Synthesis and Biological Activity of New 2-Substituted Analogs of Fluphenazine

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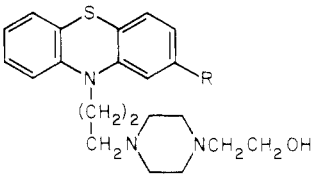
A series of 2-substituted analogs of fluphenazine, namely the nitro, cyano, dicyanoethenyl, and nitroethenyl compounds, was synthesized and tested as potential neuroleptics.

Based on a literature review of the pharmacological effectiveness of phenothiazines, it was theorized that neuroleptic activity could be greatly enhanced by replacing the trifluoromethyl substituent of fluphenazine (**1a**) with a substituent possessing greater electron-withdrawing properties either by inductive or resonance interaction.[1] A 2-substituent with greater electron-attracting properties would decrease the availability of electrons on the sulfur atom of the ring for sulfoxide formation, since the 2-substituent is para to the sulfur atom in the 5 position. Sulfoxide formation drastically reduces neuroleptic potency,[2] which may be due to an increase in water solubility.[1]



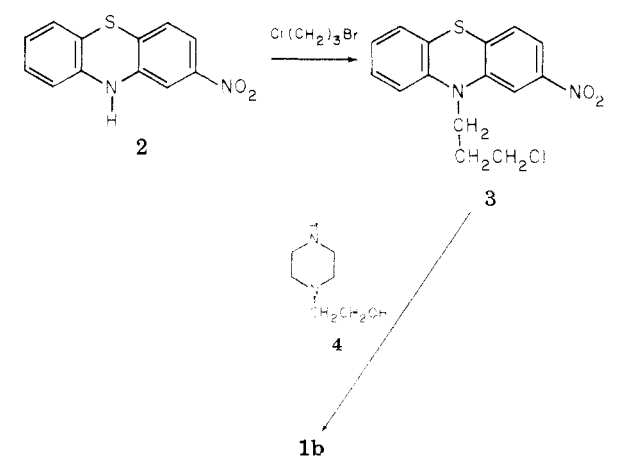
1a, R = $-CF_3$ (fluphenazine)
b, R = $-NO_2$
c, R = $-CH{=}C(CN)_2$
d, R = $-CH{=}CHNO_2$
e, R = $-CN$

Accordingly, a series of 2-substituted analogs of **1a** were prepared for biological evaluation. This series consisted of the following new compounds: 2-nitro-10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine (**1b**), 2-(2,2-dicyanoethenyl)-10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine (**1c**), and 2-(2-nitroethenyl)-10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine (**1d**). In addition, 10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine-2-carbonitrile (**1e**) was prepared following the method of Benko et al.[3] (The authors thank the Industrial Liason Office of the Chemical Laboratory for small quantities of **1e** for initial studies.)

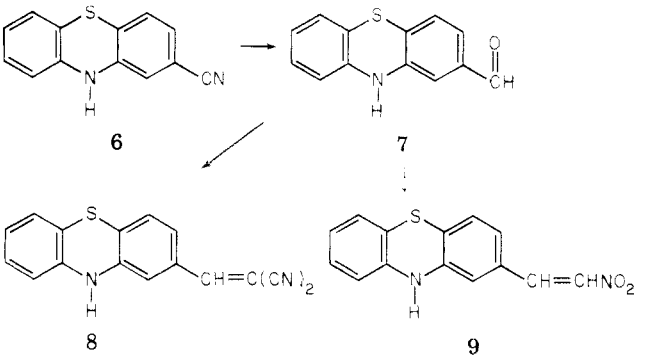
Chemistry. Compound **1b** was prepared in two synthetic steps (Scheme I). The first step consisted of alkylating 2-nitrophenothiazine[4] (**2**) with 1-bromo-3-chloropropane to produce 10-(3-chloropropyl)-2-nitrophenothiazine (**3**), alkylation being accomplished by first converting **2** to the anionic species using the strong base, sodamide. Following the procedure of Yale and Sowinski,[5] **3** was treated with 1-(2-hydroxyethyl)piperazine (**4**) in refluxing 2-butanone, in the presence of sodium iodide, to yield **1b**, which was then characterized as the dimaleate salt.

The most direct method to prepare **1c** and **1d** was to synthesize the common precursor 10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine-2-carboxaldehyde (**5**), followed by a Knoevenagel-type condensation with malononitrile and nitromethane, respectively. Following the facile one-step nitrile-to-aldehyde reduction procedure of van Es and Staskun,[6] phenothiazine-2-carbonitrile[7] (**6**) was converted to phenothiazine-2-carboxaldehyde (**7**) in a refluxing Raney alloy–aqueous formic acid mixture (Scheme II). In addition, a sample of **7** was prepared from phenothiazine 2-carboxyhydrazide by the procedure of McFadyen–Stevens[8] as modified by Fattorusso.[9] The samples prepared by the two different methods were identical. In the same manner, the conversion of **1e** appropriately yielded **5** (Scheme III). The Knoevenagel condensation of **7** with malononitrile and with nitromethane gave 1,1-dicyano-2-(2-phenothiazinyl)ethene (**8**) and 1-nitro-2-(2-phenothiazinyl)ethene (**9**), respectively. Piperidine was used as the catalyst in the preparation of **8**. The procedure of Burger and Schmalz[10] satisfactorily produced the β-nitrovinyl derivative **9**.

Scheme I



Scheme II



Scheme III

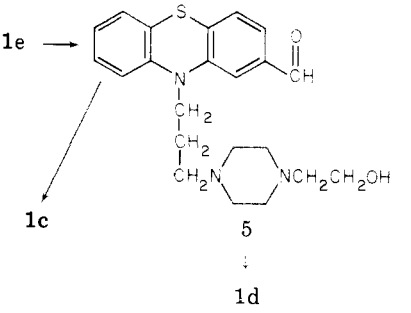


Table I. Cataleptogenic Potency Data of Phenothiazines in Male Albino Mice

No.	Form	CD_{50}, μmol/kg	Mean rel potency[a,d]	No. of determinations	Compd significantly exceeded one-sided t test, $p < 0.05$
1a (standard)	Dihydrochloride	1.6 (28)[b] 0.29 (10)[b] 0.62 (14)[b]	1.0		1c, 1d
1b	Dimaleate	2.2 2.3	0.92	2	1c, 1d
1c	Dimaleate	28	0.078	1	Indeterminate
1d	Dimaleate[c]	>57	<0.011	1	
1e	Dihydrochloride	0.99 0.41	0.70	2	1c, 1d

[a] Relative potencies are based on fluphenazine as 1.0; dosages are in terms of free base. [b] Mean values (number of determinations) for each of three different observers (*not* the value obtained in each bioassay and used to estimate relative potency). [c] 1d injected in PEG 200. [d] Standard error (common log) of a single determination of relative potency = 0.3993 with 29 degrees of freedom ($N = 48$, including the determinations of **1b** and **1e** above).

Table II. Cataleptogenic Potency Data of Phenothiazines in Male Albino Rats

No.	Form	Mean CD_{50},[a] μmol/kg	Mean rel potency[b,d]	No. of determinations	Compd significantly exceeded one-sided t test, $p < 0.05$
1a	Dihydrochloride	0.39[c]	1.0		None
1b	Dimaleate	0.17	3.2	3	1a, 1e
1e	Dihydrochloride	0.61	0.65	1	

[a] CD_{50} dosage to induce 50% of the maximum possible catalepsy score (9 points/18 points maximum) in a group of six rats. [b] Relative potencies are compared to fluphenazine as 1.0; dosages are in terms of free base. [c] Mean value of 52 determinations (*not* the value obtained in each bioassay and used to estimate relative potency). [d] Standard error (common log) of a single determination of relative potency = 0.2908 with 32 degrees of freedom ($N = 51$, including the determinations on **1b** above).

Following the same procedure used in the preparation of the 10-unsubstituted β,β-dicyanoethylene (8), **5** gave **1c**. The condensation between nitromethane and **5**, following the procedure of Burger and Schmalz,[10] produced the β-nitrovinyl derivative but only as the acetyl ester. However, the procedure of Ash and Wragg,[11] utilizing benzylamine as the catalyst in refluxing nitromethane, effectively yielded **1d**. In each case, the piperazine-substituted phenothiazines were characterized as their dimaleate salts.

Pharmacology. All compounds were initially tested for neuroleptic potency in mice using catalepsy as the end point. The most promising compounds were further studied using the more sensitive rat catalepsy test. Both procedures are based on the inherent dislike of untreated four-legged animals to placement in a sitting position with forelegs elevated and measures immobility without loss of the ability to move. The results of the rat test reportedly correlate well with neuroleptic potency in man.[12] Bioassays in both mice and rats were conducted using fluphenazine as the reference standard.

Probit analysis[13] was applied to the assay data to obtain dosage estimates to use in calculating relative potencies. Three observers carried out the mouse assays while only one did those on rats. In the mouse tests the median catalepsy doses, CD_{50}'s, of different observers were significantly different ($p < 0.05$). However, relative potency estimates were not significantly different. Accordingly, for the mouse tests, it appeared valid to average only the relative potency values (not CD_{50}'s) from different observers.

Small regression coefficient (slope) values and large variation in animal responsiveness most often made error estimates in individual assays indeterminate. An error estimate for relative potency values was determined from validly pooled (Bartlett's test[14]) estimates of variance obtained from assay repetitions, most of which were done on commercially available compounds and are not herein reported.

To compare the members of a series of cataleptogens, their relative potencies were listed in descending order of magnitude and a one-sided t test[15] was applied to assess statistical significance of differences between each relative potency value and each of those below it in the list.

Discussion

The results of the mouse test are given in Table I.

Compounds **1c** and **1d**, each possessing the ethenyl moiety between the phenothiazine tricyclic system and the strongly electron-withdrawing CN and NO_2 groups, respectively, were practically inactive. This may possibly be due to steric rather than electronic factors of the vinyl system.

Since compounds **1b** and **1e** showed much greater potency than **1c** and **1d** in mice, they were subjected to a more detailed study in rats. The results of the rat test are given in Table II.

The results shown in Table II indicate that the biological activity may not be solely related to the electron-withdrawing properties of the 2-substituent. Of the compounds tested in rats, nitro-substituted **1b**, possessing the most strongly electron-attracting 2-substituent, was over three times more potent than fluphenazine (**1a**). However, cyano-substituted **1e**, possessing a 2-substituent which has electron-attracting properties intermediate between that of the nitro and the trifluoromethyl moiety, was the least potent of the three compounds tested. Other parameters appear to be important. Along with the σ value it is felt that differential lipid–water solubility is probably a very important factor in the potencies observed, especially when using intramuscular injections.

Experimental Section

The ir spectra were recorded on a Perkin-Elmer Infracord Model 237B. The NMR spectra were obtained on a Varian A-60 spectrometer. Mass spectra were obtained on a Hitachi Perkin-Elmer RMU-6E spectrometer. All spectral data observed were in agreement with the assigned structures.

Melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected.

Elemental analyses were performed in the Micro-Analytical Section, Analytical Chemistry Branch, Chemical Laboratory.

10-(3-Chloropropyl)-2-nitrophenothiazine (3). To a dark red solution of 5.0 g (0.020 mol) of **2** in 50 ml of dry DMF (distilled from CaH) was added 1.3 g (0.033 mol) of sodamide suspended in 20 ml of dry DMF. The resulting dark green solution, containing the sodium salt of **2**, was added dropwise over 2 h to a solution of 6.3 g (0.040 mol) of 1-bromo-3-chloropropane in 70 ml of dry DMF, which was kept under a dry N_2 atmosphere. The resulting red mixture was stirred at room temperature overnight. The reaction mixture was filtered to remove inorganic salts, and the filtrate was concentrated to ~25 ml under reduced pressure. The concentrate was diluted with 800 ml of H_2O and then extracted with several portions of $CHCl_3$. The combined $CHCl_3$ extracts were dried over Drierite, filtered, and concentrated to give ~5 g of dark red tar. This material was chromatographed on neutral alumina, elution being achieved with 50–75% dichloromethane in petroleum ether (bp 30–60°), to yield, upon trituration with petroleum ether, 3.7 g (56% yield) of dark red solid, mp 82–86°. An analytical sample, recrystallized from *i*-PrOH, melted at 86.5–87.5°. Anal. ($C_{15}H_{13}ClN_2O_2S$) C, H, Cl.

2-Nitro-10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine Dimaleate (1b). A stirred mixture of 3.2 g (0.010 mol) of **3** and 2.6 g (0.020 mol) of **4** dissolved in 25 ml of dry 2-butanone and 1.5 g (0.010 mol) of NaI was refluxed overnight. The solvent was removed under reduced pressure, and the resulting residue was digested with 125 ml of 3 N HCl. The acid digest was extracted with $CHCl_3$ (4 × 30 ml) to remove starting material. The combined $CHCl_3$ extracts were washed with 3 N HCl (40 ml). The acid wash was added to the original acid solution, which was then made basic with concentrated NaOH. The resulting basic solution was extracted with $CHCl_3$ (5 × 30 ml). The combined $CHCl_3$ extracts were washed with H_2O, dried over Drierite, filtered, and concentrated to give 3.5 g (85% yield) of crude product (**1b**) as a red viscous oil. This oil was taken up in 50 ml of Me_2CO and added to a stirred solution of 2.5 g (0.022 mol) of maleic acid in 25 ml of EtOH and 50 ml of Me_2CO. The resulting precipitate was filtered and dried to give 5.1 g (80% yield) of dark orange powder, mp 161–163.5°. An analytical sample recrystallized twice from EtOH melted at 168.5–169.5°. Anal. ($C_{29}H_{34}N_3O_{11}S$) C, H, O.

Phenothiazine-2-carboxaldehyde (7). A mixture of **6** (4.5 g, 20 mmol), Raney alloy (50:50, 7.5 g), and 75% by volume of aqueous formic acid (450 ml) was refluxed, with stirring, for 5 h. The mixture was filtered while hot, and the residue was washed with a small quantity of hot formic acid. The cooled filtrate was diluted with H_2O, and the crude, orange product precipitated. The material was collected and recrystallized three times from MeCN to give bright orange needles: 2.0 g (44%); mp 200–201° (lit.[9] mp 197–198°). Anal. ($C_{13}H_9NOS$) C, H, N, S.

1,1-Dicyano-2-(2-phenothiazinyl)ethene (8). A mixture of **7** (400 mg, 1.80 mmol), malononitrile (120 mg, 1.80 mmol), and a few drops of piperidine in 20 ml of butyl alcohol was stirred at room temperature for 4 h. The mixture was then poured into ice water, filtered, dried, and recrystallized from AcOH to yield reddish-purple crystals: 350 mg (70.6%); mp 227.5–229°. Anal. ($C_{16}H_9N_3S$) C, H, N, S.

1-Nitro-2-(2-phenothiazinyl)ethene (9). A mixture of **7** (1.3 g, 5.7 mmol), NH_4OAc (6.5 g), CH_3NO_2 (19 ml), and AcOH (130 ml) was refluxed for 2 h. Upon cooling, the mixture was poured into 500 ml of ice water. The precipitated solid was collected and washed with H_2O. Three recrystallizations from 70% EtOH yielded glittering, maroon crystals: 0.29 g (19%); mp 202–204°. Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N, S.

10-[3-[4-(2-Hydroxyethyl)-1-piperazinyl]propyl]phenothiazine-2-carboxaldehyde Dimaleate (5). A mixture of **1e** (1.5 g, 3.8 mmol) and Raney alloy (50:50, 12 g) in 75% by volume, aqueous formic acid (225 ml) was refluxed, with rapid stirring, for 5 h. Upon cooling, the alloy was filtered off, and the formic acid was removed under reduced pressure. Potassium carbonate solution (5%, 200 ml) was added to the residue to form a slurry, which was then extracted three times with small portions of benzene. The combined benzene extracts were washed with H_2O and then with saturated NaCl. After drying over anhydrous Na_2SO_4, the mixture was filtered and the filtrate evaporated to dryness under reduced pressure. The yellow, gummy residue was dissolved in a mixture of MeOH (45 ml) and H_2O (3 ml). To this solution was added 0.15 g of K_2CO_3, and the solution was refluxed on a water bath for 20 min. Upon cooling, the solution was diluted with H_2O (150 ml) and extracted three times with small portions of benzene. The combined benzene extracts were washed with H_2O and then with saturated NaCl; after drying over anhydrous Na_2SO_4, the mixture was filtered, and the filtrate was taken to dryness under reduced pressure. The yellow, oily residue was chromatographed on Florisil, elution being achieved with 1% MeOH–$CHCl_3$, to yield the product as a yellow oil (0.52 g, 35%). The dimaleate salt was prepared as described above in the preparation of **1b**. The salt was recrystallized twice from EtOH to yield orange-yellow crystals: 0.47 g (20%); mp 157–158.5°. Anal. ($C_{30}H_{35}N_3O_{10}S$) C, H, N, S.

2-(2,2-Dicyanoethenyl)-10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine Dimaleate (1c). A mixture of **5** (23 mg, 0.36 mmol), malononitrile (26 mg, 0.39 mmol), and a few drops of piperidine in 200 ml of EtOH was stirred at room temperature for 16 h, and then the solvent was removed under reduced pressure. Potassium carbonate solution (0.2%, 100 ml) was added to the gummy, purple residue, and the resultant slurry was extracted three times with small portions of benzene. The combined benzene extracts were washed with water and then with saturated NaCl. The benzene solution was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under reduced pressure to yield crude product as a purple gum (98 mg, 62%). The dimaleate salt was prepared as described above in the preparation of **1b**. Two recrystallizations from EtOH yielded purple crystals: 65 mg (27%); mp 157–159°. Anal. ($C_{33}H_{35}N_5O_9S$) C, H, N, S.

2-(2-Nitroethenyl)-10-[3-[4-(2-hydroxyethyl)-1-piperazinyl]propyl]phenothiazine Dimaleate (1d). A solution of **5** (0.14 g, 0.22 mmol) in 25 ml of CH_3NO_2 was heated to reflux. A drop of benzylamine was added, and the mixture was allowed to continue to reflux, with stirring, for 5 min. The reaction mixture rapidly turned from light yellow to dark red. The mixture was then cooled to room temperature by immersion of the reaction flask in an ice bath, and the nitromethane was removed under reduced pressure. The dark-red residue was dissolved in a hot solution of 0.14 g of maleic acid in 50 ml of EtOH. Upon cooling, the precipitated red dimaleate salt was collected and dried to give 76 mg: mp 173–177°. Two recrystallizations from absolute ethanol gave **1d** as red crystals: 38 mg (20%); mp 175–177.5°. Anal. ($C_{31}H_{36}N_4O_{11}S$) C, H, N, S.

Bioassay Methods. To bioassay each compound in the mouse catalepsy test, six 20–25-g male albino mice were used for each dose of test compound or reference standard. Except for **1d**, for which polyethylene glycol 200 (PEG 200) solutions were used, all compounds were given in aqueous solutions. For negative controls, distilled water was used. All injections were intravenous and were made with 27 gauge needles. An average of four dose levels, spaced at quarter log intervals, was used per test compound, with doses selected on the basis of a preliminary gross observation screen. At 60, 120, and 180 min postinjection, each mouse was tested for catalepsy by placing it with both forepaws elevated 5 cm, an almost vertical stance for these animals. A normal mouse will almost immediately return to its normal four-legged stance or climb onto the support, while a cataleptic mouse will remain where it is placed. The protocol used for these studies requires that the animal remain in the imposed position for at least 10 s. At each test time the subjects were given up to three trials, with the response being considered quantal. Although the use of three trials is arbitrary, it was chosen because initial studies with known cataleptogens indicated that threshold cataleptic animals require more than one placement before maintaining the imposed stance. On the other hand, employing more than three trials per test period raises the question of a possible training effect.

The rat catalepsy test used was a modification of the procedure of Morpurgo[16] and is essentially the same as the mouse test. Two elevations were used, however, and the responses were scored in the following way: one-half point for the low elevation (1.5 cm) and one point for the high elevation (9 cm). The scores for each forepaw in turn were obtained, allowing a maximum score of 3. Another difference was that the rats were injected intramuscularly in the gastrocnemius using a 27-gauge, 0.25-in. needle. The total dose was usually delivered in a volume equivalent to 0.1 ml/kg of body weight; the average weight of rat used was 180 g. Each animal was treated only once to avoid possible carryover effects,

and saline-injected animals served as controls.[17]

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Synthesis and Biological Activity of Camel and Bovine β-Melanotropins

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Two natural occurring melanotropins, camel β_{C2}-MSH and bovine β-MSH, have been synthesized by improved solid-phase procedures. The coupling reaction of *tert*-butyloxycarbonylamino acids was achieved by using their preformed symmetrical anhydrides. The synthetic hormones were purified by gel filtration on Sephadex G-10 and G-25, chromatography on carboxymethylcellulose, and partition chromatography on Sephadex G-25 with final yields of 56 and 35% for β_{C2}-MSH and β_b-MSH, respectively. They were then shown to be identical with their natural hormones in amino acid analysis, paper electrophoresis, disc electrophoresis, thin-layer chromatography, enzymic digests, and bioassays. Bioassay data of these two synthetic melanotropins indicate that the replacement of Ser by Gly in β_b-MSH does not change its melanocyte-stimulating activity.

Recently, the complete amino acid sequences of two β-melanotropins from the camel (*Camelus dromedarius*) have been reported.[1] One of them, β_{C1}-MSH,[2,3] was synthesized[4] using standard procedures for solid-phase peptide synthesis.[5] In this paper, we report the solid-phase synthesis of β_{C2}-MSH with the following primary structure: H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH. Bovine β-MSH, which has already been synthesized by solution methods,[6] differs from β_{C2}-MSH only by its serine residue in position 2.[7] We decided to undertake its solid-phase synthesis in connection with our synthetic studies on ovine β-lipotropin.[8] Furthermore, the β-melanotropins afford a convenient opportunity to test recent improvements in solid-phase techniques.

Esterification of Boc-Asp(OBzl)-OH to the chloromethylated polymer was achieved following some modifications[4] of the Loffet procedure.[9] The resulting Boc-Asp(OBzl)-resin was subjected to the procedure for solid-phase peptide synthesis.[5] Coupling reactions were exclusively achieved by using symmetrical anhydrides of *tert*-butyloxycarbonylamino acids[10] preformed with dicyclohexylcarbodiimide.[11] The advantages of this method in the elimination of side products were recently pointed out.[8] All amino acids were protected on the α-amino position with the Boc group and the following side-chain blocking groups were used: Glu(OBzl), Asp(OBzl), Ser(Bzl), Lys(*o*-Br-Z),[12] Tyr(*o*-Br-Z),[13] Arg(Tos), Trp(HCO),[14] and His(Boc).[15] Removal of the N^{α}-Boc protecting group was carried out by treatment for 15 min with 50% trifluoroacetic acid in CH_2Cl_2.[16]

After completion of the syntheses, the fully protected β_{C2}-MSH resin was treated with 50% trifluoroacetic acid to remove the Boc group, whereas this group was not removed by this procedure in the protected β_b-MSH resin. The advantage in yield gained by this initial removal of the Boc group will be subsequently pointed out for β_{C2}-MSH. Evidence shows that this treatment can be used on methionine-containing peptides to avoid side products.[17]

The two peptides were cleaved from their respective resins and deprotected with liquid HF,[18–20] with the exception of the formyl-protecting group for tryptophan.[21] They were partly purified by gel filtration on Sephadex G-10 and G-25. Preliminary experiments demonstrated that the deformylation of tryptophan can be achieved very rapidly (ca. 3 min) at pH 11.5. The peptides deformylated in this manner were submitted to chromatography on CMC[22] as shown in Figures 1A and 2A. Further purification by partition chromatography[23] on Sephadex G-25 gave the results shown in Figures 1B and 2B. The highly purified synthetic β_{C2}-MSH and β_b-MSH were obtained in overall yields of 56 and 35%, respectively, based on starting Boc-Asp(OBzl)-resin. When the Boc group of the fully protected β_{C2}-MSH resin was not initially removed with the trifluoroacetic acid treatment, β_{C2}-MSH was subsequently isolated in only 32% yield. It may be noted that these yields represent improvements over our previous yields of 25% for β_{C1}-MSH where tryptophan was protected and 10% where this protection was not employed.[4]

The synthetic hormones were homogeneous on thin-layer chromatography and on paper and disc gel elec-