A Selective HIV-Protease Assay Based on a Chromogenic Amino Acid

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Dedicated to Professor *Dieter Seebach* on the occasion of his 65th birthday

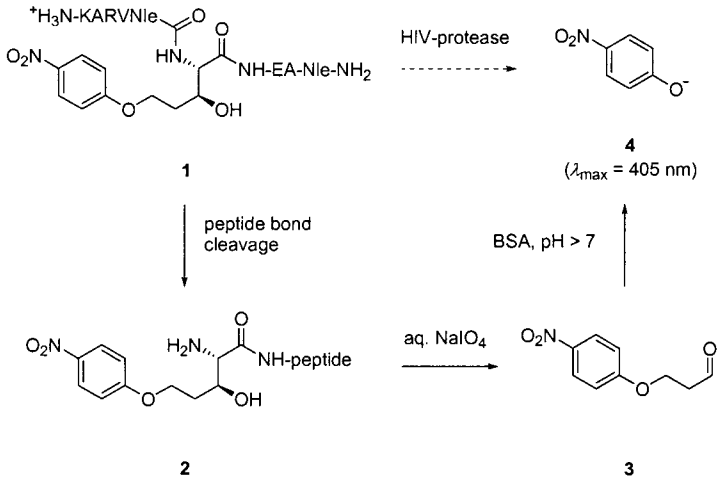
(2*S*,3*S*)-2-Amino-3-hydroxy-5-(4-nitrophenoxy)pentanoic acid (**5**) was prepared stereoselectively as the *N*-Fmoc, *O*-(*tert*-butyl)-protected derivative **5a** in eleven steps from ethyl (*E*)-4-benzyloxypent-2-enoate (**6**). This protected amino acid was used for the solid-phase peptide synthesis of oligopeptides, which serve as sequence-specific chromogenic protease substrates when used in the presence of $NaIO_4$ and bovine serum albumin. The peptide **1** (KRAVNle–**5**–EANleNH$_2$ (Nle = norleucine)) allows detection of HIV-protease activity spectrophotometrically at 405 nm.

Introduction. – Enzyme assays play a crucial role in a variety of applications. Particularly, one important assay concerns the measurement of the activity of HIV-protease and other retroviral proteases, which provide a tool to discover and monitor their inhibitors as anti-retroviral drugs [1]. Protease inhibitors have proven superior to reverse-transcriptase inhibitors at reducing viral loads in the case of HIV-infections [2]. A series of cumulative mutations can render HIV-protease resistant to its inhibitors such as indinavir. Monitoring the onset of resistance in patients enables better adjustment of the drug cocktail by removing inhibitors to which the protease has become resistant, thereby promoting reappearance of the wild-type-sensitive strain [3].

HIV-protease, whose function is to process the Gag-polyprotein (source of structural viral proteins) and the Gag/Pol-polyprotein (source of enzymes for replication, including HIV-protease itself), cleaves peptide bonds preferentially between aromatic amino acids and proline and between pairs of hydrophobic and/or aromatic amino acids [4]. Its activity can be measured by monitoring the decrease of fluorescence-resonance-energy transfer (FRET) between 4-[4-(dimethylamino)phenyldiazo]benzoic acid (DABCYL) and 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) attached at the N- and C-termini of the octapeptide SQNYPIVQ, upon cleavage of the Tyr–prolyl peptide bond [5]. This assay is quite selective but would deliver a positive signal for cleavage of any of the nine different amide bonds in the substrate. Alternatively, the peptide KARVNleF′EANle-NH$_2$ (F′ = 4-nitrophenylalanine) [6] and related sequences with 4-nitrophenylalanine either at the P1 [7] or the P1′ [8] position are reported as chromogenic HIV-protease substrates. Peptide cleavage is supposed to induce a detectable modulation of UV-absorbance at peptide bonds next to 4-nitrophenylalanine, which are specifically attacked by HIV-protease in these peptides. This method has the advantage of absolute chemoselectivity,

but the signal modulation produced is only marginal, and, therefore, quite difficult to observe. Herein, we report a related assay for HIV-protease based on the use of peptide **1** incorporating a non-natural *allo*-threonine analog at the amino-side of the scissile peptide bond (*Scheme 1*). This amino acid undergoes a chromogenic reaction when its amino-group is free, following an oxidation/β-elimination sequence.

Scheme 1. *Principle of HIV-Protease Assay with Peptide* **1**



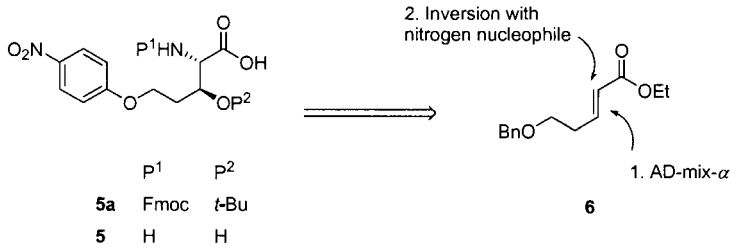
K = lysine, A = alanine, R = arginine, V = valine, Nle = norleucine, E = glutamate.

Results and Discussion. – Recently, we reported that a variety of hydrolytic enzymes can be assayed spectrophotometrically with substrates that produce periodate-sensitive vicinal diols or amino alcohols related to **2** as hydrolysis products from unoxidizable precursors [9]. The chemoselective oxidation by $NaIO_4$ is followed by a rapid β-elimination in the presence of bovine serum albumin (BSA) [10], thus yielding either umbelliferone (= 7-hydroxycoumarine) as a highly-fluorescent product or 4-nitrophenol as a colored product (*Scheme 1*). Compared to assays based on direct esters of these phenols, the key advantage of this assay is its resilience to nonspecific processes, which allows us to address enzymatic activity with high specificity, even in relatively crude extracts [11]. Given the general importance of proteases, we decided to synthesize the protected form **5a** of β-hydroxy-α-amino acid **5**, which would be suitable for Fmoc-based solid-phase peptide synthesis. Its incorporation into peptides would provide a variety of sequence-specific chromogenic probes for proteases.

Since proteases are generally highly stereoselective, an enantioselective synthesis of the L-form was required in any of the two possible diastereoisomers. We envisioned that the (2*S*,3*S*)-diastereoisomer may be accessible from ethyl (*E*)-4-benzyloxypent-2-enoate (**6**) *via Sharpless*'s asymmetric dihydroxylation with AD-mix-α as the chirality inducing step followed by inversion at C(2) with a *N*-nucleophile (*Scheme 2*). The functionalization with the 4-nitrophenyl group at C(5) and the introduction of a *t*-Bu

protecting group on the β-OH group would be achieved along the synthesis by standard methods.

Scheme 2. *Retrosynthetic Analysis for the Target Amino Acid* **5**



	P^1	P^2
5a	Fmoc	*t*-Bu
5	H	H

6

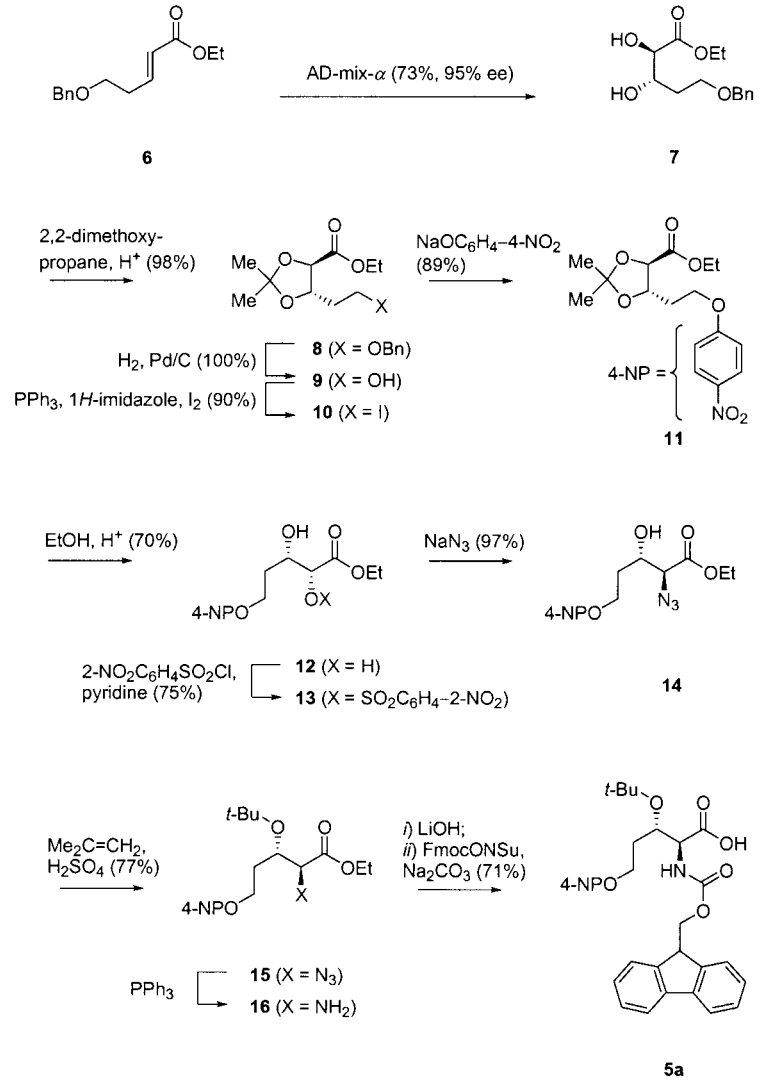
The synthesis was realized as follows (*Scheme 3*). Asymmetric dihydroxylation of **6** with AD-mix-α gave diol **7** in 73% and good optical purity (95% ee) [12]. The diol function was then protected as an acetonide to provide ester **8** (98%). The cyclic acetal locked the conformation of the molecule and prevented any interaction between the ester function and the O–C(5) group, which could then be deprotected by hydrogenation to give **9** quantitatively without any lactonization. *Mitsunobu* reaction to the corresponding iodide **10** (90%) [13] and reaction with sodium 4-nitrophenolate in DMF gave ether **11** (89%). The acetonide was then removed by acidic treatment in absolute EtOH to give diol **12** with a disappointing yield of 70% despite many attempts to optimize. According to a known sequence [14], this diol reacted with 2-nitrophenylsulfonyl chloride to give **13** (75%), which was treated with NaN_3 to give azido alcohol **14** (97%). At that stage, the OH group was protected as *tert*-butyl ether to give **15** *via* reaction with 2-methylpropene in the presence of H_2SO_4 (77%). The azido group was then reduced with PPh_3 to give **16**. Finally, the ester function was saponified by treatement with LiOH, and the crude amino acid was converted to the Fmoc-derivative **5a**, which was isolated as pure product in 71% yield over the last three steps. The desired chromogenic amino acid was thus obtained in a total of eleven steps and in 16% overall yield.

Two chromogenic peptide substrates were prepared by Fmoc-solid-phase peptide synthesis on *Rink*-amide polystyrene resin incorporating the protected amino acid **5a**. An HIV-protease-specific sequence **1** was obtained by simply substituting amino acid **5** for 4-nitrophenylalanine in a known HIV-protease substrate [6] to give KRAVNle-**5**-EANleNH$_2$[1]). As a positive control, the chromogenic tetrapeptide H_2N-Ala-Arg-**5**-Ala-NH_2 (**17**) was also prepared as a trypsin substrate. Both peptides were obtained pure after reverse-phase-HPLC purification in 9 and 24% yield, respectively.

The reactivity of proteases with peptides **1** and **17** was investigated next. The peptides were used as 1–5 mM stock solution in H_2O. We examined the reactivity of the peptides with the proteases trypsin, chymotrypsin, papain, and HIV-protease at their respective pH-optima. The assays were conducted as endpoint measurements. Thus, the peptides were first incubated with the proteases. Peptide cleavage was then assessed by adjusting the pH of the samples to *ca.* pH 9.0, and treating with $NaIO_4$ and BSA. The

[1]) For abbreviations of amino acids, see *Scheme 1*.

Scheme 3. *Stereoselective Synthesis of Chromogenic Amino Acid* **5a**



amount of 4-nitrophenol produced was then determined spectrophotometrically at 405 nm. The entire assay sequence was conveniently carried out in a total volume of 0.15 ml in individual wells of 96-well microtiter plates.

The production of 4-nitrophenol could clearly be detected in the presence of several proteases, while there was no reaction at all in the absence of enzyme, as expected from the stability of the native peptide bond in the substrates (*Table*). A reaction was clearly detected in the presence of HIV-protease with its corresponding sequence **1**. This sequence also showed a positive signal upon incubation with papain at

its optimal pH. Although we used relatively high quantities of this enzyme in comparison with HIV-protease, which was expressed from a plasmid at an estimated level of 1–10 μg/ml only, it was evident that the selectivity was not achieved with this sequence. It should be mentioned that papain activity with **1** was suppressed selectively by addition of the oxidant $NaIO_4$ at the start of the reaction, this probably by oxidation of the catalytic thiol. By contrast, both HIV-protease and trypsin retained their activity when incubated in presence of $NaIO_4$. The reference trypsin-like sequence **17** bearing an arginine at the P1-site reacted with trypsin only, while there was no reaction observed with any of the other three proteases.

Table. *Chromogenic Reactions of Peptides* **1** *and* **17** *with Different Proteases*[a])

Protease	Conditions	Cleavage [%]	
		1	**17**
HIV-Protease	100 mM AcOH (pH 4.5), 1 mM EDTA, 1 mM DTT	26	5
Trypsin	20 mM borate (pH 8.8), 2 h, 37°	< 1	75
Chymotrypsin	10 mM phosphate (pH 7.4), 160 mM NaCl, 2 h, 37°	< 1	0
Papain	20 mM phosphate (pH 6.5), 2 h, 37°	40[b])	0

[a]) Conditions: 100 μM substrate **1** or **17**, HIV-protease: 10 μg ml^{-1}, other proteases: 0.1 mg ml^{-1}, incubation for 2 h at 37°, then addition of aq. 0.2M Na_2CO_3 to pH 9, BSA (2 mg ml^{-1} final conc.) and $NaIO_4$ (final conc. 1 mM, 20 mM with HIV protease) and incubation for 60 min. The OD at 405 nm (0.05–0.60) was used to calculate the 4-nitrophenol conc. according to a calibration curve. [b]) In contrast to trypsin, papain showed no activity when BSA and $NaIO_4$ were added from the start, most likely due to oxidation of the catalytic thiol in this enzyme.

Conclusions. – The chromogenic amino acid building block **5** has been prepared stereoselectively in eleven steps in protected form suitable for Fmoc-based peptide synthesis. Its incorporation into peptide sequences provides specific chromogenic probes for proteases. The amino acid sequence may be selected at all positions relative to the cleavage site, with the exception of the P′1-site, which is occupied by the chromogenic amino acid. Compared with FRET-type substrates the key advantage of this approach is that only cleavage at the desired position delivers a signal, which makes a much higher selectivity possible. Furthermore, the 4-nitrophenol signal is very strong and readily detected even at low conversion rates.

Whether the cross-reactivity observed between our HIV-protease sequence and other proteases would be significant in clinical samples remains to be tested, and a similar cross-reactivity probably occurs with other HIV-protease probes. The problem of selectivity can be fine-tuned by adjusting the experimental conditions. Thus, the reactivity of papain with peptide **1** is suppressed by adding IO_4^- at the beginning of the reaction, which oxidizes the reactive cysteine in this thiol protease. Furthemore, working at the acidic pH of the HIV-protease should also diminish the activity of trypsin-like proteases relative to that of HIV-protease. The main drawback of the assay is the low reactivity of the peptide towards cleavage, which is probably due to the presence of the unnatural and sterically demanding chromogenic amino acid **5** at the P′1-site.

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Experimental Part

General. All reagents were purchased from *Aldrich* or *Fluka* and used without further purification. All reactions were controlled by TLC on *Alugram SIL G/UV$_{254}$* silica-gel sheets (*Macherey-Nagel*) with detection by UV or with 0.5% phosphomolybdic acid soln. in 95% EtOH. Silica gel *60* (*Macherey-Nagel*, 230–400 mesh) was used for flash chromatography (FC). Anal. RP-HPLC was done on a *Waters 600 Controller* with a *Waters 996* photodiode-array detector with a *Vydac 218TP-54* (C18, pore size 300 Å, 0.45 × 22 cm) column, and four different eluents: *A* (0.1% TFA in H_2O), *B* (H_2O/MeCN 50:50), *C* (H_2O), and *D* (MeCN/H_2O/TFA 60:40:0.1). Prep. HPLC was performed on a *Waters Prep-LC* and *Delta Prep-4000* with a *Waters 486* tunable absorbance detector. The ee of diol **7** was determined by analysis on a chiral HPLC column *OD-H* (*Daicel:* 25 cm × 0.46 cm i.d.; hexane/i-PrOH 85:15). M.ps. were determined on a *Kofler* apparatus and are uncorrected. OR were measured with a *Perkin-Elmer 241* digital polarimeter with a 1-dm cell. IR Spectra: *Mattson 3000* FT-IR spectrophotometer; ν in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AC-200* or *-300*; δ = in ppm, *J* in Hz.

*Ethyl (2*R*,3*S*)-5-Benzyloxy-2,3-dihydroxypentanoate* (**7**). A soln. of AD-mix-α [12] (34.51 g), $NaHCO_3$ (6.21 g, 73.95 mmol), and $MeSO_2NH_2$ (2.34 g, 24.65 mmol) in *t*-BuOH/H_2O 1:1 (250 ml) was stirred at r.t. until both phases were clear and then cooled to 0°. Ethyl (*E*)-5-benzyloxypent-2-enoate (**6**) [15] (5.77 g, 24.65 mmol) was added and the slurry was stirred at 0° for 24 h (TLC). Solid $Na_2S_2O_5$ (37.0 g) was added at 0°, and the resulting mixture was warmed to r.t. and stirred for 1 h. Extraction with AcOEt and evaporation of the washed (2N NaOH) org. soln. gave a solid crude residue, which was subjected to FC (hexane/AcOEt 7:3; R_f 0.2) to give pure **7** (4.79 g, 73%, 95% ee). White waxy solid. $[\alpha]_D^{20} = -4.5$ ($c = 0.42$, $CHCl_3$). IR (neat): 3446*s* (OH), 3387*s* (OH), 1726*s* (C=O). ^{1}H-NMR (200 MHz, $CDCl_3$): 7.43–7.17 (*m*, 5 H); 4.53 (*s*, 2 H); 4.28 (*q*, *J* = 7.2, 2 H); 4.14–4.07 (*m*, 1 H); 4.07–3.97 (br. *s*, 1 H); 3.81–3.59 (*m*, 2 H); 2.15–1.93 (*m*, 1 H); 1.93–1.72 (*m*, 1 H); 1.31 (t, *J* = 7.1, 3 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 173.16; 137.85; 128.43; 127.72; 127.68; 73.56; 73.25; 71.37; 67.96; 61.87, 33.25; 14.13. Anal. calc. for $C_{14}H_{20}O_5$: C 62.67, H 7.51; found: C 62.51, H 7.33.

*Ethyl (4*R*,5*S*)-5-(2-Benzyloxyethyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylate* (**8**). A soln. of **7** (2.40 g, 8.95 mmol) in anh. acetone (32 ml) was treated with 2,2-dimethoxypropane (9.33 g, 89.55 mmol, 10.98 ml) and TsOH (12 mg), and the resulting mixture was stirred at r.t. for 1.5 h. Na_2CO_3 (0.57 g) was added, and stirring was prolonged for 10 min. Evaporation of the filtered (*Celite*) org. soln. afforded a crude product consisting of practically pure **8** (2.71 g, 98%), which was used in the next step without further purification. An anal. sample was purified by FC (hexane/AcOEt 8:2; R_f 0.3) to give pure **8**. Colorless oil. $[\alpha]_D^{20} = -16.0$ ($c = 0.70$, $CHCl_3$). IR (neat): 1757 *s* (C=O), 1734 *s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 7.40–7.23 (*m*, 5 H); 4.51 (*s*, 2 H); 4.41–4.25 (*m*, 1 H); 4.25–4.09 (*m*, 1 H); 4.21 (*q*, *J* = 7.2, 2 H); 3.72–3.54 (*m*, 2 H); 2.15–2.03 (*m*, 1 H); 2.03–1.89 (*m*, 1 H); 1.45 (*s*, 3 H); 1.43 (*s*, 3 H); 1.30 (*t*, *J* = 7.1, 3 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 170.62; 138.28; 128.35; 127.51; 110.70; 79.14; 76.41; 72.96; 66.61; 61.32; 33.60; 27.10; 25.69; 14.11. Anal. calc. for $C_{17}H_{24}O_5$: C 66.21, H 7.84; found: C 66.10, H 7.79.

*Ethyl (4*R*,5*S*)-5-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolidine-4-carboxylate* (**9**). A soln. of **8** (2.69 g, 8.75 mmol) in abs. EtOH (60 ml) and in the presence of 10% Pd/C (0.54 g) was vigorously stirred under H_2 at r.t. for 18 h. Evaporation of the filtered (*Celite*) org. soln. afforded a crude liquid product consisting of practically pure **9** (1.91 g, 99%), which was used in the next step without further purification. An anal. sample of **9** was purified by FC (hexane/AcOEt 1:1; R_f 0.3) to give pure **9**. Colorless liquid. $[\alpha]_D^{20} = -10.0$ ($c = 0.48$, $CHCl_3$). IR (neat): 3446*s* (OH), 1753*s* (C=O), 1734*s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 4.32–4.19 (*m*, 4 H); 3.83 (*t*, *J* = 5.7, 2 H); 2.12–2.00 (*m*, 1 H); 1.98–1.89 (*m*, 1 H); 1.47 (*s*, 3 H); 1.43 (*s*, 3 H); 1.30 (*t*, *J* = 7.2, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.75; 111.01; 79.00; 77.85; 61.52; 60.01; 35.82; 27.05; 25.55; 14.13. Anal. calc. for $C_{10}H_{18}O_5$: C 55.03, H 8.31; found: C 55.29, H 8.56.

*Ethyl (4*R*,5*S*)-5-(2-Iodoethyl)-2,2-dimethyl-1,3-dioxolane-4-carboxylate* (**10**). A soln. of PPh_3 (3.127 g, 11.92 mmol) in anh. CH_2Cl_2 (37 ml) under N_2, was treated with 1*H*-imidazole (0.81 g, 11.92 mmol) and then with I_2 (3.03 g, 11.92 mmol) [13]. A soln. of **9** (2.00 g, 9.17 mmol) in anh. CH_2Cl_2 (9 ml) was added, and the mixture was stirred at r.t. for 2 h. Evaporation of the solvent gave a crude product, which was filtered through a short silica-gel column (hexane/AcOEt 9:1; R_f 0.5) to give pure **10** (2.70 g, 90%). Yellow oil. $[\alpha]_D^{20} = -30.0$ ($c = 0.39$,

$CHCl_3$). IR (neat): 1759*s* (C=O), 1730*s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 4.25 (*q*, *J* = 7.1, 2 H); 4.21–4.16 (*m*, 1 H); 4.13 (*d*, *J* = 7.3, 1 H); 3.36–3.20 (*m*, 2 H); 2.37–2.26 (*m*, 1 H); 2.24–2.11 (*m*, 1 H); 1.45 (*s*, 3 H); 1.44 (*s*, 3 H); 1.31 (*t*, *J* = 7.2, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.72; 126.89; 111.64; 79.07; 78.77; 61.92; 38.13; 27.45; 25.99; 14.58; 0.89. Anal. calc. for $C_{10}H_{17}IO_4$: C 36.30, H 5.22; found: C 36.17, H 5.01.

*Ethyl (4*R,5S*)-2,2-Dimethyl-5-[2-(4-nitrophenoxy)ethyl]-1,3-dioxolane-4-carboxylate* (**11**). A soln. of **10** (2.680 g, 8.17 mmol) in anh. DMF (32 ml) was treated with sodium 4-nitrophenolate (1.710 g, 10.62 mmol), and the mixture was stirred at 50° for 16 h. Aq. workup (Et_2O/H_2O) and evaporation of the org. phase afforded a crude product consisting of practically pure **11** (2.46 g, 89%), which was used in the next step without further purification. An anal. sample of **11** was purified by FC (hexane/AcOEt 8:2; R_f 0.3) to give pure **11**. Pale yellow oil. $[\alpha]_D^{20} = -19.4$ (*c* = 0.52, $CHCl_3$). IR (neat): 1755*s* (C=O), 1732 *s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.20 (*d*, *J* = 9.2, 2 H); 6.96 (*m*, *J* = 9.2, 2 H); 4.38–4.32 (*m*, 1 H); 4.30–4.16 (*m*, 5 H); 2.40–2.29 (*m*, 1 H); 2.23–2.12 (*m*, 1 H); 1.47 (*s*, 3 H); 1.44 (*s*, 3 H); 1.27 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.32; 163.66; 141.64; 125.87; 114.43; 111.15; 79.04; 75.71; 65.15; 61.48; 33.10; 27.09; 25.68; 14.12. Anal. calc. for $C_{16}H_{21}NO_7$: C 56.63, H 6.24, N 4.13; found: C 56.32, H 6.08, N 3.92.

*Ethyl (2*R,*3*S*)-2,3-Dihydroxy-5-(4-nitrophenoxy)pentanoate* (**12**). A soln. of **11** (0.83 g, 2.439 mmol) in abs. EtOH (40 ml) was treated with TsOH (0.23 g, 1.22 mmol), and the mixture was stirred at 50° for 20 h. After cooling, Na_2CO_3 (0.232 g) was added, and the mixture was stirred at r.t. for 10 min. Evaporation of the org. solvent afforded a crude product, which was subjected to FC (hexane/AcOEt 1:1; R_f 0.39) to give pure **12** (0.51 g, 70%). White solid. M.p. 103–104°. $[\alpha]_D^{20} = -29.5$ (*c* = 0.22, $CHCl_3$). IR (nujol): 3391*s* (OH), 3277*s* (OH), 1730*s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.20 (*d*, *J* = 9.2, 2 H); 6.97 (*d*, *J* = 9.2, 2 H); 4.35–4.18 (*m*, 5 H); 4.14 (*dd*, *J* = 4.78, 2.21, 1 H); 2.19–2.10 (*m*, 2 H); 1.33 (*t*, *J* = 7.2, 3 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 172.97; 163.78; 141.62; 125.92; 114.49; 73.28; 69.29; 65.43; 62.45; 33.31; 14.17. Anal. calc. for $C_{13}H_{17}NO_7$: C 52.17, H 5.73, N 4.68; found: C 52.33, H 6.01, N 4.74.

*Ethyl (2*R,*3*S*)-3-Hydroxy-5-(4-nitrophenoxy)-2-[(2-nitrophenylsulfonyl)oxy]pentanoate* (**13**). A soln. of **12** (1.38 g, 4.63 mmol) in anh. pyridine (23 ml) was treated at 0° with 2-nitrophenylsulfonyl chloride (1.03 g, 4.63 mmol), and the mixture was left at this temp. for 24 h [14]. After dilution in Et_2O, evaporation of the washed (H_2O, 1N aq. HCl, sat. aq. NaCl) org. soln. gave a crude product, which was purified by FC (hexane/AcOEt 6:4; R_f 0.3) to give pure **13** (1.68 g, 75%). Pale yellow solid. M.p. 111–112°. $[\alpha]_D^{20} = -27.0$ (*c* = 0.3, $CHCl_3$). IR (nujol): 3493 *s* (OH), 1715 *s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.40 (*d*, *J* = 8.5, 2 H); 8.18 (*d*, *J* = 9.2, 4 H); 6.96 (*d*, *J* = 9.2, 2 H); 5.11 (*d*, *J* = 2.94, 1 H); 4.49–4.44 (*m*, 1 H); 4.32–4.07 (*m*, 4 H); 2.16–2.09 (*m*, 2 H); 1.21 (*t*, *J* = 7.2, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 167.04; 164.10; 151.65; 142.53; 142.51; 130.25; 126.63; 124.97; 115.15; 81.61; 69.22; 65.44; 63.31; 33.21; 14.68. Anal. calc. for $C_{19}H_{20}N_2O_{11}S$: C 47.11, H 4.16, N 5.78; found: C 47.32, H 4.29, N 5.60.

*Ethyl (2*S,*3*S*)-2-Azido-3-hydroxy-5-(4-nitrophenoxy)pentanoate* (**14**). A soln. of **13** (1.21 g, 2.51 mmol) in anh. DMF (43 ml) was treated with NaN_3 (1.01 g, 15.61 mmol), and the mixture was stirred under N_2 at 50° for 24 h. After cooling, dilution with Et_2O and evaporation of the washed (sat. aq. NaCl) mixture afforded a crude product (0.810 g, 97%) consisting of practically pure **14**, which was used in the next step without further purification. An anal. sample of **14** was subjected to FC (hexane/AcOEt 6:4; R_f 0.35) to give pure **14**. Pale yellow oil. $[\alpha]_D^{20} = -47.6$ (*c* = 0.42, $CHCl_3$). IR (neat): 3509*m* (OH), 2114*s* (N_3), 1738*s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.20 (*d*, *J* = 9.2, 2 H); 6.97 (*d*, *J* = 9.2, 2 H); 4.42–4.12 (*m*, 5 H); 4.05 (*d*, *J* = 5.9, 1 H); 2.18–2.06 (*m*, 1 H); 2.10–1.97 (*m*, 1 H); 1.35 (*t*, *J* = 7.2, 3 H). ^{13}C-NMR (50 MHz, $CDCl_3$): 168.94; 163.68; 141.77; 126.03; 114.53; 68.73; 66.11; 65.12; 62.47; 32.32; 14.24. Anal. calc. for $C_{13}H_{16}N_4O_6$: C 48.15, H 4.97, N 17.28; found: C 48.26, H 5.21, N 17.37.

*Ethyl (2*S,*3*S*)-2-Azido-3-(*tert*-butyloxy)-5-(4-nitrophenoxy)pentanoate* (**15**). A soln. of **14** (1.075 g, 3.23 mmol) in anh. CH_2Cl_2 (9 ml), placed in a 25-ml pressure tube cooled at −50°, was treated with liquid 2-methylpropene (8 ml) and 98% H_2SO_4 (34 µl). After the tube was plugged, the temp. was raised to 0°, and the mixture was then stirred at r.t. for 72 h. The mixture was cooled again to 0° and, after neutralization (solid $NaHCO_3$), was stirred at r.t. while N_2 was blown through the soln. to remove the unreacted 2-methylpropene. After taking up the residue with CH_2Cl_2, evaporation of the washed (H_2O) org. phase gave a crude product (1.33 g), which was subjected to FC (hexane/AcOEt 9:1; R_f 0.25) to give pure **15** (0.972 g, 77%). Pale yellow solid. M.p. 45–49°. $[\alpha]_D^{20} = -9.92$ (*c* = 1.23, $CHCl_3$). IR (nujol): 2110*s* (N_3), 1751*s* (C=O). ^{1}H-NMR (300 MHz, $CDCl_3$): 8.20 (*d*, *J* = 9.2, 2 H); 6.94 (*d*, *J* = 9.2, 2 H); 4.39–4.10 (*m*, 6 H); 2.15–2.04 (*m*, 1 H); 1.90–1.79 (*m*, 1 H); 1.31 (*t*, *J* = 7.2, 3 H); 1.22 (*s*, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 168.63; 164.32; 142.28; 126.62; 114.99; 76.13; 69.62; 67.84; 65.16; 62.60; 31.40; 29.11; 14.84. Anal. calc. for $C_{17}H_{24}N_4O_6$: C 53.68, H 6.36, N 14.73; found: C 53.93, H 6.48, N 14.96.

*Ethyl (2S,3S)-Amino-3-(*tert*-butyloxy)-5-(4-nitrophenoxy)pentanoate* (**16**). A soln. of **15** (0.57 g, 1.50 mmol) in THF (20 ml) was treated with PPh_3 (0.39 g, 1.50 mmol) and H_2O (2.5 ml), and the mixture was stirred at r.t. for 2 h, until N_2 evolution stopped, and was then stirred at 60° for 19 h. After cooling, dilution in Et_2O and evaporation of the washed (sat. aq. NaCl) org. soln. gave a crude mixture (0.95 g) consisting of Ph_3PO and **16** (^{1}H-NMR) as the only reaction product, which was used in the next step without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.20 (*d*, *J* = 9.2, 2 H); 6.95 (*d*, *J* = 9.2, 2 H); 4.30–4.08 (*m*, 5 H); 3.87 (*d*, *J* = 4.6, 1 H); 2.10–1.92 (*m*, 1 H); 1.88–1.70 (*m*, 1 H); 1.38 (*t*, *J* = 7.2, 3 H); 1.2 (*s*, 9 H).

*(2S,3S)-3-(*tert*-Butyloxy)-2-[N-[(9H-fluoren-9-ylmethoxy)carbonyl]amino]-5-(4-nitrophenoxy)pentanoic Acid* (**5a**). A soln. of the above described crude mixture (0.474 g) consisting of **16** (0.75 mmol) and Ph_3PO in 1,4-dioxane (2 ml) was treated with a soln. of LiOH (0.054 g, 2.25 mmol) in H_2O (2 ml), and the mixture was stirred at r.t. for 2 h. After dilution with H_2O (3 ml) and neutralization (5N HCl), solid Na_2CO_3 (0.525 g) was added to achieve pH = 8–9. The mixture was cooled to 0°, treated with a soln. of FmocONSu (= *N*-[(9*H*-fluoren-9-ylmethoxy)carbonyloxy]succinimide; 0.28 g, 0.83 mmol) in 1,4-dioxane (1.5 ml) and then stirred at r.t. for 18 h. After cooling to 0°, 5N HCl was added to achieve pH = 2, and the mixture was extracted with Et_2O. Evaporation of the combined Et_2O extracts gave a crude product, which was purified by FC (AcOEt/hexane/AcOH 4:5.9:0.1; R_f 0.26) to give pure **5a** (0.293, 71%), which, after trituration with CH_2Cl_2/hexane, was obtained as a pale yellow solid. M.p. 107–109°. $[\alpha]_D^{20} = +14.87$ (*c* = 0.39, $CHCl_3$). IR (nujol): 1717*s*, 1668*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.17 (*d*, *J* = 8.8, 2 H); 7.75 (*d*, *J* = 7.7, 2 H); 7.58 (*d*, *J* = 7.0, 2 H); 7.39 (*t*, *J* = 7.3, 2 H); 7.30 (*d*, *J* = 7.3, 2 H); 6.93 (*d*, *J* = 7.7, 2 H); 4.75–4.63 (*m*, 1 H); 4.72 (*d*, *J* = 7.0, 2 H); 4.23 (*t*, *J* = 6.8, 2 H); 4.14 (br. *s*, 2 H); 2.12–1.88 (*m*, 2 H); 1.20 (*s*, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.80; 163. 49; 156.26; 143.62; 141.67; 141.31; 127.76; 127.05; 125.91; 124.98; 120.01; 114.39; 75.38; 68.61; 67.24; 64.70; 58.18; 47.14; 31.66; 28.29. EI-MS (pos.): m/z (relative intensity): 549 (4), 493 (15), 271 (11), 179 (100), 178 (48). Anal. calc. for $C_{30}H_{32}N_2O_8$: C 65.68, H 5.88, N 5.11; found: C 65.83, H 6.11, N 5.29.

Solid-Phase Peptide Synthesis (SPPS). *General*. Nonapeptide KRAVNle-**5**-EANleNH$_2$[1] (**1**; HIV-protease substrate) and tetrapeptide H_2N-Ala-Arg-**5**-Ala-NH_2 (**17**; trypsin substrate) were prepared by SPPS with *Rink*-resin (*Bachem*) (200 mg, 0.47 mmol g^{-1}, 200–400 mesh). The following protected amino acids, purchased from *Novabiochem* or *Bachem*, were used: Fmoc-Nle, Fmoc-Ala, Fmoc-Glu(*t*-BuO)-OH, Fmoc-Val, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH. The peptides were prepared according to a standard protocol for Fmoc-SPPS and purified by prep. reverse-phase HPLC.

Nonapeptide **1**. Nonapeptide **1** was obtained as a white solid (9%) (t_R = 27.7 min, *A*/*D* 80:20–20:80 with 1% min^{-1} gradient). The structure of the peptide was confirmed by ^{1}H-NMR (300 MHz, CD_3OD) and ES-MS (pos.; 1150 ($[M-H]^+$)).

Tetrapeptide **17**. Tetrapeptide **17** was obtained as a white solid (24%) (t_R 18.6 min, *A*/*D* 95:5–35:65 with 1% min^{-1} gradient). The structure of the peptide was confirmed by ^{1}H-NMR (300 MHz, CD_3OD) and ES-MS (pos.; 567 ($[M-H]^+$)).

Enzyme Measurements. The peptides **1** and **17** were diluted from 1 mM stock solns. in deionized *milliQ*-filtered H_2O. The proteases trypsin (from hog pancreas, 1650 U/mg, *Fluka* 93615), α-chymotrypsin (from bovine pancreas, 37 U/mg, *Sigma* SC-4129), and papain (from *Papaya latex*, 30 U/mg, *Sigma* SR-4762) were diluted from 1 mg ml^{-1} stock solns. in phosphate buffer saline (PBS, 10 mM phosphate pH 7.2, 160 mM NaCl). HIV-Protease was obtained by *in vitro* expression from a plasmid by means of the *Phenomics*® expression system (*Protéus SA*, Nîmes, France), and the soln. also contained glycerol, which required additional IO_4^- for detection (see below). The reactions were run in polystyrene 96-well half-area cell-culture plates and recorded on a *Spectramax 250* microplate spectrophotometer (*Molecular Devices*). Reactions were initiated by addition of the substrate stock soln. (10 µl) to the prediluted enzyme soln. (90 µl) in their respective buffer (see the *Table*), and incubated at 37° for 2 h. The pH was then adjusted to pH 9 by addition of 0.2M Na_2CO_3 (20 µl), BSA (final conc. 2 mg ml^{-1}) and $NaIO_4$ (final conc. 1 mM, 20 mM with HIV protease). The assay was then incubated at 25° for 60 min, and the OD was recorded at 405 nm as endpoint. The OD at 405 nm (0.05–0.60) was used to calculate the 4-nitrophenol conc. according to a calibration curve under the same conditions.

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