Synthesis and Biological Activities of Photoaffinity Labeling Analogues of Substance P

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Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$, SP) is an undecapeptide with important properties as a neurotransmitter and with other functions. No specific antagonists and no long-acting analogues of this peptide hormone are known to date. In order to reach these goals, analogues of SP have been prepared which contain potential affinity, as well as photoaffinity labeling functions, suitable for irreversible attachment to SP receptors. We report here the synthesis of SP analogues which have the Phe residues in positions 7 or 8 replaced with (4'-NO$_2$)Phe, (4'-NH$_2$)Phe, (4'-N$_2$$^+$)Phe, and (4'-N$_3$)Phe. Some of these peptides are used for photoaffinity labeling studies using various bioassays. The synthesis of the (NO$_2$)Phe-containing peptide was carried out on solid phase using Nle instead of Met and the Boc strategy up to residue 4; the remaining amino acids were added using an Fmoc strategy. The protected undecapeptide was cleaved by ammonolysis, purified by chromatography on silica gel with chloroform/methanol, and deprotected afterwards. The amino, diazonium, and azido peptides were obtained in this sequence by chemical modification of the nitro peptides. On guinea pig ileum the modified peptides in position 8 had close to maximal activity, whereas modifications in position 7 produced some reduced activity, especially the nitro modification. No diazonium peptide produced any irreversible effects on guinea pig ileum. Photoinactivation studies were carried out on strips of guinea pig trachea, but no irreversible effects have been observed, neither permanent stimulation nor permanent inactivation. The biological activities and effects are discussed in view of the molecular properties of the synthesized analogues.

Among the classical peptide hormones, such as angiotensin (AT), bradykinin (BK), adrenocorticotropin (ACTH), and vasopressin/oxytocin, substance P (SP) has a special status; it was the first to be discovered (1931), but it is the least understood. No clear physiological role has been established until very recently, and no potent specific and no competitive inhibitors are known. Earlier structure–activity studies showed the low importance of the N-terminal portion and the prominent importance of the C-terminal pentapeptide,[1] including the two phenylalanines in positions 7 and 8. The apparent similarities between kinins, like BK and SP, in size, chemical nature, and pharmacological actions and the recent results obtained with AT and BK photoaffinity labels,[2,3] encouraged us to try the same technique on SP in order to develop (a) specific and irreversible antagonists of SP and (b) labeling tools which might help to isolate the unknown SP receptor from target tissues.

Syntheses. The syntheses of SP and of its analogues are notorious for solubility and purification problems.[4] This peptide family very readily aggregates in different solvents and renders even the most trivial purification step often into a difficult operation. The pure peptide itself is quickly oxidized, most probably on its Met residue; therefore, it is almost impossible to rely over a longer period of time on a concentrated standard solution of this peptide. We therefore decided to try a new approach which will eliminate these two principal problems. The synthesis was laid out to produce at the end a fully protected peptide, which would be soluble in organic solvents and could be purified with classical silica gel chromatography before a final and mild deprotection. The oxidation problem was eliminated by the already reported substitution of Met by Nle,[5,6] a change which in the literature

in one case gave 100%[5] and in another case 80%[6] of the biological potency of SP. [Nle^{11}]SP synthesized on several occasions in our laboratories[7] gave, in the same pharmacological tests, 50% relative affinity if measured in comparison to very freshly prepared SP. [Nle^{11}]SP did not lose its activity upon storage as SP does,[7] which would also explain the observed activity discrepancies with the above-mentioned literature reports. The Nle^{11} substitution was also necessary because the conditions needed to modify the precursor nitro peptides into the amino, diazonium, and azido analogues exclude Met or create serious problems. For example, hydrogenation on palladium/charcoal is difficult because the thioether of Met absorbs to noble metals and poisons the catalyst. The condition of diazotization which is the only reasonable way for obtaining the diazonium and azido analogues[8] oxidizes Met completely. A subsequent reduction of Met sulfoxide back into Met is always difficult but possible; however, it would certainly reduce also the azido function back to the amine.

The synthesis was designed to produce fully protected, soluble peptides; therefore, the synthesis on solid phase was not started on benzhydrylamine resin but on chloromethylated polystyrene. The resulting peptide-resin ester can undergo ammonolysis[9] and produce the fully protected peptide amide. Deprotection of the final peptide amide required protecting groups which can be cleaved under mild conditions, avoiding harsh conditions like anhydrous hydrogen fluoride (HF). The sequence Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-NH$_2$ ([Nle^{11}]SP) was prepared up to residue 4 (Pro) by the classical solid-phase method using the Boc protection group and Boc-(4'-NO$_2$)Phe instead of Phe^7 or Phe^8. After residue 4 the synthesis procedure was switched over to the Fmoc procedure[10] for the next two residues, leaving the N^ϵ-Boc protection of Lys intact. Incorporation of the last amino

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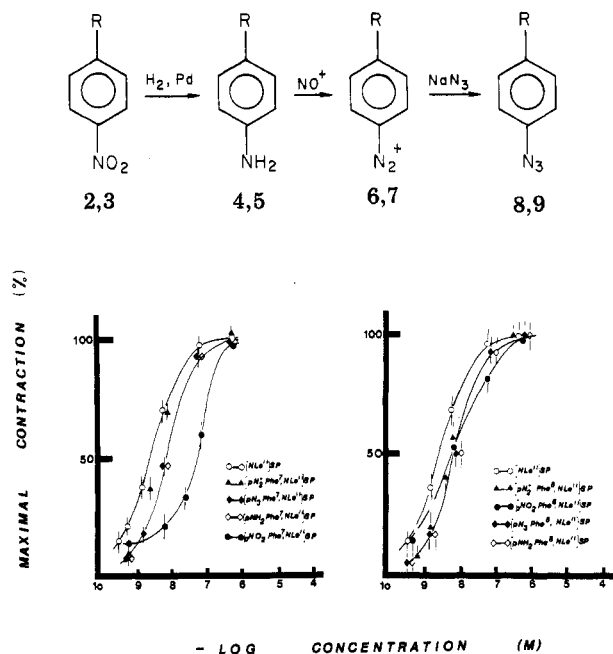
Scheme I[a]

Figure 1. Dose–response curves of [Nle[11]]SP and analogues. On the left panel are the biological activities of the modifications in position 7, and on the right are the modifications in position 8.

acid, Arg, was attempted as the bis(Boc) or even the tris(Boc) derivative; however, this failed completely. Unluckily, no other mild acid cleavable Arg derivative was available; the syntheses had to be finished with N^α-Boc-N^g-Tos-Arg, and the final deprotection after purification had to be carried out in HF anyway. For future syntheses, adamantyloxycarbonyl-protected Arg[11] will be used, but this was not available at that time.

Ammonolysis at room temperature in DMF/2-propanol was achieved in about 10 days, and the resulting crude, protected peptide was purified by chromatography on silica gel, eluted with chloroform/methanol. The pure protected peptide was treated for several minutes with small quantities of HF, and the resulting products were subjected to gel filtration on Sephadex LH20, eluted with DMF.

The two analogues [(4'-NO$_2$)Phe[7],Nle[11]]SP and [(4'-NO$_2$)Phe[8],Nle[11]]SP, respectively, were further modified according to Scheme I, a pathway already well established with angiotensin II (AT),[12] bradykinin (BK),[13] and recently neurotensin.[14] The nitro peptide was hydrogenated to the corresponding (4'-NH$_2$)Phe peptide, which in turn was diazotized to the (4'-N$_2$[+])Phe analogue and converted to the final photolabel [(4'-N$_3$)Phe[7],Nle[11]]SP and [(4'-N$_3$)-Phe[8],Nle[11]]SP, respectively (see Scheme I).

Biological Activities. All substances were tested on guinea pig ileum for their biological activities in vitro, and the results are presented in Tables I and II and Figure 1. All compounds retained much of their relative affinity compared to [Nle[11]]SP, the only exception being [(4'-NO$_2$)Phe[7],Nle[11]]SP which had only about 10% of relative affinity; all compounds retained full intrinsic activity. It has been shown earlier that both positions 7 and 8 are important for binding SP to its receptor,[7] but changes in position 8 reduce activity less than the same modifications

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Table I

no.	substance	M_r	formula	TLC BAW, R_f	TLC BAWP, R_f	TLC R_f	yield, %	biological activity (guinea pig ileum) $a^{E\,b}$	biological activity (guinea pig ileum) $pD_2{}^f$	biological activity (guinea pig ileum) RA[d]	n^e
0	SP										
	[Nle[11]]SP		$C_{63}H_{98}N_{18}O_{13}S + (C_2H_4O_2)_3$	0.38	0.43	0.22		1.0	8.78	195	60
1	[(4'-NO$_2$)Phe[7],Nle[11]]SP	1347.66 + 180.16	$C_{64}H_{100}N_{18}O_{15} + (C_2H_4O_2)_3$	0.40	0.43	0.16		1.0	8.49	100	8
2	[(4'-NO$_2$)Phe[8],Nle[11]]SP	1329.62 + 180.16	$C_{64}H_{99}N_{19}O_{15} + 3HF$	0.36	0.36	0.31	9.7[a]	1.0	7.51	10	9
3	[(4'-NH$_2$)Phe[7],Nle[11]]SP	1374.62 + 60.02	$C_{64}H_{99}N_{19}O_{15} + 3HF$	0.33	0.35	0.28	13.0[a]	1.0	8.46	93	9
4	[(4'-NH$_2$)Phe[8],Nle[11]]SP	1374.62 + 60.02	$C_{64}H_{101}N_{19}O_{13} + (C_2H_4O_2)_3$	0.31	0.26	0.56	90.0	1.0	8.23	55	8
5	[(4'-NH$_2$)Phe[8],Nle[11]]SP	1344.64 + 180.16	$C_{64}H_{101}N_{19}O_{13} + (C_2H_4O_2)_3$	0.29	0.25	0.56	95.0	1.0	8.33	69	8
6	[(4'-N$_2$[+])Phe[7],Nle[11]]SP	1344.64 + 180.16	$C_{64}H_{99}N_{20}O_{13} + (HCl)_3 \cdot Cl$					1.0	8.40	81	11
7	[(4'-N$_2$[+])Phe[8],Nle[11]]SP	1355.62 + 144.84	$C_{64}H_{99}N_{20}O_{13} + (HCl)_3 \cdot Cl$					1.0	8.35	72	8
8	[(4'-N$_3$)Phe[7],Nle[11]]SP	1355.62 + 144.84	$C_{64}H_{99}N_{21}O_{13} + (C_2H_4O_2)_3$	0.34	0.35	0.24	67.0	1.0	8.17	48	10
9	[(4'-N$_3$)Phe[8],Nle[11]]SP	1370.64 + 180.16	$C_{64}H_{99}N_{21}O_{13} + (C_2H_4O_2)_3$	0.36	0.36	0.24	33.0	1.0	8.19	50	10

[a] Yield is expressed from the amount of crude product processed after ammonolysis to the purified, deprotected peptide. The quantity of crude product obtained from the resin by ammonolysis after prolonged incubation (26 days) was 67% of the theoretical value for 2 and 85% for 3, respectively. For explanations for TLC and for RPTLC, see Experimental Section. [b] aE is the intrinsic activity. [c] pD_2 if the negative logarithm of the dose of agonist (in M) that produces half-maximal effect. [d] RA is the relative affinity (in percent) of an analogue compared with [Nle[11]]SP (=100% RA). [e] Number of experiments.

Table II. Comparison of Structure, Biological Activity, and Physicochemical Parameters of [Nle¹¹]SP Analogues

modification of [Nle¹¹]SP	position 7		position 8		physicochemical parametersʰ		
	no.	RA,ᵃ	no.	RAᵃ	σᵉ	πᶠ	MRᵃ
Phe	1	100	1	100	0.00	1.96	25.36
(4'-NO₂)Phe	2	10	3	93	0.78	1.68	31.69
(4'-NH₂)Phe	4	55	5	69	-0.66	0.73	29.75
(4'-N₂⁺)Phe	6	81	7	72	1.91	<<0.00	30ᵈ
(4'-N₃)Phe	8	48	9	50	0.15	2.42	34.53
Tyr(OMe)	b	31	b	100	-0.27	1.94	32.20
Cha	b	20	b	100		2.51	26.69
Leu	b	2.3	b	68		1.53	14.98
Car	c	<0.1	c	<0.1	-1.0-2.0ᵈ	3.00ᵈ	45ᵈ

ᵃ RA is the relative affinity of an analogue compared to [Nle¹¹]SP (or SP if indicated by b). ᵇ Literature values of [Met¹¹]SP analogues on the same bioassay; the values are expressed as RA compared to SP.⁷ ᶜ Literature values of (4-11) (4-11)SP analogues,²⁶ compared to (4-11)SP. ᵈ Approximative values, not from ref 27. ᵉ σ is the Hammett factor for electronegativity. ᶠ π is the expression for hydrophobicity. ᵍ MR is the molar refractoriness of a given residue. ʰ σ, π, and MR are determined from ref 27.

in position 7. This trend is also visible in this study for the nitro compounds but interestingly not for the other modifications which have very small activity differences between the same modification in position 7 or 8 of [Nle¹¹]SP.

These substances were prepared for photolabeling SP receptors on target tissues and cells and to act as specific and irreversible blockers of SP, because no specific and potent antagonists are available for this peptide. Both goals are of importance, especially for SP, since recent research has shown that SP is a peptide neurotransmitter,[15] but no pharmacological or biochemical tools are available to show the localization, the specificity, and the nature of the SP receptors in the CNS and elsewhere.

Other applications of the presented SP analogues are secondary but have much more immediate benefit. Due to the chemical character of the modifications, some conclusion can be drawn about the nature of the chemical interactions of the hormone with its receptors: for example, are the aromatic residues acting through their aromaticity by forming π complexes or charge-transfer complexes with some part of the receptor or are these aromatic residues acting through lipophilicity or not directly at all with the receptor? If evidence could be found for one or the other pattern it would be much easier to design analogues with enhanced capacities like duration of action, potency, reduced intrinsic activity (partial agonists), and eventually pure antagonists.

The nitro-, amino-, diazonio-, and azidophenylalanine analogues are rather similar in size and shape compared to other residues such as Leu (smaller) or iodinated tyrosine (larger), but they differ very much in their electronegativity and hydrophobicity (see Table II). It is well founded that the hydrophobic character of a pharmacologically active substance is one of the major forces contributing to bind a hormone to its receptor.[16] However, the electronegativity of an aromatic side chain has been shown to contribute to a large extent to the affinity of AT (angiotensin II) to its receptor.[17,18] When the SP analogues described above are analyzed (see Table II) with regard to the parameters for lipophilicity, for electronegativity, and MR (molecular refractoriness, a measure for

the "bulk" of a substitution), it can be seen immediately that hydrophobicity is certainly not the driving force, because hydrophilic substitutions like (4'-NH₂)Phe and even the ionic (4'-N₂⁺)Phe have almost the same activities as the unsubstituted Phe.

Compared to other analogues modified in position 7 or 8 of SP, the following conclusions can be drawn: (1) Modifications in position 7 affect the relative affinity more than does the same modification in position 8. (2) Aromaticity is not absolutely needed either in position 7 or 8; cyclohexylalanine (Cha) analogues have reasonable (position 7) or full (position 8) activity.⁷ (3) Electronegativity is not the driving force either for position 7 or for position 8. A seemingly possible influence of electronegativity in position 7 can be ruled out due to the high activity of the diazonium peptide 6. The formation of tyrosine, which is a normal reaction product of (N₂⁺)Phe at physiological pH, cannot account for the high activity of 6 and 7 because the formation of Tyr is rather slow and does not interfere as it has been shown with the same analogue of angiotensin II, [Sar¹,(4'-N₂⁺)Phe⁴]AT.[17] This analogue had 0.7% relative affinity compared to [Sar¹,Tyr⁴]AT, which has 100% relative affinity and would be the presumed reaction product. (4) In position 7 a partial influence is probably due to steric factors, residues with an MR factor (see Table II) between 20 and 35 are seemingly permitted, and analogues that are smaller, like Leu (MR = 14.98), or larger, like Car (MR ≈ 45), loose much or all affinity. (5) None of the above-presented analogues has reduced intrinsic activity nor complete antagonistic properties. Increased biological activity (superagonism) was not observed either.

Inactivation Studies. The main purpose of this study was to obtain irreversibly acting analogues of SP. The nitro, diazonium, and azido peptides were therefore tested for such eventual properties, as affinity labels (diazonium peptides 6 and 7), or under ultraviolet influence as photoaffinity labels (nitro analogues 2 and 3; azido analogues 8 and 9). Aromatic diazonium ions are quite stable at low temperature (~0 °C) and low pH but are very reactive toward tyrosine and histidine residues at physiological pH and can react also with other protein side chains: the diazonium forms an azo link to the protein and becomes irreversible. If such reactive side chains like Tyr or His are located on the receptor at or near the binding locus of the concerned side chain, then the cross reaction becomes highly favored, and the receptor site becomes irreversibly occupied. However, both SP analogues with diazonium modifications in position 7 or 8 do not show any prolonged or irreversible action on guinea pig ileum, even if they were successively applied in rather high doses (see

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Table III. Inactivation Experiments[a]

guinea pig bioassay	substance no.	treatments	concn, 10^{-7} M	residual act. %	n^b
trachea	1	3	1.50	94 ± 16	6
trachea	2	3	7.50	94 ± 9	6
trachea	3	3	3.75	97 ± 5	9
trachea	8	3	1.50	99 ± 12	6
trachea	9	3	1.50	104 ± 5	6
ileum	6	2	7.50	111 ± 12	6
ileum	7	2	7.50	97 ± 9	5

[a]Photoinactivation experiments were carried out on guinea pig trachea and the affinity labeling experiments with the diazonium peptides 6 and 7 on guinea pig ileum. The residual activity is the percent of maximal contraction of the treated tissues compared to the untreated references. [b] n is the number of determinations.

Table III). This results resembles very much that obtained with AT[17] and BK.[3] It is therefore reasonable to assume that in the immediate vicinity of the Phe[7] or Phe[8] binding locus on the SP receptor there are no Tyr or His residues.

The photosensitive analogues 2, 3, 8, and 9 were initially tested for irreversible effects on guinea pig ileum. Upon irradiation with the usual irradiation equipment, producing 18 mW/cm^2 of ultraviolet radiation around 365 nm,[3] the tissues produced variable and unreproductive results due to enhanced spontaneous contractions, both in the absence of SP and its analogues. As already mentioned, SP acts on guinea pig ileum partially through acetylcholine,[7] a mechanism which is only partially understood. Such a dual action is, however, highly unfavorable if inactivation studies have to be carried out. A sizeable inactivation of the SP receptors could be hidden by the acetylcholine-mediated response and would need additionally an atropinization of the tissues. A third inconvenience of the SP standard assay guinea pig ileum is the fast desensitization and relaxation of the tissues in the presence of SP. We therefore decided to use a bioassay which (a) is stable against irradiation, (b) is 100% specific for SP, and (c) displays a prolonged contraction or even maintains a plateau in the contracted state like rabbit aorta for AT or [des-Arg^9]BK. The only SP-specific in vitro assay that fulfills all three points is the guinea pig trachea strip, a recent bioassay developed in our laboratories.[19]

In a first series of experiments it was shown that in the absence of irradiation none of our SP analogues had any irreversible effect on the tissue even after prolonged (10 min) and repeated (3) incubations. No changes in the contractile capacity and in the base-line tension of the tissues were observed after three 10-min irradiations.

The guinea pig trachea strips were subjected to three consecutive treatments consisting of a dose of photolabel, producing maximal contraction, followed by 10 min of irradiation and extensive washing after each treatment. However, of the four potential photolabels, 2, 3, 8, and 9, none produced any significant inactivation of the trachea strips compared to the nonirradiated controls (see Table III). This negative result does not mean that no labeling takes place; it merely indicates that no pharmacologically important number of receptors has been touched. In a simple pharmacological system with no spare receptors and under the assumption that the receptor occupation is proportional to the biological response, around 10 to 15% labeling could occur without being detected pharmacologically. Low labeling yields are quite frequent with photoaffinity labeling,[20] but a successful receptor isolation

could still be possible with a few percent of labeling, if labels with sufficiently high radioactivity are available.[21] The reasons for the low amount of labeling or for nonlabeling might lay in the arrangement of the two Phe residues in positions 7 and 8 of SP. Former structure–activity studies showed that replacement of one or the other Phe in position 7 or 8 with Leu reduces the biological activity on guinea pig ileum to a relative affinity of 2.3% and 68%, respectively. However, if both positions 7 and 8 are substituted with Leu, the relative affinity drops lower than 0.1%.[7] This could suggest that the two aromatic rings act together (a conformation which is not very favorable but possible), and at least one aromatic interaction is necessary to obtain biological activity. Such a hypothesis deserves further investigations and could explain the low amount of labeling or the nonlabeling, because the activated photolabel is in close contact with the neighboring Phe and could therefore react with it instead of with the receptor.

Studies are under progress with radioactive photolabels for an eventual isolation of SP receptors from different tissues.

Experimental Section

Syntheses. N^α-Butyloxycarbonyl-protected amino acids, N^α-fluorenylmethyloxycarbonyl-protected amino acids, peptide reagents, and chloromethylated resin (copolystyrene-1% divinylbenzene; 0.75 mmol of Cl/g of resin) were obtained either from Bachem Fine Chemicals Inc. or from Chemalog, Chemical Dynamics Corp. Dicyclohexylcarbodiimide (DCC) was purified by dissolving the commercial product (Aldrich Inc.) in dry diethyl ether; the insoluble material (urea) was removed by filtration, and the ether was evaporated in vacuo. All solvents and reagents used for solid-phase synthesis were of "analytical" quality and were redistilled before use. TLC was performed on Merck precoated silica gel plates (Type G60-F254) in solvent system BAW (1-butanol–acetic acid–water, 10:2:3) or BAWP (1-butanol–acetic acid–water–pyridine, 30:6:20:12). Reversed-phase TLC was carried out on Whatman KC18 plates in the solvent system acetonitrile–2-propanol–1 M ammonium acetate–water, 30:7:50:13, at pH 7.5. Peptides were visualized by UV fluorescence and by a modified Reindel–Hoppe procedure.[22] Elution of columns was controlled by TLC in order to avoid photolysis of the products by a UV monitor. The purity of the peptides was further assessed by isocratic analytical HPLC on an Altex-ODS C18, 5-μm, reversed-phase column, eluted with 20% acetonitrile–2.5% 2-propanol in 0.25 M ammonium acetate at pH 4.5. All products produced symmetrical single peaks. Peptide samples for amino acid analyses were hydrolyzed during 24 h at 110 °C in 6 N HCl plus 0.2% ethanethiol in vacuum-sealed tubes. The amino acid analyses were performed on a Technicon TMS analyzer equipped with an Autolab integrator by Dr. P. Schiller of the Institute for Clinical Research in Montreal. Under these conditions (4'-NO$_2$)Phe and (4'-N$_3$)Phe can be monitored as (4'-NH$_2$)Phe. All

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operations were carried out under exclusion of all ultraviolet radiation in order to prevent decomposition of the nitro and azido compounds.[12]

Peptide synthesis was carried out with a Burrel shaker and glass reaction vessels using procedures previously described.[23] Dry Boc-Nle Cs salt (5 mmol) was coupled with 1 equiv of chloromethylated resin,[24] resulting in a substitution of 0.45 mmol of Nle per gram of dry resin.[25] The sequence Boc-Gly-Leu-Nle was built up on 6 g of Nle-resin, and the tetrapeptide resin was separated into three portions.

[(4'-NO₂)Phe⁷,Nle¹¹]substance P (2). Boc-Phe, Boc-(4'-NO₂)Phe, 2 × Boc-Gln, and Boc-Pro were condensed to 3 g of the above peptide-resin. After completion of the 4–11 fragment which was made up with the classical Boc and TFA strategy already described,[23] the procedure was changed for the Fmoc strategy.[10] The amino acids N^α-Fmoc-N^ϵ-Boc-Lys and Fmoc-Pro were coupled with the following schedule: coupling of 4-fold symmetrical anhydride in DMF/CH₂Cl₂ (1:1) during 45 min; 2-propanol, 3 × 5 min; CH₂Cl₂, 2 × 2 min; deprotection with piperidine, 1 × 5 and 1 × 25 min; CH₂Cl₂, 2 × 2 min; DMF, 2 × 2 min; dioxane/water (1:1), 2 × 2 min; DMF, 2 × 2 min; CH₂Cl₂, 2 × 2 min; next coupling. The synthesis was terminated with a last coupling of N^α-Boc-N^g-Tos-Arg. The undecapeptide resin ester was placed in a 350-mL pressure vessel and left for 9 days with occasional shaking at 22 °C with DMF/2-propanol, 1:1, saturated at 0 °C with gaseous ammonia. This procedure resulted in 938 mg of crude 2; an additional 619 mg was recovered after a second period of 17 days. The product was filtered over Sephadex LH20 (1.5 × 50 cm), eluted with DMF, and evaporated to dryness. The fully protected peptide (871 mg) was dissolved in a minimum of DMF in CHCl₃, diluted with CHCl₃, applied onto a Lobar Type C silica gel column (Merck), and eluted with CHCl₃/MeOH. The purest fractions were pooled and gave 140 mg of pure protected 2, which was subjected to deprotection in 2 mL of anhydrous hydrogen fluoride for 25 min at 0 °C. after HF evaporation, the product was treated with a last gel filtration on LH 20 with DMF, evaporated, and lyophilized from 0.2 M acetic acid, producing 69.8 mg pure 2. Amino acid analysis: Glx, 2.01; Pro, 2.01; Gly, 1.03; Leu, 1.05; Nle, 0.98; Phe, 1.00; (4'-NH₂)Phe, 1.01; Lys, 0.98; Arg, 0.95.

[(4'-NO₂)Phe⁸,Nle¹¹]substance P (3). This peptide was prepared with exactly the same procedures and substances described above, except that (4'-NO₂)Phe was coupled into position 8 instead of 7. The ammonolysis produced after 9 days 1.16 g of crude product; 15 additional days produced another 818 mg. The first portion was purified as shown for 2 and produced, after three subsequent chromatographies on Lobar silica gel, 165.8 mg of pure protected 3; after HF deprotection and LH20 gel filtration, 125.0 mg pure 3 was produced. Amino acid analysis: Glx, 1.94; Pro, 1.93; Gly, 1.11; Leu, 1.09; Nle, 0.99; Phe, 1.05; (4'-NH₂)Phe, 0.98; Lys, 1.01; Arg, 0.90.

[(4'-NH₂)Phe⁷,Nle¹¹]substance P (4). Compound 2 (30 mg) was dissolved in 0.5 mL of 50% acetic acid and 10% palladium on charcoal (~2 mg; Alfa inorganics) was added and placed in a microvial (Wheaton). Hydrogenation was carried out under a pressure of 8 atm at room temperature for 90 min according to a recently described procedure.[13] The catalyst was filtered off, washed with 2 mL of 10% mercaptoethanol, and lyophilized twice, producing 28.4 mg of pure 4.

[(4'-NH₂)Phe⁸,Nle¹¹]substance P (5). Compound 3 (30 mg) was hydrogenated to yield 24.4 mg of pure 5, exactly as described for 4.

[(4'-N₂⁺)Phe⁷,Nle¹¹]- and [(4'-N₂⁺)Phe⁸,Nle¹¹]substance P (6 and 7) were prepared immediately before the biological tests and not isolated. A solution of 1 mg of either 4 or 5 in 1 mL of 100 mM HCl was reacted at 0 °C with 50 μL of a 0.1 M solution

of NaNO₂ for 10 min. After a positive iodine–starch test, 50 μL of 0.2 M sulfamic acid was added in order to destroy excessive nitrite. This preparation was directly used for the biological assays and stored at 0 °C for no longer than 2 h; it was not further characterized.

[(4'-N₃)Phe⁷,Nle¹¹]substance P (8). Compound 4 (12 mg) was dissolved in 1.3 mL of 100 mM HCl at 0 °C. A 1 M solution of NaNO₂ (25 μL) was added under stirring for 10 min. After a positive iodine–starch test, 30 μL of a 1 M solution of sulfamic acid was added in order to destroy any excess nitrite. After 10 min and a negative iodine–starch test, 20 μL of a 1 M solution of NaN₃ was added, and 5 min later the pH was increased to 7 by adding solid NH₄OAc. The product was lyophilized and subjected to a partition chromatography on Sephadex G-25 in a Teflon tube of 6 mm × 50 cm, eluted with the upper phase of the solvent system butanol–acetic acid–water, 4:1:5. The product was collected and lyophilized, and 8.0 mg of 8 was obtained. IR (KBr pellet) showed N₃ at 2110 cm⁻¹.

[(4'-N₃)Phe⁸,Nle¹¹]substance P (9). Compound 5 (12 mg) was treated in the same manner as above and produced 3.9 mg of pure 9.

Biological Activities. Guinea pigs of either sex weighing between 250 and 400 g were killed by stunning and exsanguination. For the guinea pig ileum assay, the peritoneal cavity was opened, and a segment of ileum was excised 10 mm before the ileocaecal junction. The segment of ileum was washed with Krebs' solution and then cut into portions of about 3 cm, which were pulled over a small glass rod and incised along the mesenteric line. The longitudinal muscle layer was stripped off and cut into 2.5 to 2.7 cm long strips, which were suspended in organ baths containing 10 mL of Krebs' solution at 37 °C and bubbled with CO₂/O₂, 5:95. An initial tension of 0.5 g was applied and adjusted several times during the 1-h incubation time. Tissue contractions were monitored with isometric force transducers and registered on a polygraph (both from Grass Co., Quincy, MA). SP and the tested analogues were dissolved in isotonic saline at a concentration of 1 mg/mL and diluted to the desired range before each experiment.

For the guinea pig trachea assay, the animals were killed as already mentioned. The skin of the ventral side of the neck and thorax was opened, and the trachea was excised between the glottis and the bronchial separation. A glass rod was inserted into the tracheal tube, and the tissue was defatted, cut into a helical band of about 4 mm, and further divided into four strips of about 2.0 cm. These tissues were incubated in the same way as the ileum strips; however, an initial tension of 3 g was applied during the 2 to 3 h incubation time, and 1 μg/mL indomethacin was present throughout the experiment in the tissue bath (prevention of prostaglandin synthesis).

Concentrated solutions were stored at −20 °C, for up to 2 weeks; diluted solutions were discarded at the end of every experiment. SP and analogues were applied in single doses at 15-min intervals, and the bath's solution was changed several times between two applications of peptides. Concentration–response curves were obtained on guinea pig ileum by injecting initially an amount of peptide of 5 × 10⁻¹⁰ M and by increasing this amount by a factor of 2.5 untile a maximal response was obtained. Specificity of the analogues for SP receptors was determined with a desensibilization procedure and using specific inhibitors for other myotropic agents according to earlier studies.[7]

Inactivation Studies. Guinea pig ileum strips were challenged with 7.5 × 10⁻⁸ M SP to produce a maximal contraction, and after washing and relaxation they were incubated three times with 7.5 × 10⁻⁸ M 6 or 7, respectively, during 10 min, followed by a 15-min relaxation and washing period. After the second application, the tissues were relaxed and washed for 45 min, and the residual response was determined with the initial dose of SP.

Guinea pig trachea strips were challenged several times with 1.5 × 10⁻⁷ M 1 after the incubation period until a stable maximal contraction had developed. After the tissues were washed and relaxed for 45 min, they were challenged with the photolabel (2: 7.5 × 10⁻⁷ M, 3: 3.8 × 10⁻⁷ M, 8 and 9: 1.5 × 10⁻⁷ M each) in the presence of 10 mM (4'-NH₂)Phe as scavenger, and after the maximal contraction was obtained, they were irradiated for 10 min with the already described apparatus.[3] After the tissues were washed and relaxed, the treatment was repeated twice, and upon completion of the third irradiation the tissues were washed for

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at least 45 min. The residual activity was tested twice with 1.5 × 10^{-7} M 1 about 45 min apart. Reference tissues were treated exactly the same way; however, the irradiation was omitted.

Notes

Lysine and Ornithine Analogues of Methotrexate as Inhibitors of Dihydrofolate Reductase[1]

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The ornithine (6a) and lysine (6b) analogues of methotrexate (1) have been synthesized via condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (2) with N^δ-carbobenzoxy-L-ornithine *tert*-butyl ester (3a) and N^ϵ-carbobenzoxy-L-lysine *tert*-butyl ester (3b), respectively. Removal of the protecting groups gave 6a and 6b. Compounds 6a and 6b and their precursor Cbz acids (5a and 5b) show significant inhibition of dihydrofolate reductase.

Methotrexate (MTX, 1) has been employed in the

1 (methotrexate)
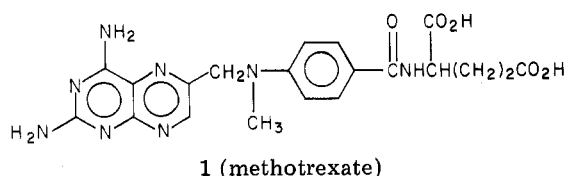
chemotherapeutic treatment of leukemias, lymphomas, psoriasis, and other clinical disorders.[2] The major intracellular receptor for MTX appears to be dihydrofolate reductase (DHFR). Inhibition of the enzyme by MTX depletes the tetrahydrofolate pool, resulting in a decreased synthesis of thymidylate and, in turn, an inhibition of DNA synthesis. One of the major clinical problems associated with MTX therapy is the development of resistance to the drug.[3,4] Furthermore, MTX in vivo forms polyglutamates via the γ-carboxylate, resulting in prolonged intracellular retention of the drug and additional toxicity.[5] These clinical findings have prompted the synthesis of a variety of analogues of MTX, with a significant portion of the work directed toward modification of the glutamate moiety in MTX. We wish to report the synthesis of the ornithine (6a) and lysine (6b) analogues of methotrexate and preliminary studies of the ability of these compounds and their precursors to inhibit dihydrofolate reductase.

Chemistry. Carboxypeptidase G_1 cleavage of methotrexate afforded the starting compound for the syntheses, 4-amino-4-deoxy-N^{10}-methylpteroic acid (APA, 2).[6] We, like others,[7] encountered difficulty in getting APA to dissolve in organic solvents to any appreciable extent. The acid has shown the greatest solubility in *N,N*-dimethylformamide (DMF) but only to the extent of about 1 g/100 mL at room temperature.[8] We have found, however, that APA is approximately three times as soluble in *N*-methyl-2-pyrrolidinone as it is in DMF, and on this basis the former solvent was chosen for the following reactions.

Scheme I
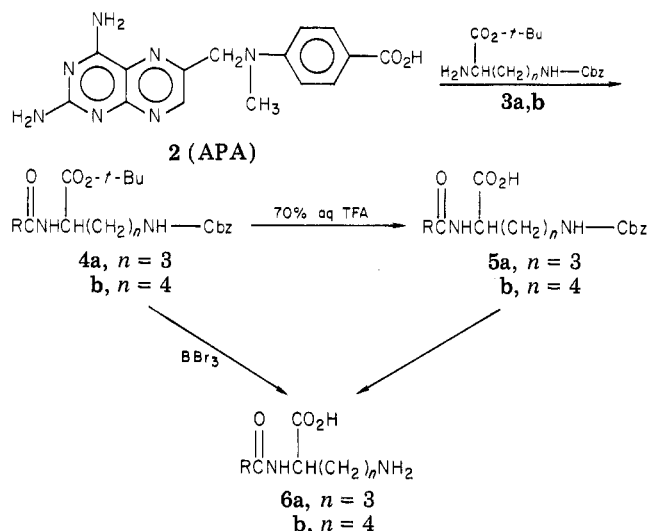
Treatment of APA (2) with N^δ-carbobenzoxy-L-ornithine *tert*-butyl ester (3a)[9] and N^ϵ-carbobenzoxy-L-lysine *tert*-

(1) Presented in part at the 28th IUPAC Congress, Vancouver, British Columbia, Canada, Aug 16–21, 1981.

(2) J. R. Bertino and D. Johns in "Cancer Chemotherapy", I. Brodsky, Ed., Grune and Stratton, New York, 1972, pp 9–22.

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(7) M. Chaykovsky, B. L. Brown, and E. J. Modest, *J. Med. Chem.*, **18**, 909 (1975).

(8) Dr. Andre Rosowsky has recently reported that the solubility of APA in DMF is greatly improved if the acid is isolated by precipitation from aqueous solution using acetic acid rather than hydrochloric acid. We thank Dr. Rosowsky for this personal communication.

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