Design of Potent Linear α-Melanotropin 4-10 Analogues Modified in Positions 5 and 10

Fahad Al-Obeidi,[†] Victor J. Hruby,[*,†] Ana M. de L. Castrucci,[‡,§] and Mac E. Hadley[‡]

Departments of Chemistry and Anatomy, University of Arizona, Tucson, Arizona 85721. Received April 25, 1988

α-Melanocyte stimulating hormone (α-MSH) is a linear tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) that has diverse physiological functions in addition to its reversible darkening of amphibian skins by stimulating melanosome dispersion within melanophores. On the basis of theoretical and experimental results from our laboratory and others, we have designed a group of 1-13, 4-13, and especially 4-10 analogues related to the superpotent analogue [Nle[4],D-Phe[7]]α-MSH in which the Glu[5] has been replaced with Asp[5], and the Gly[10] has been replaced with Lys[10] and other basic amino acid residues in the 4-10 analogues, and in which Gly[10] and Lys[11] were interchanged in the longer peptide analogues. In the 1-13 and 4-13 series the Lys[10], Gly[11] analogues generally retained superpotency for the D-Phe[7]-containing analogues. Most interestingly, synthesis of Ac-[Nle[4],Xxx[5],Yyy[7],Zzz[10]]α-MSH_{4-10}-NH_2 analogues where Xxx = Asp or Glu, Yyy = Phe or D-Phe, and Zzz = basic amino acids (Lys, Orn, α,γ-diaminobutyric acid (Dab), and α,β-diaminopropionic acid (Dpr)) provided melanotropins with potencies up to 10 times that of the native hormone in stimulating frog (*Rana pipiens*) skin darkening and 8-50 times more potent than α-MSH in stimulating lizard (*Anolis carolinensis*) skin melanophores in vitro. To our knowledge, Ac-[Nle[4],Asp[5],D-Phe[7],Dab[10]]α-MSH_{4-10}-NH_2, the most potent analogue, is the most potent melanotropin obtained thus far for the *Anolis* assay system. These results provide new insights into the structural and conformational requirements for biological potency of α-MSH and the differential structural and conformational requirements of α-MSH and its analogues at two different types of pigment cell receptors.

The tridecapeptide α-melanotropin (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, α-MSH) is an important regulatory hormone that is distributed throughout the central and peripheral nervous system and exhibits a broad range of biological activities.[2-7] These physiological activities of α-MSH have been shown to be related to the primary structure as well as to the conformational properties of the peptide.[2] It has been shown that the sequence Met-Glu-His-Phe-Arg-Trp-Gly is the minimal sequence required to obtain full melanotropic activity in the classical peripheral bioassay.[7] The 4-10 analogue Ac-[Nle[4],D-Phe[7]]α-MSH_{4-10}-NH_2 has been synthesized and was found to be 1-10 times more potent than α-MSH in various bioassays.[2,8-12] In addition, it has been shown that the presence of Lys[11] in the [4-11]-analogue Ac-[Nle[4],D-Phe[7]]α-MSH_{4-11}-NH_2 resulted in a several-fold enhancement in biological potency.[2]

While the physiological role of α-MSH in regulating pigmentation in vertebrates was the first described and has been the most intensively investigated,[2,13] the involvement of α-MSH in melanoma cells has only recently been appreciated.[14,15] In fact, in terms of structure-function analysis it has been found that for α-MSH-related peptides, high potency in the lizard skin bioassay is more closely correlated with high tyrosinase stimulation in melanoma cells[2,15] than is potency in the classical frog skin bioassay system.

In order to further investigate structure-activity relationships of Ac-α-MSH_{4-10}-NH_2 fragments and to obtain analogues highly potent in mammalian systems, we have systematically investigated the structural requirements for melanotropic activity of a number of Ac-α-MSH_{4-10}-NH_2 analogues with emphasis on the effect of substitutions in positions 5 and 10. In this paper we report the design and synthesis of several Ac-α-MSH_{4-10}-NH_2 analogues; some of these fragment heptapeptide analogues display a potency much higher than the native tridecapeptide hormone in a number of bioassay systems.

* To whom reprint requests should be addressed at the Department of Chemistry.

† Department of Chemistry, University of Arizona.

‡ Department of Anatomy, University of Arizona.

§ Permanent address: Departamento de Fisiologia, Universidade de Sao Paulo CR 11176, Sao Paulo 05499, Brazil.

Results and Discussion

Previously, it has been suggested that the side-chain moieties of Glu[5] and Lys[11] in α-MSH may be involved in formation of a salt bridge that is important for interaction with the melanotropin receptor.[2] However, the results of experimental studies[2] and of theoretical calculations[16] suggest that this may not be the case. In order to further understand the conformational properties of α-MSH,

(1) symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of L variety unless otherwise stated. Other abbreviations include: α-MSH, α-melanotropin; ACTH, adrenal cortical stimulating hormone; Nle, norleucine; Dab, 2,4-diaminobutyric acid; Dpr, 2,3-diaminopropionic acid; 2-ClZ, 2-chlorobenzyloxycarbonyl; 2-BrZ, 2-bromobenzyloxycarboxyl; Bzl, benzyl; For, formyl; Tos, tosyl; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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Table I. Relative in Vitro Potencies of α-MSH Analogues in the Frog (*Rana pipiens*) and Lizard (*Anolis carolinensis*) Skin Bioassays

no.	peptide	potency relative to α-MSH[a]		residual activity[b]	
		frog skin	lizard skin	frog skin	lizard skin
I	α-MSH	1.0	1.0	p (−)	p (−)
II	Ac-[Nle⁴,D-Phe⁷,Lys¹⁰,Gly¹¹]α-MSH$_{1-13}$-NH_2	6.0	8.0	p (+)	p (−)
III	Ac-[Nle⁴,Asp⁵,D-Phe⁷,Lys¹⁰,Gly¹¹]α-MSH$_{1-13}$-NH_2	9.0	8.0	p (+)	p (−)
IV	Ac-[Nle⁴,D-Phe⁷,Lys¹⁰,Gly¹¹]α-MSH$_{4-13}$-NH_2	0.8	8.0	p (+)	p (+)
V	Ac-[Nle⁴,Asp⁵,D-Phe⁷,Lys¹⁰,Gly¹¹]α-MSH$_{4-13}$-NH_2	1.0	10.0	p (+)	p (+)
VI	Ac-[Nle⁴,Asp⁵,D-Phe⁷]α-MSH$_{4-10}$-NH_2	0.1	8.0	p (−)	p (−)
VII	Ac-[Nle⁴,D-Phe⁷,Lys¹⁰]α-MSH$_{4-10}$-NH_2	0.2	8.0	p (−)	p (−)
VIII	Ac-[Nle⁴,Asp⁵,D-Phe⁷,Lys¹⁰]α-MSH$_{4-10}$-NH_2	0.7	8.0	p (−)	p (−)
IX	Ac-[Nle⁴,D-Phe⁷,Orn¹⁰]α-MSH$_{4-10}$-NH_2	0.6	8.0	p (−)	p (−)
X	Ac-[Nle⁴,Asp⁵,D-Phe⁷,Orn¹⁰]α-MSH$_{4-10}$-NH_2	0.9	10.0	p (−)	p (−)
XI	Ac-[Nle⁴,D-Phe⁷,Dab¹⁰]α-MSH$_{4-10}$-NH_2	0.9	10.0	p (+−)	p (+−)
XII	Ac-[Nle⁴,Asp⁵,D-Phe⁷,Dab¹⁰]α-MSH$_{4-10}$-NH_2	0.9	50.0	p (−)	p (−)
XIII	Ac-[Nle⁴,Asp⁵,D-Phe⁷,Dpr¹⁰]α-MSH$_{4-10}$-NH_2	0.2	8.0	p (−)	p (−)
XIV	Ac-[Nle⁴,Lys¹⁰]α-MSH$_{4-10}$-NH_2	0.001	0.08	p (+)	p (−)
XV	Ac-[Nle⁴,Asp⁵,Lys¹⁰]α-MSH$_{4-10}$-NH_2	0.004	0.08	p (+)	p (−)

[a] Values derived from parallel dose–response curves. [b] A prolonged response that continues for more than 4 h following washout of the hormone analogue from the bathing fluid.

Table II. Physicochemical Properties of α-MSH Fragment Analogues

no.	TLC R_f values in various solvent systems					HPLC K'[a]	$[\alpha]^{23}_{546}$ in 10% HOAc
	A	B	C	D	E		
II	0.34	0.62	0.0	0.0	0.39	1.86	−51.6° (*c* 0.31)
III	0.32	0.63	0.0	0.0	0.38	1.82	−52.3° (*c* 0.33)
IV	0.43	0.67	0.0	0.0	0.42	3.05	−49.14° (*c* 0.35)
V	0.41	0.69	0.0	0.0	0.40	2.98	−50.0° (*c* 0.32)
VI	0.01	0.76	0.74	0.29	0.62	3.32	−44.8° (*c* 0.25)
VII	0.35	0.39	0.01	0.05	ND	2.56	−50.0° (*c* 0.12)
VIII	0.36	0.40	0.0	0.07	ND	2.25	−28.0° (*c* 0.05)
IX	0.37	0.40	0.0	0.08	ND	2.24	−12.0° (*c* 0.04)
X	0.34	0.41	0.0	0.07	ND	2.14	−20.0° (*c* 0.03)
XI	0.44	0.65	0.03	0.0	ND	2.12	−30.0° (*c* 0.05)
XII	0.43	0.64	0.03	0.0	ND	2.21	−32.7° (*c* 0.11)
XIII	0.41	0.63	0.02	0.0	ND	2.18	−53.7° (*c* 0.03)
XIV	0.34	0.38	0.0	0.1	0.0	2.20	−25.0° (*c* 0.02)
XV	0.32	0.39	0.08	0.08	0.0	2.17	−24.6° (*c* 0.16)

[a] HPLC K' = (retention time − solvent front/solvent front) − solvent system was 15% acetonitrile in 0.1% trifluoroacetic acid; isocratic for 5 min, then gradient to 45% acetonitrile over 15 min. Then 5 min to 90% acetonitrile and additional 5 min to go to 15% acetonitrile and equilibration for 5 min.

molecular mechanics and molecular dynamics calculations have been carried out on α-MSH[17] at 300 K with a bulk dielectric constant of 80. No explicit solvent molecules were included. These studies indicated that the formation of a salt bridge between Glu⁵ and Lys¹¹ side chain groups has a low probability. However, on the basis of these molecular dynamics studies, we recently have found[17] under the same conditions that the replacement of Gly¹⁰ by a Lys¹⁰ residue brought the Glu⁵ and Lys¹⁰ side chains in close proximity, such that their interactions were predominant during the entire period of a 20-ps molecular dynamics simulation. On the basis of these results and the observation that the presence of Lys¹¹ in Ac-[Nle⁴,D-Phe⁷]α-MSH$_{4-11}$-NH_2 had a profound effect on the biological potency of these fragment analogues, a new series of peptides with a basic amino acid residue in position 10 was designed and synthesized and their biological activities investigated. We report here the results of these studies.

The Ac-α-MSH$_{4-10}$-NH_2 analogues listed in Table I were synthesized by the solid-phase peptide method[18-21] using a *p*-methylbenzhydrylamine resin as the solid support, and the products were purified by methods similar to those used previously for melanotropin analogues.[22,23] The

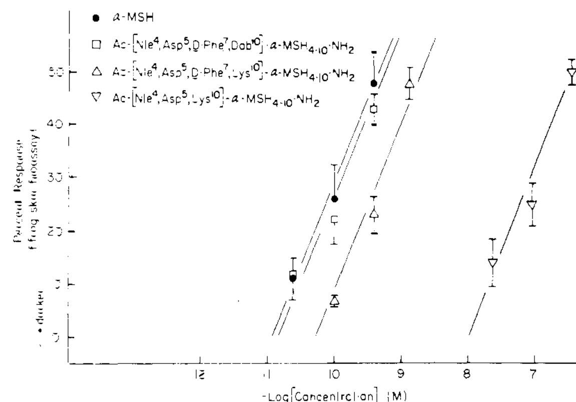

Figure 1. Comparative in vitro dose–response curves of a number of linear Ac-[Nle⁴,Asp⁵,Xxx⁷,Yyy¹⁰]α-MSH$_{4-10}$-NH_2 determined by the frog (*Rana pipiens*) skin bioassay. Each value represents the mean (±SE) darkening response of skins (N = 6 or more in all experiments) to the melanotropins at the concentrations noted. Xxx = L-Phe or D-Phe; Yyy = Lys or Dab.

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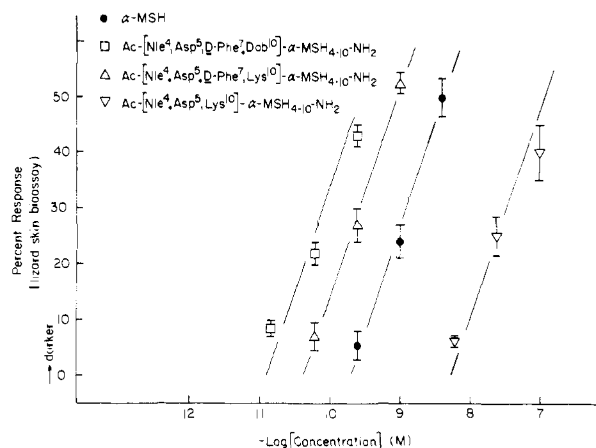

Figure 2. As in Figure 1, for the lizard (*Anolis carolinensis*) skin bioassay.

details of the synthetic methods are given in the Experimental Section, and the analytical results are given in Table II. The in vitro biological activities of the peptides were determined in both the frog (*Rana pipiens*)[8,9] and lizard (*Anolis carolinensis*)[10-12] skin assays over the full range of the log dose–response curves for the peptides. Representative dose–response curves for some analogues are shown in Figures 1 and 2, and the relative potencies for both assays are summarized in Table I. Previously, it was reported that the addition of Lys^{11} to Ac-[Nle^4,D-Phe^7]α-MSH_{4-10}-NH_2 resulted in an analogue with about one-tenth the potency of the native hormone α-MSH in frog skin bioassay and about 8 times the potency of the native hormone in the lizard skin assay.[23] Also, it has been shown that replacement of Glu in position 5 with either Gln or Gly amino acid residues resulted in equipotent or slightly less potent MSH analogues.[2,24] Replacement of Gly in position 10 by β-alanine reduces the potency of the peptide.[24]

In this study we have found that replacement of the Glu in position 5 with the shorter side chain amino acid aspartic acid leads to a slight enhancement in the biological potency, as can be seen by comparison of peptides II, VII, and IX with peptides III, VIII, and X in Table I. In addition, replacing the Gly in position 10 with the basic amino acids Lys, Orn, Dab, or Dpr gave analogues with enhanced potency in comparison to the native hormone, α-MSH, or the fragment analogue Ac-[Nle^4,D-Phe^7]α-MSH_{4-10}-NH_2 (see Table I). These results may be attributed to the potentiating effect of shorter side chain basic amino acids in position 10. Compounds II–V were designed and synthesized to examine all of the combinations in this series and to study the full effect of exchange of amino acids in position 5, 10, and 11. Though previous studies had suggested that the Gly^{10} residue is primarily important as a spacer amino acid,[2,24] potencies of these four compounds clearly demonstrate that this residue can play an important role in conjunction with residue 5 in hormone–receptor interaction, since highly potent analogues with up to 9 times the potency of the native α-MSH had resulted as in compound III. In addition to the enhanced potency, some of these analogues also showed prolonged activity in the frog skin and lizard skin bioassay systems. This is effected by the first three N-terminal amino acids in the lizard skin bioassay, as in compounds II–V (Table I), and apparently is related to the hydrophilic side chains in these three amino acids (Ser-Tyr-Ser) since II and III are not prolonged but IV and V are. This affect was not noticed in previous studies.[2]

In previous work[2] it was found that Ac-[Nle^4,D-Phe^7]α-MSH_{4-10}-NH_2 had prolonged biological activity on the lizard but not on the frog skin assay system. This property of prolongation on one system but not on the other is interesting, because it has been shown that the response in lizard skin bioassay goes in parallel with the response in the mammalian melanoma adenylate cyclase system.[2,23] The latter two systems appear to have quite similar structural requirements for hormone–receptor binding and signal transduction, on the basis of our results and those of others.[2,23]

Substitution of Gly^{10} with Lys^{10} in the heptapeptide VI resulted in an analogue with one-tenth the potency of α-MSH in the frog skin assay; however, the analogue was 8 times more potent in the lizard skin assay. There was no prolongation in either bioassay system. This pattern of reactivity was retained in the series of compounds VII–XIII (Table I), which clearly demonstrates the enhanced potency that results from substitution of Glu^5 with Asp^5 and Lys^{10} with the shorter side chain basic amino acids ornithine, 2,4-diaminobutyric acid (Dab), and 2,3-diaminopropionic acid (Dpr). The effect of the length of the side chain of the basic amino acids on biological activity is clearly seen in going from Lys (4 CH_2) to Dpr (1 CH_2) with the maximum potency at compound XII (2 CH_2) where we have potentiation of both the effect of Asp at the 5-position and that of Dab in position 10. Compound XII was equipotent to α-MSH in the frog assay system, but was 50 times more potent than α-MSH in the lizard skin assay system. This is the most potent α-melanotropin in the lizard skin assay reported thus far.

The large changes in biological potency that accompany the above substitutions cannot be explained only on the basis of the lipophilicity–hydrophilicity of these analogues, since all of the analogues have the same residual charge through differing number of methylene groups. Rather our design suggests that the high potency for XII may be due to topographical relationships of the side-chain groups in positions 5 and 10 in such a way that a strong ionic interaction occurs between the basic side-chain group of Dab^{10} (NH_2) and the acidic side-chain group of Asp^5 (COOH). The ionic interaction in this particular analogue may maximally stabilize the molecule in its proper topographical conformation for interaction with the receptor. Modifications in position 7 in which D-Phe^7 replaced the native L-Phe^7 gave analogues with increased potency in agreement with the previously reported effect of this substitution on the potency of α-MSH and most of its analogues.[2,23,25] As a result, compounds XIV and XV (Table I) were less potent than their D-Phe^7 analogues VII and VIII, respectively, with a noticeable difference of prolongation in the frog skin assay. These results confirmed what has previously been reported from our laboratory; that is, the substitution of Phe^7 by its D enantiomer yields the most potent analogues of α-MSH.[2,23,25-27]

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In conclusion, this systematic structure–function investigation provides the first extensive examination for the stereostructural requirements of positions 5 and especially 10 on the melanotropic potency of the primary active site of α-MSH, Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}. The potency range for the active-site heptapeptide analogues of α-MSH examined here was 4 orders of magnitude. Structural substitution of Glu^5 by Asp^5 and Gly^{10} by Dab^{10} in Ac-[Nle^4,D-Phe^7]α-MSH_{4-10}-NH_2 to give Ac-[Nle^4,Asp^5,D-Phe^7,Dab^{10}]α-MSH_{4-10}-NH_2 resulted in an analogue with markedly increased ($\geq$45-fold) melanotropic potency. Interestingly, substitution of Gly-10 by basic amino acids (Lys, Orn, Dab, Dpr) resulted in more potent analogues in both the lizard and frog skin bioassay systems. These results are very important for the future potential of these analogues as drug carriers to target melanoma cells. Conjugation of drugs active against melanoma cells using these acidic or basic side chains may prove very important for medicinal use of α-MSH and its analogues.[2,28-30]

Experimental Section

General Methods. Amino acid analysis was performed on a Beckman Model 120C amino acid analyzer after acid hydrolysis in sealed vials, with either 4 M methanesulfonic acid or 6 M HCl/propionic acid containing 0.5% phenol, at 110 °C for 22 h in vacuo. The amino acid analysis for all new analogues gave results within ±10% of theoretical values without correction for degradation of amino acids during peptide hydrolysis. Ascending TLC was performed on Baker 250-nm analytical silica gel glass plates in the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4 v/v), (B) 2-propanol/25% aqueous ammonia/water (3:1:1 v/v), (C) ethyl acetate/pyridine/acetic acid/water (5:5:1:3 v/v), (D) 1-butanol/acetic acid/water (4:1:5 v/v, upper phase). Peptides were visualized by ninhydrin, fluorescamine, and iodine vapor. Optical rotations were performed at the mercury green line (546 nm) with a Rudolph Research Polarimeter (Autopol). (Carboxymethyl)cellulose (CMC) cation-exchange fine-mesh resin with 0.72 mequiv/g capacity was purchased from Sigma Chemical Co., St. Louis, MO. HPLC was performed on Perkin-Elmer Series 313 chromatograph, equipped with a Model LCI 100 laboratory computing integrator using a Vydac 218TP15-16 C_{18}RP column (25 cm × 4.6 mm for analytical and 25 × 2.5 cm for preparative purposes), with a 0.1% trifluoroacetic acid/CH_3CN gradient (30:20 to 50:50) over 20 min and a flow rate of 3 mL/min. Peaks were monitored at 223 and 280 nm. HPLC solvents (Burdick and Jackson) were purchased from American Scientific Products (Phoenix, AZ). In the solid-phase synthesis, N^α-Boc-protected amino acids were N^α-deprotected with 48% TFA in methylene chloride containing 2% anisole for 5 and 20 min each. After completion of coupling of all amino acids, the N-terminal amino group of each peptide was acetylated with a 6-fold excess of N-acetylimidazole or a 2-fold excess of 1:1 mixture of acetic anhydride/pyridine in methylene chloride. The protected peptides were deprotected and removed from the resin with anhydrous liquid HF (10 mL/g of peptide resin) containing 10% anisole and 6–10% 1,2-dimercaptoethane at 0 °C for 30–60 min as previously developed by Matsueda.[31] UV spectroscopy was used to determine that the formyl group was completely removed under these conditions. In addition, the HPLC conditions used could distinguish the peptides that had a formyl group from the desired compounds. After removal of the HF in vacuo, the peptides with the resin were washed with ether or ethyl acetate (3 × 30 mL) and the free peptide extracted with 30% aqueous acetic acid and lyophilized.

***p*-Methylbenzhydrylamine Resin.** The synthetic resin was prepared and purified according to the procedure previously reported.[21,23] All syntheses were performed on a semiautomatic peptide synthesizer of our own design[20] or on Vega Coupler Model 1000 or 250 Peptide Synthesizers (Vega Biochemicals, Tucson, AZ).

Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Lys-Gly-Pro-Val-NH_2 (II). The title compound was prepared by coupling N^α-Boc-Val to a *p*-methylbenzhydrylamine resin (2.0 g of pMBHA resin, 0.7 mmol of NH_2/g of resin) with a 3-fold excess of amino acid and by standard methods of peptide synthesis in adding additional amino acid residues. After 90 min the resin was washed with dichloromethane and neutralized, and the residual amino group was acetylated with the acetic anhydride/pyridine mixture. No reactive amino groups on the resin were detected by the ninhydrin test[32] after 30 min. A cycle for coupling of each amino acid residue into the growing peptide chain consisted of the following: (1) washing with four 30-mL portions of CH_2Cl_2, 2 min/wash; (2) cleavage of Boc group by 30 mL of 48% trifluoroacetic acid in dichloromethane containing 2% anisole, one treatment for 5 min, and a second for 20 min; (3) washing with four 30-mL portions of dichloromethane, 2 min/wash; (4) neutralization by addition of two 30-mL portions of 10% diisopropylethylamine in dichloromethane and shaking for 2 min each; (5) washing with four 30-mL portions of dichloromethane, 2 min/wash; (6) addition of 3-fold excess of the N^α-Boc amino acid derivative (in this case 4.2 mmol) in 10 mL in dichloromethane, 4.2 mL of *N*-hydroxybenzotriazole (1 mmol/mL) in DMF (except in the case of N^α-Boc-N^{im}-Tos-His),[33] followed by 4.2 mL of a 1 mmol/mL solution of dicyclohexylcarbodiimide (DCC) in DMF. The mixture was shaken for 2–3 h (in case of the Trp, Arg, and His amino acid residues, DMF was used as the coupling solvent); (7) after completion of the coupling (ninhydrin negative test), the resin was washed with three 30-mL portions of dichloromethane, 2 min/wash; (8) washing with three 30-mL portions of 100% ethanol, 2 min/wash; (9) washing with four 30-mL portion of dichloromethane, 1 min/wash. The protected peptide resin corresponding to the title compound was obtained after stepwise coupling of the following N^α-Boc amino acid derivatives (in order of addition): N^α-Boc-Pro, N^α-Boc-Gly, N^α-Boc-Lys(N^ϵ-2-ClZ); N^α-Boc-Trp(N^i-For), N^α-Boc-Arg(N^g-Tos), N^α-Boc-D-Phe, N^α-Boc-His(N^{im}-Tos). The resulting Boc-His(N^{im}-Tos)-D-Phe-Arg-(N^g-Tos)-Trp(N^i-For)-Lys(N^ϵ-2-ClZ)-Gly-Pro-Val-*p*-MBHA resin was divided into four portions. One-quarter (1.0 g, ca. 0.35 mmol) of the protected peptide resin was converted to the protected title peptide resin after coupling N^α-Boc-Glu(γ-O-Bzl), N^α-Boc-Nle, N^α-Boc-Ser(O-Bzl), N^α-Boc-Tyr(O-2-BrZ), and N^α-Boc-Ser(O-Bzl). After coupling of the last amino acid, the N^α-Boc protecting group was removed, the amino group was neutralized, and the protected peptide was N^α-acetylated with a 10-fold excess of *N*-acetylimidazole in 20 mL of dichloromethane. The resulting protected peptide resin, Ac-Ser(O-Bzl)-Tyr(O-2-BrZ)-Ser(O-Bzl)-Nle-Glu(O-Bzl)-His(N^{im}-Tos)-D-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Lys(N^ϵ-2-ClZ)-Gly-Pro-Val-*p*-MBHA resin, was dried in vacuo. The protected peptide resin (1.0 g) was cleaved from the resin by liquid HF (vide supra). After evaporation of the volatile materials in vacuo at 0 °C, the dried product was washed with diethyl ether (3 × 30 mL), extracted with 30% aqueous HOAc (3 × 30 mL), and lyophilized. The peptide powder (about 530 mg) was divided into two portions (260 mg each) and one portion was dissolved in 1.5 mL of ammonium acetate buffer (pH 4.5) and filtered through a cartridge filter into the top of a CMC column (2.0 × 30.0 cm) and eluted with 250 mL each of 0.01 M (pH 4.5), 0.1 M (pH 6.8), and 0.2 M (pH 6.8) aqueous NH_4OAc. The major peak detected at 280 nm, which eluted during the end of the 0.1 M buffer and the first half of the 0.2 M NH_4OAc buffer, was pooled and lyophilized to give 142.3 mg of white powder. Then 80.0 mg of this peptide powder was purified on preparative HPLC, and the major peak was collected and lyophilized to give 63 mg

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of the title peptide. The analytical data for the purified compound are given in Table II and the biological activities are given in Table I.

Ac-Ser-Tyr-Ser-Nle-Asp-His-D-Phe-Arg-Trp-Lys-Gly-Pro-Val-NH$_2$ (III). The title compound was prepared from 1.0 g (ca. 0.35 mmol) of Boc-His(N^{im}-Tos)-D-Phe-Arg(N^g-Tos)-Trp-(N^i-For)-Lys-N^ϵ(2-ClZ)-Gly-Pro-Val-p-MBHA resin (prepared above), by stepwise coupling of N^α-Boc-Asp(β-O-Bzl), N^α-Boc-Nle, N^α-Boc-Ser(O-Bzl), N^α-Boc-Tyr(O-2-BrZ), and N^α-Boc-Ser(O-Bzl) to give Ac-Ser(O-Bzl)-Tyr(O-2-BrZ)-Ser(O-Bzl)-Nle-Asp(β-O-Bzl)-His(N^{im}-Tos)-D-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Lys(N^ϵ-2-ClZ)-Gly-Pro-Val-p-MBHA resin (1.8 g). A 1.0 g portion, after deprotection and purification as for II, gave the title peptide. The analytical data for this peptide are given in Table II, and a summary of the biological potencies is given in Table I.

Ac-Nle-Glu-His-D-Phe-Arg-Trp-Lys-Gly-Pro-Val-NH$_2$ (IV). From 1.0 g (ca. 0.35 mmol) of the Boc-His(N^{im}-Tos-D-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Lys(N^ϵ-2-ClZ)-Gly-Pro-Val-p-MBHA resin prepared above, the protected title peptide resin was synthesized by the solid-phase method by further stepwise coupling of N^α-Boc-Glu(γ-O-Bzl) and N^α-Boc-Nle. Each coupling reaction was achieved as previously reported for II, except that the acetylation of the N-terminus was achieved by a 2-fold excess of 1:1 acetic anhydride/pyridine in dichloromethane for 1 h. The peptide IV was obtained in purified form as outlined for compound III. The analytical data for the compound IV are given in Table II, and the biological activities are given in Table I.

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-Gly-Pro-Val-NH$_2$ (V). From 1.0 g (ca. 0.35 mmol) of Boc-His(N^{im}-Tos)-D-Phe-Arg-(N^g-Tos)-Trp(N^i-For)-Lys(N^ϵ-2-ClZ)-Gly-Pro-Val-p-MBHA resin prepared as in II, the title peptide was prepared by stepwise coupling of N^α-Boc-Asp(β-O-Bzl) and N^α-Boc-Nle. Workup and purification as used for compounds II and III gave compound V. The analytical data for V are given in Table II, and the biological activities are given in Table I.

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Gly-NH$_2$ (VI). To 1.0 g of p-methylbenzhydrylamine resin (0.7 mmol of NH$_2$/g resin) was coupled the following amino acid derivatives: N^α-Boc-Gly, N^α-Boc-Trp(N^i-For), N^α-Boc-Arg(N^g-Tos), N^α-Boc-D-Phe,N^α-Boc-His(N^{im}-Tos), N^α-Boc-Asp(β-O-Bzl), and N^α-Boc-Nle. Each coupling reaction was achieved with a 3-fold excess of N^α-Boc amino acid derivative, a 2.4-fold excess of DCC, and a 2.4-fold excess of HOBt following the same method as previously discussed (vida supra). After coupling of the last amino acid, the N^α-Boc protection group was removed, the amino group neutralized, and the N^α-amino group acetylated with a 2-fold excess of 1:1 mixture of acetic anhydride/pyridine in dichloromethane for 1 h. The finished protected peptide resin weighed 1.8 g. Following workup and purification as given for II, VI was obtained as a white powder. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Glu-His-D-Phe-Arg-Trp-Lys-NH$_2$ (VII). To a 2.7-g portion of p-methylbenzhydrylamine resin (0.7 mmol of NH$_2$/g of resin) were coupled the following amino acid derivatives (in order of their coupling): N^α-Boc-Lys(N^ϵ-2-ClZ), N^α-Boc-Trp-(N^i-For), N^α-Boc-Arg(N^g-Tos), N^α-Boc-D-Phe, N^α-Boc-His-(N^{im}-Tos). The resulting Boc-His(N^{im}-Tos)-D-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Lys(N^ϵ-2-ClZ)-p-MBHA resin was divided into two equal portions. Part of the resin was coupled stepwise with N^α-Boc-Glu(γ-O-Bzl) and N^α-Boc-Nle. This peptide resin was N^α-deprotected and the N-terminus acetylated with a 2-fold excess of a 1:1 mixture of acetic anhydride/pyridine in dichloromethane for 1 h (2.1 g). A 1.7-g amount of the protected peptide resin was cleaved by anhydrous liquid HF/anisole/dithioethane (17 mL of HF, 2 mL of anisole, 2 mL of dimercaptoethane). After evaporation of the volatile materials, the dried peptide resin mixture was washed with 3 × 30 mL of anhydrous diethyl ether and extracted with 3 × 30 mL of 30% aqueous HOAc. The aqueous extract of the peptide was lyophilized to give 700 mg of crude peptide. A 300-mg portion of the crude peptide was dissolved in 2 mL of 0.1 N aqueous NH$_4$OAc buffer (pH 4.5) and filtered through a cartridge filter on the top of CMC column as outlined under compound II. The major peak was collected and lyophilized to give 172 mg of the peptide as a white powder. Then, 110 mg of the crude peptide was purified on HPLC (as outlined for compound II) to give 58 mg of pure title peptide. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH$_2$ (VIII). The protected peptide resin to the title compound was prepared from 1.8 g of Boc-His(N^{im}-Tos)-D-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Lys-(N^ϵ-2-ClZ)-p-MBHA (prepared above) by stepwise coupling of N^α-Boc-Asp(β-O-Bzl) and N^α-Boc-Nle followed by acetylation as discussed for VII. Workup and purification was as described for VII. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Glu-His-D-Phe-Arg-Trp-Orn-NH$_2$ (IX). The protected peptide resin to the title compound was synthesized as reported for VII, except that N^α-Boc-Orn(N^δ-Z) was used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) in the coupling scheme. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Orn-NH$_2$ (X). The protected peptide resin to the title compound was synthesized as reported for IX, except that N^α-Boc-Asp(β-O-Bzl) was used instead of N^α-Boc-Glu(γ-O-Bzl) in the coupling scheme. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Glu-His-D-Phe-Arg-Trp-Dab-NH$_2$ (XI). The protected peptide resin to the title compound was synthesized as reported for VII, except that N^α-Boc-Dab(N^γ-Z) was used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) in the coupling scheme. The N^α-Boc-Dab(N^γ-Z) was synthesized as reported in the literature.[34,35] The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Dab-NH$_2$ (XII). The protected peptide resin to the title compound was synthesized as reported for VII, except that N^α-Boc-Dab(N^β-Z) and N^α-Boc-Asp(β-O-Bzl) were used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) and N^α-Boc-Glu(γ-O-Bzl), respectively, in the coupling scheme. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Asp-His-D-Phe-Arg-Trp-Dpr-NH$_2$ (XIII). The protected peptide resin to the title compound was synthesized as reported for VII, except that N^α-Boc-Dpr(N^β-Z) was used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) and N^α-Boc-Asp(β-O-Bzl) for N^α-Boc-Glu(γ-O-Bzl) in the coupling scheme. The N^α-Boc-Dpr(N^β-Z) was synthesized as reported in the literature.[34,35] The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Glu-His-Phe-Arg-Trp-Lys-NH$_2$ (XIV). The protected peptide resin to the title compound was synthesized as reported for VII, with the exception that N^α-Boc-Phe was used instead of N^α-Boc-D-Phe in the coupling scheme. The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Ac-Nle-Asp-His-Phe-Arg-Trp-Lys-NH$_2$ (XV). The protected peptide resin to the title compound was synthesized as reported for VII, with the exception that N^α-Boc-Phe and N^α-Boc-Asp(β-O-Bzl) instead of N^α-Boc-Glu(γ-O-Bzl). The analytical data for the compound are given in Table II, and the biological potencies are given in Table I.

Frog and Lizard Skin Bioassays. The frog (*R. pipiens*) and lizard (*A. carolinensis*) skin bioassays[8-12] were utilized to determine the relative potencies of the synthetic melanotropins. These assays measure the amount of light reflected from the surface of the skins in vitro. In response to melanotropic peptides, melanosomes within integumental melanophores migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar dispersion results in a change in color (darkening), which is measured by a Photovolt Reflectometer and is expressed as the percent response compared to the initial (time = 0) reflectance value. For α-MSH, subsequent removal of the melanotropin (by washing out the skin) results in a rapid perinuclear (centripetal) reaggregation of melanosomes, leading to a lightening of the skins back to their original (base) value. The presence of darkening after washing the skin indicated the pro-

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longation effect of the peptide. The frogs used in these studies were obtained from Lemberger Co., Germantown, WI, and the lizards were from the Snake Farm, La Place, LA.

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Registry No. II, 117499-47-5; III, 117499-48-6; IV, 117499-49-7; V, 117499-50-0; VI, 117499-52-2; VII, 117499-51-1; VIII, 117499-53-3; IX, 117499-54-4; X, 117499-55-5; XI, 117526-36-0; XII, 117499-56-6; XIII, 117499-57-7; XIV, 117603-86-8; XV, 117603-87-9; BOC-Val-OH, 13734-41-3; BOC-Pro-OH, 15761-39-4; BOC-Gly-OH, 4530-20-5; BOC-Lys(2-Clz)-OH, 54613-99-9; BOC-Trp-(For)-OH, 47355-10-2; BOC-Arg(Tos)-OH, 13836-37-8; BOC-D-Phe-OH, 18942-49-9; BOC-His(Tos)-OH, 35899-43-5; BOC-Glu-(OBzl)-OH, 13574-13-5; BOC-Nle-OH, 6404-28-0; BOC-Ser-(Bzl)-OH, 23680-31-1; BOC-Tyr(2-BrZ)-OH, 47689-67-8; BOC-Asp(OBzl)-OH, 7536-58-5; BOC-Orn(Z)-OH, 2480-93-5; BOC-Dab(Z)-OH, 3350-20-7; BOC-Dpr(Z)-OH, 65710-57-8; BOC-Phe-OH, 13734-34-4.

Synthesis and α_2-Adrenoceptor Antagonist Activity of Some Disulfonamidobenzoquinolizines

Terence J. Ward,*[†] Graham J. Warrellow,[†] John A. Stirrup,[†] Norman Lattimer,[‡] and Keith F. Rhodes[‡]

Departments of Chemistry and Biomedical Research, Wyeth Research (UK), Taplow, Maidenhead, England.
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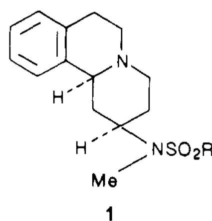
A series of disulfonamidobenzo[a]quinolizines were synthesized and evaluated for their α_2- and α_1-adrenoceptor antagonist activity on the rat vas deferens and anococcygeus muscle, respectively. N-((2β,11bα)-1,3,4,6,7,11b-Hexahydro-2H-benzo[a]quinolizin-2-yl)-N-[2-[(methylsulfonyl)amino]ethyl]methanesulfonamide (4) and its N-[2-[(methylsulfonyl)amino]ethyl]ethanesulfonamide (**22**), N-[2-[(ethylsulfonyl)amino]ethyl]ethanesulfonamide (**27**), and N-[2-[(methylsulfonyl)amino]ethyl]-4-methylbenzenesulfonamide (**30**) analogues showed 400-fold or greater selectivity in favor of α_2- over α_1-adrenoceptor blockade.

The therapeutic potential of agents which selectively block α_2-adrenoceptors has prompted the search for such agents in a number of laboratories, and selective agents from a variety of chemical classes have been reported in recent years.[1] In a previous publication we described the chemistry and biological activity of a series of 2-sulfonamidobenzoquinolizines of general structure **1** possessing selective α_2-adrenoceptor antagonist activity.[2]

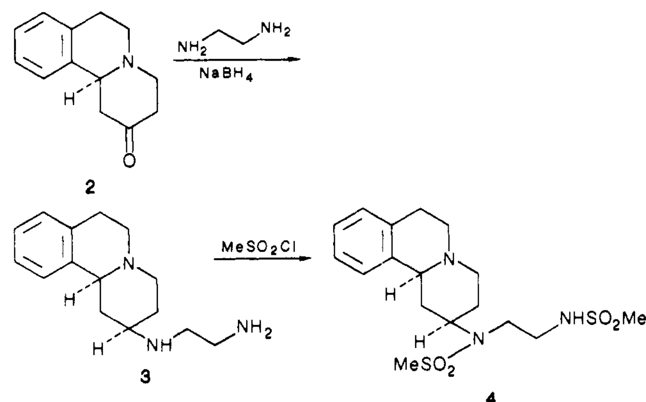


The importance of the N-methyl substituent for activity in this series, observed in our previous study, prompted us to investigate further modifications at this site in detail and led to the discovery of further analogues having enhanced selectivity in favor of the α_2-adrenoceptor. These new analogues differ from our earlier series in that they bear a second sulfonamide group on the nitrogen-linked side chain.

Chemistry

Reductive amination of the 2-oxohexahydrobenzoquinolizine (**2**) with ethylenediamine gave the key intermediate **3** (Scheme I).[3] Interestingly, reductive amination of **2** with ethylenediamine, or its homologues, did not require the use of sodium cyanoborohydride[4] as generally employed for reductive aminations, but was readily achieved by simple treatment of the ketone with ethylenediamine and sodium borohydride in ethanol. Symmetrical disulfonamide derivatives of **3** were prepared by treatment of **3** with slightly over 2 equiv of a sulfonyl chloride. The primary and secondary amine centers

Scheme I



present in **3** differ sufficiently in their reactivity to allow their differential sulfonation (Scheme II). Accordingly, although reaction of **3** with 1 equiv of methanesulfonyl chloride gave an intractable mixture of mono- and disulfonamides, the use of the more sterically demanding reagent methanesulfonic anhydride gave monosulfonamide **5**. Selective sulfonation was also achieved with the more bulky ethane-, propane-, and benzenesulfonyl chlorides. Intermediates monosulfonated on the secondary amine function of **3** were prepared following protection of the primary amine group. Accordingly, **3** was reacted with methyl acetate to form monoacetamide **6**, which was then sulfonated and deacetylated to yield monosulfonamide **7**. Monosulfonamides derived from **3** enabled the synthesis of unsymmetrical disulfonamides by reaction with a second equivalent of a sulfonyl chloride. Intermediate amines

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[†] Department of Chemistry.
[‡] Department of Biomedical Research.

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