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# STUDIES ON UREMIC TOXINS; STRUCTURE-ACTIVITY CORRELATION IN H-Asp(Gly)-OH*

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SUMMARY

The discovery of dipeptide, H-Asp(Gly)-OH, having an inhibition activity of PHA-induced lymphocyte transformation is reported. The seven analogs of H-Asp(Gly)-OH are described in which H-β-aspartyl, H-D-β-aspartyl, and Z-β-aspartyl respectively. The H-Gly-OH residue of H-Asp(Gly)-OH is also substituted for H-Sar-OH, H-β-Ala-OH, and H-Gly-OMe respectively. The biological activity of the seven analogs is compared with that of H-Asp(Gly)-OH on a PHA-induced lymphocyte transformation.

## INTRODUCTION

During work on the separation of the uremic toxins of uremic serum, H-Asp(Gly)-OH was isolated from uremic serum[1]. Sppression test of PHA-induced lymphocyte transformation was shown this dipeptide to have an inhibition activity by amounts more than 3.0mg/ml[2]. The plan of the present work was to vary independently the two amino acids of this dipeptide and the N- and C-terminal functional groups and to use these results, if definite structural requirements for a suppression test of PHA-induced lymphocyte transformation were discovered, in the design of additional compounds. The first change

* Symbols for amino acid derivatives and peptides used in this text are those recommended by IUPAC-IUB Commission on Biochmical Nomenclature: Biochem. J., 126, 773 (1972). Other abbreviations: HOBt= 1-hydroxybenztriazole, DCC= dicyclohexylcarbodiimide, DMF= dimethyformamide, MSA= methanesulfonic acid, TFA= trifluoroacetic acid, DCHA= dicyclohexylamine, PHA= phytohemaglutinin, Tos= p-toluenesulfonic acid.

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studies was the replacement of H-Gly-OH by other amino acid and it's ester: H-Sar-OH, H-β-Ala-OH and H-Gly-OMe. The reasons for the study on glycine analogs as follows: sarcosine is only the existence of the N-methyl group of peptide chain and substitution for H-β-Ala-OH is elongated peptide chain of H-Asp(Gly)-OH by one methylene goup. N-terminal β-aspartyl residue was replaced by H-α-aspartyl, H-D-β-aspartyl and Z-β-aspartyl. The reason for the study on aspartic acid analogs as follows: the necessary distance between amino acid carboxyl groups was studied by moving the peptide bond to the α-carboxyl of aspartic acid and the required stereochemistry of the molecule was determined by synthesizing optical isomer of H-Asp(Gly)-OH. On the other hand, it is founded that H-Asp(Gly)-OH is produced from hemoglobin by the action of proteolytic enzyme during the uremia[2,3]. In view of this investigation it was of interest to examine the suppression test of PHA-induced lymphocyte transformation of H-Ser-Asp-Gly-Leu-OH which was modified the C- and N-terminal vicinity being adjacent to H-Asp(Gly)-OH moiety in hemoglobin (β chain: position 72-75)[3].

The conventional method for the peptide synthesis is used in this investigation. The synthetic rout for the tetrapeptide, H-Ser-Asp-Gly-Leu-OH, is illustrated in Fig. 1. H-Leu-OBzl Tos[4] was condensed with Boc-Gly-OH[5] by the HOBt-DCC method[6] to yield Boc-Gly-Leu-OBzl (I). After removal of the Boc group of I with TFA, the resulting dipeptide ester was condensed with Boc-Asp(OBzl)-OH[6] by the similar manner as described I to yield Boc-Asp(OBzl)-Gly-Leu-OBzl (II). After removal of the Boc group of II with TFA, the resulting tripeptide ester was condensed with Boc-Ser(Bzl)-OH by the similar manner as described I to yield Boc-Ser(Bzl)-Asp(OBzl)-Gly-Leu-OBzl (III). The fully protected tetrapeptide (III) was hydrogenated over 5% palladium-carbon in AcOH solution overnight. The hydrogenated product was treated with MSA in the presence

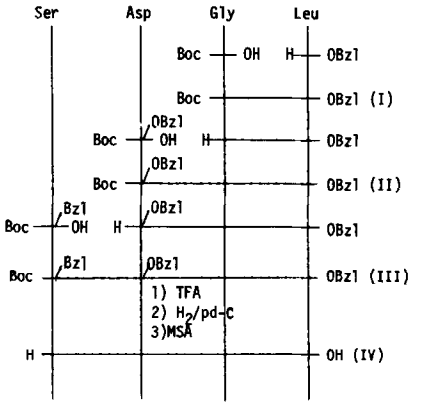


Fig. 1. Synthetic route to tetrapeptide (IV).

of anisole[7] and the resulting tetrapeptide was passed through Amberlite IRA-410 ( acetate type ) ( 1.8X10.0cm ) column. The tetrapeptide (IV) so obtained was found to be a unity from the result of paper chromatography using two different solvent systems. The amino acid ratios in the acid hydrolysate of IV and aminopeptidase-M digest agreed with theoretical values.

For the synthesis of H-Asp(Gly)-OH (VIII) the following siries of reactions was carried out. H-Gly-OBzl Tos[8] was condensed with Z-Asp(αONb)-OH DCHA[9] by the HOBt-DCC method to yield Z-Asp(Gly-OBzl)-ONb (VII). The protected dipeptide was hydrogenated over 5% palladium-carbon for 8 hr to yield H-Asp(Gly)-OH (VIII). H-D-Asp(Gly)-OH (X), H-Asp(Sar)-OH (XII), H-Asp(β-Ala)-OH (XIV), H-Asp(Gly-OMe)-OH (XVI), and H-Asp-Gly-OH (VI)[10], were prepared as similar manner described above.

For the synthesis of Z-Asp(Gly)-OH (XVII) the following reaction was carried out. Saponification of Z-Asp(Gly-OMe)-ONb (XV) with 1N NaOH solution afforded Z-Asp(Gly)-OH (XVII). The physical properties of protected dipeptides and free dipeptides are shown in Table II. The amino group free peptides were chromatographed on a filter paper Toyo Roshi No. 51, at room temperature. Rf[1] values refer to the Partridge

Table I. Physical Constants and Analytical Date of Synthesized H-Asp(Gly)-OH Analogs and Intermediates.

| Compounds | yield (%) | Recryst. Solv. | mp(°C) | $[\alpha]_D^{28°}$ (conc., solv.) |
|---|---|---|---|---|
| Boc-Asp(OBzl)-Gly-OBzl (V) | 83 | EtOAc-Pet ether | 65-67° | -9.0(1.0,DMF) |
| H-Asp-Gly-OH (VI) | 79 | $H_2O$ | 181-185° | +28°(1.0, 1N HCl) |
| Z-Asp(Gly-OBzl)-ONb (VII) | 96 | EtOAc-Pet ether | 95-99° | -9.5°(1.0,DMF) |
| H-Asp(Gly)-OH (VIII) | 87 | $H_2O$ | 160-168° | +18.1°(1.0, 1N HCl) |
| Z-D-Asp(Gly-OBzl)-OBzl (IX) | 54 | EtOAc | 134° | +1.5°(1.0,DMF) |
| H-D-Asp(Gly)-OH (X) | 69 | $H_2O$ | 210-228° | -8.9(1.0,50%AcOH) |
| Z-Asp(Sar-OBzl)-ONb (XI) | 74 | EtOAc-Pet ether | 50-52° | 0°(1.0,DMF) |
| H-Asp(Sar)-OH (XII) | 94 | $H_2O$-EtOH | 150-162° | +8.5°(1.0,$H_2O$) |
| Z-Asp(β-Ala-OBzl)-ONb (XIII) | 71 | EtOAc | 137-138° | -6.0°(1.0,DMF) |
| H-Asp(β-Ala)-OH (XIV) | 83 | $H_2O$ | 198-202° | +9.0°(1.0,50%AcOH) |
| Z-Asp(Gly-OMe)-ONb (XV) | 63 | EtOAc | 143-144° | -8.0°(1.0,DMF) |
| H-Asp(Gly-OMe)-OH (XVI) | 88 | EtOH | 215-217° | -9.5°(1.0,$H_2O$) |
| Z-Asp(Gly)-OH (XVII) | 73 | EtOAc-Pet ether | 52-55° | -8.5°(1.0,DMF) |

a) Y.Liwschitz, E. Names, and E. Levi, J. Org. Chem., 27, 3555 (1962).

system[11] and $R_f$[2] values refer to the system of BuOH-pyridine-AcOH-$H_2O$ (30:20:6:24)[12]. The chromatograms stained with ninhydrin.

These synthetic peptides were tested quantitatively for the effect of uremia on immune function. The degree of inhibition effect was measured by the incorporation of tritiated thymidine into DNA of normal lymphocyte stimulation by PHA. Result of biological examination is given in Table II. The inhibition activity on PHA-induced lymphocyte transformation of H-Asp(Sar)-OH (XII), H-Asp(β-Ala)-OH (XIV) is higher than that of H-Asp(Gly)-OH (VIII). An ester on the C terminal carboxyl (H-Asp(Gly)-OMe XVI), which causes a marke decrease the inhibition activity. Also, the aspartic acid amino group had to be unsubstituted for inhibition activity (Z-Asp(Gly)-OH XVII). Substitution of L-aspartic acid by D-aspartic acid (H-D-Asp(Gly)-OH X) shown no inhibition activity. Also, H-Asp-Gly-OH (VI) and H-Ser-Asp-Gly-Leu-OH (IV) shown no inhibition activity.

Table 1 (Continued)

| Formula | Analysis | | | | | | $Rf^1$ | $Rf^2$ | lit |
|---|---|---|---|---|---|---|---|---|---|
| | Calcd | | | Found | | | | | |
| | C | H | N | C | H | N | | | |
| $C_{25}H_{30}O_7N_2$ | 63.81 | 6.03 | 5.95 | 63.47 | 6.61 | 6.57 | | | |
| $C_6H_{10}O_5N_2$ | 37.90 | 5.30 | 14.73 | 38.36 | 5.72 | 14.89 | 0.17 | 0.18 | a |
| $C_{28}H_{27}O_9N_3$ | 61.20 | 4.95 | 7.69 | 60.81 | 5.41 | 7.35 | | | |
| $C_6H_{10}O_5N_2$ | 37.90 | 5.30 | 14.73 | 38.41 | 5.80 | 14.28 | 0.21 | 0.20 | |
| $C_{28}H_{26}O_{11}N_4$ | 56.56 | 4.41 | 9.43 | 56.18 | 4.90 | 9.03 | | | |
| $C_6H_{10}O_5N_2$ | 37.90 | 5.30 | 14.73 | 37.40 | 5.78 | 14.98 | 0.12 | 0.19 | |
| $C_{29}H_{29}O_9N_3$ | 61.80 | 5.19 | 7.46 | 62.20 | 5.54 | 7.21 | | | |
| $C_7H_{12}O_5N_2$ | 41.17 | 5.92 | 13.72 | 40.90 | 5.99 | 13.24 | 0.18 | 0.19 | |
| $C_{29}H_{29}O_9N_3$ | 61.80 | 5.19 | 7.46 | 62.29 | 5.66 | 7.82 | | | |
| $C_7H_{12}O_5N_2$ | 41.17 | 5.92 | 13.72 | 40.83 | 6.31 | 14.08 | 0.18 | 0.44 | |
| $C_{22}H_{23}O_9N_3$ | 55.81 | 4.90 | 8.88 | 56.27 | 5.26 | 9.20 | | | |
| $C_7H_{12}O_5N_2$ | 41.17 | 5.99 | 13.17 | 40.92 | 5.87 | 13.61 | 0.16 | 0.20 | |
| $C_{14}H_{16}O_7N_2$ | 49.70 | 5.36 | 7.10 | 49.69 | 5.24 | 7.46 | | | |

## EXPERIMENTAL

Melting points are uncorrected. Rotations were determined with a Atago Polax. Amino acid analyses were performed with a JEOL JLC-8AH amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 35-40°. The purity of the products was tested by paper chromatography on a filter paper Toyo Roshi No. 51, at room temperature. For paper chromatography, Boc group of the protected peptides were deblocked with TFA and Z group of the protected peptides were deblocked with catalytic hydrogenation on the palladium-carbon. Solvent systems used were Partridge system ($Rf^1$)[11] and the system of BuOH-AcOH-pyridine-$H_2O$ (30:20:6:24) ($Rf^2$)[12].

Boc-Gly-Leu-OBzl (I) ---- DCC (2.1g) was added to a mixture of Boc-Gly-OH[5] (1.9g), HOBt (1.4g) and H-Leu-OBzl(prepared from 3.9g of the p-toluenesulfonate[4] with 1.5ml of $Et_3N$) in DMF (30ml) and the mixture was stirred at 4° for 24 hr. The solution was filtered, the

Table II. Biological Activity of H-Asp(Gly)-OH Analogs[a)]

| | Inhibition activity of[b)] PHA-induced lymphocyte transformation |
|---|---|
| H-Asp(Gly)-OH (VIII) | 1.0 |
| H-D-Asp(Gly)-OH (X) | 0.0 |
| H-Asp(Sar)-OH (XII) | 2.9 |
| H-Asp(β-Ala)-OH (XIV) | 2.9 |
| H-Asp(Gly-OMe)-OH (XVI) | 0.0 |
| H-Ser-Asp-Gly-Leu-OH (IV) | 0.0 |
| H-Asp-Gly-OH (VI) | 0.0 |
| Z-Asp(Gly)-OH (XVII) | 0.0 |

a) The amount of $^{3}$H-thymidine incorporated into DNA measured in a scintilator.

b) assayed on the inhibition to PHA-induced lymphocyte transformation by H-Asp(Gly)-OH Analogs (3mg/ml).

filtrate was diluted with EtOAc, which was washed with 1N citric acid, $H_2O$, 1N $NaHCO_3$ and $H_2O$, dried over $MgSO_4$ and then evaporated. The residue was precipitated from EtOAc and petroleum ether: 3.7g (oil) (91%), $[\alpha]_D^{29}$ -9.6° (c=1.0, DMF), $Rf^1$ 0.83, $Rf^2$ 0.94, single ninhydrin positive spot, Anal. Calcd. for $C_{20}H_{30}O_5N_2$: C, 63.47; H, 7.99; N, 7.40. Found: C, 63.01; H, 8.36; N, 6.98.

Boc-Asp(OBzl)-Gly-Leu-OBzl (II) ---- Boc-Gly-Leu-OBzl (3.8g) was treated with TFA (7ml) at room temperature for 30min. The mixture was evaporated in vacuum and the resulting residue was dried over KOH pellets in vacuum and then dissolved in DMF (30ml), $Et_3N$ (1.4ml), Boc-Asp(OBzl)-OH (3.6g)[5], HOBt (1.5g) and DCC (2.2g) were combined and the mixture was stirred at 4° for 24 hr. The mixture was filtered in vacuum and the filtrate was diluted with EtOAc. The EtOAc solution was washed successively with 1N $NaHCO_3$, $H_2O$, 1N citric acid and $H_2O$, and dried over $MgSO_4$. The solution was concentrated to a small volume and petroleum ether was added to the residue: yield 4.0g (68%), mp 75-85°, $[\alpha]_D^{29}$ -7.0° (c= 1.0, DMF), $Rf^1$ 0.89, $Rf^2$ 0.95, single ninhydrin

positive spot, Anal. Calcd for $C_{31}H_{42}O_8N_3$: C, 63.68; H, 7.24; N, 7.19. Found: C, 63.49; H, 7.66; N, 7.08.

Boc-Ser(Bzl)-Asp(OBzl)-Gly-Leu-OBzl (III) ---- The compound was prepared from II (2.9g) and Boc-Ser(Bzl)-OH (1.7g) essentially in the same manner as described in the preparation of II. The product was reprecipitated from $Et_2O$ and petroleum ether: yield 3.1g (82%), mp 170-175°, $[\alpha]_D^{29}$ -7.7° (c= 1.0, DMF), $Rf^1$ 0.90, $Rf^2$ 0.96, single ninhydrin positive spot, Anal. Calcd for $C_{41}H_{52}O_{10}N_4$: C, 64.72; H, 6.89; N, 7.36. Found: C, 64.37; H, 7.31; N, 6.95.

H-Ser-Asp-Gly-Leu-OH (IV) ---- III (300mg) was dissolved in TFA (2ml) and the solution was kept at room temperature for 30min and evaporated in vacuum. The residue was dried over KOH pellets in vacuum and hydrogenated in 50% AcOH (15ml) over 5% palladium-carbon for 12 hr. The catalyst was removed by the aid of Cellite. The solution was evaporated to dryness and the residue was dried over KOH pellets in vacuum. The residue was recrystallized from EtOH and $H_2O$. Analysis by paper chromatography revealed the presence of two ninhydrin positive spot. The crude tetrapeptide (150mg) thus obtained was treated with MSA (1ml) in the presence of anisole (0.2ml) at room temperature for 40 min. To the reaction mixture was added dry $Et_2O$ at 0° and precipitate thereby formed was washed with $Et_2O$. The precipitate was dissolved in 3N AcOH (10ml) and the solution was passed through a column (1.8X12.0 cm) of Amberlite IRA-410 (acetate type). The eluate was evaporated in vacuum and was recrystallized from hot $H_2O$: yield 106mg (69%), mp 160-170°, $[\alpha]_D^{29}$ -25.0° (c= 1.0, 50% AcOH), $Rf^1$ 0.33, $Rf^2$ 0.29, single ninhydrin positive spot, Amino acid ratios in the acid hydrolysate: Ser 0.81, Asp 0.89, Gly 1.04, Leu 1.01. Amino acid ratios in aminopeptidase M hydrolysate: Ser 0.91, Asp 0.87, Gly 0.91, Leu 1.01.

General procedure for synthesis of H-Asp(Gly)-OH analogs ---- Amino acid methyl or benzyl ester salt (0.002mole) in DMF (5ml) was added

10% excess N-benzyloxycarbonylamino acid or N-tert-butyloxycarbonyl-amino acid (0.0022mole), HOBt (0.002mole), DCC (0.0022mole) followed by addition of $Et_3N$ to keep the solution slightly alkaline at $4^{\circ}$ and treated for purification as described above. The fully protected peptides (0.002mole) so obtained were hydrogenated as usual manner to yield the desired peptides. On the other hand, the fully protected dipeptides (0.002mole) was saponified with 1N NaOH (0.006mole) in dioxane (4ml) to yield the N-benzyloxycarbonyldipeptide.

Inhibition effect of these synthetic peptides on lymphocyte stimulation by PHA

Culture conditions and inhibition assay for DNA synthesis

The PHA (Difco) used was PHA-P, and it was added at the optimal concentration of 10$\mu$g/ml. Cells were cultured in 0.2ml of MEM in micro-titer plates (Falcon# 3040). 0.02ml (final 1$\mu$g/ml) of PHA is added, either 0.02ml of PHA-induced lymphocyte transformation inhibiting peptides. Triplicate cultures of each combination of $5X10^{-5}$ cells per well were incubated at $37^{\circ}C$ in a humidified atmosphere of 5% $CO_2$ in air for three days. Twenty-four hrs before harvest, 5$\mu$Ci of $^3H$-thymidine was added per culture. The amount of thymidine incorporated into DNA measured in a scintilator.

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