Structure−Activity Relationship Studies for the Peptide Portion of the Bladder Epithelial Cell Antiproliferative Factor from Interstitial Cystitis Patients

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We performed comprehensive structure−activity relationship (SAR) studies on the peptide portion of antiproliferative factor (APF), a sialylated frizzled-8 related glycopeptide that inhibits normal bladder epithelial and urothelial carcinoma cell proliferation. Glycopeptide derivatives were synthesized by solid-phase methods using standard Fmoc chemistry and purified by RP-HPLC; all intermediate and final products were verified by HPLC-MS and NMR analyses. Antiproliferative activity of each derivative was determined by inhibition of ^{3}H-thymidine incorporation in primary normal human bladder epithelial cells. Structural components of the peptide segment of APF that proved to be important for biological activity included the presence of at least eight of the nine N-terminal amino acids, a negative charge in the C-terminal amino acid, a free amino group at the N-terminus, maintenance of a specific amino acid sequence in the C-terminal tail, and *trans* conformation for the peptide bonds. These data provide critical guidelines for optimization of structure in design of APF analogues as potential therapeutic agents.

Introduction

Interstitial cystitis (IC[a]) is a devastating disease of the urinary bladder that is characterized by thinning or even focal obliteration of the bladder epithelium. It affects approximately 1 million Americans, and evidence suggests it occurs eight to nine times more frequently in women than in men.[1,2] The cause of this disorder remains unknown. Several possible causes of IC have been proposed including changes in neuronal function, autoimmune reactivity, infection, and toxin production.[3−6] Urine from IC patients has been shown to contain an antiproliferative factor (APF) that decreases ^{3}H-thymidine incorporation by human bladder epithelial cells.[6] A variety of techniques including total synthesis were previously used to identify APF as a nonapeptide (TVPAAVVVA) containing a 2,3-sialylated core 1 α-O-linked disaccharide (Galβ1-3GalNAc, the Thomsen−Friedenreich antigen, or "TF_{ag}") linked to the N-terminal threonine residue (i.e., Neu5Acα2-3Galβ1-3GalNAcα-O-TVPAAVVVA, Figure 1).[7] The peptide sequence of APF is identical to a segment of the sixth transmembrane domain of the frizzled-8 protein, a Wnt ligand receptor.[7,8]

Early studies indicated that purified native APF increased E-cadherin expression and decreased proliferation of bladder epithelial cells in vitro,[9] and both native and synthetic APF were shown to inhibit the proliferation of normal bladder epithelial as well as cells derived from urothelial carcinomas at picomolar to low nanomolar concentrations.[7,10] Therefore, APF and perhaps more efficacious synthetic derivatives potentially represent an innovative group of antitumor agents with a novel mode of action. In addition, pull-down experiments utilizing a biotinylated derivative of APF identified its bladder epithelial cell receptor as the cytoskeleton associated protein 4, CKAP4.[11] Preliminary SAR information obtained during the original complete characterization and synthesis of APF indicated that the terminal sialic acid residue is not necessary for activity but that the α-linked TF-disaccharide of the peptide is required (i.e., Galβ1-3GalNAcβ-O-TVPAAVVVA and the nonglycosylated nonapeptide were completely inactive).[7]

We have now performed additional extensive SAR studies on the peptide portion of the APF molecule to better understand the structural elements that are required for antiproliferative activity. The synthesis of congeners containing structural modifications to the peptide portion of APF (TVPAAVVVA) and the effects of these modifications on the biological activity of APF are presented.

Results

We determined whether changes to certain structural aspects of the peptide segment influenced the biological activity of APF by systematically replacing or modifying amino acids residues from the N-to-C termini of the sequence. Derivatives are grouped on the basis of amino acid substitutions or modifications made in three separate segments of APF: the N-terminal Thr-Val segment (Table 1), the Pro-Ala segment (Table 2), and the

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[a] Abbreviations: IC, interstitial cystitis; 12-Ado, 12-aminododecanoic acid; APF, antiproliferative factor; 2ClTrt resin, 2-chlorotrityl resin; Ac_2O, acetic anhydride; AcOH, acetic acid; Aib, aminoisobutyric acid; Aze, azetidine 2-carboxylic acid; BEP, 2-bromo-1-ethylpyridinium tetrafluoroborate; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; Hyp, *trans*-4-hydroxyproline; NMP, 1-methyl-2-pyrrolidinone; Hyp(^{t}Bu), *O-tert*-butyryl-*trans*-4-hydroxyproline; PyAOP, (7-azabenzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate; Pip, pipecolic acid; TF_{ag}, Thomsen−Friedenreich antigen; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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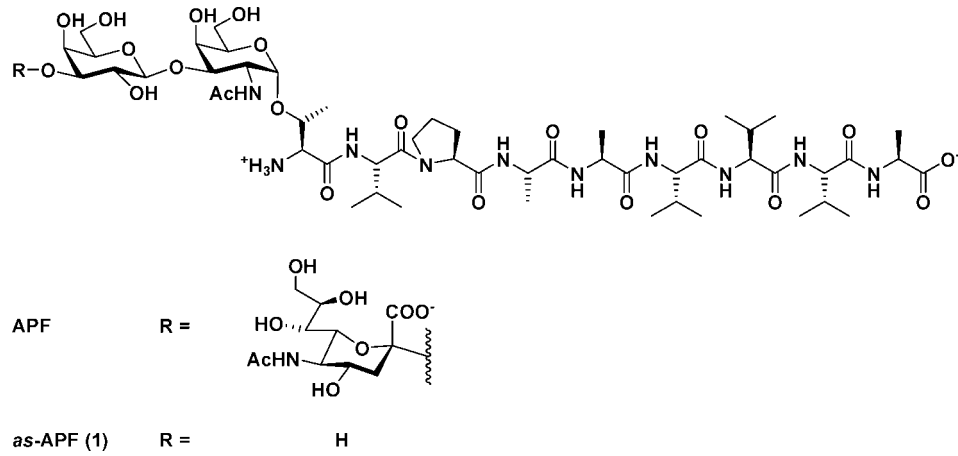


Figure 1. Structures of APF and *as*-APF.

C-terminal tail (AVVVA) segment (Tables 3−5). L-Amino acids were used for the synthesis of all derivatives unless otherwise indicated.

We previously determined that the endobiotic factor (Neu5Acα2-3Galβ1-3GalNAcα-O-TVPAAVVVA, Figure 1), the nonsialylated analogue (Galβ1-3GalNAcα-O-TVPAAVVVA, **1**), the sialylated compound with a lactosamine unit in place of the TF_{ag} disaccharide α-O-linked to threonine (Neu5Acα2-3Galβ1-4GlcNAcα-O-TVPAAVVVA), the sialylated compound with a lactosamine unit and substitution of the first two amino acids Thr-Val with Ser-Leu (Neu5Acα2-3Galβ1-4GlcNAcα-O-SLPAAVVVA), and the same compound in nonsialylated form (Galβ1-4GlcNAcα-O-SLPAAVVVA) are all essentially equipotent in biological antiproliferation assays. On the basis of these results, activity for each analogue described in this report was compared simultaneously to activity of the most synthetically accessible of these analogues, the nonsialylated form of the endobiotic (Galβ1-3GalNAcα-O-TVPAAVVVA, **1**), hereafter referred to as asialo-APF, or "*as*-APF".

Modifications to the N-Terminus (Thr^1-Val^2). We began by first replacing threonine−valine in **1** with serine−leucine, an "isosteric" substitution that maintained identical atomic mass while essentially "transferring" a methylene unit from the N-terminal threonine to Val^2 (**2**, Table 1), resulting in a derivative similar to the Galβ1-4GlcNAcα-O-SLPAAVVVA derivative described in the previous paper.[7] This modification resulted in 2 orders of magnitude loss of potency (Table 1) (Figure 2). In comparison, simple removal of the threonine methyl group in this location (i.e., the lone substitution of Thr^1 with Ser, **3**) resulted in even greater (4 orders of magnitude) loss of activity compared to the parent *as*-APF molecule **1**, and the lone substitution of Val^2 with Leu (**4**) resulted in inactivation (Table 1, Figure 2). These results indicate that the number and positioning of methyl groups in this location are important for *as*-APF activity.

Certain other minor modifications to the N-terminal two amino acids also affected *as*-APF activity (Table 1). For example, acetylation of the N-terminal threonine (**5**) and extension of the peptide sequence with Tyr (a preceding amino acid in the sequence of frizzled-8 protein,[8] **6**) both resulted in approximately 2 orders of magnitude loss of potency, providing evidence for the importance of the threonine amino group. Interestingly, replacement of Val^2 with Tyr (**7**) resulted in complete inactivation of *as*-APF, indicating that the side chain of the amino acid in this location is critical for activity.

Modifications to Pro^3-Ala^4. Except for the N-terminal glycosylated threonine, the amino acid residues of *as*-APF are made up of only three different amino acids, one of these being proline. The cyclic nature of the proline side chain is often a source of conformational adjustment in a peptide/protein sequence, being involved in turns and changes in directionality of the sequence following this residue. The proline in APF appears to be important for its activity, as substitution of proline with all but one of the modified amino acids tested was detrimental to biological function (Table 2). For example, substitution of L-proline with D-proline (**8**), pseudoproline (Ser($\Psi^{Me,Me}$ pro), **9**) (Figure 3), or azetidine 2-carboxylic acid (Aze) (**10**) (Figure 3) completely abolished activity (Table 2). While certain other substitutions did not completely destroy activity, substitution of Pro^3 with Ala (**11**), *trans*-4-hydroxyproline (**12**) (Figure 3), or *O*-*tert*-butyryl-*trans*-4-hydroxyproline (**13**) resulted in 2−3 orders of magnitude decrease, and substitution of Pro^3 with *N*-methylalanine (**14**) resulted in 4 orders of magnitude decrease in biological activity. Only substitution of Pro^3 with pipecolic acid, the six-membered ring analogue of proline (**15**), resulted in complete retention of *as*-APF's biological activity (Figure 3).

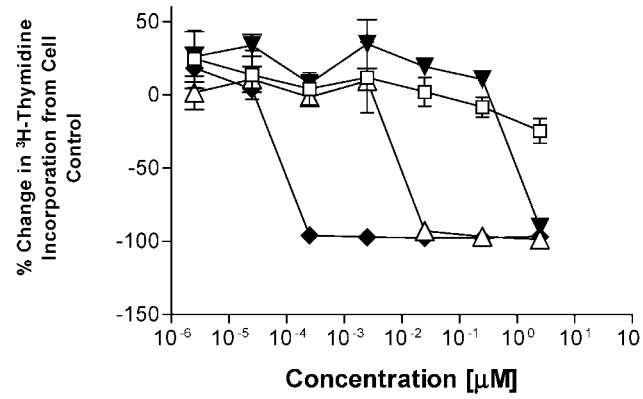
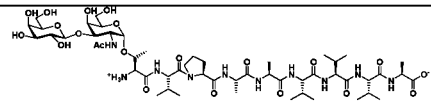
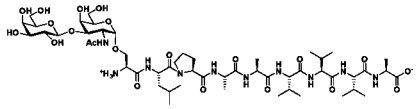
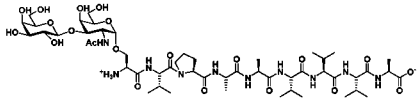
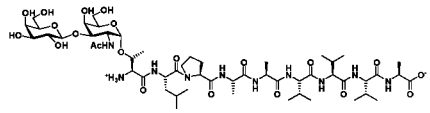
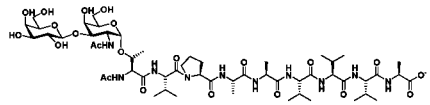
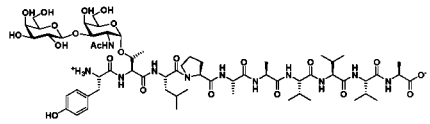
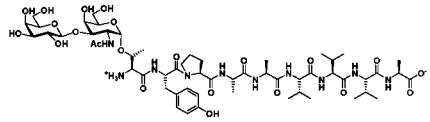


Figure 2. Antiproliferative activity of specific APF derivatives (modifications to the N terminus). Inhibition of tritiated thymidine incorporation by primary normal bladder epithelial cells was determined for each derivative at the concentrations indicated. Experiments were run in triplicate on two separate occasions. Data are expressed as the mean percent change in thymidine incorporation relative to a cell control treated with diluent (acetonitrile/H_2O at 1:1) alone; bars indicate standard error of the mean for all six data points: (◆) Galβ1-3GalNAc-O-TVPAAVVVA; (□) Galβ1-3GalNAcα-O-TLPAAVVVA; (△) Galβ1-3GalNAcα-O-SLPAAVVVA; (▼) Galβ1-3GalNAcα-O-SVPAAVVVA.

Substitution of AAVVVA with 12-Aminododecanoic Acid. Because APF is a highly hydrophobic peptide with only the N-terminal glycosylated threonine offering any measure of

Table 1. Substitutions or Modifications of the N-Terminus (Thr^1-Val^2)

No	Derivative	% of activity [a]	P value [b]
1	 Galβ1-3GalNAcα-O-TVPAAVVA	*	*
2	 Galβ1-3GalNAcα-O-SLPAAVVA	1%	< 0.001
3	 Galβ1-3GalNAcα-O-SVPAAVVA	0.01%	< 0.001
4	 Galβ1-3GalNAcα-O-TLPAAVVA	inactive	
5	 Ac-T(Galβ1-3GalNAcα-O-)VPAAVVA	0.7%	< 0.001
6	 YT(Galβ1-3GalNAcα-O-)VPAAVVA	1%	< 0.001
7	 Galβ1-3GalNAcα-O-TYPAAVVA	inactive	

[a] As a result of the variability of cell response in the biological assay, the activity of each congener was normalized to the activity of **1** run simultaneously on the same plate according to the following equation: % = [IC_{50} (*as*-APF)/IC_{50} (derivative)] × 100; the average IC_{50} value of **1** was ~1 nM. Percent of activity is expressed relative to **1**, which served as a standard control on each plate. Derivatives with no significant activity at <25 μM concentration (the cutoff limit for the biological assay) were considered to be inactive. [b] NS = not significant at $p > 0.05$.

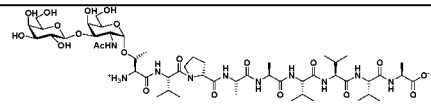
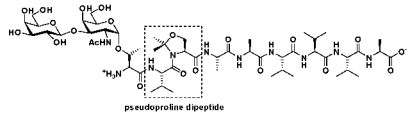
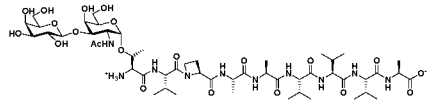
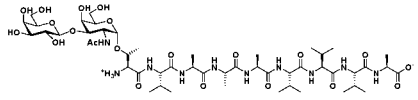
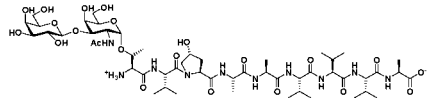
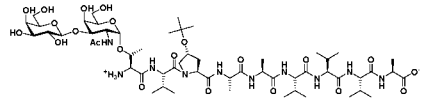
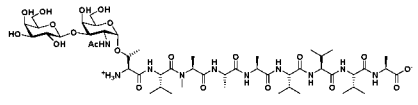
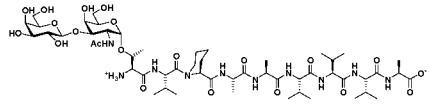
hydrophilicity, we next considered the possibility that the three N-terminal amino acids (TVP) may be important for specific interaction with the receptor while the following hydrophobic C-terminal amino acids may interact nonspecifically with (e.g., intercalate into) the lipid-containing cell membrane. We therefore determined whether complete replacement of AAVVVA with the amino-substituted fatty acid 12-aminododecanoic acid (12-Ado) (**16**) affected biological activity. This derivative proved to be completely inactive, however (Table 3), indicating a requirement for one or more additional specific structural characteristics of the carboxy-terminal peptide segment.

Modifications of Carboxy-Terminal Amino Acids 5−9 (AVVVA). To determine the length of the C-terminal tail that is necessary for activity, we first tested *as*-APF containing only five of the nine amino acids (i.e., truncated by four amino acids at the carboxy terminal end (**17**)) and determined that this derivative was completely inactive. We therefore next began truncating *as*-APF beginning at the carboxy terminal end. *as*-APF containing all but the carboxy-terminal alanine (**18**) had full activity, but *as*-APF truncated by only one additional amino acid (**19**) proved to be completely inactive (Table 3). Taken together, these findings indicate that a minimum of the eight N-terminal amino acids is necessary to maintain a required structural element of *as*-APF.

We noted that the 5 amino acid C-terminal "tail" of APF contains the AXXXA sequence, a common α-helical motif in proteins.[12] We therefore hypothesized that the amino acid sequence of this segment of *as*-APF may also be important for interaction with its receptor because similar motifs have been shown to function in protein−protein dimerization.[13−15] To test this hypothesis, we reversed Val^8 and Ala^9 (**20**) or replaced either Ala^9 or Ala^5 with more branched but similarly charged amino acids (such as Leu^9, **21**; or Val^5, **22**), all of which changes resulted in loss of most or all biological activity (Table 4). While Ala^9 is not required for *as*-APF activity, these findings provide evidence that Ala^5 and Ala^9 may both be important for optimal activity of APF containing nine amino acids.

The phenomenon of protein−protein dimerization can also occur for GXXXG or SXXXS motifs, so we next tested *as*-APF derivatives containing GXXXG or SXXXS in place of

Table 2. Substitutions or Modifications of the Pro[3]-Ala[4] Segment

No	Derivative	% of activity [a]	P value [b]
8	 Galβ1-3GalNAcα-O-TV-D-Pro-AAVVA	inactive	
9	 Galβ1-3GalNAcα-O-TVS($\Psi^{Me,Me}$ pro)AAVVA	inactive	
10	 Galβ1-3GalNAcα-O-TV-Aze-AAVVA	inactive	
11	 Galβ1-3GalNAcα-O-TVAAAVVVA	0.3%	< 0.001
12	 Galβ1-3GalNAcα-O-TV-Hyp-AAVVA	0.3%	< 0.001
13	 Galβ1-3GalNAcα-O-TV-Hyp(^{t}Bu)-AAVVA	0.2%	< 0.001
14	 Galβ1-3GalNAcα-O-TV-*N*-MeAla-AAVVA	0.05%	< 0.001
15	 Galβ1-3GalNAcα-O-TV-Pip-AAVVA	100%	NS

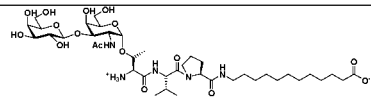
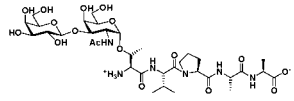
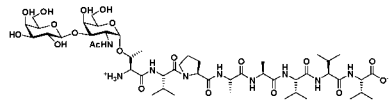
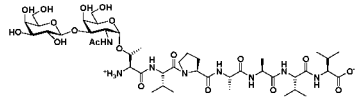
[a] As a result of the variability of cell response in the biological assay, the activity of each congener was normalized to the activity of **1** run simultaneously on the same plate according to the following equation: % = [IC_{50} (*as*-APF)/IC_{50} (derivative)] × 100; the average IC_{50} value of **1** was ∼ 1 nM. Percent of activity is expressed relative to **1**, which served as a standard control on each plate. Derivatives with no significant activity at <25 μM concentration (the cutoff limit for the biological assay) were considered to be inactive. [b] NS = not significant at $p > 0.05$.

AXXXA. Additional evidence for the importance of alanine in the fifth and ninth positions was provided by the partial inactivation resulting from replacement of both Ala[5] and Ala[9] with serine (Ser[5,9], "SXXXS", **23**) and complete inactivation resulting from replacement with glycine (Gly[5,9], "GXXXG", **24**) (Table 4). However, substitution of Val[6−8] with alanine residues with retention of Ala[5] (**25**) also resulted in decreased *as*-APF activity, while substitution of Val[6−8] with glycine residues (**26**), substitution of Val[7] with D-valine (**27**), or substitution of Val[6] and Val[8] with isoleucines (**28**) resulted in complete inactivation. Taken together, all of the above findings suggest that both the presence of alanine in the fifth position and the presence of valine in the sixth through eighth positions are important for optimal activity of *as*-APF.

Carboxyamidation of Ala[9] in *as*-APF (**29**) also resulted in decreased activity (Table 5), suggesting the possibility that a negative charge in the ninth position may be important for APF activity. Interestingly, the addition of cysteine to the carboxy terminus (**30**) resulted in a decrease in APF activity of 2 orders of magnitude while the addition of lysine with a much larger N^{ϵ}-attached dansyl group in the 10th position (**31**) resulted in no loss of activity (Figure 5). Whether this latter finding indicates additional interaction between the dansyl group and the APF receptor remains to be determined. However, the addition of either Glu (**32**) or Lys (**33**) (Figure 5) to the carboxy terminus in the 10th position (for possible subsequent cyclization, see below) resulted in the complete loss of activity, some of which was restored by neutralizing the charge on the Glu or Lys side chains while maintaining the C-terminal carboxylate [(**34**) and (**35**) (Figure 5)] (Table 5). These findings suggest that the presence of either a positively or negatively charged side chain in the 10th position is detrimental to APF activity.

Finally, we synthesized an *as*-APF derivative in which the entire peptide portion was cyclized from the amino group on

Table 3. Substitution of AAVVVA with 12-Aminododecanoic Acid and Truncated Glycopeptides

No	Derivative	% of activity [a]	P value [b]
16	 Galβ1-3GalNAcα-O-TVP-12-Ado	inactive	
17	 Galβ1-3GalNAcα-O-TVPAA	inactive	
18	 Galβ1-3GalNAcα-O-TVPAAVVV	100%	NS
19	 Galβ1-3GalNAcα-O-TVPAAVV	inactive	

[a] As a result of the variability of cell response in the biological assay, the activity of each congener was normalized to the activity of **1** run simultaneously on the same plate according to the following equation: % = [IC_{50} (*as*-APF)/IC_{50} (derivative)] × 100; the average IC_{50} value of **1** was ~1 nM. Percent of activity is expressed relative to **1**, which served as a standard control on each plate. Derivatives with no significant activity at <25 μM concentration (the cutoff limit for the biological assay) were considered to be inactive. [b] NS = not significant at $p > 0.05$.

the N-terminus to the carboxyl group on the C-terminus (**36**). Although the complete inactivity of head-to-tail cyclized APF peptide could be evidence for the importance of both C- and N-terminal charges, it is also possible that this derivative's inactivity resulted from conformational changes occurring as a result of cyclization.

Discussion

We previously showed that glycosylation of APF is necessary for biological activity[7] and have now determined that several structural aspects of the 9 amino acid peptide segment are also important for structural integrity of the active compound.

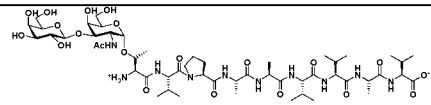
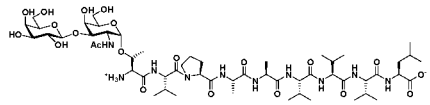
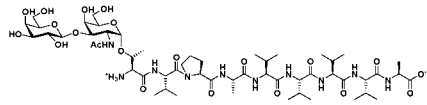
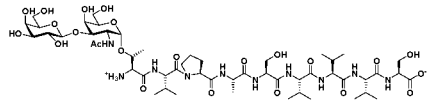
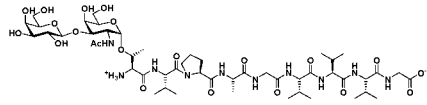
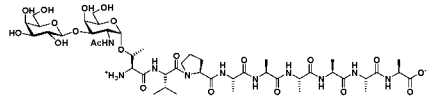
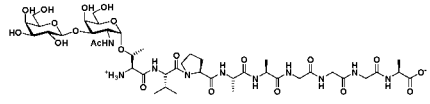
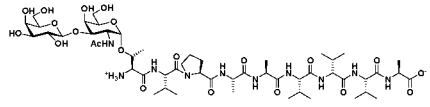
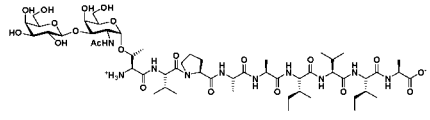
Our data clearly show that the biological activity of APF is very sensitive to changes in the N-terminus of the glycopeptide. The N-terminal two amino acids (Thr-Val) can be substituted with Ser-Leu with 2 orders of magnitude loss of activity, but sole substitution of Thr with Ser (**3**) resulted in even greater loss of potency, indicating that the number and positioning of the methyl groups in this location are important for *as*-APF activity. A recent report showed that the Ψ angle preferences are very different in simple GalNAc-containing glycoaminoacids depending on whether the amino acid is serine or threonine.[16] Whether this is operational in the N-terminal-substituted APF derivatives is at present unknown, but NMR studies are in progress to address this issue. However, the lone substitution of Val^2 with Leu resulted in complete inactivation, indicating that an additional methyl group in this location might prevent optimal interaction between *as*-APF and its receptor. In addition, the 100-fold decrease in activity caused by either extension of the N-terminus with tyrosine (the amino acid preceding threonine in the frizzled 8 protein sequence[8]) or acetylation of the N-terminal amino group provides evidence for the functional importance of the very specific positioning of a positively charged N-terminal amino group relative to the sugar moieties for maintenance of *as*-APF activity.

Conformation of *as*-APF in the area of the proline residue also appears to be very important, as substitution of L-proline with various other modified amino acids that can affect conformation also resulted in complete, or substantial, loss of activity. Ring size, functionality, and polarity can affect the conformation and potencies of proline-substituted APF derivatives, as shown by the decreased activity following substitution with D-proline, pseudoproline, azetidine 2-carboxylic acid, *trans*-4-hydroxyproline, *O-tert*-butyryl-*trans*-4-hydroxyproline, alanine, and *N*-methylalanine. In the D-proline derivative, the internal backbone torsion angle φ effectively changes sign, which in turn changes the orientation of the peptide segments on either side of the Pro residue relative to native APF, resulting in complete inactivation. Inactivation of *as*-APF following substitution of proline with pseudoproline (the latter of which often results in a high percentage of *cis* amide bonds preceding this residue[17]) may indicate a requirement for a *trans* conformation, a finding compatible with NMR data showing that APF does not contain any *cis* peptide bonds (unpublished observations). In comparison, substitution of L-proline with pipecolic acid had no apparent effect on activity, suggesting this derivative maintains a similar conformation to *as*-APF.

The decreased activity of *as*-APF following replacement of the proline with alanine or *N*-methylalanine might be explained by the fact that these derivatives, while likely to be more flexible than the parent APF congener, can have a small number of the angles in that location topochemically identical with bioactive APF, allowing some activity. In addition, the relatively greater activities of **11**−**14** or the parent congener as compared to **10** is most likely explained by the unfavorable restriction of conformation for the azetidine derivative.

Our data also indicate that Ala^5 and Val^{6-8} are very important for optimal biological activity and that a minimum of eight amino acids is required for biological activity. The decrease in activity resulting from replacement of Ala^8 or Ala^9 with amino acids containing larger side chains is compatible with the hypothesis that both amino acids may form a flat surface on an α-helix that may be important for interaction with the APF receptor.[12] However, the equal activity of **1** and **18** indicates

Table 4. Substitutions of C-Terminal Amino Acids 5−9 (AVVVA)

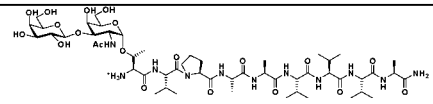
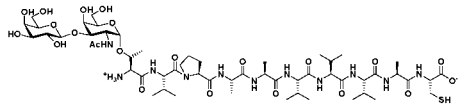
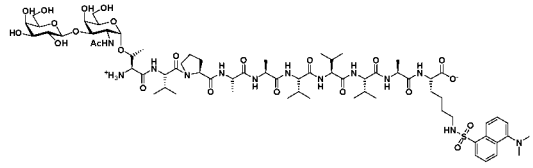
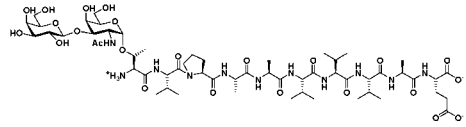
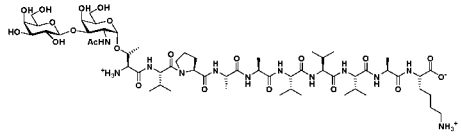
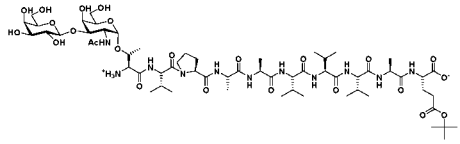
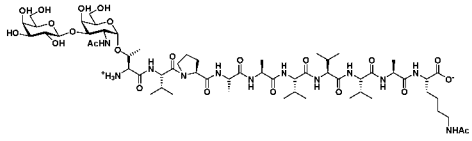
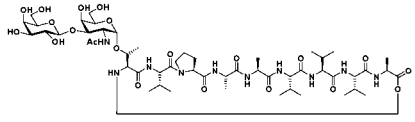
No	Derivative	% of activity [a]	P value [b]
20	 Galβ1-3GalNAcα-O-TVPAAVVAV	inactive	
21	 Galβ1-3GalNAcα-O-TVPAAVVVL	0.01%	< 0.001
22	 Galβ1-3GalNAcα-O-TVPAVVVVA	inactive	
23	 Galβ1-3GalNAcα-O-TVPASVVVS	0.2%	< 0.001
24	 Galβ1-3GalNAcα-O-TVPAGVVVG	inactive	
25	 Galβ1-3GalNAcα-O-TVPAAAAAA	1%	<0.001
26	 Galβ1-3GalNAcα-O-TVPAAGGGA	0.01%	<0.001
27	 Galβ1-3GalNAcα-O-TVPAAV-D-Val-VA	inactive	
28	 Galβ1-3GalNAcα-O-TVPAAIVIA	inactive	

[a] As a result of the variability of cell response in the biological assay, the activity of each congener was normalized to the activity of **1** run simultaneously on the same plate according to the following equation: % = [IC_{50} (*as*-APF)/IC_{50} (derivative)] × 100; the average IC_{50} value of **1** was ∼1 nM. Percent of activity is expressed relative to **1**, which served as a standard control on each plate. Derivatives with no significant activity at <25 μM concentration (the cutoff limit for the biological assay) were considered to be inactive. [b] NS = not significant at $p > 0.05$.

that this interaction, if it occurs, may only be required for Ala^5. CD measurements have not revealed an ordered structure of the parent congener **1** in water solution (see Supporting Information), and comprehensive NMR studies of *as*-APF in water solution confirm the lack of ordered structure in water (data not shown). However, there is some evidence for an ordered structure in higher concentrations (45%) of trifluoroethanol, making it reasonable to hypothesize that the carboxy terminal tail of *as*-APF may be able to adopt an α-helical-like conformation in a cellular milieu and/or upon interaction with its receptor. Extensive molecular dynamics studies based on the limited NMR restraints available clearly show that the C-terminal stretch of amino acids (AVVVA) in *as*-APF can adopt folded structures with concomitant adjustments in the rotamer distribution about the anomeric bond of the disaccharide after 1 ns (data to be published elsewhere). NMR studies of *as*-APF and several less potent analogues in the presence of its receptor in both aqueous and lipid environments are in progress to determine the precise structural features associated with maximum activity of these glycopeptides.

In addition, a negatively charged species at the carboxy-terminal end appears to be very important for *as*-APF activity. Moreover, when a negative charge state is maintained in that location, additional steric bulk can be tolerated at this end of

Table 5. Modifications of the C-Terminus

No	Derivative	% of activity [a]	P value [b]
29	 Galβ1-3GalNAcα-O-TVPAAVVVA-$CONH_2$	0.3%	< 0.001
30	 Galβ1-3GalNAcα-O-TVPAAVVVAC	1%	< 0.001
31	 Galβ1-3GalNAcα-O-TVPAAVVVAK(Dansyl)	100%	NS
32	 Galβ1-3GalNAcα-O-TVPAAVVVAE	inactive	
33	 Galβ1-3GalNAcα-O-TVPAAVVVAK	inactive	
34	 Galβ1-3GalNAcα-O-TVPAAVVVAE(O^{t}Bu)	0.01%	< 0.001
35	 Galβ1-3GalNAcα-O-TVPAAVVVAK(Ac)	0.05%	< 0.001
36	 *cyclo*(1-9)Galβ1-3GalNAcα-O-TVPAAVVVA	inactive	

[a] As a result of the variability of cell response in the biological assay, the activity of each congener was normalized to the activity of **1** run simultaneously on the same plate according to the following equation: % = [$\overline{IC_{50}}$ (*as*-APF)/$\overline{IC_{50}}$ (derivative)] × 100; the average IC_{50} value of **1** was ~1 nM. Percent of activity is expressed relative to **1**, which served as a standard control on each plate. Derivatives with no significant activity at <25 μM concentration (the cutoff limit for the biological assay) were considered to be inactive. [b] NS = not significant at $p > 0.05$.

the peptide, allowing for the synthesis of active derivatives containing fluorescent labels on the C-terminus to follow temporal and spatial aspects of the APF−cellular receptor interaction.

Inhibition of bladder epithelial cell proliferation by several of the APF derivatives appeared to be fairly abrupt for each dilution series, showing greater than 90% inhibition at the first active concentration. There are several possible explanations for this observation. Because the primary cells and derivatives used for these studies were very precious and small changes in structure generally resulted in dramatic changes in activity (to <1% of the original synthetic APF), the assay for these studies used 10-fold dilutions of the APF derivatives. It is possible that smaller increments in the dilution series for each derivative might have uncovered additional midpoints of activity. However, it is also possible that a critical concentration is required for each derivative to reach a threshold for activity and that this concentration changes as a result of a change in structure. Such a dependence might occur if APF aggregation is needed for binding to the receptor and/or if a critical APF/receptor ratio is

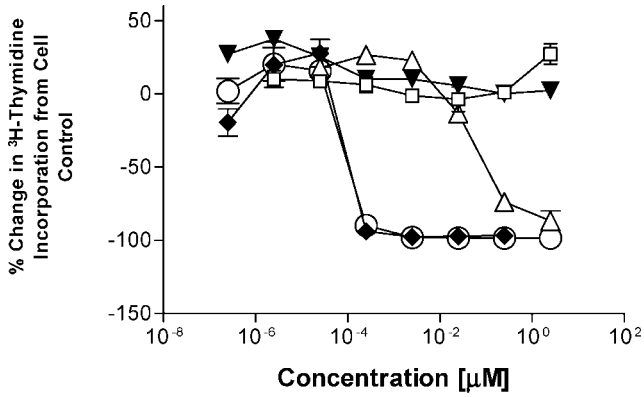


Figure 3. Antiproliferative activity of specific APF derivatives (modifications to Pro^3-Ala^4). Inhibition of tritiated thymidine incorporation by primary normal bladder epithelial cells was determined for each derivative at the concentrations indicated. Experiments were run in triplicate on two separate occasions. Data are expressed as the mean percent change in thymidine incorporation relative to a cell control treated with diluent (acetonitrile/H_2O at 1:1) alone; bars indicate standard error of the mean for all six data points: (○) Galβ1-3GalNAc-O-TVPAAVVVA; (□) Galβ1-3GalNAcα-O-TLS($^{\Psi Me,Me}$pro)-AAVVVA; (△) Galβ1-3GalNAcα-O-TV-Hyp-AAVVVA; (▼) Galβ1-3GalNAcα-O-TV-Aze-AAVVVA; (◆) Galβ1-3GalNAcα-O-TV-Pip-AAVVVA.

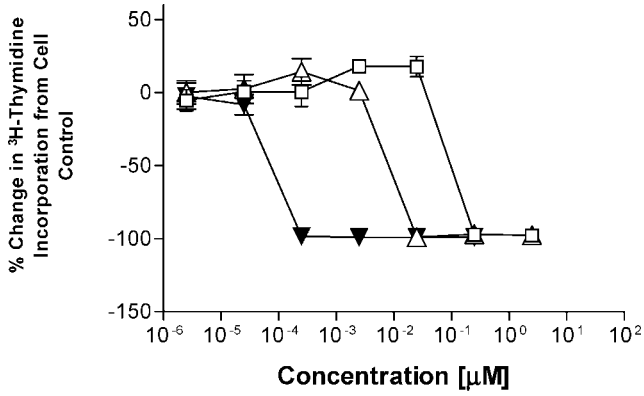


Figure 4. Antiproliferative activity of specific APF derivatives (changes in amino acids 6−8 (VVV)). Inhibition of tritiated thymidine incorporation by primary normal bladder epithelial cells was determined for each derivative at the concentrations indicated. Experiments were run in triplicate on two separate occasions. Data are expressed as the mean percent change in thymidine incorporation relative to a cell control treated with diluent (acetonitrile/H_2O at 1:1) alone; bars indicate standard error of the mean for all six data points: (▼) Galβ1-3GalNAc-O-TVPAAVVVA; (□) Galβ1-3GalNAcα-O-TVPAAGGGA; (△) Galβ1-3GalNAcα-O-TVPAAAAAA.

required for each derivative to mediate a cell signaling response. We have performed concentration-dependent ^{1}H NMR experiments on *as*-APF that showed no change in chemical shift over a 1000-fold concentration range, indicating that the endobiotic form does not aggregate (data not shown). However, additional studies to determine whether aggregation might be important for the activity of other APF derivatives, as well as to determine the mode of APF/receptor interaction(s), are in progress.

Conclusions

On the basis of these data, we were able to arrive at some definitive, and some tentative, conclusions on the requirements of the peptide portion of *as*-APF for its antiproliferative activity in bladder epithelial cells. We previously determined that *as*-APF must have an α-linked carbohydrate for biological activity

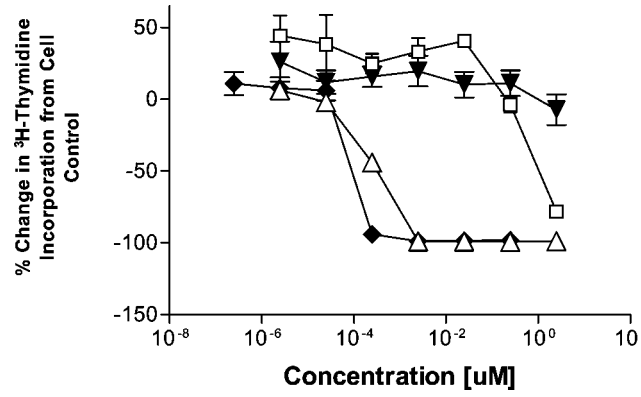


Figure 5. Antiproliferative activity of specific APF derivatives (modifications to carboxy-terminal alanine). Inhibition of tritiated thymidine incorporation by primary normal bladder epithelial cells was determined for each derivative at the concentrations indicated. Experiments were run in triplicate on two separate occasions. Data are expressed as the mean percent change in thymidine incorporation relative to a cell control treated with diluent (acetonitrile/H_2O at 1:1) alone; bars indicate standard error of the mean for all six data points: (◆) Galβ1-3GalNAc-O-TVPAAVVVA; (□) Galβ1-3GalNAcα-O-TV-PAAVVVAK(Ac); (△) Galβ1-3GalNAcα-O-TVPAAVVVAK(dansyl) (▼) Galβ1-3GalNAcα-O-TVPAAVVVAK.

to be present.[7] This report demonstrates that the peptide portion of *as*-APF must also have at least eight N-terminal amino acids. A summary of these requirements is illustrated in Figure 6A. Our data also suggest the possible additional requirement for a *trans* conformation for the Pro-Ala peptide bond. In addition, for optimal activity the peptide portion of *as*-APF must contain (1) a specific amino acid sequence with alanine in position 5 and valines in positions 6−8, (2) the conformation allowed by proline or pipecolic acid in position 3, (3) a very specific arrangement of methyl groups on the two N-terminal amino acids, (4) an amino acid no bulkier than alanine in the ninth position, and (5) a free N-terminal amino group and a free C-terminal carboxy group. These features are highlighted in Figure 6B.

Materials and Methods

General. Amino acids and resins were purchased from AnaSpec, Inc. (San Jose, CA) or EMD Chemicals (San Diego, CA), PyAOP, AcOH, and Ac_2O from Sigma Aldrich (St. Louis, MO), HOAt and HATU from AK Scientific, Inc. (Mountain View, CA), and solvents from American Bioanalytical (Natick, MA). Peptide synthesis was performed on a Nautilus 2400 Parallel synthesizer (Argonaut, Technologies, Foster City, CA). Preparative HPLC was performed on a Waters 600 instrument with UV detection (Waters 2487) on reverse phase C_{18} or C_8 silica (mobile phase: solvent A, H_2O/0.1% TFA, solvent B, CH_3CN in 0.1% TFA). NMR analyses were performed on a Varian INOVA instrument operating at 500 MHz for proton from 15 to 40 °C in either D_2O or H_2O/D_2O, 9:1. Water suppression was accomplished by standard WATERGATE or WET pulse sequences for observation of amide protons. CD measurements were performed on an AVIV 202 spectrometer in water (50 μM, pH 6.0) and neat TFE (50 μM).

Patients. Normal controls who were asymptomatic for urinary tract disease and undergoing cystoscopy following abdominal or pelvic surgery as standard of care consented to provide biopsy for the generation of normal bladder epithelial cell explants. These participants were all at least 18 years old and enrolled in accordance with guidelines of the Institutional Review Board of the University of Maryland School of Medicine.

Synthesis of APF Derivatives. The synthesis of the peptide segments of the glycopeptides were carried out in 0.1 mM scale by solid-phase methods by using standard Fmoc chemistry on

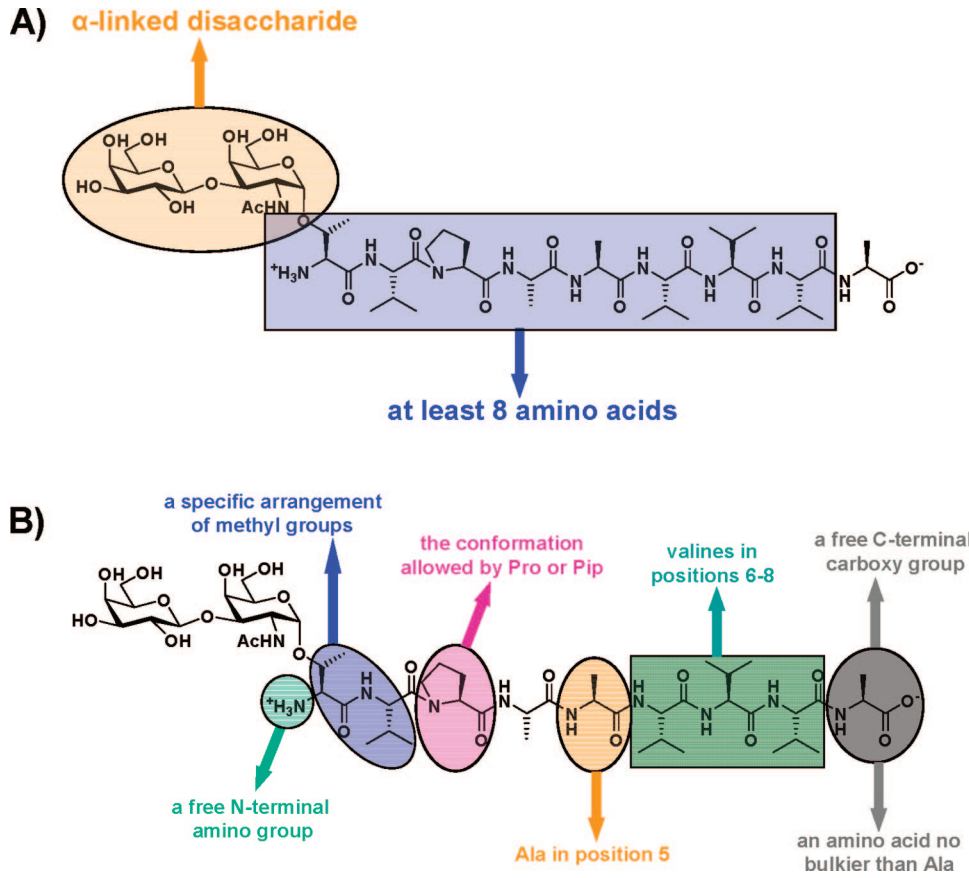


Figure 6. Schematic diagram indicating (A) essential and (B) important structural requirements for *as*-APF activity.

2ClTrt resin. Protected amino acids (0.5 mmol) were coupled using HATU (0.5 mmol) and HOAt (0.5 mmol) reagents in the presence of DIPEA (1.0 mmol). The Fmoc group was removed with 20% piperidine in NMP, and a mixture of BEP/HOAt/DIPEA (0.05 mmol/0.05 mmol/0.15 mmol) in NMP was used for coupling of Fmoc-Thr($Ac_4Gal\beta$1-3Ac_2GalNAcα-O-)-OH or Fmoc-Ser($Ac_4Gal\beta$1-3Ac_2GalNAcα-O-)-OH (0.05mmol) to the peptide chain. Acetyl groups were removed on the solid support using 10% hydrazine monohydrate in MeOH,[18] and each glycopeptide was cleaved from the resin with TFA/DCM/H_2O (50/49/1) or TFA/H_2O (95/5). All intermediates and the final products were verified by HPLC−MS; purity of >95% was confirmed for all compounds by HPLC trace analysis at 227 nm (see Supporting Information). Glycopeptides were purified by RP-HPLC on either a C_8 or C_{18} column with gradient elution with H_2O (0.1% TFA) and MeCN (0.1% TFA).

Synthesis of Glycopeptide 5. The glycopeptide was synthesized using the general procedure described above. After the attachment of Fmoc-Thr($Ac_4Gal\beta$1-3Ac_2GalNAcα-O-)-OH, the Fmoc group was removed with 20% piperidine in NMP and the deprotected amino group was acetylated using Ac_2O/DIPEA (2:5) in DCM.

Synthesis of Glycopeptide 9. The synthesis of glycopeptide **9** was performed using the same general procedure described above with the exception that the Val-Ser($\Psi^{Me,Me}$pro) segment was coupled as a dipeptide unit. To protect the pseudoproline unit, the glycopeptide was cleaved from the resin using a TFE/DCM (2/8) mixture.

Synthesis of Glycopeptide 13. The general procedure described above was used to synthesize **13**, which was then cleaved from the resin using a TFE/DCM (2:8) mixture to maintain the protective groups on the Hyp moiety.

Synthesis of Glycopeptide 14. The glycopeptide was synthesized as described above with the exception that coupling of the amino acid that precedes the *N*-methylamino acid in the sequence was repeated twice.

Synthesis of Glycopeptide 29. This compound was synthesized using the general procedure described above except that it was performed on a Rink amide resin. Prior to the first coupling step, the Fmoc group was removed with 20% piperidine in NMP.

Synthesis of Glycopeptide 35. After the acylation of the 2ClTrt resin with Dde-Lys(Fmoc)-OH, the Fmoc group was removed and the deprotected amino group was acetylated using a Ac_2O (2 mmol)/DIPEA (5 mmol) mixture in dry DCM. The Dde group was then removed with 2% hydrazine in DMF, and synthesis of the remaining glycopeptide was performed using the general method described above.

Synthesis of Cyclic Glycopeptide 36. $Ac_4Gal\beta$1-3Ac_2GalNAcα-O-TVPAAVVVA was synthesized using the general procedure described above on 2ClTrt resin. The Fmoc group was removed with 20% piperidine in NMP, and the glycopeptide was cleaved using TFA/DCM/H_2O (50/49/1). After HPLC purification of the crude glycopeptide, 30 mg (0.02 mmol) was dissolved in 2:1 DCM/DMF (45 mL) and stirred for 24 h in the presence of PyAOP/HOAt/DIPEA (0.1 mmol/0.1 mmol/0.1 mmol). The formation of **33** was then confirmed by HPLC−MS. Following evaporation of the solvent, the glycopeptide was dissolved in H_2O/MeCN and lyophilized. After additional HPLC purification the glycopeptide was dissolved in 10% hydrazine monohydrate in MeOH. All acetyl groups were then removed, and the solution was neutralized with AcOH and evaporated. The dry glycopeptide was then dissolved in AcOH/H_2O/MeCN and lyophilized. Further HPLC purification led to pure **36** (5.5 mg, 23% yield).

Cell Culture. Cystoscopy was performed under general anesthesia, and 4 mm^2 pieces of transitional epithelium with submucosal bladder tissue were obtained for the growth of primary bladder epithelial cells, as previously described.[6,7] Primary normal bladder epithelial cells were propagated in DMEM-F12 (Media-Tech, Herndon, VA) with 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic solution, 1% L-glutamine, 0.25 units/mL insulin (all from Sigma, St. Louis, MO), and 5 ng/mL hEGF (R&D Systems, Minneapolis, MN) at 37 °C in a 5% CO_2 atmosphere and characterized by binding of AE-1/AE-3 pancytokeratin antibodies (Signet, Dedham, MA).

^{3}H-Thymidine Incorporation. Cell proliferation was measured by ^{3}H-thymidine incorporation into explanted normal human bladder epithelial cells, plating 1.5×10^4 cells/well onto a 96-well cell culture plate (VWR 29442-054), in 150 μL/well MEM containing 10% heat inactivated FBS, 1% antibiotic/antimycotic solution, and 1% L-glutamine (all from Sigma), resulting in a doubling time of 48−72 h, as previously described.[6,7] Each purified lyophilized synthetic APF congener was resuspended in acetonitrile/distilled water (1:1) and applied to the cells in serum-free MEM (containing only L-glutamine and antibiotics/antimycotics); cell controls received acetonitrile/distilled water diluted in serum-free MEM alone. Cells were then incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. The cell contents were harvested and methanol-fixed onto glass fiber filter paper, and the amount of radioactivity incorporated was determined. Significant inhibition of ^{3}H-thymidine incorporation was defined as a mean decrease in counts per minute of greater than 2 standard deviations from the mean of control cells for each plate. Inhibition of cell proliferation was determined from a semilog plot of dose−response for each APF derivative; IC_{50} was determined as the concentration of each derivative that caused a mean 50% inhibition of thymidine incorporation compared to the mean of untreated cell controls.

Statistical Analysis. The thymidine incorporation (APF biological activity) assay was performed in triplicate on at least two separate runs, with **1** run simultaneously in triplicate on the same plate. The significance of the difference between mean values for each congener vs mean values for compound **1** was determined by an analysis of variance.

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Supporting Information Available: HPLC traces, NMR spectra, and CD data of selected derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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