Design and Discovery of a Novel Dipeptidyl-peptidase IV (CD26)-Based Prodrug Approach

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Here we describe a novel type of enzyme-based prodrug approach in which a dipeptide moiety is linked to a nonpeptidic therapeutic drug through an amide bond which is specifically cleaved by the dipeptidyl-peptidase IV (DPP IV/CD26) enzyme activity present in plasma and on the surface of certain cells. DPP IV has high substrate selectivity for peptides with a proline (or an alanine) at the penultimate amino acid position at the N-terminus but tolerates a wide range of natural amino acids at the amino terminal end. A variety of dipeptidyl amide prodrugs of anti-HIV TSAO molecules were synthesized and evaluated for their ability to act as substrates for the enzyme. Our data revealed that DPP IV/CD26 can efficiently recognize such prodrugs as substrates, releasing the parent compound. Moreover, it is possible to modify the half-life and the lipophilicity of the prodrugs by changing the nature of the dipeptide. All conjugates have shown marked in vitro antiviral activities irrespective the the nature of the terminal and/or the penultimate amino acid moiety.

Introduction

The lymphocyte surface glycoprotein CD26, originally described as a T-cell activation/differentiation marker,[1] belongs to a unique class of membrane-associated peptidases. It is characterized by an array of diverse functional properties and it is identical to dipeptidyl-peptidase IV (DPP IV).[2−4] DPP IV is a member of the prolyl oligopeptidase family, a group of atypical serine proteinases able to hydrolyze a prolyl peptide bond. CD26 is strongly expressed on a variety of leukocyte cell subsets (e.g. T cells, B cells, natural killer cells, and macrophages), but also on several types of epithelial, endothelial, and fibroblast cells. A soluble form of this enzyme also exists and is detected in plasma and cerebrospinal fluid at lower amounts.[2−4] CD26 is endowed with an interesting (dipeptidyl)peptidase catalytic activity, and it has high selectivity for peptides with a proline or alanine at the penultimate position of the N-terminus of a variety of natural peptides but tolerates a wide range of amino acyl residues at the ultimate N-terminal end. A free amino group on the ultimate amino acid is a prerequisite for substrate activity by the enzyme. DPP IV truncates several bioactive peptides of medical importance.[2−4]

In 2005, it was demonstrated for the first time that this enzyme plays a direct regulatory and indispensable role in the eventual antiretroviral activity of small synthetic molecules such as the antiretroviral prodrug GlyProGly-NH_2 (inactive) releasing glycinamide (Gly-NH_2) (active).[5] This is the first demonstration that a differentiation/activation leukocytic marker, abundantly present on activated T-lymphocytic cells, acts as a highly specific and obligatory activator of a synthetic anti(retro)viral prodrug that is otherwise inactive as such.

These findings prompted us to explore an entirely novel enzyme-based prodrug approach that provides conjugates of therapeutic agents with a di- (or oligo) peptide moiety, containing a proline at the penultimate position of the N-terminus, as a carrier, wherein the conjugate [(Xaa-Pro)$_n$]−[drug] (**I**, Figure 1) is specifically cleavable by the endogenous dipeptidyl-peptidase IV enzyme (DPP IV/CD26) present on the surface of certain cells or in plasma.

Many prodrug technologies have already been developed depending on the kind of drug that has to be converted.[6,7] Coupling of peptides or amino acids as carriers to a therapeutic agent has already been pursued in the past. Examples of amino acid coupling to drugs are valacyclovir and valgancyclovir, the valyl ester prodrugs of the antiherpetic acyclovir and ganciclovir drugs, respectively.[8−10] The 3−5-fold increased oral bioavailability of the prodrugs have been shown to be caused by their affinity for the human peptide transporter hPEPT-1 located in the membrane of the upper small intestine epithelial cells.[11] The prior art for ameliorating solubility and bioavailability reveals however only amino acid prodrugs (only one amino acid coupled) of small organic molecules whereby the amino acid is usually coupled through ester bonds, allowing easy back conversion to the free therapeutic agent by esterases. However, the prodrugs have low stability at physiological pH and their delivery is often not optimal.[8a]

Our novel dipeptide-prodrug approach would allow us to ameliorate the solubility and/or bioavailability of therapeutic agents in a potentially more successful manner since coupling of a di- (or oligo)peptide moieties (instead of one single amino acid) to a therapeutic agent is performed. Moreover, the therapeutic drug is linked to the peptide through a more stable amide bond (instead of an ester bond) which is specifically cleaved by DPP IV/CD26. The presence of a proline near the N-terminus may also serve as efficient structural protection against nonspecific proteolytic degradation.[12]

Design. The novel prodrug approach should be used on drugs that contain a free amino group that can be directly coupled with the carboxyl group of amino acids via an amide bond. Since human immunodeficiency virus (HIV), the ethiological agent of AIDS, mainly infects lymphocytes or macrophages that abundantly express CD26/DPP IV enzyme in their membrane we focused on anti-HIV compounds, in particular on TSAO derivatives. These compounds belong to an unique family of

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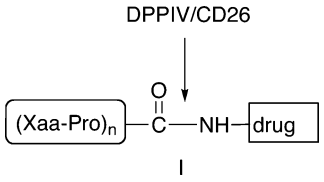


Figure 1. [Xaa-Pro]−[drug] conjugates of general formula **I** cleavable by CD26.

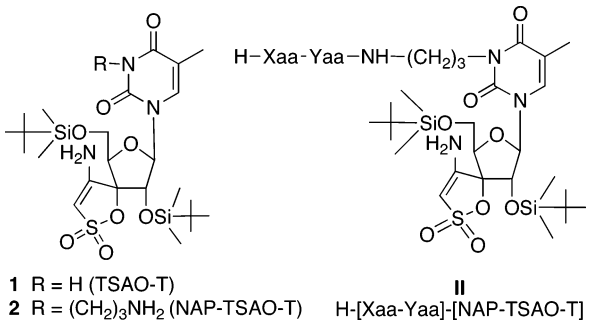


Figure 2. Structures of TSAO-T (**1**), NAP-TSAO-T (**2**), and [Xaa-Yaa]−[NAP-TSAO-T] conjugates of general formula **II**.

nonnucleoside reverse transcriptase inhibitors discovered in our laboratories. The prototype compound is the thymine derivative designated as TSAO-T (**1**, Figure 2).[13−15] A lot of experience has been gained over the past years with regard to the chemical synthesis and the efficient introduction of modifications in its structure. Therefore, the TSAO-T molecule was chosen as a prototype compound for proof of concept. Due to the low reactivity of the 4″-amino group of the spirosultone moiety of TSAO derivatives,[16] the N-3 aminopropyl TSAO-T derivative (NAP-TSAO-T, **2**, Figure 2) has been chosen as a model compound because its primary amine functionality would allow the formation of an amide bond between the peptide and the TSAO molecule. Also, it is known, in the whole series of N-3 substituted TSAO derivatives, that attachment of the $(CH_2)_3$-NH_2 substituent to the N-3 atom of the thymine of the prototype TSAO-T preserves the antiviral activity of the compound.[17] It has been shown that the N-3-substituted moiety of the TSAO derivatives run parallel to the subunit interface and is exposed to the solvent. This explains why sometimes large substituents at N-3 of TSAO-T, such as an AZT molecule linked to N-3 by a methylene spacer, do not compromise the anti-HIV (and anti-RT) activity of those compounds.[17,18]

We designed and synthesized a variety of dipeptidyl amide prodrugs of TSAO molecules deprotected at the peptide N-terminus (**II**, Figure 2) and evaluated their ability to act as efficient substrates for the enzyme DPPIV/CD26. The aim for the design of the dipeptide−TSAO conjugates is to explore whether (1) DPP IV/CD26 is able to efficiently recognize dipeptide sequences when linked through an amide bond to a molecule (i.e. TSAO) different from a natural peptide, and if so, whether (2) it is possible to modify the enzymatic and serum hydrolysis rate (half-life) of the conjugates changing the nature (Xaa, Yaa) of the dipeptide promoieties, and (3) to study the influence of the nature of the dipeptide moiety on the overall lipophilicity of the prodrugs. In addition, we have also evaluated the TSAO−dipeptide prodrugs against HIV-1 RT in a cell-free enzyme assay and against HIV-1 replication in cell culture.

Results and Discussion

Chemistry. For the synthesis of the target conjugates we designed a two-step procedure that consisted of the coupling of NAP-TSAO-T **2** with the appropriate C-deprotected dipeptide derivative Z-Xaa-Yaa-OH followed by deprotection of the amino group of the intermediate N-protected conjugates by catalytic hydrogenation. The presence of *tert*-butyldimethylsilyl groups (TBDMS) at positions 2′ and 5′, sensitive to basic and acid media, respectively, but essential for the antiviral activity of TSAO compounds, implies the selection of a benzyloxycarbonyl protecting strategy due to the smooth deprotection reaction reaction conditions compatible with TBDMS groups. The precursor NAP-TSAO-T (**2**) was obtained as described previously.[17]

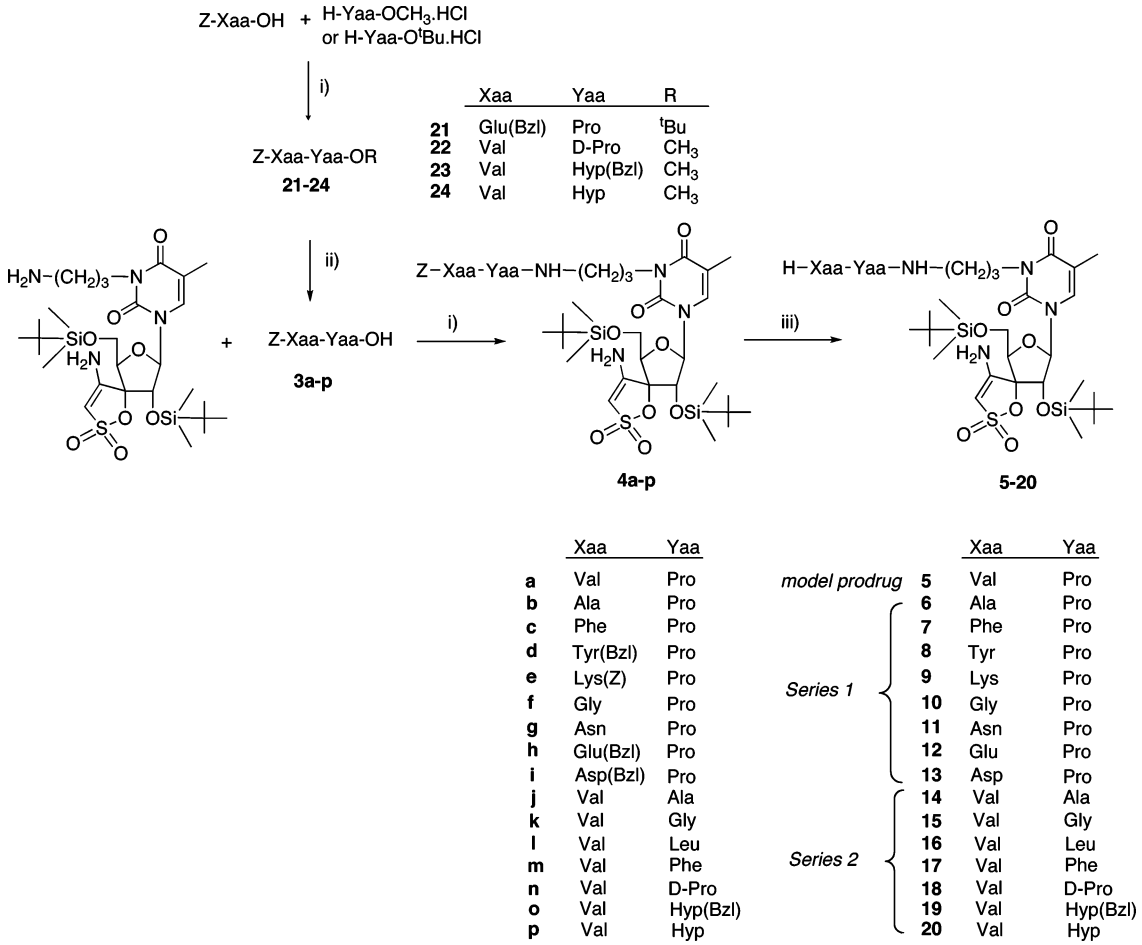
First, we focused on the synthesis of the model prodrug **5** (Scheme 1) bearing the Val-Pro dipeptide sequence, easily recognized by the enzyme DPPIV/CD26 in natural peptides, as the promoiety. Thus, coupling of compound **2** with the commercially available dipeptide Z-Val-Pro-OH (**3a**) in CH_2Cl_2, in the presence of BOP and TEA, gave the corresponding fully protected dipeptide **4a** in 54% yield after purification by flash column chromatography. Catalytic hydrogenation of **4a** in the presence of 10% Pd/C in methanol afforded the deprotected model prodrug **5** in 83% yield. No deprotection of the TBDMS groups was observed.

The biological results (see below) obtained with the model prodrug **5** encouraged us to prepare a novel series of conjugates [Xaa-Pro]−[NAP-TSAO-T] (compounds **6**−**13**, Scheme 1) where the proline of the model conjugate **5** was maintained and the valine was replaced by a broad range of different natural amino acids (Xaa) such as hydrophobic (alanine, phenylalanine, tyrosine), basic (lysine), neutral (glycine, asparagine) or acid (aspartic acid, glutamic acid) residues.

The synthesis of compounds **6**−**13** (Series 1, Scheme 1) was carried out in a similar way to that described for the model prodrug **5** starting from the appropriate dipeptide derivative Z-Xaa-Yaa-OH (**3b**−**i**). Noncommercially available dipeptides Z-Tyr(OBzl)-Pro-OH (**3d**),[19] Z-Lys(Z)-Pro-OH (**3e**),[20] Z-Gly-Pro-OH (**3f**),[21] Z-Asn-Pro-OH (**3g**),[22] and Z-Asp(OBzl)-Pro-OH (**3i**),[23] were synthesized as previously described. Novel dipeptide intermediate Z-Glu(OBzl)-Pro-OH (**3h**) (Scheme 1) was prepared using standard coupling/deprotection techniques as follows. Thus, coupling of Z-Glu(Bzl)-OH in CH_2Cl_2 in the presence of BOP and TEA gave the corresponding fully protected dipeptide Z-Asp(OBzl)-Pro-OH (**21**) (Scheme 1) in 63% yield after purification by flash column chromatography. Acid hydrolysis of compound **21** in a TFA solution yielded the C-deprotected derivative **3h** in 87% yield. Next, the appropriate dipeptide derivatives Z-Xaa-Yaa-OH (**3b**−**i**) were reacted with NAP-TSAO-T (**2**) in the presence of BOP and TEA to give the protected intermediate conjugates **4b**−**i** in moderate to good yields (40−89%). Removal of the benzyl groups by catalytic hydrogenation in the presence of 10% Pd/C in CH_3OH afforded the desired final deprotected conjugates **6**−**13** in good yields (66−97%).

To further explore the substrate activity requirements for DPPIV/CD26 a second series of conjugates [Val-Yaa]−[NAP-TSAO-T] (Series 2, Scheme 1) was also prepared. In these conjugates the valine of the model conjugate **5** is maintained and the proline is changed by an alanine (**14**), also recognized although less efficiently by the enzyme in natural peptide substrates, or a glycine, leucine, or phenylalanine (**15**−**17**) as negative control compounds. Also, unnatural amino acids such as D-proline (**18**) or modified prolines [*trans*-4-hydroxy-L-proline (Hyp, **19**, **20**) and 2,3-*trans*-3,4-*cis*-3,4-dihydroxy-L-proline (DHP, **29**, Scheme 2)] whose substrate specificity is unknown in natural peptides, were introduced. Conjugates **14**−**20** (Scheme 1) were prepared by coupling of the appropriate C-deprotected

Scheme 1. Synthesis of [Xaa-Yaa]−[NAP-TSAO-T] Conjugates **5**−**20**[a]



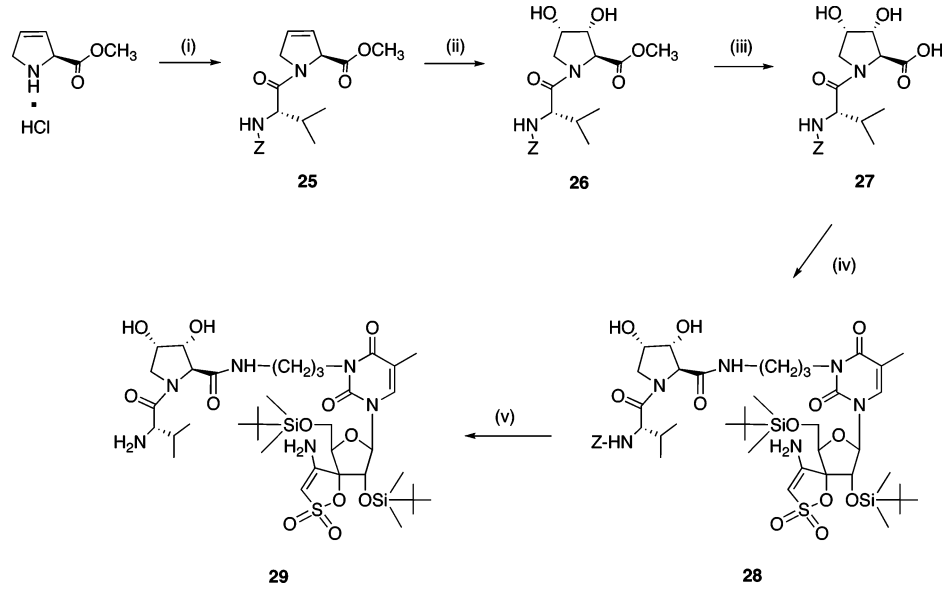
	Xaa	Yaa	R
21	Glu(Bzl)	Pro	^{t}Bu
22	Val	D-Pro	CH_3
23	Val	Hyp(Bzl)	CH_3
24	Val	Hyp	CH_3

	Xaa	Yaa
a	Val	Pro
b	Ala	Pro
c	Phe	Pro
d	Tyr(Bzl)	Pro
e	Lys(Z)	Pro
f	Gly	Pro
g	Asn	Pro
h	Glu(Bzl)	Pro
i	Asp(Bzl)	Pro
j	Val	Ala
k	Val	Gly
l	Val	Leu
m	Val	Phe
n	Val	D-Pro
o	Val	Hyp(Bzl)
p	Val	Hyp

		Xaa	Yaa
model prodrug	**5**	Val	Pro
Series 1	**6**	Ala	Pro
	7	Phe	Pro
	8	Tyr	Pro
	9	Lys	Pro
	10	Gly	Pro
	11	Asn	Pro
	12	Glu	Pro
	13	Asp	Pro
Series 2	**14**	Val	Ala
	15	Val	Gly
	16	Val	Leu
	17	Val	Phe
	18	Val	D-Pro
	19	Val	Hyp(Bzl)
	20	Val	Hyp

[a] Reagents: (i) BOP, TEA, CH_2Cl_2; (ii) 2 N NaOH or TFA; (iii) H_2, Pd(C), CH_3OH.

Scheme 2. Synthesis of [Val-DHP]−[NAP-TSAO-T] Conjugate **29**[a]

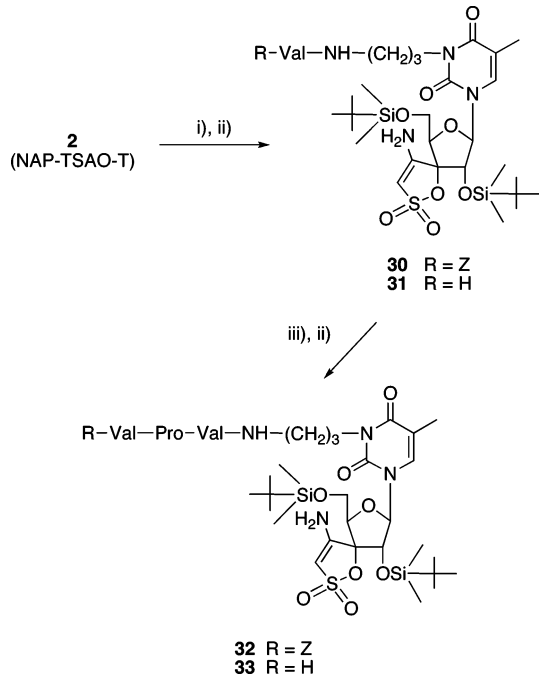


[a] Reagents: (i) Z-Val-OH, BOP, TEA, CH_2Cl_2; (ii) OsO_4, NMO, 1,4-dioxane/H_2O; (iii) 2 N NaOH, CH_3OH; (iv) NAP-TSAO-T (**2**), BOP, TEA, CH_2Cl_2; (v) H_2, Pd(C), CH_3OH.

dipeptide derivatives Z-Xaa-Yaa-OH **3j**−**p** with NAP-TSAO-T (**2**) in the presence of BOP/TEA to afford the Z-conjugates **4j**−**p** in moderate to good yields (37−89%) that were subsequently hydrogenated (H_2, Pd/C, CH_3OH) to give the final deprotected prodrugs **14**−**20**.

Conjugate **29**, bearing a 2,3-*trans*-3,4-*cis*-3,4-dihydroxy-L-proline (DHP) amino acid residue, was prepared from the commercially available 3,4-dehydroproline amino acid as shown in Scheme 2. Coupling of H-3,4-dehydro-Pro-OCH_3·HCl with Z-Val-OH gave the N-protected dipeptide ester derivative **25**

Scheme 3. Synthesis of [Val-Pro-Val]−[NAP-TSAO-T] Conjugates **33**



Reagents: (i) Z-Val-OH, BOP, TEA, CH_2Cl_2; (ii) H_2, Pd(C), CH_3OH; (iii) Z-Val-Pro-OH, BOP, TEA, CH_2Cl_2.

in 68% yield. The stereoselective osmium tetroxide oxidation of compound **25** with *N*-methyl-morpholine-*N*-oxide (NMO)[24] afforded the 2,3-*trans*-3,4-*cis*-3,4-dihydroxy-L-proline dipeptide ester derivative **26** as the major diasteromeric derivative in good yield (82%). Cleavage of the methyl ester group under standard saponification conditions yielded the desired C-deprotected dipeptide derivative **27** in 89% yield. Compound **27** and NAP-TSAO-T (**2**) were similarly coupled in the presence of BOP/TEA to give Z-conjugate **28** in 40% yield which was subsequently hydrogenated in the presence of 10% Pd/C in CH_3OH to give the final desired conjugate **29** in good yields (87%).

The structure of intermediate conjugates Z-[Xaa-Yaa]−[NAP-TSAO-T] (**4a**−**p**, **28**) and final deprotected conjugates H-[Xaa-Yaa]−[NAP-TSAO-T] (**5**−**20** and **29**) was determined by analytical and spectroscopic data. Racemization was not observed by HPLC or NMR except in the Val-Ala (**4j**, **14**), Val-Leu (**4l**, **16**) and Val-Phe (**4m**, **17**) conjugates where an epimerization of the C-terminal amino acid residue by 15%, 30%, and 24%, respectively, was observed. These results are in agreement with previous studies on racemization during coupling reactions that showed that Phe or Leu are the most susceptible residues to a-inversion.[25]

Finally, we prepared the valyl-prolyl-valyl derivative **33** (Scheme 3) in which the dipeptide model sequence Val-Pro was linked to the primary amino group of the valyl-NAP-TSAO-T intermediate **31** in order to investigate whether this compound would be also an efficient substrate of the DPP IV/CD26 enzyme. Coupling of **2** with Z-Val-OH in the presence of BOP and TEA gave the protected valyl TSAO derivative **30** in 72% yield. Subsequent standard catalytic hydrogenation of **30** (H_2, Pd/C) yielded the corresponding deprotected derivative **31** (72%). Compound **31** was finally coupled with the commercially available Z-Val-Pro-OH in the presence of BOP and TEA to give the Z-conjugate **32** in 70% yield and hydrogenated (H_2, Pd/C) to afford the deprotected tripeptide conjugate **33** (59%).

Biological Studies

Anti-HIV Activity of Dipeptidyl Prodrugs of NAP-TSAO-T. Stock solutions of the compounds were made in DMSO prior to dilution in cell culture medium and initiation of the antiviral experiment. The [Xaa-Pro]−[NAP-TSAO] derivatives **5** to **13** and the [Val-Yaa]−[NAP-TSAO-T] derivatives **14** to **20**, **29**, **31**, and **33** were evaluated for their antiviral activity against HIV-1 and HIV-2 in CEM and MT-4 cell cultures (Table 1). The parent NAP-TSAO-T derivative **2** was active at an EC_{50} of 0.14 μM in CEM and 0.52 μM in MT-4 cell cultures against HIV-1, but not active against HIV-2. As a rule, none of the prodrug compounds were inhibitory to HIV-2 in CEM and MT-4 cell cultures. In contrast, the dipeptidyl NAP-TSAO prodrugs containing a penultimate Pro as the amino acid (**5** to **13**) invariably inhibited HIV-1-induced syncytium formation. The prodrugs were virtually as active as the parent compound **2**. Taking all [Xaa-Pro]−[NAP-TSAO-T] prodrugs together (**5** to **13**), the EC_{50}s against HIV-1 ranged between 0.10 μM and 1.1 μM in CEM, and between 0.14 μM and 1.2 μM in MT-4 cell cultures. Also the cellular toxicity of compounds **5** to **13** was invariably in the same order of magnitude against MT-4 and CEM cells (CC_{50}s ranging between 10 μM and $\geq$125 μM). Similar data were obtained for the dipeptide prodrugs in which the NH_2-terminal Val was kept, but the penultimate proline had been replaced by a variety of natural (**14**−**17**), but also other, less common or synthetic amino acids including the Val D-Pro (**18**), Val Hyp(Bzl) (**19**), Val Hyp (**20**), and Val DHP (**29**) derivatives. Thus, as a rule, [Xaa-Yaa]−[NAP-TSAO-T] derivatives have shown marked in vitro antiviral activities irrespective the nature of the terminal and/or the penultimate amino acid moiety. Interestingly, the [Val-Pro-Val]−[NAP-TSAO-T] derivative **33** proved also at least as inhibitory to HIV as the [Val]−[NAP-TSAO-T] derivative **31** and the parent compound **2** (Table 1).

Inhibitory Activity of Dipeptidyl Prodrugs of NAP-TSAO-T on Recombinant Purified HIV-1 Reverse Transcriptase. The compounds were also examined on their inhibitory activity against HIV-1 RT. The parent NAP-TSAO-T derivative **2** inhibited the reaction at an IC_{50} of 8.1 μM. All dipeptidyl prodrug derivatives inhibited the RT reaction at a similar extent (Table 1).

Inhibitory Effect of [Val-Pro]−[NAP-TSAO-T] (5) against CD26-Catalyzed Conversion of GlyPro-*p*-nitroanilide (pNA) to GlyPro and pNA. The enzymatic activity of purified CD26 has been measured by conversion of the artificial substrate GlyPro-pNA to GlyPro and pNA. During the hydrolysis reaction, yellow-colored pNA is released from the uncolored GlyPro-pNA. The formation of pNA can be monitored by spectrophotometric absorption measurements at 400 nm. Compound **5** dose-dependently inhibited the hydrolysis of 1 mg/mL (3.4 mM) GlyPro-pNA in the presence of 1.5 mUnits of purified CD26. At 100 μM **5**, the reaction was inhibited by ~70% (Figure 3). Also, 20 μM **5** could still inhibit the reaction by ~50%. The corresponding Z-blocked analogue **4a** was completely inactive in this assay. The dose-dependent inhibition of the CD26-catalyzed conversion of GlyPro-pNA to GlyPro and pNA by compound **5** is obviously due to competition of **5** with the artificial substrate for the enzyme.

Conversion of [Val-Pro]−[NAP-TSAO-T] (5) to NAP-TSAO-T (2) and Val-Pro by Purified CD26 and Human and Bovine Serum. When 50 μM **5** was exposed to purified CD26, conversion to compound **2** took place as a function of incubation time (Table 2). After 1 h, 37% conversion was observed, and after 4 and 24 h, ~60% of prodrug derivative was hydrolyzed.

Table 1. Antiviral, Cytostatic, and Anti-RT Activity of Dipeptidyl Prodrugs of NAP-TSAO-T (**2**)

		EC_{50} (HIV-1) (μM)[a]		CC_{50} (μM)[b]		IC_{50} (μM)[c]
compd	amino acids at N-3 position	MT-4	CEM	MT-4	CEM	HIV-1 RT (polyrC.dG)
2		0.52 ± 0.31	0.14 ± 0.09	14 ± 7.8	12 ± 8.2	8.1 ± 4.6
5	Val-Pro	0.30 ± 0.12	0.13 ± 0.08	11 ± 0.99	10 ± 0.64	6.6 ± 4.2
6	Ala-Pro	0.30 ± 0.27	0.42 ± 0.18	20 ± 0.17	19 ± 4.2	
7	Phe-Pro	0.20 ± 0.12	0.10 ± 0.03	19 ± 0.14	14 ± 6.6	
8	Tyr-Pro	0.14 ± 0.03	0.15 ± 0.04	11 ± 13	10 ± 0.28	8.1 ± 5.1
9	Lys-Pro		0.16 ± 0.06	87 ± 54	47 ± 7.4	4.7 ± 0.5
10	Gly-Pro	0.39 ± 0.11	0.20 ± 0.10	13 ± 0.57	51 ± 12	13 ± 6.0
11	Asn-Pro	0.59 ± 0.08	0.37 ± 0.24	22 ± 6.1	25 ± 3.0	4.0 ± 0.2
12	Glu-Pro	0.49 ± 0.04	0.26 ± 0.01	64 ± 19	30 ± 8.8	
13	Asp-Pro	1.2 ± 1.0	1.1 ± 0.64	69 ± 23	≥125	7.2 ± 4.3
14	Val-Ala	0.23 ± 0.03	0.08 ± 0.01	22 ± 2.6	11 ± 0.71	5.4 ± 1.2
15	Val-Gly	0.14 ± 0.02	0.05 ± 0.01	9.9 ± 6.3	10 ± 0.19	3.8 ± 0.2
16	Val-Leu	0.21 ± 0.06	0.19 ± 0.11	13 ± 6.5	11 ± 0.78	4.6 ± 0.1
17	Val-Phe	0.26 ± 0.09	0.09 ± 0.01	14 ± 5.3	10 ± 1.4	6.8 ± 3.0
18	Val-DPro	0.49 ± 0.25	0.09 ± 0.01	22 ± 0.78	11 ± 0.78	22 ± 6.0
19	Val-Hyp(Bzl)	0.27 ± 0.12	0.09 ± 0.01	2.0 ± 0.12	2.1 = 0.26	28 ± 1.0
20	Val-Hyp	0.41 ± 0.09	0.40 ± 0.0	10 ± 0.14	15 ± 1.6	16 ± 10
29	Val-DHP	0.77 ± 0.02	0.35 ± 0.21	46 ± 0.85	51 ± 3.0	4.8 ± 0.3
31	Val	0.30 ± 0.06	0.06 ± 0.03	8.2 ± 3.3	9.6 ± 0.38	6.7 ± 2.7
33	Val-Pro-Val	0.22 ± 0.07	0.07 ± 0.01	11 ± 9.4	11 ± 0.78	4.4 ± 0.3

[a] 50% effective concentration required to inhibit HIV-induced cytopathicity by 50%. [b] 50% cytostatic concentration required to inhibit MT-4 or CEM cell proliferation by 50%. [c] Inhibitory concentration = 50% of the compound concentration required to inhibit recombinant HIV-1 RT activity by 50%.

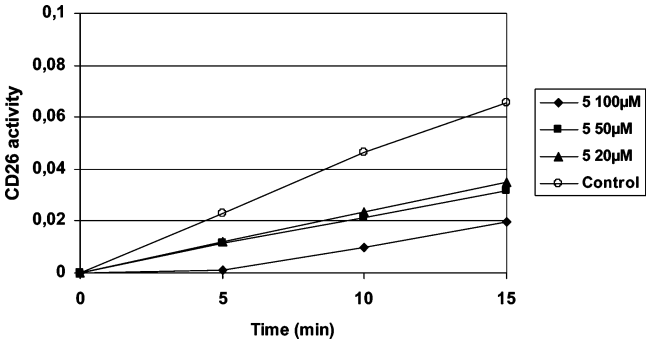


Figure 3. Inhibitory effect of different concentrations of [Val-Pro]−[NAP-TSAO-T] **5** against CD26-catalyzed conversion of GlyPro-pNA to GlyPro and pNA at 5, 10, or 15 min of reaction.

Table 2. Conversion of Dipeptidyl Prodrugs of NAP-TSAO-T (**2**) by Purified CD26 (1.5 mUnits) after 1, 4, or 24 h of Incubation

	percent of conversion to parent compound **2**		
	1 h	4 h	24 h
2 (NAP-TSAO-T)			
4a	0	0	0
5	37	62	61
6		81	88
7		37	58
8	43	66	79
9		85	99
10	5	20	58
11		27	73
12		48	92
13		7.9	30
14	0	6.5	35
15	0	0	0
16	0	0	0
17	0	0	0
18	0	2.2	4.2
19		0	0
20	4.1	18	43
29	0	0	0

Higher (i.e. 4-fold) CD26 concentrations did not further increase the amount of conversion (data not shown). The fact that the reaction did not further proceed after a 4 h exposure time nor in the presence of higher CD26 enzyme concentrations is most likely due to feedback inhibition of the reaction by the hydrolysis product ValPro. Indeed, when ValPro was exposed to CD26-catalyzed GlyPro-pNA conversion, it dose-dependently inhibited the reaction (Figure 4). At 40 μM, the release of pNA was prevented by ~50%.

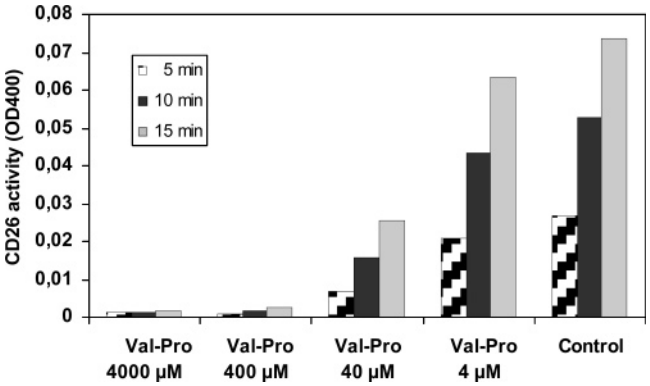


Figure 4. Inhibitory effect of different concentrations of the dipeptide Val-Pro against CD26-catalyzed conversion of GlyPro-pNA to GlyPro and pNA at 5, 10, or 15 min of reaction.

Interestingly, conjugate **4a** containing a benzyloxycarbonyl group (Z) at the terminal amino group of valine completely lacked substrate activity for CD26 (Table 2). Even after 24 h of incubation, no traces of NAP-TSAO-T (**2**) could be observed. Thus, a free amino group on the NH_2-terminal amino acid is a prerequisite for substrate activity by CD26.

Human serum (HS) and bovine serum (BS) also hydrolyzed compound **5** to ProVal and compound **2**. Indeed, when diluted to 5, 2.5, 1, or 0.5% in PBS, both HS and BS converted **5** to **2** in a serum concentration-dependent manner (Figure 5). A human serum concentration as low as 2.5% in PBS hydrolyzed **5** up to 30% after 3 h and up to 50% after 6 h. BS had a slower conversion rate than HS. After 6 h, **2** was formed from **5** by almost 20%, and 50% of **5** had converted to **2** after overnight (24 h) incubation (Figure 5).

Inhibition of CD26-Catalyzed Conversion of Compound 5 to Compound 2 by CD26 Inhibitors. Both Diprotin A and IlePyr are known inhibitors of CD26.[26−28] When **5** was added to the reaction mixture containing purified CD26, 2.5% HS or 2.5% BS in PBS and 500 μM or 50 μM IlePyr or 1000 μM or 100 μM Diprotin A, the conversion of **5** to **2** after 5 h was markedly inhibited. In fact, at the highest inhibitor concentration,

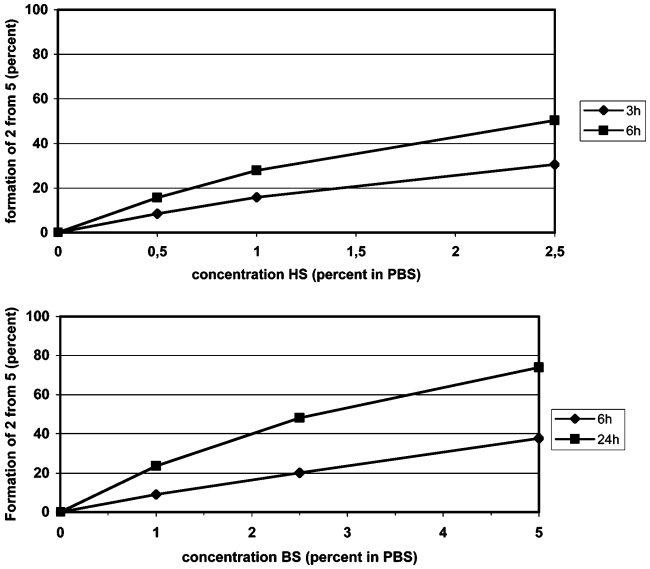


Figure 5. Conversion of [Val-Pro]−[NAP-TSAO-T] **5** to the parent compound [NAP-TSAO-T] **2** by human and bovine serum.

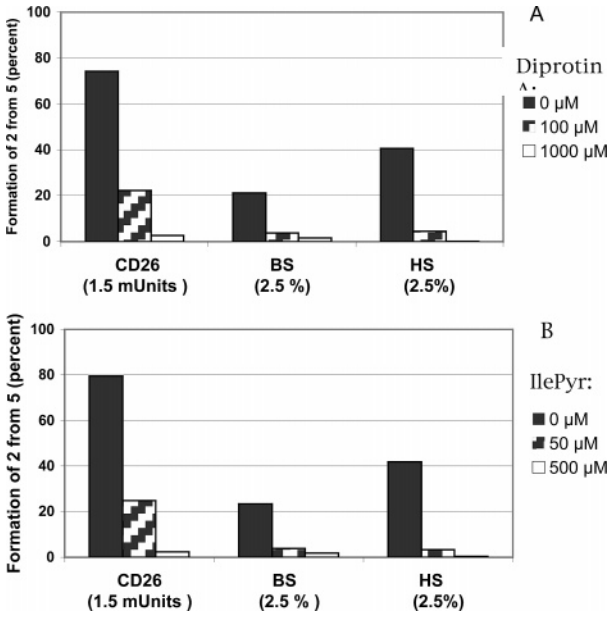


Figure 6. Effect of the specific CD26 inhibitors diprotin A and IlePyr on the conversion of [Val-Pro]−[NAP-TSAO-T] **5** to [NAP-TSAO-T] **2**.

the reaction was completely blocked in the presence of CD26, HS, or BS. At the lowest inhibitor concentration, ∼70% of the reaction was inhibited for CD26, and >80% for HS and BS (Figure 6). These observations point to CD26 as the main and predominant enzyme responsible in HS and BS to remove the dipeptide part from the prodrug.

Conversion of Dipeptidyl Prodrugs of Compound 2 by Purified CD26. A variety of dipeptides were introduced on the parent compound **2**. In a first series of compounds, the second amino acid consisted of a proline (compounds **5** to **13**). All prodrugs proved to be a substrate for CD26 (Table 2). Compounds **6** (AlaPro) and **9** (LysPro) ranked among the most efficient substrates whereas **10** (GlyPro) and **13** (AspPro) were among the least efficient substrates of CD26. In none of the cases except for compound **9**, conversion was complete after 24 h. Obviously, as earlier demonstrated for ValPro, the released dipeptide may feedback inhibit the enzymatic reaction, and the potency of inhibition may depend on the nature of the released dipeptide.

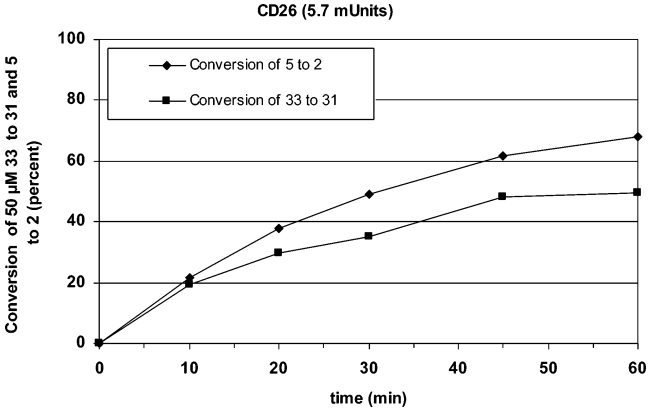


Figure 7. Conversion of [Val-Pro-Val]−[NAP-TSAO-T] **33** to [NAP-TSAO-T] **5** in the presence of purified CD26 in function of time.

In the second series of compounds, the penultimate proline was replaced by another natural amino acid and the first (NH_2-terminal) amino acid consisted of a Val (compounds **14** to **20** and **29**). None of the compounds, except for the ValAla NAP-TSAO-T derivative **14**, showed significant substrate activity against CD26. Interestingly, the Val D-pro derivative **18** showed measurable but rather marginal substrate activity. Instead, Val-Hydroxyproline (ValHyp) was markedly converted to the parent **2**, albeit at a lower efficiency than the corresponding ValPro derivative. The Val-dihydroxyproline (ValDHP) derivative **29** was devoid of any CD26 substrate activity.

The ValProVal tripeptide derivative of **2** (compound **33**) has also been synthesized and investigated for substrate activity against purified CD26. Interestingly, the tripeptide prodrug was efficiently converted by CD26 to the Val derivative **31**, which itself is not a substrate for CD26. When the substrate activity of **33** was compared with compound **5** in the presence of 5.7 mUnits CD26, it proved to be slightly less efficiently converted to its hydrolysis product than compound **5** (Figure 7).

Modulation of the Lipophilicity of the Dipeptidyl Derivatives of NAP-TSAO-T. The cLogP values for the different dipeptidyl derivatives of NAP-TSAO-T have been calculated and compared with the parent compound **2** (Supporting Information). We also correlated the cLogP data with the retention time of the prodrug derivatives on a reverse phase HPLC column. There was a good correlation between both values. We found a correlation coefficient of $r = 0.73$ between cLogP and t_R (compounds **6**, **12**, and **19** were excluded because their t_R values were obtained through a different HPLC gradient system). Interestingly, when considering the Xaa Pro prodrugs **5** to **13**, the lipophilicity of the prodrugs could be markedly modulated depending the nature of the NH_2-terminal amino acids. Indeed, the AspPro and GluPro derivatives (cLogP: −0.27 and −0.69) had a 1000-fold lower lipophilicity than parent drug **2** (cLogP: 2.69). Several other dipeptidyl prodrugs showed a cLogP value between these extremes. However, lipophilicity could be also increased by at least 1 order of magnitude by the PhePro prodrug (cLopP: 3.88). Also, among the ValYaa prodrugs (i.e. **14** to **20** and **29**), the lipophilicity could be markedly modulated depending the nature of the penultimate amino acid. Thus, the lipophilicity of the parent drug can be easily optimized depending on the eventual needs and nature of the drug.

Discussion

The dipeptidyl-peptidase IV occurs as a membrane-bound enzyme present on several cell types, but also as a soluble form

present in plasma.[2] It predominantly cleaves the dipeptidyl part from the amino terminal side of peptides when the penultimate amino acid is preferentially a proline.[2] However, peptides with penultimate alanine are also known to be a substrate for CD26 albeit at a lower efficiency as when a proline is present.[2,3] In fact, the structure (amino acid)−activity relationship for CD26/DPP IV has also been demonstrated among the XaaPro-prodrugs of NAP-TSAO-T. The stringent selectivity conferred by the penultimate amino acid, but also the freedom to have a broad variety of natural amino acids at the amino terminal end of the peptide, let us to apply these principles in a novel type of prodrug approach in which a dipeptide is linked to the free amino-terminal end of a nonpeptidic drug. Our data revealed that CD26 can indeed recognize these prodrugs as efficient substrates to be converted to the parent compound.

In fact, the known substrate specificity of CD26 to cleave natural peptides[29] also proved valid when a synthetic compound such as TSAO-T had been linked to the dipeptide: Xaa-Pro is recognized as well as Xaa-Ala; hydrophobic aliphatic residues are favored at the amino terminal position, and negatively charged Xaa such as Asp is among the least favored amino acids, whereas the positively charged Xaa Lys is usually a well-accepted substrate for CD26. Also, CD26 is strongly stereospecific. The scissile and Xaa-Pro bonds must be in trans configuration.[29] Thus, the substrate activity and selectivity of dipeptidyl derivatives of synthetic compounds to be cleaved by CD26 can be more or less predicted as based on the known SAR of CD26 for its natural peptidic substrates.

The dipeptidyl derivatives of NAP-TSAO generally showed a pronounced anti-HIV activity in cell culture irrespective the nature of the amino acids in the dipeptide part of the molecule. These findings are not unexpected since these TSAO derivatives invariably inhibited HIV RT in cell-free assays, due to the flexibility that is allowed on the N-3 position of the thymine ring to introduce bulky substituents at this position.[14,15] It is therefore not clear whether the anti-HIV activity observed in the cell culture assays is due to the presence of the prodrug or to the released parent NAP-TSAO-T compound. However, when 10^7 CEM cells were suspended in PBS and exposed for 3 h at 37 °C to compound **5**, up to 63.5% of the prodrug has been converted to the parent NAP-TSAO-T compound in the PBS supernatant within this short time period, and 62% of the parent compound has been found intracellularly after 3 h. This conversion had been obviously due to the CD26 present in the CEM cell membrane. Given the fact that there is also CD26 present on the culture medium of HIV-infected CEM cell cultures, it is reasonable to assume that the eventual antiviral activity recorded at day 4 postinfection is most likely due to the parent compound released from the prodrug.

In this study, the major aims were to demonstrate that dipeptidyl (particularly Xaa-Pro) prodrugs of synthetic small molecules can be cleaved to the parent compound by the selective action of CD26. This has now been clearly demonstrated with purified enzyme, as well as human and bovine serum. Although we have also shown preservation of antiviral activity of the prodrugs in cell culture, these data do not reveal whether the prodrugs are efficiently converted to the parent drug since the prodrugs also proved inhibitory to the TSAO target HIV-1 reverse transcriptase.

In conclusion, the advantages of this approach are multiple. First, upon conversion of the prodrug to its parent compound, a natural product (a dipeptide) is released. Therefore, the prodrug would not be expected to create additional side effects after cleavage to the parental drug and the prodrug part. Second, the nature of the different amino acids at the amino terminal end, as well as the presence of a proline, a hydroxyproline or an alanine as the second amino acid of the prodrug part clearly determines the half-life of the prodrug in the plasma. This will allow modulation of the release of the parent compound as a function of the particular needs. Third, if required, the lipophilicity of the prodrug can be profoundly modulated by the choice of the first amino acid (i.e. aspartic acid or glutamic acid on the one extreme versus phenylalanine on the other extreme). Fourth, it is also obvious that the nature of the amino-terminal amino acid will also modulate the solubility of the parent drug and may allow more efficient or optimized formulation of the particular parent compound. The fact that CD26 is abundantly present in plasma in its soluble form and on several cell types will guarantee that the prodrug will be eventually converted to the parent drug.

Experimental Section

Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadropole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). ^{1}H NMR spectra were recorded with a Varian XL-300 and a Bruker AM-200 spectrometer operating at 300 and at 200 MHz with Me_4Si as internal standard. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck). Separations on silica gel were performed by flash column chromatography with silica gel 60 (230−400 mesh) (Merck) or preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron (Kiesegel 60 PF_{254} gipshaltig (Merck), layer thickness (1 mm), flow rate (5 mL/min). The dipeptide derivatives Z-Val-Pro-OH, Z-Ala-Pro-OH, Z-Phe-Pro-OH and Z-Val-Ala-OH were purchased from Bachem Feinchemikalien. Z-Tyr(OBzl)-Pro-OH,[19] Z-Lys(Z)-Pro-OH,[20] Z-Gly-Pro-OH,[21] Z-Asn-Pro-OH,[22] Z-Asp(OBzl)-Pro-OH,[23] Z-Val-Gly-OH,[30] Z-Val-Leu-OH,[31] and Z-Val-Phe-OH[32] were synthesized as previously described.

Synthesis of Novel Dipeptide Derivatives Z-Xaa-Yaa-OH (3h,n,o,p). General Coupling Procedure for the Synthesis of Z-Xaa-Yaa-OR. To a solution of the corresponding Z-Xaa-OH (1 equiv) in CH_2Cl_2 (2−3 mL) were successively added (benzotriazol-1-yl-oxy)-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.2 equiv), the corresponding C-protected amino acid H-Yaa-OR·HCl (1.2 equiv), and TEA (2.2 equiv), and the mixture was stirred at room temperature for 15 h. After removal of the solvent in vacuo, the residue was dissolved in ethyl acetate (50 mL) and washed with 10% aqueous citric acid (3 × 20 mL), 10% aqueous $NaHCO_3$ (3 × 20 mL), water (3 × 20 mL), and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was purified by flash column chromatography. The chromatography eluent, yield of the isolated products, analytical and spectroscopic data are indicated below for each compound.

Z-Glu(Bzl)-Pro-O^{t}Bu (21). According to the general procedure, Z-Glu(OBzl)-OH (0.55 g, 1.48 mmol) was reacted with H-Pro-O^{t}Bu·HCl (0.21 g, 1.24 mmol). The residue was purified by flash column chromatography (hexane:ethyl acetate, 7:3) to give 0.40 g (63%) of **21** as a white foam. MS (ESI$^+$): m/z 483.3 $(M + 1)^+$. Anal. for $C_{29}H_{36}N_2O_7$: C, H, N.

Z-Val-D-Pro-OCH$_3$ (22). Via the general coupling procedure, Z-Val-OH (0.47 g, 1.90 mmol) was treated with H-D-Pro-OMe·HCl (0.20 g, 1.58 mmol). The residue was purified by flash column chromatography (hexane:ethyl acetate, 2:1) to give 0.43 g (75%) of **22** as a white foam. MS (ESI$^+$): m/z 363.3 $(M + 1)^+$. Anal. for $C_{19}H_{26}N_2O_5$: C, H, N.

Z-Val-Hyp(Bzl)-OCH$_3$ (23). Z-Val-OH (0.30 g, 1.21 mmol) was reacted with H-Hyp(Bzl)-OMe·HCl (0.39 g, 1.46 mmol) according to the general coupling procedure. The final residue was purified by flash column chromatography (hexane:ethyl acetate, 1:1) to give

0.51 g (90%) of **23** as a white foam. MS (ESI^+): *m/z* 469.3 $(M + 1)^+$. Anal. for $C_{26}H_{32}N_2O_6$: C, H, N.

Z-Val-Hyp-OCH_3 (24). Following the general procedure, Z-Val-OH (0.33 g, 1.33 mmol) was reacted with H-Hyp-OCH_3·HCl (0.16 g, 1.11 mmol). The final residue was purified by flash column chromatography (hexane:ethyl acetate, 1:2) to give 0.22 g (45%) of **24** as a white foam. MS (ESI^+): *m/z* 379.2 $(M + 1)^+$. Anal. for $C_{19}H_{26}N_2O_6$: C, H, N.

General C-Deprotection Procedure for the Synthesis of Z-Xaa-Yaa-OH. Method A (saponification). A solution of the corresponding Z-Xaa-Yaa-OCH_3 (1 equiv) in CH_3OH (7−9 mL) was treated with a 2 N NaOH solution (1.5 equiv). The mixture was stirred at room-temperature overnight, and the solvent was evaporated to dryness. The residue was dissolved in water (15 mL) and acidified with a 1 N HCl solution (pH = 3), and the product was extracted with ethyl acetate (3 × 15 mL). The organic layer was washed with brine (3 × 20 mL), dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was dissolved in water and liophilized.

Method B (acid hydrolysis). A solution of the corresponding Z-Xaa-Yaa-OtBu (1 equiv) in CH_2Cl_2 (4−5 mL) was reacted with TFA (1.5 equiv), and the mixture was stirred at room temperature for 3 h. The solvent was removed in vacuo, and the residue was coevaporated several times with CH_2Cl_2. The final residue was dissolved in water and liophilized.

The deprotection method, yield of the isolated products, analytical and spectroscopic data are indicated below for each compound.

Z-Glu(Bzl)-Pro-OH (3h). Following the general C-deprotection procedure (Method B), compound 21 (0.40 g, 1.48 mmol) was reacted with TFA to give 0.31 g (87%) of 3h as a colorless syrup. MS (ESI^+): *m/z* 483.3 $(M + 1)^+$. Anal. for $C_{25}H_{28}N_2O_7$: C, H, N.

Z-Val-D-Pro-OH (3n). According to the general deprotection procedure (Method A), Z-Val-D-Pro-OCH_3 (**22**) (0.37 g, 0.95 mmol) was treated with a 2 N NaOH solution to give 0.31 g, (86%) of **3n** as a white foam. MS (ESI^+): *m/z* 349.1 $(M + 1)^+$. Anal. for $C_{18}H_{24}N_2O_5$: C, H, N.

Z-Val-Hyp(Bzl)-OH (3o). Z-Val-Hyp(Bzl)-OCH_3 (**23**) was deprotected with a 2 N NaOH solution via the general procedure (Method A) to yield 0.34 g, (75%) of **3o** as an amorphous white solid. MS (ESI^+): *m/z* 455.3 $(M + 1)^+$. Anal. for $C_{25}H_{30}N_2O_6$: C, H, N.

Z-Val-Hyp-OH (3p). Z-Val-Hyp-OCH_3 (**24**) (0.20 g, 0.54 mmol) was deprotected with a 2 N NaOH solution according to the general procedure (Method A) to yield 0.20 g, (98%) of **3p** as a white foam. MS (ESI^+): *m/z* 365.2 $(M + 1)^+$. Anal. for $C_{18}H_{24}N_2O_6$: C, H, N.

Z-Val-3,4-dehydro-Pro-OCH_3 (25). Z-Val-OH (0.31 g, 1.26 mmol) was reacted with H-3,4-dehydro-Pro-OCH_3·HCl (0.24 g, 1.51 mmol) according to the general procedure. The final residue was purified by flash column chromatography (hexane:ethyl acetate, 1:1) to give 0.22 g (45%) of **25** as a white foam. ^1H NMR (300 MHz, $CDCl_3$): δ 0.96 (d, 3H, γ-CH_3a, Val, $J = 6.8$ Hz), 1.05 (d, 3H, γ-CH_3b, Val, $J = 6.8$ Hz), 2.01 (m, 1H, β-CH, Val), 3.70 (s, 3H, OCH_3), 4.27 (m, 1H, α-CH, Val), 4.43 (dd, 1H, δ-CH_2a, Pro, $J = 2.8$ Hz, $J = 14.5$ Hz), 4.60 (dd, 1H, δ-CH_2b, Pro, $J = 2.2$ Hz, $J = 14.5$ Hz), 5.05 (s, 2H, CH_2, Z), 5.18 (m, 1H, α-CH, Pro), 5.42 (d, 1H, NH, Val, $J = 9.3$ Hz), 5.81 (m, 1H, γ-CH, Pro), 5.96 (m, 1H, β-CH, Pro), 7.31 (m, 5H, Ar, Z). MS (ESI^+): *m/z* 361.2 $(M + 1)^+$. Anal. for $C_{19}H_{24}N_2O_5$: C, H, N.

Z-Val-(3*R*,4*S*)-3,4-dihydroxy-Pro-OCH_3 (26). A solution of compound **25** (0.13 g, 0,27 mmol) was placed in a mixture of 1,4-dioxane (3 mL) and water (1 mL) to which was added *N*-methylmorpholine oxide (NMO, 50% aqueous solution, 93.3 mL, 0.45 mmol) followed by a *tert*-butyl alcohol solution of OsO_4 (2.5% w/w, 61.88 μL, 0.01 mmol). The reaction was stirred under an argon atmosphere for 2 days, and then it was quenched with a 10% solution of $Na_2S_2O_3$ (2 mL). The reaction was concentrated to remove THF, and the aqueous fraction was extracted with ethyl acetate (3 × 20 mL). The combined organic fractions were washed with 10% aqueous $NaHCO_3$ (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was purified by CCTLC on the Chromatotron (dichloromethane−methanol, 40:1) to give 0.12 g (82%) of compound **26** as a syrup. ^1H NMR (200 MHz, $CDCl_3$): δ 0.95 (d, 3H, γ-CH_3a, Val, $J = 6.8$ Hz), 1.01 (d, 3H, γ-CH_3b, Val, $J = 6.8$ Hz), 2.01 (m, 1H, b-CH, Val), 3.70 (m, 6H, OCH_3, δ-CH_2, γ-CH, Hyp), 4.28 (m, 2H, α-CH, Val, Hyp), 4.41 (d, 1H, β-CH, Hyp, $J = 5.3$ Hz), 5.07 (s, 2H, CH_2, Z), 5.60 (d, 1H, NH, Val, $J = 8.6$ Hz), 7.34 (m, 5H, Ar, Z). MS (ESI^+): *m/z* 395.2 $(M + 1)^+$. Anal. for $C_{19}H_{26}N_2O_7$: C, H, N.

Z-Val-(3*R*,4*S*)-3,4-dihydroxy-Pro-OH (27). Following the general deprotection procedure (Method A), compound **26** (0.12 g, 0.31 mmol) was treated with a 2 N NaOH solution to yield 0.10 g (89%) of dipeptide derivative **27** as a white foam. ^1H NMR (300 MHz, DMSO-d_6): δ 0.95 (d, 3H, γ-CH_3a, Val, $J = 6.8$ Hz), 1.01 (d, 3H, γ-CH_3b, Val, $J = 6.8$ Hz), 2.01 (m, 1H, β-CH, Val), 3.59 (dd, 1H, δ-CH_2a, Hyp, $J = 5.0$ Hz, $J = 10.0$ Hz), 3.72 (dd, 1H, δ-CH_2b, Hyp, $J = 5.4$ Hz, $J = 10.0$ Hz), 4.02 (m, 4H, α-CH, Val, α-CH, β-CH, γ-CH, Hyp), 5.01 (dd, 2H, CH_2, Z, $J = 12.6$, $J = 17.6$ Hz), 5.14 (bs, 1H, OH), 5.43 (bs, 1H, OH), 7.43 (m, 5H, Ar, Z), 7.43 (d, 1H, NH, Val, $J = 8.4$ Hz). MS (ESI^+): *m/z* 381.2 $(M + 1)^+$. Anal. for $C_{18}H_{24}N_2O_7$: C, H, N.

General Coupling Procedure for the Synthesis of Compounds 4a−p, 28, 30, and 32. A solution of the corresponding C-deprotected dipeptide (1.5 equiv) in CH_2Cl_2 (2 mL) was successively treated with BOP (1.5 equiv), the N-3-aminopropyl TSAO derivative (NAP-TSAO-T) **2** (1 equiv), and triethylamine (2.5 equiv). The reaction mixture was stirred at room temperature for 24 h, and the solvent was evaporated to dryness. The residue was dissolved in ethyl acetate 26(50 mL), washed with 10% aqueous citric acid (3 × 20 mL), 10% aqueous $NaHCO_3$ (3 × 20 mL), water (3 × 20 mL), and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was purified by flash column chromatography or by CCTLC on the Chromatotron. The chromatography eluent, yield of the isolated products, and analytical and spectroscopic data are indicated below for each compound.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4a). According to the general coupling procedure, NAP-TSAO-T **2** (99 mg, 0.15 mmol) was reacted with Z-Val-Pro-OH **3a** (64 mg, 0.18 mmol). The final residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 81 mg (54%) of **4a** as a white foam. ^1H NMR (300 MHz, CDCl3) d 0.92 (d, 3H, γ-CH_3a, Val, $J = 6.7$ Hz), 0.96 (d, 3H, γ-CH_3b, Val, $J = 6.7$ Hz), 1.76 (m, 2H, -CH_2-), 2.00 (m, 8H, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.14 (m, 2H, CH_2NHCO), 3.58 (m, 2H, δ-CH_2, Pro), 3.95 (m, 4H, 2H-5′, N^3-CH_2), 4.29 (m, 1H, H-4'), 4.32 (dd, 1H, α-CH, Val, $J = 6.1$ Hz, $J = 9.0$ Hz), 4.49 (m, 2H, H-2′, α-CH, Pro), 5.05 (s, 2H, CH_2, Z), 5.45 (d, 1H, NH, Val, $J = 9.2$ Hz), 5.55 (s, 1H, H-3″), 5.58 (bs, 2H, NH_2-4″), 5.89 (d, 1H, H-1′, $J = 8.0$ Hz), 7.03 (m, 1H, NHCO), 7.15 (s, 1H, H-6), 7.32 (m, 5H, Ar, Z). MS (ESI^+): *m/z* 977.2 $(M + 1)^+$. Anal. for $C_{45}H_{72}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-alanyl-prolylamino]propyl]-thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4b). Following the general coupling procedure, nucleoside **2** (100 mg, 0.15 mmol) was reacted with Z-Ala-Pro-OH **3b** (59 mg, 0.18 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 125 mg (85%) of **4b** as a white foam. MS (ESI^+): *m/z* 949.3 $(M + 1)^+$. Anal. for $C_{43}H_{68}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-phenyalanyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4c). Nucleoside **2** (100 mg, 0.15 mmol) was reacted with Z-Phe-Pro-OH **3c** (74 mg, 0.18 mmol) according to the general coupling procedure. The residue was purified by flash column chromatography (dichloromethane:methanol, 70:1) to give 141 mg (89%) of **4c** as a white foam. MS (ESI^+): *m/z* 1026.2 $(M + 1)^+$. Anal. for $C_{49}H_{72}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-*O*(benzyl)-tyrosyl-prolylamino]-propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4d). Nucleoside **2** (75 mg, 0.15 mmol) was reacted with Z-Tyr(Bzl)-Pro-OH **3d** (116 mg, 0.30 mmol) according to the general coupling procedure. The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 80 mg (61%) of **4d** as a white foam. MS (ESI$^+$): *m*/*z* 1131.6 (M + 1)$^+$. Anal. for $C_{56}H_{78}N_6O_{13}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a},*N*^{e} *bis*(benzyloxycarbonyl)-lysyl-prolylamino]-propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4e). Nucleoside **2** (109 mg, 0.15 mmol) was reacted with Z-Lys(Z)-Pro-OH **3e** (148 mg, 0.31 mmol) following the general coupling procedure. The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 76 mg (40%) of **4e** as a white foam. MS (ESI$^+$): *m*/*z* 1141.3 (M + 1)$^+$. Anal. for $C_{54}H_{81}N_7O_{14}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-glycyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4f). Following the general coupling procedure, nucleoside **2** (75 mg, 0.15 mmol) was reacted with Z-Gly-Pro-OH **3f** (71 mg, 0.18 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 57 mg (53%) of **4f** as a white foam. MS (ESI$^+$): *m*/*z* 935.3 (M + 1)$^+$. Anal. for $C_{42}H_{66}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-asparaginyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4g). Following the general coupling procedure, nucleoside **2** (100 mg, 0.15 mmol) was reacted with Z-Asn-Pro-OH **3g** (84 mg, 0.24 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 30:1) to give 76 mg (50%) of **4g** as a white foam. MS (ESI$^+$): *m*/*z* 1083.4 (M + 1)$^+$. Anal. for $C_{51}H_{74}N_6O_{14}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-*O*-benzyl-glutamyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4h). According to the general coupling procedure, nucleoside **2** (100 mg, 0.15 mmol) was reacted with Z-Glu(Bzl)-Pro-OH **3h** (86 mg, 0.18 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 50:1) to give 110 mg (65%) of **4h** as a white foam. MS (ESI$^+$): *m*/*z* 1096.2 (M + 1)$^+$. Anal. for $C_{52}H_{76}N_6O_{14}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-*O*-benzyl-aspartyl-prolylamino]-propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4i). Nucleoside **2** (86 mg, 0.13 mmol) was reacted with Z-Asp(Bzl)-Pro-OH **3i** (73 mg, 0.18 mmol) following the general coupling procedure. The residue was purified by by flash column chromatography (dichloromethane:methanol, 70:1) to give 89 mg (61%) of **4i** as a white foam. MS (ESI$^+$): *m*/*z* 1083.4 (M + 1)$^+$. Anal. for $C_{51}H_{74}N_6O_{14}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-alanylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4j). Via the general coupling procedure, Z-Val-Ala-OH **3j** (132 mg, 0.60 mmol) was treated with NAP-TSAO-T **2** (132 mg, 0.30 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 105 mg (54%) of **4j** as a white foam. MS (ESI$^+$): *m*/*z* 951.3 (M + 1)$^+$. Anal. for $C_{43}H_{70}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-glycylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4k). Following the general procedure, Z-Val-Gly-OH **3k** (80 mg, 0.26 mmol) was reacted with NAP-TSAO-T **2** (85 mg, 0.13 mmol). The final residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 88 mg of **4k** (73%) as a white foam. MS (ESI$^+$): *m*/*z* 937.3 (M + 1)$^+$. Anal. for $C_{42}H_{68}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-leucylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4l). Via the general coupling procedure, Z-Val-Leu-OH **3l** (102 mg, 0.28 mmol) was treated with NAP-TSAO-T **2** (90 mg, 0.14 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 70:1) to give 61 mg (39%) of **4l** as a white foam. MS (ESI$^+$): *m*/*z* 993.5 (M + 1)$^+$. Anal. for $C_{46}H_{76}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-phenylalanylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4m). According to the general procedure, Z-Val-Phe-OH **3m** (116 mg, 0.29 mmol) was reacted with NAP-TSAO-T **2** (94 mg, 0.14 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 70:1) to give 68 mg (37%) of **4m** as a white foam. MS (ESI$^+$): *m*/*z* 1027.3 (M + 1)$^+$. Anal. for $C_{49}H_{74}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-D-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4n). Via the general coupling procedure, Z-Val-D-Pro-OH **3n** (64 mg, 0.18 mmol) was treated with NAP-TSAO-T **2** (99 mg, 0.15 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 70:1) to give 72 mg (48%) of **4n** as a white foam. MS (ESI$^+$): *m*/*z* 977.3 (M + 1)$^+$. Anal. for $C_{45}H_{72}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-(2S,4R)-4-benzyloxyprolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4o). Following the general procedure, Z-Val-Hyp(Bzl)-OH **3o** (105 mg, 0.23 mmol) was reacted with NAP-TSAO-T **2** (75 mg, 0.11 mmol). The final residue was purified by flash column chromatography (dichloromethane:methanol, 70:1) to give 112 mg of **4o** (89%) as a white foam. MS (ESI$^+$): *m*/*z* 1083.6 (M + 1)$^+$.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-(2S,4R)-4-*O*-hydroxyprolylaminoprolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (4p). Via the general coupling procedure, Z-Val-Hyp-OH **3p** (59 mg, 0.16 mmol) was treated with NAP-TSAO-T **2** (70 mg, 0.11 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 50:1) to give 43 mg (40%) of **4p** as a white foam.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[(2S,3R,4S)-*N*^{a}(benzyloxycarbonyl)-3,4-dihydroxyprolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (28). According to the general procedure, Z-dipeptide derivative **27** (63 mg, 0.16 mmol) was reacted with NAP-TSAO-T **2** (72 mg, 0.11 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 40:1) to give 44 mg (40%) of compound **28** as a white foam. MS (ESI$^+$): *m*/*z* 1009.5 (M + 1)$^+$. Anal. for $C_{45}H_{72}N_6O_{14}SSi_2$: C, H, N, S.

General N-Deprotection Procedure for the Synthesis of Compounds 5−20, 29, 31, and 33. A solution of the corresponding protected conjugate intermediate [Z-Xaa-Yaa]−[TSAO-T] (1 equiv) in methaanol (8 mL) containing Pd/C (10%) (40wt %/wt) was hydrogenated at 25 psi at room temperature for 2 h. The reaction mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water and lyophilized to give the final deprotected conjugates **5**−**20**, **29**, **31**, and **33**. The yield of the isolated products and spectroscopic and analytical data are indicated below for each compound.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (5). According to the general N-deprotection procedure, conjugate **4a** (49 mg, 0.05 mmol) was

hydrogenated to give **5** (40 mg, 83%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.92 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.96 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.82 (m, 10H, -CH_2-, CH_3-5, β-CH, Val, β-CH_2,γ-CH_2, Pro), 2.95 (m, 1H, CH_2aNHCO), 3.15 (m, 1H, CH_2bNHCO), 3.45 (m, 2H, δ-CH_2, Pro), 3.82 (m, 4H, α-CH, Val, N^3-CH_2, H-5′a), 3.91 (dd, 1H, H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.21 (m, 2H, H-4′, α-CH, Pro), 4.75 (d, 1H, H-2′, J = 8.4 Hz), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.4 Hz), 6.97 (bs, 2H, NH_2-4″), 7.62 (s, 1H, H-6), 7.78 (m, 1H, NHCO). MS (ESI$^+$): m/z 843.2 $(M + 1)^+$. Anal. for $C_{37}H_{66}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[alanyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (6). Following the general N-deprotection procedure, conjugate **4b** (76 mg, 0.08 mmol) was hydrogenated to give **6** (59 mg, 87%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.21 (d, 3H, β-CH_2, Ala, J = 7.0 Hz), 1.58−2.16 (m, 9H, -CH_2-, CH_3-5, β-CH_2, γ-CH_2, Pro), 3.10 (m, 2H, CH_2NHCO), 3.57 (m, 2H, δ-CH_2, Pro), 3.80 (m, 4H, 2H-5′, N^3-CH_2), 3.92 (m, 1H, α-CH, Ala), 4.22 (m, 1H, H-4′), 4.32 (m, 1H, α-CH, Pro), 4.54 (d, 1H, H-2′, J = 8.3 Hz), 5.73 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.3 Hz), 7.03 (bs, 2H, NH_2-4″), 7.76 (s, 1H, H-6), 7.91 (m, 1H, NHCO). MS (ESI$^+$): m/z 815.3 $(M + 1)^+$. Anal. for $C_{35}H_{62}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[phenyalanyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (7). Conjugate **4c** (82 mg, 0.08 mmol) was hydrogenated following the general N-deprotection procedure to give **7** (55 mg, 80%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.62 (m, 2H, -CH_2-), 1.80−2.08 (m, 8H, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.75 (m, 1H, β-CH_2a, Phe), 3.06 (m, 3H, CH_2NHCO, β-CH_2b, Phe), 3.63 (m, 2H, δ-CH_2, Pro), 3.77 (m, 3H, N^3-CH_2, H-5′a), 3.92 (dd, 1H, H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.21 (m, 1H, H-4′), 4.29 (m, 1H, α-CH, Phe), 4.43 (m, 1H, α-CH, Pro), 4.56 (d, 1H, H-2′, J = 8.3 Hz), 5.75 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 8.3 Hz), 6.97 (bs, 2H, NH_2-4″), 7.26 (m, 5H, Ar, Phe), 7.65 (m, 2H, H-6, NHCO). MS (ESI$^+$): m/z 891.2 $(M + 1)^+$. Anal. for $C_{41}H_{66}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[tyrosyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (8). Conjugate **4d** (57 mg, 0.05 mmol) was hydrogenated following the general N-deprotection procedure to give **8** (34 mg, 66%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.61 (m, 2H, -CH_2-), 1.80−1.92 (m, 8H, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.03 (m, 1H, β-CH_2, Pro), 2.65 (m, 1H, β-CH_2a, Tyr), 2.94 (dd, 1H, β-CH_2b, Tyr, J = 5.0, J = 13.7 Hz), 3.00 (m, 1H, CH_2aNHCO), 3.11 (m, 1H, CH_2bNHCO), 3.62 (m, 2H, δ-CH_2, Pro), 3.77 (m, 4H, α-CH, Tyr, N^3-CH_2, H-5′a), 3.93 (dd, 1H, H-5′b, J = 7.5 Hz, J = 11.7 Hz), 4.25 (m, 2H, α-CH, Pro, H-4′), 4.56 (d, 1H, H-2′, J = 8.3 Hz), 5.74 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 8.3 Hz), 6.65 (m, 2H, Ar, Tyr), 6.98 (m, 4H, NH_2-4″, Ar, Tyr), 7.65 (s, 1H, H-6), 8.08 (m, 1H, NHCO), 9.22 (s, 1H, OH-4, Tyr). MS (ESI$^+$): m/z 907.3 $(M + 1)^+$. Anal. for $C_{41}H_{66}N_6O_{13}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[lysyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (9). Following the general N-deprotection procedure, conjugate **4e** (57 mg, 0.05 mmol) was hydrogenated to give **9** (42 mg, 93%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.33 (m, 6H, β-CH_2, γ-CH_2, δ-CH_2, Lys), 1.61 (m, 3H, β-CH_2a, Pro, -CH_2-), 1.82 (m, 2H, γ-CH_2, Pro), 1.88 (s, 3H, CH_3-5), 2.00 (m, 1H, β-CH_2b, Pro), 2.62 (m, 2H, ϵ-CH_2, Lys), 2.92 (m, 1H, CH_2aNHCO), 3.11 (m, 1H, CH_2bNHCO), 3.43 (m, 1H, δ-CH_2a, Pro), 3.57 (m, 1H, δ-CH_2b, Pro), 3.86 (m, 4H, α-CH, Lys, H-5′a, N^3-CH_2), 3.93 (dd, 1H, H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.25 (m, 2H, α-CH, Pro, H-4′), 4.58 (d, 1H, H-2′, J = 8.2 Hz), 5.72 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.2 Hz), 6.98 (bs, 2H, NH_2-4″), 7.67 (s, 1H, H-6), 7.78 (m, 1H, NHCO). MS (ESI$^+$): m/z 872.3 $(M + 1)^+$. Anal. for $C_{38}H_{69}N_7O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[glycyl-prolylamino]propyl]thymine]-3′-spiro-″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (10). Via the general N-deprotection procedure, conjugate **4f** (47 mg, 0.05 mmol) was hydrogenated to give **10** (47 mg, 97%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.59 (m, 2H, -CH_2-), 1.88 (m, 8H, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.04 (m, 2H, CH_2NHCO), 3.49 (m, 2H, δ-CH_2, Pro), 3.81 (m, 4H, α-CH, Gly, N^3-CH_2, H-5′a), 3.92 (dd, 1H, H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.22 (m, 1H, H-4′), 4.34 (m, 1H, α-CH, Pro), 4.53 (d, 1H, H-2′, J = 8.3 Hz), 5.74 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 8.3 Hz), 6.96 (bs, 2H, NH_2-4″), 7.62 (s, 1H, H-6), 7.86 (m, 1H, NHCO). MS (ESI$^+$): m/z 801.3 $(M + 1)^+$. Anal. for $C_{34}H_{60}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[asparaginyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (11). According to the general N-deprotection procedure, conjugate **4g** (54 mg, 0.05 mmol) was hydrogenated to give **11** (35 mg, 81%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.52−1.98 (m, 9H, CH_3-5, -CH_2-, β-CH_2, γ-CH_2, Pro), 2.24 (m, 1H, β-CH_2a, Asn), 2.50 (m, 1H, β-CH_2b, Asn), 3.09 (m, 2H, CH_2NHCO), 3.57 (m, 2H, δ-CH_2, Pro), 3.83 (m, 4H, 2H-5′, N^3-CH_2), 4.22 (m, 2H, α-CH, Asn, H-4′), 4.57 (m, 2H, α-CH, Pro, H-2′), 5.74 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 7.7 Hz), 6.97 (bs, 3H, $CONH_2$a, Asn, NH_2-4″), 7.65 (m, 2H, H-6, $CONH_2$b, Asn), 7.78 (m, 1H, NHCO). MS (ESI$^+$): m/z 858.3 $(M + 1)^+$. Anal. for $C_{36}H_{63}N_7O_{11}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[glutamyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (12). According to the general N-deprotection procedure, conjugate **4h** (55 mg, 0.05 mmol) was hydrogenated to give **12** (28 mg, 72%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.52−2.04 (m, 13H, CH_3-5, -CH_2-, β-CH_2, γ-CH_2, Pro, Glu), 2.93 (m, 1H, CH_2aNHCO), 3.07 (m, 1H, CH_2bNHCO), 3.52 (m, 2H, δ-CH_2, Pro), 3.79 (m, 3H, N^3-CH_2, H-5′a), 3.91 (dd, 1H, H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.21 (m, 2H, α-CH, Glu, H-4′), 4.27 (m, 1H, α-CH, Pro), 4.52 (d, 1H, H-2′, J = 8.3 Hz), 5.73 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.3 Hz), 6.94 (bs, 2H, NH_2-4″), 7.60 (s, 1H, H-6), 7.82 (m, 1H, NHCO). MS (ESI$^+$): m/z 873.2 $(M + 1)^+$. Anal. for $C_{37}H_{64}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[aspartyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (13). Conjugate **4i** (54 mg, 0.05 mmol) was hydrogenated following the general N-deprotection procedure to give **13** (19 mg, 60%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.57 (m, 2H, -CH_2-), 1.62−1.75 (m, 7H, CH_3-5, β-CH_2, γ-CH_2, Pro), 2.13 (dd, 1H, β-CH_2a, Asp, J = 9.6 Hz, J = 15.8 Hz), 2.50 (m, 1H, β-CH_2b, Asp), 3.02 (m, 2H, CH_2NHCO), 3.62 (m, 2H, δ-CH_2, Pro), 3.81 (m, 3H, H-5′a, N^3-CH_2), 3.94 (m, 2H, α-CH, Asp, H-5′b), 4.24 (m, 2H, α-CH, Pro, H-4′), 4.56 (d, 1H, H-2′, J = 7.8 Hz), 5.57 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 7.8 Hz), 6.98 (bs, 2H, NH_2-4″), 7.65 (s, 1H, H-6), 7.88 (m, 1H, NHCO). MS (ESI$^+$): m/z 859.0 $(M + 1)^+$. Anal. for $C_{36}H_{62}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-alanylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (14). Conjugate **4j** (67 mg, 0.07 mmol) was hydrogenated following the general N-deprotection procedure to give **14** (45 mg, 75%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.91 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.95 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.19 (d, 3H, β-CH_2, Ala, J = 7.1 Hz), 1.92 (m, 4H, CH_3-5, β-CH, Val), 3.03 (m, 1H, CH_2-aNHCO), 3.13 (m, 1H, CH_2bNHCO), 3.82 (m, 4H, α-CH, Val, H-5′a, N^3-CH_2), 3.92 (dd, 1H, H-5′b, J = 7.1 Hz, J = 11.7 Hz), 4.22 (t, 1H, H-4′, J = 2.7 Hz), 4.26 (m, 1H, α-CH, Ala), 4.55 (d, 1H, H-2′, J = 7.8 Hz), 5.75 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 7.8 Hz), 6.98 (bs, 2H, NH_2-4″), 7.65 (s, 1H, H-6), 7.98 (m, 1H, NHCO), 8.07 (m, 1H, NH, Ala). MS (ESI$^+$): m/z 817.3 $(M + 1)^+$. Anal. for $C_{35}H_{64}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-glycylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-

1″,2″-oxathiole-2″,2″-dioxide] (15). Conjugate **4k** (47 mg, 0.05 mmol) was hydrogenated via the general N-deprotection procedure to give **15** (53 mg, 90%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.81 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.85 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.24 (m, 2H, -CH_2-), 1.89 (s, 4H, CH_3-5, β-CH, Val), 3.07 (m, 2H, CH_2NHCO), 3.68 (m, 2H, α-CH_2, Gly), 3.82 (m, 4H, α-CH, Val, 2H-5′a, N^3-CH_2), 3.92 (dd, 1H, 2H-5′b, J = 7.1 Hz, J = 11.7 Hz), 4.21 (t, 1H, H-4′, J = 2.7 Hz), 4.55 (d, 1H, H-2′, J = 7.8 Hz), 5.75 (s, 1H, H-3″), 5.94 (d, 1H, H-1′, J = 7.8 Hz), 6.96 (bs, 2H, NH_2-4″), 7.64 (s, 1H, H-6), 7.82 (m, 1H, NHCO), 8.12 (m, 1H, NH, Gly). MS (ESI$^+$): m/z 803.3 (M + 1)$^+$. Anal. for $C_{34}H_{62}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-leucylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (16). Following the general N-deprotection procedure, conjugate **4l** (50 mg, 0.05 mmol) was hydrogenated to give **16** (44 mg, 95%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.88 (m, 12H, γ-CH_3, Val, δ-CH_3, Leu), 1.59 (m, 6H, -CH_2-, β-CH_2, γ-CH_2, Leu), 1.97 (m, 4H, β-CH_3, Val, CH_3-5), 3.09 (m, 1H, CH_2aNHCO), 3.11 (m, 1H, CH_2bNHCO), 3.82 (m, 4H, α-CH, Val, 2H-5′a, N^3-CH_2), 3.92 (dd, 1H, 2H-5′b, J = 7.1 Hz, J = 11.7 Hz), 4.21 (t, 1H, H-4′, J = 2.7 Hz), 4.35 (m, 1H, α-CH, Leu), 4.55 (d, 1H, H-2′, J = 8.5 Hz), 5.74 (s, 1H, H-3″), 5.95 (d, 1H, H-1′, J = 8.5 Hz), 6.96 (bs, 2H, NH_2-4″), 7.64 (s, 1H, H-6), 8.02 (m, 2H, NHCO, NH, Leu). MS (ESI$^+$): m/z 859.0 (M + 1)$^+$. Anal. for $C_{38}H_{70}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-phenylalanylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (17). Conjugate **4m** (51 mg, 0.05 mmol) was hydrogenated following the general N-deprotection procedure to give **17** (44 mg, 90%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.66 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.68 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.60 (m, 2H, -CH_2-), 1.89 (s, 4H, CH_3-5, β-CH, Val), 2.82 (m, 1H, CH_2aNHCO), 3.00 (m, 3H, CH_2bNHCO, β-CH_2, Phe), 3.82 (m, 4H, α-CH, Val, 2H-5′a, N^3-CH_2), 3.92 (dd, 1H, 2H-5′b, J = 7.1 Hz, J = 11.7 Hz), 4.21 (t, 1H, H-4′, J = 2.7 Hz), 4.55 (m, 2H, H-2′, α-CH, Phe), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.3 Hz), 6.97 (bs, 2H, NH_2-4″), 7.18 (m, 5H, Ar, Phe), 7.66 (s, 1H, H-6), 8.05 (m, 1H, NHCO), 8.12 (m, 1H, NH, Phe). MS (ESI$^+$): m/z 893.3 (M + 1)$^+$. Anal. for $C_{41}H_{68}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-D-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (18). Conjugate **4n** (68 mg, 0.07 mmol) was hydrogenated according to the general N-deprotection procedure to give **18** (57 mg, 98%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.90 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.94 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.62 (m, 2H, -CH_2-), 1.97 (m, 8H, CH_3-5, β-CH, Val, β-CH_2,γ-CH_2, Pro), 3.04 (m, 2H, CH_2NHCO), 3.45 (m, 2H, δ-CH_2, Pro), 3.78 (m, 4H, α-CH, Val, H-5′a, N^3-CH_2), 3.95 (dd, 1H, H-5′b, J = 7.1 Hz, J = 11.7 Hz), 4.20 (t, 1H, H-4′, J = 2.7 Hz), 4.38 (m, 1H, α-CH, Pro), 4.61 (d, 1H, H-2′, J = 8.4 Hz), 5.72 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 8.4 Hz), 6.99 (bs, 2H, NH_2-4″), 7.71 (s, 1H, H-6), 7.81 (m, 1H, NHCO). MS (ESI$^+$): m/z 843.3 (M + 1)$^+$. Anal. for $C_{37}H_{66}N_6O_{10}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-(2S,4R)-4-*O*-benzyloxyprolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (19). According to the general N-deprotection procedure, conjugate **4o** (54 mg, 0.05 mmol) was hydrogenated to give **19** (28 mg, 60%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.87 (m, 6H, γ-CH_3, Val), 1.57 (m, 1H, -CH_2a-), 1.65 (m, 1H, -CH_2b-), 1.93 (m, 5H, β-CH_2a, Hyp, CH_3-5, β-CH, Val), 2.21 (m, 1H, β-CH_2b, Hyp), 2.97 (m, 1H, CH_2aNHCO), 3.12 (m, 1H, CH_2bNHCO), 3.61-(m, 2H, δ-CH_2, Hyp), 3.82 (m, 4H, α-CH, Val, N^3-CH_2, H-5′a), 3.92 (dd, 1H, H-5′b, J = 7.3 Hz, J = 11.7 Hz), 4.22 (m, 2H, g-CH, Hyp, H-4′), 4.31 (m, 1H, α-CH, Hyp), 4.49 (dd, 2H, CH_2, Bzl, J = 11.7 Hz, J = 17.1 Hz), 4.54 (d, 1H, H-2′, J = 8.3 Hz), 5.75 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 8.3 Hz), 6.95 (bs, 2H, NH_2-4″), 7.30 (m, 5H, Ar, Z), 7.63 (s, 1H, H-6), 7.94 (m, 1H, NHCO). MS (ESI$^+$): m/z 949.5 (M + 1)$^+$. Anal. for $C_{44}H_{72}N_6O_{11}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-(2S,4R)-4-hydroxyprolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (20). Following the general N-deprotection procedure, conjugate **4p** (50 mg, 0.05 mmol) was hydrogenated to give **20** (26 mg, 58%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.86 (m, 6H, γ-CH_3, Val), 1.61 (m, 2H, -CH_2-), 1.79−2.14 (m, 6H, CH_3-5, β-CH_2, Hyp, β-CH, Val), 2.93 (m, 1H, CH_2aNHCO), 3.12 (m, 1H, CH_2bNHCO), 3.60 (m, 2H, δ-CH_2, Hyp), 3.83 (m, 4H, α-CH, Val, N^3-CH_2, H-5′a), 3.91 (dd, 1H, H-5′b, J = 7.1 Hz, J = 11.7 Hz), 4.21 (m, 1H, H-4′), 4.26 (m, 2H, α-CH, g-CH, Hyp), 4.56 (d, 1H, H-2′, J = 8.3 Hz), 5.02 (m, 1H, γ-OH), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.3 Hz), 6.96 (bs, 2H, NH_2-4″), 7.64 (s, 1H, H-6), 7.86 (m, 1H, NHCO). MS (ESI$^+$): m/z 859.5 (M + 1)$^+$. Anal. for $C_{37}H_{66}N_6O_{11}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[(2S,3R,4S)-3,4-dihydroxyprolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (29). Conjugate **28** (30 mg, 0.04 mmol) was hydrogenated according to the general N-deprotection procedure to give **29** (20 mg, 87%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.86 (m, 6H, 2γ-CH_3, Val), 1.64 (m, 2H, -CH_2-), 1.90 (m, 4H, CH_3-5, β-CH, Val), 3.09 (m, 2H, CH_2NHCO), 3.61 (m, 1H, δ-CH_2a, DHP), 3.90 (m, 6H, 2H-5′, N^3-CH_2, δ-CH_2b, γ-CH, DHP), 4.05 (m, 3H, α-CH, Val, DHP, β-CH, DHP), 4.21 (m, 1H, H-4′), 4.60 (d, 1H, H-2′, J = 8.4 Hz), 5.10 (m, 2H, β-OH, γ-OH), 5.73 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, J = 8.4 Hz), 7.00 (bs, 2H, NH_2-4″), 7.70 (s, 1H, H-6), 7.86 (m, 1H, NHCO). MS (ESI$^+$): m/z 875.5 (M + 1)$^+$. Anal. for $C_{37}H_{66}N_6O_{12}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (30). According to the general coupling procedure, Z-Val-OH (80 mg, 0.32 mmol) was reacted with NAP-TSAO-T **2** (138 mg, 0.21 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 70:1) to give **30** (134 mg, 72%) as a white foam. ^{1}H RMN (300 MHz, $CDCl_3$): δ 0.92 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.96 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.57 (m, 2H, -CH_2-), 1.95 (s, 3H, CH_3-5), 2.13 (m, 1H, β-CH, Val), 2.99 (m, 1H, CH_2aNHCO), 3.33 (m, 1H, CH_2bNHCO), 3.92 (m, 5H, 2H-5′, N^3-CH_2, α-CH, Val), 4.30 (t, 1H, H-4′, J = 2.7 Hz), 4.49 (d, 1H, H-2′, J = 7.8 Hz), 5.09 (dd, 2H, CH_2, Z, J = 12.2 Hz, J = 14.9 Hz), 5.38 (d, 1H, NH, Val, J = 9.0 Hz), 5.55 (s, 1H, H-3″), 5.61 (bs, 2H, NH_2-4″), 5.88 (d, 1H, H-1′, J = 7.8 Hz), 7.18 (s, 1H, H-6), 7.32 (m, 5H, Ar, Z). MS (ESI$^+$): m/z: 880.3 (M + 1)$^+$. Anal. for $C_{40}H_{65}N_5O_{11}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (31). According to the general N-deprotection procedure, compound **30** (123 mg, 0.14 mmol) was hydrogenated to yield **31** (100 mg, 96%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.76 (d, 3H, γ-CH_3a, Val, J = 6.7 Hz), 0.80 (d, 3H, γ-CH_3b, Val, J = 6.7 Hz), 1.62 (m, 2H, -CH_2-), 1.86 (m, 4H, CH_3-5, β-CH, Val), 3.15 (m, 2H, CH_2NHCO), 3.82 (m, 4H, α-CH, Val, N^3-CH_2, 2H-5′a), 3.91 (dd, 1H, 2H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.22 (t, 1H, H-4′, J = 2.7 Hz), 4.53 (d, 1H, H-2′, J = 7.8 Hz), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 8.3 Hz), 6.98 (bs, 2H, NH_2-4″), 7.62 (s, 1H, H-6), 7.92 (m, 1H, NHCO). MS (ESI$^+$): m/z 746.3 (M + 1)$^+$. Anal. for: $C_{32}H_{59}N_5O_9SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[*N*^{a}(benzyloxycarbonyl)-valyl-prolyl-valylamino]propyl]-thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (32). Via the general coupling procedure, Z-Val-Pro-OH (59 mg, 0.17 mmol) was treated with compound **31** (84 mg, 0.11 mmol). The residue was purified by flash column chromatography (dichloromethane:methanol, 70:1) to give 85 mg (70%) of tripeptide derivative **32** as a white foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ

0.92 (d, 6H, γ-CH_3a, Val, J = 6.7 Hz), 0.96 (d, 6H, γ-CH_3b, Val, J = 6.7 Hz), 1.97 (m, 9H, CH_3-5, -CH_2-, β-CH_2, γ-CH_2, Val, Pro), 2.23 (m, 1H, β-CH, Pro), 3.00 (m, 1H, CH_2aNHCO), 3.29 (m, 1H, CH_2bNHCO), 3.59 (m, 1H, δ-CH_2, Pro), 3.72 (m, 1H, δ-CH_2, Pro), 3.83 (dd, 1H, H-5'a, J = 7.5 Hz, J = 12.4 Hz), 3.95 (m, 3H, H-5'b, N^3-CH_2), 4.19 (dd, 1H, α-CH, Val, J = 5.6 Hz, J = 8.3 Hz), 4.28 (m, 1H, H-4′), 4.34 (dd, 1H, α-CH, Val, J = 6.2 Hz, J = 8.9 Hz), 4.48 (d, 1H, H-2′, J = 7.8 Hz), 4.66 (m, 1H, α-CH, Pro), 5.06 (dd, 2H, CH_2, Z, J = 12.4 Hz, J = 15.6 Hz), 5.50 (d, 1H, NH, Val, J = 8.8 Hz), 5.55 (s, 1H, H-3″), 5.66 (bs, 2H, NH_2-4″), 5.85 (d, 1H, H-1′, J = 7.8 Hz), 6.91 (m, 1H, NHCO), 7.17 (s, 1H, H-6), 7.32 (m, 5H, Ar, Z). MS (ESI^+): m/z: 1076.4 $(M + 1)^+$. Anal. for $C_{50}H_{81}N_7O_{13}SSi_2$: C, H, N, S.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3-*N*-[3-*N*′-[valyl-prolyl-valylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (33). Via the general N-deprotection procedure, **32** (65 mg, 0.06 mmol) was hydrogenated to give 31 mg (59%) of **33** as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.92 (d, 6H, γ-CH_3a, Val, J = 6.7 Hz), 0.96 (d, 6H, γ-CH_3b, Val, J = 6.7 Hz), 1.67 (m, 2H, -CH_2-), 2.05 (m, 9H, CH_3-5, β-CH_2,γ-CH_2, Pro, β-CH, Val), 3.00 (m, 1H, CH_2aNHCO), 3.04 (m, 2H, CH_2NHCO), 3.46 (m, 1H, δ-CH_2, Pro), 3.61 (m, 1H, δ-CH_2, Pro), 3.78 (m, 4H, α-CH, Val, N^3-CH_2, 2H-5′a), 3.91 (dd, 1H, 2H-5′b, J = 7.5 Hz, J = 12.4 Hz), 4.02 (m, 1H, α-CH, Val), 4.28 (t, 1H, H-4′, J = 5.8 Hz), 4.38 (m, 1H, α-CH, Pro), 4.45 (d, 1H, H-2′, J = 7.8 Hz), 5.72 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, J = 7.8 Hz), 6.99 (bs, 2H, NH_2-4″), 7.71 (s, 1H, H-6), 7.81 (m, 1H, NHCO), 8.06 (m, 1H, NH, Val). MS (ESI^+): m/z 942.3$(M + 1)^+$. Anal. for: $C_{42}H_{75}N_7O_{11}SSi_2$: C, H, N, S.

Biological Methods. Compounds and Enzymes. GlyPro-pNA was purchased from Sigma-Aldrich (Bornem, Belgium). CD26 was purified as described[33] and kindly provided by I. De Meester and A.-M. Lambeir (Antwerp, Belgium). DPP IV/CD26 inhibitor Diprotin A (Ile-Pro-Ile) was obtained from Bachem. DPP IV/CD26 inhibitor L-isoleucyl pyrrolidine (Ile-Pyr) was synthesized using a modified procedure (see Supporting Information) to that described by Christoffers Jens.[34] Foetal bovine serum (FBS) was obtained from Integro (Dieren, The Netherlands) and human serum provided by the Blood Bank, Leuven, Belgium.

Evaluation of the Inhibitory Effect of Test Compound 5 and Dipeptide ValPro against Purified CD26 Catalyzed Conversion of GlyPro pNA to GlyPro and pNA. All enzyme activity assays were performed in 96-well microtiter plates (Falcon, Becton Dickinson, Franklin Lakes, NJ). To each well were added 5 μL of CD26 (at a final concentration of 0.237 milliUnits/200 μL-well), 20 μL of appropriate concentrations of test compound or ValPro solution in PBS (containing 1% DMSO), and PBS to reach a total volume of 150 μL. The pH of the reaction mixture was 7.5, which is virtually identical to the physiological pH of plasma. The reaction was started by the addition of 50 μL of GlyPro-pNA substrate at 50 μM and carried out at 37 °C. Enzyme activity was examined as the amount of (yellow-colored) *p*-nitroaniline (pNA) released from GlyPro-pNA in function of time. The pNA release was recorded by the increase of absorption [optical density (OD)] at 400 nm in a Spectramax microplate spectrometer (Molecular Devices, Sunnyvale, CA). Under our experimental conditions, the reaction proceeded linearly for at least 60 min. The 50% inhibitory concentrations of the test compounds against CD26 were defined as the compound concentrations required to inhibit the enzyme (CD26)-catalyzed hydrolysis of GlyPro-pNA to pNA and GlyPro by 50%. The OD_{400} values of blank reaction mixtures (lacking the CD26 enzyme) were subtracted from the obtained OD_{400} values to represent the real increase of OD_{400} value as a measurement of the enzyme activity.

Conversion of Dipeptidyl Prodrugs of NAP-TSAO-T to the Dipeptides and the Parent Compound. A variety of test compounds have been evaluated for their substrate activity against purified CD26, human serum (HS), and bovine serum (BS) in eppendorf tubes. The 400 μL reaction mixture contained 50 μM of test compound (dipeptidyl prodrugs of NAP-TSAO-T) in PBS (containing 0.1% DMSO). The reaction was started by the addition of purified CD26 (1.5 mU) or different concentrations of HS (in PBS) or BS (in PBS) at 37 °C. At different time points (as indicated in the tables and figures), 100 μL was withdrawn from the reaction mixture, added to 200 μL methanol, and put on ice for ∼10 min. Then, the mixture was centrifuged at 13.000 rpm for 5 min at 4 °C, and 250 μL of supernatant was analyzed by HPLC on a reverse phase RP-8 column, using following gradients:

Gradient A (Buffer A: 50 mM NaH_2PO_4 + 5 mM heptanesulfonic acid pH3.2; buffer B: acetonitrile): 2 min 98% A + 2% B; 6 min linear gradient to 80% A + 20% B; 2 min linear gradient to 75% A + 25% B; 2 min linear gradient to 65% A + 35% B; 18 min linear gradient to 50% A + 50% B; 5 min 50% A + 50% B; 5 min linear gradient to 98% A + 2% B; 5 min equilibration at 98% A + 2% B.

Gradient B: 2 min 98% A + 2% B; 6 min linear gradient to 80% A + 20% B; 2 min linear gradient to 75% A + 25% B; 2 min linear gradient to 65% A + 35% B; 8 min linear gradient to 50% A + 50% B; 10 min 50% A + 50% B; 10 min linear gradient to 20% A + 80% B; 5 min 20% A + 80% B; 15 min linear gradient to 98% A + 2% B; 5 min 98% A + 2% B.

Evaluation of the Inhibitory Effect of IlePyr and DiprotinA on Conversion of Compound 5 to 2 by CD26, Human, and Bovine Serum. The inhibition assays were performed in eppendorf tubes. The reaction mixture (RMix) contains 50 μM ValPro-NAP-TSAO-T (**5**) and inhibitor (IlePyr 500 or 50 or 0 μM) in PBS. The reaction is started by addition of 32 μL of CD26 (1.5 mU) or 10 μL of BS or HS (total volume reaction mixture: 400 μL) at 37 °C. After 5 h, 100 μL of the RMix was withdrawn and added to 200 μL methanol (on ice). The precipitate was then centrifuged at 13.000 rpm during 5 min, and 250 μL of the supernatant was injected on HPLC (Reverse phase RP-8, Merck) to quantify the reaction products as described above.

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Supporting Information Available: Results from elemental analysis for all compounds and ^{1}H NMR chemical shift assignments of novel dipeptide derivatives **21**−**24**, **3h**,**n**−**p** and protected conjugates **4b**−**p** and **30**. The chemical procedure for the synthesis of the DPP IV/CD26 inhibitor IlePyr and a table showing the cLogP and t_R values for dipeptidyl prodrugs of NAP-TSAO-T are also included. This material is available free of charge via the Internet at http://pubs.acs.org.

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