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Amino Acids and Peptides

XLI. A Possible Active Site Homology Between Carbonic Anhydrase and β-Lipotropin Hormone

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The hexapeptide L-histidyl–L-phenylalanyl–L-histidyl–L-tryptophanyl–glycyl–L-serine was synthesized because of its possible relationship to the known active-site residues in both carbonic anhydrase and β-lipotropin. The compound possessed weak anhydrase activity.

In connection with a homology study devoted to intestinal and pancreatic hormones (*1*), an amino acid alignment table was constructed and used to search for significant common structural and biological features in these compounds (*2*). Among the proteins listed were the various melanotropins (MSH), adrenocorticotropins (ACTH), and lipotropins (LPH). The positioning employed for these related peptides was not entirely satisfactory and a new study was begun for other possible archetype relationships.

It will be recalled that the α-MSH sequence is contained within ACTH, while the related β-MSH pattern is repeated in β-LPH. In turn, γ-LPH consists of a major portion of β-LPH. Thus, β-LPH, or an equivalent generalized sequence, can be considered to incorporate the main features of the adenohypophyseal hormones. Based on the above alignment pattern, the histidine located at position 6 in ACTH, position 13 in β-MSH, and position 49 in β-LPH is found to be the same one. This specific residue is known to play a major role in biological function of these compounds. The so-called "core fragment," histidyl–phenylalanyl–arginyl–tryptophanyl–glycyl, is required in MSH

TABLE 1

COMMON PARTIAL AMINO ACID SEQUENCES

Compound	Residues	Sequence
Human carbonic anhydrase B	84–102	*D S Y R L* F *Q F H F H W G S* T N E H
Human carbonic anhydrase C	84–102	G T *Y R L* I *Q F H F H W G S* L D *G* Q
Ovine β-lipotropin	41–58	*D S G P Y K M E H F R W G S P P K* D
Porcine β-lipotropin	41–58	*D* E *G P Y K M E H F R W G S P P K* D
Dogfish β-melanotropin	1–18	*D G* I D *Y K M* G *H F R W G* A *P* M D K
Rat pancreatic ribonuclease	114–127	*D G* N *P Y* V P V *H F* D A S V
Porcine luteinizing releasing hormone	1–10	*<E H* W S Y *G* L R *P G*

for minimum activity; by contrast, other, more distant, portions of ACTH are needed for a full physiological response (*3*, *4*).

The sequences of two human carbonic anhydrases (CA) were recently completed and the histidines located at positions 64 and 94 were specifically implicated in the chelation of a zinc atom (*5–8*). Attention is called now to the point that the residues around the penultimate histidine in CA, histidyl–phenylalanyl–histidyl–tryptophanyl–glycyl–seryl, closely resemble the core set found in the LPH series. At the same time, one can also note a resemblance to similar regions in ribonuclease and luteinizing releasing hormone (Table 1).

Unfortunately, no other isologous regions seem to exist, but it is possible that the sequencing of the equivalent ovine and/or porcine CA, as well as human β-LPH, plus other mammalian releasing hormones, can provide additional tests for this hypothesis. It should not be forgotten that some evidence for the existence of large corticotropin precursors has been supplied and these proteins may help contribute other common structural features (*9*, *10*). In the event no additional correlation exists, then one may be seeing a possible example of convergent evolution.

An interesting way to test this idea would be to prepare the short, common segment found at the CA catalytic site, His–Phe–His–Trp–Gly–Ser, and to evaluate it for physiological activity. Note that the similar sequence found in α-MSH, His–Phe–Arg–Trp–Gly–Ser, is the smallest synthetic fragment known to possess biological activity in the frog skin assay (*11*). Thus, the proposed new peptide might have previously unsuspected dual properties.

The synthetic task was accomplished by solution phase methods in a facile fashion. To begin, N^{α}-benzyloxycarbonyl-L-tryptophan (**I**) was joined with methyl glycinate (**II**) by a mixed anhydride procedure to give methyl N^{α}-benzyloxycarbonyl-L-tryptophyl-glycinate (**III**). Hydrolysis of dipeptide **III** using aqueous sodium hydroxide afforded N^{α}-benzyloxycarbonyl-L-tryptophyl–glycine (**IV**), which was found to exist in a polymorphoic state. In order to extend the chain length, t-butyl N^{α}-benzyloxycarbonyl-*O*-t-butyl-L-serinate (**V**) was hydrogenated with 10% palladium-on-charcoal catalyst to produce t-butyl *O*-t-butyl-L-serinate (**VI**). Amine **VI** was combined with the acid **IV** by a mixed anhydride step to yield t-butyl N^{α}-benzyloxycarbonyl-L-tryptophyl–glycyl–*O*-t-butyl-L-serinate (**VII**). Deprotection of tripeptide **VII** by hydrogenation provided the corresponding acid (**VIII**), which was coupled with N^{α}-benzyloxycarbonyl-N^{im}-t-butyloxycarbonyl-L-histidine (**IX**) via a mixed anhydride to supply t-butyl N^{α}-benzyloxycarbonyl-N^{im}-t-butyloxycarbonyl-L-histidyl–L-tryptophyl–glycyl–*O*-t-butyl-L-serinate (**X**).

Removal of the N^{α}-benzyloxycarbonyl protecting group in the usual fashion generated the tetrapeptide amine (**XI**), and a condensation with N^{α}-benzyloxycarbonyl-L-phenylalanine (**XII**) furnished t-butyl N^{α}-benzyloxycarbonyl-L-phenylalanyl-N^{im}-t-butyloxycarbonyl-L-histidyl–L-tryptophyl–glycyl–*O*-t-butyl-L-serinate (**XIII**). Hydrogenation formed the equivalent pentapeptide amine (**XIV**) and a mixed anhydride reaction with N^{α}-benzyloxycarbonyl-L-histidine (**XV**) provided t-butyl N^{α}-benzyloxycarbonyl-L-histidyl–L-phenylalanyl–N^{im}-t-butyloxycarbonyl-L-histidyl–L-tryptophyl–glycyl–*O*-t-butyl-L-serinate (**XVI**). Another hydrogenolysis of hexapeptide **XVI** gave t - butyl - L - histidyl – L - phenylalanyl – N^{im} - t - butyloxycarbonyl - L - histidyl – L - tryptophyl–glycyl–*O*-t-butyl-L-serinate (**XVII**). When compound **XVII** was dissolved

in a solution of trifluoroacetic acid–indole there was obtained the deblocked hexapeptide trifluoroacetate salt (**XVIII**). Finally, passage of the salt **XVIII** through an ion-exchange resin converted it to L-histidyl–L-phenylalanyl–L-histidyl–L-tryptophyl–glycyl–L-serinate triacetate (**XIX**). The route followed is summarized in the following diagram (Fig. 1).

The desired hexapeptide **XIX** existed as a fluffy white powder and possessed an excellent amino acid analysis, as determined by both acidic and basic hydrolysis methods. As expected, thin-layer chromatography showed the material to be homogeneous using a variety of detection reagents (fluorescence; iodine; ninhydrin). The

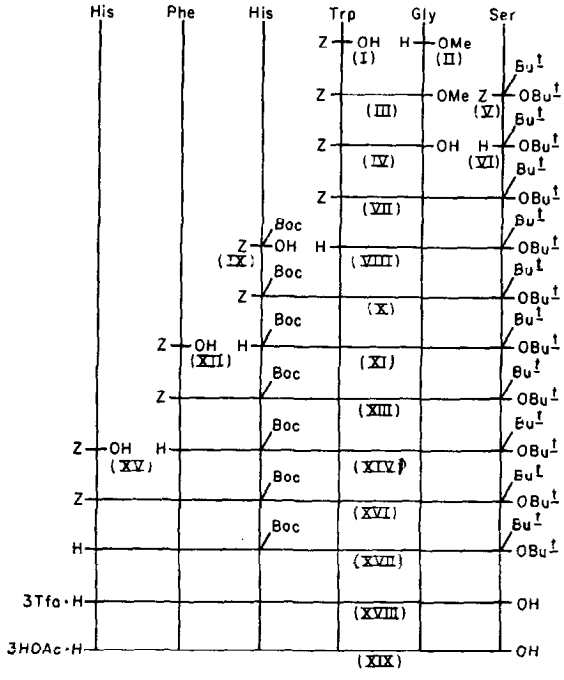

FIG. 1. Synthesis of the hexapeptide L-histidyl–L-phenylalanyl–L-histidyl–L-tryptophanyl–glycyl–L-serine.

ultraviolet and nuclear magnetic resonance spectra fully supported the assigned structure.

A sample submitted to Professor H. Yajima was found to be inactive by the standard MSH assay method; by contrast, the peptide exhibited weak CA response as measured by Professor Y. Pocker. Although the former result is somewhat disappointing, it must be remembered that the compound only approximates the minimum fragment necessary for an adenohypophysic response. By contrast, the positive response for anhydrase activity is interesting, since no information is currently available on model anhydrase systems. Thus, viewed as a first step, the homolog concept is a useful idea but has yet to be proven. The next approach would be to synthesize a longer fragment that might produce higher physiological responses, and better data.

EXPERIMENTAL

Methyl N$^{\alpha}$*-benzyloxycarbonyl-*L*-tryptophyl-glycinate* (**III**). N^{α}-benzyloxycarbonyl-L-tryptophan (13.5 g, 0.04 mol) and *N*-methylmorpholine (4.40 ml, 4.04 g, 0.04 mol) were dissolved in anhydrous tetrahydrofuran (100 ml) and the solution cooled to −20°C. Isobutyl chloroformate (5.20 ml, 5.46 g, 0.04 mol) was added and the mixture kept at −20°C for 5 min. A suspension of methyl glycinate hydrochloride (5.03 g, 0.04 mol) and *N*-methylmorpholine (4.40 ml, 4.04 g, 0.04 mol) in tetrahydrofuran (50 ml) and *N,N'*-dimethylformamide (25 ml) was introduced and the reaction stirred at −20°C for 2 hr and at room temperature for another 3 hr. The tetrahydrofuran was evaporated under reduced pressure at 30°C, and the *N,N'*-dimethylformamide was evaporated *in vacuo* at 50°C. The residue was taken into ethyl acetate and washed successively with 10% citric acid (three times), 10% sodium bicarbonate (three times), water (two times), and brine. The organic layer was dried over magnesium sulfate, and the solvent was evaporated until crystals began to form. The crystals were dissolved by warming and upon the addition of a little petroleum ether, crystallization proceeded at room temperature. The product was filtered and recrystallized from ethyl acetate–petroleum ether. Yield, 12.7 g (77.4%); mp, 155–157°C; R_f, 0.64 (chloroform–methanol, 9.5/0.5); $[\alpha]_D^{27}$, −11.9° (*c* 2, glacial acetic acid); lit. (*12*): mp, 158–159°C, $[\alpha]_D^{27}$, −11.0° (*c* 2, glacial acetic acid).

N$^{\alpha}$*-benzyloxycarbonyl-*L*-tryptophyl-glycine* (**IV**). Methyl N^{α}-benzyloxycarbonyl-L-tryptophyl–glycinate (12.55 g, 0.031 mol) in methanol (315 ml) was stirred for 1.5 hr with sodium hydroxide (37.7 ml, 1 *N*). At this time thin-layer chromatography (silica G, chloroform–methanol, 9.5/0.5) showed the completion of the reaction. The methanol was evaporated under reduced pressure at 25°C. One must take care to remove all the methanol, for on the addition of ethyl acetate, an emulsion will form and the layers will not separate. Water (200 ml) was added to the resulting aqueous solution, and the aqueous layer was extracted with ethyl acetate (3 × 100 ml). The aqueous solution was acidified to Congo red with dilute hydrochloric acid (2 *N*), extracted with ethyl acetate (3 × 600 ml), washed with brine, and dried over magnesium sulfate. The solvent was evaporated and the residue taken into a minimum amount of ethyl acetate with heating on the steam bath. Petroleum ether was added to the cloud point, and crystallization was allowed to proceed in the freezer at −20°C overnight. The product was filtered and recrystallized from ethyl acetate–petroleum ether. Crystals were allowed to form at room temperature for 3 hr and then in the refrigerator at 4°C overnight. Yield, 8.2 g (70%); mp, 155–157°C; $[\alpha]_D^{24}$, −24.6° (*c* 1, methanol); nmr (DMSO): δ 7.28 (singlet, aromatic on benzyloxycarbonyl), 6.77–7.18 (complex, indole of tryptophan), and 5.00 (singlet, CH_2 in benzyloxycarbonyl; ir: 1420 cm^{-1} (broad, strong intensity, carboxyl group), and 741 cm^{-1} (sharp, strong intensity, 4 adjacent hydrogens on the indole of tryptophan); lit. (*12*): mp, 158–159°C; lit. (*13*): D isomer, $[\alpha]_D^{24}$, +19.8°C (*c* 1, methanol).

In the first synthesis of this compound, a polymorph was observed to melt at 85–87°C. Resolidification occurred at 145°C, and the crystals remelted at 157°C. Comparison samples of N^{α}-benzyloxycarbonyl-L-tryptophyl–glycine were obtained and the ir spectra found to be identical to that of the above compound (*14*).

t-Butyl O-*t-butyl*-L-*serinate* (**VI**). A solution of t-butyl N^{α}-benzyloxycarbonyl-*O*-t-butyl-L-serinate (11.2 g, 0.032 mol) in methanol (100 ml) containing 10% palladium-on-charcoal catalyst was hydrogenated at room temperature for 5.5 hr. Thin-layer chromatography (silica G, chloroform–methanol, 9.5/0.5) showed the reaction was complete at the end of this period. The catalyst was removed by filtration, and evaporation of the solvent gave an oil (ninhydrin positive).

t-Butyl N^{α}-*benzyloxycarbonyl*-L-*tryptophyl–glycyl*-O-*t-butyl*-L-*serinate* (**VII**). N^{α}-benzyloxycarbonyl-L-tryptophyl–glycine (8.2 g, 0.021 mol) was dissolved in anhydrous tetrahydrofuran (100 ml) and *N*-methylmorpholine (2.3 ml, 2.1 g, 0.021 mole). The solution was cooled to –20°C and isobutyl chloroformate (2.7 ml, 2.9 g, 0.021 mole) added. The solution was stirred at –20°C for 5 min, and then t-butyl *O*-t-butyl-L-serinate (6.5 g, 0.03 mol, 1.4 equiv) in tetrahydrofuran (50 ml) was introduced and the reaction stirred at –20°C for 1.5 hr and at room temperature overnight. The solvent was evaporated and the residue taken into ethyl acetate and washed successively with 10% citric acid (three times), 10% sodium bicarbonate (three times), and brine. The organic layer was dried over magnesium sulfate and the solvent evaporated to about one-third of its original volume. Petroleum ether was added to the cloud point while heating on a steam bath. Crystallization was allowed to proceed at room temperature for 5 hr and then in the refrigerator at 4°C for 4 days. The products were filtered and washed with petroleum ether. Yield, 9.8 g (78.4%); mp, 80–82°C; R_f, 0.66 (chloroform–methanol, 9.5/0.5); $[\alpha]_D^{24}$, –8.7° (*c* 1, methanol); nmr ($CDCl_3$): δ 7.30 (singlet, aromatic in benzyloxycarbonyl), 7.27–6.98 (complex, indole of tryptophan), 5.17 (singlet, CH_2 in benzyloxycarbonyl), 1.47 (singlet, t-butyl ether), and 1.18 (singlet, t-butyl ester).

Anal. Calcd for $C_{32}H_{43}N_4O_7$ (595.69): C, 64.52; H, 7.28; N, 9.41. Found: C, 64.87; H, 7.03; N, 9.19.

t-Butyl L-*tryptophyl–glycyl*-O-*t-butyl*-L-*serinate* (**VIII**). A solution of t-butyl N^{α}-benzyloxycarbonyl-L-tryptophyl–glycyl–*O*-t-butyl-L-serinate (3.30 g, 0.0055 mol) in methanol containing 10% palladium-on-charcoal catalyst was hydrogenated at room temperature for 1 hr. Thin-layer chromatography (silica G, chloroform–methanol, 9.5/0.5) showed reaction to be complete at the end of this period. The catalyst was removed by filtration, and evaporation of the solvent gave the product in quantitative yield (ninhydrin positive).

t-Butyl N^{im}-*t-butyloxycarbonyl*-N^{α}-*benzyloxycarbonyl*-L-*histidyl*-L-*tryptophyl–glycyl*-O-*t-butyl*-L-*serinate* (**X**). N^{α}-benzyloxycarbonyl-N^{im}-t-butyloxycarbonyl-L-histidine (0.78 g, 0.002 mol) was dissolved in anhydrous tetrahydrofuran and *N*-methylmorpholine (0.22 ml, 0.20 g, 0.002 mol) added. The solution was cooled to –20°C and isobutyl chloroformate (0.26 ml, 0.27 g, 0.002 mol) added. After the solution was stirred at –20°C for 5 min, t-butyl L-tryptophyl–glycyl–*O*-t-butyl-L-serinate (1.11 g, 0.0024 mol, 1.2 equiv) dissolved in anhydrous tetrahydrofuran was added and the reaction stirred at –20°C for 1.5 hr and at room temperature overnight. The solvent was evaporated and the residue taken into ethyl acetate. The ethyl acetate solution was chilled to –20°C and washed with 10% citric acid (three times) that had been cooled to 0°C. The organic layer was subsequently washed with 10% sodium bicarbonate (three times), and water (two times), then dried over magnesium sulfate, filtered, and evaporated. The residue was dissolved in ethyl acetate on the steam bath and petroleum ether added to the cloud point. Recrystallization in this manner was performed twice.

Yield, 1.1 g (66%); mp, 142–144°C; R_f, 0.53 (chloroform–methanol, 9.5/0.5); $[\alpha]_D^{20}$, −13.8° (*c* 1, methanol); nmr ($CDCl_3$); δ 7.35 (singlet, aromatic in benzyloxycarbonyl), 7.29, 7.25–6.99 (singlet and complex, imidazole in histidine), 5.00 (singlet, CH_2 in benzyloxycarbonyl), 1.60 (singlet, N^{im}-t-butyloxycarbonyl), 1.47 (singlet, t-butyl ether), and 1.18 (singlet, t-butyl ester).

Anal. Calcd for $C_{43}H_{58}N_7O_{18}$ (832.95): C, 62.00; H, 7.02; N, 11.77. Found: C, 61.75; H, 6.67; N, 11.33.

t-Butyl N^{im}-*t-butyloxycarbonyl*-L-*histidyl*-L-*tryptophyl*-*glycyl*-O-*t-butyl*-L-*serinate* (**XI**). A solution of t-butyl N^{α}-benzyloxycarbonyl-N^{im}-t-butyloxycarbonyl-L-histidyl-L-tryptophyl-glycyl-*O*-t-butyl-L-serinate (1.00 g, 0.0012 mol) in methanol containing 10% palladium-on-charcial catalyst was hydrogenated at room temperature for 3 hr and at 35°C for 3 more hr. Thin-layer chromatography (silica G, chloroform–methanol, 9.5/0.5) showed the reaction to be complete at the end of this period. The catalyst was removed by filtration, and evaporation of the solvent gave the product in quantitative yield (ninhydrin positive).

t-Butyl N^{α}-*benzyloxycarbonyl*-L-*phenylalanyl*-N^{im}-*t-butyloxycarbonyl*-L-*histidyl*-L-*tryptophyl*-*glycyl*-O-*t-butyl*-L-*serinate* (**XIII**). N^{α}-benzyloxycarbonyl-L-phenylalanine (0.40 g, 0.0013 mole) was dissolved in anhydrous tetrahydrofuran (25 ml) and *N*-methylmorpholine (0.14 ml, 0.13 g, 0.0013 mol) added. The solution was cooled to −20°C and isobutyl chloroformate (0.17 ml, 0.18 g, 0.0013 mol) added. The solution was stirred at −20°C for 5 min, t-butyl N^{im}-t-butyloxycarbonyl-L-histidyl-L-tryptophyl-glycyl-*O*-t-butyl-L-serinate (0.83 g, 0.0012 mol, 1.1 equiv) added and the reaction stirred for 1 hr at −20°C and at room temperature overnight. The solvent was evaporated and the residue taken into ethyl acetate. The ethyl acetate solution was chilled to −20°C and washed with 10% citric acid (three times) that had been cooled to 0°C. The organic layer was subsequently washed with 10% sodium bicarbonate (three times) and water (two times), then dried over magnesium sulfate, filtered, and evaporated. The glassy solid was recrystallized from ethyl acetate–petroleum ether. Yield, 0.80 g (67%); mp, 107–110°; R_f, 0.58 (chloroform–methanol, 9.5/0.5); $[\alpha]_D^{20}$, −22.3° (*c* 1, methanol); nmr ($CDCl_3$): δ 7.29 (singlet, aromatic in benzyloxycarbonyl), 7.20 (singlet, aromatic in phenylalanine), 7.18–6.90 (complex, imidazole of histidine), 4.95 (singlet, CH_2 in benzyloxycarbonyl), 1.60 (singlet, N^{im}-t-butyloxycarbonyl), 1.43 (singlet, t-butyl ether), and 1.18 (singlet, t-butyl ester).

Anal. Calcd for $C_{52}H_{67}N_8O_{11}$ (980.13): C, 63.72; H, 6.89; N, 11.43. Found: C, 63.95; H, 6.92; N, 11.11.

t-Butyl L-*phenylalanyl*-N^{im}-*t-butyloxycarbonyl*-L-*histidyl*-L-*tryptophyl*-*glycyl*-O-*t-butyl*-L-*serinate* (**XIV**). A solution of t-butyl N^{α}-benzyloxycarbonyl-L-phenylalanyl-N^{im}-t-butyloxycarbonyl-L-histidyl-L-tryptophyl-glycyl-*O*-t-butyl-L-serinate (0.40 g, 0.00041 mol) in methanol containing 10% palladium-on-charcoal catalyst was hydrogenated at 35°C for 6 hr. Thin-layer chromatography (silica G, chloroform–methanol, 9.5/0.5) showed the reaction to be complete at the end of this period. The catalyst was removed by filtration and evaporation of the solvent gave an oil in quantitative yield (ninhydrin positive).

t-Butyl N^{α}-*benzyloxycarbonyl*-L-*histidyl*-L-*phenylalanyl*-N^{im}-*t-butyloxycarbonyl*-L-*histidyl*-L-*tryptophyl*-*glycyl*-O-*t-butyl*-L-*serinate* (**XVI**). N^{α}-benzyloxycarbonyl-L-histidine (0.15 g, 0.00052 mol, 1.3 equiv) was dissolved in anhydrous tetrahydrofuran and

N-methylmorpholine (0.06 ml, 0.053 g, 0.00052 mol). The solution was cooled to −20°C and isobutyl chloroformate (0.07 ml, 0.07 g, 0.00052 mol) added. After the solution was stirred at −20°C for 5 min, t-butyl L-phenylalanyl-N^{im}-t-butyloxycarbonyl-L-histidyl-L-tryptophyl-glycyl-*O*-t-butyl-L-serinate (0.35 g, 0.00041 mol) was introduced and the reaction mixture stirred at −20°C for 1.5 hr and at room temperature overnight. The solvent was evaporated and the residue taken into ethyl acetate. The ethyl acetate solution was chilled to −20°C and washed with 10% citric acid (three times) that had been cooled to 0°C. The organic layer was subsequently washed with 10% sodium bicarbonate (three times) and with water (two times), then dried over magnesium sulfate, filtered, and evaporated. The residue was recrystallized from ethyl acetate–petroleum ether. Yield, 0.12 g (40%); mp, 157–160°C; $[\alpha]_D^{20}$, −26.8° (*c* 1, chloroform); nmr (DMSO): δ 7.30 (singlet, aromatic in benzyloxycarbonyl), 7.16 (singlet, aromatic in phenylalanine), 7.22–6.90 (complex, imidazole of histidine), 5.00 (singlet, CH_2 in benzyloxycarbonyl), 1.57 (singlet, N^{im}-t-butyloxycarbonyl), 1.42 (singlet, t-butyl ether), and 1.18 (singlet, t-butyl ester).

t-Butyl L-*histidyl*-L-*phenylalanyl*-N^{im}-*t-butyloxycarbonyl*-L-*histidyl*-L-*tryptophyl-glycyl*-O-*t-butyl*-L-*serinate* (**XVII**). A solution of t-butyl N^{a}-benzyloxycarbonyl-L-histidyl – L - phenylalanyl – N^{im} - t - butyloxycarbonyl - L - histidyl – L - tryptophyl – glycyl-*O*-t-butyl-L-serinate (0.050 g, 0.00045 mol) in *N,N'*-dimethylformamide (20 ml) and glacial acetic acid (15 ml) containing 10% palladium-on-charcoal catalyst was hydrogenated at room temperature for 4 hr. Thin-layer chromatography (silica G, chloroform–methanol, 9.5/0.5) showed the reaction to be complete at the end of this period. The catalyst was removed by filtration, and evaporation of the solvent gave the product in quantitative yield (ninhydrin positive); nmr (undeuterated DMF): absence of singlets at δ 7.30 (aromatic in benzyloxycarbonyl), and δ 5.00 (CH_2 in benzyloxycarbonyl).

L-*Histidyl*-L-*phenylalanyl*-L-*histidyl*-L-*tryptophyl-glycyl*-L-*serine trifluoroacetate* (**XVIII**). t-Butyl L-histidyl-L-phenylalanyl-N^{im}-t-butyloxycarbonyl-L-histidyl-tryptophyl-glycyl-*O*-t-butyl-L-serinate (0.04 g, 0.00045 mol) was dissolved in anhydrous trifluoroacetic acid (1.0 ml) and indole (0.050 g, 0.00042 mol) added. The reaction was stirred at room temperature for 45 min; at the end of this period, the nmr showed that the three t-butyl singlets had coalesced into one singlet (δ 1.59), whose position was essentially identical with t-butyl trifluoroacetate (δ 1.60). The solvent was removed *in vacuo* to leave an oil (ninhydrin and Pauly positive).

L-*Histidyl*-L-*phenylalanyl*-L-*histidyl*-L-*tryptophyl-glycyl*-L-*serine acetate* (**XIX**). The aforementioned trifluoroacetate salt (**XVIII**) was dissolved in dilute acetic acid (5 ml, 0.01 *N*) and passed through an ion-exchange column (Bio-Rad AG1-X2, 10 × 1.5 cm) using dilute acetic acid (0.01 *N*) as the elutant. Lyophilization gave a light brown solid that was contaminated with indole. This material was chromatographed on a preparative thin-layer plate (silica PF-254, 40 × 20 cm, *n*-butanol–pyridine–acetic acid–water, 15/10/3/12), the desired strip detected by fluorescence, a silica band removed, and washed with dilute acetic acid (50 ml, 0.01 *N*). The resulting solution was lyophilized, then the residual solid redissolved in dilute acetic acid (5 ml, 0.01 *N*) and passed through a Sephadex column (G-10, 10 × 1 cm) to remove traces of silica. Lyophilization gave a white solid. Yield, 0.028 g (80%); mp, 175–177°C; R_f, 0.55 (*n*-butanol–pyridine–acetic acid–water, 15/10/3/12); $[\alpha]_D^{20}$, +10.0°; nmr (D_2O): absence

of t-butyl signal in the region δ 1.00–1.70; ir: absence of t-butyl signals at 1939 and 1356 cm^{-1}; uv (dilute acetic acid, 0.1 *N*): 287.5 mμ (ε 5762), and 279.1 mμ (ε 6762); under the same conditions tryptophan possessed two characteristic absorptions: 287.5 mμ (ε 4550) and 278.5 mμ (ε 5550).

Amino acid analysis (*acid hydrolysis*). Gly, 1.00; His, 2.00; Phe, 1.18; Ser, 1.00.

Amino acid analysis (*basic hydrolysis*). Gly, 1.00; His, 1.97; Phe, 1.04; Ser, 0.87; Trp, 0.98.

The above hexapeptide was tested by Dr. A. Tanaka and Dr. A. Makamura of the Shionogi Research Laboratory, Shionogi and Co., Ltd., Osaka. *In vivo* assay was performed on a frog by injecting 1 mg of the compound into the subcutis of the thigh through the lymph sac of the dorsum. The change in the dorsal skin brightness was measured by a photoreflectometer (*15*). Negative results were seen with the above dosage.

The carbonic anhydrase activity was performed by Dr. Joan Ng, University of Washington. The reaction medium consisted of a Veronal buffer, which is a 0.0167 *M* solution of sodium diethyl barbiturate containing bromothymol blue and adjusted to pH 8.2 with hydrochloric acid. The peptide was dissolved in this medium and carbon dioxide was bubbled at a steady rate. The rate of hydration of the carbon dioxide is a function of the rate of proton formation, which, in turn, is measured by a change in the indicator color from blue to yellow (*16*). Such a procedure is extremely sensitive to traces of acid that may be present in any sample. Therefore, the compound was redissolved in distilled water and lyophilized six times before the activity was measured on it. The results are summarized in Table 2.

TABLE 2

CARBONIC ANHYDRASE ACTIVITIES

Compound	Quantity	End point (sec)
Blank	—	61.4
H-His–Phe–His–Trp–Gly–Ser-OH	3 mg	46.6
Carbonic anhydrase C	2 μg	12.0

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