carefully made acidic with 1% HCl at 0°. The depositing precipitate was recrystallized from Me_2CO to yellow plates, mp 232° (decomp.). *Anal.* Calcd. for $C_{15}H_8O_6$: C, 63.39; H, 2.84. Found: C, 63.57; H, 2.99. IR cm^{-1}: 3250, 3080, 1688, 1626. This product was identified with an authentic sample of I derived from II,[3] and a sample[10] isolated from *Rubia chinensis* Regel et Maack var. *glabrescence* Kitagawa by mixed mp determination and IR comparison.

Methyl 1,3-Dimethoxy-2-anthraquinonecarbonate (XIII) (1,3-di-O-methylmunjistin 2-methyl ester)——(1) A mixture of I (0.5 g), Ag_2O (3 g), CH_3I (3 ml), and Me_2CO (100 ml) was refluxed for 10 hr. (2) A mixture of I (0.5 g), K_2CO_3 (10 g), $MeSO_4$ (3 ml), and Me_2CO (100 ml) was refluxed for 10 hr.

The product obtained by treatment as shown in (1) and (2) run was recrystallized from MeOH to give pale yellow needles, mp 147—148°. *Anal.* Calcd. for $C_{18}H_{14}O_6$: C, 66.25; H, 4.32. Found: C, 66.66; H, 4.47. IR cm^{-1}: 1734, 1678, 1664.

1,3-Di-O-methylmunjistin (XII) (1,3-dimethoxy-2-anthraquinonecarboxylic Acid)——1) XIII (0.2 g) was refluxed with 2% ethanolic KOH (50 ml) for 3 hr. The product obtained by the usual way was recrystallized from MeOH to pale yellow needles, mp 212—213°. *Anal.* Calcd. for $C_{17}H_{12}O_6$: C, 65.38; H, 3.87. Found: C, 64.95; H, 3.91. IR (Nujol) cm^{-1}: 1760, 1720, 1700, 1673, 1646. IR ($CHCl_3$) cm^{-1}: 1760, 1720, 1689. IR (dioxane) cm^{-1}: 1750, 1682. IR ($CHCl_3$+1 gtt. piperidine) cm^{-1}: 1674.

2) To a solution of 1,3-dimethoxy-2-anthraquinonecarbaldehyde (XI)[13] (200 mg) in AcOH (1):$(AcO)_2O$ (1) (10 mg) was added dropwise with stirring a solution of CrO_3 (100 mg) in AcOH (10 ml) and H_2O (0.1 ml) at 55—60°, and the stirring was continued keeping at this temperature for 2 hr when the reaction was complete changing the reaction mixture color from brown to green. The reaction mixture was poured into hot water (500 ml) and left at room temperature overnight. The depositing precipitate was recrystallized from MeOH to pale yellow needles, mp 212—213°. The IR spectrum of this product was shown to be superimposable with that of the foregoing product (XII), and their mixed mp showed no depression.

3) To a solution of 1,3-dimethoxy-2-hydroxymethylanthraquinone (X)[12] (200 mg) in AcOH (1):$(AcO)_2O$ (1) (10 ml) was added dropwise with stirring a solution of CrO_3 (200 mg) in AcOH (10 ml) and H_2O (0.1 ml) at 55—60°. The product obtained by the working as described above was shown to be identical with the foregoing product by mixed mp determination and IR comparison.

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Studies on Peptides. XVII. Synthesis of N-(N^{α}-Acetylseryltyrosylseryl-methionylglutamylhistidylphenylalanylarginyl)-5-methoxy-tryptamine and Its Physiological Properties on Frog Melanocyte *in vitro*[1,2]

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Recently we have replaced the acetyl group of melatonin[4,5] which is known as the most powerful but nonspecfic inhibitior of α-melanocyte-stimulating hormone (MSH)[6] with a histi-

1) The Part XVI of this series: *Chem. Pharm. Bull.* (Tokyo), **16**, 919 (1968).
2) The peptides and peptide derivatives mentioned in this communication are of the L-configuration and their abbreviated designations are those recommended by IUPAC-IUB commission for biological nomenclature in July 1965 and July 1966: *Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967).
3) Location: *Sakyo-ku, Kyoto.*
4) A.B. Lerner, J.D. Case, and R.V. Heinzelman, *J. Am. Chem. Soc.*, **81**, 6084 (1959).
5) A.B. Lerner, J.D. Caes, and Y. Takahashi, *J. Biol. Chem.*, **235**, 1992 (1960).
6) J.I. Harris and A.B. Lerner, *Nature*, **179**, 1346 (1957).

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dylphenylalanylarginyl moiety and have observed that this synthetic peptidyl-5-methoxytryptamine, N-(histidylphenylalanylarginyl)-5-methoxytryptamine (I)[7,8] reversed the action of α-MSH but not of caffeine which is known as a darkening agent of melanocyte. This behavior is in contrast to that of the closely related tetrapeptide, histidylphenylalanylarginyltryptophan[9] which exhibited intrinsic melanotropic activity. However the potency of I was about one millionth of that of melatonin.

We have now added the N-terminal portion of α-MSH to I in order to compare the potency and physiological property of such a compound, N-(N^{α}-acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyl)-5-methoxytryptamine (II) with that of I. For the synthesis of II, N^{α}-benzyloxycarbonyl-γ-benzylglutamate *p*-nitrophenyl ester[10] was allowed to react with I and the resulting product was subsequently hydrogenated. The product, N-(glutamylhistidylphenylalanylarginyl)-5-methoxytryptamine (III) was purified by column chromatography on carboxymethylcellulose (CM-cellulose) using pyridine acetate buffers. This was condensed with N^{α}-acetylseryltyrosylserylmethionine[1] by the azide procedure[11] to give II, which was purified by column chromatography on CM-cellulose. Pyridine acetate buffers were used to elute the desired compound. Homogeneity of the synthetic II was demonstrated by paper and thin-layer chromatographies. The acid hydrolysate of the product contained the constituent amino acids in the ratios predicted by theory except for 5-methoxytryptamine which was destroyed during the acid treatment.

The *in vitro* bioassay was conducted as described previously[8] using frog skins from *Rana pipiens* and the identical result was obtained in *Rana nigromaculata* H. It was found that the compound III was a lightening agent as I, exhibiting the potency of approximately one millionth that of melatonin. Next, when 0.1 ml of a solution of II (2.0 mg/ml) was added to a Ringer solution containing the frog skins predarkened by 10 MSH U. of the standard α-MSH, a very week lightening of the skins was observed (weeker than that of III). However, it was found that this compound II itself possessed an ability to darken the color of the frog skins and this potency was estimated as 2.6×10^4 MSH U/g. The structurally related N-terminal portion of α-MSH, N^{α}-acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycine which was prepared at present for comparison with II, exhibited only the MSH activity of 7.0×10^4 U/g. It is known that some modified peptide hormone derivatives exhibit the minor but normal harmonal activity as well as inhibitory power to a native hormone. For example, 2-[O-methyl or O-ethyltyrosyl]-oxytocin[12] possesses low oxytocic activity but when they were administered to the tissues stimulated previously by oxytocin, some inhibition was recorded. Such observation is also known in bradykinin analogues[13] and seems to offer an additional subject of investigation in regards to the relationship between srtucture and function of peptide hormones. Recently Schulz and du Vigneaud[14] modified the N-terminal cysteine residue of oxytocin and found that their synthetic 1-[penicillamine]-oxytocin possessed the powerful inhibitory action. Thus chemical modification of a peptide hormone molecule seems to serve as an approach to look into a peptide which possesses the inhibitory action. For establishment of generality to prepare peptide hormone inhibitors, further studies are required.

7) H. Yajima, K. Kawasaki, Y. Okada, and S. Lande, *Biochim. Biophys. Acta*, **107**, 141 (1965).
8) H. Yajima, K. Kawasaki, M. Koida, and S. Lande, *Chem. Pharm. Bull.* (Tokyo), **14**, 884 (1966).
9) H. Otsuka and K. Inouye, *Bull. Chem. Soc. Japan*, **37**, 289, 1465 (1964).
10) G. Losse, H. Jeschkeit, and W. Langenbeck, *Chem. Ber.*, **96**, 204 (1963).
11) T. Curtius, *J. Prakt. Chem.*, **70**, 57 (1902); *Chem. Ber.*, **35**, 3226 (1902).
12) J. Rudinger and I. Krejci, *Experientia*, **18**, 585 (1962); Z. Beranková, I. Pychlik, K. Jost, J. Rudinger, and F. Sorm, *Coll. Czech. Chem. Commun.*, **26**, 2673 (1961).
13) J.M. Stewart, "Hypotensive Peptides," ed. by E. Erdos, N. Back, and F. Sicuteri, Springer-Verlag, N.Y., 1966, p. 23.
14) H. Schulz and V. du Vigneaud, *J. Med. Chem.*, **9**, 647 (1966).

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Synthetic peptidyl–melatonin derivatives.

I H-His-Phe-Arg-NH-CH_2-CH_2-(5-methoxyindol-3-yl) [5-OCH3, 1-NH]

III H-Glu-His-Phe-Arg-NH-CH_2-CH_2-(5-methoxyindol-3-yl) [5-OCH3, 1-NH]

II Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-NH-CH_2-CH_2-(5-methoxyindol-3-yl) [5-OCH3, 1-NH]

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-NH-CH(-CH_2-indol-3-yl)-CO-Gly-OH

Experimental

The general experimental methods are essentially the same as described in the Part IV[15] of this series. Rf_1 values refer to the system of AcOH, *n*–BuOH, H_2O (1:4:5) on paper and Rf_2 values refer to the system of *n*–BuOH, AcOH, Pyridine, H_2O (4:1:1:2) on silica gel (Kieselgel G, Merck) thin–layer chromatography.

N-(Glutamylhistidylphenylalanylarginyl)-5-methoxytryptamine——N–(Histidylphenylalanylarginyl)–5–methoxytryptamine[8] (0.17 g) was dissolved in H_2O and the solution, after addition of 1 N HCl (0.7 ml) was lyophilized. The resulting hydrochloride and N^{α}–benzyloxycarbonyl–γ–benzylglutamate *p*–nitrophenyl ester[10] (0.20 g) were dissolved in dimethylformamide (DMF) (10 ml) and triethylamine (0.03 ml) was added. The solution was stirred at room temperature for 72 hr until the ninhydrin test of the reaction mixture became negative. After evaporation of the solvent, the oily residue was treated with H_2O and the resulting solid powder was then washed with AcOEt. This powder in 95% MeOH (35 ml) containing glacial AcOH (1 ml) was hydrogenated over a Pd catalyst in the usual manner. The catalyst was removed by filtration, the filtrate was evaporated to dryness *in vacuo* and the residue, after drying over KOH pellets *in vacuo*, was dissolved in H_2O (150 ml). The solution was applied to a CM–cellulose column (2×7 cm), which was eluted with successively with the following pH 5.0 pyridine acetate buffers: 0.01 M (600 ml), 0.025 M (1200 ml), 0.05 M (600 ml), 0.1 M (600 ml) and 0.2 M (350 ml). Individual fractions, 17 ml each, were collected at a flow rate of 4 to 5 ml per min and the absorbancy of individual tubes was determined at 280 mμ. The 0.025 to 0.05 M eluates (tube 79—190) containing the desired product were pooled, the bulk of the solvent was removed *in vacuo*, and the concentrate was lyophilized to constant weight; yield 0.11 g (59%), $[\alpha]_D^{25}$ −12.3° (c=0.6, H_2O), Rf_1 0.30, single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot; amino acid ratios in an acid hydrolysate $Glu_{1.06}His_{1.00}Phe_{1.00}Arg_{0.86}$(5–methoxytryptamine was destroyed, average recovery 96%). *Anal.* Calcd. for $C_{37}H_{49}O_7N_{11} \cdot 2CH_3COOH \cdot 5H_2O$: C, 50.8; H, 7.0; N, 15.9. Found: C, 51.0; H, 7.1; N, 16.7.

N-(N^{α}-Acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyl)-5-methoxytryptamine—— This entire operation was carried out in a cold room at 4° and the solutions were ice–cooled prior to their use. To a solution of N^{α}–acetylseryltyrosylserylmethionine hydrazide[1] (0.29 g) in 0.5 N HCl (2.6 ml), there was added a 5% solution of $NaNO_2$ (0.83 ml) and the mixture was kept in an ice–bath for 5 min. The pH of the solution was then adjusted to 7.0 by addition of triethylamine. This solution was added to a solution of N–(glutamylhistidylphenylalanylarginyl)–5–methoxytryptamine (0.25 g) in 90% pyridine (20 ml) containing triethylamine (0.07 ml). The mixture was kept at 4° for 50 hr with stirring. The solvents were removed by lyophilization and the residue was dissolved in H_2O (300 ml). The solution was applied to a CM–cellulose column (2×15 cm), which was first eluted with H_2O (850 ml) and then the following pH 5.0 pyridine acetate buffers: 0.01 M (700 ml), 0.02 M (1700 ml) and 0.1 M (1700 ml). Individual fractions of 17 ml each were collected at a flow rate of 3 to 4 ml per min with an automatic fraction collector and the absorbancy of each

15) H. Yajima and K. Kubo, *Chem. Pharm. Bull.* (Tokyo), **13**, 759 (1965).

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fraction was determined at 280 mμ. The 0.02 M pyridine acetate eluates (tubes 116—174) containing the desired product were pooled, concentrated *in vacuo* and lyophylized to give colorless fluffy material; yield 0.12 g (33%), $[\alpha]_D^{28}$ −43.8° (c=0.3, 30% AcOH); ninhydrin negative, single methionine, Pauly, Sakaguchi and Ehrlich positive spot; Rf_1 0.71, Rf_2 0.59; amino acid ratios in an acid hydrolysate $Ser_{1.78}Tyr_{0.90}Met_{0.81}Glu_{1.05}His_{1.12}Phe_{1.00}Arg_{0.88}$(5-methoxytryptamine was destroyed, average recovery 95%). *Anal.* Calcd. for $C_{59}H_{79}O_{15}N_{15}S\cdot CH_3COOH\cdot 3.5H_2O$: C, 53.4; H, 6.6; N, 15.3. Found: C, 53.4; H, 6.3; N, 14.9.

Glutamylhistidylphenylalanylarginyltryptophylglycine——The title compound was prepared according to the same manner as described in the corresponding 5-methoxytryptamine derivative, but this differs from those of literatures.[16–18] $[\alpha]_D^{32}$ −28.6° (c=0.6, 1 M AcOH). (lit.[16]) −15° in 1 N AcOH, lit.[17]) −17.3° in AcOH, lit.[18]) −18.0° in 1 N AcOH); Rf_1 0.36, Rf_2 0.67, single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot; amino acid ratios in an acid hydrolysate $Glu_{0.96}His_{1.05}Phe_{1.00}Arg_{1.01}Gly_{0.98}$ (Trp was destroyed, average recovery 82%). *Anal.* Calcd. for $C_{39}H_{50}O_9N_{12}\cdot 5H_2O$: C, 50.9; H, 6.6; N, 18.3. Found: C, 51.2; H, 7.0; N, 17.2.

N^α-Acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycine——N^α-acetylseryltyrosylserylmethionine azide (prepared from 0.17 g of the hydrazide[1]) as previously described) was added to an ice-cold solution of glutamylhistidylphenylalanylarginyltryptophylglycine (0.15 g) in 80% pyridine (9 ml) containing triethylamine (0.04 ml). The mixture was stirred at 4° for 24 hr and then the second portion of the azide (prepared from 0.09 g of the hydrazide) was added. After the solution was stirred for an additional 24 hr, the solvent was evaporated and the residue was dissolved in H_2O (250 ml). The solution was applied to a column of Amberlite CG-50 (H^+ form, 1.5×15 cm), which was eluted first with H_2O (1600 ml) and then 10% AcOH (800 ml). Individual fractions (15 ml each) were collected at a flow rate of 4 to 5 ml per min and absorbancy at 280 mμ was determined for each fraction. Two peaks were present, the former was in the H_2O eluate and the latter in 10% AcOH eluate (tubes 111 to 162). The contents of the latter eluate were pooled, the solvent was evaporated *in vacuo* and the residue was dried over KOH pellets *in vacuo*. This fraction contained one major (Rf_1 0.64) and one minor component (Rf_1 0.46, glutamylhistidylphenylalanylarginyltryptophylglycine). For further purification, the product was dissolved in H_2O (200 ml) and the solution was applied to a CM-cellulose column (2×20 cm), which was eluted with H_2O (1500 ml) and then 0.02 M pyridine acetate buffer (1000 ml). Individual fractions (15 ml each) were collected. The H_2O eluates (tubes 25 to 55) were pooled and lyophilized to give colorless fluffy powder; yield 0.11 g (45%), $[\alpha]_D^{28}$ −34.4° (c=0.3, 30% AcOH). Rf_1 0.64, ninhydrin negative and single methionine, Pauly, Sakaguchi and Ehrlich positive spot. Amino acid ratios in an acid hydrolysate $Ser_{1.72}Tyr_{0.98}Met_{0.75}Glu_{1.01}His_{0.99}Phe_{1.00}Arg_{1.01}Gly_{1.01}$ (Trp was destroyed, average recovery 90%); weight difference before and after drying of the sample was 4.9% which corresponds to $4H_2O$; Calcd. value was 5.1%. *Anal.* Calcd. for $C_{61}H_{80}O_{17}N_{16}S$ (dried at 100° for 4 hr): C, 54.6; H, 6.0; N, 16.3. Found: C, 54.3; H, 6.2; N, 16.0.

In the 0.02 M pyridine acetate buffer eluate, two peaks were obtained. The former located in the front portion of this eluate, yield 18 mg Rf_1 0.56, ninhydrin and methionine negative, Pauly, Sakaguchi and Ehrlich positive spot, presumably the sulfoxide of the acetyldecapeptide. The latter (Rf_1 0.46) was identical with glutamylhistidylphenylalanylarginyltryptophylglycine.

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16) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).
17) C.H.Li and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).
18) K. Inouye, *Bull. Chem. Soc. Japan*, **38**, 1148 (1965).

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