Use of β-Methylphenylalanine (βMeF) Residues To Probe the Nature of the Interaction of Substance P with Its Receptor: Effects of βMeF-Containing Substance P Analogs on Rabbit Iris Smooth Muscle Contraction

David M. Birney,*,§ Derek C. Cole,§ Craig E. Crosson,† Brenda F. Kahl,§ Bart W. Neff,§ Ted W. Reid,*,§ Kaijun Ren,‡ and Robert D. Walkup*,§

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409, Department of Ophthalmology and Visual Sciences, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, Texas Tech University Institute for Biotechnology, Lubbock, Texas 79409, and Texas Biotechnology Corp., 7000 Fannin, Suite 1920, Houston, Texas 77030

Received November 17, 1994[⊗]

The effects of substituting (2*S*,3*S*)-β-methylphenylalanine (*S*-βMeF) or (2*S*,3*R*)-β-methylphenylalanine (*R*-βMeF) for the Phe[7] and/or Phe[8] residues of the tachykinin substance P (SP, RPKPQQFFGLM-NH_2) upon the ability of SP to stimulate contraction of the rabbit iris smooth muscle were investigated. The eight βMeF-containing SP analogs (four monosubstituted analogs, four disubstituted analogs) **1**–**8** were synthesized and found to be agonists of SP in the smooth muscle contraction assay, having EC_{50} values ranging from 0.15 to 10.0 nM. Three analogs are significantly more active than SP [8*R*-(βMeF)SP (**4**), 7*S*,8*S*-(βMeF)$_2$SP (**5**), and 7*R*,8*S*-(βMeF)$_2$SP (**6**)], three analogs are approximately equipotent with SP [7*S*-(βMeF)SP (**1**), 7*R*-(βMeF)SP (**2**), and 7*S*,8*R*-(βMeF)$_2$SP (**8**)], and two analogs are significantly less active than SP [8*S*-(βMeF)SP (**3**) and 7*R*,8*R*-(βMeF)$_2$SP (7)]. The effects of the βMeF substitutions upon the activity of SP are not additive and cannot be explained using simple conformational models which focus only on the side chain conformations of the βMeF residues. It is postulated that the βMeF residues induce minor distortions in the peptide backbone with resultant consequences upon peptide–receptor binding which are not dictated solely by the side chain conformations. This idea is consistent with ^{1}H-NMR data for the monosubstituted analogs **1**–**4**, which imply that the βMeF substitutions cause slight distortions in the peptide backbone and that the βMeF side chains are assuming trans or gauche(−) conformations.

Substance P (SP), a ubiquitous mammalian neuropeptide having the structure RPKPQQFFGLM-NH_2, has been found to have many physiological activities among which are the ability to lower blood pressure,[1] to stimulate smooth muscle contraction[2] and cytokine release,[3] and to promote cell growth.[4,5] It is a member of a family of peptides (tachykinins) which have a common carboxy terminal sequence (...FXGLM-NH_2, where X is a hydrophobic amino acid). The three known mammalian tachykinins (SP, neurokinin A, and neurokinin B) each utilize individual receptors—NK1, NK2, and NK3, respectively—for their physiological activities. Although each of the tachykinins will bind to each of the three receptors, the ability of each to activate its own receptor occurs at a significantly lower concentration than that found for the stimulation of the other receptors.[6]

Because of the apparent role which SP plays in physiological processes relevant to various pathological conditions, an important goal for eventual chemotherapies which target the SP receptor, via SP antagonists or agonists, is to understand the structural requirements for SP's biological activity. Assays of various SP analogs have indicated that the six amino acids at the carboxy terminal end of the molecule are required for most of SP's binding to its receptor and that the two phenylalanine residues at positions 7 and 8 are particularly important for this interaction.[7] The importance of Phe[7] and Phe[8] to the activity of SP has been recently reemphasized by Josien *et al.*, who reported the activities of various SP analogs which contained conformationally restricted phenylalanine residues.[8] Their results led them to propose that the active conformation of SP, with respect to its interactions with the NK1 receptor, is a backbone whose Lys[3]-Phe[8] core has adopted a helical secondary structure (as indicated by earlier NMR studies[9]) whose Phe[7] side chain favors the t conformation ($\chi_1 = 180°$; see Figure 1 for the structural definitions of these terms) and whose Phe[8] side chain favors either the g$^-$ ($\chi_1 = -60°$) or t conformation (Figure 1).

We now report a systematic study of SP analogs in which a methyl group has been introduced onto the β carbon of one or both Phe residues (*i.e.*, β-methylphenylalanine (βMeF) substitutions for Phe[7] and/or Phe[8]) in a stereodefined manner. Such apparently minor structural alterations of the side chains result in significant variations in the biological activity of the resulting SP analogs. The trends observed for the effects of these βMeF substitutions on the biological activity cannot be explained on the basis of side chain conformations alone, and it is postulated that the βMeF residues induce distortions in the peptide backbone with resultant consequences upon peptide–receptor binding.

The eight βMeF-containing substance P analogs **1**–**8**, where either the *R*-βMeF or *S*-βMeF diastereomer (having the *R* or *S* absolute configuration at the β carbon, respectively) of the L-β-methylphenylalanine residues is employed, were synthesized and evaluated in a smooth muscle contraction assay using the rabbit iris sphincter. This tissue was chosen because it is

† Texas Tech University Health Sciences Center.
‡ Texas Biotechnology Corp.
§ Texas Tech University, Texas Tech University Health Sciences Center, and Texas Tech University Institute for Biotechnology.
[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.

0022-2623/95/1838-2478$09.00/0

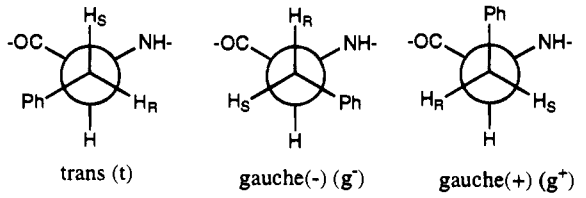


Figure 1. Newman projections, from C_β to C_α, for the three staggered conformations of phenylalanine. H_S refers to the β hydrogen which, upon replacement with a methyl group, gives (2*S*,3*S*)-β-methylphenylalanine, and H_R refers to the β hydrogen which, upon replacement with a methyl group, gives (2*S*,3*R*)-β-methylphenylalanine.

known to contain only the NK1 and NK3 receptors[10] and because evidence exists for SP only utilizing the NK1 receptor in it.[11] Furthermore, SP appears to act on the rabbit iris sphincter directly, without the involvement of nerve-mediated release of secondary myotropic substances, which could complicate the interpretation of the results.[12]

1: R-P-K-P-Q-Q-[SβMeF]-F-G-L-M-NH_2
(7S-(βMeF)SP

2: R-P-K-P-Q-Q-[RβMeF]-F-G-L-M-NH_2
(7R-(βMeF)SP

3: R-P-K-P-Q-Q-F-[SβMeF]-G-L-M-NH_2
(8S-(βMeF)SP

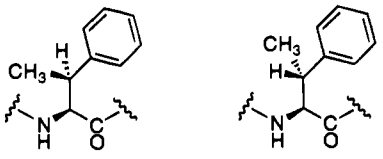
4: R-P-K-P-Q-Q-F-[RβMeF]-G-L-M-NH_2
(8R-(βMeF)SP

5: R-P-K-P-Q-Q-[SβMeF]-[SβMeF]-G-L-M-NH_2
(7S,8S-$(\beta MeF)_2$SP

6: R-P-K-P-Q-Q-[RβMeF]-[SβMeF]-G-L-M-NH_2
(7R,8S-$(\beta MeF)_2$SP

7: R-P-K-P-Q-Q-[RβMeF]-[RβMeF]-G-L-M-NH_2
(7R,8R-$(\beta MeF)_2$SP

8: R-P-K-P-Q-Q-[SβMeF]-[RβMeF]-G-L-M-NH_2
(7S,8R-$(\beta MeF)_2$SP



The 2*S*,3*S* and 2*S*,3*R* isomers of β-methylphenylalanine were prepared according to the procedure of Dharanipragada *et al.*,[13] protected as the N-Fmoc derivatives, and then used in the solid phase syntheses of peptides **1**–**8**. Each synthetic peptide was purified by HPLC before use, and its identity was confirmed by high-resolution FAB mass spectrometry. The smooth muscle contraction assays were measured using iris sphincter muscle strips disected from New Zealand white rabbits, bathed in pH 7.4 Ringer's solution (containing indomethacin, thiophorphan, phosphoramidon, captopril, leupeptin, and chymostatin) at 37 °C, and connected to a Grass force-displacement transducer. Dose–response curves (from 2×10^{-11} to 7×10^{-7} M) were determined for SP and for each of the SP analogs **1**–**8**, and a minimum of four measurements were made for each peptide. The dose–response curves (Figure 2)

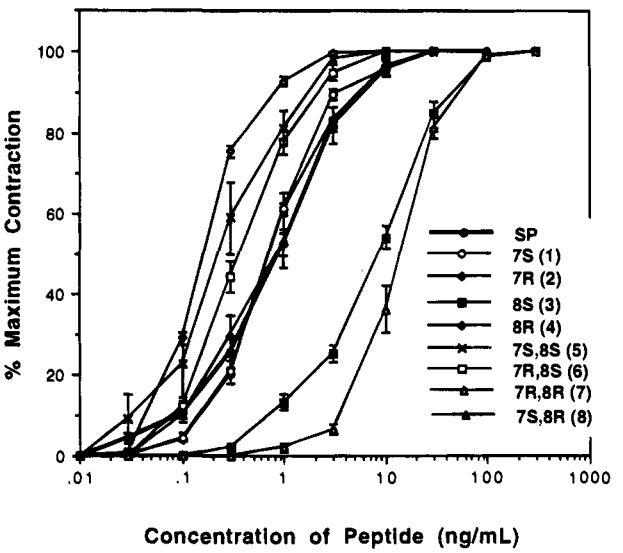


Figure 2. Dose–response curves for SP and the βMeF-containing SP analogs **1**–**8**. See text for experimental details.

Table 1. Smooth Muscle Contraction Activity of Substance P and β-Methylphenylalanine-Containing Substance P Analogs **1**–**8** (Rabbit iris sphincter, 37 °C)

peptide	EC_{50} (nM)	Hill coefficient
SP	0.61 ± 0.031	1.1 ± 0.055
7*S*-(βMeF)SP (**1**)	0.54 ± 0.016	1.5 ± 0.056
7*R*-(βMeF)SP (**2**)	0.59 ± 0.024	1.4 ± 0.071
8*S*-(βMeF)SP (**3**)	5.76 ± 0.29	1.1 ± 0.057
8*R*-(βMeF)SP (**4**)	0.13 ± 0.003	1.8 ± 0.075
7*S*,8*S*-$(\beta MeF)_2$SP (**5**)	0.18 ± 0.024	1.2 ± 0.17
7*R*,8*S*-$(\beta MeF)_2$SP (**6**)	0.28 ± 0.013	1.4 ± 0.080
7*R*,8*R*-$(\beta MeF)_2$SP (**7**)	10.0 ± 0.43	1.8 ± 0.13
7*S*,8*R*-$(\beta MeF)_2$SP (**8**)	0.58 ± 0.049	1.1 ± 0.088

were fit by means of a nonlinear regression analysis program and yielded the EC_{50} values and Hill coefficients listed in Table 1.

These results indicate that all of the βMeF-substituted SP analogs are SP agonists, that the presence of either *R*- or *S*-β-methyl groups on one or both of the phenylalanine residues of SP results in varying activities in this assay, and, unexpectedly, that the functional consequences of having either stereoisomer of βMeF (*R* versus *S*) in a single position (monosubstituted analogs) are not additive (*cf.* data for the disubstituted analogs). The muscle contraction responses induced by the βMeF-containing SP analogs ranged from EC_{50} values of 0.13 to 10 nM. Three of the analogs are significantly more active than SP [8*R*-(βMeF)SP (**4**), 7*S*,8*S*-$(\beta MeF)_2$SP (**5**), and 7*R*,8*S*-$(\beta MeF)_2$SP (**6**)], three analogs are approximately equipotent with SP [7*S*-(βMeF)SP (**1**), 7*R*-(βMeF)SP (**2**), and 7*S*,8*R*-$(\beta MeF)_2$SP (**8**)], and two analogs are significantly less active than SP [8*S*-(βMeF)-SP (**3**) and 7*R*,8*R*-$(\beta MeF)_2$SP (**7**)].

The use of βMeF residues to probe the conformational aspects of peptide activity has been applied to several important biologically active peptides, including angiotensin II,[14] enkephalin,[15] and somatostatin.[16] Such studies have indicated that βMeF residues in a peptide affect biological activity in a measurable manner. NMR studies of a βMeF-containing cholecystokinin peptide subunit implied that the βMeF residues affect the backbone conformation of a peptide only locally, at the amino acid residues adjacent to the βMeF residue.[17] Simple modeling studies of each diastereomer of the

Table 2. Selected ^{1}H-NMR Chemical Shifts for SP and Peptides **1**–**4**[a]

structure	5,6-Q-α	5,6-Q-β	7-F-α	7-F-β	8-F-α	8-F-β	9-G-α
SP	4.15[b]	1.85–2.10[c]	4.48	2.91	4.50	3.19	3.93, 3.83[c]
7*S*-(βMeF)SP (**1**)	4.29, 4.53	1.85–2.03	4.51 ($J_{\alpha\beta}$ = 11.9 Hz)	2.87	4.55	2.67, 3.32	3.97, 3.77
7*R*-(βMeF)SP (**2**)	4.20, 4.41	2.10–2.15	4.39 ($J_{\alpha\beta}$ = 9.6 Hz)	3.19	4.40	2.84, 3.18	3.83, 3.71
8*S*-(βMeF)SP (**3**)	4.49	2.45–2.60	4.32	2.85, 2.57	4.49 ($J_{\alpha\beta}$ = 9.5 Hz)	3.25	3.92, 3.88
8*R*-(βMeF)SP (**4**)	4.39	2.48–2.60	4.56	3.1, 2.95	4.53 ($J_{\alpha\beta}$ = 8.1 Hz)	3.33	3.81, 3.76

[a] NMR chemical shifts in ppm relative to the signal for methanol. [b] Overlapping signals. [c] The dash between the numbers indicates a chemical shift range where overlapping signals made discrete assignments impossible, and a comma between two values indicates that those signals were distinguishable.

βMeF monomer predict that the *S*-βMeF (the 2*S*,3*S* stereoisomer) residue will tend to favor the gauche(−) (g^-) conformation and that the *R*-βMeF residue will tend to favor the trans (t) conformation;[18] however, NMR studies suggest that these preferences are not always followed in βMeF-containing peptides.[17]

The effects which βMeF substitutions have upon the activity of SP cannot be explained using simple conformational models. For example, if we were to assume that SP's peptide backbone conformation is not altered by the introduction of a βMeF residue and that the βMeF residue adopts the predicted side chain conformations (*S* isomer favors g^-, *R* isomer favors t), then we would expect to observe consistent biological effects associated with a βMeF substitution which correspond to the predicted side chain conformation of the given βMeF substituent and at least some degree of additivity in these effects in the bis-βMeF-substituted SP analogs. Neither is the case. On the basis of Josien *et al.*'s predictions concerning the biologically active side chain conformations for Phe^7 and Phe^8 in substance P (*i.e.*, Phe^7 in t conformation, Phe^8 in either t or g^- conformation),[8] we would predict that there would be no significant differences between the activities of the 8*R*- and 8*S*-βMeF-containing SP analogs and that those 7-βMeF-containing analogs which favor the t conformation at position 7—the 7*R*-βMeF-containing analogs—would be more active than the other analogs. In contrast to these predictions, (1) 8*R*-(βMeF)SP (**4**) and 8*S*-(βMeF)SP (**3**) are, respectively, the most active and one of the least active of the eight analogs, (2) 7*R*-(βMeF)SP (**2**) and 7*S*-(βMeF)SP (**1**) have activities similar to each other and to SP, and (3) 7*R*,8*R*-(βMeF)$_2$SP (**7**) is the least active analog, despite its bearing a 7*R*-βMeF residue. Furthermore, our results with the disubstituted analogs do not indicate that the effects of βMeF residues upon activity are additive: (1) even though an 8*R*-βMeF monosubstitution (**4**) enhances activity nearly 5-fold (relative to SP) and a 7*R*-βMeF monosubstitution (**2**) does not change the activity, a combination of the two [7*R*,8*R*-(βMeF)$_2$SP, **7**] results in a 16-fold reduction in activity and (2) 7*S*,8*S*-(βMeF)$_2$SP (**5**) is significantly more active than SP, even though 8*S*-(βMeF)SP (**3**) is much less active than SP and 7*S*-(βMeF)SP (**1**) has activity approximately equal to that of SP.

A preliminary 300 MHz 1-D ^{1}H-NMR survey of the four monosubstituted βMeF-containing SP analogs **1**–**4** in CD_3OD (a solvent which mimics the cell membrane-like environment in which SP interacts with its receptor[9]) indicates that these analogs assume a gross structure similar to that of SP (*i.e.*, a peak-for-peak comparison of the spectra of **1**–**4** with the spectrum of SP indicates an overall similarity). However, slight changes in the signals for protons as far as three residues removed from the site of the βMeF substitution were observed (Table 2). β-Methyl substitution at either Phe^7 or Phe^8 resulted in a change in the chemical shifts of the α and β protons on both Gln^5 and Gln^6, as well as a change in the chemical shifts of both α protons on Gly^9, and these changes were accompanied by changes in the shifts of the protons located on the Phe^7 and Phe^8 residues. The observed H_α–H_β proton–proton coupling constants for the βMeF residues in peptides **1**–**4** are observed to be 8–12 Hz, suggesting an anti relationship between these two protons, in accordance with the t or g^- side chain conformations predicted from the reported conformational analysis of β-methylphenylalanine.[18] (The alternative eclipsed side chain conformations predicted by these coupling constants according to the Karplus equation are deemed to be less likely on the basis of their inherent steric strains.)

Our studies, the first to use β-methylphenylalanine as a conformational probe in a tachykinin,[19] indicate that such a subtle alteration of the substance P structure as the introduction of a single methyl group onto the side chain of a phenylalanine residue results in profound changes in the biological activity, suggesting that a βMeF residue will change the preferred conformation of SP toward either a more or less active conformation. Our observations suggest that βMeF substitutions in SP affect the peptide backbone sufficiently to alter the biological activity of SP in a manner which may be unrelated to the side chain conformation. It is possible that such backbone alterations, which might occur in reponse to steric repulsions between the branched side chains of the βMeF residues and other groups in the peptide, result in projections of the βMeF side chain phenyl groups into orientations, relative to the overall shape of the SP molecule, which correspond to the active or inactive conformations inferred by Josien *et al.*'s findings,[8] regardless of the particular χ_1 angle favored by the βMeF residues [as indicated, for example, by the observed NMR coupling constants for the α and β protons of the βMeF residues in **1**–**4** (Table 2) discussed above]. This scenario would particularly explain the observation that the disubstituted analogs **5**–**8** showed activities which could not be predicted on the basis of the activities of the monosubstituted analogs **1**–**4**: the presence of the two branched side chains in the disubstituted analogs would result in steric repulsions and resultant backbone distortions, which would be different from those caused by one branched side chain. In some cases (according to this scenario), these distortions are compensatory, allowing the disubstituted

Table 3. Analytical Data for the β-Methylphenylalanine-Containing Substance P Analogs 1–8[a]

structure	HPLC t_R (min)	HRMS m/z (M + 1)	amino acid content (mol %) Arg	Pro	Lys	Glx	Phe	SβMeF	RβMeF	Gly	Leu	Met	other[b]
7S-(βMeF)SP (1)	17.32	1361.7459[c]	9.0[d]	18.0[e]	8.4[d]	17.2[e]	9.0[d]	9.3[d]	0.4[f]	10.0[d]	9.9[d]	8.8[d]	ND[f,g]
7R-(βMeF)SP (2)	17.05	1361.7474[c]	9.5[d]	18.2[e]	9.0[d]	17.0[e]	9.2[d]	0.8[f]	7.8[d]	9.9[d]	9.7[d]	8.6[d]	0.2% Ser[f]
8S-(βMeF)SP (3)	17.00	1361.7456[c]	8.2[d]	17.6[e]	7.9[d]	16.7[e]	9.4[d]	9.5[d]	0.6[f]	10.1[d]	10.0[d]	8.1[d]	0.6% Ser[f] 0.3% Asx[f] 0.4% Thr[f] 0.3% Val[f] 0.3% Ala[f]
8R-(βMeF)SP (4)	17.00	1361.7478[c]	10.4[d]	18.5[e]	6.6[d]	18.5[e]	9.5[d]	ND[f,g]	8.1[d]	10.8[d]	9.6[d]	8.1[d]	ND[f,g]
7S,8S-(βMeF)$_2$SP (5)	17.87	1375.7669[h]	9.1[d]	18.2[e]	9.1[d]	18.2[e]	ND[f,g]	18.2[e]	1.5[f]	9.1[d]	9.1[d]	9.1[d]	ND[f,g]
7R,8S-(βMeF)$_2$SP (6)	17.42	1375.7625[h]	10.3[d]	18.2[e]	10.0[d]	19.5[e]	ND[f,g]	6.6[d]	6.8[d]	10.4[d]	9.5[d]	8.7[d]	0.1% Tyr[f]
7R,8R-(βMeF)$_2$SP (7)	17.38	1375.7617[h]	10.0[d]	17.2[e]	9.2[d]	18.4[e]	ND[f,g]	1.3[f]	16.6[e]	9.9[d]	8.7[d]	8.6[d]	ND[f,g]
7S,8R-(βMeF)$_2$SP (8)	17.30	1375.7637[h]	11.3[d]	19.3[e]	10.0[d]	15.8[e]	ND[f,g]	6.8[d]	6.4[d]	11.4[d]	9.9[d]	9.2[d]	ND[f,g]

[a] See the Experimental Section for conditions used for analyses. [b] Other amino acids which were detected in the HPLC trace of the PTC amino acid mixtures obtained from the sample. [c] $C_{64}H_{101}N_{18}O_{13}S$ calcd: 1361.7516. [d] Calcd: 9.1%. [e] Calcd: 18.2%. [f] Calcd: 0.0%. [g] ND = none detected. [h] $C_{65}H_{103}N_{18}O_{13}S$ calcd: 1375.7672.

peptide to bind to and activate the NK1 receptor as well as or better than SP (*e.g.*, the (βMeF)$_2$SP analogs **5** and **6**), and in other cases they are not (*e.g.*, analog **7**). Ongoing research is aimed at elucidating such subtle, yet profound, effects of β-methylphenylalanine residues upon the structure and activity of substance P.

Experimental Section

Syntheses of the Fmoc-Protected (2S,3S)-β-Methylphenylalanine and (2S,3R)-β-Methylphenylalanine Monomers. (2*S*,3*S*)-β-Methylphenylalanine and (2*S*,3*R*)-β-methylphenylalanine were synthesized using the method of Dharanipragada *et al.*[13] The *N*-fluorenylmethoxycarbonyl (Fmoc)-protected derivatives of each amino acid were prepared using the following representative procedure: (2*S*,3*S*)-β-Methylphenylalanine (203 mg, 0.94 mmol) was stirred in 9% aqueous sodium carbonate (3.4 mL) at 0 °C, and *N*-[9-fluorenylmethoxycarbonyl)oxy]succininide (266 mg, 0.788 mmol) in dioxane (2 mL) was added. After stirring at room temperature for 1 h, water (20 mL) was added, and the solution was extracted with ether (3 × 10 mL). The aqueous phase was then acidified with 10% HCl and extracted with ethyl acetate (3 × 10 mL). The ethyl acetate extracts were dried over $MgSO_4$, filtered, and concentrated to give the crude (~90%) product, which could be further purified by flash chromatography on silica gel using ethanol/chloroform (5:95) as the eluent to give 194 mg (60%) of pure *N*-Fmoc-(2*S*,3*S*)-β-methylphenylalanine: ^{1}H NMR ($CDCl_3$) δ 1.38 (d, J = 7.0 Hz, 3 H), 3.44 (dd, J = 6.8, 5.6 Hz, 1 H), 4.19 (t, J = 6.8 Hz, 1 H), 4.29–4.51 (m, 2 H), 4.62 (dd, J = 8.9, 5.0 Hz, 1 H), 5.0 (d, J = 9.0 Hz, 1 H), 7.11–7.46 (m, 9 H), 7.46–7.60 (m, 2 H), 7.76 (d, J = 9.0 Hz, 2 H); ^{13}C NMR ($CDCl_3$) δ 17.80, 41.75, 47.09, 58.94, 66.90, 119.92, 124.95, 127.03, 127.37, 127.68, 128.62, 140.24, 141.27, 143.59, 156.30, 176.43; IR (neat film) 3650–3130, 3130–2840, 2341, 1710, 1514 cm^{-1}; $[\alpha]_D$ +4.8° (c = 0.003 g/mL, CH_2Cl_2); HRMS m/z 401.1631 (calcd for $C_{25}H_{23}NO_4$ [M$^+$], 401.1627).

This procedure was followed using (2*S*,3*R*)-β-methylphenylalanine to yield *N*-Fmoc-(2*S*,3*R*)-β-methylphenylalanine in 71% yield: ^{1}H NMR ($CDCl_3$) δ 1.40 (d, J = 6.4 Hz, 3 H), 3.34 (t, J = 8.4 Hz, 1 H), 4.11–4.50 (m, 3 H), 4.62 (dd, J = 8.4, 5.2 Hz, 1 H), 5.39 (d, J = 8.8 Hz, 1 H), 7.11–7.48 (m, 9 H), 7.48-7.52 (m, 2 H), 7.18–7.32 (m, 2 H); ^{13}C NMR ($CDCl_3$) δ 16.17, 42.09, 47.13, 59.14, 67.11, 120.00, 125.03, 127.10, 127.29, 127.74, 128.52, 140.89, 141.30, 143.72, 155.89, 175.84; IR (neat film) 3636–2813, 2359, 1714, 1515 cm^{-1}; $[\alpha]_D$ +3.5° (c = 0.026 g/mL, CH_2Cl_2); HRMS m/z 401.1628 (calcd for $C_{25}H_{23}NO_4$ [M$^+$], 401.1627).

Syntheses of Peptides 1–8. The eight substance P analogs bearing each of the β-methylphenylalanine residues in positions 7 and/or 8 were synthesized from the Fmoc-protected amino acids. The side chain-protecting groups for the trifunctional residues Arg^1 and Lys^3 were the Pmc and Boc groups, respectively. The peptides were prepared by solid phase synthesis on a Rink resin using HBTU chemistry procedure using a Protein Technologies, Inc. Symphony peptide synthesizer. The peptides were cleaved from the resin with a TFA cocktail (4% thioanisol, 4% thiophenol, and 4% EDT) for 1.5 h at room temperature and then purified using preparative HPLC (C_{18}, 300 Å, 21.4 mm × 25 cm, 5 μm spherical packing at a flow rate of 10 mL/min), with UV detection (214 nm). Two HPLC mobile phases, solutions A (5% deionized water in acetonitrile containing 0.1% TFA) and B (5% acetonitrile in deionized water containing 0.15% TFA), were used in a programmed gradient of 5–70% A over 60 min. The purity and identity of each peptide was ascertained by analytical HPLC (C_{18}, 300 Å, 4.6 mm × 25 cm, 5 μm spherical packing at a flow rate of 1 mL/min, same mobile phase as for the preparative runs), amino acid analyses (6 N HCl hydrolysis, PTC derivatization, triplicate HPLC analysis, identification, and quantitation by comparison with known standards), and fast atom bombardment (FAB) mass spectrometry (Table 3).

Smooth Muscle Contraction Assay. New Zealand white rabbits were sacrificed by an iv overdose of sodium pentobarbital. The eyes were then enucleated, and the iris/ciliary body was isolated. The iris sphincter muscle was then isolated and the tissue bisected, producing two iris sphincter muscle preparations. The muscle strips were connected to a Grass force-displacement transducer with an initial load of 100 mg placed on the muscle. The tissue was then allowed to equilibrate for 60 min. Contractions were recorded by a Grass 7 physiograph. Tissues were bathed at 37 °C in a 50 mL modified Ringer's solution [90 mM NaCl, 3.6 mM KCl, 20 mM HEPES (sodium salt), 0.6 mM MgCl, 7.5 mM Na_2SO_4, 1.4 mM Ca-gluconate, 20 mM $NaHCO_3$, 0.6 mM $MgSO_4$, 0.6 mM K_2HPO_4, and 26 mM glucose]. The Ringer's solution was gassed with 95% air/5% CO_2 (pH 7.4). To suppress the endogenous production of prostaglandins which have been shown to contract the iris sphincter muscle, the incubating solutions contained indomethacin at a concentration of 1 μg/mL. To block potential proteolytic effects, the following proteolysis inhibitors were added, 1 μM thiorphan, 1 μM phosphoramidon, 1 μM captopril, 3 μg/mL leupeptin, and 3 μg/mL chymostatin. To evaluate the viability of each muscle preparation, maximum contraction was determined by a 10^{-5} M dose of carbachol at the beginning and end of each experiment. Following the initial carbachol dose, tissues were washed by three exchanges of Ringer's solutions. Previous studies have shown that this is sufficient to remove carbachol from these solutions and return the resting tension to normal values. Each peptide was then evaluated for its ability to induce sphincter muscle contraction, and dose–response curves (from 2×10^{-11} to 7×10^{-7} M) were determined. A minimum of four determinations was performed for each experimental condition. The dose–response curves were fit by means of a nonlinear regression analysis program (Sigma Plot, Jandel Scientific). The following equation was used to analyze the data: $R = 100\text{-}[A]^H/([A]^H + EC_{50})$, where R is the percent maximal response,

[A] is the agonist dose, EC_{50} is the agonist concentration producing a half-maximal response, and H is the Hill coefficient.

^{1}H-NMR Measurements of Peptides 1–4. ^{1}H-NMR data for the four monosubstituted βMeF-substituted SP analogs were obtained using a Brucker AF-300 spectrometer (300 MHz). Samples were prepared by dissolving 4 mg of the peptide in 0.5 mL of CD_3OD, giving a 3 mM solution. Proton assignments were made by analogy to published assignments for SP[9b] and from the COSY spectra (data not shown). Selected ^{1}H-NMR chemical shifts are indicated in Table 2.

Acknowledgment. This research was made possible by grants from the Texas Tech University Biotechnology Institute, Research to Prevent Blindness, the National Eye Institute (Grant No. EY09741), and (with the assistance of our colleague, John N. Marx) the National Institute for Allergy and Infectious Diseases (Grant No. AI32727). Assistance from the Midwest Center for Mass Spectrometry, with partial support by the National Science Foundation, Biology Division (Grant DIR9017262), is acknowledged. The authors thank Professor Rickey P. Hicks of Mississippi State University for providing data from his research on the structure of substance P, Professor Victor J. Hruby of the University of Arizona for providing information about the synthesis of β-methylphenylalanine, and Dr. Mark S. Wright of Texas A&M University (Department of Entomology) for assistance with the amino acid analyses.

References

(1) (a) Crossman, D. C.; Dashwood, M. R.; Davies, G. J.; Larkin, S.; Maseri, A.; Spyer, K. M.; Yacoub, M. Coronary Vasodilatation in vivo by Substance P and the Distribution of Substance P and Calcitonin Gene-Related Peptide-(CGRP)-Binding Sites in Human Coronary Artery. *J. Physiol.* **1988**, *407*, 15P. (b) Hakason, R.; Byne, G.; Beding, B.; Wahlestedt, C. Tachykinin Antagonists Suppress Responses to Ocular Injury in the Rabbit. In *Tachykinin Antagonists*; Hakanson, R., Sundler, Eds.; Elsevier Science: New York, 1985; pp 91–96. (c) Losay, J.; Mroz, E. A.; Tregear, G. W.; Leeman, S. E.; Gamble, W. J. Action of Substance P on the Coronary Blood Flow in the Isolated Dog Heart. In *Substance P*; Von Euler, U. S., Pernow, B., Eds.; Raven: New York, 1977; pp 287–293. (d) Zhang, G.; Yamamoto, Y.; Miwa, K.; Suzuki, H. Vasodilation Induced by Substance P in Guinea Pig Carotid Arteries. *Am. J. Physiol.* **1994**, *266*, H1132–1137.

(2) (a) Holzer, P.; Lembeck, F. Neurally Mediated Contraction of Ileal Longitudinal Muscle by Substance P. *Neurosci. Lett.* **1980**, *17*, 101–105. (b) Leander, S.; Hakanson, R.; Rosell, S.; Folkers, K.; Sundler, F.; Tornqvist, K. A Specific Substance P Antagonist Blocks Smooth Muscle Contractions Induced by Non-cholinergic, Non-adrenergic Nerve Stimulation. *Nature (London)* **1981**, *294*, 467–469.

(3) (a) Lotz, M.; Vaughan, J. H.; Carson, D. A. Effect of Neuropeptides on Production of Inflammatory Cytokines by Human Monocytes. *Science* **1988**, *241*, 1218–1221. (b) Kimball, E. S.; Persico, F. J.; Vaught, J. L. Substance P, Neurokinin A, and Neurokinin B Induce Generation of IL-1-like Activity in P388D1 Cells. Possible Relevance to Arthritic Disease. *J. Immunol.* **1988**, *141*, 3564–3569.

(4) (a) Nilsson, J.; Von Euler, A. M.; Dalsgaar, C. J. Stimulation of Connective Tissue Cell-growth by Substance P and Substance K. *Nature* **1985**, *315*, 61–63. (b) Payan, D. G.; Brewster, D. R.; Goetzl, E. J. Specific Stimulation of Human T Lymphocytes by Substance P. *J. Immunol.* **1983**, *131*, 1613–1615. (c) Reid, T. W.; Murphy, C. J.; Iwahashi, C. K.; Foster, B.; Mannis, M. J. Stimulation of Epithelial Cell Growth by the Neuropeptide Substance P. *J. Cell. Biochem.* **1993**, *52*, 476–485.

(5) Sandberg, B.; Iversen, L. Substance P. *J. Med. Chem.* **1982**, *25*, 1009–1015.

(6) Guard, S.; Watson, S. P. Tachykinin Receptor Types: Clasification and Membrane Signalling Mechanisms. *Neurochem Int.* **1991**, *18*, 149–165.

(7) (a) Cascieri, M. A.; Huang, R.-R. C.; Fong, T. M.; Cheung, A. H.; Sadowski, S.; Ber, E.; Strader, C. D. Determination of the Amino Acid Residues in Substance P Conferring Selectivity and Specifity for the Rat Neurokinin Receptors. *Mol. Pharmacol.* **1992**, *41*, 1096–1099. (b) Wang, J.; Dipasquale, A.; Bray, A.; Maeji, N.; Spellmeyer, D.; Geysen, H. Systematic Study of Substance P Analogs. II. Rapid Screening of 512 Substance P Stereoisomers for Binding to NK1 Receptor. *Int. J. Pept. Protein Res.* **1993**, *42*, 392–399. (c) Fournier, A.; Couture, R.; Regoli, D.; Gendreau, M.; Saint-Pierre, S. Synthesis of Peptides by the Solid-phase Method. 7. Substance P and Analogues. *J. Med. Chem.* **1982**, *25*, 64–68.

(8) Josien, H.; Lavielle, S.; Brunissen, A.; Saffroy, M.; Torrens, Y.; Beaujouan, J.-C.; Glowinski, J.; Chassaing, G. Design and Synthesis of Side-Chain Conformationally Restricted Phenylalanines and Their Use for Structure-Activity Studies on Tachykinin NK-1 Receptor. *J. Med. Chem.* **1994**, *37*, 1586–1601.

(9) (a) Chassaing, G.; Convert, O.; Lavielle, S. Preferential Conformation of Substance P in Solution. *Eur. J. Biochem.* **1986**, *154*, 77–85. (b) Young, J. K.; Anklin, C.; Hicks, R. P. Nuclear Magnetic Resonance and Molecular Modeling Investigations of the Neuropeptide Substance P in the Presence of 15 mM Sodium Dodecyl Sulfate Micelles. *Biopolymers* **1994**, *34*, 1449–1462.

(10) Wang, Z.; Hakanson, R. The rabbit iris sphincter contains NK1 and NK3 but not NK2 receptors: a study with selective agonists and antagonists. *Regul. Pept.* **1993**, *44*, 269–275.

(11) Reid, T. W.; Kahl, B. F. Unpublished results.

(12) Too, H. P.; Unger, W. G.; Hanley, M. R. Evidence for Multiple Tachykinin Receptor Subtypes on the Rabbit Iris Sphincter Muscle. *Mol. Pharmacol.* **1988**, *33*, 64–71.

(13) Dharanipragada, R.; VanHulle, K.; Bannister, A.; Bear, S.; Kennedy, L.; Hruby, V. J. Asymmetric Synthesis of Unusual Amino Acids: An Efficient Synthesis of Optically Pure Isomers of β-Methylphenylalanine. *Tetrahedron* **1992**, *48*, 4733–4748.

(14) Samanen, J.; Narindray, D.; Cash, T.; Brandeis, E.; Adams, W., Jr.; Yellin, T.; Eggleston, D.; DeBrosse, C.; Regoli, D. Potent Angiotensin II Antagonists with Non-β-branched Amino Acids in Position 5. *J. Med. Chem.* **1989**, *32*, 466–472.

(15) Hruby, V. J.; Toth, G.; Gehrig, C. A.; Kao, L. F.; Knapp, R. J.; Lui, G. K.; Yamamura, H. I.; Kramer, T. H.; Davis, P.; Burks, T. F. Topographically Designed Analogues of [D-Pen,D-Pen^5]-enkaphalin. *J. Med. Chem.* **1991**, *34*, 1823–1830.

(16) Huang, Z.; He, Y.-B.; Raynor, K.; Tallent, M.; Reisine, T.; Goodman, M. Main Chain and Side Chain Chiral Methylated Somatostatin Analogs: Syntheses and Conformational Analyses. *J. Am. Chem. Soc.* **1992**, *114*, 9390–9401.

(17) Kover, K. E.; Jiao, D.; Fang, S.; Hruby, V. J. Conformational Properties of the Unnatural Amino Acid β-Methylphenylalanine in a Linear Octapeptide System; Correlations of ^{13}C-NMR Chemical Shifts with the Side-Chain Stereochemistry of These Amino Acid Residues. *J. Org. Chem.* **1994**, *59*, 991–998.

(18) Hruby, V. J.; Pettitt, B. M. Conformation Biological Activity Relationships for Receptor-Selective, Conformationally Constrained Opioid Peptides. In *Computer-Aided Drug Design: Methods and Applications;* Perun, T. J., Propst, C. L., Eds.; Marcel Dekker: New York, 1989; pp 405–460.

(19) A bradykinin analog bearing βMeF residues (along with other unnatural amino acid residues) has been synthesized and assayed, but no systematic studies of the effects of the various βMeF residues upon the activity of the peptide were reported. Reissmann, S.; Kirsche, B.; Arold, H.; Suli-Vargha, H.; Medzihradszky, K. Synthese von Tritiierbaren Bradykininanaloga. (Synthesis of Bradykinin Analogs Designed for the Tritium Labelling.) *J. Prakt. Chem.* **1984**, *326*, 111–120.

JM940779L