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Application of the Dipeptidyl Peptidase IV (DPPIV/CD26) Based Prodrug Approach to Different Amine-Containing Drugs

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Here we explore the applicability of the dipeptidyl peptidase IV (DPPIV/CD26) based prodrug approach to a variety of amine-containing drugs. Efficient procedures have been developed for the synthesis of dipeptide and tetrapeptide amide prodrugs including N-acylation protocols of the exocyclic amino function of cytidine and adenosine nucleosides. Our studies demonstrated that XaaPro dipeptides linked to a free amino group present on an aromatic ring or on a sugar entity are prodrugs that efficiently release the parent drug upon conversion by purified DPPIV/CD26 as well as soluble DPPIV/CD26 in bovine and human serum. Vildagliptin, a specific inhibitor of DPPIV/CD26, was able to completely block the hydrolysis of the prodrugs in the presence of purified CD26 but also in human and bovine serum. When the amino group is present on a pyrimidine or purine ring, the dipeptide derivatives are chemically unstable, whereas the tetrapeptide derivatives (i.e., ValProValPro or ValAlaValPro) were much more stable in solution and efficiently converted to the parent drug by the action of DPPIV/CD26. This DPPIV/CD26-directed prodrug technology can be useful to increase solubility of the parent drug molecules and/or to allow better formulation properties.

Introduction

The lymphocyte surface glycoprotein CD26,[a] 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 (DPPIV).[2−4] DPPIV/CD26 is a member of the prolyl oligopeptidase family, a group of atypical serine proteases able to hydrolyze the prolyl bond. It is endowed with an interesting (dipeptidyl) peptidase catalytic activity, and it has high selectivity for peptides with a proline or to a lesser extent an alanine at the penultimate position of the N-terminus of a variety of natural peptides. Instead, it tolerates a much wider range of amino acid residues at the N-terminal end. A free, nonsubstituted amino group on the ultimate (amino terminal) amino acid position is one of the prerequisites for substrate recognition by the enzyme.[2−4] DPPIV/CD26 truncates several bioactive peptides of medical importance.[2−4] It is expressed not only on a variety of leukocyte cell subsets but also on several types of epithelial, endothelial, and fibroblast cells. A soluble form of the enzyme is detected in plasma and cerebrospinal fluid at low amounts.[5]

In 2005, it was demonstrated for the first time that a synthetic small molecule (GPG-NH_2) can be converted to an antiviral drug through the specific action of DPPIV/CD26.[6] This was the first demonstration that a differentiation/activation leukocytic marker acts as a highly specific and obligatory activator of a synthetic anti(retro)viral prodrug that is otherwise inactive as such. On the basis of this study, we recently reported[7,8] a novel type of prodrug approach that could be applied to mediate the solubility, formulation, and potentially also the bioavailability of therapeutic agents. In our approach a di- (or oligo)peptide moiety was linked to a free amino group of a nonpeptidic drug through an amide bond that is specifically cleaved by the endogenous DPPIV/CD26 enzyme (Figure 1).[7,8]

Many prodrug technologies have already been developed depending on the nature of the drug that has to be released.[9−11] Coupling of peptides or amino acids as carriers of 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, respectively,[12−14] and most recently also the valine derivative of the highly specific antivaricella zoster virus bicyclic nucleoside analogue (BCNA) drug Cf1743 (designated FV-100).[15] The markedly increased oral

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[a] Abbreviations: 6-AQ, 6-aminoquinoline; ara-A, 9-β-D-arabinofuranosyladenosine; ara-C, 1-β-D-arabinofuranosylcytosine; BCNA, bicyclic nucleoside analogue; BOP, 1-benzotriazolyloxy-tris-dimethylaminophosphonium hexafluorophosphate; BS, bovine serum; CCTLC, centrifugal circular thin layer chromatography; CD26, cluster of differentiation 26; DIC, diisopropylcarbodiimide; DKP, diketopiperazine; Dox, doxorubicin; DPPIV, dipeptidyl peptidase IV; HATU, *O*-(7-azabenzotriazolyl)tetramethyluronium hexafluorophosphate; HCTU [bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate; hPEPT1, human intestinal oligopeptide transporter 1; HIV-1, human immunodeficiency virus type 1; HOBt, 1-hydroxybenzotriazole; HS, human serum; NAP, *N*-3-aminopropyl; SPE, solid phase extraction; TFE, 2,2,2-trifluoroethanol; TSAO, (*tert*-butyldimethylsilyl-β-D-ribofuranosyl)-3′-spiro-4″-amino-1″,2″-oxathiole-2″,2″-dioxide.

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bioavailability of these prodrugs has been shown to be caused through their recognition by the human peptide transporter hPEPT-1 located in the membrane of the upper small intestine epithelial cells.[16] The prior art for ameliorating solubility and bioavailability predominantly reveals amino acid prodrugs (only one amino acid coupled) of small organic molecules whereby the amino acid is usually linked through ester bonds, allowing easy back-conversion to the free therapeutic parental agent by esterases. However, such prodrugs have low stability at physiological pH and their delivery is often not optimal.[12]

The peptide−prodrug approach would allow us to ameliorate the solubility and formulation of therapeutic agents in a conceptually entirely different and potentially more successful manner, since coupling of di- (or oligo)peptide moieties (instead of one single amino acid) to a therapeutic agent is performed through a more stable amide bond (instead of an ester bond) which is specifically cleaved by DPPIV/CD26 (Figure 1). The presence of a proline near the N-terminus serves as structural protection against nonspecific proteolytic degradation (many exopeptidases do not recognize such sequences),[17] which restricts the use of peptides as promoieties. Moreover, the fact that DPPIV/CD26 is present in plasma in its soluble form and in the membrane of several cell types guarantees that the prodrug will eventually be converted to the parent drug. It is also worth noticing that upon conversion of the prodrug to the parent compound, a natural product (di- or oligopeptide) is released that is not expected to generate any undesired side effects.

For proof of the concept of our novel prodrug technology, we initially focused on the lipophilic TSAO compounds[18−20] that inhibit HIV-1 replication. This retrovirus infects lymphocytes that abundantly express DPPIV/CD26 in their membrane. In particular, the *N*-3-aminopropyl TSAO-T derivative (NAP-TSAO-T, Figure 2) was chosen as model compound because its primary amine functionality would allow the formation of an amide bond between the peptide and the TSAO molecule. A variety of dipeptide and tetrapeptide amide prodrugs of NAP-TSAO-T molecules of general formulas **I** and **II** deprotected at the peptide N-terminus (Figure 2) were synthesized and studied. Our data[7,8] revealed that purified DPPIV/CD26 could specifically recognize these prodrugs as efficient substrates to be converted to the parent compound. Tetrapeptides of formula **II** were cleaved in two successive dipeptidylpeptidase-directed steps to release the parent drug. Soluble DPPIV/CD26 activity present in human and bovine serum was the predominant and often sole enzyme responsible for removing the dipeptide part of the prodrugs in these biological media. Our studies indicated that it was possible to modify the enzymatic and serum hydrolysis rate (half-life) of the prodrug conjugates by changing the nature and length of the peptide promoiety. Also, the nature of the peptide promoiety markedly influenced the overall lipophilicity of the prodrugs, resulting in some cases in a substantial higher water solubility than the parent compound.[8]

Whereas in the previous studies the peptidic sequence in the [peptide]-[NAP-TSAO-T] conjugates was linked to a primary amino group on an aliphatic alkyl chain, we now extend the applicability of this prodrug strategy to a variety of amine-containing drugs of different nature (Figure 3). As an amine-containing aromatic compound, the fluorescent 6-aminoquinoline (6-AQ) bearing a primary amino group bound to the aromatic ring was selected.[21] Antracycline antibiotics containing an amino sugar such as doxorubicin (Dox), the most commonly prescribed intercalating agent for the treatment of cancer, were next chosen.[22] Finally, in order to study whether the strategy was feasible in primary amino groups bound to ring heterocyclic systems, the anti-HIV-1 TSAO-cytosine derivative (TSAO-m^{5}C)[23] and the anticancer drug cytarabine (ara-C)[24] were selected as model pyrimidine nucleosides and the antiviral drug vidarabine (ara-A)[25] as model purine nucleoside (Figure 3). As the promoiety, we focused on the dipeptide ValPro sequence that is efficiently recognized by DPPIV/CD26 in natural peptides[2,3] and in the [peptide]-[NAP-TSAO-T] conjugates.[7] The synthesis and stability of the corresponding prodrugs (general formula **III**, Figure 3), their ability to act as efficient substrates for DPP-IV/CD26, and their human and bovine serum hydrolysis profiles and solubility in aqueous solutions are herein described.

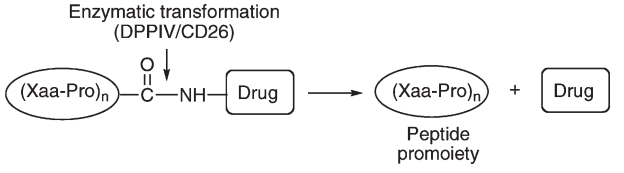


Figure 1. [(Xaa-Pro)$_n$]-[drug] conjugates cleavable by DPPIV/CD26.

Results and Discussion

Chemistry. The target 6-aminoquinoline (6-AQ) dipeptide conjugate **4a** (Scheme 1) was prepared following our previously described two-step procedure[7] that consisted of the coupling of **4a** with the commercially available dipeptide Z-Val-Pro-OH followed by catalytic hydrogenation of the N-protected conjugate intermediate. Thus, coupling of 6-AQ with Z-Val-Pro-OH in CH_2Cl_2 in the presence of (benzotriazol-1-yloxy)-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and TEA gave the corresponding protected

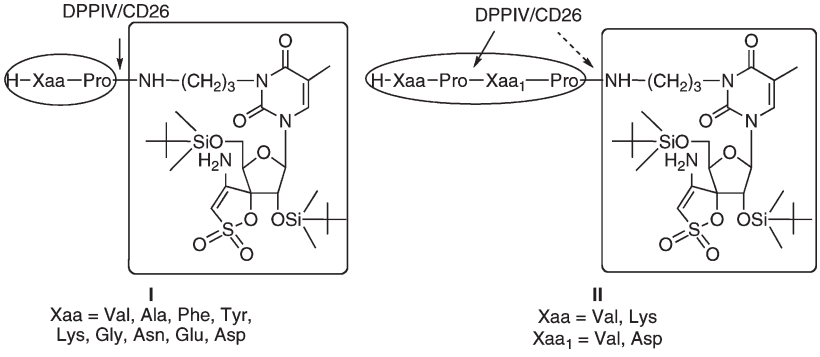


Figure 2. Structures of [Xaa-Pro]-[NAP-TSAO-T] and [Xaa-Pro-Xaa$_1$-Pro]-[NAP-TSAO-T] conjugates of general formulas **I** and **II**.

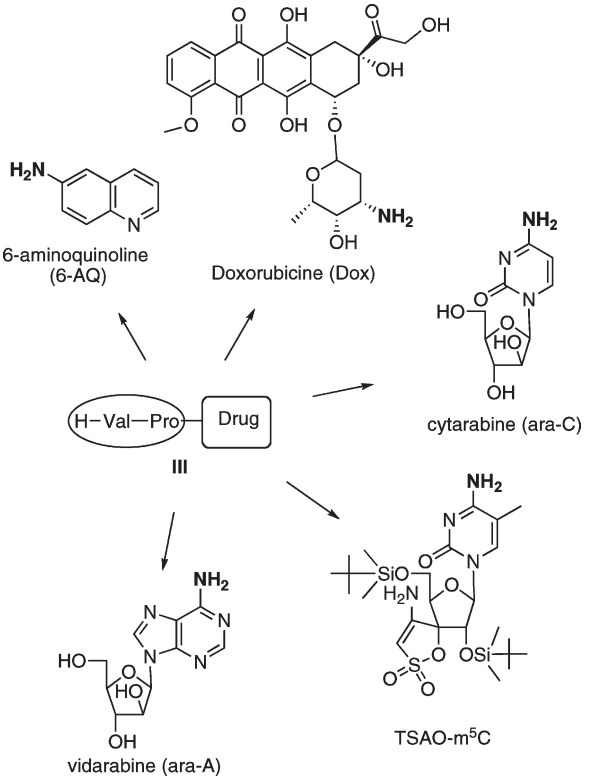


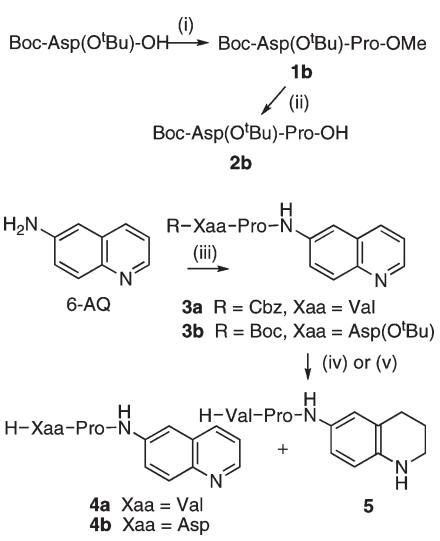
Figure 3. Structures of target [Val-Pro]-[drug] conjugates of general formula **III**.

dipeptide conjugate **3a** (Scheme 1) in 65% yield, after purification by flash column chromatography. Catalytic hydrogenation of **3a** in the presence of 10% Pd/C in methanol afforded the N-deprotected prodrug **4a** in 48% yield together with the partially reduced tetrahydroquinoline derivative **5** (Scheme 1) as minor compound (12% yield). Examples of catalytic hydrogenation of quinolines at the pyridine ring generating tetrahydroquinoline derivatives in the presence of H_2, Pd/C have been described.[26,27]

The conjugate **4b** bearing the Asp-Pro dipeptide sequence, which showed a much lower conversion rate to the parent compound by CD26 in previous studies with [Xaa-Yaa]-[NAP-TSAO-T] conjugates,[7] was next prepared (Scheme 1). The synthesis of compound **4b** was carried out in a way similar to that described above following a *tert*-butoxycarbonyl (BOC) strategy in order to avoid the partial catalytic hydrogenation of the quinoline system in the final deprotection step. The noncommercially available dipeptide Boc-[Asp(O^{t}Bu)-Pro]-OH (**2b**) was prepared using standard coupling/deprotection techniques (Scheme 1). Coupling of Boc-[Asp(O^{t}Bu)-Pro]-OH (**2b**) with 6-aminoquinoline in the presence of BOP and TEA gave the protected intermediate **3b** in 45%. Acid hydrolysis of compound **1b** in TFA/CH_2Cl_2 solution yielded exclusively the fully deprotected conjugate **4b** in 83% yield.

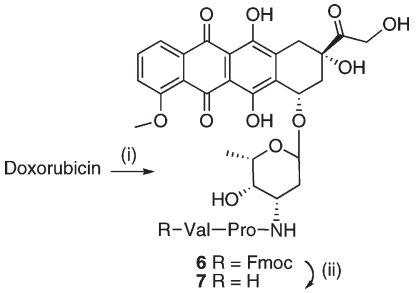
The base-labile 9-fluorenylmethoxycarbonyl (Fmoc) protecting group was used in the preparation of the doxorubicin (Dox) dipeptide conjugate **7** (Scheme 2). Thus, commercially available Fmoc-Val-Pro-OH was coupled with Dox in DMSO in the presence of hexafluorophosphate salt of the *O*-(7-azabenzotriazolyl)tetramethyluronium hexafluorophosphate (HATU) as the coupling agent[28] and DIEA as base to afford the protected conjugate **6** in 45% yield. The Fmoc protecting group was removed by treatment of **6** with 50% piperidine in DMF. The final deprotected conjugate **7**

Scheme 1. Synthesis of [Xaa-Pro]-[6-AQ] Conjugates **4a**,**b**[a]



[a] Reagents: (i) H-Pro-OMe·HCl, BOP, TEA, CH_2Cl_2, room temp; (ii) 2 N NaOH; (iii) Cbz-Val-Pro-OH (**2a**) or Boc-Asp(O^{t}Bu)-Pro-OH (**2b**), BOP, TEA, CH_2Cl_2, room temp; (iv) H_2, Pd (C), CH_3OH, room temp; (v) TFA, CH_2Cl_2, room temp.

Scheme 2. Synthesis of [Val-Pro]-[Dox] Conjugate **7**[a]



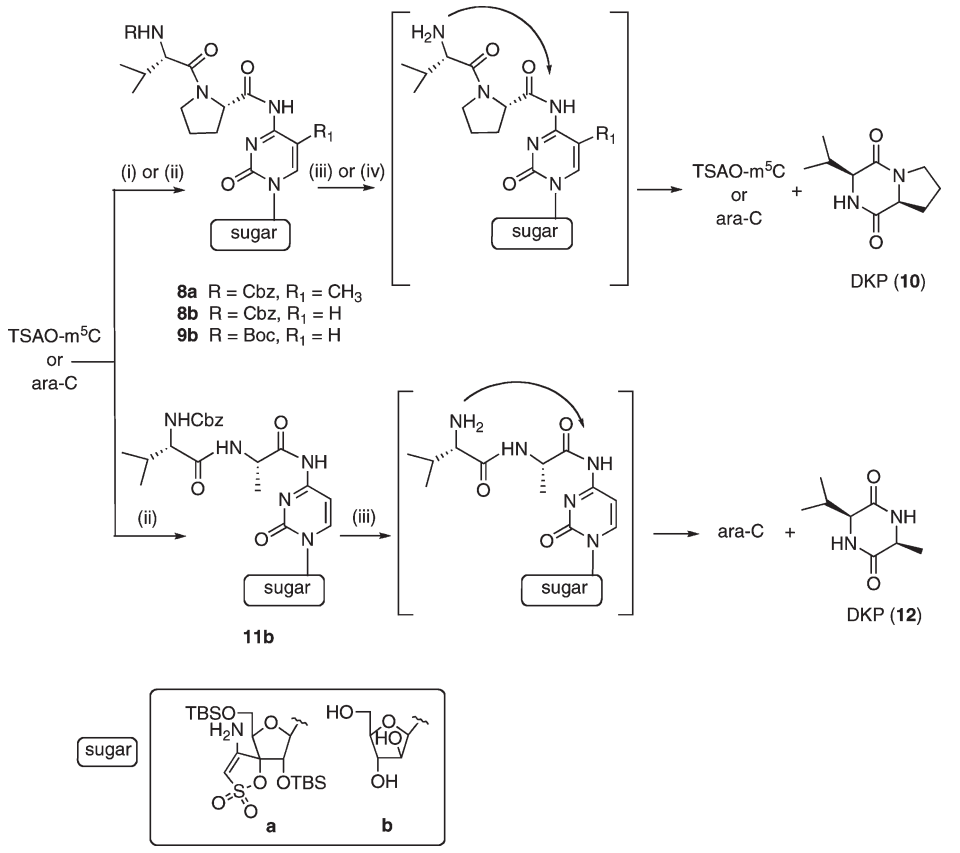
[a] Reagents: (i) Fmoc-Val-Pro-OH, HATU, DIEA, DMSO, room temp; (ii) piperidine 50%, DMF, room temp.

was obtained in 50% yield after purification by reverse phase chromatrography using solid-phase extraction (SPE) cartridges.

Next, we focused on the synthesis of pyrimidine nucleoside prodrugs bearing the model Val-Pro dipeptide sequence as promoiety (Scheme 3). A benzyloxycarbonyl (Cbz)-protecting strategy was selected for the synthesis of dipeptide prodrugs of TSAO-m^{5}C. Thus, TSAO-m^{5}C was reacted with the commercially available dipeptide Cbz-Val-Pro-OH in CH_2Cl_2, in the presence of BOP and TEA at room temperature, to give the corresponding protected dipeptide conjugate **8a** in 53% yield (Scheme 3). Because of the low nucleophilicity of the cytosine amino group, an extra amount of coupling reagent and base was required to drive the reaction to completion.

Peptide bond formation at the N^4-position of ara-C proved to be more complicated by the lower solubility of the compound due to the presence of three free hydroxyl groups. Peptide prodrugs of ara-C at the N^4-position have already been described to overcome the drawbacks for the clinical use of ara-C such as toxicity, low plasma levels, and rapid enzymatic degradation.[29−32] In most of the examples protection of the glycoside moiety of the nucleosides was required. Interestingly, a mild procedure for the synthesis of peptidyl-ara-C derivatives, which does not require protection of

Scheme 3. Attempted Synthesis of Dipeptide Conjugates of Cytidine Nucleosides TSAO-m^{5}C or ara-C[a]



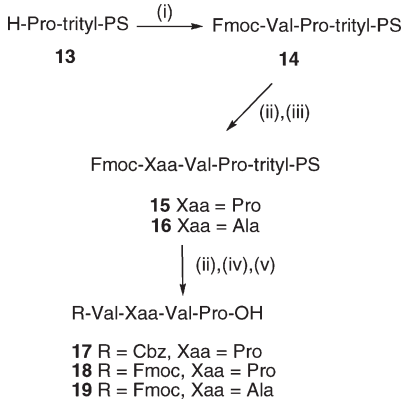
[a] Reagents: (i) Cbz-Val-Pro-OH, BOP, TEA, CH_2Cl_2, room temp; (ii) R-Val-Pro-OH or Cbz-Val-Ala-OH, HOBt, DIC, TEA, DMF, room temp; (iii) H_2, Pd (C) 10%, CH_3OH; (iv) HCl, AcOEt, room temp.

ara-C, has also been reported.[31] Following this protocol, ara-C was reacted with dipeptide Cbz-Val-Pro-OH in DMF using 1,3-diisopropylcarbodiimide (DIC) as coupling agent and *N*-hydroxybenzotriazole (HOBt) as additive to give the Cbz-protected conjugate intermediate **8b** in 27% yield.

Catalytic hydrogenation of protected conjugates **8a** and **8b** in the presence of 10% Pd/C in methanol showed that parent nucleosides TSAO-m^{5}C and ara-C were spontaneously released from the N-deprotected conjugates with concomitant formation of the known diketopiperazine (DKP) such as compound **10**[33] (Scheme 3). It is well-documented in the literature[34−37] that dipeptide esters including Val-Pro-derived dipeptide inhibitors of DPP IV[38] readily cyclize to a DKP derivative (to a much lesser extent in dipeptide amides owing to their lower electrophilic reactivity). It is noted, however, that the spontaneous intramolecular cyclization of the Val-Pro dipeptide promoiety had not been observed, neither in the N-deprotected dipeptide conjugates of 6-AQ and Dox **4a**,**b** and **7** herein described nor in the previously described NAP-TSAO-T dipeptide conjugates that proved to be chemically stable.[7] A much lower electrophilicity of the N-4 amide in the cytidine conjugates could explain why the cytidine drugs are better leaving groups than 6-AQ, Dox, or NAP-TSAO-T. In order to enhance the chemical stability of the dipeptide prodrugs of ara-C and to suppress spontaneous release of the parent drug, several approaches were attempted. Boc was first selected as protecting group, instead of a Cbz-protecting strategy, because it could be removed under acidic conditions, affording the corresponding salts that may prevent spontaneous cyclization.[30] Thus, treatment of ara-C with the commercially available Boc-Val-Pro-OH under the above-mentioned coupling conditions gave the intermediate conjugate **9b** in 28% yield. Conjugate **9b** was deprotected with HCl/ethyl acetate. However, the desired hydrochloride salt of the dipeptide-ara-C conjugate was not chemically stable and only ara-C and DKP **10**[33] were isolated after chromatographic purification. Since it is known that a proline at the penultimate position significantly facilitates the rate of DKP formation owing to the greater propensity to adopt a *cis*-amide conformation,[36] the Val-Pro dipeptide was next replaced by the Val-Ala sequence (which is also recognized by DPP IV/CD26 as previously described).[7] Thus, commercially available Cbz-Val-Ala-OH was coupled with ara-C in DMF in the presence of DIC and HOBt to give the intermediate conjugate **11b** (Scheme 3) in 26% yield. However, in the catalytic hydrogenation of **11b** only ara-C and the corresponding DKP derivative **12**[39] were isolated. Our findings indicate that the Val-Pro and Val-Ala dipeptide sequences cannot be used as useful promoieties in cytidine nucleoside drugs because of an extremely high tendency for cyclization. On the other hand, there is one example reported in the literature where other dipeptide sequences with non-proteinogenic amino acids (such as α,α-disubstituted amino acids) have been successfully employed as carriers in prodrugs of ara-C.[30] Therefore, our results point to a key role of the nature of the dipeptide on the rate of release of the parent drug via DKP formation.

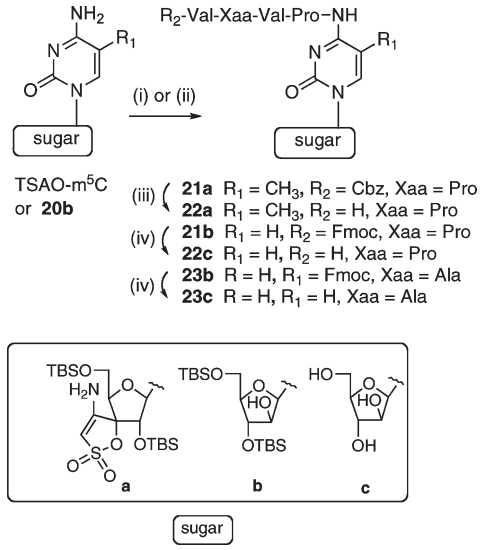
To further investigate whether the DPP IV/CD26 prodrug strategy could be applied to cytidine nucleoside drugs, tetrapeptide prodrugs of TSAO-m^{5}C and ara-C were

Scheme 4. Solid-Phase Synthesis of Tetrapeptides **17**−**19**[a]



[a] Reagents: (i) Fmoc-Val-OH, HCTU, DIEA, DMF; (ii) piperidine 20%, DMF; (iii) Fmoc-Pro-OH or Fmoc-Ala-OH, HCTU, DIEA, DMF; (iv) R-Val-OH, HCTU, DIEA, DMF; (v) AcOH, TFE, DCM.

Scheme 5. Synthesis of Tetrapeptide Conjugates of Cytidine Nucleosides **22a**,**c** and **23c**[a]

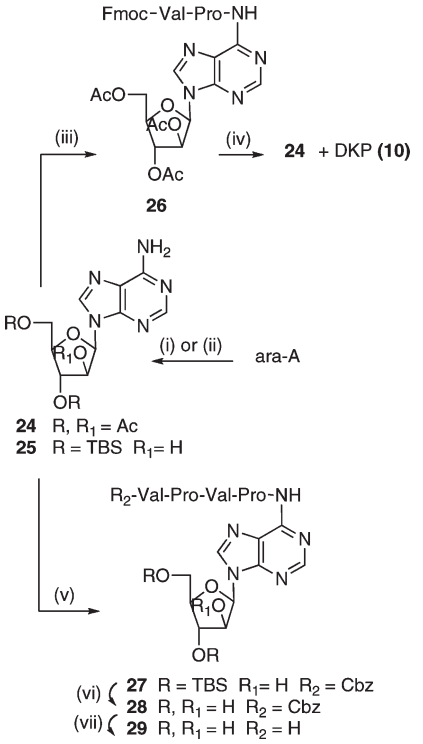


[a] Reagents: (i) R-Val-Pro-Val-Pro-OH (**17**), BOP, TEA, CH_2Cl_2, room temp; (ii) Fmoc-Val-Xaa-Val-Pro-OH (**18** or **19**), BOP, TEA, DMF, room temp or 45 °C; (iii) H_2, Pd (C) 10%, CH_3OH ; (iv) TBAF, THF, room temp.

designed (Scheme 5). On the basis of our previous studies[8] showing that tetrapeptide derivatives of NAP-TSAO were efficiently converted to the parent compound in two steps by DPPIV/CD26, the tetrapeptide sequences Val-Pro-Val-Pro and Val-Ala-Val-Pro (with different conversion rates to the parent compound) were selected.[8]

The synthesis of the target tetrapeptide conjugates **22a**,**c** and **23c** (Scheme 5) utilized a combination of solid-phase and solution chemistry. The tetrapeptide promoieties **17**−**19** were prepared by solid-phase synthesis using a Fmoc strategy on a 2-chlorotritylpolystyrene resin (Scheme 4). This solid support does not favor the on-resin formation of diketopiperazines because of steric hindrance of the bulky 2-chlorotrityl. Resin, preloaded with the first amino acid (H-Pro), was coupled to Fmoc-Val-OH in the presence of 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HCTU)/DIEA to give intermediate dipeptide **14**. Fmoc-Pro-OH or Fmoc-Ala-OH was then similarly incorporated after deprotection of the Fmoc N-protecting group to afford tripeptide derivatives **15** and **16**. The N-terminal residue Cbz-Val-OH or Fmoc-Val-OH was finally incorporated under similar conditions. Final tetrapeptides **17**−**19** were obtained by cleavage from the resin with AcOH/2,2,2-trifluoroethanol (TFE). The tetrapeptides were isolated in excellent yields (92−96%) and at a high purity (> 95%) and were used without further purification in coupling with nucleosides. This solid-phase strategy afforded the tetrapeptides in better yields and enhanced efficacy than the previous solution methodology.[8]

Scheme 6. Synthesis of the Tetrapeptide Conjugate of ara-A **29**[a]



[a] Reagents: (i) Ac_2O, py, room temp; (ii) TBSCl, imidazole, DMF, room temp; (iii) Fmoc-Val-Pro-OH, BOP, TEA, CH_2Cl_2, room temp; (iv) piperidine 20%, DMF, room temp, 5 min; (v) Cbz-Val-Pro-Val-Pro-OH (**17**), BOP, TEA, CH_2Cl_2, 70 °C; (vi) (a) TBAF, THF, room temp; (b) $CaCO_3$, Dowex 50Wx8, CH_3OH, room temp; (vii) H_2, Pd (C) 10%, CH_3OH, room temp.

Coupling of tetrapeptide **17** with TSAO-m^{5}C in the presence of excess amounts of BOP and TEA gave the corresponding protected intermediate conjugate **21a** in moderate yield (41%) (Scheme 5). Removal of the benzyl group by catalytic hydrogenation in the presence of 10% Pd/C in CH_3OH afforded the final desired deprotected conjugate of TSAO-m^{5}C **22a**, which proved to be chemically stable, in excellent yield (95%). Initial attempts to couple tetrapeptides **18** and **19** with unprotected ara-C in DMF in the presence of DIC and HOBt (under similar conditions described above for the dipeptide conjugates **8b**, **9b**, and **11b**) afforded the desired tetrapeptide conjugates **21b** and **23b** in very low yields (6% and 9%, respectively). However, when silyl protected ara-C (**20b**)[40] was condensed with tetrapeptide **18** or **19** in the presence of excess amounts of BOP and TEA, a much higher yield of the fully protected conjugates **21b** and **23b** was obtained (56% and 43%, respectively) (Scheme 5).

Removal of both the *tert*-butyldimethylsilyl (TBS) and Fmoc groups of compounds **21b** and **23b** was carried out by

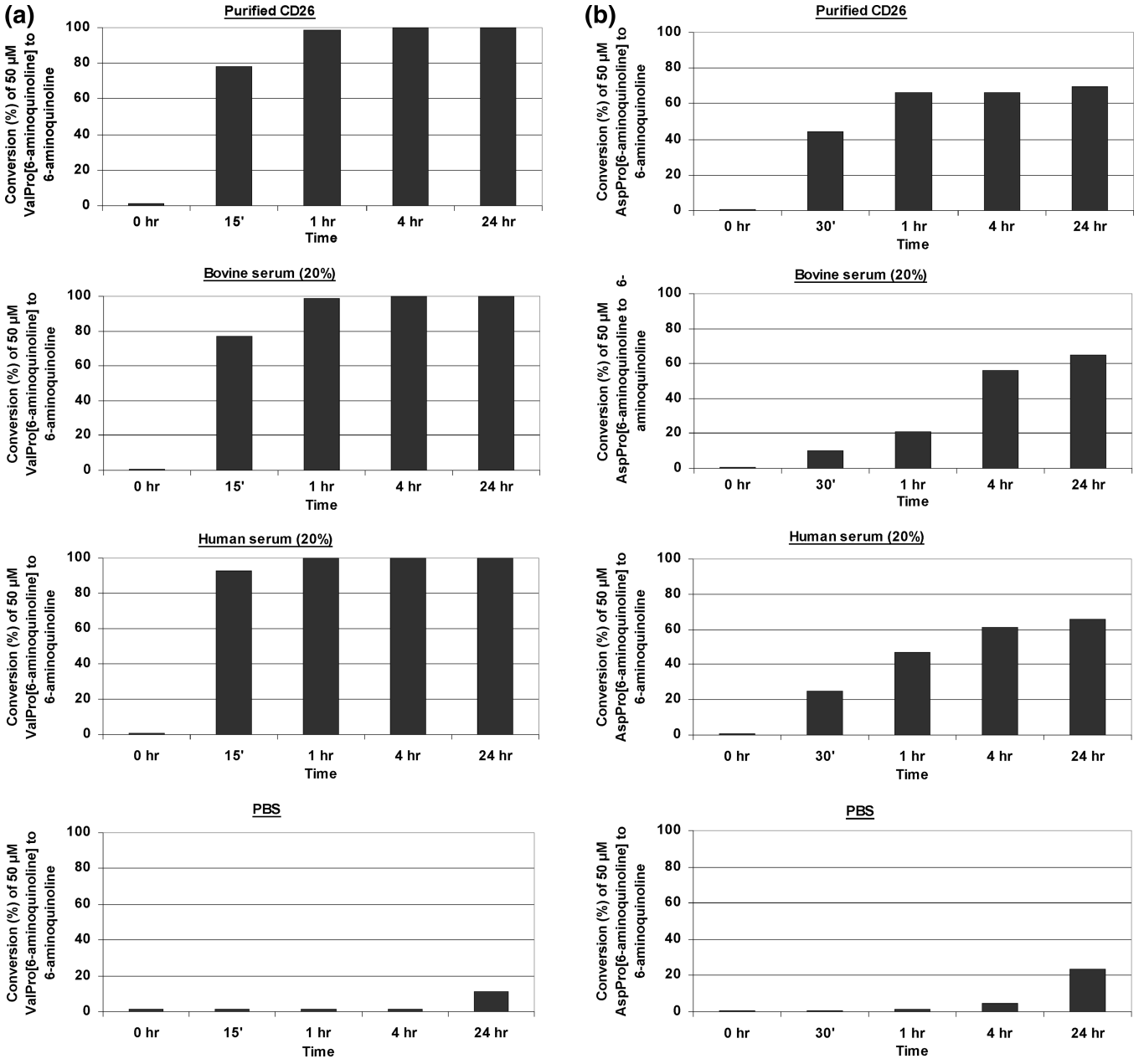


Figure 4. (a) Conversion of [ValPro]-[6-AQ] (**4a**) to parent 6-AQ in the presence of purified CD26, bovine and human serum (20%), and PBS. (b) Conversion of [AspPro]-[6-AQ] (**4b**) to parent 6-AQ in the presence of purified CD26, bovine and human serum (20%), and PBS.

treatment with tetrabutylammonium fluoride (TBAF) to give the target fully deprotected conjugates **22c** and **23c** (Scheme 5) in good yields (60% and 63%, respectively).

Finally, the synthesis of purine nucleoside prodrugs of the antiviral vidarabine (ara-A) bearing di- and tetrapeptide sequences as promoieties was performed (Scheme 6). The preparation of the fully blocked dipeptide derivative **26** (Scheme 6) was carried out in moderate yields (56%) by coupling of the acetyl protected nucleoside **24**[41] with Fmoc-Val-Pro-OH in the presence of excess amounts of BOP and TEA at room temperature. A higher reaction temperature (90 °C) was required for an efficient coupling of ara-A with tetrapeptide sequences. After evaluation of a range of N-terminal protecting groups including Fmoc, Boc, or Cbz, the final candidate for protection of the peptide promoiety was the Cbz group that could be easily removed under mild conditions.[42] Thus, treatment of tetrapeptide **17** with the conveniently protected nucleoside **25**[43] in the presence of excess amounts of BOP and TEA at 90 °C gave the fully protected intermediate conjugate **27** in moderate yield (59%) (Scheme 6).

Similar to that observed with cytidine dipeptide prodrugs, N-deprotection of dipeptide conjugate **26** with 20% piperidine showed that parent nucleoside ara-A was spontaneously released with concomitant formation of the known DKP **10**[33] whereas the tetrapeptide prodrug was much more stable. The Cbz-tetrapeptide conjugate derivative **27** was first subjected to TBAF-promoted TBS removal in THF. A recently described simple and efficient workup to remove excess TBAF and TBAF-derived materials,[44] which avoids the conventional aqueous-phase extraction protocol (precluded in our water-soluble compound), was used. This method involves addition of a commercially available sulfonic acid resin and calcium carbonate, followed by filtration

and evaporation. Thus, treatment of **27** with excess of TBAF in THF, followed by this workup method, afforded the desilylated conjugate **28** in 76% yield after filtration, evaporation, and chromatography purification of the crude residue. Finally, removal of the Cbz group by catalytic hydrogenation in the presence of 10% Pd/C in CH_3OH afforded the desired fully deprotected conjugate **29** in 85% yield (Scheme 6).

In addition, water solubility studies were carried out with the 6-aminoquinoline (6-AQ) dipeptide prodrugs **4a** and **4b** and the vidarabine tetrapeptide prodrug **29** and compared to that of the parent compounds. The solubility of [ValPro]-[6-AQ] **4a** and the most hydrophilic [AspPro]-[6-AQ] prodrug **4b** in aqueous solution were 33.18 and 47.82 mg/mL, respectively, while the solubility of 6-AQ was 7.01 mg/mL. Thus, it is possible to modify the water solubility of the conjugates by changing the nature of the dipeptide promoiety. Furthermore, the vidarabine Val-Pro-Val-Pro tetrapeptide prodrug **29** improved the water solubility (34.74 mg/mL) more than 60-fold in comparison with the parent compound (0.526 mg/mL). Thus, the solubility of peptide-based prodrugs increased between 5- and more than 60-fold compared with their parent compounds. This result suggests that the DPPIV/CD26 prodrug approach could be useful for increasing the water solubility of poorly soluble parent drug molecules.

Biological Studies. Among the aromatic dipeptide prodrugs, the [ValPro]-[6-AQ] (**4a**) and the [AspPro]-[6-AQ] (**4b**) prodrugs have been evaluated for their conversion to the parent 6-AQ compound by purified CD26 and in the presence of 20% bovine serum (BS) and 20% human serum (HS) in PBS (Figure 4). In the absence of enzyme, 50 μM [ValPro]-[6-AQ] (**4a**) (Figure 4a) and [AspPro]-[6-AQ] (**4b**) (Figure 4b) solutions in PBS were fairly stable for 4 h and slightly (spontaneously) converted to the parent compound 6-AQ after 24 h ($\sim$10$-$20%) (Figures 4). Instead, both prodrugs were efficiently converted to 6-AQ by purified CD26 and by 20% bovine and human serum. Complete conversion was noticed for [ValPro]-[6-AQ] within 1 h of incubation, whereas [AspPro]-[6-AQ] needed at least 4 h (in BS and HS) for optimal conversion. It is noted that [ValPro]-[6-AQ] could be fully converted to the parent compound, whereas the maximal conversion of [AspPro]-[6-AQ] to its parent compound was around 70%, even after 24 h. This is obviously due to the feedback inhibitory activity of the released dipeptide AspPro against CD26, an observation that has also earlier been made for the [AspPro]-[NAP-TSAO-T] prodrug.[7] The higher efficiency (rate) of conversion for [ValPro]-[6-AQ] (**4a**) than [AspPro]-[6-AQ] (**4b**) is also in agreement with earlier findings[7] and with the known cleavage specificities of CD26 against natural peptides.[2,3] The presence of a positively charged (i.e., Lys) or lipophilic (i.e., Val) amino acid as N-terminal amino acid is preferred over a negatively charged (i.e., Asp) amino acid for hydrolytic cleavage after the penultimate proline by CD26.

The ValPro derivative of doxorubicin (**7**) in which the dipeptide is linked to the molecule through the sugar amide moiety proved to be a good substrate for CD26 (Figure 5). While fully stable in PBS for at least 24 h, CD26 was able to substantially convert the prodrug to its parent compound within 1 h of incubation. Also BS and HS time-dependently and efficiently hydrolyzed the prodrug (Figure 5).

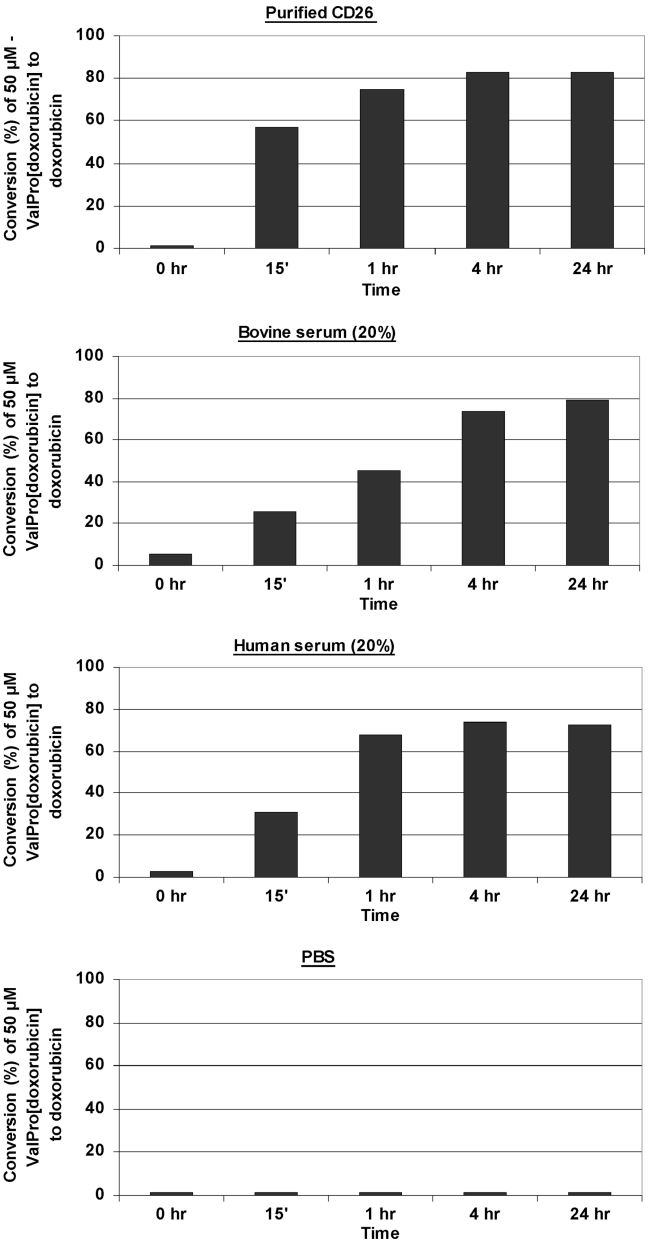


Figure 5. Conversion of [ValPro]-[Dox] (**7**) to parent doxorubicin in the presence of purified CD26, bovine and human serum (20%), and PBS.

Two peptide prodrug derivatives were prepared using the amine function of cytosine as the entity to link the peptide moiety. [ValProValPro]-[TSAO-m^5C] (**22a**) was not very stable in PBS. However, it proved to be a substrate for CD26 ($\sim$50% conversion after 2 h) and was also efficiently converted to the parent compound in 20% HS ($\sim$20% conversion after 2 h) and 20% BS ($\sim$40% conversion after 2 h). The dipeptide intermediate has not been detected during the TSAO-m^5C release process (data not shown). The tetrapeptide derivatives of ara-C, in which the ValProValPro (**22c**) and ValAlaValPro (**23c**) tetrapeptides were linked to the amine at the cytosine heterocyclic ring, proved to be much more stable in PBS (Figure 6). Indeed, upon incubation during the first 4 h in PBS, release of hardly any parent compound (a few percent) could be detected. After 24 h, 18$-$24% of the tetrapeptide prodrug was converted to parental ara-C. However, in the presence of purified CD26, HS, or BS, the ara-C prodrug **22c**

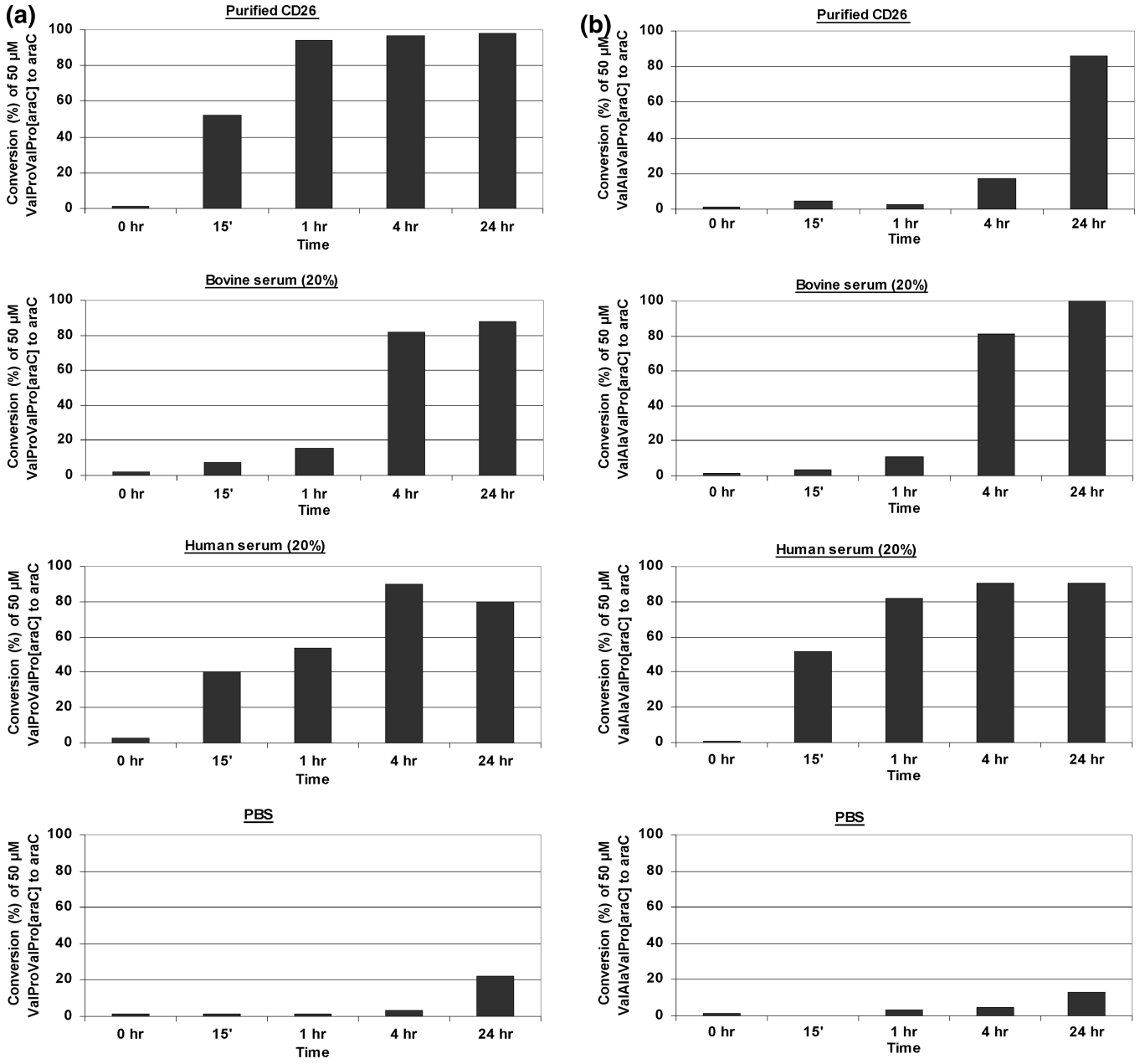


Figure 6. (a) Conversion of [ValProValPro]-[ara-C] (**22c**) to parent ara-C in the presence of purified CD26, bovine and human serum (20%), and PBS. (b) Conversion of [ValAlaValPro]-[ara-C] (**23c**) to parent ara-C in the presence of purified CD26, bovine and human serum (20%), and PBS.

was converted to ara-C by 80−90% within 4 h (Figure 6a). No ara-C dipeptide intermediate could be detected by HPLC at any time point investigated, an observation that is in agreement with the observed high instability of the ara-C dipeptide prodrug. Therefore, although it cannot be excluded that the intermediate ara-C dipeptide prodrug is a substrate for CD26 and can also be catalytically (very rapidly) cleaved a second time, we may assume that once the ara-C tetrapeptide prodrug has been enzymatically converted to the ara-C dipeptide prodrug by the action of CD26, this intermediate would be instantly converted to ara-C by spontaneous nonenzymatic hydrolysis. Similar data were observed for the [ValAla-ValPro]-[ara-C] prodrug **23c** (Figure 6b).

The stability in PBS and susceptibility of the ValProVal-Pro tetrapeptide derivative of ara-A **29** upon exposure to CD26 or 20% serum was investigated. Whereas this compound was fully stable in PBS for up to 24 h, the tetrapeptide ara-A derivative converted to ara-A by CD26, as well as by 20% HS and BS (Figure 7). No dipeptide ara-A could be detected during the conversion process by CD26, presumably because of the noted instability of this intermediate metabolite (as also observed for the ara-C dipeptide prodrug (see above)). The tetrapeptide ara-A compound **29** seemed to be less efficiently converted to the parent drug than the corresponding ara-C prodrug **22c** (Figure 6a). It is mentioned that no formation of ara-Hx (deamination product of ara-A) was observed in the CD26-catalyzed reaction or under the 20% HS and 20% BS experimental conditions. Since the disappearance of the prodrug in the samples was also measured (instead of appearance of parental compound), potential deamination of ara-A to ara-Hx would not influence the conversion kinetic measurements.

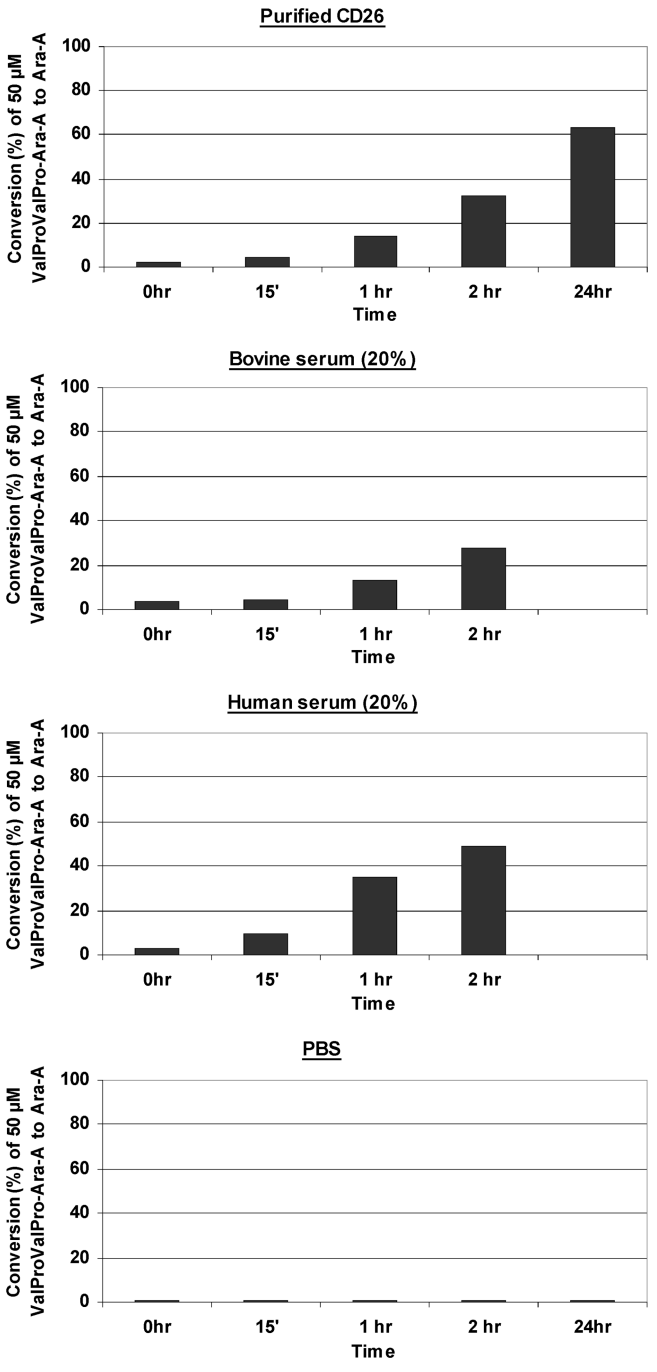


Figure 7. Conversion of [ValProValPro]-[ara-A] (**29**) to parent ara-A in the presence of purified CD26, bovine and human serum (20%), and PBS.

To ascertain that the observed conversions of the prodrugs to their parental compounds were solely due to the action of CD26 but not to other enzymatic activities, the conversion of the [ValPro]-[6-aminoquinoline], [AspPro]-[6-aminoquinoline], [ValPro]-[doxorubicine], and [ValProValPro]-[ara-A] by CD26, 20% HS, and 20% BS was studied in the presence of vildagliptin, a specific inhibitor of CD26.[45−47] At 20 μM vildagliptin, no signs of hydrolysis of any of the prodrugs was observed, neither in the presence of purified CD26 nor in the presence of 20% HS or 20% BS. These findings clearly indicate that the observed conversions of the prodrugs to their parent compounds were exclusively due to the action of CD26. When the ValProValPro prodrug of ara-A was exposed to CD26 in the presence of a variety of the vildagliptin inhibitor concentrations, a dose-dependent inhibition of prodrug conversion was observed. At 2 μM, the inhibitor was still able to fully prevent prodrug conversion to ara-A. At a concentration as low as 0.05 μM vildagliptin, the CD26-catalyzed reaction was inhibited by ~50% (Figure 8). This vildagliptin concentration closely corresponded to the 50% inhibitory concentration of this drug reported against DPP-4 activity in vitro (IC_{50} = 62 nM).[47]

The doxorubicin and ara-C prodrugs have been evaluated for their inhibitory activity against the proliferation of murine leukemia L1210, human lymphocyte CEM, and cervix carcinoma HeLa cells (Table 1). The prodrugs virtually kept their cytostatic activity compared with the parental compounds doxorubin and ara-C. This observation points to an efficient conversion of the prodrug derivatives to the parent drugs by CD26 activity present in the cell cultures and is in agreement with the kinetic conversion data. Also, the TSAO-m^5C prodrug kept its anti-HIV-1 activity in CEM cell cultures when compared to TSAO-m^5C (EC_{50} = 0.07 μM), and the [ProValProVal]-[ara-A] prodrug was virtually equally antivirally active against herpes simplex virus type 1 (HSV-1) and vaccinia virus (VV) in HEL cell cultures as the parent ara-A (Table 2). However, in the presence of 20 μM vildagliptin, the antiviral activity was unaltered for ara-A but 5- to 10-fold decreased for the prodrug of ara-A. These findings indicate that the CD26 inhibitor could prevent conversion of [ValProValPro]-[ara-A] to ara-A by at least 80−90% under the experimental conditions (addition of one single administration of the inhibitor at the time of initiation of the virus infection).

The kinetic parameters (K_m, V_{max}) were also determined for [ValPro]-[6-aminoquinoline], [AspPro]-[6-aminoquinoline], [ValPro]-[doxorubicine], and [ValProValPro]-[ara-A] against purified CD26 (Table 3). Whereas the K_m values were rather similar for the three ValPro prodrugs (irrespective the nature of the parent drug linked to the ValPro-moiety (K_m = 25−56 μM)), [AspPro]-[6-aminoquinoline] was endowed with a much higher K_m value (524 μM). Instead, the V_{max} values differ much more depending the nature of the parent drug: the highest V_{max} values were observed for the 6-aminoquinoline prodrugs and the lowest V_{max} values (7-fold lower) for the [ValProValPro]-[ara-A] prodrug (Table 3). The overall hydrolyzing capacity of CD26 (ratio V_{max}/K_m) differed up to ~10-fold for the four prodrugs investigated. The K_m values of the prodrugs were somewhat higher than reported for a variety of natural substrates including SDF-1α, IP-10, RANTES, and LD78β (1−4 μM).[48] However, the K_m for eotoxin was 25 μM,[48] a value comparable to that found for [ValPro]-[doxorubicine] and [ValPro]-[6-aminoquinoline] (Table 3).

In conclusion, we could earlier demonstrate that purified CD26 (as well as human and bovine serum) could efficiently recognize dipeptide derivatives where the dipeptide is linked to a nonpeptidic drug through an aminoalkyl side chain. We now could extend this observation to drugs at which a free amino group is located at an aromatic ring (i.e., 6-aminoquinoline) or an amino sugar (i.e., doxorubicine). Whereas linkage of a dipeptide to an amino group at a heterocyclic ring such as in cytosine and adenine is highly unstable, tetrapeptide derivatives of ara-C and ara-A instead afford fairly stable prodrugs that are efficiently recognized by CD26 and well-cleaved in bovine and human serum. Thus, our studies revealed that a wide variety of drug types containing a

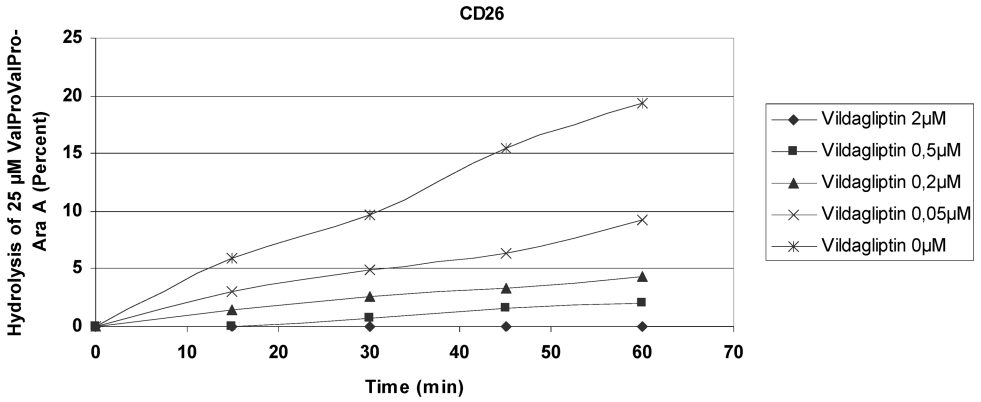


Figure 8. Inhibition of CD26-catalyzed [ValProValPro]-[ara-A] (**29**) hydrolysis in the presence of different concentrations of vildagliptin.

Table 1. Inhibitory Activity of the ara-C and Its Prodrug against Tumor Cell Proliferation

	IC_{50}[a] (μM)		
compd	L1210	CEM	HeLa
ara-C	0.024 ± 0.009	0.024 ± 0.001	0.26 ± 0.01
[ValProValPro]-[ara-C] (**22c**)	0.10 ± 0.04	0.057 ± 0.016	0.63 ± 0.04
[ValAlaValPro]-[ara-C] (**23c**)	0.082	0.050 ± 0.005	0.56 ± 0.07

[a] 50% inhibitory concentration, or compound concentration required to inhibit tumor cell proliferation by 50%.

Table 2. Antiviral Activity of ara-A and [ValProValPro]-[ara-A] in HEL Cell Cultures

	EC_{50}[a] (μM)			
	HSV-1		VV	
compd	as such	+vildagliptin	as such	+vildagliptin
ara-A	35 ± 15	42 ± 11	8.3 ± 4.0	10 ± 0.7
[ValProValPro]-[ara-A]	54 ± 7.5	238 ± 20	16 ± 7.5	135 ± 50

[a] EC_{50} values, or compound concentrations required to reduce the virus-induced cytopathicity by 50%.

Table 3. Kinetic Parameters of Prodrugs against Purified CD26

	kinetic parameters		
prodrug	K_m (μM)	V_{max} (nmol/μg/h)	V_{max}/K_m
[ValPro]-[6-aminoquinoline]	25 ± 8.5	32 ± 3.2	1.3
[AspPro]-[6-aminoquinoline]	524 ± 207	70 ± 22	0.13
[ValPro]-[doxorubicin]	30 ± 8.1	15 ± 3.5	0.50
[ValProValPro]-[ara-A]	56 ± 19	10 ± 12	0.18

free amino moiety on an aromatic, carbohydrate, or heterocyclic pyrimidine or purine ring can be derivatized with di- or tetrapeptides to become efficient substrates for the dipeptidyl peptidase activity of CD26. Furthermore, the 6-AQ and vidarabine peptide prodrugs exhibited a considerably higher water solubility than the parent compounds, a property that can be beneficial for drug formulation modalities.

Experimental Section

Chemical Procedures. Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument, and the analytical results were within 0.4% of the theoretical values. Electrospray mass spectra were measured on a quadropole mass spectrometer equipped with an electrospray source (Hewlett-Packard, LC/MS HP 1100). Spectra were recorded with a Varian Inova-300 or Varian Inova-400 spectrometers operating at 300 or at 400 MHz for ^{1}H NMR and at 75 or at 100 MHz for ^{13}C NMR 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 (Kieselgel 60 PF_{254} gipshaltig (silica gel containing gypsum) (Merck), layer thickness of 1 mm, flow rate of 5 mL/min). Preparative reverse phase purification was carried out using reverse phase SPE cartridges. The dipeptide derivatives Cbz-Val-Pro-OH, Fmoc-Val-Pro-OH, Boc-Val-Pro-OH, and Cbz-Val-Ala-OH were purchased from Bachem Feinchemikalien. 3′,5′-TBS-ara-C (**20c**),[39] 2′,3′,5′-Ac-ara-A (**24**),[40] and 3′-5′-TBS-ara-A (**25**)[42] were synthesized as previously described. H-Pro-2-chlorotrityl resin and HCTU were purchased from GL Biochem (Shanghai) Ltd. The purity of novel compounds was determined to be >95% by elemental analysis.

6-*N*-[*N*$^{\alpha}$-(Bencyloxycarbonyl)valylprolyl]quinoline (3a). To a solution of Cbz-Val-Pro-OH (0.58 g, 1.66 mmol) in CH_2Cl_2 (3 mL) was successively added BOP (0.73 g, 1.66 mmol), TEA (0.231 mL, 1.66 mmol), and 6-aminoquinoline (0.20 g, 1.38 mmol). 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 CCTLC on the Chromatotron (hexane/ethyl acetate, 1:4) to give **3a** (0.39 g, 65%) as a yellow foam. MS (ESI^+): m/z 475.3 ($M + 1^+$). Anal. for $C_{27}H_{30}N_4O_4$: C, H, N.

6-*N*-[*N*$^{\alpha}$-(*tert*-Butoxy)-*O*^{4}-*tert*-butylaspartylprolyl]quinoline (3b). Following the coupling procedure described for compound **3a**, 6-aminoquinoline (0.068 g, 0.474 mmol) was reacted with Boc-Asp(O^{t}Bu)-Pro-OH (0.22 g, 0.57 mmol) in the presence of BOP (0.252 g, 0.57 mmol) and TEA (0.079 mL, 0.57 mmol) in CH_2Cl_2 (5 mL). The final residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/CH_3OH, 40:1) to give **3b** (0.105 g, 45%) as a white foam. MS (ESI^+): m/z 513.3 ($M + 1^+$). Anal. for $C_{27}H_{36}N_4O_6$: C, H, N.

6-*N*-(Valylprolyl)quinoline (4a). A solution of Cbz-[Val-Pro]-[6-AQ] (**3a**) (0.42 g, 0.88 mmol) in methanol (10 mL) containing Pd/C (10%) (40 wt %/wt) (0.12 g) was hydrogenated at 1 atm at room temperature for 2 h. The reaction mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 2:1). From the fastest running fractions, 0.035 g (12%) of **5** was isolated as a yellow oil. ^{1}H NMR (300 MHz, acetone-d_6): δ 0.83 (d, 3H, γ-CH_3a, Val, J = 6.6 Hz), 0.95 (d, 3H, γ-CH_3b, Val, J = 6.6 Hz), 1.80−2.30 (m, 7H, H-3, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.66 (t, 2H, H-4, J = 6.3 Hz), 3.22 (t, 2H, H-2, J = 5.4 Hz), 3.49−3.78 (m, 2H, δ-CH_2, Pro), 3.96 (d, 1H, α-CH, Val, J = 8.1 Hz), 4.57 (m, 1H, α-CH, Pro), 6.38 (d, 1H, H-8, J = 8.4 Hz), 7.06 (m, 1H, H-7), 7.14 (s, 1H, H-5), 9.08 (s, 1H, NHCO). MS (ESI^+): m/z 345.1 ($M + 1^+$),

367.1 (M + Na^+). The slowest moving band afforded 0.14 g (48%) of 6-*N*-(valylprolyl)quinoline (**4a**) as a yellow foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.87 (d, 3H, γ-CH_3a, Val, J = 6.6 Hz), 0.95 (d, 3H, γ-CH_3b, Val, J = 6.6 Hz), 1.82−2.08 (m, 4H, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.21 (m, 1H, β-CH_2, Pro), 3.84 (m, 3H, α-CH, Val, δ-CH_2, Pro), 4.52 (m, 1H, α-CH, Pro), 7.46 (m, 1H, H-3), 7.78 (d, 1H, H-7, J = 2.2 Hz, J = 9.0 Hz), 7.97 (d, 1H, H-8, J = 9.0 Hz), 8.28 (d, 1H, H-4, J = 7.8 Hz), 8.36 (d, 1H, H-5, J = 2.2 Hz), 8.77 (dd, 1H, H-2, J = 1.7 and 4.1 Hz), 10.39 (bs, 1H, NHCO). MS (ESI$^+$): m/z 341.2 (M + 1$^+$). Anal. for $C_{19}H_{24}N_4O_2$: C, H, N.

6-*N*-(Aspartylprolyl)quinoline (4b). To a solution of **3b** (0.091 g, 0.18 mmol) in CH_2Cl_2 (3 mL) was added TFA (0.40 mL), and the mixture was stirred for 5 h. After removal of the solvent in high vacuum, the salt formed was liberated with 1 equiv of TEA. The crude was purified by CCTLC on the Chromatotron (ethyl acetate/MeOH, 2:1) to give **4b** (0.052 g, 83%) as a white foam. MS (ESI$^+$): m/z 357.2 (M + 1$^+$). Anal. for $C_{18}H_{20}N_4O_4$: C, H, N.

***N*-[*N*$^{\alpha}$-(9-Fluorenylmethoxycarbonyl)valylprolyl]doxorubicine (6).** A solution of Fmoc-Val-Pro-OH (0.07 g, 0.16 mmol) and doxorubicin hydrochloride (0.10 g, 0.16 mmol) was dissolved in DMSO (8 mL). DIEA (59.0 μL, 0.34 mmol) was next added, and the mixture was stirred for 15 min at room temperature (protected from light). A solution of HATU (0.06 g, 0.18 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at room temperature for 15 h, and the solvent was lyophilized. 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 dissolved in hot CH_2Cl_2, filtered, and evaporated to dryness to give **6** (0.08 g, 45%) as a red foam. MS (ESI$^+$): m/z 984.3 (M + Na^+). Anal. for $C_{52}H_{55}N_3O_{15}$: C, H, N.

***N*-(Valylprolyl)doxorubicine (7).** The Fmoc-protected conjugate **6** (0.06 g, 0.06 mmol) was treated with 50% piperidine in DMF (4 mL). The reaction mixture was stirred at room temperature for 5 min, and the solvent was evaporated to dryness. The residue was purified by reverse phase chromatrography using SPE cartridges (acetonitrile/water, 70:1) to give **7** (0.02 g, 50%) as a red foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.81 (m, 6H, 2γ-CH_3, Val), 1.11 (d, 3H, H-6′, J = 6.4 Hz), 1.43 (m, 1H, H-2'), 1.74−1.89 (m, 6H, H-2′, H-8, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.14 (m, 3H, H-8, β-CH_2, Pro), 2.95 (m, 2H, H-10), 3.51 (m, 3H, H-4′, δ-CH_2, Pro), 3.95 (m, 5H, H-3′, OCH_3, α-CH, Val), 4.14 (m, 1H, H-5′), 4.35 (m, 1H, α-CH, Pro), 4.56 (bs, 2H, H-14), 4.78 (d, 1H, OH-4′, J = 5.6 Hz), 4.85 (m, 1H, OH-14), 4.93 (bs, 1H, H-7), 5.21 (bs, 1H, H-1'), 5.46 (s, 1H, OH-9), 7.53 (d, 1H, NH-3′, J = 8.0 Hz), 7.64 (m, 1H, H-1), 7.91 (m, 2H, H-2, H-3). MS (ESI$^+$): m/z 740.2 (M + 1$^+$). Anal. for $C_{37}H_{45}N_3O_{13}$: C, H, N.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4-*N*-[*N*$^{\alpha}$-(benzyloxycarbonyl)valylprolyl]cytosine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (8a). Following a similar coupling procedure described for compound **3a**, TSAO-m^{5}C[23] (0.13 g, 0.22 mmol) was reacted with Cbz-Val-Pro-OH (0.11 g, 0.32 mmol) in the presence of BOP (0.14 g, 0.32 mmol) and TEA (0.066 mL, 0.43 mmol) in dry CH_2Cl_2 (4 mL) at room temperature. After 1 h of reaction, an extra amount of BOP (0.14 g, 0.32 mmol) and TEA (0.066 mL, 0.43 mmol) were added to drive the coupling to completion. The final residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 50:1) to give **8a** (0.107 g, 53%) as a white foam. MS (ESI$^+$): m/z 919.3 (M + 1$^+$). Anal. for $C_{42}H_{66}N_6O_{11}SSi_2$: C, H, N, S.

4-*N*-[*N*$^{\alpha}$-(Benzyloxycarbonyl)valylprolyl]-1-β-D-arabinofuranosylcytosine (8b). A solution of Cbz-Val-Pro-OH (0.17 g, 0.49 mmol) (1.2 equiv) in DMF (2 mL) was successively reacted with HOBt (0.066 g, 0.49 mmol), DIC (0.062 g, 0.49 mmol), and ara-C (0.10 g, 0.41 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 15 h, and the solvent was evaporated to dryness. 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 CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 10:1) to give **8b** (0.06 g, 27%) as a white foam. MS (ESI$^+$): m/z 574.3 (M + 1$^+$). Anal. for $C_{27}H_{35}N_5O_9$: C, H, N.

4-*N*-[*N*$^{\alpha}$-(*tert*-Butoxycarbonyl)valylprolyl]-1-β-arabinofuranosylcytosine (9b). Following a similar coupling procedure described for compound **8b**, Boc-Val-Pro-OH (0.17 g, 0.49 mmol) was treated with ara-C (0.10 g, 0.41 mmol) to give **9b** (0.06 g, 28%) as a white foam. MS (ESI$^+$): m/z 540.3 (M + 1$^+$). Anal. for $C_{24}H_{37}N_5O_9$: C, H, N.

4-*N*-[*N*$^{\alpha}$-(Benzyloxycarbonyl)valylalanyl]-1-β-D-arabinofuranosylcytosine (11b). Cbz-Val-Ala-OH (0.17 g, 0.49 mmol) was treated with ara-C (0.10 g, 0.41 mmol), via a similar coupling procedure described for compound **8b**, to give **11b** (0.065 g, 26%) as a white foam. MS (ESI$^+$): m/z 547.3 (M$^+$). Anal. for $C_{25}H_{33}N_5O_9$: C, H, N.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4-*N*-[*N*$^{\alpha}$-(benzyloxycarbonyl)valylprolylvalylprolyl]cytosine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (21a). Following the coupling procedure described for compound **3a**, TSAO-m^{5}C[23] (0.094 g, 0.16 mmol) was reacted with Cbz-Val-Pro-Val-Pro-OH (0.13 g, 0.24 mmol) in the presence of BOP (0.106 g, 0.24 mmol) and TEA (0.037 mL, 0.24 mmol) in dry CH_2Cl_2 (4 mL). The final residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 50:1) to give **21a** (0.07 g, 41%) as a white foam. MS (ESI$^+$): m/z 1115.4 (M + 1$^+$). Anal. for $C_{52}H_{82}N_8O_{13}SSi_2$: C, H, N, S.

3′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-4-*N*-[*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)valylprolylvalylprolyl]-1-β-D-arabinofuranosylcytosine (21b). 3′,5′-TBS-ara-C (**20b**)[39] (0.10 g, 0.21 mmol) was treated with Fmoc-Val-Pro-Val-Pro-OH (0.16 g, 0.25 mmol), via a similar coupling procedure described for compound **3a**, to give **21b** (0.13 g, 56%) as a white foam. MS (ESI$^+$): m/z 1086.6 (M$^+$), 1109.3 (M + Na^+). Anal. for $C_{56}H_{83}N_7O_{11}Si_2$: C, H, N.

[1-[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-4-*N*-[(valylprolylvalylprolyl)cytosine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (22a). A solution of the protected tetrapeptide conjugate **21a** (0.045 g, 0.039 mmol) in methanol (6 mL) containing Pd/C (10%) (40 wt %/wt) (0.021 g) 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 liophilized to give **22a** (0.038 g, 95%) as a white foam. MS (ESI$^+$): m/z 981.5 (M$^+$). Anal. for $C_{44}H_{76}N_8O_{11}SSi_2$: C, H, N, S.

4-*N*-[Valylprolylvalylprolyl]-1-β-D-arabinofuranosylcytosine (22c). To a solution of Fmoc-[Val-Pro-Val-Pro]-[3′,5′-TBS-ara-C] (**21b**) (0.11 g, 0.10 mmol) in THF (1 mL) was added a 1.1 M solution of TBAF in THF (0.28 mL, 0.31 mmol). The reaction mixture was stirred at room temperature for 8 h, and the solvent was evaporated to dryness. The residue was dissolved in isobutanol (50 mL) and washed with brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was purified by flash cromatography (^{i}PrOH:/H_2O/NH_3, 20:1:0.5) to give **22c** (0.039 g, 60%) as a white foam. ^{1}H NMR (300 MHz, acetone-d_6): δ 0.84−0.93 (m, 12H, 4γ-CH_3, 2Val), 1.71−2.19 (m, 10H, 2β-CH, 2Val, 2β-CH_2, 2Pro, 2γ-CH_2, 2Pro), 3.44−3.82 (m, 8H, 2H-5′, H-4′, α-CH, Val, 2δ-CH_2, 2Pro), 3.92 (m, 1H, H-3′), 4.06 (m, 1H, H-2'), 4.32 (m, 1H, α-CH, Val), 4.42 (m, 1H, α-CH, Pro_1), 4.51 (m, 1H, α-CH, Pro_2), 6.06 (d, 1H, H-1′, J = 3.6 Hz), 7.15 (d, 1H, H-5, J = 7.2 Hz), 7.99 (d, 1H, NH, Val, J = 8.4 Hz), 8.07 (d, 1H, H-6, J = 7.5 Hz), 11.04 (bs, 1H, NH, ara-C). MS (ESI$^+$): m/z 636.5 (M + 1$^+$), 658.5 (M + Na^+). Anal. for $C_{29}H_{45}N_7O_9$: C, H, N.

3′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-4-*N*-[*N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)valylalanylvalylprolyl]-1-β-D-arabinofuranosylcytosine (23b). To a solution of Fmoc-Val-Ala-Val-Pro-OH (0.085 g,

0.14 mmol) in CH_2Cl_2 (3 mL) was successively added BOP (0.062 g, 0.14 mmol), TEA (0.033 mL, 0.23 mmol), and 3′,5′-TBS-ara-C (**20b**) (0.055 g, 0.12 mmol). The mixture was stirred at 45 °C for 15 h. After removal of the solvent in vacuo, the residue was dissolved in ethyl acetate (15 mL) and washed with 10% aqueous citric acid (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL), water (3 × 10 mL), and brine (3 × 10 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The final residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 20:1) to give **23b** (0.05 g, 43%) as a white solid. MS (ESI^+): m/z 1060.9 ($M + 1^+$). Anal. for $C_{54}H_{81}N_7O_{11}Si_2$: C, H, N.

4-*N*-[Valylalanylvalylprolyl]-1-β-D-arabinofuranosylcytosine (23c). Via a similar deprotection procedure described for compound **22c**, Fmoc-[Val-Ala-Val-Pro]-[3′,5′-TBS-ara-C] (**23b**) (0.040 g, 0.039 mmol) was reacted with 1.0 M solution of TBAF in THF (0.31 mL, 0.31 mmol) for 8 h to afford **23c** (0.015 g, 63%) as a white foam. MS (ESI^+): m/z 610.5 ($M + 1^+$). Anal. for $C_{27}H_{43}N_7O_9$: C, H, N.

2′,3′,5′-Tris-*O*-(acetyl)-4-*N*-[N^{α}-(9-fluorenylmethoxycarbonyl)valylprolyl]1-β-D-arabinofuranosyladenosine (26). 2′,3′,5′-AcO-ara-A (**24**)[40] (0.10 g, 0.21 mmol) was treated with Fmoc-Val-Pro-OH (0.16 g, 0.25 mmol), via a similar coupling procedure described for compound **3a**, to give **26** (0.13 g, 56%) as a white foam. MS (ESI^+): m/z 812.8 ($M + 1^+$). Anal. for $C_{41}H_{45}N_7O_{11}$: C, H, N.

4-*N*-[N^{α}-(Benzyloxycarbonyl)valylprolylvalylprolyl]-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-1-β-D-arabinofuranosyladenosine (27). 3′,5′-TBS-ara-A (**25**)[42] (0.055 g, 0.11 mmol) was treated with Cbz-Val-Pro-Val-Pro-OH (0.071 g, 0.13 mmol) at 90 °C, via a similar coupling procedure described for compound **23b**, to give **27** (0.066 g, 59%) as a white foam. MS (ESI^+): m/z 1023.0 ($M + 1^+$). Anal. for $C_{50}H_{79}N_9O_{10}Si_2$: C, H, N.

4-*N*-[N^{α}-(Benzyloxycarbonyl)valylprolylvalylprolyl]-1-β-D-arabinofuranosyladenosine (28). To Cbz-[Val-Pro-Val-Pro]-[3′,5′-TBS-ara-A] (**27**) (0.040 g, 0.039 mmol) was added a 1.0 M solution of TBAF in THF (0.31 mL, 0.31 mmol) at room temperature and under argon atmosphere. The reaction mixture was stirred for 3 h, and then $CaCO_3$ (0.065 g), DOWEX 50Wx8 (0.194 g), and MeOH (0.5 mL) were added. The resulting suspension was stirred for 1 h, and the solvent was evaporated to dryness. The crude was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 20:1) to give **28** (0.023 g, 76%) as a white solid. MS (ESI^+): m/z 794.7 ($M + 1^+$). Anal. for $C_{38}H_{51}N_9O_{10}$: C, H, N.

4-*N*-[Valylprolylvalylprolyl]-1-β-D-arabinofuranosyladenosine (29). Cbz-[Val-Pro-Val-Pro]-[ara-A] (**28**) (0.020 g, 0.025 mmol) was hydrogenated at 1 atm at room temperature for 24 h, via a similar hydrogenation procedure described for compound **4a**, to give **29** (0.014 g, 85%) as a white foam. ^1H NMR (300 MHz, DMSO-d_6): δ 0.82−0.96 (m, 12H, 4γ-CH_3, 2Val), 1.53−2.27 (m, 10H, 2β-CH, 2Val, 2β-CH_2, 2Pro, 2γ-CH_2, 2Pro), 3.16 (d, 2H, δ-CH_2, Pro, J = 5.0 Hz), 3.58−3.83 (m, 5H, H-4′, H-5′, δ-CH_2, Pro), 4.11−4.24 (m, 3H, H-2′, H-3′, α-CH, Pro), 4.31−4.53 (m, 2H, 2α-CH, 2Val), 4.85 (bs, 1H, α-CH, Pro), 5.11 (t, 1H, OH-5′, J = 5.4 Hz), 5.58 (d, 1H, OH-3′, J = 4.6 Hz), 5.66 (d, 1H, OH-3′, J = 5.3 Hz), 6.37 (d, 1H, H-1′, J = 5.0 Hz), 7.92−7.98 (m,1H, NH, Val), 8.51 (s, 1H, H-8), 8.62 (s, 1H, H-2), 10.87 (bs, 1H, NH, ara-A). MS (ESI^+): m/z 660.7 ($M + 1^+$). Anal. for $C_{30}H_{45}N_9O_8$: C, H, N.

Water-Solubility Studies. Water solubility of the prodrugs and the parent compounds was determined by HPLC analysis. HPLC was carried out on a Waters 484 system using Novapack C18 reverse phase column: flow rate, 1 mL/min; detection, UV 254 nm; gradient solvent system A/B (acetonitrile/water), initial 15% A + 85% B; 5 min linear gradient to 25% A + 75% B; 5 min linear gradient to 35% A + 65% B; 10 min linear gradient to 45% A + 55% B; 5 min linear gradient to 60% A + 40% B; 5 min linear gradient to 100% A. Excess amount of the prodrug or of the parent drug was suspended in deionized water, sonicated for 10 min at room temperature, and then equilibrated overnight at room temperature. The samples were centrifuged at 14 000 rpm in an Eppendorf microcentrifuge for 1.5 min at room temperature. An aliquot of the clear supernatant was removed and diluted to a concentration within the range of a five-point standard curve. Water solubility was calculated from each peak area of the solution by HPLC compared with the sample dissolved in dimethyl sulfoxide as the standard, the exact concentration of which is known.

Biological Methods. Compounds and Enzymes. Human soluble CD26 was purified as described.[49,50] Human recombinant CD26, expressed in Sf9 cells, was obtained from Sigma (St. Louis, MO) (1.4 mg protein/mL, 48 U/mg). Vildagliptin (a specific CD26 inhibitor)[48−50] was kindly provided by I. De Meester (Antwerp, Belgium). Fetal bovine serum (FBS) was obtained from Integro (Dieren, The Netherlands), and human serum was provided by the Blood Bank, Leuven, Belgium.

Conversion of Di- and/or Tetrapeptide Prodrugs to the Corresponding Parent Compound. The 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 mixtures contained 50 μM test compound (di- or tetrapeptide prodrugs of 6-aminoquinoline (6-AQ), doxorubicin, TSAO-m^5C, ara-C, and ara-A) in PBS (containing 0.1% DMSO). The reaction was started by the addition of purified CD26 (1.5 mU) or 20% of HS (in PBS) or BS (in PBS) at 37 °C. Stability of the prodrugs was performed in PBS in the absence of enzyme or serum. At different time points (as indicated in the figures) 100 μL was withdrawn from the reaction mixture, added to 200 μL of cold methanol, and put on ice for 10 min. Then the mixtures were centrifuged at 13 000 rpm for 5 min at 4 °C and 250 μL supernatant was analyzed by HPLC on a reverse phase RP-8 column, using the following buffers and gradients: buffer A, 50 mM NaH_2PO_4 + 5 mM heptanesulfonic acid pH 3.2; buffer B, acetonitrile). Gradient A: 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. The gradients allowed us to separate the di- and/or tetrapeptide prodrugs from the corresponding parent drugs.

For the determination of the kinetic parameters (K_m, V_{max}), CD26 (human recombinant CD26 derived from Sigma) was exposed to different concentrations of the test compounds (i.e., 50, 25, 10, 5, and 2 μM for [ValPro]-[6-aminoquinoline], 1000, 750, 500, 250, 100, and 50 μM for [AspPro]-[6-aminoquinoline], 50, 25, 10, 5, and 2 μM for [ValPro]-[doxorubicine], and 50, 25, 10, 5, and 2 μM for [ValProValPro]-[ara-A]. Hydrolysis (removal of the prodrug moiety) was measured after 15, 30, and 45 min as described above. K_m and V_{max} values were calculated at each time point, and average values were made for the data obtained from the three time points. The kinetic values are the average of at least two to three independent experiments.

[ValPro]-[6-aminoquinoline], [AspPro]-[6-aminoquinoline], [ValPro]-[doxorubicine], and [ValProValPro]-[ara-A] (25 μM) were exposed to CD26, 20% HS, or 20% BS (as described above) in the presence or absence of different concentrations of the DPPIV/CD26 inhibitor vildagliptin (ranging between 50 and 0.05 μM). Conversion of the prodrugs to the parent compounds was evaluated after 15, 30, 45, and 60 min and analyzed as described above.

Antiviral Assays. Human T lymphocytic CEM cells were cultured in RPMI-1640 medium (Gibco, Paisley, Scotland)

supplemented with 10% fetal bovine serum (FBS) (BioWittaker Europe, Verviers, Belgium), 2 mM L-glutamine (Gibco), and 0.075 M $NaHCO_3$ (Gibco). HIV-1 (IIIB) was obtained from Dr. R. C. Gallo and Dr. M. Popovic (at that time at the National Cancer Institute, NIH, Bethesda, MD).

The CEM cells ($\sim 4.5 \times 10^5$ cells/mL) were suspended in fresh cell culture medium and infected with HIV (IIIB and ROD) at 100 $CCID_{50}$ (1 $CCID_{50}$ being the virus dose infective for 50% of the cell cultures) per milliliter of cell suspension. Then 100 μL of the infected cell suspension was transferred to microplate wells, mixed with 100 μL of appropriate (freshly prepared) dilutions of the test compounds (i.e., final concentrations of 2000, 400, 80, 16, 3.2, and 0.62 μM), and further incubated at 37 °C. After 4−5 days, giant cell formation was recorded microscopically in the CEM cell cultures. The 50% effective concentration (EC_{50}) corresponded to the compound concentrations required to prevent syncytium formation in the virus-infected cell cultures by 50%.

The antiherpes simplex virus type 1 [HSV-1 (KOS)] and vaccinia virus [VV (Lederle)] activity was determined by infecting HEL cell cultures with 100 $CCID_{50}$/mL in 96-well microtiter plates in the presence or absence of the prodrug [ValProValPro]-[ara-A] and 20 μM CD26 inhibitor (vildagliptin) (added as one single administration at the initiation of the virus infection). The antiviral activity was scored at day 3 after infection and based on estimation of the virus-induced cytopathicity.

Proliferation Assays. The cytostatic activity against murine leukemia L1210, human lymphocyte CEM, and cervix carcinoma HeLa cells was measured in 200 μL wells of a 96-well microtiter plate (initial cell number of $(5-7.5) \times 10^4$ cells/well). After 48 (L1210) or 72 (CEM, HeLa) h, the tumor-cell number was determined by a Coulter counter. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to inhibit tumor cell proliferation by 50%.

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Supporting Information Available: Elemental analysis data for novel compounds **1b**, **2b**, **3a**,**b**, **4a**,**b**, **5**−**7**, **8a**,**b**, **9b**, **11b**, **18**, **19**, **21a**,**b**, **22a**,**c**, **23b**,**c**, and **26**−**29**; chemical procedures for the synthesis of di- and tetrapeptide derivatives **1b**, **2b**, and **17**−**19**; ^{1}H NMR and ^{13}C NMR chemical shifts assigments of compounds **3a**,**b**, **4b**, **6**, **8a**−**c**, **11b**, **21a**,**b**, **22a**, **23b**,**c**, and **26**−**28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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