3$-glycyl-L-2,3-diaminopropionic acid replacing an ethylene group in the side chain of lysine by a peptide bond were synthesized. The susceptibility of each derivative to trypsin was compared with that of the corresponding $N^2$-acetyl-L-lysine derivative. Each derivative was hydrolyzed by trypsin, but was less susceptible than the corresponding L-lysine derivative.

The specificity of trypsin is restricted. The enzyme hydrolyzes selectively the amide and ester bonds containing a carboxyl group of L-lysine or L-arginine. Several lysine-like derivatives such as L-4-thialysine[1] or L-4-oxalysine[2] are susceptible to trypsin; a methylene group in the side chain of lysine is replaced by a sulfur or oxygen atom. In order to examine the influence of replacement of an ethylene group in the side chain of lysine by a peptide bond, we undertook to synthesize an amide (**1**) and an ethyl ester (**2**) of $N^2$-acetyl-$N^3$-glycyl-L-2,3-diaminopropionic acid (Ac-L-$A_2$pr(Gly)-OH[3]) and to subject them to the action of trypsin. Ac-L-Lys-$NH_2$ (**3**) and Ac-L-Lys-OEt (**4**) were used for references.

We attempted to synthesize a key intermediate, Ac-L-$A_2$pr(Z-Gly)-$NH_2$ (**12**), by the action of methanolic $NH_3$ on Ac-L-$A_2$pr(Z-Gly)-OEt (**5**). A crystalline compound (mp 209 °C) was isolated, but the result of elemental analysis was not in line with the calculated value **12**. The structure of the compound with mp 209 °C was determined to be L-2-acetamido-3-[$N'$-(carbamoylmethyl)ureido]propionamide (**7**) by




means of nuclear magnetic resonance and infrared spectroscopy. The result of elemental analysis also agreed with the calculated value as **7**. We assume that the starting compound **5** could be converted into an intermediate hydantoin **6** which is subsequently converted into **7**.

The key intermediate **12** was synthesized as follows. Ac-L-$A_2$pr(Z)-$NH_2$ (**10**) was prepared from Ac-L-$A_2$pr(Z)-OEt (**9**) by the action of methanolic $NH_3$, **10** being transformed into Ac-L-$A_2$pr-$NH_2$ (**11**) by hydrogenolysis. Z-Gly-OH was coupled with $\beta$-amino group in **11** by the carbodiimide method, the intermediate **12** being obtained in a good yield. Finally, the desired Ac-L-$A_2$pr(Gly)-$NH_2$ (**1**) was prepared by hydrogenolysis of **12**. The ester substrate, Ac-L-$A_2$pr(Gly)-OEt (**2**) was easily prepared from compound **5** by hydrogenolysis.

TABLE 1. OPTIMUM pH AND $C_{max}$ VALUES OF SUBSTRATES BY TRYPSIN AT 30 °C

| Substrate | Optimum pH | $C_{max}$[a] |
|---|---|---|
| **1** | 8.0 | 0.0049 |
| **3** | 7.8 | 0.37 |
| **2** | 8.2 | 12.7 |
| **4** | 8.0 | 246 |

a) $C_{max}$ values were estimated from $C_{max}=k_3/2.3\ K_m$, where $K_m$ is a Michaelis constant and $k_3$ a rate constant.[4]

In order to determine the hydrolytic rates of the amide **1** and the ester **2** by trypsin, the effect of pH was measured for the four substrates. The optimum pH values are given in Table 1. For a comparison of the hydrolytic rates, maximum proteolytic coefficients ($C_{max}$)[4] were determined at the optimum pH. Ac-L-$A_2$pr(Gly)-$NH_2$ (**1**) is hydrolyzed at *ca.* 1.3/100 times the rate for Ac-L-Lys-$NH_2$ (**3**), and Ac-L-$A_2$pr(Gly)-OEt (**2**) at *ca.* 5.2/100 times that of Ac-L-Lys-OEt (**4**). The lysine-like derivative Bz-L-4-thialysinamide is hydrolyzed at 16/100 times Bz-L-Lys-$NH_2$.[1] Replacement of a methylene group in L-lysine by an atom such as sulfur and that of an ethylene group by a CONH lead to a decrease in the susceptibility for trypsin.

## Experimental

All melting points are uncorrected. Homogeneity of each compound was confirmed by TLC carried out on silica gel G (Merck) with various solvent systems, only the $R_f$ value with *n*-BuOH–AcOH–pyridine–$H_2O$ (4:1:1:2, v/v) being presented. Hydrolytic rates were determined by means of a Hitachi amino acid analyzer KLA-5 under the following conditions: flow rate, 30 ml/h; jacket temperature, 55 °C. Trypsin was salt free, crystalline sample from Nutritional Biochemicals Corporation, Ohio, U.S.A. $^1$H-NMR spectra were measured in DMSO-$d_6$ with a Hitachi R-20B spectrometer (60 MHz) using tetramethylsilane as an internal standard.

*H-L-$A_2$pr(Z)-OEt·HCl (**8**·HCl).* *N*-Carboxy anhydride (NCA) of H-L-$A_2$pr(Z)-OH was synthesized according to the method of Takagi *et al.*[5] The NCA (0.26 g, 1 mmol) was dissolved in ethanolic 1 M HCl (1.7 ml), and the solution

was left to stand at 50 °C for a few min and at room temperature for 2 d. The solvent was evaporated *in vacuo*, and the residue was recrystallized from hot acetone; yield, 0.27 g (89%); mp 165—166 °C; $[\alpha]_D^{20}$ +5.7° (*c* 2, EtOH); $R_f$ 0.77. Found: C, 51.39; H, 6.30; N, 9.24%. Calcd for $C_{13}H_{19}O_4N_2Cl$: C, 51.57; H, 6.33; N, 9.25%.

*Ac-L-A$_2$pr(Z)-OEt (**9**).* Acetic anhydride (0.51 ml, 5 mmol) was added with stirring to a chilled solution of **8**·HCl (0.30 g, 1 mmol) in pyridine (1.6 ml). After being stirred at 0 °C for 30 min and at room temperature for 3 h, the solvent was evaporated. After the residue had been dissolved in EtOAc, the solution was washed with water, dried ($Na_2SO_4$), and evaporated. The residue was recrystallized from EtOAc–ether; yield, 0.28 g (91%); mp 115—116 °C; $[\alpha]_D^{20}$ +24.2° (*c* 2, $CHCl_3$); $R_f$ 0.90. Found: C, 58.32; H, 6.51; N, 9.09%. Calcd for $C_{15}H_{20}O_5N_2$: C, 58.43; H, 6.54; N, 9.09%.

*Ac-L-A$_2$pr(Z)-NH$_2$ (**10**).* Compound **9** (1 mmol) was dissolved in methanol (6 ml) saturated with $NH_3$ at 0 °C, and the solution was left to stand at room temperature for 3 d. The solvent was evaporated, and the residue was recrystallized from EtOH–ether; yield, 97%; mp 187—188 °C; $[\alpha]_D^{20}$ −11.7° (*c* 2, DMF); $R_f$ 0.73. Found: C, 55.82; H, 6.07; N, 14.99%. Calcd for $C_{13}H_{17}O_4N_3$: C, 55.90; H, 6.14; N, 15.05%.

*Ac-L-A$_2$pr-NH$_2$·HCl (**11**·HCl).* Compound **10** (0.28 g, 1 mmol) dissolved in ethanolic 0.2 M HCl (5.5 ml) was treated with hydrogen in the presence of Pd black. After the completion of hydrogenolysis, the filtrate from the catalyst was evaporated to dryness: yield of hygroscopic powder (**11**·HCl), 0.18 g (*ca.* 100%); $R_f$ 0.43.

*Ac-L-A$_2$pr(Z-Gly)-NH$_2$ (**12**).* Compound **11**·HCl (0.18 g, *ca.* 1 mmol) and Z-Gly-OH (0.31 g, 1.5 mmol) were dissolved in DMF (8 ml). To the solution were added at 0 °C $Et_3N$ (0.14 ml, 1.0 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl salt (0.29 g, 1.5 mmol). After being stirred at 0 °C for 3 h and at room temperature overnight, the solvent was evaporated. The residue was dissolved in a mixture of $H_2O$–MeOH (1:1). The solution was put on a column (0.8×3.5 cm) of Dowex 50X8 ($H^+$ form) and eluted with the same solvent. The eluate was evaporated, and the residue was recrystallized from EtOH–EtOAc; yield, 0.26 g (74%); mp 144—146 °C; $[\alpha]_D^{20}$ −6.4° (*c* 1, EtOH); $R_f$ 0.71. Found: C, 51.40; H, 5.86; N, 16.17%. Calcd for $C_{15}H_{20}O_5N_4\cdot 3/4H_2O$: C, 51.50; H, 6.19; N, 16.01%.

*Ac-L-A$_2$pr(Gly)-NH$_2$·HCl (**1**·HCl).* This was prepared from **12** (0.35 g, 1 mmol) in the same way as for **11**·HCl; yield of hygroscopic powder, 0.24 g (*ca.* 100%); $R_f$ 0.31.

*Ac-L-A$_2$pr-OEt·HCl (**13**·HCl).* Compound **9** (2 mmol) was hydrogenated in the same way as for **11**·HCl. The residue was recrystallized from DMF–ether; yield, 85%; mp 176—178 °C (dec); $[\alpha]_D^{20}$ −41.8° (*c* 2, EtOH); $R_f$ 0.76. Found: C, 39.90; H, 7.18; N, 13.32%. Calcd for $C_7H_{15}O_3N_2Cl$: C, 39.90; H, 7.18; N, 13.30%.

*Ac-L-A$_2$pr(Z-Gly)-OEt (**5**).* Z-Gly-OH (1.5 mmol) was coupled with **13**·HCl (1 mmol) in the same way as for **12**. The solvent was evaporated, and the residue was dissolved in EtOAc. The solution was washed successively with 4% $NaHCO_3$, 2% HCl, and water, dried, and evaporated. The residue was recrystallized from EtOH–ether; yield, 71%; mp 116—118 °C; $[\alpha]_D^{20}$ −6.1° (*c* 2, EtOH); $R_f$ 0.77. Found: C, 55.78; H, 6.34; N, 11.44%. Calcd for $C_{17}H_{23}O_6N_3$: C, 55.88; H, 6.35; N, 11.50%.

*Ac-L-A$_2$pr(Gly)-OEt·HCl (**2**·HCl).* Compound **5** was hydrogenated in the same way as for **11**·HCl. The residue was recrystallized from 2-propanol–ether; yield, 77%; mp 194—196 °C (dec); $[\alpha]_D^{20}$ −20.2° (*c* 2, EtOH); $R_f$ 0.58. Found: C, 39.89; H, 6.71; N, 15.53%. Calcd for $C_9H_{18}O_4N_3Cl\cdot 1/4H_2O$: C, 39.71; H, 6.85; N, 15.44%.

*Ac-L-A$_2$pr(Gly)-OH (**14**).* 1 M NaOH (0.22 ml) was added to a solution of **2**·HCl (27 mg, 0.1 mmol) in water (0.2 ml). The solution was left to stand at room temperature for 15 min. It was then put on a column of Dowex 50X8 ($H^+$ form), and the resin was washed with water and eluted with 2 M $NH_4OH$. The eluate was evaporated; yield of powder, 18 mg (*ca.* 90%); $R_f$ 0.15.

*L-2-Acetamido-3-[N′-(carbamoylmethyl)ureido]propionamide (**7**).* Compound **5** (1 mmol) was treated with methanolic $NH_3$ (6 ml) in the same way as for **10**. The solvent was evaporated, and the residue was recrystallized from DMF–ether; yield, 94%; mp 208—209 °C; $[\alpha]_D^{20}$ −2.4° (*c* 2, $H_2O$); $R_f$ 0.76. IR (KBr) 3370, 3210 ($NH_2$), 3300 (NH), 1610—1670, 1570, 1300 (amide), 1660 (substituted urea CO), 1360 ($CH_3$) and 1420 $cm^{-1}$ ($CH_2$); $^1$H-NMR (DMSO-$d_6$) $\delta$=7.90 (1H, d, $J$=7.5 Hz, -CONHCH-), 7.35 (2H, brd s, -CONH$_2$), 7.04 (2H, brd s, -CONH$_2$), 6.29 (1H, t, $J$=6 Hz, -CONHCH$_2$-), 6.19 (1H, t, $J$=6 Hz, -CONHCH$_2$-), 4.20 (1H, m, -NHCHCH$_2$-), 3.63 (2H, d, $J$=6 Hz, -NHCH$_2$CO-), 3.28 (2H, m, -NHCH$_2$CH-) and 1.90 (3H, s, CH$_3$CO-). Found: C, 38.97; H, 6.14; N, 28.32%. Calcd for $C_8H_{15}O_4N_5$: C, 39.18; H, 6.17; N, 28.56%.

*Ac-L-Lys-NH$_2$·HCl (**3**·HCl), Ac-L-Lys-OEt·HCl (**4**·HCl), and Ac-L-Lys-OH (**15**).* These reference compounds were synthesized according to the methods reported.[6-8] $R_f$ values for the 3 compounds were found to be 0.35, 0.76, and 0.28, respectively.

*Determination of Hydrolytic Rates.* An aliquot (0.2 ml)[9] of trypsin solution in 0.001 M HCl and 0.5 ml of Tris buffer with a certain pH were placed in a 2-ml assay flask containing either an amide or ester substrate in a certain concentration. The solution was made up to 2.0 ml with water, the final concentration of the buffer being 0.1 M. An aliquot sample (0.2 ml) was withdrawn at certain intervals and placed in a flask containing 0.2 M citrate buffer (0.8 ml) at pH 2.2. A portion of the solution was subjected to assay by means of an amino acid analyzer with a 0.6×10 cm column using 0.35 M citrate buffer at pH 5.28.[10] The extent of % hydrolysis at certain intervals was calculated by the amount of either **14** or **15**. The enzymic hydrolysis of the substrates at various intervals followed the first-order kinetics within experimental error.

## References

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9) The final concentrations of the enzyme in test solution were as follows: 0.059 mg enzyme $N$/ml for **1**, 0.0091 for **3**, 0.000074 for **2**, and 0.000037 for **4**.

10) Elution volume and relative color intensity were determined as 8.0 ml and 30% for **14** and 8.0 ml and 100% for **15**, respectively.