3460 (br), 3200–2500 (NH_3^+), 2050 (C≡N), 1580 (aromatic), 1530–1460 (NH_3^+), 1335, 1150, 1110, 1065, 1040 (sh), 750 (aromatic) cm^{-1}; ^1H NMR (Me_2SO-d_6/$CHCl_3$) (mixture of isomers) δ 10.33–8.67 (br, 1.5 H, NH_3^+), 7.33 (s, 4 H, aromatic), 7.25 (m, 1.5 H, NH_3^+), 3.88 (m, 0.5 H, bridgehead), 3.75 (m, 0.5 H, bridgehead), 3.23 (m, 1 H, bridgehead), 2.48–0.75 (m, 6 H, C-3, C-9, and C-10 CH_2). Anal. ($C_{13}H_{15}N_2Cl \cdot 1H_2O$) C, H, N.

Phenylalanine Decarboxylase Assay. L-Phenylalanine decarboxylase (PAD, EC 4.1.1.53) activity was determined[22] by measuring the $^{14}CO_2$ produced from L-[1-^{14}C]phenylalanine in the presence of tyrosine decarboxylase.[31] The reactions were performed in 10-mL Kontes reaction flasks with a side arm and a plastic center well containing Hyamine 10-X hydroxide to absorb the $^{14}CO_2$. The standard reaction mixture (total volume 0.5 mL) consisted of 5×10^{-4} M pyridoxal 5-phosphate in 0.2 M Na_2HPO_4 buffer (pH 5.5), 1×10^{-2} M L-[1-^{14}C]phenylalanine (10^{-2} μCi/μmol) in 0.2 M Na_2HPO_4 buffer (pH 5.5), and 0.5 mg of tyrosine decarboxylase (crude powder also contains L-phenylalanine decarboxylase activity) in 75 mM citrate–0.15 M phosphate buffer (pH 5.5). Inhibitor concentrations were 1.76 mM in citrate–phosphate buffer (pH 5.5), unless otherwise noted. Reactions were incubated for 20 min in a shaking water bath at 37 °C, and the reactions were stopped by injection of 0.1 mL of 50% trichloroacetic acid. $^{14}CO_2$ was absorbed for 30 min, whereupon the plastic center wells were transferred to scintillation vials. The radioactivity was counted and compared with controls containing no inhibitor.

Phenylalanine Hydroxylase Assay. Phenylalanine hydroxylase (PH, EC 1.14.3.1) activity was determined by measuring the phenylalanine-dependent change in absorbance of the tetrahydropteridine cofactor, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$), as it is oxidized to the dihydro form.[23] The reaction mixture (total volume 1.0 mL) consisted of 0.17 mM $DMPH_4$, 1 mM substrate (L-phenylalanine or test compound) plus or minus 1 mM inhibitor, phenylalanine hydroxylase [2 mg of protein, 45% $(NH_4)_2SO_4$ fraction from guinea pig liver] in 0.1 M Tris-HCl, pH 7.4, at 30 °C. L-Phenylalanine, phenylalanine hydroxylase, and the compound to be tested were preincubated together for 2.5 min at 30 °C before initiation of the reaction by the addition of the cofactor. The change of absorbance at 330 nm was monitored, and the inhibition was measured as the decrease in absorbance in the presence of the compound relative to the control incubation.

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Bradykinin Analogues Containing N^{α}-Methyl Amino Acids

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Analogues of bradykinin were synthesized containing single substitutions by N^{α}-methyl amino acids in the 1, 4, 5, 8, and 9 positions. [MeArg^1]Bradykinin possessed 60% of the muscle-contracting activity of the parent compound in a guinea pig ileum assay. The other analogues were very weak agonists (<2%) and, disappointingly, failed to show blocking activity except at very high doses.

Bradykinin (1) was one of the first peptides to be in-

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
1

vestigated in what might be called the modern era of peptide synthesis, i.e., since du Vigneaud's preparation of oxytocin in 1953.[1] In fact, the structure of the isolated substance was reported incorrectly,[2] and the correct structure was first obtained by synthesis based on what must have been an inspired hunch by Boissonnas.[3-5]

The pharmacological effects of bradykinin are numerous, while its physiological role is still poorly understood. A major stumbling block to elucidation of the latter has been the lack, despite the preparation of a hundred or two analogues, of an effective in vivo inhibitor. Thus, finding a potent bradykinin blocker is still, after nearly 20 years, a challenging problem in medicinal chemistry. The biological activities of bradykinin and the need for an inhibitor have been reviewed in an excellent fashion by Marshall[6] and by Regoli.[7]

Our approach to the search for a bradykinin blocker was to prepare a series of analogues in which each of the six amino acids bearing an α-NH_2 group was successively replaced by an *N*-methyl amino acid. Although the *N*-methyl group certainly profoundly influences the conformation of the peptide backbone, its effect on receptor binding really cannot be predicted, since the latter could depend largely on the various side groups attached to the main chain. In a small linear peptide such as bradykinin, there is great uncertainty about the effects of N-methylation because the parent compound has a random conformation in solution.[8] N-Methylation of both angiotensin[9] and enkephalin[10] analogues has given compounds with improved pharmacological properties, such as enhanced potency and duration of action. It was assumed this was due to resistance to proteolysis[11] and, in fact, [Sar^1]angiotensin II has been shown to be completely stable in the presence of angiotensinase.[12] Also, in an

(1) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953).
(2) D. F. Elliott, G. P. Lewis, and E. W. Horton, *Biochem. J.*, **76**, 16P (1960).
(3) R. A. Boissonnas, S. Guttmann, and P. A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).
(4) R. A. Boissonnas, S. Guttmann, and P. A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1481 (1960).
(5) S. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, **44**, 1713 (1961).
(6) J. Turk, P. Needleman, and G. R. Marshall, *J. Med. Chem.*, 18, 1135 (1975).
(7) W. K. Park, S. A. St.-Pierre, J. Barabe, and D. Regoli, *Can. J. Biochem.*, **56**, 92 (1978).
(8) V. T. Ivanov, M. P. Filatova, Z. Reissman, T. O. Reutova, E. S. Efremov, V. S. Pashkov, S. G. Galaktionov, G. L. Grigoryan, and Y. A. Ovchinnikov, "Peptides: Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Eds., Ann Arbor Science Publishers, Ann Arbor, Michigan, 1975, p 151; R. E. London, J. M. Stewart, J. R. Cann, and N. A. Matwiyoff, *Biochemistry*, **17**, 2270 (1978).
(9) D. T. Pals, F. D. Masucci, G. S. Denning, Jr., F. Sipos, and D. C. Fessler, *Circ. Res.*, **29**, 673 (1971).
(10) D. Roemer and J. Pless, *Life Sci.*, **24**, 612 (1979).
(11) R. K. Turker, M. M. Hale, M. Yamamoto, C. S. Sweet, and F. M. Bumpus, *Science*, **177**, 1203 (1972).
(12) M. M. Hall, M. C. Khosla, P. A. Khairallah, and F. M. Bumpus, *J. Pharmacol. Exp. Ther.*, **188**, 222 (1974).

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Table I. Synthesis of H-MeArg(Tos)-OH and Derivatives

Bzl-Arg(Tos)-OH **2** H-MeArg(Tos)-OH **4** Z-MeArg(Tos)-OH **6**
Bzl-MeArg(Tos)-OH **3** H-MeArg(Tos)-OBzl **5**

no.	scale, mmol	yield, %	TLC R_f	TLC solvent system	mp, °C	$[\alpha]_D$, deg (solvent)	formula[a]
2	20	93	0.04	B	138-141 (MeOH)[b]	+15 (MeOH)	$C_{20}H_{26}N_4O_4S\cdot CH_3OH$
3	3	60	0.41	G	amorph	+1 (MeOH)	$C_{21}H_{28}N_4O_4S\cdot H_2O$
4	78	94	0.33	G	210-212 (MeOH)	+7 (MeOH)	$C_{14}H_{22}N_4O_4S$
5[c]	20	77	0.54	G	106-108 (EtOAc)	−1 (MeOH)	$C_{21}H_{28}N_4O_4S$
6	15	73	0.30	E	amorph	−19 (DMF)	$C_{22}H_{28}N_4O_6S$

[a] All compounds were analyzed for C, H, N, and S. [b] The crystallization solvent is shown in parentheses. [c] Procedure of R. H. Mazur and J. M. Schlatter, *J. Org. Chem.*, **28**, 1025 (1963).

Table II. [MeArg[1]]Bradykinin

Z-MeArg(Tos)-Pro-Pro-Gly-OMe **7** Z-MeArg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(Tos)-OH **9**
Z-MeArg(Tos)-Pro-Pro-Gly-OH **8** H-MeArg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH **10**

no.	scale, mmol	yield, %	TLC R_f	TLC solvent system	purifn	$[\alpha]_D$, deg (solvent)	formula[a]
7[b]	10	57	0.10	D	LPLC[c]	−58 (DMF)	$C_{35}H_{47}N_7O_9S\cdot H_2O$
8	5.4	94	0.27	C		−76 (DMF)	$C_{34}H_{45}N_7O_9S\cdot 2.5H_2O$[d]
9[e]	1.3	12	0.61	F	LPLC[f]		$C_{73}H_{93}N_{15}O_{17}S_2\cdot 4.5H_2O$
10	0.12	75	0.05	G	Dowex 2[g]	−77 (MeOH)	$C_{51}H_{75}N_{15}O_{11}\cdot 2CH_3CO_2H\cdot 4H_2O$[h]

[a] All compounds were analyzed for C, H, N, and S, except for **10** which was analyzed for C, H, and N only. [b] The intermediates were Z-Pro-Pro-OH, Z-Pro-Pro-Gly-OMe, and H-Pro-Pro-Gly-OMe·HBr. [c] LPLC = low-pressure liquid chromatography. Woelm silica, 5% EtOH–$CHCl_3$. [d] N: calcd, 12.68; found, 12.12. [e] Z-MeArg(Tos)-Pro-Pro-Gly-OTc prepared in situ was coupled with H-Phe-Ser-Pro-Phe-Arg(Tos)-OH.[12] [f] CC-4 silica, 2% MeOH–CH_2Cl_2. Irreversible adsorption partly accounted for the low yield. [g] Acetate form; eluted with 1 N HOAc. [h] Amino acid analysis: MeArg, not determined; Pro, 3.3; Gly, 1.1; Phe, 1.9; Ser, 0.8; Arg, 0.9.

especially striking example, [MePhe[8]]angiotensin II, replacement of phenylalanine by *N*-methylphenylalanine transformed the natural hormone to a powerful antagonist.[13] Therefore, we hoped that our bradykinin derivatives would not only be inhibitors but would also benefit from the other favorable effects of N-methylation.

Thus, our objective was the synthesis of six compounds:[14] [MeArg[1]]-, [Sar[4]]-, [MePhe[5]]-, [MeSer[6]]-, [MePhe[8]]-, and [MeArg[9]]bradykinin. In fact, we were unable to obtain [MeSer[6]]bradykinin but were able to prepare the other five. The chief difficulty in this work was the surprising degree of steric hindrance at the amino group caused by mono-N-methylation. In our hands, successful coupling to an *N*-methyl amino acid or peptide could only be achieved by carbodiimide. Active ester and mixed anhydride procedures simply failed. The *N*-methyl amino acids were synthesized by the method of Quitt.[15] Since this sequence required catalytic hydrogenation, tosyl was chosen to protect the side chain of arginine.[17,18] Removal of the tosyl groups was carried out with liquid hydrogen fluoride.[19] Tosylarginine gave satisfactory yields on benzylation, methylation, and debenzylation. Additionally, the carbobenzoxy derivative and benzyl ester of H-MeArg(Tos)-OH were prepared. The properties of these compounds are given in Table I.

The syntheses broadly followed the scheme of Boissonnas[18] in that a final 4,5 coupling was carried out. As will be explained below, this was modified for the [MePhe[5]] analogue. Standard coupling procedures were generally used. Of particular value was the azide method under strictly anhydrous conditions[20] when the amino component was a peptide in the form of its triethylamine salt. Many intermediates were satisfactorily purified by reprecipitation. When this simple method did not succeed, chromatography and countercurrent distribution were employed.

[MeArg[1]]Bradykinin (Table II). Formation of Z-MeArg(Tos)-OH proceeded in satisfactory yield. Crude intermediates (all oils) were carried through to Z-MeArg-(Tos)-Pro-Pro-Gly-OMe—also an oil—which was conveniently purified by low-pressure liquid chromatography on silica gel using ethanol–chloroform for elution. Z-

(13) C. Pena, J. M. Stewart, and T. C. Goodriend, *Life Sci.*, 14, 1331 (1974).

(14) Standard abbreviations are used. For the sake of simplicity, N^{α}-methylarginine is written as MeArg, etc. Amino acids have the L configuration unless otherwise noted. LPLC = low-pressure liquid chromatography; CCD = countercurrent distribution; ONp = *p*-nitrophenyl ester; OTc = 2,4,5-trichlorophenyl ester.

(15) P. Quitt, J. Hellerbach, and K. Vogler, *Helv. Chim. Acta*, **46**, 327 (1963). Our *N*-methyl amino acids were synthesized before the procedure of Benoiton[16] appeared. The latter undoubtedly is the method of choice from the standpoint of negligible racemization, and we use it routinely for amino acids with unreactive side chains. Since the bradykinin analogues did not have the desired biological activity, it did not seem worthwhile to repeat the work. Also, there is the possibility that successive purifications during the syntheses eliminated any small amount of D isomer originally present.

(16) S. T. Cheung and N. L. Benoiton, *Can. J. Chem.*, **55**, 906, 916 (1977).

(17) E. Schnabel and C. H. Li, *J. Am. Chem. Soc.*, **82**, 4576 (1960).

(18) S. Guttmann, J. Pless, and R. A. Boissonnas, *Helv. Chim. Acta*, **45**, 170 (1962).

(19) R. H. Mazur and G. Plume, *Experientia*, **24**, 661 (1968).

(20) R. H. Mazur and J. M. Schlatter, *J. Org. Chem.*, **29**, 3212 (1964). H. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

Table III. [Sar⁴]Bradykinin

Z-Arg(Tos)-Pro-Pro-Sar-OMe
11
Z-Arg(Tos)-Pro-Pro-Sar-OH
12
Z-Arg(Tos)-Pro-Pro-Sar-Phe-Ser-Pro-Phe-Arg(Tos)-OH
13
H-Arg(Tos)-Pro-Pro-Sar-Phe-Ser-Pro-Phe-Arg(Tos)-OH
14
H-Arg-Pro-Pro-Sar-Phe-Ser-Pro-Phe-Arg-OH
15

no.	scale, mmol	yield, %	TLC R_f	TLC solvent system	purifn	$[\alpha]_D$, deg (solvent)	formula[a]
11[b]	10	52	0.53	B	$K = 0.36$[c]	−86 (MeOH)	$C_{35}H_{47}N_7O_9S\cdot(C_2H_5)_2O$
12	10	87			$CHCl_3$-Et_2O[d]	−79 (MeOH)	$C_{34}H_{45}N_7O_9S$
13[e]	4	45	0.56	G	$K = 0.54$	−76 (MeOH)	$C_{73}H_{93}N_{15}O_{17}S_2$
14	3.6	32	0.26	G	$K = 1.50$	−67 (CH_3COOH)	$C_{65}H_{87}N_{15}O_{15}S_2\cdot 2H_2O$[f]
15	0.72	94	0.02	G	IRC-50		$C_{51}H_{75}N_{15}O_{11}\cdot 2CH_3CO_2H\cdot 2H_2O$[g]

[a] All compounds were analyzed for C, H, N, and S, except for 15 which was analyzed for C, H, and N only. [b] The intermediates were Z-Pro-Pro-OH, Z-Pro-Pro-Sar-OMe, and Pro-Pro-Sar-OMe·HBr. [c] The partition coefficient, K, was calculated from the position of the peak tube after countercurrent distribution. [d] The product was reprecipitated from the solvents indicated. [e] Z-Arg(Tos)-Pro-Pro-Sar-OTc prepared in situ was coupled with H-Phe-Ser-Pro-Phe-Arg(Tos)-OH.[12] [f] Amino acid analysis: Arg, 2.2; Pro, 3.0; Sar, 1.0; Phe, 2.0; Ser, 0.9. [g] Amino acid analysis: Arg, 1.9; Pro, 3.1; Sar, 1.1; Phe, 2.0; Ser, 0.9.

Table IV. [MePhe⁵]Bradykinin

Z-Gly-MePhe-Ser-OMe
16
Z-Gly-MePhe-Ser-$NHNH_2$
17
Z-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
18
H-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
19
Z-Pro-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
20
H-Pro-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
21
Z-Pro-Pro-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
22
H-Pro-Pro-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
23
Z-Arg(Tos)-Pro-Pro-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
24
H-Arg(Tos)-Pro-Pro-Gly-MePhe-Ser-Pro-Phe-Arg(Tos)-OH
25
H-Arg-Pro-Pro-Gly-MePhe-Ser-Pro-Phe-Arg-OH
26

no.	scale, mmol	yield, %	TLC R_f	TLC solvent system	purifn[a]	$[\alpha]_D$, deg (solvent)	formula[b]
16[c]	53	44	0.36	A	$K = 0.48$	−16 (MeOH)	$C_{24}H_{29}N_3O_7\cdot 2.5H_2O$
17	19	59	0.10	A	$CHCl_3$-Et_2O	−60 (MeOH)	$C_{23}H_{29}N_5O_6$
18[d]	45	78	0.68	G	$CHCl_3$-Et_2O	−51 (MeOH)	$C_{50}H_{61}N_9O_{12}S$
19	28	94	0.38	G	MeOH-Et_2O	−46 (MeOH)	$C_{42}H_{55}N_9O_{10}S\cdot H_2O$
20[e]	10	78	0.75	G	$CHCl_3$-Et_2O	−72 (MeOH)	$C_{55}H_{68}N_{10}O_{13}S\cdot 0.5H_2O$
21	15	95	0.30	G	MeOH-Et_2O	−58 (MeOH)	$C_{47}H_{62}N_{10}O_{11}S\cdot 1.5H_2O$[f]
22[e]	10	74	0.73	G	$CHCl_3$-Et_2O	−96 (MeOH)	$C_{60}H_{75}N_{11}O_{14}S\cdot H_2O$
23	6.6	95	0.25	G	MeOH-Et_2O	−134 (MeOH)	$C_{52}H_{69}N_{11}O_{12}S$
24[g]	6.0	90	0.75	G	$CHCl_3$-Et_2O	−63 (MeOH)	$C_{73}H_{93}N_{15}O_{17}S_2\cdot 1.5H_2O$
25	5.2	30	0.25	G	$K = 2.0$	−71 (MeOH)	$C_{65}H_{87}N_{15}O_{15}S_2$[h]
26	0.72	42	0.05	G	IRC-50	−74 (MeOH)	$C_{51}H_{75}N_{15}O_{11}\cdot 2CH_3CO_2H\cdot 3H_2O$[i]

[a] See footnotes c and d, Table III. [b] All compounds were analyzed for C, H, N, and S, except for 16, 17, and 26 which were analyzed for C, H, and N only. [c] The intermediates were Z-MePhe-OH, Z-MePhe-ONp, Z-MePhe-Ser-OMe, and MePhe-Ser-OMe. [d] Hydrazide 17 was coupled with H-Pro-Phe-Arg(Tos)-OH.[12] [e] Z-Pro-ONp was used. [f] N: calcd, 13.98; found, 13.38. [g] Z-Arg(Tos)-OTc was used. [h] Amino acid analysis: Arg, 2.0; Pro, 3.0; Gly, 1.0; MePhe, 1.0; Ser, 0.9; Phe, 1.0. [i] Amino acid analysis: Arg, 1.9; Pro, 3.0; Gly, 1.1; MePhe, 1.1; Ser, 0.9; Phe, 1.0.

MeArg(Tos)-Pro-Pro-Gly-OH was converted to the trichlorophenyl ester, which was used without isolation for the final coupling. The yield in this step was very bad but the desired product was obtained. Because of the small amount of material available, all protecting groups were removed in one operation. Final purification was achieved by ion-exchange chromatography on Dowex 2, a strong anion exchanger, in the acetate form.

[Sar⁴]Bradykinin (Table III). As can be seen from the table, three sarcosine-containing intermediates were purified by countercurrent distribution. The two-phase system used in all CCD separations was methanol–water–chloroform–carbon tetrachloride[21] in the ratios 37:10:26:27. These were chosen to give phases of equal volumes at equilibrium, thus using the solvents as efficiently as possible. Yields of homogeneous material were moderate. The final deprotected 9-peptide was subjected to gradient elution chromatography on IRC-50.[18,19] A straight-line gradient from 0.1 N acetic acid to glacial acetic acid proved effective and was used routinely. [Sar⁴]Bradykinin has been reported[22] without experimental details, and serine may have been in the *O*-acetyl form. The compound was not tested for blocking activity.

[MePhe⁵]Bradykinin (Table IV). Because of steric problems associated with *N*-methyl amino acids, MePhe

(21) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, 41, 1273 (1958).

(22) E. Nicolaides and M. Lipnik, *J. Med. Chem.*, 9, 958 (1966). We thank a referee for telling us that [Sar⁴]bradykinin was synthesized by J. Turk (Thesis, Washington University, St. Louis, 1976). In rat uterus and rat pressor assays, the compound had 0.1% of the agonist activity of bradykinin and was not an inhibitor.

Table V. [MePhe^8]Bradykinin

Z-Arg(Tos)-Pro-Pro-Gly-OEt
27
Z-Arg(Tos)-Pro-Pro-Gly-NHNH$_2$
28
Z-Pro-MePhe-OMe
29
Z-Pro-MePhe-OH
30
Z-Pro-MePhe-Arg(Tos)-OH
31
H-Pro-MePhe-Arg(Tos)-OH
32
Z-Phe-Ser-Pro-MePhe-Arg(Tos)-OH
33
H-Phe-Ser-Pro-MePhe-Arg(Tos)-OH
34
Z-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-MePhe-Arg(Tos)-OH
35
H-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-MePhe-Arg(Tos)-OH
36
H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-MePhe-Arg-OH
37

no.	scale, mmol	yield, %	TLC R_f	TLC solvent system	purifin[a]	$[\alpha]_D$, deg (solvent)	formula[b]
27[c]	200	92	0.22	A	EtOAc-Et_2O	−80 (MeOH)	$C_{35}H_{47}N_7O_9S$
28	10	98	0.63	G	$CHCl_3$-Et_2O	−60 (MeOH)	$C_{33}H_{45}N_9O_8S\cdot 0.5(C_2H_5)_2O$
29	300	31	0.60	A	$K = 0.25$[d]	−111 (MeOH)	$C_{24}H_{28}N_2O_5$
30	50	91	0.90	G		−74 (MeOH)	$C_{23}H_{26}N_2O_5\cdot 0.66H_2O$
31	40	60	0.74	G	$CHCl_3$-Et_2O	−37 (MeOH)	$C_{36}H_{44}N_6O_8S$
32	24	83	0.35, 0.39	G	$K = 4.3$	−19 (MeOH)	$C_{28}H_{38}N_6O_6S\cdot 0.5H_2O$
33[e]	25	71	0.64, 0.68	G	$K = 0.52$	−32 (MeOH)	$C_{48}H_{58}N_8O_{11}S$
34	24	97	0.52, 0.56	G	MeOH	−31 ($CHCl_3$)	$C_{40}H_{52}N_8O_9S\cdot 0.5H_2O$
35	24	67	0.44	G	$K = 0.43$	−48 (MeOH)	$C_{73}H_{93}N_{15}O_{17}S_2\cdot 1.5H_2O$
36	6.5	53	0.26	G	$K = 1.37$	−50 (MeOH)	$C_{65}H_{87}N_{15}O_{15}S_2\cdot 2H_2O$[f]
37	0.72	73	0.07	G	IRC-50	−82 (H_2O)	$C_{51}H_{75}N_{15}O_{11}\cdot 3CH_3CO_2H\cdot 2H_2O$[g]

[a] See footnotes *c* and *d*, Table III. [b] All compounds were analyzed for C, H, N, and S, except for **29**, **30**, and **37** which were analyzed for C, H, and N only. [c] The intermediates were Z-Pro-Pro-OH, Z-Pro-Pro-Gly-OEt, and H-Pro-Pro-Gly-OEt·HBr. [d] Crystallized from benzene-cyclohexane, mp 82–84 °C. [e] Z-Phe-Ser-NHNH$_2$[3] was used. [f] Amino acid analysis: Arg, 2.2; Pro, 3.0; Gly, 0.9; Phe, 1.0; Ser, 0.9; MePhe, 1.0. [g] Amino acid analysis: Arg, 1.9; Pro, 3.1; Gly, 1.0; Phe, 1.1; Ser, 1.0; MePhe, 1.0.

was insulated between glycine and serine. Although Z-MePhe-Ser-OMe was homogeneous according to CCD, the oily product would not give a good analysis. Z-Gly-MePhe-Ser-OMe was also purified by CCD and yielded a hydrazide which was coupled to H-Pro-Phe-Arg(Tos)-OH. The synthesis was completed by adding the remaining amino acids stepwise using active esters.

[MeSer^6]Bradykinin. A number of attempts were made to use MeSer to prepare a suitably protected derivative of H-Phe-MeSer-OH or H-Phe-MeSer-Pro-OH. We were unable to find a satisfactory solution to the combination problem of unreactivity of the methylamino group and the need for reversible protection of the hydroxyl group. In addition, some experiments suggested that MeSer and its derivatives underwent elimination reactions to dehydroalanine compounds much more readily than the corresponding serine derivatives.

[MePhe^8]Bradykinin (Table V). Like the [MePhe^5] analogue, MePhe was sandwiched between its neighbors, proline and arginine. Z-Pro-MePhe-OMe was a convenient intermediate, since it was actually crystalline. The next steps were saponification to Z-Pro-MePhe-OH, conversion to the *p*-nitrophenyl ester, and coupling with H-Arg-(Tos)-OH. It was recognized that some racemization might occur in this sequence, but it has been reported that *N*-methyl amino acids racemize less readily than their corresponding nonmethylated homologues.[23] The whole subject of amino acid racemization has been thoroughly reviewed.[23,24]

In fact, however, the product probably contained racemic MePhe as shown by a double spot on TLC after removal of the carbobenzoxy group. The next two compounds, Z-Phe-Ser-Pro-MePhe-Arg(Tos)-OH and H-Phe-Ser-Pro-MePhe-Arg(Tos)-OH, also looked like approximately 1:1 mixtures of diastereoisomers as would be predicted for DL-MePhe. It would appear, therefore, that Z-Pro-MePhe-OH did not yield an optically homogeneous product and that possibly some racemization mechanism other than the azlactone process is operating. The synthesis was completed to give [DL-MePhe^8]bradykinin. [MePhe^8]Bradykinin has been prepared by an entirely different procedure[25] and was not tested for inhibitory activity.[26a]

[MeArg^9]Bradykinin (Table VI). The major problem was the very pronounced tendency of dipeptides of the form A-MeB to cyclize to a diketopiperazine. This, for example, has caused difficulties in the solid-phase synthesis of peptides bearing a C-terminal *N*-methyl amino acid. When the intermediate A-MeB-[P][26b] is liberated from its salt, cyclization cleaves the dipeptide off the resin and A-MeB simply does not appear in the final product.[27,28] An extreme case of this reaction was encoutered with Arg-Sar-NH$_2$, where no coupling conditions could be found that would produce Boc-Gly-Arg-Sar-NH$_2$ in competition with diketopiperazine formation.[29] In the present work, Z-Pro-Phe-MeArg(Tos)-OBzl was obtained, although in low yield. The rest of the synthesis proceeded unevent-

(23) M. Goodman and C. Glaser, "Peptides: Chemistry and Biochemistry", B. Weinstein and S. Lande, Eds., Marcel Dekker, New York, 1970, p 267.
(24) E. Wünsch, "Methoden der Organischen Chemie. Synthese von Peptiden. I", E. Muller, Ed., Georg Thieme Verlag, Stuttgart, 1974, pp 34–41.
(25) N. A. Krit, G. A. Ravdel, and V. T. Ivanov, *Bioorg. Khim.*, **2**, 1455 (1976).
(26) (a) G. A. Popkova, M. B. Astapova, Y. I. Lisunkin, G. A. Ravdel, and N. A. Krit, *Bioorg. Khim.*, **2**, 1606 (1976). (b) Because of problems with the composition system, the usual symbol of the polymer support resin, a circled P, will be represented by [P].
(27) M. C. Khosla, R. R. Smeby, and F. M. Bumpus, *J. Am. Chem. Soc.*, **94**, 4721 (1972).
(28) C. Pena and J. M. Stewart, *Life Sci.*, **14**, 1331 (1974).
(29) R. A. Mikulec, "Abstracts of Papers", 8th Great Lakes Regional Meeting of the American Chemical Society, West Lafayette, Ind., 1974, American Chemical Society, Washington, D.C., 1974, Abstr BIOL 56.

Table VI. [MeArg^9]Bradykinin

Z-Phe-MeArg(Tos)-OBzl
38
Z-Pro-Phe-MeArg(Tos)-OBzl
39
H-Pro-Phe-MeArg(Tos)-OH
40
Z-Phe-Ser-Pro-Phe-MeArg(Tos)-OH
41
H-Phe-Ser-Pro-Phe-MeArg(Tos)-OH
42
Z-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-MeArg(Tos)-OH
43
H-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-MeArg(Tos)-OH
44
H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-MeArg-OH
45

no.	scale, mmol	yield, %	TLC R_f	TLC solvent system	purifin[a]	$[\alpha]_D$, deg (solvent)	formula[b]
38	10	72	0.49	A	K = 0.18	−32 (MeOH)	$C_{38}H_{43}N_5O_7S$
39[c]	80	48	0.29	A	K = 0.29	−54 (MeOH)	$C_{43}H_{50}N_6O_8S$
40	19	88	0.35	G	MeOH-Et_2O	−46 (MeOH)	$C_{28}H_{38}N_6O_6S$
41[d]	10	85	0.05	B	$CHCl_3$-Et_2O	−50 (MeOH)	$C_{48}H_{58}N_8O_{11}S\cdot 0.5H_2O$
42	7.4	92	0.30	G	MeOH-Et_2O	−46 (MeOH)	$C_{40}H_{52}N_8O_9S\cdot H_2O$
43	6.5	83	0.75	G	$CHCl_3$-Et_2O	−64 (MeOH)	$C_{73}H_{93}N_{15}O_{17}S_2$
44	4.6	45	0.27	G	K = 3.0	−61 (MeOH)	$C_{65}H_{87}N_{15}O_{15}S_2$[e]
45	0.72	83	0.02	G	IRC-50	−79 (MeOH)	$C_{51}H_{75}N_{15}O_{11}\cdot 2CH_3CO_2H\cdot 4H_2O$[f]

[a] See footnotes *c* and *d*, Table III. [b] All compounds were analyzed for C, H, N, and S, except 45 which was analyzed for C, H, and N only. [c] The intermediate was H-Phe-MeArg(Tos)-OBzl·2HBr. [d] Z-Phe-Ser-$NHNH_2$[3] was used. [e] Amino acid analysis: Arg, 1.1; Pro, 3.0; Gly, 0.9; Phe, 2.1; Ser, 0.9; MeArg, 1.0 (not a reliable value). [f] Amino acid analysis: Arg, 0.9; Pro, 3.0; Gly, 0.9; Phe, 2.1; Ser, 0.9; MeArg, 1.2 (not a reliable value).

Table VII. Smooth Muscle Stimulating Potencies of Analogues Determined on Isolated Guinea Pig Ilea[a]

analogue	stimulating potency	95% fiducial limits
bradykinin triacetate	1.000	standard
[MeArg^1]bradykinin	0.61	0.526-0.722
[Sar^4]bradykinin	0.0039	0.0036-0.0043
[MePhe^5]bradykinin	0.010	0.009-0.011
[MePhe^8]bradykinin	0.015	0.013-0.018
[MeArg^9]bradykinin	0.0046	0.0040-0.0054

fully. Since the experimental details of peptide synthesis are well known or readily available, it seems desirable in the interest of conserving space to limit the number of examples to a representative few. Thus, only the preparation of [MeArg^9]bradykinin is described under Experimental Section.

Table VII shows smooth-muscle stimulating potencies of the analogues relative to bradykinin triacetate, as determined on isolated guinea pig ilea. All of the compounds demonstrated some stimulating activity with dose–response curves essentially parallel to those produced by bradykinin triacetate. [Sar^4]Bradykinin and [MeArg^9]-bradykinin demonstrated only weak stimulation activity (about 0.4% of bradykinin). The [MePhe^5] and [MePhe^8] analogues were 1 and 1.5% as potent as bradykinin. [MeArg^1]Bradykinin demonstrated considerable bradykinin-like activity, with a relative potency of 61%.

In the tests for inhibition, all of the analogues produced initial contractions of guinea pig ileum smooth muscle as anticipated from their stimulatory properties. The tissue gradually relaxed during the next 4–12 min with the analogues still in the bath. Table VIII shows the effects of the analogues on contractions produced by bradykinin, acetylcholine, and prostaglandin E_2 when the agonists were added to the bath after the tissue tone had returned to essentially control levels. Some generalized reductions in contractions were seen at this time in the presence of all of the analogues. This inhibitory effect was the most potent with [MeArg^1]bradykinin, and it appeared to be somewhat specific against bradykinin-induced contractions with all of the analogues except [Sar^4]bradykinin.

In related experiments, we tested bradykinin itself as an inhibitor of contractions produced by further additions of bradykinin, by acetylcholine, and by prostaglandin E_2.

Table VIII. Effect of Analogues on Contractions of Isolated Guinea Pig Ilea Produced by Bradykinin (Bdkn), Acetylcholine (ACh), and Prostaglandin E_2 (PGE_2)

compound	concn, μg/mL	no. of expts	mean % change from control contractions produced by: Bdkn	ACh	PGE_2
[MeArg^1]-Brdkn	0.01	1	−88	−29	−28
	0.10	1	−100	−67	−88
	1.00	1	−100	−100	−95
	10.00	1	−100	−97	−83
[Sar^4]-Brdkn	1	1	+5	−32	−20
	3	1	+21	−9	−27
	10	2	−25	−58	−85
	30	1	−40	−70	−69
[MePhe^5]-Brdkn	10	1	−32	−31	+1
[MePhe^8]-Brdkn	1	1	−27	−6	−17
	3	1	−81	−41	−60
	10	2	−97	−75	−88
[MeArg^9]-Brdkn	10	1	−64	−28	+7

The results of these experiments were similar to those we obtained with the *N*-methylbradykinin analogues: after the initial contractions subsided, bradykinin-induced contractions were specifically inhibited as long as the initial bradykinin remained in the bath (at least 45 min). If the initial bradykinin was rinsed out of the bath after a contact time of 11 or 12 min, the sensitivity to bradykinin quickly returned to normal. This suggests that the depressant effect of bradykinin, and probably of the analogues, is best described as autoinhibition. It may be due to continued occupation of bradykinin receptors by the compound even after the contractions have subsided; this would prevent stimulation by the addition of more bradykinin. These results support the conclusion that the analogues (with the possible exception of [Sar^4]bradykinin) cause contractions of guinea pig ileal smooth muscle through stimulation of bradykinin receptors.

Experimental Section

All products were essentially homogeneous, as determined by TLC on neutral silica. The following eluting solvents were used:

A, 5% MeOH–$CHCl_3$; B, 10% MeOH–$CHCl_3$; C, 10% EtOH–$CHCl_3$; D, CH_2Cl_2–MeOH–H_2O (84:15:1); E, CH_2Cl_2–MeOH–HOAc–H_2O (83:15:1:1); F, $CHCl_3$–MeOH–H_2O–NH_4OH (64:30:4:2); G, *n*-BuOH–HOAc–H_2O (7:1:2). Spots were detected by the *tert*-butyl hypochlorite–starch iodide method.[30] Solvents for rotation and reprecipitation were the following: CH_3COOH, acetic acid; $CHCl_3$, chloroform; DMF, dimethylformamide; EtOAc, ethyl acetate; Et_2O, ethyl ether; MeOH, methanol; H_2O, water. Rotations were measured at room temperature at approximately 1% concentration. Where analyses are indicated only by symbols of the elements, analytical results were within 0.4% of theoretical values.

Z-Arg(Tos)-Pro-Pro-Gly-OEt (27). In 500 mL of CH_2Cl_2 were dissolved 75.6 g (0.20 mol) of H-Pro-Pro-Gly-OEt·HBr and 97.0 g (0.21 mol) of Z-Arg(Tos)-OH. After the solution cooled to −5 °C, *N*-methylmorpholine (22.4 mL, 0.20 mol) and dicyclohexylcarbodiimide (45.3 g, 0.22 mol) were added with stirring. After the solution stirred overnight at room temperature, the dicyclohexylurea was removed by filtration. The CH_2Cl_2 filtrate was washed with 1 N HCl, water, and 1 N $KHCO_3$, dried over Na_2SO_4, and the CH_2Cl_2 was distilled off under vacuum. The residue turned to a powder upon trituration with Et_2O: yield 145.5 g (98%); mp 81–91 °C; TLC (A) R_f 0.22 with trace impurities at R_f 0.00, 0.43, 0.95.

Z-Arg(Tos)-Pro-Pro-Gly-$NHNH_2$ (28). Ester **27** (7.42 g, 10 mmol) was dissolved in 40 mL of MeOH, and 4.9 mL (100 mmol) of 100% hydrazine hydrate was added. Conversion to the hydrazide was complete after 24 h at room temperature. The solution was cooled in an ice bath and neutralized with 100 mmol of glacial HOAc. The MeOH was removed under vacuum. The residual oil was dissolved in $CHCl_3$, the $CHCl_3$ was washed with water and dried over Na_2SO_4, and the solution was taken to dryness. The residue solidified upon trituration with Et_2O: yield 7.12 g (98%); TLC (G) R_f 0.63 with trace impurities at R_f 0.75, 0.95.

Z-Pro-Phe-MeArg(Tos)-OBzl (39). In 300 mL of CH_2Cl_2 were dissolved Z-Pro (19.9 g, 80 mmol) and H-Phe-MeArg-(Tos)-OBzl·2HBr (60.0 g, 80 mmol). The solution was cooled to 0 °C and *N*-methylmorpholine (20.4 mL, 182 mmol) and dicyclohexylcarbodiimide (16.5 g, 80 mmol) were added. After the solution was left standing overnight at room temperature, the reaction was worked up as described for compound **27**: TLC (A) R_f 0.29 with major impurities at R_f 0.19, 0.41. The total crude product was purified in two batches by countercurrent distribution in a 200 tube Craig–Post machine having 40-mL phases; 400 transfers using MeOH–H_2O–$CHCl_3$–CCl_4 (37:10:26:27) gave a good separation: yield 31.1 g (48%), oil; K = 0.29; TLC (A) R_f 0.29 with trace impurities at R_f 0.12 (K = 0.21), 0.17 (K = 0.21), 0.52 (K = 0.27), 0.78 (K = 0.25).

H-Pro-Phe-MeArg(Tos)-OH (40). Compound **39** (15.4 g, 19 mmol) in 150 mL of 90% HOAc was hydrogenated at room temperature and 60 psi over 1.5 g of palladium metal. The theoretical amount of hydrogen was absorbed in 1.5 h. The catalyst was removed by filtration and the filtrate taken to dryness under vacuum. The crude product was precipitated from MeOH–Et_2O: yield 9.81 g (88%); TLC (G) R_f 0.35 with trace impurities at R_f 0.20, 0.50, 0.68.

Z-Phe-Ser-Pro-Phe-MeArg(Tos)-OH (41). Z-Phe-Ser-$NHNH_2$ (4.97 g, 12 mmol) was suspended in 50 mL of DMF and brought into solution with 9.5 mL (60 mmol) of 6.3 N HCl in dioxane. The solution was cooled to −30 °C and 1.76 mL (13 mmol) of isoamyl nitrite was added dropwise with good stirring. After 5 min, *N*-methylmorpholine (6.7 mL, 60 mmol) was added slowly, keeping the temperature below −30 °C. Compound **40** (5.87 g, 10 mmol) in 10 mL of DMF containing 1.2 mL (10 mmol) of *N*-methylmorpholine was added to the azide mixture at −30 °C. The mixture was stirred overnight at 5 °C and poured into a large volume of 1 N HCl. The crude solid product was precipitated from $CHCl_3$–Et_2O: yield 8.07 g (85%); TLC (B) R_f 0.05, trace impurity at R_f 0.53.

H-Phe-Ser-Pro-Phe-MeArg(Tos)-OH (42). Hydrogenation of 7.07 g (7.4 mmol) of Z-Phe-Ser-Pro-Phe-MeArg(Tos)-OH was carried out as described for compound **40**. The crude product was precipitated from MeOH–Et_2O: yield 5.61 g (92%); TLC (G) R_f 0.30, homogeneous.

Z-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-MeArg(Tos)-OH (43). Compound **28** (5.68 g, 7.8 mmol) was converted to the azide at −30 °C using 39 mmol of 6.3 N HCl in dioxane, 8.2 mmol of isoamyl nitrite, and 39 mmol of *N*-methylmorpholine as described for compound **41**. Compound **42** (5.36 g, 6.5 mmol) in DMF containing 6.5 mmol of *N*-methylmorpholine was added, and the reaction was carried out as described for compound **41**: yield 8.20 g (83%) after precipitation from $CHCl_3$–Et_2O; TLC (G) R_f 0.75 with trace impurities at R_f 0.58, 0.90.

H-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-MeArg-(Tos)-OH (44). Compound **43** (7.00 g, 4.6 mmol) was hydrogenated as described for compound **40**. The crude product was purified by countercurrent distribution, 200 transfers: yield 2.86 g (45%); K = 3.0; TLC (G) R_f 0.27, trace impurity at R_f 0.47 (K = 2.5).

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-MeArg-OH·2HOAc·4H_2O (45). Compound **44** (1.00 g, 0.72 mmol) in a polypropylene weighing bottle was cooled to −78 °C and 30 mL of anhydrous HF condensed in. The HF was originally trapped in another vessel and then redistilled into the reactor. The cold bath was replaced by an ice bath, and the solution was stirred 0.5 h at 0 °C. The HF was then blown off under nitrogen (internal temperature about −20 °C), and the last traces were removed in a vacuum desiccator over KOH. The residue was dissolved in a small amount of 0.1 N HOAc and loaded onto 100 g of IRC-50 previously equilibrated with 0.1 N HOAc. The bradykinin analogue was eluted with a linear gradient constructed from 1.5 L of 0.1 N HOAc and 1.5 L of glacial acetic acid. The appropriate fractions were pooled, and the solvents were removed under vacuum. The residue was dissolved in water and the solution lyophilized: yield 0.75 g (83%), powder; TLC (G) R_f 0.02, homogeneous.

Pharmacology. Pharmacological evaluations for smooth-muscle stimulatory and inhibitory effects were conducted on segments of isolated guinea pig ilea set up as described previously.[31]

Four-point parallel-line bioassays[32] were conducted to determine the smooth-muscle stimulating potencies of the test compounds relative to bradykinin triacetate, calculated on a weight basis. A randomized block design was used for the administration of the doses at 4-min intervals, and the bath was rinsed after the peak contraction was reached. The magnitudes of the tissue contractions were used to estimate relative potencies and fiducial limits by the method of Finney.[33] Only assays in which there was no significant deviation from parallel bradykinin and test substance dose–response curves are shown.

Tests for inhibition of contractions produced by bradykinin triacetate, acetylcholine chloride, and prostaglandin E_2 were conducted as described previously.[31] Briefly, this method consisted of eliciting two control contractions in response to each agonist at regular 3- or 4-min intervals, followed by three sets of contractions elicited in the presence of the test compound. The last two sets of treated responses were compared with the two sets of control responses to obtain percent change in the mean of the treated contractions from the mean of the control contractions. The first set of treated contractions was used to maintain the timed sequence of injections during the period allowed for the tissue to become equilibrated with the antagonist. The analogues caused contractions of the tissue during this period. Averages of the mean changes are shown when more than one experiment was conducted on a concentration of test compound.

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(30) R. H. Mazur, B. W. Ellis, and P. Cammarata, *J. Biol. Chem.*, **237**, 1619 (1962).

(31) J. H. Sanner, *Arch. Int. Pharmacodyn. Ther.*, **180**, 46 (1969).

(32) D. J. Finney, "Statistical Method in Biological Assay", 2nd ed, Griffin, London, 1971, p 99.

(33) Bioassay results were analyzed by means of a PARLIN 7 computer program obtained from D. J. Finney and adapted for the Honeywell 16 computer.