4 of hydroxyproline are in the cis configuration, this amino acid partially forms a lactone upon treatment with acids. The existence of this lactone was detected as a cathodic band on paper electrophoresis (pyridine–acetic acid–water, 10:1:89 v/v/v) at pH 6.5, a position where the neutral hydroxyproline does not migrate.

cis-Hydroxyproline as an amino acid was observed clearly as a mixed peak with aspartic acid in the amino acid analysis at pH 3.2 and 2.8.

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Registry No. [7-*trans*-4-Hyp]AVP, 108666-16-6; d[7-*trans*-4-Hyp]AVP, 108666-17-7; d[7-*cis*-4-Hyp]AVP, 108739-48-6; H-*cis*-Hyp-OH, 618-27-9; BOC-*cis*-Hyp-OH, 87691-27-8; BOC-Gly-OH, 4530-20-5; BOC-Tyr-OH, 3978-80-1; BOC-Arg(N^G-tosyl)-OH, 13836-37-8; BOC-Asn-OH, 7536-55-2; BOC-Gln-OH, 13726-85-7; BOC-Hyp(*O*-benzyl)-OH, 54631-81-1; (BOC-Phe)$_2$O, 33294-54-1; [BOC-Cys(MeBzl)]$_2$O, 85097-53-6.

Structure–Activity Studies on the C-Terminal Hexapeptide of Substance P with Modifications at the Glutaminyl and Methioninyl Residues

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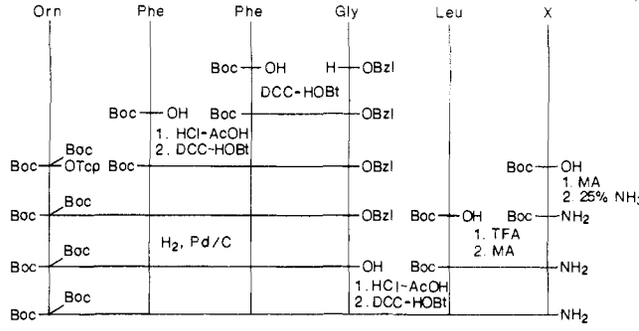
Analogues of [Orn^6]-SP$_{6-11}$ have been synthesized in which the SCH_3 group of the Met^{11} side chain is replaced by other functional groups, such as $(CH_2)_2NH_2$, COOH, $CONH_2$, and COOR, which have basic, acidic, or neutral character and which may act as either H-bonding donors or H-bonding acceptors. These analogues were tested in guinea pig ileum and rat colon muscularis mucosae, in vitro. Substitution of Lys, Gln, or Glu at position 11 caused a marked reduction in biological activity in both tissues. In contrast, the glutamate benzyl ester analogue had only slightly reduced activity in the guinea pig ileum and an increased (4.7 times) activity in the rat colon. It is concluded that charged groups in the side chain at position 11 of SP$_{6-11}$ reduce the biological activity of SP hexapeptide.

The C-terminal hexapeptide amide of substance P (SP$_{6-11}$, H-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is the minimal peptide fragment that retains substantial SP-like agonist activity in most pharmacological tests.[1] This applies to gastrointestinal smooth muscle[2,3] and salivary secretion[4] and to the hypotensive effects of this group of peptides.[5] In neuronal preparations (rat spinal cord and rat superior cervical ganglion, in vitro), the C-terminal hepta- and hexapeptides are somewhat more active than the parent undecapeptide.[6,7] However, the N-terminal residues should not be considered redundant since they may well contribute to the potency of the compound in some test systems, while in others, there is an absolute requirement for the N-terminal basic residues.[8,9] In view of the importance of the C-terminal hexapeptide for biological activity, this sequence should provide a basis for examining some aspects of the structure–activity relationship for tachykinin agonists.

In previous studies, it has been shown that the methionine residue is one of the most important elements for activity of SP and related peptides.[1,10] Two key elements of this amino acid, the α-carboxamide group and the side chain, appear to be important in determining the active conformation of the molecule. In the present work, we investigated the nature of the side chain of methionine at position 11 and its contribution to the biological activity of SP$_{6-11}$. In our investigation, we used as a model peptide the analogue [Orn^6]-SP$_{6-11}$, which is almost equipotent with the parent hexapeptide in smooth muscle preparations, has high solubility, and avoids problems associated with formation of the pyroglutaminyl derivative from the Gln^{11} residue at position 6. We designed analogues in which the SCH_3 group of methionine was replaced by acidic, basic, or neutral groups that can also act as either H-bonding donors or H-bonding acceptors.

Scheme I. Synthesis of the C-Terminal Hexapeptide of SP Analogues[a]



[a] X = Lys(Z), Glu(OBzl), Gln.

The synthesized analogues were tested in two smooth muscle preparations (guinea pig ileum and rat colon muscularis mucosae, in vitro) representative of the proposed "SP-P" and "SP-E" subtypes of tachykinin recep-

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Table I. Biological Activity of SP_{6-11} Analogues in Smooth Muscle Preparations

no.	compound	relative activities (SP = 1) guinea pig ileum[a]	rat colon[a]
1	SP_{1-11}	1 ($EC_{50} = 2 \times 10^{-9}$ M)	1 ($EC_{50} = 2 \times 10^{-7}$ M)
2	SP_{6-11}[b]	0.30 [0.24–0.39]	0.076 [0.058–0.096]
3	[Orn^6]-SP_{6-11}	0.39 [0.32–0.51]	0.36 [0.30–0.43]
16	[Orn^6,Lys^{11}]-SP_{6-11}	inactive to 10 μM	inactive to 10 μM
14	[Orn^6,Gln^{11}]-SP_{6-11}	0.0022 [0.0013–0.0031]	<0.01
17	[Orn^6,Glu^{11}]-SP_{6-11}	0.00058 [0.00035–0.00085]	<0.01
15	[Orn^6,Glu(OBzl)^{11}]-SP_{6-11}	0.14 [0.12–0.2]	0.61 [0.47–0.81]

[a] Figures in brackets are 95% confidence limits. [b] The tendency for Gln to cyclize to form pyroglutamic acid means that the compound SP_{6-11} is a mixture of the glutaminyl and pyroglutaminyl forms. Estimates based on HPLC measurements suggest that the ratio of the two forms is approximately 65:35.

tor,[11,12] and structure–activity correlations are reported.

Results and Discussion

Syntheses of the fully protected analogues of the C-terminal hexapeptide amide of SP were performed by conventional solution-phase methods (Scheme I). Final products were obtained from the fully protected hexapeptides by removing the Z and benzyl ester groups with hydrogenation over 10% Pd/C and the Boc groups with 1 N HCl in acetic acid followed by purification with gel filtration on Sephadex G-15 and partition chromatography on Sephadex G-25F.

Structural modifications in the model hexapeptide amide H-Orn-Phe-Phe-Gly-Leu-Met-NH_2[13] involved replacement of the SCH_3 group of methionine by $(CH_2)_2NH_2$ (Lys), $CONH_2$ (Gln), COOH (Glu), and COOBzl (Glu(OBzl)). The resulting analogues were then tested in two bioassay preparations, the guinea pig ileum and rat colon muscularis mucosae in vitro, and their relative activities to substance P are summarized in Table I.

The lysine analogue was inactive in both systems up to 10 μM, demonstrating that a basic character in the side chain at position 11 is undesirable. An acidic (glutamic acid analogue) or a neutral polar character (glutamine analogue) in the side chain at position 11 results in dramatic loss of potency relative to the parent analogue. In contrast, the activity of the benzyl ester analogue is only slightly reduced in the guinea pig ileum (threefold relative to [Orn^6]-SP_{6-11}) but is increased twofold on the rat colon.

In addition to their basic, acidic, or neutral character, the substituents we have employed may act as H-bonding donors or acceptors. Thus they may contribute to the conformation of the molecule via H bonding between elements of the peptide backbone and side chains.

The dramatic loss of potency observed in most of the analogues may be an indication that the side chain of methionine is directly interacting with lipophilic pockets of the receptor. Such an interaction may be further stabilized or mediated, specifically in the case of methionine, by metal chelation through the lone pair of electrons of sulfur, or may depend on the length and size of the group attached at the δ-position of the side chain of the amino acid in position 11. The latter prompted us to investigate the behavior of the analogue **15** in two different types of SP receptors. The increased potency observed for the analogue [Orn^6,Glu(OBzl)^{11}]-SP_{6-11} in rat colon is rather surprising and may be associated with the increased lipophilic character at position 11 and/or the bulkiness of the benzyl ester group. An analogue of the C-terminal heptapeptide of substance P, [(*S*-Bzl)Cys^{11}]-Sp_{5-11}, with a benzyl group at position 11 has been previously reported[14] and is biologically acceptable in guinea pig ileum. Any correlation of the two analogues, for the moment, would be unsuccessful because of the different structures of the side chains (CH_2SCH_2Ph, $CH_2CH_2COOCH_2Ph$) and the different positions of the benzyl groups in the side chains of the two analogues. The latter may be an important factor closely associated with the active conformation of the molecule.

It is concluded that charged groups in the side chain at position 11 of SP_{6-11} cause dramatic loss of potency. The side chain at this position is probably responsible for effective intramolecular interactions and/or receptor activation.

The lipophilic character and size of the position 11 side chain may also be important determinants of biological activity at tachykinin receptors.

The behavior of the analogue [Orn^6,Glu(OBzl)^{11}]-SP_{6-11} in rat colon is encouraging in that the Met^{11} residue in SP analogues may be replaced by the Glu(OBzl) residue, thus avoiding sulfoxide formation.

Experimental Section

Chemistry. Capillary melting points were determined on a Büchi SMP-20 apparatus and are reported uncorrected. Optical rotations were measured with the Carl Zeiss precision polarimeter (0.005°). Analysis by TLC was on precoated plates of silica gel F 254 (Merck) with the following solvent systems: (A) chloroform–methanol (6:1), (B) chloroform–methanol (9:1), (C) 1-butanol–acetic acid–water (4:1:1), (D) 1-butanol–acetic acid–water (4:1:5, upper phase), and (E) 1-butanol–acetic acid–water–pyridine (30:6:24:20). The products on TLC plates were detected by UV light and either chlorination followed by *o*-tolidine or ninhydrin. The elemental analyses are within ±0.40% of the calculated values. Amino acid analysis of the final products was performed on an LKB 4400 amino acid analyzer. Samples were hydrolyzed by boiling in 6 N HCl containing 0.1% phenol at 110 °C for 18 h in evacuated sealed ampules.

FAB mass spectral analyses were made with a VARIAN 311A double-focusing reversed-geometry mass spectrometer equipped with an M-Scan FAB gun. Data recording: Finnigan INCOS data system or UV photosensitive chart paper. Scan function: linear, 50 amu to maximum in 10–20 s. Primary atom beam: xenon, Xe^0 energy 6–9 keV (flux equivalent 10 μA). Solvents: formic acid–H_2O (1:1), acetic acid–H_2O (1:1). Matrix: glycerol or thioglycerol. Method: Approximately 0.1 mg of peptide was dissolved in 3–5 μL of acid–water. Target (stainless steel) was smeared with 1–2 μL of matrix to produce an even film. Approximately 1 μL of peptide solution was spotted into matrix film and was spread manually to give an even coating. Loaded target (on probe) was introduced into fast atom beam immediately prior

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Table II. Physical Constants of Peptides Boc-Y-X-NH_2

no.	X	Y	formula	mp, °C	recrystn solvent	yield, %	$[\alpha]^{22}_D$, deg	TLC: R_f (solvent)	anal.
4	Gln	bond	$C_{10}H_{19}N_3O_4$	132–133	EtOAc	78	+2.1[a]	0.06 (B), 0.56 (C)	C, H, N
5	Glu(OBzl)	bond	$C_{17}H_{24}N_2O_5$	122–123	EtOAc–pet. ether	74	+3.0[b]	0.52 (B), 0.88 (C)	C, H, N
6	Lys(Z)	bond	$C_{19}H_{29}N_3O_5$	138–139	EtOAc–pet. ether	70	−1.4[c]	0.79 (A), 0.76 (C)	C, H, N
7	Gln	Leu	$C_{16}H_{30}N_4O_5$	169–170	EtOAc–pet. ether	78	−19.1[d]	0.10 (B), 0.65 (C)	C, H, N
8	Glu(OBzl)	Leu	$C_{23}H_{35}N_3O_6$	113–114	EtOAc–pet. ether	69	−31.0[d]	0.63 (B), 0.91 (C)	C, H, N
9	Lys(Z)	Leu	$C_{25}H_{40}N_4O_6$	119–120	EtOAc–pet. ether	72	−18.6[d]	0.81 (C), 0.79 (C)	C, H, N

[a] c = 1% ethanol. [b] c = 2.5% DMF. [c] c = 5% DMF. [d] c = 1% DMF.

Table III. Physical Constants of Peptides Boc-Orn(Boc)-Phe-Phe-Gly-Leu-X-NH_2

no.	X	formula	mp, °C	recrystn solvent	yield, %	$[\alpha]^{22}_D$,[a] deg	TLC: R_f (solvent)	anal.
10	Gln	$C_{46}H_{69}N_9O_{11}$	223–224[b]	DMF–Et_2O	71	−24.2	0.80 (C), 0.84 (E)	C, H, N
11	Glu(OBzl)	$C_{53}H_{74}N_8O_{12}$	205–206	DMF–Et_2O	75	−36.0	0.81 (C), 0.79 (E)	C, H, N
12	Lys(Z)	$C_{55}H_{79}N_9O_{12}$	192–194	DMF–Et_2O	74	−28.5	0.82 (C), 0.87 (E)	C, H, N
13	Glu	$C_{46}H_{68}N_8O_{12}$	210–212	–	95	−34.0	0.75 (C), 0.69 (E)	C, H, N

[a] c = 1% DMF. [b] Decomposition.

Table IV. Physical Constants of Peptides H-Orn-Phe-Phe-Gly-Leu-X-NH_2

no.	X	formula	mp, °C	yield, %	$[\alpha]^{22}_D$, deg	TLC: R_f (solvent)	FAB-MS: m/z	amino acid analysis					
								Leu	Gly	Phe	Glu	Orn	Lys
14	Gln	$C_{36}H_{53}N_9O_7$	162–165	38	−33.4[b]	0.24 (A), 0.22 (C), 0.42 (E)	724	1.02	1.05	1.94	0.99	0.98	
15	Glu(OBzl)	$C_{43}H_{58}N_8O_8$	135–137	56	−28.5[b]	0.33 (A), 0.28 (C), 0.55 (C)	815	1.03	1.00	1.96	1.02	1.00	
16	Lys	$C_{37}H_{57}N_9O_6$	260–268[a]	32	−58.9[c]	0.06 (A), 0.08 (C), 0.48 (E)	745	1.05	1.01	1.97		0.97	0.99
17	Glu	$C_{36}H_{52}N_8O_8$	150–152	41	−37.2[b]	0.50 (A), 0.47 (C), 0.79 (E)	721	1.04	1.01	1.97	0.98	0.99	

[a] Decomposition. [b] c = 0.5% DMF. [c] c = 0.2% DMF.

to acquisition of spectra. All amino acids are of the L configuration.

The following abbreviations are used: DCC, *N,N*-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; EtOAc, ethyl acetate; pet. ether, petroleum ether, bp 60–80 °C; EtOH, ethanol; MeOH, methanol; Et_2O, diethyl ether; DMF, dimethylformamide; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid.

Preparation of Boc-X-NH_2. To a solution of Boc-X-OH (15 mmol) in THF (50 mL) cooled to −10 °C were added NMM (15 mmol) and isobutyl chloroformate (15 mmol). After 2 min, a 25% solution of ammonia (22.5 mmol) was added and the mixture stirred for 0.5 h at 10 °C and then for 1 h at room temperature. The solvent was removed in vacuo, and the residue was partitioned in EtOAc–H_2O. The organic layer was washed with 5% $NaHCO_3$ and water and dried (Na_2SO_4). The final product was obtained after evaporation of the solvent.

The compound **3** (Table I) showed high solubility in water; thus, the isolation procedure was modified as follows. At the end of the reaction, the solvent was evaporated in vacuo, and the residue was triturated with ethanol in which the desired product was dissolved. The ethanol was removed, and the residue was crystallized from EtOAc. The crude products were further purified by recrystallization (Table II).

Preparation of Boc-Leu-X-NH_2. A sample of Boc-X-NH_2 (10 mmol) was deprotected with TFA–anisole (10:1 v/v) for 45 min at room temperature. Evaporation of TFA yielded an oily residue, which was solidified by the addition of dry ether. The solid was washed several times with dry ether and dried over P_2O_5 in vacuo. The trifluoroacetic acid salt (TFA, H-X-NH_2) was dissolved in THF (20 mL) and neutralized with NMM at −10 °C.

To a solution of Boc-Leu-OH·H_2O (10 mmol) in THF (20 mL) cooled to −10 °C were added NMM (10 mmol) and isobutyl chloroformate (10 mmol). After 2 min, the solution of the neutralized trifluoroacetic salt was added, and the mixture was stirred for 0.5 h at −10 °C and then for 1 h at room temperature. The solvent was then removed in vacuo, and the residue was partitioned in EtOAc–H_2O. The organic layer was washed with 5% $NaHCO_3$, water, 10% citric acid, and water and dried (Na_2SO_4). Evaporation of the solvent yielded the desired products, which were further purified by recrystallization (Table II).

Preparation of Boc-Orn(Boc)-Phe-Phe-Gly-Leu-X-NH_2. A portion of Boc-Leu-X-NH_2 (5 mmol) was deprotected with 1 N HCl in acetic acid for 1 h at room temperature, and the resultant hydrochloride salt was dissolved in DMF (6 mL), neutralized with NMM, and allowed to react with a sample of Boc-Orn(Boc)-Phe-Phe-Gly-OH[13] (5 mmol) dissolved in DMF (10 mL) and preactivated at 0 °C for 0.5 h with HOBt (9 mmol) and DCC (5 mmol). The reaction mixture was left to stand for 2 h at 0 °C and then for 24 h at room temperature. The precipitated DCU was filtered, and the solvent was removed in vacuo. The remaining residue was solidified by trituration with 5% $NaHCO_3$. The resulting solid was washed several times, on the filter, with 5% $NaHCO_3$, water, 10% citric acid, and water and dried over P_2O_5 in vacuo. The products were further purified by recrystallization (Table III).

Preparation of Boc-Orn(Boc)-Phe-Phe-Gly-Leu-Glu-NH_2 (12). A sample of Boc-Orn(Boc)-Phe-Phe-Gly-Glu(OBzl)-NH_2 (3 mmol) was dissolved in 40 mL of DMF–H_2O (9:1 v/v) and hydrogenated over 10% Pd/C at atmospheric pressure. The progress of the reaction was monitored by TLC. At the end of the reaction, the solvent was removed in vacuo, and the residue was solidifed upon addition of ether. The solid was filtered, washed with ether, and dried over P_2O_5 under reduced pressure. For physical constants see Table III.

Preparation of H-Orn-Phe-Phe-Gly-Leu-X-NH_2. A sample of Boc-Orn(Boc)-Phe-Phe-Gly-Leu-X-NH_2 (150–400 mg) was deprotected with 1 N HCl in acetic acid for 1 h at room temperature. The solvent was removed in vacuo, and the residue was solidified by the addition of dry ether. The solid was filtered, washed several times with dry ether, and dried over KOH pellets under reduced pressure.

The hydrochloride salt resulting from the deprotection of **11** (X = Lys(Z)) was dissolved in glacial acetic acid and was subjected to catalytic hydrogenation over 10% Pd/C in the presence of 1 equiv of HCl. The reaction was monitored by TLC. At the end of the reaction, the catalyst was filtered and the solvent was evaporated. The residue was solidified by the addition of dry ether, and the solid was isolated as described above.

The fully deprotected hexapeptides were dissolved in water, filtered through a Millipore filter, and lyophilized. The products were further purified by gel filtration on Sephadex G-15 (2 × 85 cm) using 1 M acetic acid as eluant for peptides **13**, **14**, and **16** and 2 M acetic acid for peptide **15** followed by partition chromatography on Sephadex G-25F (2 × 85 cm) with 1-butanol–acetic acid–water (4:1:5 v/v, upper phase). Yields of the final products were 32–56% based on the protected peptides. For physical constants see Table IV.

Bioassays. Sources of drugs were as follows: substance P and the C-terminal hexapeptide of substance P (SP_{6-11}), Merseyside Laboratories U.K.; atropine sulfate and indomethacin, Sigma; methylsergide bimaleate, Sandoz; mepyramine maleate, generous gift of May and Baker Ltd. All other compounds were of Analar quality.

Indomethacin was dissolved in 10% $NaHCO_3$; all other drugs were dissolved in physiological salt solution.

Bioassays were performed on the guinea pig ileum and rat colon muscularis mucosae preparations as previously described[15,16] but with the following modifications: (i) atropine (1 μM), mepyramine (1 μM), methysergide (1 μM), and indomethacin (1 μM) were included in the bathing media for all experiments; and (ii) the time cycle for administration of agonist doses was arranged such that there was a 7-min interval between doses in the guinea pig ileum and 10 min in the rat colon. Pilot experiments had demonstrated that between-dose interactions were minimized for SP when these schedules were used.

Formal 3 + 3 assays (guinea pig ileum) or 2 + 2 assays (rat colon) were performed on all analogues by using a randomized block design. Results were analyzed by analysis of variance, and only those assays that satisfied tests for parallelism, linearity, difference curvature, and regression were accepted for estimates of relative activities. In the presence of the antagonists described above, the EC_{50} for SP is 2×10^{-9} M in the guinea pig ileum and 2×10^{-7} M on the rat colon muscularis mucosae. All results are expressed relative to the SP EC_{50}, taking SP_{1-11} as 1.0.

Samples of peptides were prepared as stock solutions of 1-10 mM. SP_{6-11} was dissolved in Me_2SO. All other peptides were dissolved in 0.01 M acetic acid. The stock solution was divided into aliquots, which were stored at -30 °C until required for use. A sample of solution was dried under nitrogen and prepared for amino acid analysis to provide an accurate estimate of peptide concentration. A further sample of each compound was subjected to FAB mass spectrometry to confirm its structure. Thus, molecular ions (M^+) were observed at m/z 724, 815, 745, and 721 for the new peptides **14-17**, respectively.

During the experiments, dilutions were made in physiological salt solution. Control experiments with Me_2SO showed that, at the concentrations used in the present experiments, this compound did not affect the response of the tissue to any of the peptides tested.

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Synthesis of 5-Chloro-5,8-dideaza Analogues of Folic Acid and Aminopterin Targeted for Colon Adenocarcinoma[1]

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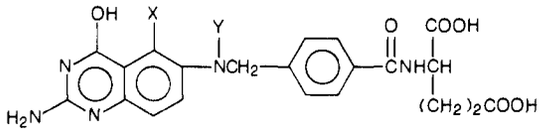
Several classical quinazoline analogues of folic acid bearing chloro or methyl substituents at position 5 were evaluated as inhibitors of the growth of four human gastrointestinal adenocarcinoma cell lines in vitro. The preparation of two of these, 5-chloro-5,8-dideazaisofolic acid, **1e**, and 5-chloro-5,8-dideazaisoaminopterin, **2a**, is reported for the first time. In addition, a new synthetic route to 5-chloro-5,8-dideazaaminopterin, **2b**, is described. For compounds having a 2,4-diamino configuration, the presence of chlorine at position 5 afforded superior growth inhibitory potency. However, compound **1e** was substantially less effective than its 5-methyl counterpart.

The folate analogue 5,8-dideazaisofolic acid (IAHQ), **1a**, has generated considerable interest as a potential agent for treating solid tumors that are unresponsive to methotrexate (MTX). Recent studies revealed that IAHQ was an effective inhibitor of the growth of human colon adenocarcinoma cells (HCT-8) in vitro.[2] Significant activity



	X	Y
1a	H	H
b	CH_3	H
c	H	CH_3
d	CH_3	CH_3
e	Cl	H

against colon tumor 38 in mice was also demonstrated, while MTX was not effective in this model.[2,3] It was also found that IAHQ protected newborn hamsters from mortality due to transplantable human osteosarcoma cells, whereas MTX was without effect against this xenograph at maximally tolerated dose.[4] Recently, we reported the syntheses of the 5-CH_3, **1b**, 9-CH_3, **1c**, and 5,9-$(CH_3)_2$, **1d**, modifications of IAHQ.[5] Each of these derivatives was found to have a modest level of growth inhibitory activity similar to that of IAHQ toward four human gastrointestinal adenocarcinoma cell lines in vitro.[5]

It was of interest, therefore, to prepare the 5-Cl analogue of IAHQ, 5-chloro-5,8-dideazaisofolic acid, **1e**, in order to assess the effect of an electron-withdrawing lipophilic substituent located at position 5 upon antitumor activity. In the case of 5,8-dideaza analogues of aminopterin, the 5-Cl modifications were found to display greater inhibitory activity toward the growth of L1210 leukemia cells in vitro than their corresponding 5-CH_3 or 5-H counterparts.[6] It

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