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Efficient conversion of tetrapeptide-based TSAO prodrugs to the parent drug by dipeptidyl-peptidase IV (DPPIV/CD26)

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Abstract

A novel prodrug approach has been evaluated using the anti-HIV-active TSAO molecule as the prototype drug to prove the kinetics with purified enzyme and the principles of conversion to the parent compound in sera and cell culture. When a variety of tetrapeptidyl amide prodrugs of NAP-TSAO were synthesized and exposed to purified dipeptidyl-peptidase IV (DPPIV/CD26) as well as human and bovine sera, they are converted to the parent NAP-TSAO drug in two successive steps by both purified CD26 and human and bovine serum. The efficiency of conversion strongly depends on the nature of the amino acid that has to be cleaved-off from the prodrug molecule. The tetrapeptidyl prodrug **20** showed a more than 10-fold improved water-solubility in comparison to that of the parent compound NAP-TSAO. The antiviral activity of the prototype NAP-TSAO could also be modulated by introducing different tetrapeptide moieties on the molecule resulting, in some cases, in a superior antiviral potential in cell culture than the parent drug.

Keywords: Dipeptidyl-peptidase IV (DPPIV); CD-26; Prodrugs; TSAO conjugates; TSAO-T compounds

1. Introduction

The lymphocyte surface glycoprotein CD26, also designated dipeptidyl-peptidase IV (DPPIV), is a transmembrane serine peptidase that can also occur in a soluble form (De Meester et al., 1999; Lambeir et al., 2003; Yaron and Naider, 1993). Dipeptidyl-peptidase IV/CD26 (DPPIV/CD26) belongs to the prolyloligopeptidase family (De Meester et al., 2003), which selectively cleaves X-Pro or to a lesser extent X-Ala dipeptides from the N-termini of polypeptides and proteins with a penultimate proline or alanine residue (De Meester et al., 1999, 2003; Yaron and Naider, 1993; Mentlein, 1999). However, it tolerates a wide range of amino acid residues at the ultimate N-terminal end. A free amino group on the ultimate amino acid is one of the prerequisites for substrate recognition by the enzyme. DPPIV/CD26 is unable to hydrolyze substrates with proline, hydroxyproline or *N*-methyl glycine on the third amino acid position (De Meester et al., 1996, 1999; Lambeir et al., 2003; Mentlein, 1999, 2004). Tripeptides with proline (Augustyns et al., 1999) are DPPIV inhibitors. DPPIV truncates several bio-active peptides of medical importance (De Meester et al., 1999, 2003; Lambeir et al., 2003; Yaron and Naider, 1993). DPPIV/CD26 occurs as a membrane-bound enzyme present on several cell types, but also as a soluble form present in plasma (De Meester et al., 1999; Yaron and Naider, 1993; Mentlein, 1999).

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 (Balzarini et al., 2004). 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. Based on this study, we have recently reported (García-Aparicio et al., 2006) a novel type

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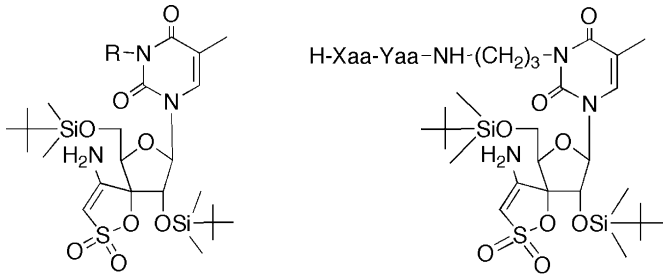


	Xaa	Yaa
3a	Val	Pro
3b	Lys	Pro
3c	Val	Ala
3d	Asp	Pro

Fig. 1. Structures of TSAO-T (**1**), NAP-TSAO (**2**) and H-[Xaa-Yaa]-[NAP-TSAO] conjugates of general formula **3**.

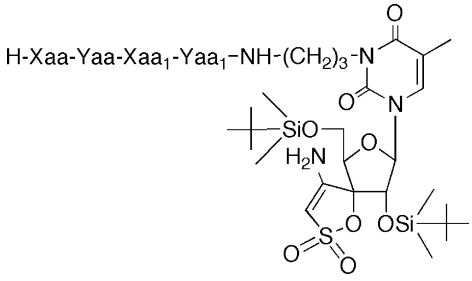
of prodrug approach that can be applied to mediate the solubility, and potentially also the bioavailability of therapeutic agents. In our approach a dipeptide moiety is linked to the free amino terminal of a non-peptidic drug through an amide bond which is specifically cleaved by DPPIV/CD26. The presence of a proline near the N-terminus serves as structural protection against non-specific proteolytic degradation (many exopeptidases do not recognize such sequences) (Vanhoof et al., 1995). For the initial studies to validate the novel DPPIV/CD26-based prodrug approach, we focused on the anti-HIV lipophilic TSAO derivatives as a prototype model compound, earlier developed in our laboratories (Camarasa et al., 1992, 2004; Balzarini et al., 1992, 1993). Thus the TSAO-T molecule (**1**, Fig. 1) was chosen as prototype compound for proof of the concept.

A variety of dipeptidyl amide prodrugs of TSAO molecules deprotected at the peptide N-terminus of general formula **3** (Fig. 1) bearing dipeptides of different nature were synthesized and evaluated for their ability to act as efficient substrates for the enzyme DPPIV/CD26 (García-Aparicio et al., 2006). Our data revealed that CD26 could specifically recognize these prodrugs as efficient substrates to be converted to the parent compound (García-Aparicio et al., 2006).

Thus, the known substrate specificity of CD26 to cleave natural peptides (De Meester et al., 2003) proved also 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 favoured at the amino terminal position, and negatively charged Xaa such as Asp is among the least favoured amino acids, whereas the positively charged Xaa Lys is usually a well-accepted substrate for CD26. Thus, the substrate activity and selectivity of dipeptidyl derivatives of synthetic compounds to be cleaved by CD26 can be more or less predicted based on the known SAR of CD26 for its natural peptidic substrates.

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, it is possible to modify the half-life of the prodrug in the plasma by changing the nature of the dipeptide. This will allow to modulate the release of the parent compound in 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. 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.

Since, according to the above mentioned, it is possible to modify the hydrolysis rate (half-life) of the conjugates by modifying the nature of the amino acids (Xaa, Yaa), now, in a further step, we considered of interest to study how the length and nature of the peptides would influence the half-life and the physicochemical properties of the conjugates. In this paper we describe for the first time the synthesis and study of tetrapeptidyl amide prodrugs of TSAO-T molecules of general formula H-[Xaa-Yaa-Xaa$_1$-Yaa$_1$]-[NAP-TSAO] (Fig. 2). The tetrapeptide sequences Val-Pro-Val-Pro, Val-Ala-Val-Pro, Val-Ala-Val-Ala and Lys-Pro-Asp-Pro were chosen because in our previous study with [Xaa-Yaa]-[NAP-TSAO] conjugates those bearing the dipeptides Val-Pro, Lys-Pro, Val-Ala and Asp-Pro showed different conversion rates to the parent compound, after 24 h, in the presence of DPPIV/CD26 (García-Aparicio et al., 2006). Thus, Val-Pro was the sequence of the model conjugates (**3a**, Fig. 1) (61% conversion to the parent compound after 24 h). Conjugates bearing Lys-Pro sequences (**3b**) ranked among the most efficient substrates for CD26 (conversion to the parent compound was complete (∼100%) after 24 h, whereas those bearing Val-Ala (**3c**, 35% conversion after 24 h) or Asp-Pro (**3d**, 30% conversion after 24 h) were among the least efficient substrates.



Xaa	Yaa	Xaa_1	Yaa_1
Val	Pro	Val	Pro
Val	Ala	Val	Pro
Val	Ala	Val	Ala
Lys	Pro	Asp	Pro

Fig. 2. Structures of tetrapeptidyl NAP-TSAO prodrugs of general formula H-[Xaa-Yaa-Xaa$_1$-Yaa$_1$]-[NAP-TSAO].

2. Materials and methods

2.1. Synthesis

2.1.1. General methods

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. Deuterated DMSO was used as a solvent unless otherwise noted. All chemical shifts (δ) are expressed in ppm. Coupling constants (J) are given in Hertz (Hz). 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 and Z-Val-Ala-OH and H-Val-Pro-O^{t}Bu·HCl were purchased from Bachem Feinchemikalien. The tetrapeptide derivatives Z-Val-Ala-Val-Ala-OH and Z-Lys(Z)-Pro-Asp(Bzl)-Pro-OH were purchased from Neosystem.

2.1.2. Z-Val-Pro-Val-Pro-O^{t}Bu (7)

A solution of Z-Val-Pro-OH (García-Aparicio et al., 2006) (0.68 g, 1.94 mmol) in CH_2Cl_2 (5 mL) was successively treated at room temperature with (benzotriazol-1-yl-oxy)-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) (1.02 g, 2.32 mmol), H-Val-Pro-O^{t}Bu·HCl (0.60 g, 1.94 mmol) and TEA (2.2 equiv.) and the mixture was stirred at room temperature overnight. After removal of the solvent in vacuo, the residue was dissolved in CH_2Cl_2 (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 residue was purified by flash column chromatography (hexane:ethyl acetate, 1:3) to give **7** (0.78 g, 68% yield) as a white foam; ^{1}H NMR (300 MHz): δ = 0.89 (d, 12H, γ-CH_3, Val), 1.37 (s, 9H, ^{t}Bu), 1.71–1.96 (m, 9H, β-CH_2, γ-CH_2, Pro, β-CH, Val), 2.11 (m, 1H, β-CH_2, Pro), 3.64 (m, 4H, δ-CH_2, Pro), 4.02 (m, 1H, α-CH, Val), 4.14 (m, 1H, α-CH, Pro), 4.36 (m, 1H, α-CH, Val), 4.43 (m, 1H, α-CH, Pro), 5.01 (AB system, 2H, CH_2, Z, J = 12.2 Hz, J = 14.9 Hz), 7.32 (m, 5H, Ar, Z), 7.42 (d, 1H, NH, Val, J = 8.6 Hz), 7.95 (d, 1H, NH, Val, J = 8.8 Hz); MS: m/z = 601.3 (M + 1$^+$); elemental analysis calcd. (%) for $C_{32}H_{48}N_4O_7$: C 63.98, H 8.05, N 9.33; found: C 64.10, H 8.15, N 9.40.

2.1.3. Z-Val-Ala-Val-Pro-O^{t}Bu (8)

Following a similar procedure to that described for compound **7**, Z-Val-Ala-OH (García-Aparicio et al., 2006) (0.63 g, 1.96 mmol) was reacted with H-Val-Pro-O^{t}Bu·HCl (0.61 g, 1.96 mmol). The residue was purified by flash column chromatography (hexane:ethyl acetate, 1:3) to give **8** (0.83 g, 75% yield) as a syrup; ^{1}H NMR (300 MHz): δ = 0.89 (d, 12H, γ-CH_3, Val), 1.16 (d, 3H, β-CH_3, Ala, J = 6.8 Hz), 1.37 (s, 9H, ^{t}Bu), 1.68–1.89 (m, 5H, β-CH_2, γ-CH_2, Pro, β-CH, Val), 2.14 (m, 1H, β-CH_2, Pro), 3.55 (m, 1H, δ-CH_2, Pro), 3.70 (m, 1H, δ-CH_2, Pro), 3.86 (m, 1H, α-CH, Val), 4.20 (m, 1H, α-CH, Pro), 4.33 (m, 2H, α-CH, Ala, Val), 5.02 (s, 2H, CH_2, Z), 7.29 (d, 1H, NH, Val, J = 8.8 Hz), 7.35 (bs, 5H, Ar, Z), 7.86 (d, 1H, NH, Val, J = 8.8 Hz), 8.00 (d, 1H, NH, Ala, J = 6.8 Hz)); MS: m/z = 574.3 (M+); elemental analysis calcd. (%) for $C_{30}H_{46}N_4O_7$: C 67.70, H 8.07, N 9.75; found: C 67.80, H 8.16, N 9.84.

2.1.4. Z-Val-Pro-Val-Pro-OH (9)

A solution of Z-Val-Pro-Val-Pro-O^{t}Bu (**7**) (0.66 g, 1.10 mmol) was treated with TFA (2.76 mL, 3.58 mmol) in CH_2Cl_2 (5 mL). The reaction was stirred at room temperature for 3 h. The solvent was removed in vacuo, and the residue was co-evaporated several times with CH_2Cl_2. The residue was dissolved in water and lyophilized to give **9** (0.49 g, 84%) as a syrup. The compound was used in the next step without further purification; ^{1}H NMR (200 MHz): δ = 0.89 (d, 12H, γ-CH_3, Val), 1.65–2.18 (m, 10H, β-CH_2, γ-CH_2, Pro, β-CH, Val), 3.64 (m, 4H, δ-CH_2, Pro), 4.19 (m, 2H, α-CH, Pro, Val), 4.32 (m, 1H, α-CH, Val), 4.40 (m, 1H, α-CH, Pro), 5.01 (AB system, 2H, CH_2, Z, J = 12.2 Hz, J = 14.9 Hz), 7.34 (m, 5H, Ar, Z), 7.45 (d, 1H, NH, Val, J = 8.6 Hz), 7.97 (d, 1H, NH, Val, J = 8.8 Hz); MS: m/z = 545.1 (M + 1$^+$).

2.1.5. Z-Val-Ala-Val-Pro-OH (10)

Following a similar procedure to that described for compound **9**, compound **8** (0.98 g, 1.71 mmol) was reacted with TFA (4.3 mL, 5.57 mmol) in CH_2Cl_2 (5 mL) to give **10** (0.73 g, 83% yield) as a syrup. The compound was used in the next step without further purification; ^{1}H NMR (300 MHz): δ = 0.86 (d, 12H, γ-CH_3, Val), 1.16 (d, 3H, β-CH_3, Ala, J = 6.8 Hz), 1.74–1.97 (m, 5H, β-CH_2, γ-CH_2, Pro, β-CH, Val), 2.14 (m, 1H, β-CH_2, Pro), 3.55 (m, 1H, δ-CH_2, Pro), 3.70 (m, 1H, δ-CH_2, Pro), 3.86 (m, 1H, α-CH, Val), 4.20 (m, 1H, α-CH, Pro), 4.33 (m, 2H, α-CH, Ala, Val), 5.02 (s, 2H, CH_2, Z), 7.29 (d, 1H, NH, Val, J = 8.8 Hz), 7.35 (m, 5H, Ar, Z), 7.86 (d, 1H, NH, Val, J = 8.8 Hz), 8.00 (d, 1H, NH, Ala, J = 6.8 Hz); MS: m/z = 519.4 (M + 1$^+$).

2.1.6. General coupling procedure for the synthesis of compounds 13–16

A solution of the corresponding C-deprotected tetrapeptide (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) **2** (Bonache et al., 2005) (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 (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 residue was purified by CCTLC on the Chromatotron.

*2.1.7. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[N$^{\alpha}$-(benzyloxycarbonyl)valyl-prolyl-valyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**13**)*

According to the general coupling procedure compound **2** (Bonache et al., 2005) (0.10 g, 0.15 mmol) was reacted with Z-Val-Pro-Val-Pro-OH **9** (0.11 g, 0.22 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 40:1) to give 0.06 g (41%) of **13** as a white foam; ^{1}H NMR (300 MHz): δ = 0.73, 0.86 (2s, 18 H, 2*t*-Bu) 0.90 (m, 12H, γ-CH_3, Val), 1.69 (m, 15H, –CH_2–, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.91 (m, 1H, CH_2NHCO), 3.15 (m, 1H, CH_2NHCO), 3.57 (m, 4H, δ-CH_2, Pro), 3.77 (m, 4H, α-CH, Val, 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, 2H, H-4′, α-CH, Val), 4.31 (m, 1H, α-CH, Pro), 4.41 (m, 1H, α-CH, Pro), 4.54 (d, 1H, H-2′, *J* = 8.0 Hz), 5.00 (s, 2H, CH_2, Z), 5.75 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, *J* = 8.0 Hz), 6.96 (bs, 2H, NH_2-4″), 7.34 (m, 5H, Ar, Z), 7.45 (d, 1H, NH, Val, *J* = 8.6 Hz), 7.62 (s, 1H, H-6), 7.81 (bt, 1H, NHCO), 7.93 (d, 1H, NH, Val, *J* = 8.6 Hz); MS: *m/z* = 1173.3 (M+); elemental analysis calcd. (%) for $C_{55}H_{88}N_8O_{14}SSi_2$: C 56.29, H 7.56, N 9.55; found: C 56.41, H 7.39, N 9.62.

*2.1.8. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[N$^{\alpha}$-(benzyloxycarbonyl)valyl-alanyl-valyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**14**)*

Following the general coupling procedure, nucleoside **2** (Bonache et al., 2005) (0.15 g, 0.23 mmol) was reacted with Z-Val-Ala-Val-Pro-OH **10** (0.17 g, 0.34 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 40:1) to give **14** (0.11 g, 44%) as a white foam; ^{1}H NMR (300 MHz): δ = 0.73, 0.86 (2s, 18H, 2*t*-Bu), 0.86 (m, 12H, γ-CH_3, Val), 1.15 (d, 3H, β-CH_3, Ala, *J* = 7.0 Hz), 1.61 (m, 2H, –CH_2–), 1.74–2.04 (m, 9H, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.93 (m, 1H, CH_2NHCO), 3.10 (m, 1H, CH_2NHCO), 3.60 (m, 2H, δ-CH_2, Pro), 3.78 (m, 4H, α-CH, Val, 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, 2H, H-4′, α-CH, Val), 4.31 (m, 2H, α-CH, Pro, Ala), 4.53 (d, 1H, H-2′, *J* = 8.3 Hz), 5.01 (s, 2H, CH_2, Z), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, *J* = 8.0 Hz), 6.94 (bs, 2H, NH_2-4″), 7.31 (m, 6H, NH, Val, Ar, Z), 7.61 (s, 1H, H-6), 7.83 (m, 2H, NH, Val, NHCO), 7.99 (d, 1H, NH, Ala, *J* = 7.0 Hz); MS: *m/z* = 1147.3 (M+); elemental analysis calcd. (%) for $C_{53}H_{86}N_8O_{14}SSi_2$: C 55.47, H 7.55, N 9.76; found: C 55.39, H 7.42, N 9.89.

*2.1.9. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[N$^{\alpha}$-(benzyloxycarbonyl)valyl-alanyl-valyl-alanylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**15**)*

Following the general coupling procedure, nucleoside **2** (Bonache et al., 2005) (0.10 g, 0.15 mmol) was reacted with Z-Val-Ala-Val-Ala-OH **11** (0.10 g, 0.22 mmol). The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 40:1) to give **15** (0.11 g, 69%) as a white solid; mp: 237–239 °C; ^{1}H NMR (300 MHz): δ = 0.73, 0.86 (2s, 18H, 2*t*-Bu), 0.86 (m, 12H, γ-CH_3, Val), 1.19 (2d, 6H, β-CH_3, Ala, *J* = 7.0 Hz), 1.61 (m, 2H, –CH_2–), 1.89 (bs, 3H, CH_3-5), 1.95 (m, 2H, β-CH, Val), 2.97 (m, 1H, CH_2NHCO), 3.11 (m, 1H, CH_2NHCO), 3.77 (m, 4H, α-CH, Val, N^3–CH_2, H-5′a), 3.92 (dd, 1H, H-5′b, *J* = 7.5 Hz, *J* = 12.4 Hz), 4.18 (m, 3H, H-4′, α-CH, Val, Ala), 4.36 (m, 1H, α-CH, Ala), 4.60 (d, 1H, H-2′, *J* = 8.3 Hz), 5.02 (s, 2H, CH_2, Z), 5.73 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, *J* = 8.0 Hz), 7.00 (bs, 2H, NH_2-4″), 7.33 (m, 6H, NH, Val, Ar, Z), 7.71 (m, 2H, H-6, NH, Ala), 7.87 (t, 1H, NHCO, *J* = 5.6 Hz), 7.94 (d, H, NH, Val, *J* = 8.6 Hz), 8.04 (d, 1H, NH, Ala, *J* = 7.0 Hz); MS: *m/z* = 1121.7 (M+); elemental analysis calcd. (%) for $C_{51}H_{84}N_8O_{14}SSi_2$: C 54.62, H 7.55, N 9.99; found: C 54.73, H 7.66, N 9.82.

*2.1.10. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[N$^{\alpha,\varepsilon}$-(dibenzyloxycarbonyl)lysyl-prolyl-(O-benzyl)aspartyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**16**)*

Nucleoside **2** (Bonache et al., 2005) (0.10 g, 0.15 mmol) was reacted with Z-Lys(Z)-Pro-Asp-Pro-OH **12** (0.17 g, 0.22 mmol) according to the general coupling procedure. The residue was purified by CCTLC on the Chromatotron (dichloromethane:methanol, 40:1) to give **16** (0.11 g, 52%) as a white foam; ^{1}H NMR (300 MHz): δ = 0.71, 0.85 (2s, 18 H, 2*t*-Bu), 1.33–1.96 (m, 15H, –CH_2–, CH_3-5, β-CH_2, γ-CH_2, δ-CH_2, Lys, β-CH_2, γ-CH_2, Pro), 2.50 (m, 1H, β-CH_2, Asp), 2.85 (m, 1H, β-CH_2, Asp), 3.15 (m, 4H, ε-CH_2, Lys, CH_2NHCO), 3.63 (m, 1H, δ-CH_2, Pro), 3.69 (m, 1H, δ-CH_2, Pro), 3.86 (m, 4H, 2H-5′, N^3–CH_2), 4.22 (m, 4H, H-4′, α-CH, Lys, Pro), 4.52 (d, 1H, H-2′, *J* = 7.8 Hz), 4.91 (m, 1H, α-CH, Asp), 4.98 (s, 4H, CH_2, Z), 5.03 (s, 2H, CH_2, Bzl), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, *J* = 7.8 Hz), 6.94 (bs, 2H, NH_2-4″), 7.20 (m, 1H, ζ-NHCO, Lys), 7.32 (m, 15H, Ar, Z, Bzl), 7.47 (d, 1H, NHCO, Lys, *J* = 7.8 Hz), 7.60 (m, 2H, H-6, NHCO), 8.18 (d, 1H, NH, Asp, *J* = 8.3 Hz); MS: *m/z* = 1442.3 (M+); elemental analysis calcd. (%) for $C_{70}H_{99}N_9O_{18}SSi_2$: C 58.27, H 6.92, N 8.74; found: C, 58.41; H 7.10, N 8.61.

*2.1.11. General N-deprotection procedure for the synthesis of compounds **17–20***

A solution of the corresponding protected conjugate intermediate [Z-Xaa-Yaa-Xaa$_1$-Yaa$_1$]-[NAP-TSAO] (1 equiv.) in methanol (8 mL) containing Pd/C (10%) (40%, w/w) 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 the deprotected conjugates **17–20**.

*2.1.12. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[valyl-prolyl-valyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**17**)*

According to the general *N*-deprotection procedure conjugate **13** (0.06 g, 0.05 mmol) was hydrogenated to give **17** (0.04 g,

80%) as a white foam; ^{1}H NMR (300 MHz): δ = 0.71, 0.85 (2s, 18 H, 2*t*-Bu), 0.90 (m, 12H, γ-CH_3, Val), 1.81 (m, 15H, –CH_2–, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.95 (m, 1H, CH_2NHCO), 3.09 (m, 1H, CH_2NHCO), 3.56 (m, 4H, δ-CH_2, Pro), 3.77 (m, 4H, α-CH, Val, N^3–CH_2, H-5′a), 3.92 (dd, 1H, H-5′b, *J* = 7.5 Hz, *J* = 12.4 Hz), 4.20 (m, 2H, H-4′, α-CH, Val), 4.34 (m, 1H, α-CH, Pro), 4.46 (m, 1H, α-CH, Pro), 4.58 (d, 1H, H-2′, *J* = 8.0 Hz), 5.73 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, *J* = 8.0 Hz), 7.00 (bs, 2H, NH_2-4″), 7.68 (s, 1H, H-6), 7.79 (m, 2H, NHCO, NH, Val), 8.03 (d, 1H, NH, Val, *J* = 8.6 Hz); MS: *m*/*z* = 1039.3 (M+); elemental analysis calcd. (%) for $C_{47}H_{82}N_8O_{12}SSi_2$: C 54.31, H 7.95, N 10.78; found: C 54.44, H 8.11, N, 10.67.

2.1.13. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[valyl-alanyl-valyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**18**)

Following the general *N*-deprotection procedure, conjugate **14** (0.07 g, 0.06 mmol) was hydrogenated to give **18** (0.05 g, 92%) as a white foam; ^{1}H NMR (300 MHz): δ = 0.86 (m, 12H, γ-CH_3, Val), 1.13 (d, 3H, β-CH_3, Ala, *J* = 7.0 Hz), 1.61 (m, 2H, –CH_2–), 1.74–2.04 (m, 9H, CH_3-5, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.92 (m, 1H, CH_2NHCO), 3.12 (m, 1H, CH_2NHCO), 3.60 (m, 2H, δ-CH_2, Pro), 3.79 (m, 4H, α-CH, Val, N^3–CH_2, H-5′a), 3.93 (dd, 1H, H-5′b, *J* = 7.5 Hz, *J* = 12.4 Hz), 4.20 (m, 2H, H-4′, α-CH, Val), 4.29 (m, 1H, α-CH, Pro), 4.39 (m, 1H, α-CH, Ala), 4.54 (d, 1H, H-2′, *J* = 8.3 Hz), 5.74 (s, 1H, H-3″), 5.96 (d, 1H, H-1′, *J* = 8.0 Hz), 6.94 (bs, 2H, NH_2-4″), 7.62 (s, 1H, H-6), 7.82 (bt, 1H, NHCO), 7.96 (m, 1H, NH, Val, *J* = 8.6 Hz), 8.10 (d, 1H, NH, Ala, *J* = 7.0 Hz); MS: *m*/*z* = 1013.3 (M+); elemental analysis calcd. (%) for $C_{45}H_{80}N_8O_{12}SSi_2$: C 53.33, H 7.96, N 11.06; found: C 53.21, H 8.11, N 11.01.

2.1.14. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[valyl-alanyl-valyl-alanylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**19**)

Conjugate **15** (0.08 g, 0.07 mmol) was hydrogenated, following the general *N*-deprotection procedure, to give **19** (0.05 g, 92%) as a white solid; mp: 175–177 °C; ^{1}H NMR (300 MHz): δ = 0.73, 0.86 (2s, 18 H, 2*t*-Bu), 0.86 (m, 12H, γ-CH_3, Val), 1.19 (2d, 6H, 2β-CH_3, Ala, *J* = 7.0 Hz), 1.63 (m, 2H, –CH_2–), 1.89 (bs, 3H, CH_3-5), 1.95 (m, 2H, β-CH, Val), 3.11 (m, 2H, CH_2NHCO), 3.77 (m, 4H, α-CH, Val, N^3–CH_2, H-5′a), 3.92 (dd, 1H, H-5′b, *J* = 7.5 Hz, *J* = 12.4 Hz), 4.15 (m, 3H, H-4′, α-CH, Val, Ala), 4.40 (m, 1H, α-CH, Ala), 4.56 (d, 1H, H-2′, *J* = 8.3 Hz), 5.74 (s, 1H, H-3″), 5.97 (d, 1H, H-1′, *J* = 8.0 Hz), 6.96 (bs, 2H, NH_2-4″), 7.71 (m, 2H, H-6, NH, Ala), 7.86 (m, 2H, NHCO, NH, Val), 7.94 (d, 1H, NH, Val, *J* = 8.6 Hz), 8.16 (m, 1H, NH, Ala); MS: *m*/*z* = 987.5 (M+); elemental analysis calcd. (%) for $C_{43}H_{78}N_8O_{12}SSi_2$: C 52.31, H 7.96, N 11.35; found: C 52.22, H 8.13, N 11.46.

2.1.15. [1-[2′,5′-Bis-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3-N-[3-N′-[lysyl-prolyl-aspartyl-prolylamino]propyl]thymine]-3′-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide] (**20**)

According to the general *N*-deprotection procedure, conjugate **16** (0.08 g, 0.05 mmol) was hydrogenated to give **20** (0.04 g, 85%) as a white foam; ^{1}H NMR (300 MHz): δ = 0.75, 0.84 (2s, 18 H, 2*t*-Bu), 1.33–1.98 (m, 15H, –CH_2–, CH_3-5, β-CH_2, γ-CH_2, δ-CH_2, Lys, β-CH_2, γ-CH_2, Pro), 2.40 (m, 4H, β-CH_2, Asp, ε-CH_2, Lys), 3.15 (m, 2H, CH_2NHCO), 3.63 (m, 1H, δ-CH_2, Pro), 3.69 (m, 1H, δ-CH_2, Pro), 3.86 (m, 4H, 2H-5′, N^3–CH_2), 4.21 (m, 4H, H-4′, α-CH, Lys, Pro), 4.52 (d, 1H, H-2′, *J* = 7.8 Hz), 4.98 (m, 1H, α-CH, Asp), 5.73 (s, 1H, H-3″), 5.95 (d, 1H, H-1′, *J* = 7.8 Hz), 6.97 (bs, 2H, NH_2-4″), 7.60 (m, 3H, H-6, NHCO, NH, Lys), 8.18 (d, 1H, NH, Asp, *J* = 8.3 Hz); MS: *m*/*z* = 1084.2 (M+); elemental analysis calcd. (%) for $C_{47}H_{81}N_9O_{14}SSi_2$: C 52.06, H 7.53, N 11.62; found: C 52.21, H 7.44, N 11.74.

2.2. Water-solubility studies

Water-solubility of the prodrug **20** and the parent compound **2** 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 and 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 acetonitrile as the standard, the exact concentration of which is known.

2.3. Biological methods

2.3.1. Compounds and enzymes

Human soluble CD26 was purified as described and kindly provided by I. De Meester and A.-M. Lambeir (Antwerp, Belgium) (De Meester et al., 1996, 2003). Foetal bovine serum (FBS) was obtained from Integro (Dieren, The Netherlands) and human serum provided by the Blood Bank, Leuven, Belgium.

2.3.2. Conversion of tetrapeptidyl prodrugs of NAP-TSAO to the corresponding dipeptidyl prodrug and the 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 of test compound (tetrapeptidyl prodrugs of

NAP-TSAO) 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. 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 analysed by HPLC on a reverse phase RP-8 column, using 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.

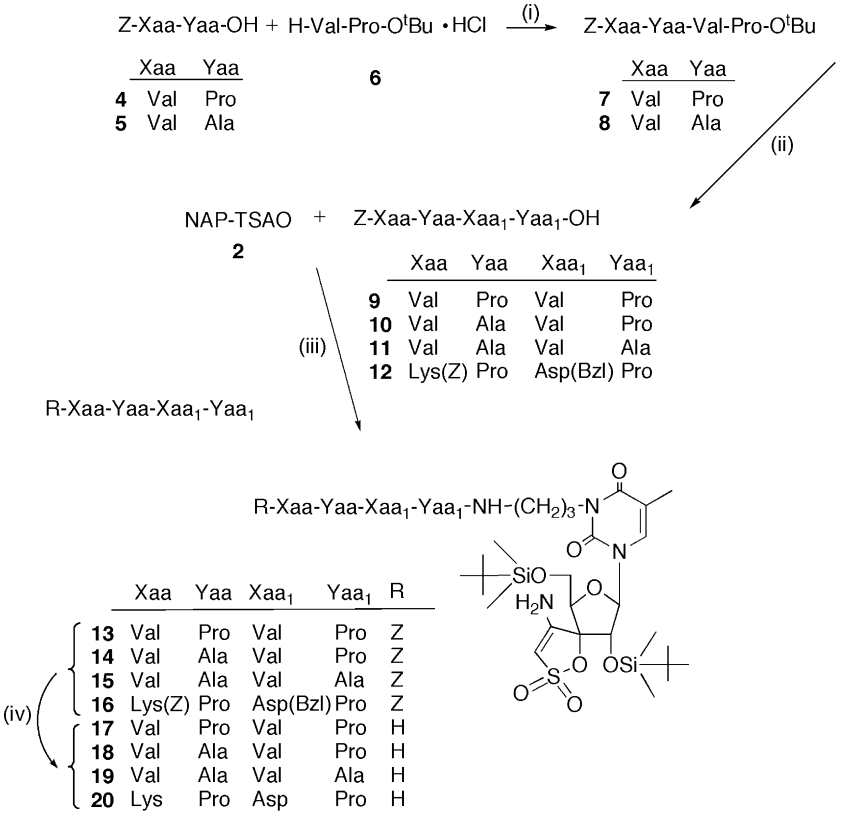
Gradient B: 2 min 98% A + 2% B; 6 min linear gradient to 80% A + 20% B; 2 min linear gradient to 75% A + 25% B; 2 min linear gradient to 65% A + 35% B; 8 min linear gradient to 50% A + 50% B; 10 min 50% A + 50% B; 10 min linear gradient to 20% A + 80% B; 5 min 20% A + 80% B; 15 min linear gradient to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

The gradients allowed to separate the tetrapeptidyl-NAP-TSAO prodrugs from the corresponding dipeptidyl-NAP-TSAO and parent NAP-TSAO.

2.3.3. Antiviral assays

Human T lymphocytic CEM and MT-4 cells were cultured in RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 10% foetal bovine serum (FBS) (BioWittaker Europe, Verviers, Belgium), 2 mM L-glutamine (Gibco) and 0.075 M $NaHCO_3$ (Gibco). HIV-1 (III_B) was obtained from Dr. R.C. Gallo and Dr. M. Popovic (at that time at the National Cancer Institute, NIH, Bethesda, MD). The HIV-2 isolate ROD was provided by Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France).

Human T lymphocytic CEM and MT-4 cells (∼4.5×10^5 cells/mL) were suspended in fresh cell culture medium and infected with HIV (III_B 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 or cell lysis in the MT-4 cell culture by trypan blue staining. The 50% effective concentration (EC_{50}) corresponded to the compound concentrations required to prevent syncytium formation or cell destruction in the virus-infected cell cultures by 50%.



Reagents: (i) BOP, TEA,CH_2Cl_2; (ii) TFA, CH_2Cl_2; (iii) BOP, TEA, CH_2Cl_2; (iv) H_2, Pd(C), CH_3OH

Scheme 1.

3. Results and discussion

3.1. Chemical results

Due to the low reactivity of the 4″-amino group of the spirosultone moiety of TSAO derivatives (De Castro et al., 2005), as for the dipeptidyl amide prodrugs of TSAO molecules (García-Aparicio et al., 2006) the N-3 aminopropyl TSAO-T derivative (NAP-TSAO, **2**, Fig. 1) 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 (Bonache et al., 2005). Moreover, from our own results, in the whole series of N-3 substituted TSAO derivatives, it is known 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 (Bonache et al., 2005). The target compound was obtained as previously described (Bonache et al., 2005). The synthesis of the target conjugates was carried out in a two-step procedure that consisted of the coupling of the appropriate *C*-deprotected tetrapeptide derivative Z-Xaa-Yaa-Xaa_1-Yaa_1-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 conditions compatible with TBDMS groups.

The synthesis of tetrapeptide conjugates was performed as follows. The tetrapeptides Z-Val-Pro-Val-Pro-OH (**9**) and Z-Val-Ala-Val-Pro-OH (**10**) were not commercially available and were prepared by coupling of the dipeptides Z-Val-Pro-OH (**4**) and Z-Val-Ala-OH (**5**) (García-Aparicio et al., 2006) (Scheme 1) with commercially available H-Val-Pro-O^{t}Bu·HCl (**6**) in the presence of BOP and TEA, giving the corresponding fully protected tetrapeptides **7** and **8** in 68% and 75% yield, respectively. Acid hydrolysis of the ^{t}Bu ester group (TFA solution in CH_2Cl_2) yielded the corresponding *C*-deprotected tetrapeptides **9** and **10** in good yields (84% and 83% yield, respectively) after purification by flash column chromatography.

Coupling of tetrapeptides **9**, **10**, or commercially available **11** and **12** with TSAO compound **2** in the presence of BOP and TEA, gave the corresponding protected intermediate conjugates **13–16** in moderate to good yields (41–70%). Removal of the benzyl groups by catalytic hydrogenation in the presence of 10% Pd/C in CH_3OH afforded the desired final deprotected conjugates **17–20** in good yields (80–90%).

3.2. Biological results

3.2.1. Conversion of *17–20* to NAP-TSAO by purified CD26

The Val-Pro-Val-Pro-NAP-TSAO (**17**) and Val-Ala-Val-Pro-NAP-TSAO (**18**) prodrug derivatives were efficiently converted to the parent NAP-TSAO (**2**) by purified CD26. After 4 h of incubation ~85% of prodrug **17** has been converted to the parent compound (Fig. 3A). A longer incubation time did not further allow the reaction to proceed. The hydrolysis reaction

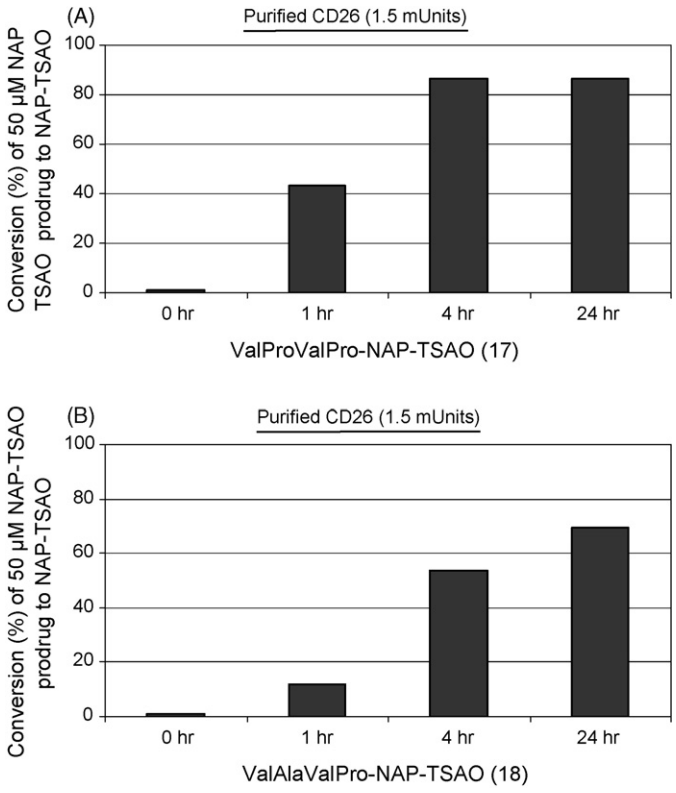


Fig. 3. Conversion of prodrugs ValProValPro-NAP-TSAO (**17**) and ValAlaValPro-NAP-TSAO (**18**) to NAP-TSAO (**2**) in the presence of purified CD26 at 1, 4, or 24 h.

with **18** was less pronounced than with **17** (Fig. 3B). After 4 h, ~55% of **18** was converted to the parent drug, but the hydrolysis further proceeded to almost 70% after 24 h. No intermediate Val-Pro-NAP-TSAO (**3a**) could be measurably detected during the enzymatic conversion reaction. This may mean that the first reaction in the two-step conversion of the prodrug to the parent compound is rate limiting and occurs slower than the second reaction step. The fact that the hydrolysis of both compounds leveled off after 75–85% conversion to the parent compound is likely due to feed-back inhibition of the reaction by the released dipeptides. Indeed, it was previously shown that the dipeptide ValPro inhibit the CD26-catalysed reaction at a 50%-inhibitory concentration of ~50 μM (García-Aparicio et al., 2006). Val-Ala shows a more pronounced inhibitory activity against CD26 than Val-Pro (Lambeir, unpublished), explaining the slightly faster leveling-off of the reaction during conversion of prodrug **18** to NAP-TSAO.

The conversion of the NAP-TSAO prodrugs **19** (Val-Ala-Val-Ala-NAP-TSAO) and **20** (Lys-Pro-Asp-Pro-NAP-TSAO) showed different hydrolysis kinetics by purified CD26 (Figs. 4 and 5). A slight but detectable appearance of the intermediate prodrug Val-Ala-NAP-TSAO could be observed after 4 h of incubation of the prodrug **19**. The presence of this metabolite was more pronounced after 24 h (Fig. 4A). However, only after this relatively long incubation period, ~30% of parental NAP-TSAO was found. An additional (minor) metabolite was found but not identified. Also with the Lys-Pro-Asp-Pro-NAP-TSAO

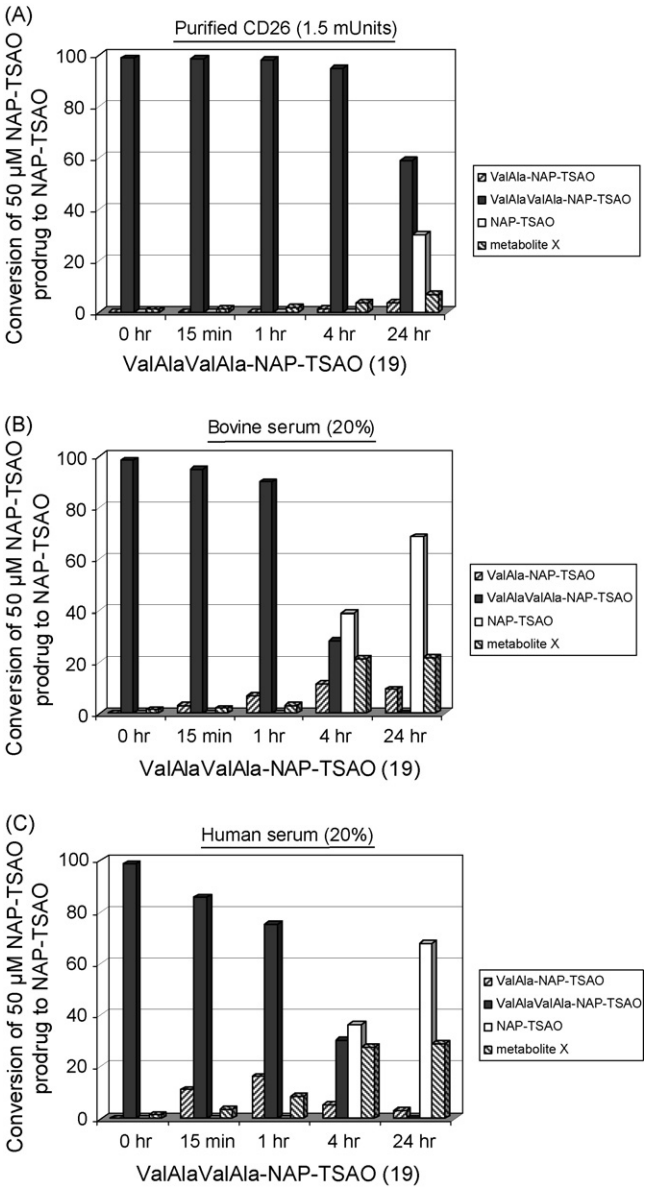


Fig. 4. Conversion of prodrug ValAlaValAla-NAP-TSAO (**19**) to NAP-TSAO (**2**) by purified CD26, Bovine and Human sera at 15 min, 1, 4, or 24 h.

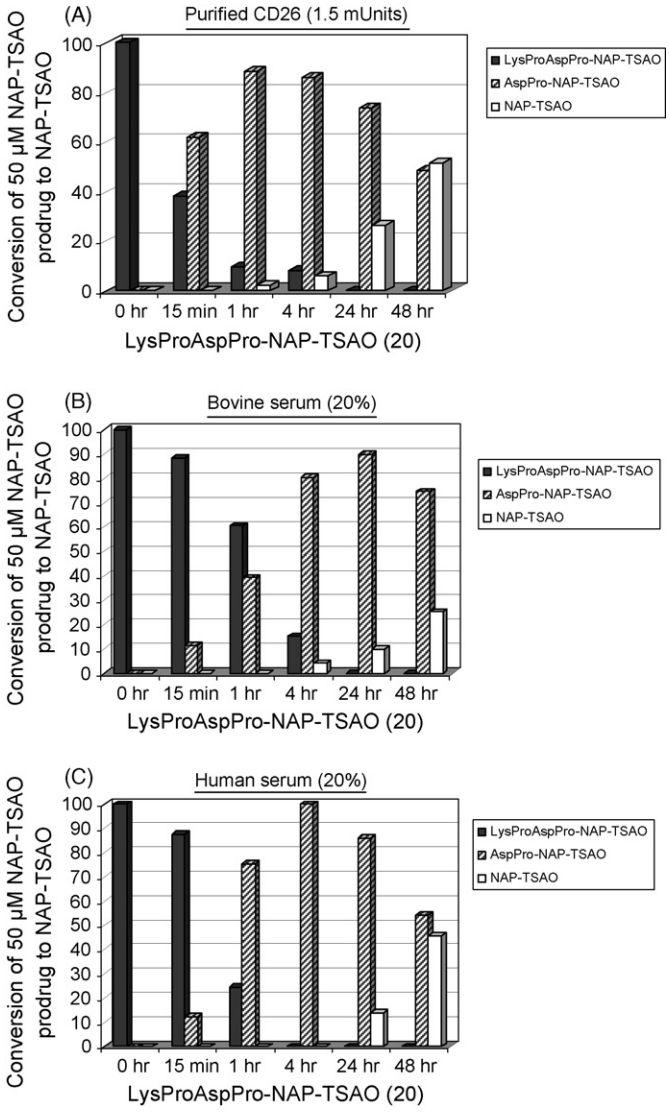


Fig. 5. Conversion of prodrug LysProAspPro-NAP-TSAO (**20**) to NAP-TSAO (**2**) by purified CD26, Bovine and Human sera at 15 min, 1, 4, 24 or 48 h.

prodrug (**20**), the intermediate dipeptidyl derivative Asp-Pro-NAP-TSAO could be detected upon exposure of purified CD26 (Fig. 5A). Here, this intermediate metabolite was already abundantly formed within 15 min of the reaction (∼60%) after which further progressive hydrolysis to parent compound occurred, showing a concomitant decrease of the intermediate (Fig. 5A). Our findings indicate that the first-step conversion of **19** and **20** to the intermediate Val-Ala-NAP-TSAO and Asp-Pro-NAP-TSAO, respectively, occurs very rapidly, whereas the eventual conversion of both the Val-Ala-NAP-TSAO and the Asp-Pro-NAP-TSAO intermediate, to parent compound proved clearly rate limiting. In fact, in the case of the tetrapeptidyl prodrug **20**, a virtual full conversion to the intermediate dipeptidyl prodrug Asp-Pro-NAP-TSAO occurs before further conversion of the dipeptidyl derivative starts to proceed to the parent drug. These observations are in full agreement with our previous findings (García-Aparicio et al., 2006), and with literature data (De Meester et al., 1999) that a NH_2-terminal Lys-Pro dipeptidyl group is by far a more efficient substrate for CD26 than a NH_2-terminal Asp-Pro dipeptidyl derivative.

The Val-Ala-Val-Ala-NAP-TSAO prodrug **19** has also been investigated on its stability in human and bovine serum. In both sera, the prodrug was progressively converted to the parent drug through the dipeptidyl intermediate as also observed in the presence of purified CD26. After 24 h, no prodrug and tiny amounts of intermediate were detectable (Fig. 4B and C). The unidentified additional metabolite, found in the purified CD26 experiments, was also found to be present in the sera conversion experiments. However, after 24 h, almost 70% of parent drug was formed. Thus, the conversion of the Val-Ala-Val-Ala-NAP-TSAO prodrug **19** to the parent drug NAP-TSAO (**2**) proceeded efficiently in the presence of 20% human and bovine serum. Therefore, it can be assumed that, if such a prodrug would have been administered intravenously *in vivo*, it would have been effi-

Table 1
Anti-HIV and cytostatic activity of tetrapeptidyl prodrugs of NAP-TSAO

Compound	MT-4[a]		CEM[a]		MT-4[b]	CEM[b]
	HIV-1	HIV-2	HIV-1	HIV-2		
18	0.32 ± 0.21	>10	0.14 ± 0.02	>10	11 ± 6.5	14 ± 6.1
19	0.084 ± 0.003	>10	0.060 ± 0.028	>10	16 ± 2.3	18 ± 3.7
20	2.5 ± 2.1	>50	2.7 ± 1.1	>50	148 ± 28	114 ± 35
NAP-TSAO (**2**)	0.52 ± 0.31	>10	0.14 ± 0.09	>10	14 ± 7.8	12 ± 8.3

[a] EC_{50} (μM). Fifty percent effective concentration or compound concentration required to inhibit virus-induced cell lysis (MT-4) or giant cell formation (CEM) by 50%.
[b] CC_{50} (μM). Fifty percent cytostatic concentration or compound concentration required to inhibit cell proliferation by 50%.

ciently converted to the parent drug by the CD26 present in the plasma.

3.2.2. *Antiviral activity of tetrapeptide prodrugs of NAP-TSAO*

Compounds **18–20** have been evaluated for their anti-HIV activity in T lymphocyte MT-4 and CEM cell cultures (Table 1) and compared with the parent drug NAP-TSAO (**2**). These studies were undertaken to provide proof-of-concept of efficient conversion of the novel tetrapeptide prodrugs to their parent compounds in virus-infected cell cultures. The prodrug derivatives invariably showed a pronounced antiviral activity in cell culture. Whereas **18** proved an equally efficient inhibitor of HIV-1 replication as the parent compound NAP-TSAO (**2**), the Val-Ala-Val-Ala-NAP-TSAO (**19**) was 2.5–6-fold more inhibitory. In contrast, Lys-Pro-Asp-Pro-NAP-TSAO (**20**) was 5–20-fold less inhibitory than the parent compound. At first glance, this observation is somewhat surprising. However, in a previous study, the antiviral activities of the NAP-TSAO derivatives containing the dipeptidyl moieties Val-Pro, Ala-Pro and Asp-Pro have been investigated (García-Aparicio et al., 2006). It was found that the Val-Pro- and Ala-Pro-NAP-TSAO derivatives showed a comparable antiviral activity as the parent compound, but the Asp-Pro-NAP-TSAO derivative was 2.5–10-fold less inhibitory to the virus replication (but also less toxic). Thus our findings on the lesser antiviral and cytostatic activity of the tetrapeptidyl prodrug **20**, releasing the Asp-Pro-NAP-TSAO intermediate is in full agreement with our previous observations (García-Aparicio et al., 2006). It is well possible that the slow release of the Asp-Pro-NAP-TSAO to parent NAP-TSAO by CD26 represents the mechanistic reason of the lower antiviral activity in cell culture. In contrast, the higher antiviral activity of **19** than parent compound **10** cannot be easily explained. The kinetics of release of the intermediate and/or the parent compound might perhaps be more optimal for displaying antiviral activity than when the NAP-TSAO parent drug is administered to the infected cell cultures from the very beginning. Studies with labeled compound may address this issue.

3.3. *Solubility studies*

The water-solubility of the most hydrophilic Lys-Pro-Asp-Pro-NAP-TSAO prodrug (**20**) was determined and compared to that of the parent drug NAP-TSAO (**2**). The prodrug **20** improved the water-solubility (0.88 mg/mL) 17-fold in comparison to that of the parent compound **2** (0,05 mg/mL). This result suggests that the CD26 prodrug approach could be useful for increasing the water-solubility of hydrophobic drugs.

4. Conclusions

In conclusion, we have demonstrated in this study that tetrapeptidyl derivatives of NAP-TSAO are efficiently converted to the parent compound by CD26 and also by human and bovine serum in two successive reaction steps. The efficiency of conversion is determined by both the first (amino terminal) amino acid (being more efficient in the presence of Val and Lys than of Asp) and the second amino acid (being more efficient in the presence of Pro than of Ala). Our studies could demonstrate that tetrapeptidyl derivatives of drugs that are linked to the tetrapeptide through a free amino group on the particular drug molecule can be useful to modulate release of the parent drug in the plasma, and to show its eventual biological (i.e. antiviral, cytostatic) activity in cell culture, that is at least equal, if not superior, to the parent drug. Furthermore, the Lys-Pro-Asp-Pro-NAP-TSAO (**20**) prodrug exhibited a markedly higher water-solubility than the parent compound NAP-TSAO (**2**), which may be a beneficial property of the peptide prodrug concept to improve formulation of hydrophobic drugs.

Acknowledgements

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