heated with anhydrous ammonia (80 mL) for 18 h in a stainless-steel bomb. The next day the ammonia was evaporated, and the residue was refluxed with ethanolic aqueous Na_2CO_3. The product was purified by silica gel column chromatography as described above to obtain 1.35 g (42%) of XI as a pale green powder: mp >250 °C; UV λ_{max} (pH 7) 259 and 316 nm (ϵ 6150 and 7200); UV λ_{max} (pH 12) 259 and 316 nm (ϵ 6100 and 7000); UV λ_{max} (pH 1) 278 and 317 (ϵ 10970 and 5700); ^{1}H NMR (Me_2SO-d_6) δ 8.20 (s, 1, C_2 H), 6.68 (t, 1, J = 6 Hz, $H_{1'}$, peak width = 13 Hz) and 5.50 (s, 1, C_7 H). Anal. ($C_{11}H_{14}N_4O_4 \cdot H_2O$) C, H, N.

6-Amino-3-(2′-deoxy-α-D-ribofuranosyl)imidazo[4,5-*c*]pyridin-4(5*H*)-one (XII). Compound VII (0.25 g, 0.5 mmol) was cyclized as described for XI above. The product (XII) was purified by preparative TLC [2-mm silica gel, developed in $CHCl_3$–MeOH (2:1)] to obtain 0.048 g (47%) of XII as a beige foam: UV λ_{max} (pH 7) 258 and 314 nm; UV λ_{max} (pH 12) 257 and 313 nm; UV λ_{max} (pH 1) 277 and 314 nm; ^{1}H NMR (D_2O) δ 8.18 (s, 1, C_2 H), 6.55 (q, 1, peak width = 10.2 Hz) and 5.77 (s, 1, C_7 H).

Cell Culture. Leukemia L1210 cells were obtained from Associated Biomedic Systems, Inc., Buffalo, NY. The cells were maintained in RPMI-1640 media containing 10% fetal calf serum and 1% (v/v) penicillin–streptomycin (supplies were obtained from Grand Island Biological Co., Grand Island, NY) in a carbon dioxide incubator at 37 °C. The cells had a doubling time of 8–10 h. Our methods for drug screening have been described previously.[22]

Animal Model Studies. All animals were obtained through C. R. Reeder, Division of Mammalian Genetics and Animal Production, National Cancer Institute, Bethesda, MD. The tumor line, C3H mammary adenocarcinoma 16/C, was obtained from Dr. T. Corbett, Southern Research Institute, Birmingham, AL, and maintained (lung-passed) in C3H female mice according to the method of Corbett et al.[23] For drug testing, the tumors (2- to 4-mm^3 freshly harvested fragments) were transplanted (subcutaneously) in 18- to 20-g B6C3F$_1$ female mice on day 0. The following day, the animals were randomized into various treatment groups. The drugs were dissolved in 0.2 M phosphate buffer and administered by intraperitoneal injections. The tumor weights were obtained by measuring the length (l) and width (w) of each tumor with a calliper and using the conventional $(l + w^2)/2$ formula.

Acknowledgment. The work was supported by National Cancer Institute Cancer Emphasis Research Grant CA 25715, Cancer Center Grant CA 14089 and Research Grant CA 32906. We are thankful to Stephanie Pentecost and Ken Leung for expert technical assistance. We are also indebted to Dr. Kenneth Service, Department of Chemistry of this University, for help in ^{13}C NMR studies, performed on a Varian XL-100 spectrometer.

Registry No. I, 56039-06-6; II (isomer 1), 56596-91-9; II (isomer 2), 58459-35-1; III (α), 83587-57-9; III (β), 83587-65-9; IV, 83587-58-0; V, 83587-59-1; VI, 83603-90-1; VII, 83587-60-4; VIII, 83587-61-5; IX, 961-07-9; X, 83587-62-6; XII, 83587-63-7; XIII, 83587-64-8.

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A Synthetic Approach to Poly(γ-glutamyl) Conjugates of Methotrexate

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Methotrexate poly(γ-L-glutamate)s bearing two and three glutamate units above that present in methotrexate have been synthesized by extension of a previously described route used to synthesize the lower conjugate bearing one added glutamate unit. Key steps in the sequence are the peptide coupling of *N*-[4-[[(benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic acid α-benzyl ester (**5**) with oligo(γ-L-glutamate) benzyl esters, removal of blocking groups by catalytic hydrogenolysis, and introduction of the (2,4-diamino-6-pteridinyl)methyl grouping by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide. Elaboration of the required oligo(γ-L-glutamate) chain was achieved one unit at a time, beginning with the coupling of L-glutamic acid dibenzyl ester with [(*tert*-butyloxy)carbonyl]-L-glutamic acid α-benzyl ester (**7**), followed by selective removal of the *tert*-butyloxycarbonyl grouping and another coupling step with **5** or **7** as required. Diphenylphosphoryl azide was used as the coupling reagent in each conversion producing a peptide linkage.

Intracellular conversion of methotrexate (MTX) to poly(γ-glutamyl) peptide derivatives has been shown to occur in a variety of animal and human tissues.[1-9] The poly(γ-glutamate)s are known to inhibit dihydrofolate reductase as strongly as MTX itself[2b,9-11] and also to inhibit thymidylate synthetase,[12] but many aspects of their biochemical actions remain to be elucidated. Current studies on their identification, extent of in vivo synthesis, transport characteristics, and role in antifolate activity led to a need for pure reference samples of authentic MTX poly(γ-glutamate)s. We describe in this paper a synthetic approach that allows unequivocal syntheses of these compounds.

The approach is based on syntheses of α- and γ-substituted peptides and amides of MTX, which involved the

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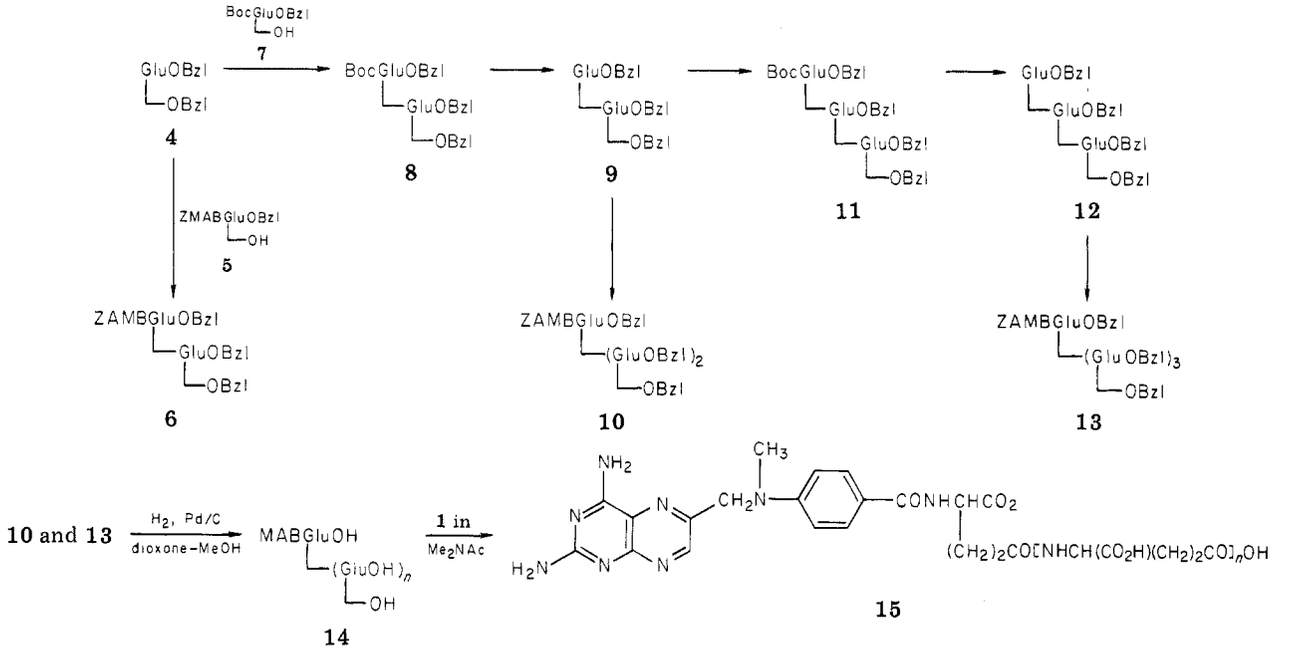
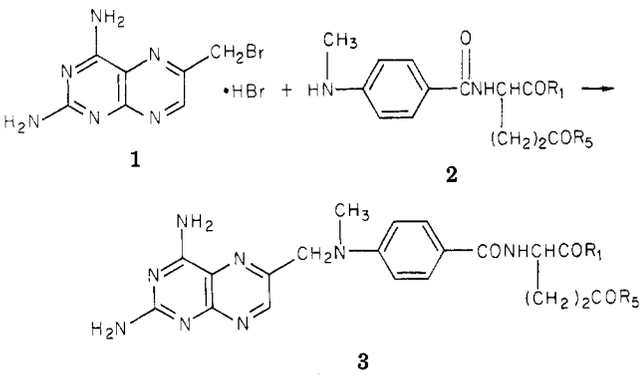
Scheme I. Synthesis of Poly(γ-glutamate) Analogues of Methotrexate[a]



[a] Bzl = $C_6H_5CH_2$; Z = $C_6H_5CH_2OCO$; MAB = 4-$CH_3NHC_6H_4CO$; Boc = $(CH_3)_3COCO$.

preparation of *N*-[4-(methylamino)benzoyl]-L-α- and -γ-glutamyl precursors (general structure **2**) and then attachment of the (2,4-diamino-6-pteridinyl)methyl grouping by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**1**).[13] The earlier work included the synthesis of the lowest MTX–γ-glutamate conjugate, *N*-[*N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]-benzoyl]-L-γ-glutamyl]-L-glutamic acid (MTX–γ-Glu).



Extension of this general approach to higher conjugates involved preparation of the appropriate oligo(γ-glutamate) benzyl esters (as shown in Scheme I), which were then coupled through their free amino group with the unprotected γ-carboxy grouping of *N*-[4-[[(benzyloxy)-carbonyl]methylamino]benzoyl]-L-glutamic acid α-benzyl ester (**5**) to give blocked intermediates of the desired chain lengths.

Oligo(γ-glutamic acid)s of up to seven units have been prepared previously as intermediates to folyl poly(γ-glutamate)s by Meienhofer et al.[14] and later by Drey and Priestly[15] who used the *tert*-butyl ester group for carboxy protection and the benzyloxycarbonyl group for temporary amine protection. In our approach, we prepared the desired oligo(γ-glutamate) derivatives using the benzyl ester group for carboxy protection and the *tert*-butyloxycarbonyl group for temporary protection of the primary amino grouping. As indicated above, the final peptide grouping to be introduced involved coupling of the oligo(γ-glutamate) benzyl esters with **5**. This approach afforded the advantage of giving the next-to-last precursor bearing benzyl and benzyloxycarbonyl blocking groups, which are smoothly and concomitantly removed by catalytic hydrogenolysis.

Each of the peptide-forming coupling steps shown in Scheme I was done with diphenylphosphoryl azide in *N*,*N*-dimethylformamide containing triethylamine according to the procedure of Yamada and co-workers.[16] L-Glutamic acid dibenzyl ester *p*-toluenesulfonate was coupled with [(*tert*-butyloxy)carbonyl]-L-glutamic acid α-benzyl ester (**7**) to give **8**. The protective Boc group of **8** was removed by treatment with dry HCl in dioxane solution.[14] Following removal of the dioxane, the residual **9**·HCl, a syrup that resisted crystallization, was converted to the crystalline *p*-toluenesulfonate. Coupling of **9** with **5** gave **10**, the blocked precursor of **15** (n = 2). The coupling of **9** with **7** gave **11**, whose Boc grouping was removed by dry HCl in dioxane to give the hydrochloride of **12**, which, in contrast to the hydrochloride of **9**, was easily obtained in solid form when the dioxane was removed. Coupling of **12** with **5** then provided **13**, the blocked precursor of **15** (n = 3). Hydrogenolysis of pure **10** and **13** in dioxane–MeOH (10:1) in the presence of 5% Pd on C at

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(17) Melting points were observed on a Mel-Temp apparatus. Analytical results indicated by element symbols were within ±0.4% of the calculated percentages. Unless indicated otherwise, products were dried in vacuo (<1 mm) at 25 °C over P_2O_5. Instruments used in spectral determinations have been described.[13]

Table I. Blocked Peptides from Coupling Reactions

no.	yield, %	mp, °C	formula
6	71[a]	88-90	$C_{47}H_{47}N_3O_{10}$
10	97[b]	150-152	$C_{59}H_{60}N_4O_{13}$[c]
13	82[d]	155-160	$C_{71}H_{73}N_5O_{16}$[c]
8	78	92-93	$C_{36}H_{42}N_2O_9$[c]
11	97	124-128	$C_{48}H_{55}N_3O_{12}$[c]

[a] Previously prepared by mixed anhydride method with isobutyl chloroformate (lit.[13] mp 87-89 °C, yield 78%). [b] Waxy solid residue from removal of $CHCl_3$ dissolved when stirred with warm EtOH (6 mL/g of residue); then white solid separated, apparently all at once, from the boiling solution. [c] Anal. C, H, N. [d] Yield was lowered to 51% when the typical procedure was used, due to emulsification during workup of the $CHCl_3$ solution and relatively poor solubility in $CHCl_3$. The better yield resulted when the residue following removal of DMF was stirred with H_2O to give a solid, which was washed on a funnel with 2 N H_2SO_4, 5% $NaHCO_3$, and H_2O. Product was then recrystallized from EtOH.

ambient conditions led to the deblocked precursors **14** (n = 2, 3) as solidified foams suitable for use in the final step; their ^1H NMR spectra revealed harmless retention of small amounts of dioxane and MeOH. Alkylation with **1** proceeded as expected. Isolation procedures for **15** (n = 2, 3) reflect an increase in solubility in water with increasing glutamate chain length. Water-diluted solutions of the reaction mixtures adjusted to near pH 3.5 were combined with ethanol to cause precipitation of **15** (n = 2, 3). Further purification was effected by a reprecipitation consisting of addition of a solution of the compound in AcOH–H_2O (1:1) to EtOH.

High-performance liquid chromatography (HPLC) assays showed the target compounds to be of high purity (99% minimum) with respect to UV-absorbing material, and both compounds moved cleanly as single spots on thin-layer chromatograms. The chromatography systems are described in Table II. Other data mentioned in Table II also support the assigned structures and the indicated solvation.

Previous syntheses of **15** (n = 2, 3) by the Merrifield solid-phase technique afforded the products in aqueous solutions eluted from ion-exchange columns.[1,10] A higher conjugate (**15**, n = 4) was prepared by coupling of 4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoic acid with glutamyltris(γ-glutamyl)glutamate hexa-*tert*-butyl ester, followed by ester hydrolysis in trifluoroacetic acid. The product, isolated in very low yield following gel filtration and ion-exchange chromatography, was characterized by its MTX-like UV spectrum and by amino acid analysis, which established the number of glutamic acid units present per molecule.[3]

Experimental Section

Preparations of 6, 10, and 13. Coupling of 5 with 4, 9, and 12. The procedure for the preparation of **6** is illustrative. Results and additional data are given in Table I. A solution of $(C_6H_5O)_2PON_3$ (4.55 g, 16.5 mmol; Aldrich Chemical Co.) in DMF (15 mL) was added dropwise during 10 min to a stirred, cold (−5 to 0 °C) solution of **5**[13] (7.57 g, 15.0 mmol) and **4**·TsOH[18] (8.24 g, 16.5 mmol) in DMF (30 mL). A solution of Et_3N (3.34 g, 33.0 mmol) in DMF (15 mL) was then added dropwise during 10–15 min, and the solution was kept near 0 °C for 5 h before it was allowed to warm to room temperature and left overnight (16 h). DMF was removed in vacuo (<1 mm, bath to 35 °C), and the residual oil was dissolved in $CHCl_3$ (75 mL). The $CHCl_3$ solution was washed successively with 50-mL portions of 1 N HCl, H_2O, saturated $NaHCO_3$ solution, and H_2O and then dried (Na_2SO_4) and evaporated. The residue was recrystallized from EtOH.

Preparation of 8 and 11. Coupling of 7 with 4 and 9. These preparations were carried out with **7** (Chemical Dynamics Corp.) in the way **5** was used in the preparation described above. Products were recrystallized from EtOH. Results and additional data are included in Table I.

***N*-(L-γ-Glutamyl)-L-glutamic Acid Tribenzyl Ester (9) *p*-Toluenesulfonate.** A solution of **8** (20.6 g, 31.9 mmol) in dry HCl–dioxane solution (80 mL of 4.4 N) was stirred at 25 °C for 45 min. Evolution of CO_2, observed by means of a H_2O-charged bubble counter, had stopped after about 30 min. The solution was then evaporated under reduced pressure, and two portions of dioxane added to the residue were evaporated to aid the removal of excess HCl. The thick syrup that remained was dissolved in warm (50 °C) H_2O (400 mL), and the solution was transferred to a beaker and then treated with stirring with a warm (40 °C) solution of an excess of TsOH·H_2O (12.6 g) in H_2O (50 mL). The salt **9**·TsOH crystallized readily, and, after being kept overnight in a refrigerator, was collected, pressed, washed with a minimum of cold H_2O, and dried. The yield of pure **9**·TsOH, mp 149–152 °C, was 98% (22.5 g). Anal. ($C_{31}H_{34}N_2O_7 \cdot HO_3SC_6H_4CH_3$) C, H, N.

***N*-[*N*-(L-γ-Glutamyl)-L-γ-glutamyl]-L-glutamic Acid Tetrabenzyl Ester (12) Hydrochloride.** Treatment of **11** (10.5 g, 12.1 mmol) with 4.4 N HCl–dioxane solution (30 mL) as described for the preparation of **9** was followed by evaporation to give a white solid, which was stirred with H_2O (60 mL) until a smooth suspension formed. The collected solid was dried to give **12**·HCl·H_2O, mp 140–144 °C, in 96% yield (9.52 g). Anal. ($C_{43}H_{47}N_3O_{10} \cdot HCl \cdot H_2O$) C, H, N.

Hydrogenolysis of 10 and 13 to Produce 14 (n = 2, 3). Conversion of **10** and **13** to **14** (n = 2, 3) was carried out in the same way. The preparation of **14** (n = 3) is illustrative. A magnetically stirred solution of **13** (5.50 g, 4.32 mmol) in dioxane (200 mL)–MeOH (20 mL) containing 5% Pd on C(1.0 g) under H_2 at ambient conditions of the laboratory absorbed 687 mL of H_2 during 18 h (662 mL during the first 2.5 h). The catalyst was removed by filtration (Celite mat), and the filtrate was evaporated (final conditions <1 mm, bath at 25 °C). The residue, a deliquescent solid foam, was left in the flask in which it had been concentrated for direct conversion to **15** (n = 3).

Table II. MTX Poly(γ-glutamate) **15** (n = 0-3)

n	molecular formula[a-c]	HPLC retention time, min[d]
0	$C_{20}H_{22}N_8O_5 \cdot 3H_2O$[e]	15.8
1	$C_{25}H_{29}N_9O_8 \cdot 1.5H_2O$[e]	10.5
2	$C_{30}H_{36}N_{10}O_{11} \cdot 0.5C_2H_5OH$[f]	9.3
3	$C_{35}H_{43}N_{11}O_{14} \cdot 1.5C_2H_5OH \cdot 2H_2O$[f]	8.7

[a] Elemental analysis results for C, H, and N were in agreement with values calculated for the indicated solvates. The ^1H NMR spectra of these samples are as expected for the assigned structures and the indicated solvation by ethanol. [b] Field-desorption mass spectra[19] showed the expected molecular ions with no indication of lower conjugates. [c] Thin-layer chromatographic analyses of **15** (n = 2,3) on DEAE-cellulose sheets (Baker-flex) with 0.5 M NaCl, 0.2 M in 2-mercaptoethanol, in 0.005 M KH_2PO_4 buffer solution at pH 7.0 produced single spots with the following R_f values: **15** (n = 2), 0.45; **15** (n = 3), 0.50. In this system, MTX gave R_f 0.41. [d] HPLC assays were done with a Model 6000A pump and a U6K injector from Waters Associates. We determined retention times by monitoring UV absorbance at 254 nm on a Waters Differential UV detector. A reverse-phase system was used, with a mobile phase of 10% CH_3CN in 0.1 M NaOAc, pH 3.6. Solutions of the samples (1.0 mg/mL in standard buffer, pH 7.0) were applied to a 30 × 0.39 cm C_{18} μBondapak column (Waters Associates) and eluted isocratically at 1.5 mL/min. Retention times and relative peak areas were determined with a Hewlett-Packard 3380 integrator at a chart speed of 0.5 cm/min. [e] Dried in vacuo (25-30 °C over P_2O_5). [f] Dried in vacuo (77 °C over P_2O_5).

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***N*-[*N*-[*N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamic Acid (15, *n* = 2).** Side-chain precursor **14** (*n* = 2) from hydrogenolysis of **10** (5.68 g, 5.50 mmol) was dissolved in Me_2NAc (25 mL). Solid **1** (4.58 mmol) was added, and the mixture was stirred at 25 °C for 6 days.[20] The yellow-orange solution was combined with cold H_2O (105 mL) to which 1 N NaOH (20 mL) had been added. The resulting solution (pH 4.7) was treated with 1 N HCl to pH 3.7, and a yellow solid precipitated. After refrigeration overnight, the precipitate was collected, but it changed to a viscous gum on the funnel. Dilution of the filtrate with EtOH (1 L) caused separation of yellow solid. The stiff gum and the EtOH-precipitated solid (1.07 g) were combined while being dissolved in H_2O (80 mL) to which NaOH solution (1 N) was added in small increments to keep the pH near 6. The solution that formed was treated dropwise with 1 N HCl, and a viscous gum-like precipitate began forming at pH 4.0 and continued until the pH was 3.2. After the mixture had been chilled in an ice-H_2O bath, the supernatant was removed by decantation; then the gum was dissolved in a 1:1 solution of AcOH-H_2O (25 mL). Dropwise addition to continuously stirred EtOH (60 mL) followed, and **15** (*n* = 2) separated as an easily managed yellow solid. The collected solid was washed successively with 5:1 (v/v) EtOH-H_2O, EtOH, and then Et_2O and dried at successive temperatures of 25, 58, and 77 °C until it reached a constant weight of 2.26 g (67% yield). Additional data are included in Table II.

***N*-[*N*-[*N*-[*N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-γ-glutamyl]-L-glutamic Acid (15, *n* = 3).** A mixture of side-chain precursor **14** (*n* = 3; 4.32 mmol) and **1** (3.60 mmol) in Me_2NAc (20 mL) was stirred at 25 °C for 6 days,[20] and then the solution that formed was combined with cold H_2O (100 mL). Dropwise addition of 1 N NaOH to raise the pH from 2.2 followed, and the precipitate began forming when the pH was 3.0. When the pH had been brought to 3.7, only a small amount of gummy precipitate had formed. Addition of 1 N NaOH was continued until the pH was 6.8 in order to redissolve the precipitate. The solution was then carefully treated with 1 N HCl to lower the pH to 3.8-4.0, where it remained clear. This solution was added dropwise to stirred EtOH (600 mL) to give a yellow solid, which was collected after the mixture had been left overnight in a refrigerator. The collected precipitate was washed with EtOH, followed by Et_2O, and dried in vacuo to give 3.50 g of material whose field-desorption mass spectrum showed peaks of *m/e* 841 (M^+) and 842 [$(M + 1)^+$]. Assay by HPLC showed high purity (98%) with respect to UV-absorbing materials, but elemental analysis results (Found: C, 41.18, 40.99; H, 4.99, 4.93; N, 13.69, 13.62) indicated contamination by inorganic material in addition to the expected solvation by EtOH. This material was dissolved in AcOH-H_2O (1:1, 20 mL), and the solution was added dropwise to stirred EtOH (500 mL). The remainder of the isolation procedure was the same as described above for **15** (*n* = 2). The yield of **15** (*n* = 3) was 2.66 g. More data are given in Table II. The yield was 78% based on the solvate indicated in Table II.

(20) The light-sensitive products were protected during the reaction period by wrapping the flasks with Al foil. Isolated products were stored in a freezer.

Acknowledgment. This work was supported by Public Health Service Contract 263-MD-104307, awarded by the National Cancer Institute, DHHS. The authors thank Sheila Campbell for HPLC analyses.

Registry No. 1, 52853-40-4; 4, 2768-50-5; 5, 79974-14-4; 6, 79974-10-0; 7, 30924-93-7; 8, 83816-89-1; 9, 83816-90-4; 10, 83816-91-5; 11, 83816-92-6; 12, 83816-93-7; 13, 83816-94-8; 14 (*n* = 2), 83816-95-9; 14 (*n* = 3), 83816-96-0; 15 (*n* = 0), 59-05-2; 15 (*n* = 1), 41600-13-9; 15 (*n* = 2), 41600-14-0; 15 (*n* = 3), 73610-81-8.

Aromatic Amidines: Comparison of Their Ability to Block Respiratory Syncytial Virus Induced Cell Fusion and to Inhibit Plasmin, Urokinase, Thrombin, and Trypsin

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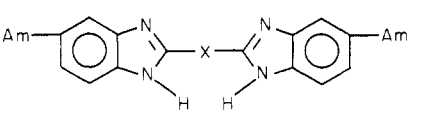
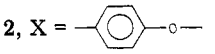
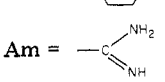
Two series of amidine derivatives consisting of a total of 24 compounds were examined for a correlation between their blocking effect on respiratory syncytial virus induced cell fusion and their inhibitory activity against selected trypsin-like protease. Although no correlation was evident between the two activities, several potentially important discoveries were made. A highly selective inhibitor of plasmin over thrombin (compound **10**) was obtained, and a potent new blocker of virus-induced cell fusion (compound **22**) was identified.

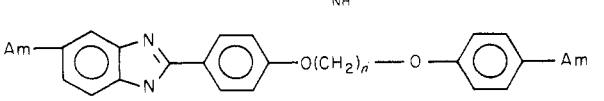
Respiratory syncytial (RS) virus is a common respiratory pathogen and the leading cause of respiratory tract infection in infancy and early childhood.[1] Although the virus commonly produces bronchiolitis and bronchopneumonia and may be life threatening in certain populations, there is no effective vaccine or chemotherapeutic agent yet available. Recently, we reported that certain aromatic amidino derivatives effectively blocked cell fusion induced by RS virus and significantly reduced the yield of RS virus.[2,3] Aromatic amidino compounds are generally recognized as potent reversible inhibitors of arginine- and lysine-specific esteroproteases. Specifically, we observed that our leading compound against virus-induced cell fusion,[2,3] bis(5-amidino-2-benzimidazolyl)methane (BABIM, compound 1), was also an outstanding inhibitor of three esteroproteases (trypsin, urokinase, and plasmin).[4,5] The



1, X = CH_2

2, X = 

Am = 



3, *n* = 2
4, *n* = 3
5, *n* = 4
6, *n* = 5

suggestion of a relationship between the antifusion potency of amidino compounds and their effectiveness against

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0022-2623/83/1826-0294$01.50/0