Melanin Concentrating Hormone Analogues: Contraction of the Cyclic Structure. 1. Agonist Activity[1]

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Melanin concentrating hormone (MCH) is a heptadecapeptide, Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val, which is synthesized in the hypothalamus and secreted by the neurohypophysis of teleost fishes. This hormone exhibits both MCH-like as well as α-MSH (α-melanocyte stimulating hormone) like activity. We have examined the role of the disulfide bond for the two contrasting melanotropic activities of MCH. Nine analogues of the parent peptide were synthesized and characterized for biological activity. The disulfide ring was contracted from the 5–14 to the 7–14, 8–14, and 10–14 residues with concomitant substitution of alanine for Cys at position 5 in each of the heptadecapeptides. Similar substitutions were made in a series of MCH_{5-17} analogues. In addition, the following cyclic peptides also were synthesized: [Cys^7]MCH_{7-17}, [Cys^8]MCH_{8-17}, and [Cys^{10}]MCH_{10-17}. The fish-skin bioassay is sensitive to MCH at a concentration of 10^{-12} M. All ring-contracted analogues were inactive at 10^{-6} M or lower concentrations; less than $^1/_{1\,000\,000}$ compared to MCH (1.0) except [Ala^5,Cys^8]MCH_{5-17} (0.0008; $^1/_{1250}$), [Cys^{10}]MCH_{10-17} (0.000 09; $^1/_{10\,000}$), and [Cys^8]MCH_{8-17} (0.000 001; $^1/_{1\,000\,000}$). In the frog-skin bioassay, [Ala^5,Cys^{10}]MCH, although lacking MCH-like activity in the fish-skin bioassay, was equipotent to MCH in its α-MSH-like component of activity. Most other analogues were either inactive or much less active than MCH in stimulating melanosome dispersion. These results demonstrate that the disulfide bond between positions 5 and 14 is essential for the MCH-like activity since contraction of the ring generally leads to inactive peptides. Contraction of the disulfide bridge does not, however, have as great an effect on the MSH-like activity of MCH.

The primary structure of a recently discovered[2] and synthesized[3-6] hormone, MCH (melanin concentrating hormone), has been shown[7] to contain certain sequential homology with a cyclic analogue of MSH, [Cys^4,Cys^{10}]-α-MSH, synthesized in our laboratory some years ago[8] when one or the other cysteine residues is aligned to the cysteine of the other peptide. No comparable homology is found, however, if other alignments of these peptides are examined (Figure 1). Moreover, it was shown that MCH also exhibits α-MSH-like activity[3] even though some differences in potencies were reported from different laboratories.[3,5,6,9]

Recently we have studied analogues of MCH shortened at either the carboxy or amino terminus of the molecule, and we were able to demonstrate that the amino terminal tetrapeptide sequence (or some sequence therein) was required for the α-MSH-like activity of the hormone.[10] Further shortening of MCH past residue 5 is not possible without modification of the disulfide bridge. We therefore decided to decrease the size of the intramolecular ring in MCH and to further shorten the amino terminal of the peptide in order to examine the structural requirements for the MCH- and α-MSH-like activities of the hormone. In the full sequence 1–17 peptide we have replaced cysteine in position 5 by alanine and the amino acid residues at positions 7, 8, and 10 by cysteine. We have also prepared these analogues in the shortest form (i.e., starting from the amino terminal cysteine) and as their 5–17 tridecapeptides. The tridecapeptides mimic the length of the cyclic α-MSH analogue, and the analogue containing cysteine in position 8 and 14 has the same ring size as the cyclic α-MSH analogue. The structures of the peptides are given in Figure 2. We studied the melanotropic activities of the hormone analogues on frog and teleost melanocytes.

Results

Chemistry. Nine disulfide bridge contracted analogues (Figure 2) were synthesized. All analogues were synthesized by the solid-phase method on 1% cross-linked Merrifield resin with dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole as the coupling agent. All couplings of

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* 1972, *247*, 977). The notation Cys refers to a half-cystine residue with a disulfide bond to another half-cystine residue in the same cyclic peptide. Hence, [Ala^5,Cys^7]MCH refers to the MCH analogue with Ala^5 and Cys^7 residues and with a disulfide bond between Cys^7 and Cys^{14} in the MCH analogue. Other abbreviations include the following: α-MSH, α-melanotropin, α-melanocyte stimulating hormone; DCC, dicyclohexylcarbodiimide; 2,6-Cl_2-Bzl, 2,6-dichlorobenzyl; 4-Me-Bzl, *p*-methylbenzyl; HOBT, *N*-hydroxybenzotriazole; For, formyl; Tos, tosyl; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; 1-BuOH, 1-butanol; Pyr, pyridine. All optically active amino acids are of the L configuration.

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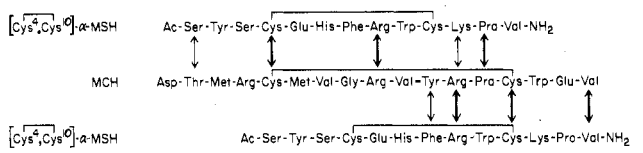

Figure 1. Sequential homology between MCH and the α-melanotropin analogue, [Cys^4,Cys^{10}]-α-MSH. Heavy double arrows, same amino acid residue; lighter double arrows, closely similar amino acid residues.

N^{α}-Boc-protected amino acids were performed in dimethylformamide. Peptides were cleaved from the resin by liquid hydrogen fluoride in the presence of anisole and dithioethane, and the disulfide bridge was formed by potassium ferricyanide oxidation. The peptides were purified by gradient ion-exchange chromatography, reversed-phase HPLC, and gel filtration. They were found homogeneous by analytical HPLC, thin-layer chromatography, and paper electrophoresis, and their structures were proven by amino acid analysis and fast atom bombardment mass spectroscopy (FAB-MS).

MCH-like Activity of MCH Analogues. The biological activities of these peptides were determined in two bioassays. As we have reported elsewhere,[3,4] MCH not only exhibits MCH-like activity (causes melanosome aggregation within integumental fish melanocytes and lightening of the skin), but the peptide also exhibits α-MSH-like activity (melanosome dispersion and skin darkening) on tetrapod (frog and lizard) skins. We will discuss these contrasting agonistic activities of MCH-related analogues individually for reasons of clarity.

The MCH-like activity of the analogues were compared to the native hormone, MCH, in the fish-skin bioassay. The ring-contracted analogues consisting of the entire heptadecapeptide sequence of MCH (analogues 1–3) were devoid of MCH activity, even at the highest concentration (10^{-6} M) of the peptides employed (Table I). Since the minimal effective dose of MCH needed to significantly lighten fish skins is 10^{-12} M, these analogues were at least 1 000 000 times less active than the native hormone.

Table I. Relative Potencies of the MCH Analogues in Assays for MCH- and α-MSH-like Biological Activities

analogue MCH[c]	MCH-like activity:[a] fish bioassay	α-MSH-like activity:[b] frog-skin bioassay
1, [Ala^5,Cys^{10}]MCH	inactive[d]	1.0
2, [Ala^5,Cys^8]MCH	inactive[d]	0.04
3, [Ala^5,Cys^7]MCH	inactive[d]	0.025
4, [Ala^5,Cys^{10}]MCH$_{5-17}$	inactive[d]	0.025
5 [Ala^5, Cys^8]MCH$_{5-17}$	0.0008	inactive[e]
6, [Ala^5,Cys^7]MCH$_{5-17}$	inactive[d]	inactive[e]
7, [Cys^{10}]MCH$_{10-17}$	0.000 091	inactive[e]
8, [Cys^8]MCH$_{8-17}$	0.000 001	inactive[e]
9, [Cys^7]MCH$_{7-17}$	inactive[d]	0.004

[a] Melanosome aggregation within melanocytes. [b] Melanosome dispersion within melanocytes. [c] The EC_{50} from parallel dose–response curves in each of the bioassays utilized. The potency of MCH was considered as 1.0. [d] Inactive at 10^{-6} M or lower. [e] Inactive at 10^{-5} M or lower.

Fragment analogues consisting of the 5–17 sequence of MCH with the ring contracted to the 10-, 8-, and 7-positions (analogues **4–6**, Figure 2) were either inactive or only minimally active (Table I). [Ala^5,Cys^{10}]MCH$_{5-17}$, **4**, and [Ala^5,Cys^7]MCH$_{5-17}$, **6**, were inactive at 10^{-6} M, although it is possible that they might exhibit some minimal activity at a higher concentration. [Ala^5,Cys^8]MCH$_{5-17}$, **5**, although

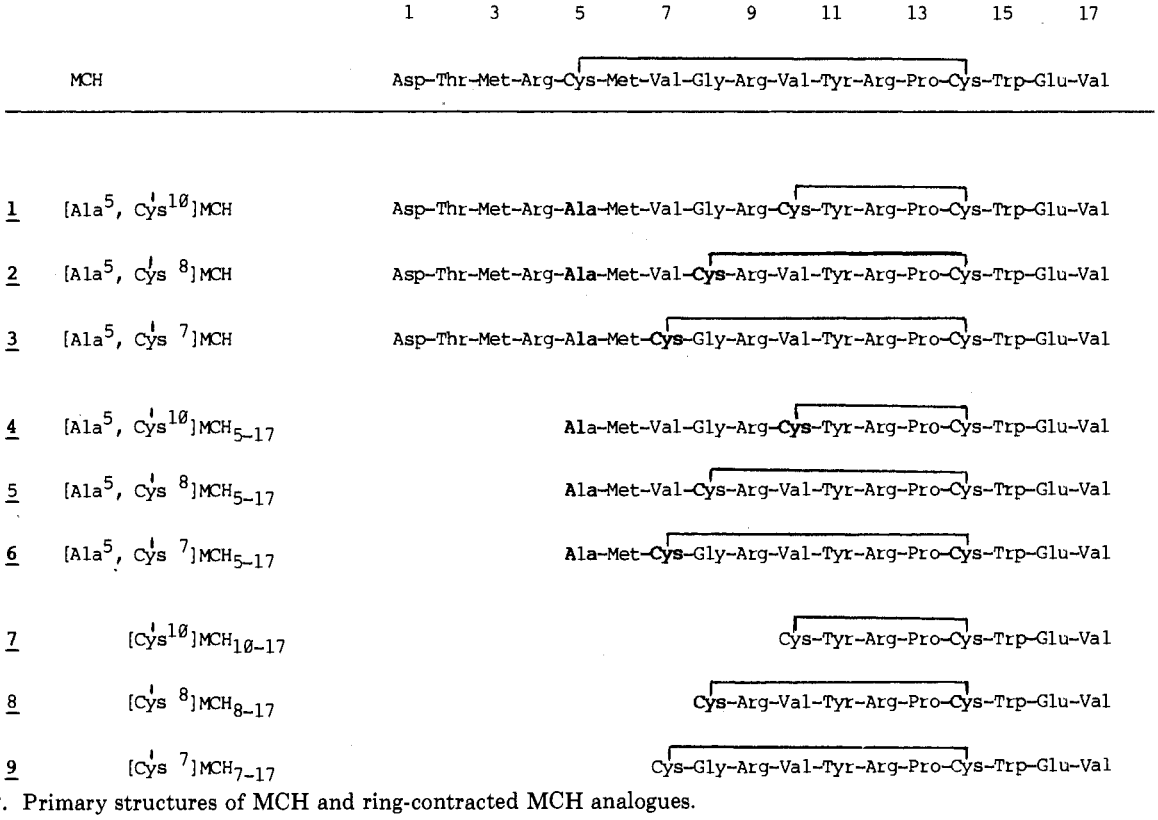

Figure 2. Primary structures of MCH and ring-contracted MCH analogues.

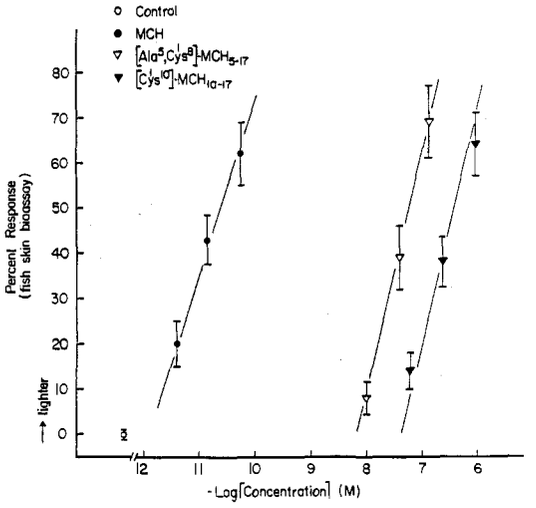

Figure 3. In vitro demonstration of the full agonist activity of [Ala^5,Cys^8]MCH$_{5-17}$ and [Cys^{10}]MCH$_{10-17}$ in the fish-skin bioassay as compared to MCH. Each value is the mean ± SE response (lightening) of the skins (N = 6–15) to the peptides at the concentrations noted.

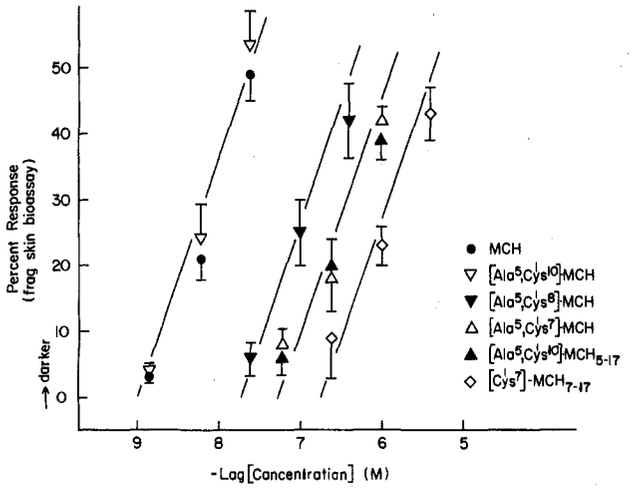

Figure 4. In vitro demonstration of the α-MSH-like activities of several ring-contracted MCH analogues in the frog-skin bioassay. Each value is the mean ± SE response (darkening) of the skins (N = 6–12) to the peptides at the concentrations noted.

about 1250 times less active than MCH, was a full agonist (Table I, Figure 3).

The shorter C-terminal cyclic fragment disulfide bridge contracted analogues **7**–**9**, Figure 2) were either inactive ([Cys^7]MCH$_{7-17}$, **9**), very minimally active ([Cys^8]MCH$_{8-17}$, **8**), or somewhat more active [Cys^{10}]MCH$_{10-17}$, **7**). The shortest fragment analogue, [Cys^{10}]MCH$_{10-17}$, **7**, was surprisingly a full agonist at the concentration employed (Figure 3).

α-MSH-like Activity of MCH Analogues. In the frog-skin bioassay (α-MSH-like activity) it was determined that [Ala^5,Cys^{10}]MCH, **1**, was a full agonist and equipotent to MCH. Previously MCH was found to be about $^1/_{600}$ as potent as α-MSH in this assay.[4] The other heptadecapeptide analogues, [Ala^5,Cys^8]MCH (**2**), possessed about $^1/_{25}$ and [Ala^5,Cys^7]MCH and [Ala^5,Cys^{10}]MCH$_{5-17}$ (**3** and **4**) about $^1/_{40}$ of the α-MSH-like activity of MCH (Figure 4). Of the three N-terminal shortened 5–17 ring-contracted peptides, only the [Ala^5,Cys^{10}]MCH$_{5-17}$ analogue, **4**, exhibited α-MSH-like activity ($^1/_{40}$ that of MCH, Figure 4). The cyclic C-terminal [Cys^{10}]MCH$_{10-17}$ and [Cys^8]-MCH$_{8-17}$ analogues (**7** and **8**) were inactive (at 10^{-5} M), but the [Cys^7]MCH$_{7-17}$ analogue, **9**, exhibited weak but full α-MSH-like agonism (Table I).

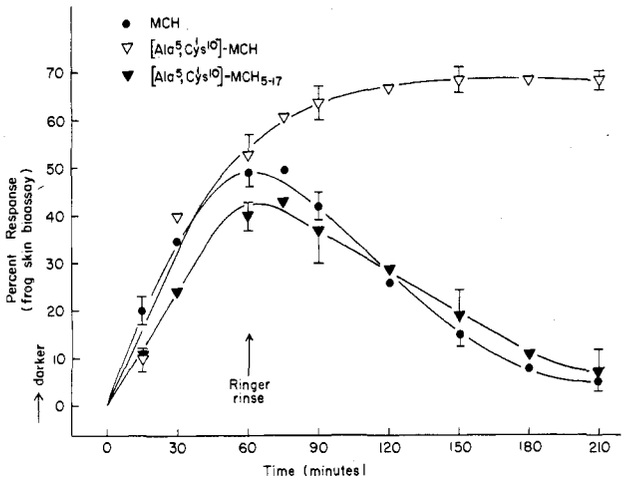

Figure 5. In vitro demonstration of the prolonged α-MSH-like activity of [Ala^5,Cys^{10}]MCH (2.5 × 10^{-8} M) compared to [Ala^5,Cys^{10}]MCH$_{5-17}$ (10^{-6} M) and to MCH (2.5 × 10^{-8} M). Each value is the mean ± SE response (darkening) of the skins (N = 6–12) to the peptides.

We have reported elsewhere that a number of α-MSH analogues exhibit prolonged (residual) biological activity; that is, after transfer of frog skins to Ringer's solution in the absence of the hormone, the darkening of the skins induced by certain analogues is maintained for a considerable length of time compared to the actions of α-MSH, which are rapidly dissipated.[11-13] It was interesting to note, therefore, that [Ala^5,Cys^{10}]MCH, 1, the analogue possessing the greatest α-MSH-like activity, also exhibited prolonged activity (Figure 5). This residual activity contrasted with that of the fragment analogue, [Ala^5,Cys^{10}]MCH$_{5-17}$, **4**, and MCH, neither of which exhibited such activity (Figure 5). Prolongation of the activity was also observed in the two other analogues containing the full 1–17 sequence and also in the [Cys^7]-MCH$_{7-17}$ analogue, **9**.

Discussion

These results from the bioassays clearly demonstrate that reduction in size of the cyclic structure of MCH results in a drastic or total loss of MCH-like activity. We have recently reported that MCH exhibits both MCH-like as well as α-MSH-like activity and that the two peptides are most likely evolutionarily related.[10,14] We also suggested that the C-terminal tripeptide sequence of MCH is im-

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portant for full potency of MCH whereas the N-terminal tripeptide sequence of MCH is important for the MSH-like activity of the hormone. What is interesting, therefore, in the present experiments is that although we have been able to totally abolish the MCH activity of MCH by ring contraction, these structural modifications have not in some cases diminished the MSH-like activity of these analogues.

MCH-like Activity (Melanosome Aggregating Activity). Contraction of the ring of MCH from the 5 to the 7-, 8-, or 10-positions within the peptide abolished the activity of the full-length (heptadecapeptide) analogues **1–3**. The 5–17 fragment analogues were inactive or minimally active, the [Ala^5,Cys^8]MCH$_{5-17}$ analogue **6**, exhibiting about $^1/_{1250}$ the activity of MCH.

Most interesting was the observation that the smallest fragment analogue, [Cys^{10}]MCH$_{10-17}$, **7**, was a full agonist with an activity about 1/10 000 that of MCH. We had previously shown that MCH$_{5-17}$ was equipotent to MCH.[10] The cyclic central sequence of MCH, MCH$_{5-14}$, is a weak but full agonist indicating that the C-terminal tripeptide sequence, Trp-Glu-Val, though not essential for receptor signal transduction, is important for potency. Our results indicate that the shortest sequence having MCH-like activity may be comprised of residues 10–14, i.e., the sequence Val-Tyr-Arg-Pro-Cys.

α-MSH-like Activity (Melanosome Dispersing Activity). Although [Ala^5,Cys^{10}]MCH is totally devoid of MCH-like activity, this analogue is equipotent to MCH relative to its α-MSH-like activity in the frog-skin bioassay (Table I). The observation that [Ala^5,Cys^{10}]MCH$_{5-17}$ exhibits about $^1/_{40}$ the MSH-like activity (in the frog-skin assay) of the heptadecapeptide analogue [Ala^5,Cys^{10}]MCH reveals, as we have discussed elsewhere,[10] that some component of the N-terminal tetrapeptide sequence is important for the α-MSH-like activity of MCH. Although contraction of the disulfide bridge from the 5- to the 10-position does not diminish the α-MSH-like activity of the peptide, shorter contractions of the ring to the 7- or 8-positions do lower the α-MSH-like activity about 40- and 25-fold, respectively. Possibly this segment of the primary structure possesses a contiguous message sequence that complements the N-terminal 1–4 sequence when it is exposed by the larger shift of the disulfide bridge toward the C-terminus of the peptide. It should be noted that MCH$_{1-14}$ possesses only about $^1/_{10}$ the α-MSH-like activity of MCH.[10] This suggests that in addition to the importance of the first four amino acids of MCH for α-MSH-like activity, full α-MSH-like activity requires contributions of two or more of the three C-terminal amino acids. It is interesting to note that although MCH$_{5-17}$ lacks α-MSH-like activity,[10] [Ala^5,Cys^{10}]MCH$_{5-17}$ does exhibit some ($^1/_{40}$ that of MCH) such activity. Ring contraction to the 10-position may expose, as in the heptadecapeptide analogue, a structural component that was previously hidden by the 5–14 disulfide bridge, which can interact with the melanotropin receptor.

The observation that some of the MCH analogues exhibit prolonged biological activity as do certain analogues of α-MSH is further evidence that MCH and α-MSH must bear some similar topological structural features that account for their MSH-like component of activity. This is further confirmation that the two peptides, which share minimal primary structural similarities, must, nevertheless, be evolutionarily related.

Experimental Section

General Methods. Amino acids used in the synthesis were obtained from Vega Biochemicals (Tucson, AZ) or Bachem (Torrence, CA) and were checked prior to the coupling by the ninhydrin test.[15] Solid-phase synthesis was performed on a Vega 1000 synthesizer. Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the systems: 2-butanol–98% formic acid–water (75:13.5:11.5) (S1) and 1-butanol–pyridine–acetic acid–water (15:10:3:6) (S4). Electrophoresis was performed on a Whatman 3MM paper in moist chamber (20 V/cm) for 1 h in 1 M aqueous acetic acid (pH 2.4) and in pyridine–acetate buffer (pH 5.7). The peptides were detected by ninhydrin or by the chlorination method.[16] Samples for amino acid analysis were hydrolyzed with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole and analyzed on a Beckman 120C automatic analyzer. High-performance liquid chromatography (HPLC) was carried out on a Spectra Physics SP-8700 instrument equipped with an SP-8400 detector (Spectra-Physics, Santa Clara, CA) with UV detection at 280 nM and with a Vydac C_{18} RP column, 10 mm × 25 cm for preparative purifications. ^{1}H NMR spectra were recorded on a Brunker AM 250-MHz spectrometer. Fast atom bombardment mass spectra were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas.

Synthesis of the Protected Peptide Resin. Merrifield resin (7 g) cross-linked with 1% divinylbenzene and containing 0.3 mmol/g of N^α-Boc-Val was deprotected by the action of 45% trifluoroacetic acid in dichloromethane (2 and 20 min) and neutralized with 10% diisopropylethylamine in dichloromethane. Then, N^α-Boc-Glu(OBzl), N^α-Boc-Trp(For), N^α-Boc-Cys(4-Me-Bzl), N^α-Boc-Pro, N^α-Boc-Arg(Tos), and N^α-Boc-Tyr(2,6-Cl_2-Bzl) were coupled to it by the action of dicyclohexylcarbodiimide (3 equiv) and *N*-hydroxybenzotriazole (3 equiv) in dimethylformamide. The following sequence was used in each step: (1) 3 × 1.5 min dichloromethane wash, (2) 4 × 1.5 min 10% diisopropylethylamine in dichloromethane, (3) 2 × 1 min dimethylformamide, (4) coupling 30 min to 15 h as monitored by the ninhydrin test (if incomplete after 15 h, 0.2 equiv of (dimethylamino)pyridine was added, and the mixture was shaken for another 1 h), (5) 2 × 1 min dimethylformamide, (6) 3 × 1.5 min dichloromethane, and (7) 45% trifluoroacetic acid and 2% anisole in dichloromethane, 2 and 20 min.

After the coupling of tyrosine, the peptide resin was divided into three equal parts (peptide resin A) and N^α-Boc-Val and N^α-Boc-Arg(Tos) were coupled to two of them (peptide resin B). This resin, in turn, was divided in two parts and to one was coupled N^α-Boc-Cys(4-Me-Bzl) (peptide resin C). One third of peptide resin C was stored, and to the rest were coupled N^α-Boc-Val, N^α-Boc-Met, and N^α-Boc-Ala (peptide resin D). Half of peptide resin D was further extended by addition of N^α-Boc-Arg(Tos), N^α-Boc-Met, N^α-Boc-Thr(Bzl), and N^α-Boc-Asp(OBzl) (peptide resin E).

To the rest of the peptide resin A was coupled N^α-Boc-Cys-(4-Me-Bzl) (peptide resin F); this peptide resin was divided into three parts, and to two of them were coupled N^α-Boc-Arg(Tos), N^α-Boc-Gly, N^α-Boc-Val, N^α-Boc-Met, and N^α-Boc-Ala (peptide resin G). This resin was divided in two parts, and to one were coupled N^α-Boc-Arg(Tos), N^α-Boc-Met, N^α-Boc-Thr(Bzl), and N^α-Boc-Asp(OBzl) (peptide resin II). To the half of peptide resin B were coupled N^α-Boc-Gly and N^α-Boc-Cys(4-Me-Bzl) (peptide resin I). This resin was divided into three parts, and to two of them were coupled N^α-Boc-Met and N^α-Boc-Ala (peptide resin K).

Preparation of Cyclic Peptides. The appropriate peptide-resin (1 g) was mixed with 2 mL of anisole and 1 mL of ethanedithiol in the reaction vessel of HF cleavage line, and approximately 20 mL of liquid HF was distilled in it. After 1 h at 0 °C,

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Table II. Physical Characteristics of the MCH Analogues Synthesized

analogue	E_{His}[a] pH 2.4 pH 5.7	R_f S1 S4	HPLC[b] k'	amino acid analysis						[FAB-MS] found calcd
				Asp Cys	Thr Val	Glu Met	Pro Tyr	Gly Arg	Ala Trp	
1	0.67	0.02	4.37	1.02	0.94	1.08	0.84	1.05	1.05	2070.8
	0.31	0.15		1.71	1.82	1.78	1.07	2.04	0.67	2070.8
2	0.69	0.02	20.1	1.06	0.88	1.00	1.06		1.10	2113
	0.29	0.16		1.63	2.75	1.73	1.09	3.01	0.72	2112.8
3	0.70	0.02	4.95	1.13	1.00	1.08	0.86	1.05	1.08	2070.8
	0.29	0.16		1.83	1.86	1.75	1.04	3.04	0.96	2070.8
4	0.67	0.05	3.28			1.07	1.01	1.06	1.12	1567.6
	0.42	0.31		1.91	1.98	0.95	0.94	2.02	0.88	1567.6
5	0.68	0.10	6.18			1.09	0.94		1.13	1609.9
	0.41	0.36		1.65	2.66	0.87	1.06	1.99	0.89	1609.7
6	0.67	0.08	4.07			1.04	0.98	1.02	1.06	1567.6
	0.40	0.35		1.72	2.03	0.97	1.03	1.99	0.82	1567.6
7	0.57	0.14	2.77			1.03	0.93			1053
	0.15	0.35		1.79	0.89		1.00	1.06	0.86	1053.3
8	0.70	0.07	3.99			1.08	1.12			1308.6
	0.36	0.28		1.77	1.78		0.99	2.13	0.81	1308.5
9	0.71	0.07	3.49			0.99	0.86	1.04		1365
	0.35	0.29		1.74	2.03		1.02	1.88	0.83	1365.5

[a] Relative electrophoretic mobility on paper at a given pH. [b] 23% of acetonitrile in 0.1% aqueous trifluoroacetic acid.

the mixture was evaporated, the residue was washed with ethyl acetate (3 × 60 mL), and the peptide was extracted by 30% acetic acid (3 × 60 mL). After lyophilization, the residue was dissolved in degassed water (600 mL), the pH of the solution was brought to 8, and a 0.01 M solution of $K_3Fe(CN)_6$ (60 mL) was added dropwise during 30 min. The yellow solution was mixed for another 1 h, the pH was brought to 4 by addition of acetic acid, and Amberlite IRA-45 (Cl^- form) was added. After 30 min, the resin was filtered off, and the solution was lyophilized. The lyophilizate was dissolved in 0.01 M aqueous acetic acid and applied onto the column of CM cellulose (25 × 2 cm). Elution was performed with a gradient composed of 0.01 M acetic acid (300 mL) and 0.3 M ammonium acetate, pH 6.5 (300 mL). The major peak was pooled and injected in three portions onto a Vydac C_{18} column, 250 × 10 mm, equilibrated with 0.1% aqueous trifluoroacetic acid. Elution was performed with a gradient running from 1% to 18% acetonitrile in 2 min and from 18% to 30% acetonitrile in 40 min, with 0.1% aqueous trifluoroacetic acid as the aqueous layer. The major peak was lyophilized, and the product was dissolved in 3 M aqueous acetic acid and applied onto the column of Bio-Gel P-4 (100 × 1 cm); elution was performed with the same solvent. Efforts were made to avoid contamination with potent α-melanotropins by performing all purifications of MCH analogues on columns that had never been exposed to α-melanotropins. After lyophilization of what was generally a single symmetrical peak, the products were evaluated for purity by analytical HPLC, TLC, and electrophoresis; single symmetrical peaks were obtained. The characteristics of prepared compounds are given in Table II.

[Ala^5,Cys^{10}]MCH (1). The peptide resin H (1 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 21.2 mg (9%) of the title compound 1 was obtained in the form of a white powder. Analytical data of the product are given in Table II.

[Ala^5,Cys^8]MCH (2). The peptide resin E (1 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 9.8 mg (4.1%) of the title compound 2 was obtained in the form of white powder. Analytical data of the product are given in Table II.

[Ala^5,Cys^7]MCH (3). The peptide resin K (1.08 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 20.2 mg (7.8%) of the title compound 3 was obtained in the form of white powder. Analytical data of the product are given in Table II.

[Ala^5,Cys^{10}]MCH$_{5-17}$ (4). The peptide resin G (0.96 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 15.0 mg (5.2%) of the title compound 4 was obtained in the form of white powder. Analytical data of the product are given in Table II.

[Ala^5,Cys^8]MCH$_{5-17}$ (5). The peptide resin D (0.93 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 21.3 mg (7.7%) of the title compound 5 was obtained in the form of white powder. Analytical data of the product are given in the Table II.

[Ala^5,Cys^7]MCH$_{5-17}$ (6). The peptide resin J (0.46 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 9.06 mg (6.6%) of the title compound 6 was obtained in the form of white powder. Analytical data of the product are given in Table II.

[Cys^{10}]MCH$_{10-17}$ (7). The peptide resin F (0.49 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 12.2 mg (12%) of the title compound 7 was obtained in the form of white powder. Analytical data of the product are given in Table II.

[Cys^8]MCH$_{8-17}$ (8). The peptide resin C (0.57 g) (the preparation of which is given above) was subjected to the HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 13.6 mg (10.6%) of the title compound 8 was obtained in the form of white powder. Analytical data of the product are given in Table II.

[Cys^7]MCH$_{7-17}$ (9). The peptide resin I (0.45 g) (the preparation of which is given above) was subjected to HF cleavage (vide supra). After purification by ion-exchange chromatography, HPLC, gel filtration, and lyophilization, 8.1 mg (7.3%) of the title compound 9 was obtained in the form of white powder. Analytical data of the product are given in Table II.

Bioassays. The teleost fish, *Synbranchus marmoratus*, an eel obtained from the Pantanal (Big Swamp) of Brazil, was used. Skins were removed and prepared[17] as originally described for the frog[18]-skin bioassay and as detailed elsewhere.[19] In the

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fish-skin bioassay, skins become light in response to MCH due to melanosome aggregation within melanocytes; the skins can then be redarkened by the addition of MSH, which causes redispersion of melanosomes within melanocytes. Movement of melanosomes within melanocytes results in color changes that can be monitored by a Photovolt reflectometer. Changes in skin color (reflectance) are recorded as percent changes from the initial base (zero) value. The frog (*Rana pipiens*) skin bioassay was utilized as previously described.[18,19]

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Registry No. 1, 112794-06-6; **2**, 112794-07-7; **3**, 112794-08-8; 4, 112794-09-9; **5**, 112794-10-2; **6**, 112794-11-3; **7**, 112794-12-4; **8**, 112794-13-5; **9**, 112794-14-6; BOC-Glu(OBzl), 13574-13-5; BOC-Trp(For), 47355-10-2; BOC-Cys(4-Me-Bzl), 61925-77-7; BOC-Pro, 15761-39-4; BOC-Arg(Tos), 13836-37-8; BOC-Tyr(2,6-Cl_2-Bzl), 40298-71-3; BOC-Val, 13734-41-3; BOC-Met, 2488-15-5; BOC-Ala, 15761-38-3; BOC-Thr(Bzl), 15260-10-3; BOC-Asp(OBzl), 7536-58-5; BOC-Gly, 4530-20-5.

Nonsteroidal Antiandrogens. Synthesis and Structure–Activity Relationships of 3-Substituted Derivatives of 2-Hydroxypropionanilides

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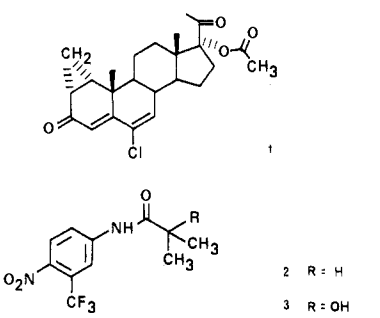
A series of 3-(substituted thio)-2-hydroxypropionanilides and some corresponding sulfones and sulfoxides of general structure **7**, in which R′ is methyl or trifluoromethyl, were prepared and tested for antiandrogen activity. Members of the trifluoromethyl series (**7**, R′ = CF_3) generally exhibited partial androgen agonist activity whereas the members of the methyl series (**7**, R′ = CH_3) were pure antagonists. Lead optimization in the methyl series has led to the discovery of novel, potent antiandrogens, which are peripherally selective. One of these, (*RS*)-4′-cyano-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methyl-3′-(trifluoromethyl)propionanilide, **40** (ICI 176334), is being developed currently for the treatment of androgen-responsive benign and malignant disease.

Cancer of the prostate is the second most common cause of death in American males with about 25 000 deaths from this condition being recorded in 1974.[1] The growth of the prostate tumors is stimulated by androgens, the male sex hormones. Since the pioneering work of Huggins and Hodges in 1941,[2] which showed the hormone dependence of this tumor, the mainstay of treatment for prostate cancer has been the withdrawal of androgens either by castration (orchidectomy) or estrogen therapy. Both of these therapies have disadvantages. Orchidectomy is unpopular with the patients and can result in psychological problems, and it exposes the elderly patients to the trauma of surgery. Estrogen therapy is effective in most patients but is accompanied by severe side effects including cardiovascular complications (cardiac failure, edema, and thromboembolism), painful gynecomastia, impotence, and loss of libido.[3] Both these approaches lead to the withdrawal of the androgens produced by the testes; however, the adrenal glands also produce androgens, so a more effective therapeutic agent would be a compound that prevented the natural androgens from interacting with their receptors, i.e., an antiandrogen.

There are two antiandrogens currently available commercially, cyproterone acetate (1) and the nonsteroidal anilide flutamide (**2**), whose active form in vivo is the hydroxylated metabolite, hydroxyflutamide (**3**). In addition to its antiandrogen activity, cyproterone acetate is also a potent progestin and inhibits gonadotrophin secretion.[4] It is effective in the treatment of prostate cancer but among its side effects may be listed loss of libido, gynecomastia, fluid retention, and thrombosis. Flutamide is a pure antiandrogen; that is, it does not exhibit other



hormonal activities. It is effective in the treatment of prostate cancer, the main side effect reported being gynecomastia.[5] One consequence of its pure antiandrogenic profile is that it prevents androgens from exerting their negative feedback mechanism on the hypothalamus, which results in an increased pituitary secretion of, interalia, luteinizing hormone (LH), which stimulates androgen production by the testes.[6] The antagonist, therefore, brings about the increased production of the natural agonist, which effectively diminishes its efficacy at the target organ. Our objective was to find a pure antiandrogen that was selective for the accessory sex organs, that is, had little or no effect on pituitary LH and consequently testosterone secretion. This paper describes our successful preparation of such a selective antiandrogen, which should have clinical advantages over existing antiandrogens in the treatment of prostate cancer.

Chemistry

Two general synthetic routes were used to prepare the anilides listed in Tables I–III. The route outlined in Scheme I was used for the preparation of anilides **7**. This involved coupling of an α-hydroxy acid chloride, prepared in situ by treating the α-hydroxy acid **5** with thionyl

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