Evaluation of a Vitamin-Cloaking Strategy for Oligopeptide Therapeutics: Biotinylated HIV-1 Protease Inhibitors

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The outstanding limitations to the oligopeptide as a therapeutic agent are poor oral availability and rapid biliary clearance. To address these concerns a series of eight peptidic HIV-1 protease inhibitors containing the structural segment of the vitamin *biotin* have been prepared. These have been evaluated with regard to the hypothesis that this vitamin would cloak the peptidic character of these oligopeptides, and thus impart to these inhibitors the potential for absorption and distribution *via* biotin transporters and receptors. By iterative optimization about a -Chaψ[CH-(OH)CH(OH)]Val- core inhibitory insert, three particularly potent inhibitors ($K_i \leq 10$ nM) of the HIV-1 protease were obtained. Although excellent cell culture antiviral activity is observed for other peptidic protease inhibitors of comparable affinity, none in this series exhibited satisfactory antiviral activity. This failure is attributed to the incompatibility of the hydrophilic and hydrogen-bonding biotin segment, with the facile membrane permeability and intracellular access presumably required for antiviral activity. The ability of the biotin to cloak the peptide, and thus render the overall appearance of the conjugate as that of a vitamin, was evaluated. Four of this series were evaluated for recognition by the Caco-2 cell intestinal biotin transporter. None inhibited competitively biotin uptake, indicating a lack of recognition. A vitamin may bind to a specific protein carrier, and thus attain an improved serum profile (by resistance to biliary clearance) and advantageous delivery to cells. Therefore, the serum concentrations of three were evaluated following an iv bolus in a rat model for serum clearance. One of the three protease inhibitors (L-idonamide, 6-cyclohexyl-2,5,6-trideoxy-2-(1-methylethyl)-5-[[3-methyl-1-oxo-2-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]butyl]amino]-*N*-[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]-, [3a*S*-[3aα,4β(1*R**,2*R**,3*R**),6α]]-) sustained a more than 5-fold increase in serum concentration at all time points relative to the benchmark structure. The remaining two had serum concentrations at least equal to the benchmark, suggestive of improved resistance to clearance. One (L-idonamide, 6-cyclohexyl-2,5,6-trideoxy-5-[[2-[[5-(hexahydro-2-oxo-1*H*-thieno-[3,4-*d*]imidazol-4-yl)pentyl]thio]benzoyl]amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]-, [3a*S*-[3aα,4β(1*R**,2*R**),6aα]]-) was prepared as a complex with the biotin-binding protein avidin. Avidin may resemble an endogenous serum biotin carrier protein. The antiviral activity (evaluated in an H9-HTLV$_{IIIB}$ acute HIV-1 infection assay) of the inhibitor and the avidin complex was identical. This suggests that the avidin–inhibitor complex is capable of cell internalization. Although the weak antiviral activity of these biotinylated inhibitors precludes consideration as practical HIV therapeutics, the overall data remain suggestive of vitamin cloaking of oligopeptides as a strategy of potential value.

The development of peptide-based therapeutic agents is limited by their low oral availability and their rapid and efficient removal from the serum by an hepatic active transporter.[1] In the particular example of inhibitors[2] of the HIV-1 protease, these difficulties are exacerbated by the requirement (for antiviral activity) of a hydrophobic peptidic character.[3] Two chemical strategies may be adopted in face of these difficulties. The first is *liquidation and replacement*, where each superfluous peptidic segment is expunged and each essential peptidic segment is replaced with a mimetic. The second strategy is that of *cloaking*. Here the peptidic character is preserved, but augmented with a second structural entity, chosen so as to confer the desired properties. Both strategies balance advantage and disadvantage. The liquidation approach has the promise of departing completely from the peptidic liabilities, but at the cost of exhaustive iterative exchange of structural segments. A cloaking strategy, on the other hand, allows the direct union of the peptide—a structure much more easily optimized—with its disguise. Its disadvantage, however, is the inherent difficulty of accommodating in a single structure *both* the activity requirements of the cloak and the peptide. Although *a priori* both strategies are of merit, the preferred choice of the medicinal chemist for peptidomimetic optimization has been generally that of liquidation and replacement. The serendipitous discovery that the Upjohn renin inhibitor U-75875 (1) was a potent HIV-1 protease inhibitor ($K_i < 1$ nM) with excellent antiviral activity[4,5] provided compelling incentive to evaluate potential cloaking strat-

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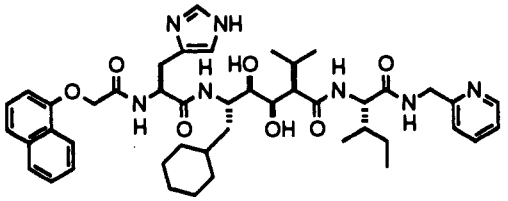
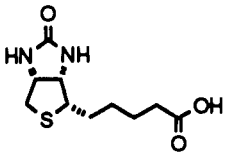
egies. Given this already promising structure, might an appropriate cloaking incisively address its peptidic shortcomings?



1

The shortcomings of 1 are those common to the oligopeptides. Peptidomimetic 1 is poorly soluble, is not well absorbed orally, and is sequestered efficiently by the biliary multisubstrate transporter. Moreover, 1 is unarguably that of a large peptide mimetic, having a central, hydrolytically-stable ersatz dipeptide insert flanked on both sides by a terminus-blocked amino acid. Under any circumstance, no further increase in the mass of 1 is possible, which in turn demands that the clock replace (rather than append to) an existing segment. An attractive strategy under these circumstances is *vitamin* conjugation.[6] Many of the vitamins attain reasonable aqueous solubility. As essential cellular nutrients, vitamins are provided with high-affinity transporters to bypass the vagaries of membrane passage.[7] Thus, vitamins are transported through the enterocyte, carried by binding proteins in the serum, and delivered to transporters for import into each cell (including[8] through the blood–brain barrier). The breadth of structure offered by the vitamins permits a choice with respect to structural and functional group compatibility with the partner. Thus, in principle, the pairing of a vitamin and the peptidic antiviral might preserve the antiviral potency *and* confer the advantages of these transport mechanisms. The disadvantage of a vitamin choice is the high affinity, recognition fidelity, and low capacity (proportional to nutritional need) of their transporters. This poses the prospect of limited recognition, limited carrying capacity, and exclusion by the endogenous vitamin. Nonetheless, the proper assessment rested in experimental evaluation.



2

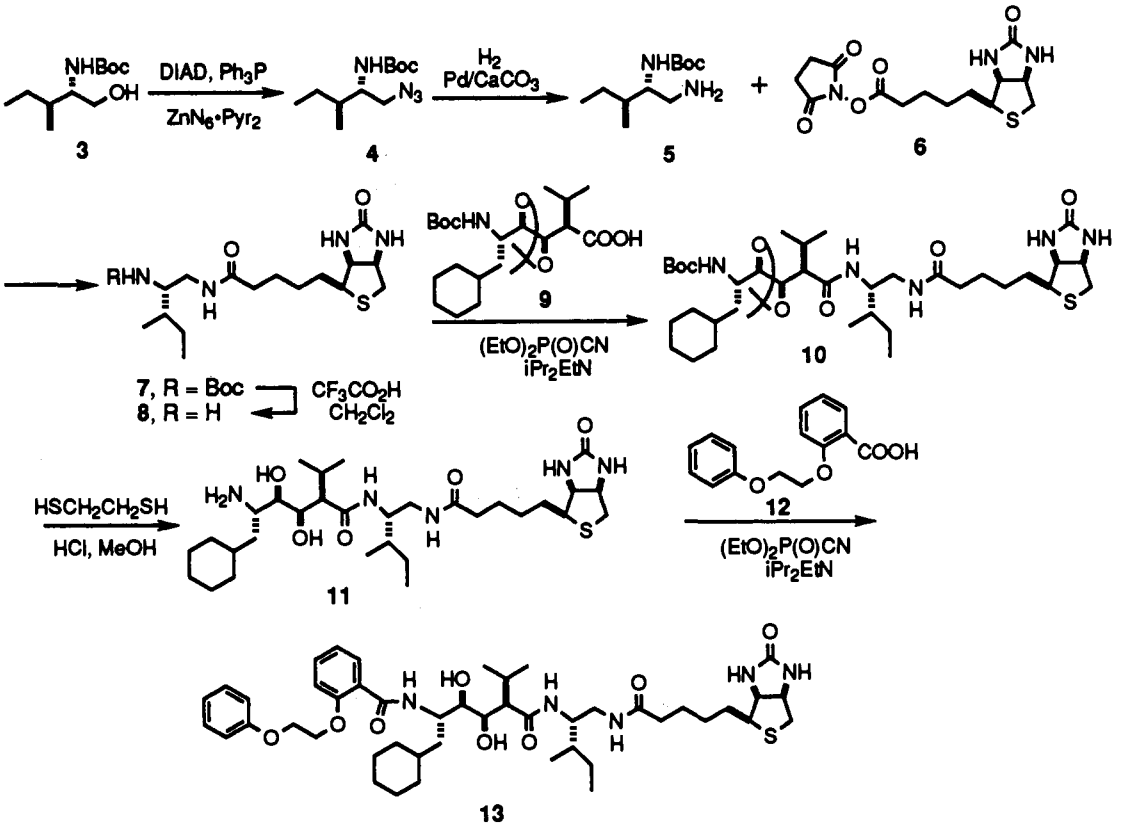
There are several choices for the vitamin. Ours, made with respect to with respect to the ease and practicality of this endeavor, was *biotin* (2). By several criteria this was an attractive choice. Its aqueous solubility is approximately 1 mM. It offers structural simplicity and stability, including a carboxylate as the point of attachment to the protease inhibitor. It is a notably well-studied vitamin with respect to *in vivo* recognition.[9-16] Moreover, a biotin–protease inhibitor hybrid would permit access to the extensive biotin–avidin technologies,[17] which have already been put to advantage with regard to the purification of a chemically-synthesized SIV protease.[18] The evaluation of biotin analogs with the egg biotin binding protein avidin suggested that functionalization of the biotin carboxylate could preserve binding affinity,[19] although similar studies with other biotin transporters were less promising (*vide infra*). There was, therefore, reason to expect promise from the use of biotin as a cloak and segment replacement for the potent antiviral 1.

Results

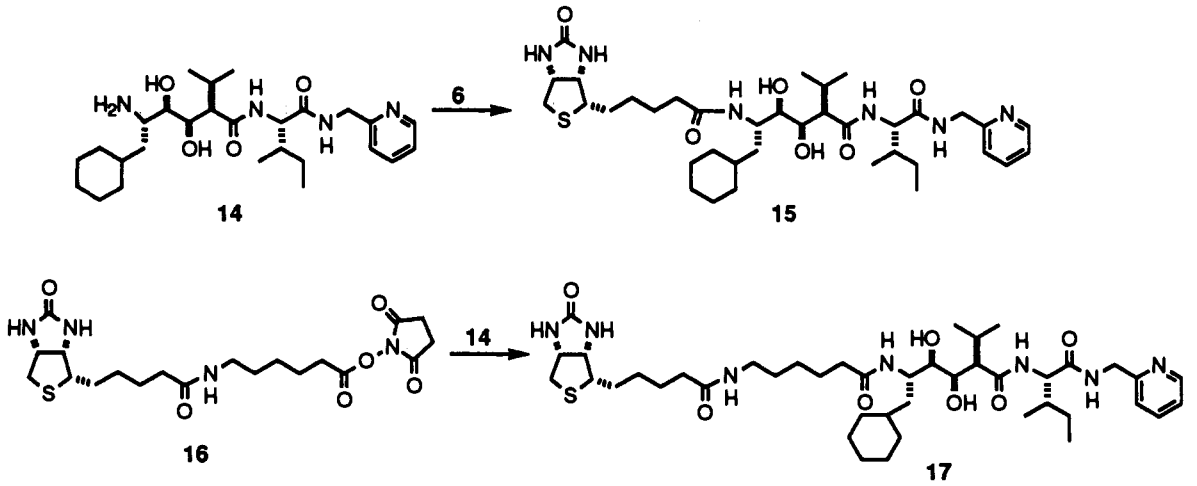
Synthesis and Biological Activity of the Biotinylated Inhibitors. The biotin carboxylate was used as the connecting tether, via straightforward amide bond formation between the biotin and an amine of the peptidomimetic. This connection may be done directly to the amino terminus (*amide*), or after appropriate transformation, to the carboxylate terminus of the peptide (*retroamide*). The latter strategy was evaluated first (Scheme 1). Thus *N*-Boc-L-isoleucinol **3** was transformed to the azide **4** by the ZnN_6-pyridine$_2$ Mitsunobu variation.[20] Reduction (H_2, Pd/$CaCO_3$) provided the amine **5**, to which biotin was coupled by reaction with *N*-hydroxysuccinimidobiotin **6**.[21] Removal of the Boc group of **7** (CF_3CO_2H, CH_2Cl_2) provided **8**, to which was coupled (iPr_2EtN, $(EtO)_2P(O)CN$) the protected insert **9**. Simultaneous removal of the acetyl and Boc protecting groups of **10** (HCl/MeOH, $HS(CH_2)_2SH$) yielded **11**. As the value of the 2-(2-phenoxyethoxy)benzoic acid **12** as a P-2 mimetic had been established previously within the –Chaψ[CH(OH)CH(OH)]Val– series,[4b] it was chosen to complete the structure. Incorporation of this segment by the standard coupling procedure gave **13**. Evaluation of this as a competitive inhibitor of the HIV-1 protease indicated exceedingly poor binding (K_i > 100 nM). Concurrent studies in another series[22] suggest the transposition of the P-2′ (isoleucine) carbonyl as the probable explanation. Crystallographic analyses of protease–inhibitor complexes show an extensive enzyme–inhibitor hydrogen bonding network,[23] and although only few of these are essential to binding, this carbonyl would appear to number among these. Although restoration of the carbonyl to its proper place would be a small synthetic excerise, we elected at this time to abandon the carboxy terminus for biotin insertion, in favor of the amino terminus. The motivation for this change was the desire to retain the L-Ile-AMP C-terminus of 1. This terminus is well accepted among many HIV-1 protease inhibitors,[4,22] and its pyridyl group has proven value for imparting aqueous solubility. Furthermore, a separation of the renin-inhibitory activity of 1, from its HIV protease activity, was needed. The removal (or replacement) of the P-2 histidine would be an important step to this objective. In addition, it would offer the possibility of less efficient removal from the serum of the resulting peptide, by the liver multisubstrate transporter, as has been observed upon replacement of the histidine imidazole in a renin inhibitor series.[1c,24]

Our first approach was the elongation of the insert–carboxy terminus core of 1 by active ester coupling with biotin (Scheme 2). Thus, condensation of **14** with **6** yielded structure **15**. The identical reaction with a longer tethered biotin segment (**16**) provided **17**. Evaluation of **15** as an enzyme inhibitor indicated a significant (K_i = 45 nM), but still not fully satisfactory, improvement relative to **13** (K_i > 100 nM). Given the inhibitor orientation within the protease,[23] the biotin segment of **15** must fall within the active site, with unfavorable consequences. The longer tether of **17** allows the biotin to escape, with dramatic improvement in binding (K_i = 4 nM). Although an *in vitro* affinity corresponding to a K_i < 10 nM is sufficient to anticipate significant antiviral activity, both **15** and **17**

Scheme 1



Scheme 2



in the vaccinia virus vVk-1/CV-1 assay (Table 1) were weakly antiviral. Accordingly, further optimization was required.

The strategy toward this objective was to retain the biotin *N*-terminus and to incorporate a specific P-2 binding segment (Scheme 3). This was done first by insertion of L-valine between the biotin and peptidomimetic. Thus, **6** was reacted with **18** to provide **19**. This product has excellent enzyme inhibition ($K_i \leq 4$ nM) and improved antiviral capability (26% inhibition at 1 μM in the vVk-1/CV-1 assay). This remained, however, significantly poorer than the activity of **1**. As noted above, an *ortho*-substituted benzoic acid is an effective P-2 surrogate.[4b] The possibility of improved affinity from this P-2 segment was examined. Reduction of *N*-hydroxysuccinimidobiotin ($NaBH_4$, HMPA/THF) gave biotinol **20**, which was converted to the mesylate **21**. Displacement of the mesylate by the phenoxide of methyl salicylate (K_2CO_3, DMF) gave **23**, which was solvolyzed to give **24**. This segment yielded **27** by coupling to **14**. Similarly, biotinol **6** was treated with $MeSO_2Cl$ in pyridine to afford a mixture of the chloro **22** and mesyl **21** derivatives, separable by flash chromatography. Reaction of **22** with methyl thiosalicylate (K_2CO_3, DMF) afforded **25**. Hydrolysis to **26**, followed by coupling to **14**, provided the cognate structure **28**. The affinity of **27** ($K_i = 10$ nM) was distinctly greater than **28** ($K_i = 53$ nM), yet **27** possessed antiviral activity no better than **19** (Table 1).

Two additional structures were made to complete this evaluation. In connection with studies on thiamine–peptidomimetic hybrids, the usefulness of the [(methylthiazolyl)ethyl]amine as the C-terminus, replacing the more customary [(pyridyl)methyl]amine, was noted. Accordingly, this substitution became the basis for the two final structures (Schemes 4 and 5). Thus, azide Mitsunobu of **29** afforded **30**, which was reduced to the amine **31**.

Table 1. Biological Data for the Biotinylated HIV-1 Protease Inhibitors

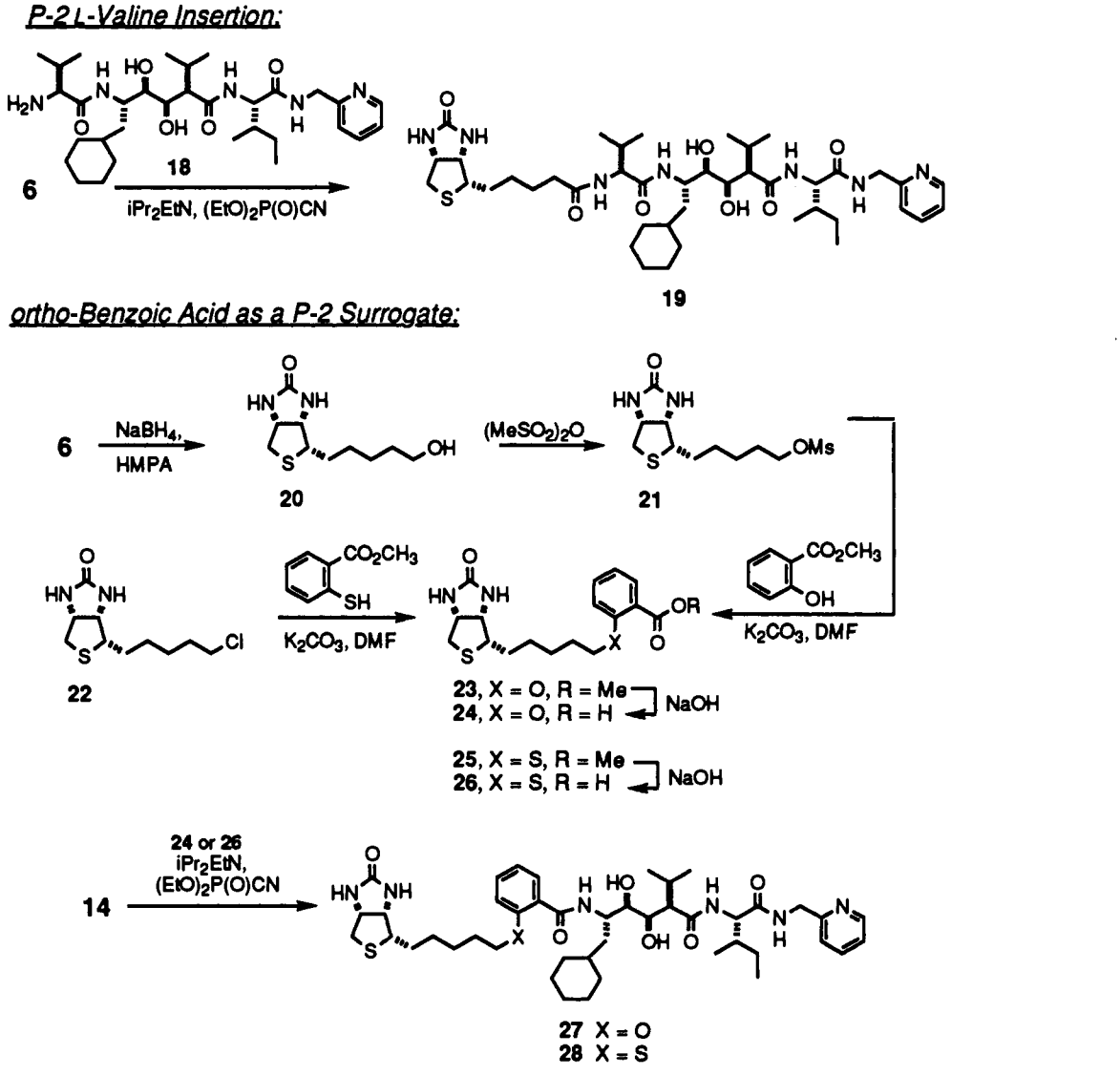
Inhibitor	K_i, HIV-1 protease (nM)	% inhibition of p24 expression at 1 μM, vVK/CV-1 Assay	% inhibition, day 4 PBMC Assay RNA (conc)		p24
1	≤1	>80	98	(100 nM)	95
			80	(10 nM)	82
			30	(1 nM)	38
AZT[a]	–	–	90	(40 nM)	81
			0	(4 nM)	2
13	>100	inactive		ND[b]	
15	45	9		ND	
17	20	inactive		ND	
19	≤4	26	69	(100 nM)	89
			22	(10 nM)	11
			18	(1 nM)	7
27	10	24	17	(100 nM)	83
			10	(10 nM)	5
			15	(1 nM)	2
28	53	ND		ND	
36	130	15		ND	
37	4	34		ND	

[a] AZT, 4′-azido-4′-deoxythymidine. [b] ND, not determined.

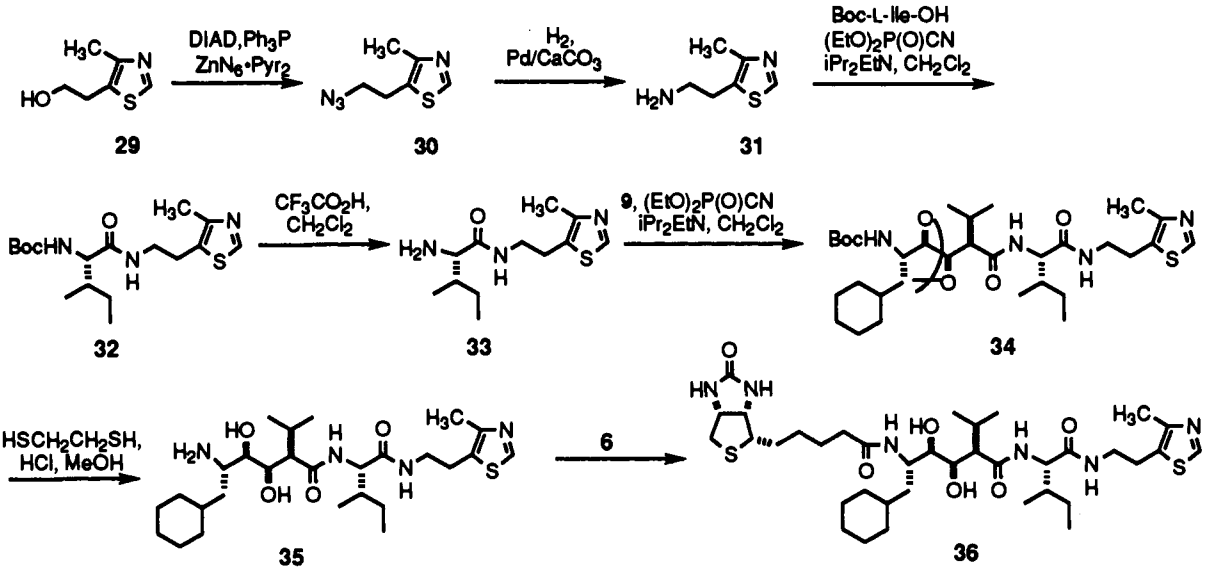
Coupling of this amine to Boc-L-isoleucine yielded **32**. Deprotection (to **33**) and coupling to **9** provided **34**, which was in turn deprotected (to **35**) and biotinylated to provide **36**, a cognate (differing only by a (2-(4-methyl-5-thiazolyl)-ethyl)amino in place of the customary (2-pyridylmethyl)-amino terminus) structure to **15**. Likewise coupling of **26** to **35** provided **37**, a cognate structure of **28**. Surprisingly, although the affinity of **36** (K_i = 130 nM) was 3-fold poorer than that of **15**, that of **37** (K_i = 4 nM) was notably better than **28** (K_i = 53 nM). A structural basis for this pattern is not obvious. In the 1–HIV-2 complex, the pyridyl segment is conformationally mobile,[23a,b] and this reversal is not likely to arise just as the consequence of the different terminus. Rather, there must be a more subtle synergy between the sulfur and oxygen bonds (very different bond lengths and angles) at P-2, transmitted to the *C*-terminus.[23c–e]

A tabulation of the antiviral activity is in Table 1. The best *in vitro* activity was found for structures **19**, **27**, and **37**. Each had an HIV-1 protease K_i of less than or equal to 10 nM. Likewise, in the CV-1 assay at 1 μM concentration, each inhibited viral growth by approximately 30%. This potency is significantly less than that shown by **1** (an IC_{50} = 0.3 μM in this assay). A significant loss (approximately 10-fold) in antiviral potency upon biotin conjugation was confirmed, for two of the biotinylated inhibitors, by measurement of p24 protein and viral RNA in the HIV_{D34}-infected PBMC. Under assay conditions where the IC_{50} for **1** and AZT (for both p24 and viral RNA on day 4) is between 1–10 and 4–40 nM, respectively, neither **19** nor **27** demonstrated appreciable activity at 10 nM.

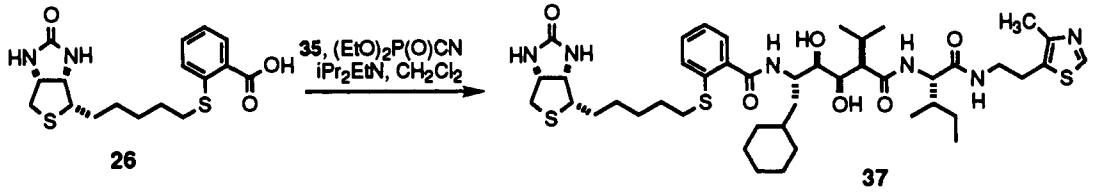
Scheme 3



Scheme 4



Scheme 5



Although antiviral activity was observed at higher concentrations (Table 1), the potency of the biotin-containing analogs clearly is comprised to the point that further exploration of biotin as a cloaking auxiliary was unwarranted.

Conceptual Evaluation of the Biotinylated Protease Inhibitors as Vitamin Conjugates. The primary shortcomings that the vitamin cloaking was intended to redress were poor oral availability and pharmacodynamics (imparting resistance to serum clearance and advantageous *in vivo* delivery to cells). Although the mediocre antiviral activity could not justify a detailed evaluation of these issues, a limited yet focused experimental effort examined these possibilities.

A. Potential for Oral Availability. Four of the inhibitors (**15**, **19**, **17**, and **27**) were evaluated for transport competence by the biotin transporter of the Caco-2 human colorectal carcinoma. This biotin transporter is a model for the human intestinal transporter[16] of which these biotinylated peptides were intended to take advantage. In the presence of 300 μM of these four biotinylated inhibitors, however, transport of 0.01 μM [^{3}H]biotin was unimpeded (Table 2). None of these four is recognized by this active transporter.

B. Serum Clearance. Three of the inhibitors were evaluated for clearance following iv administration in the rat. When peptide **1** is given at a dose of 4 mL kg^{-1}, of a 0.2 mg mL^{-1} inhibitor–vehicle solution, it showed serum concentrations of 73 ± 7, 28 ± 4, 18 ± 3, and <17 ± 3 nM at 10, 20, 40, and 80 min, respectively, after dosing. The concentrations observed for **19** (at equimolar dosing as **1**) were at 10 min, 432 ± 97 nM; 20 min, 152 ± 6 nM; 40 min, 120 ± 4 nM; and 80 min, <118 ± 10 nM; and for **28** were at 10 min, 156 ± 13 nM; 20 min, 106 ± 26 nM; 40 min, 92 ± 26 nM; and 80 min, <80 ± 18 nM. These concentrations are several-fold greater than those for **1**. The concentrations for **27** were comparable to those of **1** (10 min, 107 ± 13 nM; 20 min, 68 ± 6 nM; 40 min, <25 nM; and 80 min, <44 ± 15 nM). Each of the three maintains, or improves upon, the serum clearance of **1**. The difference is not, however, decisive. Since peptidomimetic recognition and clearance to the bile by the liver multi-substrate transporter is exquisitely sensitive to structure[1a,c] it is not possible to ascribe this benefit to the biotin alone.

Table 2. Effect of the Biotinylated HIV-1 Protease Inhibitors on the Apical-to-Basolateral Transport of 10 nM [^{3}H]-Biotin in the Caco-2 Cell Monolayer

additive (300 μM)	transport[a] [(pmol min^{-1} mg protein^{-1}) × 100]	% control
none	0.189 ± 0.009	[100]
biotin	0.000 ± 0.000	0[b]
15	0.241 ± 0.016	129
17	0.206 ± 0.035	111
19	0.227 ± 0.063	122
27	0.158 ± 0.025	85

[a] Values are the average of triplicate determinations. [b] $P < 0.01$ vs control.

C. Advantageous Delivery to Cells. Little is known concerning serum protein transport of the vitamins. Since the amount required to maintain vitamin stasis is small (for biotin, a circulating serum level of ca. 1 nM),[25] a specific binding protein (or transporter) has not been identified. Yet it is reasonable to believe that these transporters exist, for the purpose of ensuring an equitable distribution of the vitamins to all cells. A model of such a carrier is provided by avidin, the biotin-binding protein of the chicken egg white. As noted by Green,[19] one might regard avidin as a eukaryotic protein of recent vintage, were it not for its close similarity to the bacterial biotin-binding protein streptavidin. This suggests a conserved biotin-binding motif. Chalifour and Dakshinamurti[9] have established that complexation of biotin with avidin *facilitates* biotin incorporation into human HeLa and fibroblast cells, probably by adsorptive pinocytosis and lysosomal

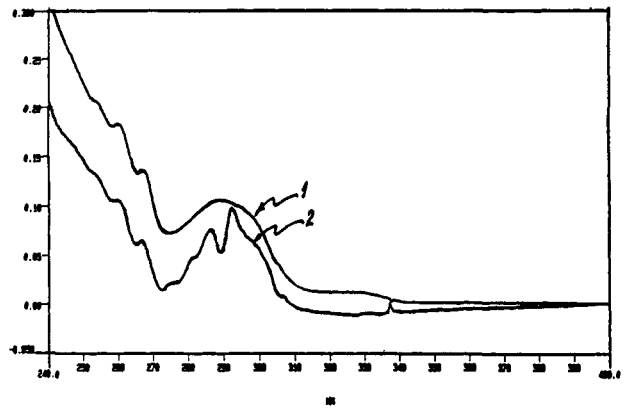


Figure 1. The absorption spectrum (A, 0.0–0.3; λ, 240–400 nm) labeled as 1 is that of 33 μM 27 in MeOH (l = 10.0 mm). This spectrum is compared to the difference spectrum 2, obtained by subtraction of the absorption spectrum of 33 μM avidin from the spectrum of 33 μM avidin–27 complex. The approximately $^1/_2$-fold diminution in intensity of the latter spectrum is taken to indicate an average stoichiometry of 2 mol of 27 bound to each avidin tetramer.[19b,28] This stoichiometry is used to calculate the concentration of 27–avidin for the measurement of antiviral activity.

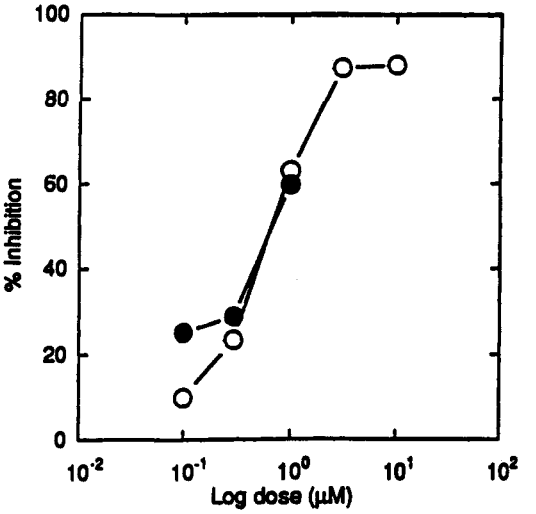


Figure 2. A comparison of the antiviral activity of 27 (○) and avidin–27 (●) in the H9-HTLV$_{IIIB}$ acute HIV-1 antiviral assay. A value for avidin–27 could not be measured above 1 μM due to interference of the avidin with the assay used to measure p24 concentration. Below 1 μM the antiviral activity of the two is, within experimental error, indistinguishable.

release (via avidin degradation). Moreover, Pardridge *et al.* have shown that the avidin complex of a biotinylated drug penetrates the bovine blood–brain barrier, whereas the biotinylated drug itself does not.[26,27] It was therefore of interest to evaluate the effect of avidin complexation on cell culture antiviral activity.

An avidin–HIV protease inhibitor complex was made by simple admixture of 27 with avidin. Purification of the complex (from unbound 27) was done by molecular sieve chromatography. The difference spectrum obtained by subtraction of the avidin spectrum, from the 27–avidin complex spectrum, indicated the presence of bound inhibitor (Figure 1). This difference spectrum resembles that of 27 alone, but with a vibrational resolution as might be expected from the protein environment. The resulting change in extinction coefficient, however, precludes the quantitative determination of relative concentrations. Nonetheless, the approximately $^1/_2$-fold decrease in absorption of avidin–27, compared to 27 equimolar with avidin monomer, suggests a stoichiometry of two 27 bound per avidin tetramer. Moreover, this suggestion is consistent with the preferential complexation of biotin derivatives by avidin in this stoichiometry.[19b,28]

The antiviral effect of the avidin–27 complex was compared to 27 alone in an acute H9-HTLV$_{IIIB}$ HIV-1 assay. Even given the uncertainty in stoichiometry, the antiviral potency of the two is experimentally indistinguishable (Figure 2). This requires one of two explanations. Either the avidin–27 complex is internalized (*vide supra*) for intracellular release of 27, or the avidin acts merely as a formulation "adjuvant" from which 27 dissociates for internalization by an avidin-independent process (the identical means of access as in the absence of avidin). Although we have no evidence to distinguish between these two, the latter explanation is argued to be the less probable. The effect of HIV protease inhibitors on viral maturation and protease-dependent cytotoxicity[29] strongly suggests that intracellular penetration of the protease inhibitor is required for effective antiviral activity. Avidin binds well a breadth of biotin derivatives. It is among the most thermally and proteolytically stable of all proteins. It is reasonable to expect that if the avidin–27 complex were not capable of internalization, it would persist as a competitive external reservoir for 27, and thereby to decrease the external concentration available for intracellular uptake—and hence antiviral activity—of 27. The antiviral activity observed for the 27–avidin complex is more suggestive of intracellular internalization of avidin–27 (with subsequent release of 27 from the avidin to the protease).

Discussion

Our ambition was the application of vitamin "cloaking" for the amelioration of the therapeutic deficiencies of oligopeptides. Although this particular effort was unsuccessful in terms of its particular objective (an antiviral HIV protease inhibitor), and therefore was also unsatisfactory with respect to the evaluation of the cloaking concept, our experience is instructive. An evaluation is made from three interrelated perspectives: the redress of the shortcomings, the success of the cloak in conferring the guise of a vitamin, and the antiviral activity.

The two peptidic shortcomings of outstanding concern are poor oral availability and efficient biliary clearance. A simultaneous solution to these problems would be the access of the transporter mechanisms believed to be available to the vitamins. The outcome with these biotinylated conjugates emphasizes the difficulty (anticipated from previous studies on biotin analogs[10–13,16]) in achieving this subterfuge. They are not recognized by the Caco-2 biotin transporter. Further, the evidence is not compelling that they diminish biliary excretion, by avoiding recognition by the hepatic multisubstrate transporter. In this latter instance it may only be remarked that the inclusion of the biotin structure—an "endogenous" structural segment—may contribute in part to the several fold increase (relative to 1) in rat serum concentration over an 80-min period following iv administration. Do then these inhibitors ever pass as vitamins? The most suggestive (but still yet equivocal) experiment is the antiviral activity of the avidin–27 complex. The very similar antiviral activity of this complex compared to 27 alone suggests that the avidin does not impede, and by analogy to the extensive studies of Dakshinamurti and Chalifour (avidin-mediated biotin uptake by cells),[9] and by Pardridge and colleagues[26,27] and Audus and colleagues[14b] (avidin-me-

diated passage of biotinylated molecules through a cultured bovine blood–brain barrier), may assist in the delivery of biotinylated peptides to certain cells.

The decreased antiviral activity (relative to 1) of these biotinylated HIV protease inhibitors may derive from the nature of the biotin structure. An orally effective HIV protease inhibitor must pass through a membrane (at least) twice. The first passage is through the mucous-coated intestinal enterocyte, so as to reach the portal vein. The second is through the membrane of the virus-infected cell. The structural characteristics for optimized membrane permeability for an oligopeptide are unknown. As a class, oligopeptides simply are not orally available, as if they possess a functional inability to pass through the mucous and/or membrane barriers of the enterocyte.[1] It has been suggested that this inability correlates with the relative difficulty in desolvation of the amide bonds of the peptide.[30] A similar problem is seen for the therapeutic nucleosides,[31] and likewise may represent an important feature of the blood–brain barrier.[8,32] The possession of antiviral activity by these (and numerous other peptidomimetic HIV protease inhibitors)[2-5,29] indicates that the structural requirements for passive membrane diffusion to the inside of the infected cell are more permissive. If the difficulty is amide bond desolvation, then it is implied that the desolvation requirement for solute passage through the enterocyte membrane is more demanding than that (for example) of the cells used here to assess antiviral potency. The outstanding debit of *these* biotinylated peptides—their 10–100-fold diminution in antiviral activity relative to structures of comparable *in vitro* inhibitory activity against the enzyme—may be rationalized in terms of this hypothesis. The ureidobiotin segment—particularly when constrained within a ring, as is the case for biotin—may be particularly difficult to desolvate. If so, a consequent decreased capability for membrane passage would translate as diminished antiviral activity.

For these peptides, and for this target, the vitamin-cloaking strategy was unsuccessful. It should not be taken, however, that these experiments have proven valueless. This study is a first iteration at the simultaneous optimization of a peptide and vitamin "cloak" pairing. Advantageous modification of recalcitrant therapeutics by endogenous structural segments will remain an area of opportunity.[33] With regard to oligopeptides, only a herculean application of the classic strategy of liquidation and replacement has identified structures with tolerable oral availability and adequate serum concentrations,[1] and even then with unpredictable structure–activity correlations.[34] In light of this challenge, alternative solutions to the therapeutic peptide conundrum will remain attractive and may well continue to be sought within the realm of the molecular cloak.

Experimental Section

Materials and Methods. Flash chromatography used a 230–400-mesh silica solid phase. TLC data are for glass-backed silica. IR and MS analyses were performed by the Upjohn Physical & Analytical staff. NMR data are recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C); coupling constants are given in hertz. The purity of the final compounds was confirmed by analytical HPLC analysis, using a C-18 reverse-phase column (5-μm support; 4.6 mm × 250 mm). The same gradient elution sequence was used for all separations (a binary solvent consisting of 83% [9:1:0.002 MeCN/H_2O/CF_3CO_2H] and 17% [7:3:0.002 MeCN/H_2O/CF_3CO_2H] at a flow of 1.50 mL min^{-1}, isocratic for 2 min by a linear gradient to 100% over 20 min. Solute elution was monitored at 220 nm. Chemical Abstracts Service registry numbers are provided by the author and given in brackets.

Carbamic Acid, [1-(Azidomethyl)-2-methylbutyl]-, 1,1-Dimethylethyl Ester, [*S*-(*R,*R**)]- [124590-30-3] ($C_{11}H_{22}N_4O_2$, 4).** ZnN_6-pyridine$_2$ (2.3 g, 7.50 mmol) was suspended in a solution of Boc-L-isoleucinol **3** (2.1 g, 9.6 mmol) and Ph_3P (5.25 g, 20 mmol) in dry toluene (90 mL). Diisopropyl azodicarboxylate (4 mL, 20 mmol) was added dropwise at room temperature. The reaction was stirred for 3 h, filtered through a pad of Celite, and concentrated. The crude product purified by flash silica chromatography (4/1 hexane/EtOAc) to afford 1.8 g (77%) of **4** as a yellow oil: TLC R_f = 0.85 (1:1 EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 4.60 (br d, 1 H, J = 6.0), 3.68–3.54 (m, 1 H), 3.44–3.40 (t, 2 H, J = 5.0), 1.66–1.52 (m, 2 H), 1.45 (s, 9 H), 1.21 (m, 1 H), 0.93–0.88 (overlayed d and t, 6 H); MS (EI) m/z 243 (M^+), 217, 187, 143, 130, 86, 74, 57 (base).

Carbamic Acid, [1-(Aminomethyl)-2-methylbutyl]-, 1,1-Dimethylethyl Ester, [*S*-(*R,*R**)]- [115654-48-3] ($C_{11}H_{24}N_2O_2$, 5).** A solution of **4** (0.70 g, 2.88 mmol) and 200 mg of Pd/$CaCO_3$ in 40 mL MeOH was hydrogenated at atmospheric pressure for 4 h. The solution was filtered and concentrated to a yellow oil, which was chromatographed (95:5 $CHCl_3$/MeOH) on silica to provide **5** (480 mg, 76%) as an oil. TLC R_f = 0.14 (9:1 $CHCl_3$/MeOH); IR (Mineral oil mull) 3352, 3211, 3120, 2957, 2927, 1688, 1600, 1523, 1279, 1209, 1069, 966, 919, 913, 879, 839, 722, 666 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 4.63 (d, 1 H, J = 15.0), 3.52–3.38 (m, 1 H, m), 2.91–2.58 (m, 2 H), 2.60 (s, 2 H), 1.58–1.44 (m, 1 H), 1.45 (s, 9 H), 1.25–1.03 (m, 1 H), 0.93–0.88 (overlayed d and t, 6 H); ^{13}C NMR ($CDCl_3$) δ 11.42, 15.30, 25.10, 28.26, 36.96, 43.17, 57.23, 79.01, 156.25. MS (FAB) m/z 217 (M + H)$^+$, 161, 117, 100, 57 (base); Anal. C, H, N.

1*H*-Thieno[3,4-*d*]imidazole-4-pentanamide, Hexahydro-2-oxo-*N*-[2-[[(1,1-dimethylethoxy)carbonyl]amino]-3-methylpentyl]-, [3a*S*-[3aα,4β(1*R),6aα]]- ($C_{21}H_{38}N_4O_4S$, 7).** To a solution of **6** [72040-63-2] (65 mg, 0.2 mmol) in 2 mL of DMF was added **3** (50 mg, 0.22 mmol). After the mixture was stirred at room temperature for 14 h, the DMF was removed *in vacuo*. The white residue was chromatographed (19:1 $CHCl_3$/MeOH) on silica gel to afford **7** (67 mg, 75%) as a white solid: TLC R_f = 0.59 (9:1 $CHCl_3$/MeOH); ^{1}H NMR ($CDCl_3$) δ 6.85 (t, 1 H, J = 5.4), 6.72 (s, 1 H), 5.83 (s, 1 H), 4.85 (d, 1 H, J = 9.4), 4.59–4.48 (m, 1 H), 4.38–4.29 (m, 1 H), 3.62–3.32 (m, 2 H), 3.22–3.09 (m, 1 H), 2.92–2.88 (dd, 1 H, J = 12.7, 4.8), 2.76 (d, 1 H, J = 12.7), 2.21 (m, 1 H), 1.69 (s, 1 H), 1.73–1.60 (m, 2 H), 1.43 (s, 9 H), 1.32–1.05 (m, 1 H), 0.90 (t, 3 H, J = 6.7); ^{13}C NMR ($CDCl_3$) δ 11.92, 15.36, 25.40, 27.90, 28.41, 29.50, 36.02, 37.52, 40.52, 42.02, 55.61, 60.20, 61.78, 79.98, 157.50, 164.42, 174.52; MS (FAB) m/z 443 (M + H)$^+$, 343, 326, 259, 227, 143, 100, 86, 69, 57 (base), 41.

1*H*-Thieno[3,4-*d*]imidazole-4-pentanamide, Hexahydro-2-oxo-*N*-[2-amino-3-methylpentyl-, (3a*S*-[3aα,4β(1*R),6aα]]- ($C_{16}H_{30}N_4O_2S$, 8).** To a solution of **7** (130 mg, 0.29 mmol) in 15 mL of CH_2Cl_2 was added CF_3CO_2H (2 mL). After 3 h at room temperature the solvent was evaporated, and the sticky white residue was purified by silica chromatography (96:4 $CHCl_3$/saturated NH_3 in MeOH) to afford **8** (100 mg, 99%) as a white solid: TLC R_f = 0.16 (9:1 $CHCl_3$/MeOH); MS (EI) m/z (rel intensity) 342 (55) [M^+], 324 (15), 323 (22), 295 (14), 268 (18), 267 (100), 257 (17), 227 (36), 181 (23), 140 (38), 100 (13), 97 (34), 95 (18), 91 (53), 87 (11), 86 (91), 85 (21).

1,3-Dioxolane-4-acetamide, 5-[2-Cyclohexyl-1-[[(1,1-dimethylethoxy)carbonyl]amino]ethyl]-2,2-dimethyl-α-(1-methylethyl)-*N*-[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]-, [3a*S*-[3aα,4β(1*R,2*R**,3*S**,4*S**),6aα]]- ($C_{39}H_{69}N_5O_7S$, 10).** To a solution of **8** (45 mg, 0.13 mmol) in 2 mL of CH_2Cl_2 and 18 μL of iPr_2EtN were added 56 mg (0.13 mmol) of **9** [112190-45-1] and 21.3 μL of $(EtO)_2P(O)CN$. After the mixture was stirred at room temperature for 18 h, the solvent was removed *in vacuo*. The residue was chromatographed on silica (98/2 $CHCl_3$/MeOH then 98:2 $CHCl_3$/saturated NH_3 in MeOH) to afford **10** (42 mg, 42%) as a white solid: TLC R_f = 0.37 (19:1 $CHCl_3$/MeOH); MS (FAB) m/z 752 (M + H)$^+$, 652, 343, 310, 227, 143, 126, 100, 86, 57, 41.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-*N*-[1-[[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]methyl]-2-methylbutyl]-2-(1-methylethyl)-5-amino-, [3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{31}H_{57}N_5O_5S$, 11).** To a solution of 15 mL of MeOH and 115 μL of acetyl chloride, stirred

at room temperature for 15 min, were added 122 mg (0.162 mmol) of 10 and then 40 μL of ethanedithiol. The reaction mixture was stirred at room temperature for 6 h and then quenched with excess solid $NaHCO_3$. After 1 h, the reaction mixture was filtered and the solvent evaporated. Silica chromatography of the residue (96:4 $CHCl_3$/saturated NH_3 in MeOH) provided 11 (68 mg, 68%) as a white solid: TLC R_f = 0.38 (9:1 $CHCl_3$/MeOH); MS (FAB) m/z 612 $(M + H)^+$, 425, 343, 326, 270, 227, 215, 143, 126, 100, 83, 55.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-*N*-[1-[[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]methyl]-2-methylbutyl]-2-(1-methylethyl)-5-[[2-(2-phenoxyethoxy)benzoyl]amino]-, [3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{46}H_{69}N_5O_8S$, 13).** To a suspension of 11 (30 mg, 0.049 mmol) in 3 mL of CH_2Cl_2 were added 10 μL of iPr_2EtN and 7.6 μL of $(EtO)_2P(O)CN$. 2-(2-Phenoxyethoxy)-benzoic acid 12 (12.9 mg, 0.050 mmol) was added, and the reaction was stirred at room temperature for 20 h. After evaporation of the solvent and purification of the residue by flash silica chromatography (94:4 $CHCl_3$/saturated NH_3 in MeOH), 13 (12 mg, 28%) was obtained as a glassy white solid: TLC R_f = 0.68 (96:4 $CHCl_3$/saturated NH_3 in MeOH); HPLC t_R = 15.0 min; MS (FAB) m/z 852 $(M + H)^+$, 834, 626, 510, 467, 366, 343, 241, 227, 147, 121, 95, 77, 55.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[(2-pyridylmethyl)amino]carbonyl]butyl]-, [3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{37}H_{56}N_6O_6S$, 15).** To a solution of 6 (25 mg, 0.073 mmol) in 3 mL of DMF was added 14 (35.8 mg, 0.073 mmol). The reaction mixture was stirred at room temperature for 14 h. The DMF was removed *in vacuo*, and the white residue was purified by silica chromatography (97:3 $CHCl_3$/MeOH, then 96:4 $CHCl_3$/MeOH) to yield 15 (39 mg, 54%) as a white solid: TLC R_f = 0.48 (9:1 $CHCl_3$/MeOH); HPLC t_R = 13.4 min; MS (FAB) m/z 717 $(M + H)^+$, 609, 496, 364, 348, 334, 290, 270, 227, 177, 126, 109, 86, 69.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-[[6-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]-1-oxohexyl]amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[(2-pyridylmethyl)amino]carbonyl]butyl]-, [3a*S*-[3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{47}H_{71}N_7O_7S$, 17).** To a solution of 16 [89889-52-1] (23.2 mg, 0.051 mmol) in 2 mL of DMF was added 14 (25 mg, 0.050 mmol). The reaction mixture was stirred at room temperature for 8 h. DMF was removed *in vacuo*, and the white residue was chromatographed (95:5 $CHCl_3$/MeOH) on silica to afford a white solid (18 mg, 42%). The compound was further purified by dissolving in 2 mL of 95:5 $CHCl_3$/MeOH and precipitation by hexanes: TLC R_f = 0.51 (9:1 $CHCl_3$/MeOH); MS (FAB) m/z 830 $(M + H)^+$, 722, 609, 348, 334, 290, 270, 222, 173, 126, 109, 86, 69, 55.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-2-(1-methylethyl)-5-[[3-methyl-1-oxo-2-[[5-(hexahydro-2-oxo-1*H*-thieno-[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]butyl]amino]-*N*-[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]-, [3a*S*-[3aα,4β(1*R,2*R**,3*R**),6α]]- ($C_{42}H_{66}N_7O_7S$, 19).** To a solution of 6 (21.5 mg, 0.062 mmol) in 3 mL of DMF was added 18 (37 mg, 0.063 mmol). The reaction mixture was stirred at room temperature for 6 h. The DMF was removed *in vacuo*, and the white residue was chromatographed (95:5 $CHCl_3$/MeOH) on silica to afford 19 (30 mg, 59%) as a white solid: TLC R_f = 0.49 (9:1 $CHCl_3$/MeOH); HPLC t_R = 14.2 min; MS (FAB) m/z 816 $(M + H)^+$, 708, 690, 595, 348, 334, 298, 270, 227, 177, 126, 109, 86, 72.

1*H*-Thieno[3,4-*d*]imidazol-2(3*H*)-one, Tetrahydro-4-(5-hydroxypentyl)-, [3a*S*-(3aα,4β,6aα)]- [53906-36-8] ($C_{10}H_{18}N_2O_2S$, 20). To a suspension of 6 (156.8 mg, 0.45 mmol) in 40 mL of dry THF and 1 mL of HMPA was added 42 mg of $NaBH_4$. The reaction was stirred at room temperature for 4 h and then the solvent evaporated. Water (0.5 mL) was added slowly with cooling. The residue was dried *in vacuo* and then purified by silica chromatography (95:5 $CHCl_3$/MeOH) to afford biotinol 20 as a white solid (70 mg, 67%). The yield for a 2-g reaction was 50%: TLC R_f = 0.39 (9:1 $CHCl_3$/MeOH); IR (mineral oil mull) 3331, 3235, 2949, 2927, 2884, 2855, 1701, 1676, 1477, 1464, 1425, 1260, 1073, 726, 699, 651, 610 cm^{-1}; ^1H NMR ($CDCl_3$) δ 4.50–4.46 (dd, 1 H, J = 7.7, 4.1), 4.31–4.27 (dd, 1 H, J = 7.8, 4.4), 3.56–3.52 (d, 2 H, J = 6.3), 3.23–3.17 (m, 1 H), 2.95–2.89 (dd, 1 H, J = 12.6, 4.9), 2.69 (d, 1 H, J = 12.7), 1.53–1.28 (m, 8 H); MS (EI) m/z 230 (M^+), 200, 170, 152, 123, 111, 97 (base), 85, 70, 61, 43.

1*H*-Thieno[3,4-*d*]imidazol-2(3*H*)-one, Tetrahydro-4-[5-(methylsulfonyl)pentyl]-, [3a*S*-(3aα,4β,6aα)]- ($C_{11}H_{20}N_2O_4S_2$, 21). To a solution of 20 (230 mg, 1.0 mmol) in 10 mL of 1:1 pyridine/CH_2Cl_2 was added $(CH_3SO_2)_2O$ (170.2 mg, 1 mmol), and the reaction was stirred at room temperature for 10 h. The solvent was evaporated to yield a yellow residue. This was dissolved in $CHCl_3$, washed sequentially with water and brine, and then dried (Na_2SO_4). Evaporation of the solvent yielded a yellow solid, which was purified by silica chromatography (98:2 $CHCl_3$/MeOH) to afford 21 (277 mg, 90%) as a white solid: TLC R_f = 0.33 (9:1 $CHCl_3$/MeOH); IR (mineral oil mull) 3426, 3217, 2946, 2924, 2855, 1699, 1688, 1647, 1345, 1311, 1177, 1160, 982, 952, 852 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.32 (br s, 1 H), 5.18 (br s, 1 H), 4.58–4.48 (m, 1 H), 4.48–4.38 (m, 1 H), 4.27–4.22 (t, 2 H, J = 6.4), 3.21–3.11 (m, 1 H), 3.03 (s, 3 Hs), 2.92–2.89 (dd, 1 H, J = 13.6, 3.4), 2.74 (d, 1 H, J = 13.4), 1.97–1.41 (m, 8 H); ^{13}C NMR ($CDCl_3$) δ 25.28, 28.13, 28.36, 28.64, 37.26, 40.46, 55.38, 59.84, 61.77, 69.96, 163.34; MS (EI) m/z 308 (M^+), 248, 164, 129, 97 (base), 85, 55, 40.

1*H*-Thieno[3,4-*d*]imidazol-2(3*H*)-one, Tetrahydro-4-(5-chloropentyl)-, [3a*S*-(3aα,4β,6aα)]- ($C_{10}H_{17}N_2ClOS$, 22). To a solution of 150 mg (0.65 mmol) of 20 in 5 mL of pyridine at 0 °C was added 0.1 mL of $MeSO_2Cl$. After 3 h at 0 °C, the solvents were evaporated to leave a yellow residue, which was dissolved in $CHCl_3$ and filtered. Evaporation of the solvent and purification of the yellow residue by flash silica chromatography (99:1, then 97:3 $CHCl_3$/MeOH) afforded 21 (28 mg, 13%) and 22 (111 mg, 68%): IR (mineral oil mull) 3207, 2954, 2927, 2855, 1700, 1464, 646, 601 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.61 (br s, 1 H), 4.56–4.50 (dd, 1 H, J = 12.4, 4.8), 4.36–4.30 (dd, 1 H, J = 12.3, 7.7), 3.56–3.52 (t, 2 H, J = 13.2), 3.25–3.12 (m, 1 H), 2.96–2.90 (dd, 1 H, J = 12.9, 5.0), 2.75 (d, 1 H, J = 12.8), 1.82–1.41 (m, 8 H); ^{13}C NMR ($CDCl_3$) δ 26.66, 28.23, 28.39, 32.17, 40.47, 44.94, 55.47, 59.98, 61.94, 163.55; MS (EI) m/z 248 (M^+), 205, 188, 164, 129, 97 (base), 85, 40.

Benzoic Acid Methyl Ester, 2-[[5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl]oxy]-, [3a*S*-(3aα,4β,6aα)]- ($C_{18}H_{24}N_2O_4S$, 23). A mixture of methyl salicylate (32.3 μL, 0.25 mmol), K_2CO_3 (35 mg), and 21 (72 mg, 0.23 mmol) in DMF (3 mL) was stirred at 70 °C for 18 h. The white residue, obtained by removal of the solvent, was dissolved in 19:1 MeOH/$CHCl_3$ (20 mL). After filtration and evaporation, the crude product was purified by silica chromatography to give 23 (80 mg, 95%) as a white solid: TLC R_f = 0.51 (9:1 $CHCl_3$/MeOH); ^1H NMR ($CDCl_3$) δ 7.78–7.75 (dd, 1 H, J = 7.9, 1.8), 7.43 (dt, 1 H, J = 8.2, 1.5), 6.97–6.93 (m, 2 H), 6.06 (br s, 1 H), 5.59 (br s, 1 H), 4.57–4.47 (m, 1 H), 4.37–4.28 (m, 1 H), 4.02 (t, 2 H, J = 6.4), 3.22–3.13 (m, 1 H), 2.92–2.86 (dd, 1 H, J = 12.7, 5.0), 2.72 (d, 1 H, J = 12.7), 1.96–1.45 (m, 8 H); MS (EI) m/z 364 (M^+), 333, 213, 152 (base), 121, 97, 81, 55, 43.

Benzoic Acid, 2-[[5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl]oxy]-, [3a*S*-(3aα,4β,6aα)]- ($C_{17}H_{22}N_2O_4S$, 24). A solution of 23 (110 mg, 0.30 mmol) in 5:1 MeOH/CH_2Cl_2 (6 mL) and 1 N NaOH (0.5 mL) was stirred at 60 °C for 12 h. After solvent evaporation, the white residue was dissolved in water and acidified (1 N HCl). The white precipitate was washed and dried to afford 24 (98 mg, 93%): ^1H NMR (CD_3OD) δ 7.80 (d, 1 H, J = 6.0), 7.51 (t, 1 H, J = 8.1), 7.09 (d, 1 H, J = 7.2), 7.0 (t, 1 H, J = 8.0), 4.56–4.46 (m, 1 H), 4.37–4.29 (m, 1 H), 4.13 (t, 2 H, J = 6.5), 3.29–3.19 (m, 1 H), 2.98–2.89 (dd, 1 H, J = 12.6, 5.0), 2.70 (d, 1 H, J = 12.7), 1.98–1.45 (m, 8 H); MS (EI) m/z 350 (M^+), 332, 306, 213, 200, 152 (base), 140, 121, 97, 85, 55, 43.

Benzoic Acid Methyl Ester, 2-[[5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl]oxy]-, [3a*S*-(3aα,4β,6aα)]- ($C_{18}H_{24}N_2O_3S_2$, 25). To a solution methyl thiosalicylate (16.5 μL, 0.11 mmol) in 1.5 mL of DMF was added 16 mg of solid K_2CO_3. The reaction was stirred at room temperature for 30 min. At this time mesylate 21 (37 mg, 0.11 mmol) was added, and the reaction mixture was stirred for 18 h. Evaporation of the solvent and silica purification (98:2 $CHCl_3$/MeOH) of the residue afforded 25 (38 mg, 90%) as a light yellow solid: TLC R_f = 0.68 (9:1 $CHCl_3$/MeOH); IR (mineral oil mull) 3115, 3071, 2952, 2922, 2867, 2852, 1726, 1706, 1599, 1491, 1465, 1461, 1455, 1303, 1288, 1272, 1246, 1086, 760 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.97–7.94 (dd, 1 H, J = 7.8, 1.1), 7.47–7.42 (dt, 1 H, J = 8.6, 1.5), 7.30 (d, 1 H, J = 7.4), 7.17–7. 12 (t, 1 H, J = 8.6), 5.63 (br s, 1 H), 5.17

(br s, 1 H), 4.53–4.49 (m, 1 H), 4.33–4.29 (m, 1 H), 3.91 (s, 3 H), 3.22–3.10 (m, 1 H), 2.98–2.88 (overlayed d and t, 3 H), 2.75 (d, 2 H, J = 12.7), 1.81–1.38 (m, 8 H); MS (EI) m/z 380 (M^+), 362, 349, 320, 288, 245, 213, 168, 152 (base), 136, 97, 85.

Benzoic Acid, 2-[[5-(Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]-imidazol-4-yl)pentyl]thio]-, [3a*S*-(3aα,4β,6aα)]- ($C_{17}H_{22}N_2O_3S_2$, 26). To a solution of 101 mg (0.26 mmol) of **22** in 5 mL of MeOH and 1 mL of CH_2Cl_2 was added 0.5 mL of 1 N NaOH. The reaction mixture was stirred at 60 °C until the disappearance of starting material (14 h). Evaporation of the solvent gave a white residue, which is dissolved in water. Addition to this solution of 1 N HCl precipitated a white solid, which was washed with water and then dried *in vacuo* to provide pure **26** (88 mg, 92%): IR (mineral oil mull) 3312, 2952, 2922, 2868, 2854, 1695, 1463, 1429, 1288, 1268, 1251, 745, 612 cm^{-1}; ^{1}H NMR (CD_3OD) δ 7.92 (d, 1 H, J = 7.8), 7.53–7.37 (m, 2H), 7.17 (t, 1 H, J = 8.0), 4.55–4.47 (m, 1 H), 4.36–4.25 (m, 1 H), 3.25–3.16 (m, 1 H), 3.03–2.89 (m, 2 H), 2.69 (d, 1 H, J = 12.7), 1.84–1.35 (m, 8 H); MS (EI) m/z 366 (M^+), 348, 306, 288, 213, 199, 152, 136, 123, 109, 97, 85, 69, 55, 43.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-[[2-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl]thio]benzoyl]-amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]-, 3a*S*-[3aα,4β(1*R,2*R**), 6aα]]- ($C_{44}H_{66}N_6O_6S_2$, 27).** To a suspension of **14** (29.9 mg, 0.061 mmol) and 11 μL of iPr_2EtN in 3 mL of CH_2Cl_2 was added 22 mg (0.060 mmol) of **24** and 10 μL of $(EtO)_2P(O)CN$. The reaction mixture was stirred at room temperature for 5 h. Evaporation of the solvents *in vacuo* gave a residue, which was purified by silica chromatography (96:4 $CHCl_3$/saturated NH_3 in MeOH) to yield a small amount of pure compound, as well as mixed fractions. Preparative TLC (9:1 $CHCl_3$/MeOH) of the mixed fractions gave pure compound, which was extracted from the silica with 98:2 $CHCl_3$/MeOH. This material was combined with the pure column fractions to afford **27** (18 mg, 35%): TLC R_f = 0.35 (9:1 $CHCl_3$/MeOH); HPLC t_R = 16.7 min; MS (FAB) m/z 839 (M + H)$^+$, 618, 491, 349, 222, 213, 109, 86.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-[[2-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl]oxy]benzoyl]-amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]-, [3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{44}H_{66}N_6O_7S$, 28).** To a suspension of **14** (61.5 mg, 0.125 mmol) and 30 μL of iPr_2EtN in 6 mL of CH_2Cl_2 were added 45.5 mg (0.125 mmol) of **26** and 21.3 μL of $(EtO)_2P(O)CN$. The reaction mixture was stirred at room temperature for 16 h. Removal of the solvent *in vacuo*, and purification of the residue by silica chromatography (97/3 $CHCl_3$/saturated NH_3 in MeOH) gave the pure compound, and some mixed fractions, which were purified by preparative TLC (88:12 $CHCl_3$/MeOH). The total recovery of pure **28** was 73 mg (70%): TLC R_f = 0.53 (9:1 $CHCl_3$/MeOH); HPLC t_R = 18.2 min; MS (FAB) m/z 823 (M + H)$^+$, 715, 602, 491, 333, 222, 213, 121, 109, 86.

5-Thiazolylethanazide, 4-Methyl- ($C_6H_8N_4S$, 30). ZnN_6-pyridine$_2$ (2.76 g, 9.0 mmol) was suspended in a solution of 2-(4-methyl-5-thiazolyl)ethanol [137-00-8] (**29**) (0.858 g, 6.0 mmol) and PPh_3 (3.14 g, 12 mmol) in 30 mL of dry toluene. Diisopropyl azodicarboxylate (2.36 mL, 12.0 mmol) was added dropwise via syringe at room temperature. The reaction mixture was stirred for 3 h, filtered through Celite, and concentrated. The crude product was purified by flash silica chromatography (80:20 $CHCl_3$/hexanes) to afford **30** (0.53 g, 54%) as a light yellow oil: TLC R_f = 0.73 (99:1 CHC_3/MeOH); IR (neat) 2949, 2927, 2110, 1544, 1450, 1433, 1515, 1378, 1351, 1294, 1279, 1263, 1240, 844, 795, 648 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.62 (s, 1 H), 3.51 (t, 2 H, J = 6.7), 3.04 (t, 2 H, J = 6.8), 2.43 (s, 3 H); MS (EI) m/z 168 (M^+), 112, 85, 69, 59, 45. Anal. C, H, N.

5-Thiazolylethanamine, 4-Methyl- [58981-35-4] ($C_6H_{10}N_2S$, 31). To a solution of **30** (83 mg, 0.49 mmol) in 12 mL of MeOH was added Pd/$CaCO_3$ (35 mg) under N_2. The solution was hydrogenated at atmospheric pressure for 2.5 h, filtered, and concentrated to afford **31** (68 mg, 97%) as an oil: TLC R_f = 0.40 (9:1 $CHCl_3$/MeOH); ^{1}H NMR ($CDCl_3$) δ 8.58 (s, 1 H), 2.98–2.87 (m, 4 H), 2.87 (s, 3 H), 1.33 (br s, 2 H); MS (EI) m/z 143 (M^+), 126, 113, 95, 83, 69, 55, 43.

5-Thiazolylethanamine, 4-Methyl-*N*-[2-[[(1,1-dimethylethoxy)carbonyl]amino]-3-methyl-1-oxopentyl]-[2*S*-(*R,*R**)]- ($C_{17}H_{29}N_3O_3S$, 32).** To a solution of **31** (71.1 mg, 0.50 mmol) in 2 mL of CH_2Cl_2 and 98 μL of iPr_2EtN were added *N*-Boc-L-isoleucine (127 mg, 0.55 mmol) and 83 μL of $(EtO)_2P(O)CN$. After the mixture was stirred at room temperature for 20 h, the solvent was removed *in vacuo* and the residue chromatographed (97:3 $CHCl_3$/satd NH_3 in MeOH) over silica to afford **32** as a white solid (190 mg, 97%): TLC R_f = 0.58 (99:1 $CHCl_3$/MeOH); IR (mineral oil mull) 3345, 3315, 2925, 2872, 2856, 1680, 1656, 1545, 1523, 1448, 1178, 630, cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.58 (s, 1 H), 6.57 (t, 1 H, J = 6.0), 5.13 (d, 1 H, J = 8.9), 3.95–3.89 (dd, 1 H, J = 9.0, 6.7), 3.55–3.39 (m, 2 H), 2.99 (t, 2 H, J = 6.7), 2.39 (s, 3 H), 1.43 (s, 9 H), 0.95–0.86 (m, 6 H); MS (FAB) m/z 356 (M + H)$^+$, 300, 256, 143, 126, 113, 86, 57. Anal. C, H, N.

5-Thiazolylethanamine, 4-Methyl-*N*-(2-amino-3-methyl-1-oxopentyl)-, [2*S*-(*R,*R**)]- ($C_{12}H_{21}N_3OS$, 33).** To a solution of **32** (103 mg, 0.29 mmol) in 15 mL of CH_2Cl_2 was added 0.5 mL of CF_3CO_2H. The reaction mixture was stirred at room temperature for 1 h, and the volatiles were then removed *in vacuo*. The reaction mixture was neutalized with aqueous $NaHCO_3$ and then extracted with CH_2Cl_2. The organic phase was dried and evaporated to afford **33** (68 mg, 91%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 8.50 (s, 1 H), 7.60 (br s, 1 H), 3.49 (q, 2 H, J = 6.7), 2.99 (t, 2 H, J = 7.0), 2.41 (s, 3 H), 2.0–0.75 (m, 12 H total).

Carbamic Acid, [1-(2,2-Dimethyl-5-[2-cyclohexyl-1-[[[2-methyl-1-[[(2-pyridinylmethyl)amino]carbonyl]butyl]amino]-carbonyl]ethyl]-1,3-dioxolan-4-yl]-2-methylpropyl]-, 1,1-Dimethylethyl Ester, [4*R*-[4α(*R),5β(*S**)]]- ($C_{38}H_{60}N_4O_6S$, 34).** To a solution of **33** (68 mg, 0.26 mmol) in 2 mL of CH_2Cl_2 and 34 μL of iPr_2EtN was added **9** (111 mg, 0.26 mmol) and 40 μL of $(EtO)_2P(O)CN$. The reaction mixture was stirred at room temperature for 14 h. Removal of the solvent *in vacuo* and purification of the residue by silica chromatography (70/30 EtOAc/$CHCl_3$) yielded **34** (137 mg, 80%) as a white solid: TLC R_f = 0.60 (4:1 EtOAc/hexanes); MS (FAB) m/z 665 (M + H)$^+$, 565, 395, 310, 256, 143, 126, 86, 57.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-amino-2-(1-methylethyl)-*N*-[2-methyl-1-[[[2-(4-methyl-5-thiazolyl)ethyl]amino]carbonyl]butyl]-, [3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{27}H_{48}N_4O_4S$, 35).** A solution of 10 mL of MeOH and 0.56 mL of acetyl chloride was stirred at room temperature for 15 min, at which time **34** (516 mg, 0.77 mmol) and 0.12 mL of ethanedithiol were added. The reaction mixture was stirred for 6 h, quenched with excess solid $NaHCO_3$, and filtered. The filtrate was evaporated *in vacuo*, and the residue was chromatographed (94:6 $CHCl_3$/saturated NH_3 in MeOH) on silica gel to afford **35** (307 mg, 75%) as a white solid: TLC R_f = 0.20 (9:1 $CHCl_3$/saturated NH_3 in MeOH); MS (FAB) m/z 525 (M + H)$^+$, 381, 364, 282, 270, 256, 143, 126, 86, 69, 55, 41.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)-1-oxopentyl]amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[[2-(4-methyl-5-thiazolyl)ethyl]amino]carbonyl]butyl]-, [3a*S*-[3aα,-4β(1*R,2*R**),6aα]]- ($C_{37}H_{62}N_6O_6S_2$, 36).** To a solution of **6** (6.82 mg, 0.02 mmol) in 2 mL of DMF was added **35** (10 mg, 0.019 mmol). The reaction mixture was stirred at room temperature for 15 h. The DMF was removed *in vacuo*, and the white residue was chromatographed (95:5 $CHCl_3$/MeOH) on silica to yield **36** (9.8 mg, 68%) as a white solid: TLC R_f = 0.44 (9:1 $CHCl_3$/saturated NH_3 in MeOH); MS (FAB) m/z 751 (M + H)$^+$, 565, 525, 496, 348, 270, 227, 143, 126, 86.

L-Idonamide, 6-Cyclohexyl-2,5,6-trideoxy-5-[[2-[[5-(hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentyl]thio]benzoyl]-amino]-2-(1-methylethyl)-*N*-[2-methyl-1-[[[(2-(4-methyl-5-thiazolyl)ethyl]amino]carbonyl]butyl]-, [3a*S*-[3a*S*-[3aα,4β(1*R,2*R**),6aα]]- ($C_{44}H_{66}N_6O_6S_3$, 37).** To a suspension of **35** (34 mg, 0.064 mmol) in 3 mL of CH_2Cl_2 and 9 μL of iPr_2EtN were added **22** (25.6 mg, 0.07 mmol) and 10.6 μL of $(EtO)_2P(O)CN$. The reaction mixture was stirred at room temperature for 16 h. Removal of the solvent *in vacuo* gave a residue, which was purified by silica chromatography (98:2 $CHCl_3$/MeOH) to afford **37** (17 mg, 30%) as a white solid: TLC R_f = 0.41 (9:1 $CHCl_3$/saturated NH_3 in MeOH); HPLC t_R = 17.0 min; MS (FAB) m/z 839 (M + H)$^+$, 731, 618, 349, 222, 213, 109, 86.

Preparation of the Avidin Complex of 27. A solution of **27** (2.01 mg, 4.06 μmol) in 0.10 mL of DMF and a solution of avidin (33.5 mg, 1.97 μmol monomeric sites) in 15 mM Tris-HCl pH 7 buffer were mixed gently. After mixing, the solution was stirred at ambient temperature for 15 min and then clarified by centrifugation, with the solids being discarded. The supernatent

was applied to a G-25 Sephadex molecular sieve filtration column equilibrated in 15 mM Tris-HCl pH 7.5 buffer. The protein-containing fractions (by the 280-nm absorption) were pooled. The concentration of the protein was estimated from the absorbance at 280 nm and the volume of each fraction. A portion of this purified protein was diluted to provide a solution of approximately 33 μM, and from this absorption spectrum was subtracted the absorption spectrum of a solution of 33 μM avidin (using ϵ = 26 350 M^{-1} cm^{-1} for the avidin monomer). This absorption spectrum was compared to that of a 33 μM solution (in MeOH) of **27** ($\epsilon_{289\ nm}$ = 3250 M^{-1} cm^{-1}). A comparison of the two spectra (Figure 1) provides an estimation of 2 mol of **27** bound per avidin tetramer. The combined avidin–**27** complex from the column was lyophilized to recover 36.4 mg of a white powder. Stock solutions were prepared after correcting this mass for buffer salts, and using M_r equal to the sum of that for **27** and that for two avidin monomers.

Evaluation in the Rat of the Serum Clearance of the Biotinylated Peptides. Each compound was formulated at the molar equivalent to 1 mg mL^{-1} **1** (U-75875) in an aqueous vehicle containing 4% EtOH, 0.08% (v/v) Tween 80, 5% (mass/v) dextrose, and 8 mM HCl. Male Sprague–Dawley rats having a mass between 194 and 290 g were fasted overnight in home cages with access to water. The animals were anesthetized with dialurethane (ip 0.65 mg kg^{-1}). Compound was administered (four rats for each compound) iv through the tail vein (4 mL kg^{-1}). Blood samples were taken from the orbital sinus at 10, 20, 40, and 80 min after dosing. The serum was harvested by centrifugation and frozen until assay. The procedures for the determination of serum protease inhibitor concentration are described elsewhere.[35]

Effect of the Biotin-Protease Inhibitor Conjugates on Biotin Transport in the Caco-2 Cell. The procedure for the growth of the Caco-2 human colorectal cell culture was as previously described.[16] The passage number for the cells used in this study was 25. Stock solutions of the biotin–protease inhibitor conjugates were prepared in MeOH and diluted into the transport medium (Hanks' balanced salt solution containing 25 mM glucose and 10 mM Hepes pH 7.35 buffer) to a final concentration of 300 μM. The final [MeOH] was 1% for **13**, **15**, **27** and 5% for **19**; control experiments showed the monolayers to be unaffected at these concentrations. All transport experiments were performed at 37 °C in the transport medium. Prior to the experiments, the culture medium of Transwell-grown Caco-2 cell monolayers were replaced with transport medium at 37 °C, and the culture was equilibrated for 30 min. In apical-to-basolateral transport studies, all wells in six-well clusters received 2.6 mL of transport medium previously equilibrated to 37 °C. Inserts containing the Caco-2 cell monolayers were positioned in the wells such that the outer surface of the insert basolateral side was immersed in the transport medium. Transport medium (1.5 mL equilibrated to 37 °C) containing 10 nM [^{3}H]biotin (Amersham) alone, or 10 nM [^{3}H]biotin in the presence of 300 μM unlabeled biotin or biotin–protease inhibitor conjugates, was applied to the apical side. At time intervals, prior to sampling, the six-well cluster cell plate was gently swirled to ensure mixing. Samples (50 μL) were removed from the basolateral side and replaced by fresh transport medium. Monolayer integrity during each transport experiment was evaluated with [^{3}H]mannitol as an impermeable marker, added to three randomly chosen cell monolayers. The true level of [^{3}H]biotin transported at time t($B_{R,t}$) was calculated as being equal to $\{B_{T,t} - B_{P,t}\}$, where $B_{T,t}$ is the total amount transported and $B_{P,t}$ is the amount passively transported, at time t. $B_{P,t}$ was estimated, using the assumption that the paracellular transport of biotin is equal to that of mannitol, by the equation $B_{P,t} = P_M B t$, where P_M is the permeability coefficient for mannitol; B is the biotin concentration; and t is the elapsed time. The statistical analysis of the data was done by the student's T-test.

Assessment of the Antiviral Activity of 27 and the Avidin–27 Complex. The protocols for the vVk-1 infected CV-1 cell and HIV_{D84}-infected PBMC (Diagen Assay) have been described previously.[4] The evaluation of the avidin complex was as follows. HIV-1 virus was obtained from the culture supernatent of H9 cells infected with $HTLV_{3B}$ virus. H9 human T-lymphoid cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.01 M Hepes buffer. Cells (5×10^4) were seeded into each well of a 96-well dish. Cultures were infected by a 90-min incubation with diluted stock virus at a low (0.01) multiplicity of infection. The avidin–**27** complex was dissolved in culture medium and **27** in $(CH_3)_2SO$. The infected cells were maintained in the continuous presence of these inhibitors for 5–7 d in a humidified CO_2 chamber. Culture supernatant was harvested, and p24 core antigen was quantitated by HIV-1 p24 antigen ELISA (Coulter). The percent inhibition was relative to control culture supernatent. Test compounds also were evaluated for H9 cytotoxicity, by trypan blue dye exclusion, after 5 d in culture. Neither **27** nor the avidin–**27** complex was cytotoxic at 10 μM concentration.

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