Synthesis and Biological Activity of the Prodrug of Class I Major Histocompatibility Peptide GILGFVFTL Activated by β-Glucuronidase[1]

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The first synthesis of a prodrug of HLA-A2.1 associated antigenic influenza peptide **2a** was accomplished. Two methods for synthesis of prodrugs of antigenic peptides activated by β-glucuronidase and comprising a self-immolative 3-nitrobenzyloxycarbonyl moiety were investigated. Reaction of β-glucuronic acid glycoside of 4-hydroxy-3-nitrobenzyl alcohol (**3**) with *N*,*N*′-disuccinimidyl carbonate (DSC) followed by conjugation with AlaOMe, Gly, Thr, Phe-Leu, and Leu-Arg gave carbamates **4a**−**4f**. Deacetylation of **4b** and **4e** with MeONa/MeOH gave β-glucuronides **5b** and **5e**. Compound **5e** was converted to β-glucuronic acid conjugate **6e** by the action of pig liver esterase (PLE). Compound **6e** is a substrate for β-glucuronidase. Method of a direct introduction of the prodrug residue into antigenic nonapeptide GILGFVFTL (**2b**) failed. Alternately, glycine conjugate **5b** was activated to pentafluorophenyl ester **10**. Model coupling of **10** with Phe-Leu gave tripeptide conjugate ester **11a** which was hydrolyzed by PLE to uronic acid **12**. Condensation of **10** with octapeptide ILGFVFTL (**9**) gave prodrug precursor **11b**. Octapeptide **9** was prepared by de novo synthesis using a racemization-free fragment coupling method. Ester hydrolysis with $Ba(OH)_2$/MeOH gave the target prodrug **2a** which is a substrate for β-glucuronidase. Prodrug **2a** does not bind to HLA-A2.1 of T2 human cells defective in major histocompatibility complex I (MHC I)-associated peptide processing. Addition of β-glucuronidase restored the binding to the level observed with parent nonapeptide **2b** although higher concentrations of prodrug **2a** and enzyme were necessary.

In an effort to design more effective and less toxic drugs against cancer, attention has turned in recent years to prodrugs of antitumor agents. Most of these approaches rely on specific enzymes capable of converting the prodrugs into active species at a tumor site. Selective drug release should reduce the side effects associated with other forms of chemotherapy. This is also the principle of antibody- and gene-directed prodrug therapy (ADEPT[2,3] and GDEPT[4]). Prodrugs comprising a carbohydrate moiety amenable to enzyme action attached through a self-immolative benzyloxycarbonyl linker to an anticancer drug (e.g., doxorubicin or daunorubicin, compounds **1a**−**1c**, Chart 1) have attracted a considerable interest.[5−9] The prodrug-activating enzymes include β-glucuronidase and α- or β-galactosidase. Prodrug **1b** ($R_2 = NO_2$, HMR 1826) activated by β-glucuronidase liberated in necrotic tumors[10] has an increased efficacy and reduced toxicity relative to that of the parent drug.[11] It is a promising candidate for clinical studies.[12]

We have reasoned that this prodrug concept can be extended to antigenic oligopeptides[13] (8−10 a.a.) which bind to an extracellular domain of class I major histocompatibility complex (MHC I) or human leukocyte antigen (HLA) and are recognized by CD8 positive cytotoxic T lymphocytes (CTL). Interaction between T cell receptor (TCR) and class I HLA/peptide complex on the target cells triggers CTL activity which mediates target cell lysis.[14] The HLA I peptides on tumor cells originate from endogenous proteins but they can be replaced exogenously by synthetic peptides. It has been shown that CTL can kill tumor cells very effectively if the latter were loaded with appropriate peptides.[15] Thus, mice immunized with tumor-associated peptides rejected tumors expressing the specific peptides.

We can then assume that tumors can be rejected if any antigenic peptide which binds to the approriate MHC is delivered to the tumor in an immunized host. Systemic delivery of such antigenic peptide is not possible because it will bind to all nucleated cells resulting in severe toxicity. Effective antitumor activity may be achieved if the peptide is converted to a suitable prodrug which will release the peptide only at the tumor site. Glucuronic acid linked through a benzyloxycarbonyl spacer to the N-terminus of the antigenic peptide seems to fulfill such requirements. The anchor residues at or near the N- and C-termini of peptides are necessary for effective interaction[16] with HLA or MHC I. Blocking one of these sites by a bulky prodrug grouping should eliminate or diminish such a binding. As indicated above for prodrugs **1a**−**c**, β-glucuronidase in the microenvironment of tumors can effectively generate an active drug. Substitution at the N-terminus should also reduce the susceptibility of the prodrug toward enzymatic degradation (peptidases) in cell culture and in vivo.

We have chosen a prodrug of nonapeptide GILGFVFTL (**2a**) as a target of this study. Parent nona-

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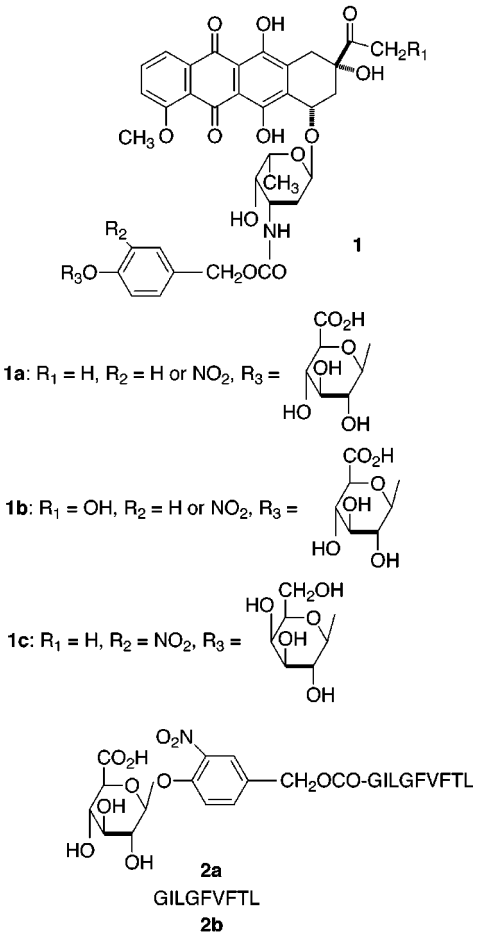
Chart 1



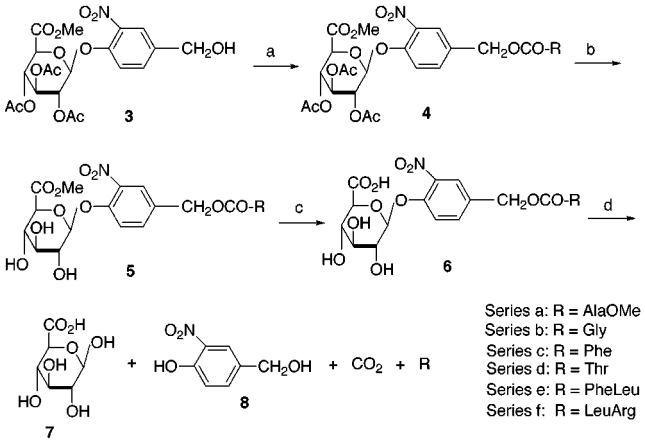
peptide **2b** is an influenza matrix peptide 58−66 which was shown to be optimal for binding to HLA-2 and presentation to cytotoxic T lymphocytes.[17] This article deals with the synthesis and biological investigation of peptide prodrug **2a**.

Synthesis

Although methods for *N*-glycopeptide assembly based on solution- or solid-phase peptide synthesis are available,[18] examples of carbohydrates linked to an N-terminus of a peptide through a spacer are rare. In one instance, a *β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside moiety was attached through a butyryl spacer to a neutral tricosapeptide in the last step of solid-phase synthesis.[19] Because nonapeptide **2b** is commercially available, a possibility of direct introduction of *β*-D-glucuronyl-3-nitrobenzyloxycarbonyl spacer to the N-terminus of **2b** was considered first.

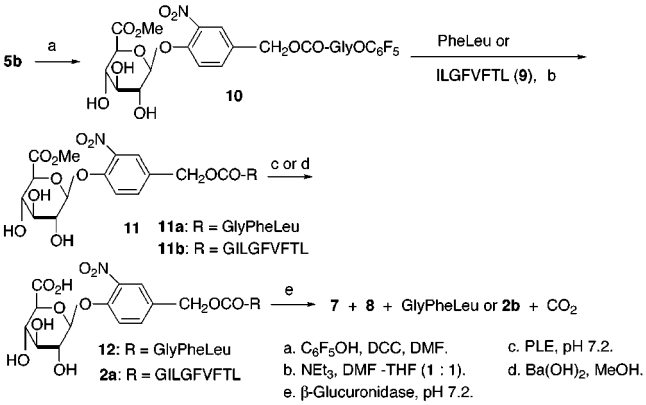
Several model experiments were conducted to determine feasibility of such an approach. Reaction of methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucuronyl-3-nitrobenzyl alcohol (**3**), *N*,*N*′-disuccinimidyl carbonate (DSC),[8] and AlaOMe in the presence of triethylamine gave smoothly compound **4a** in 62% yield (Scheme 1). In a similar vein, free amino acids Gly, Phe, and Thr were converted to conjugates **4b**−**4d** in 66−83% yield. Transformation of dipeptides Phe-Leu and Leu-Arg to compounds **4e** and **4f** in 56 and 41% yield, respectively, was also uneventful. Interestingly, in case of Leu-Arg, attachment of the prodrug moiety occurred at the N-terminus as evidenced

Scheme 1[a]



[a] Reagents and conditions: (a) (1) *N*,*N*′-disuccinyliminocarbonate (DSC), MeCN, NEt_3, (2) R, NEt_3, DMF; (b) MeONa, MeOH; (c) PLE, pH 7.2; (d) *β*-glucuronidase, pH 7.2.

Scheme 2[a]

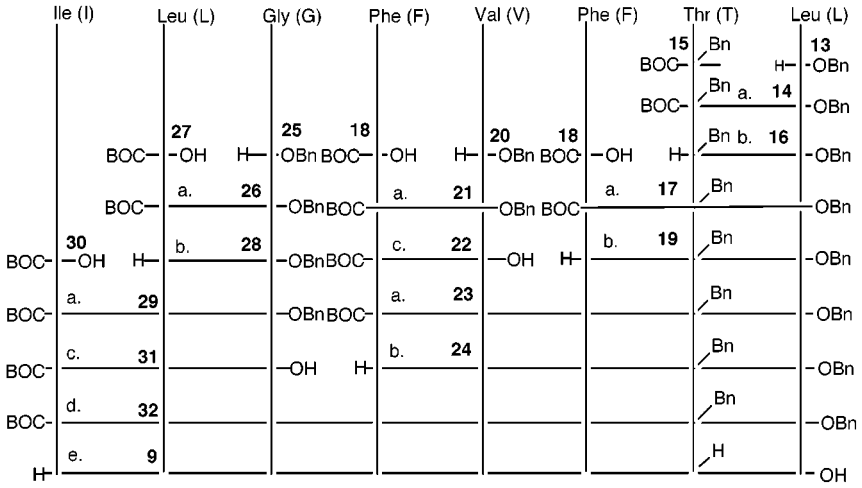


[a] Reagents and conditions: (a) C_6F_5OH, DCC, DMF; (b) NEt_3, DMF−THF (1:1); (c) PLE, pH 7.2; (d) $Ba(OH)_2$, MeOH; (e) *β*-glucuronidase, pH 7.2.

by ^1H NMR spectrum and negative reaction with ninhydrin. This can be explained by a protection of arginine moiety (stronger base than triethylamine) by protonation.[20] Deacetylation[8] of conjugates **4b** and **4e** with MeONa in MeOH was also problem-free. Compounds **5b** and **5e** were obtained in 79 and 76% yield, respectively. Methyl glucuronate **5e** was hydrolyzed by pig liver esterase (PLE) to give 91% of glucuronic acid derivative **6e**. The latter compound was quantitatively digested by *β*-glucuronidase from *Escherichia coli* to give *β*-glucuronic acid (**7**), alcohol **8**, and Phe-Leu.

Although these experiments have indicated that a direct introduction of a glucuronyl prodrug residue into N-termini of amino acids or dipeptides is possible, application to nonapeptide **2b** was beset with problems from the very beginning. Reaction of **2b** with DSC and glucuronyl linker **3** led to formation of a complex mixture of products as shown by HPLC. Although mass spectrum showed that the desired prodrug **2a** was also present, preparative separation would be extremely difficult. Therefore, an alternate synthetic approach was devised. Because unprotected carbohydrate moieties interfere neither with the active ester formation nor peptide bond synthesis,[21,22] it was anticipated that glycine conjugate **5b** can be used for introducing the prodrug function into octapeptide ILGFVFTL (**9**). As an

Scheme 3[a]



[a] Reagents and conditions: (a) DCC, HOBT, THF−DMF, 0 °C, (b) HCO_2H; (c) H_2, Pd/C, EtOH; (d) EDCl, HOBT, THF−DMF, 0 °C; (e) (1) H_2, Pd/C, AcOH, (2) TFA. [EDCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.]

additional advantage, no deprotection of the glucuronate moiety in the latter stages of prodrug synthesis would be necessary. In a model experiment, conjugate **5b** was converted to pentafluorophenyl ester **10** (91%, Scheme 2) which was then reacted with PheLeu to give intermediate **11a** in 63% yield. Digestion with PLE gave glucuronic acid conjugate **12** (91%). A similar cleavage of glucuronate prodrugs was described.[23,24] Compound **12** was readily degraded by β-glucuronidase from bovine liver to give alcohol **8** and Gly-Phe-Leu.

Next, octapeptide **9** was prepared by a racemization-free fragment coupling strategy[25,26] (Scheme 3). Joining of protected amino acid derivatives **13** and **15** gave dipeptide **14** (84%) which was deprotected to give **16** (87%). Coupling with Boc-Phe-OH (**18**) gave tripeptide **17** (77%). Deprotection afforded tripeptide fragment **19** (82%). In a similar fashion, Boc-Phe-OH (**18**) was coupled with Val-OBn (**20**) to furnish dipeptide **21** (81%). Debenzylation led to **22** (90%) which was converted to pentapeptide **23** by condensation with tripeptide **19** (70%). Deprotection then afforded pentapeptide **24** (90%), a key fragment for synthesis of octapeptide **9**. Dipeptide **26** was prepared from Gly-OBn (**25**) and Boc-Leu-OH (**27**) in 87% yield. Deprotection of **26** gave dipeptide **28** (85%) which was extended to tripeptide **29** (77%) with Boc-Ile-OH (**30**). Debenzylation afforded a key tripeptide fragment **31** (85%). Coupling of both fragments **24** and **31** gave octapeptide **32** (66%) which was totally deprotected by hydrogenolysis in AcOH followed by treatment with TFA to give ILGFVFTL (**9**) in 62% yield.

Condensation of **10** with octapeptide **9** gave the methyl ester of the prodrug **11b** (36%). In contrast to dipeptide **5e** and tripeptide **11a**, the nonapeptide ester prodrug **11b** was a poor substrate for PLE. Therefore, ester **11b** was hydrolyzed with $Ba(OH)_2$ in MeOH[24] to give target prodrug **2a** (37%). Digestion of **2a** with β-glucuronidase from bovine liver gave benzyl alcohol **8** and nonapeptide **2b** which were identical with authentic samples.

Biological Studies

Binding of nonapeptide **2b** and prodrug **2a** to HLA-A2.1 was investigated with T2 human cells defective in

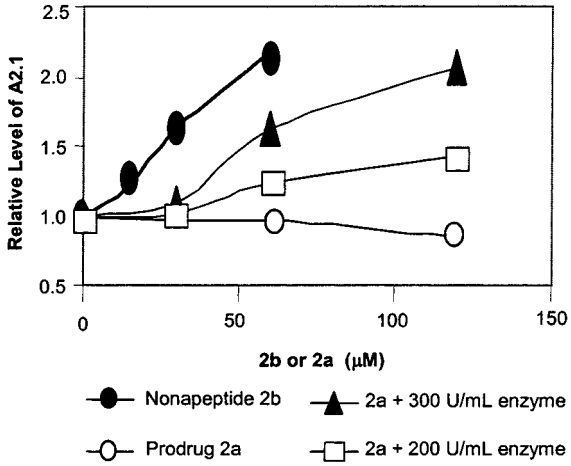


Figure 1. A total of 3×10^5 T2 cells were incubated with nonapeptide **2b** (solid diamonds) or prodrug **2a** (open circles) at concentrations of 15 to 120 μM as indicated. *E. coli* β-glucuronidase at 200 (open squares) or 300 μM (solid triangles) was added to mixtures containing cells and prodrug **2a**. For other details, see Experimental Section.

MHC I-associated peptide processing.[27] Because of the defect, HLA-A2.1 on these cells do not contain endogenous peptides, are unstable, and promptly degraded, resulting in a low level of HLA-A2.1 on the surface. Binding of exogenous peptides to A2.1 gives stable MHC/peptide complexes and increased level of surface HLA-A2.1 which can be detected by flow cytometry.

Nonapeptide **2b** binds to T2 cells in a dose-dependent manner in the range of 15 to 60 μM. At 60 μM of **2b**, the HLA-A2.1 level increased to 230% of control cells which were incubated without peptide or β-glucuronidase at pH 6.5−7.0 to approach the physiological conditions. The enzyme alone did not affect the level of HLA-A2.1 (data not shown). Incubation of nonapeptide prodrug **2a** up to the 120 μM level without β-glucuronidase had no effect on HLA-A2.1, indicating a complete absence of binding (Figure 1). In the presence of 200 and 300 units/mL of β-glucuronidase, HLA-A2.1 level increased with increasing concentration of the enzyme. At 300 units/mL of the enzyme and 120 μM of prodrug **2a**, the HLA-A2.1 level increased to 210% of the control value. Therefore, β-glucuronidase liberated active

nonapeptide **2b** from prodrug **2b**. Although this pH may not be optimal[28] for the activity of β-glucuronidase in a tumor environment, there is a significant release of active nonapeptide **2b** to mark the cells. Further biological investigations are in progress.

Conclusion

We have elaborated two basic strategies for introduction of prodrug grouping, which can be activated by β-glucuronidase, into amino acids and peptides. This effort culminated in the synthesis of the first prodrug of antigenic nonapeptide GILGFVFTL (compound **2a**). Nonapeptide **2b** released from **2a** by the action of β-glucuronidase binds to T2 cells in a dose-dependent manner but it is inactive in the absence of the enzyme. This has demonstrated that a prodrug therapy concept previously employed in the context of antitumor anthracycline antibiotics is applicable to exogenous antigenic peptides to mark tumor cells for immune destruction.

Experimental Section

General Methods. NMR spectra were determined at 400 MHz in DMSO-d_6. Optical rotations were determined with a JASCO DIP-370 digital polarimeter. Mass spectrometry was performed in electron-impact (EI) mode on KRATOS MS80, fast-atom bombardment (FAB) mode on KRATOS MS50, or electrospray ionization (ESI) mode on MICROMASS QUATTRO LC-MS. The TLC was run on 6 × 2 cm precoated aluminum sheets of silica gel 60 F_{254}, layer thickness 0.2 mm (E. Merck, Darmstadt, Germany), in the following solvents: S_1, CH_2Cl_2−MeOH (9:1); S_2, CH_2Cl_2−MeOH (4:1); S_3, CH_2Cl_2−MeOH−NH_4OH (6:4:0.1); S_4, CH_2Cl_2−MeOH (3:2); S_5, CH_2Cl_2−THF (4:1); S_6, CH_2Cl_2−MeOH (7:3); S_7, BuOH−AcOH−H_2O (8:1:1); and S_8, CH_2Cl_2−MeOH (20:1). HPLC was performed with Waters C-18 column, 3.9 × 300 mm, or Phenomenex Aqua C-18 column, 4.6 × 250 mm, detection at 220 nm, flow rate 0.5 mL/min unless indicated otherwise. With the Waters column the following solvents were used: S_9, MeCN−H_2O (3:2, 0.1% TFA); S_{10}, MeCN−0.02 M NaH_2PO_4 (pH 4.0, 2:3); S_{11}, MeOH−H_2O (4:1); S_{12}, MeOH−H_2O (9:1); S_{13}, MeOH−H_2O (65:35); S_{14}, MeCN−H_2O (4:1, 0.1% TFA). Phenomenex column was employed with solvent S_{15}, MeCN−H_2O (1:1, 0.1% TFA), and S_{16}, MeCN−H_2O (45:55, 0.1% TFA). Nonapeptide GILGFVFTL was purchased from Genemed, Inc., San Francisco, CA. Glucuronide **3** was prepared as described.[8] Pig liver esterase (PLE, EC 3.1.1.1), β-glucuronidase (EC 3.2.1.31) from *E. coli* (type X-A), and bovine liver (type B-1) were products of Sigma, St. Louis, MO.

Methyl *N*-[(2,3,4-Tri-*O*-acetyl)-β-D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-alanine Methyl Ester (4a). Triethylamine (0.45 mL, 3.29 mmol) was added to a stirred solution of glucuronide **3** (0.5 g, 1.02 mmol) and *N*,*N*′-disuccinimidyl carbonate (DSC, 0.42 g, 1.64 mmol) in MeCN (5 mL) at room temperature. The progress of reaction was monitored on TLC (CH_2Cl_2−THF, 9:1). The starting material **3** was consumed after 2 h. A solution of AlaOMe·HCl (0.15 g, 1.02 mmol) and NEt_3 (0.143 mL, 1.02 mmol) in THF (5 mL) was then added. The mixture was stirred at room temperature for 16 h, solvent was evaporated, and residue was dissolved in CH_2Cl_2 (150 mL). The organic layer was washed with brine and water (50 mL each), dried (Na_2SO_4), and evaporated. Column chromatography on silica gel using solvent S_1 gave product **4a** (0.385 g, 62%): mp 155−156 °C; $[\alpha]^{24}_D$ 36° (*c* 1, $CHCl_3$); TLC, R_f 0.82 (S_1); HPLC (S_9, flow rate 1.0 mL/min, t_R 7.98 min, purity 95%); ^{1}H NMR ($CDCl_3$) δ 7.81 (s, 1H), 7.52 (d, 1H) and 7.35 (d, 1H), 7.25 (s, 1H), 5.33 (m, 3H), 5.19 (d, 1H) and 4.19 (d, 1H), 5.09 (s, 2H), 4.36 (m, 1H), 3.74 and 3.75 (2s, 6H), 2.12, 2.06 and 2.05 (3s, 9H), 1.42 (d, 3H); FAB-MS (NaCl) 637 (M + Na). Anal. $C_{25}H_{30}N_2O_{16}$ (C, H, N).

Methyl *N*-[(2,3,4-Tri-*O*-acetyl)-β-D-glucopyranuronate-3-nitrobenzyloxycarbonyl]glycine (4b). The reaction of glucuronide **3** (0.25 g, 0.51 mmol) with DSC was performed as described for alaninate **4a**. A solution of glycine (38 mg, 0.51 mmol) and NEt_3 (71 μL, 0.51 mmol) in DMF (5 mL) was added to the mixture which was stirred at room temperature for 16 h. After evaporation, ethyl acetate (100 mL) was added, and the workup followed the procedure for **4a**. Chromatography using solvent S_2 afforded compound **4b** (0.25 g, 83%): mp 94−96 °C, $[\alpha]^{28}_D$ 15.0° (*c* 0.1, MeOH); TLC, R_f 0.56 (S_6); HPLC (S_{10}, t_R 10.3 min, purity 90%); ^{1}H NMR δ 12.59 (bs, 1H), 7.87 (s, 1H), 7.67−7.57 (m, 2H), 7.40 (d, 1H), 5.72 (d, 1H), 5.43 (t, 1H), 5.11−5.06 (m, 2H), 4.72 (d, 1H), 5.03 (s, 2H), 3.63 (m, 5H), 1.98 (bs, 9H); FAB-MS (KCl) 625 (M + K). Anal. Calcd for $C_{23}H_{26}N_2O_{16}$: C, 47.09; H, 4.47; N, 4.78. Found C, 47.15; H, 4.61; N, 4.75.

Methyl *N*-[(2,3,4-Tri-*O*-acetyl)-β-D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-phenylalanine (4c). The procedure described for compound **4a** was perfomed on a 0.41 mmol scale of L-phenylalanine. Chromatography of the crude product using solvent S_2 provided product **4c** (0.175 g, 66%): mp 126−127 °C; $[\alpha]^{25}_D$ 18° (*c* 0.5, $CHCl_3$); TLC, R_f 0.65 (S_2); HPLC (S_{10}, t_R 3.9 min, purity 94%); ^{1}H NMR ($CDCl_3$) δ 7.74 (s, 1H), 7.45 (d, 1H) and 7.32 (d, 1H), 7.26 (bs, 5H), 7.15 (d, 1H), 5.35−5.18 (m, 4H), 4.24 (d, 1H), 5.04 (s, 2H), 4.64 (m, 1H), 3.72 (s, 3H), 3.12 (m, 2), 2.17, 2.11 and 2.05 (3s, 9H); FAB-MS (KCl) 715 (M + K). Anal. $C_{30}H_{32}N_2O_{16}$ (C, H, N).

Methyl *N*-[(2,3,4-Tri-*O*-acetyl)-β-D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-threonine (4d). The reaction was performed on 0.82 mmol scale with L-threonine as described above for compound **4a**. Chromatography using solvent S_6 as eluent gave product **4d** (0.38 g, 73%): mp 90−92 °C; $[\alpha]^{24}_D$ 8.2° (*c* 0.5, $CHCl_3$); TLC, R_f = 0.50 (S_3); HPLC (S_{10}, t_R 10.0 min, purity 82%); ^{1}H NMR (300 MHz) δ 7.90 (bd, 1H), 7.68 (d, 1H) and 7.41 (d, 1H), 7.08 (d, 1H), 5.73 (d, 1H), 5.43 (t, 1H) and 5.12−5.02 (m, 4H) and 4.72 (d, 1H), 4.05 (m, 1H), 3.93 (m, 1H), 3.62 (s, 3H), 2.02, 2.01 and 1.98 (3s, 9H), 1.07 (d, 3H); FAB-MS 631 (M + H). Anal. $C_{25}H_{30}N_2O_{17}$ (C, H, N).

Methyl *N*-[(2,3,4-Tri-*O*-acetyl)-β-D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-phenylalanyl-L-leucine (4e). The procedure described above for alaninate **4a** was followed on 2.06 mmol scale of Phe-Leu. Column chromatography on silica gel using solvent S_2 afforded product **4e** (0.9 g, 56%): mp 110−111 °C; $[\alpha]^{23}_D$ 42°(*c* 1, EtOH); TLC, R_f 0.79 (S_2); HPLC (S_9), t_R 13.3, flow rate 1.0 mL/min, purity 90%; ^{1}H NMR δ 8.28 (d, 1H), 7.76 (s, 1H) and 7.56 (d, 1H), 7.45 (d, 1H), 7.15 (d, 1H), 7.34−7.18 (m, 5H), 5.72 (d, 1H), 5.44 (t, 1H), 5.10 (m, 2H) and 4.74 (d, 1H), 4.91 (s, 2H), 4.29−4.19 (m, 2H), 3.62 (s, 3H), 3.00−2.64 (m, 2H), 2.06, 1.99 and 1.97 (3s, 9H), 1.62−1.46 (m, 3H), 0.88−0.81 (dd, 6H); FAB-MS 790 (M + H). Anal. $C_{36}H_{43}N_3O_{17}$ (C, H, N).

Methyl *N*-[(2,3,4-Tri-*O*-acetyl)-β-D-glucopyranuronate-3-nitrobenzyloxycarbonyl]-L-leucyl-L-arginine (4f). The reaction was as described for alaninate **4a** on 0.2 mmol scale of Leu-Arg acetate. Chromatography using solvent S_2 gave product **4f** (67 mg, 40.9%): mp 110°; $[\alpha]^{24}_D$ 8° (*c* 0.4, $CHCl_3$); R_f 0.79 (S_2); HPLC (S_{10}, t_R 7.23 min, purity 84%); ^{1}H NMR δ 9.28 (bs, 1H), 7.88 (s, 1H), 7.66 (2d, 3H), 7.44−7.34 (d, 4H), 5.71 (d, 1H), 5.41 (t, 1H), 5.12−4.91 (m, 4H), 4.71 (d, 1H), 3.98 (d, 1H), 3.82 (d, 1H), 3.61 (s, 3H), 2.98 (bs, 2), 2.00, 1.98 and 1.97 (3s, 9H), 1.62−1.38 (m, 7H), 0.79 (dd, 6H); FAB-MS 799 (M + H). Anal. $C_{33}H_{46}N_6O_{17}\cdot 2H_2O$ (C, H, N, −0.44).

Methyl *N*-[β-D-Glucopyranuronate-3-nitrobenzyloxycarbonyl]glycine (5b). Freshly prepared MeONa (0.79 mmol) in MeOH (2 mL) was added with stirring to a solution of compound **4b** (0.5 g, 0.63 mmol) in MeOH (20 mL) at 0 °C. The progress of deacetylation was monitored by TLC (S_4) and HPLC (S_{10}, flow rate 0.8 mL/min). The starting material was consumed in 30 min. Dowex 50 ($H^{(+)}$ form, 0.25 g) was added, the mixture was filtered, and solvent was evaporated. Chromatography of the residue using solvent S_4 afforded product **5b** (0.320 g, 79%): mp 88−89 °C; $[\alpha]^{28}_D$ −25° (*c* 0.1, MeOH); TLC, R_f 0.50 (S_3); HPLC (S_{15}, t_R 5.3 min, purity 99%); ^{1}H NMR

δ 7.88 (s, 1H), 7.66 (d, 1H) and 7.52 (d, 1H), 7.15 (bs, 1H), 6.05 (d, 1H), 5.86 (d, 1H), 4.03 (t, 1H), 3.78 (t, 1H), 3.48 (d, 1H), 5.02 (s, 2H), 3.68 (s, 3H), 3.42−3.35 (m, 6H); ESI-MS (NaCl) 483 (M + Na). Anal. $C_{17}H_{20}N_2O_{13}\cdot H_2O$ (C, H, N).

Methyl *N*-[β-D-Glucopyranuronate-3-nitrobenzyloxycarbonyl)]-L-phenylalanyl-L-leucine (5e). The reaction with triacetate **4e** was performed as described for compound **5b** to give product **5e** (0.32 g, 76%): mp 142−143 °C; $[\alpha]^{24}_D$ −38.8° (*c* 0.5, EtOH); TLC, R_f 0.51 (S_3); HPLC (S_{10}, flow rate 0.8 mL/min, t_R 10.1 min, purity 85%); ^{1}H NMR δ 8.26 (d, 1H), 7.74 (s, 1H) and 7.53 (d, 1H), 7.44 and 7.37 (2d, 2H), 7.29−7.12 (m, 5H), 5.53−5.49 (3 overlapped s, 3H), 4.90 (s, 2H), 5.29 (apparent d, 2H), 4.12 (d, 1H), 4.29−4.19 (m, 2H), 3.63 (s, 3H), 3.00−2.57 (m, 2H), 1.6−1.46 (m, 3H), 0.89−0.81 (dd, 6H); FAB-MS (KCl) 702 (M + K). Anal. $C_{30}H_{37}N_3O_{14}$ (C, H, N).

***N*-[β-D-(Glucopyranuronic acid)-3-nitrobenzyloxycarbonyl]-L-phenylalanyl-L-leucine (6e).** Compound **5e** (14.5 mg, 21 μmol) in 0.02 M Na_2HPO_4 (pH 7.2, 10 mL) was incubated with PLE (1,200 units) at 37 °C. Progress of the reaction was monitored by HPLC (MeCN−NaH_2PO_4, pH 4.0, 3:7, flow rate 0.6 mL/min). The starting material was consumed in 1 h with the formation of a new peak (t_R 5.6 min). The pH was adjusted to 4.0 with HCl, and the mixture was extracted with ethyl acetate. The organic phase was washed with water, it was dried (Na_2SO_4), and the solvent was evaporated to give compound **6e** (13 mg, 91%): mp 122−124 °C; $[\alpha]^{25}_D$ −14° (*c* 0.25, EtOH); TLC, R_f 0.42 (S_3); HPLC (S_{10}, flow rate 0.8 mL/min, t_R 5.6 min, purity 95%); ^{1}H NMR δ 8.25 (d, 1H), 7.74 (s, 1H) and 7.53 (d, 1H), 7.43 (d, 1H) and 7.34 (d, 1H), 7.27−7.15 (m, 5H), 5.48 (d, 1H), 4.42 (d, 1H), 3.94 (m, 2H) and 3.25 (d, 1H), 5.24 (3 overlapped s, 3H), 4.90 (s, 2H), 4.24 (m, 2), 3.00−2.96 (m, 2H), 1.65−1.49 (m, 3H), 0.84 (dd, 6H); FAB-MS (KCl) 688 (M + K). Anal. $C_{29}H_{35}N_3O_{14}$ (C, H, N).

Digestion of Compound 6e with β-Glucuronidase. Compound **6e** (1 mg, 1.5 μmol) and *E. coli* β-glucuronidase (800 units) were incubated in 0.02 M Na_2HPO_4 (pH 7.2, 1 mL) at 37 °C. Progress of reaction was monitored by HPLC/MeCN−0.02 M NaH_2PO_4 (pH 4.0) 3:7, flow rate 0.6 mL/min). The starting material was consumed within 1 h with the formation of Phe-Leu and 4-hydroxy-3-nitrobenzyl alcohol (**8**) whose t_R's (4.8 and 9.7 min, respectively) were identical with those of the corresponding authentic samples.

Methyl *N*-[β-D-Glucopyranuronate-3-nitrobenzyloxycarbonyl]glycine Pentafluorophenyl Ester (10). DCC (0.138 g, 0.67 mmol) and pentafluorophenol (0.124 g, 0.67 mmol) were added to a solution of compound **5b** (0.31 g, 0.67 mmol) in DMF−THF (1:5, 30 mL) at 0 °C. The mixture was stirred for 2 h at 0 °C and then at room temperature for 16 h. The solvents were evaporated, the residue was dissolved in EtOAc (50 mL), the solution was filtered, and the filtrate was evaporated. Chromatography on a silica gel column using hexane:EtOAc (1:4) as eluent gave 0.385 g (91%) of product **10**, which was used for the next coupling reaction: mp 145−147 °C; $[\alpha]^{28}_D$ −31° (*c* 0.1, MeOH); TLC, R_f 0.8 (S_5); HPLC (S_{11}, flow rate 0.8 mL/min, t_R 4.8 min, purity 84%); ^{1}H NMR δ 7.80 (s, 1H), 7.59 (d, 1H) and 7.38 (d, 1H), 5.26 (d, 1H), 4.24 (bs, 2H), 4.07 (d, 1H), 3.04 (m, 3H), 5.02 (s, 2H), 3.61 (s, 3H), 3.36−3.24 (m, 3H); FAB-MS (NaCl) 649 (M + Na).

Methyl *N*-[β-D-Glucopyranuronyl-3-nitrobenzyloxycarbonyl]glycyl-L-phenylalanyl-L-leucine (11a). A solution of pentafluorophenyl ester **10** (0.1 g, 0.159 mmol) in THF (5 mL) was added with stirring to Phe-Leu (45 mg, 0.159 mmol) and NEt_3 (25 μL, 0.159 mmol) in DMF (5 mL) at room temperature. The stirring was continued for 16 h, the solvents were evaporated, and residue was chromatographed on a silica gel column using solvent S_6 to give compound **11a** (72 mg, 63%): mp 160−161 °C, $[\alpha]^{29}_D$ −27° (*c* 0.1, MeOH); TLC, R_f 0.50 (S_4); HPLC (S_{10}, t_R 11.0 min, purity 93%); ^{1}H NMR δ 8.24 (d, 1H), 8.01 (d, 1H), 7.82 (s, 1H), 7.59 (d, 1H), 7.43 (m, 2H), 7.19 (m, 5H), 5.50 (bs, 2H), 5.30 (d, 2H), 4.10 (d, 1H), 3.57 (d, 1H), 3.52 (bs, 1H), 4.98 (s, 2H), 4.53 (q, 1H), 4.35 (bs, 1H), 4.16 (q, 1H), 3.62 (s, 3H), 3.38 (bs, 2H), 3.00 (dd, 2H), 2.72 (m, 1H), 1.51−1.61 (m, 3H), 0.85 (dd, 6H); ESI-MS (NaCl) 743 (M + Na).

***N*-[β-D-(Glucopyranuronic acid)-3-nitrobenzyloxycarbonyl]glycyl-L-phenylalanyl-L-leucine (12).** Compound **11a** (20 mg, 28 μmol) and PLE (1,200 units) were incubated in Na_2HPO_4 (0.02 M, pH 7.2, 10 mL) at 37 °C. Progress of reaction was monitored by HPLC (S_{10}, flow rate 0.4 mL/min). The starting material was consumed in 1 h with the formation of a new major peak with t_R 7.2. The pH was adjusted to 4.0 with HCl, the mixture was partitioned between ethyl acetate and water, and the organic phase was dried (Na_2SO_4) and evaporated: yield 18 mg (91%) of tripeptide derivative **12**; mp 171−173 °C; $[\alpha]^{29}_D$ −14° (*c* 0.1, MeOH); TLC, R_f 0.49 (S_3); HPLC (S_{10}, flow rate 0.6 mL/min, t_R 7.4 min, purity 95%); ^{1}H NMR δ 12.62 (bs, 1H), 8.28 (d, 1H), 8.00 (d, 1H), 7.83 (s, 1H), 7.59 (d, 1H) and 7.43 (m, 2H), 7.18 (m, 5H), 5.49 (bs, 1H), 5.25 (bs, 2H), 4.98 (s, 2H), 4.54 (q, 1H), 4.21 (q, 1H), 4.01−3.92 (m, 2H), 3.62−3.44 (m, 3H), 3.00 (dd, 2H), 2.72 (m, 1H), 1.51−1.61 (m, 3), 0.85 (dd, 6H); ESI-MS (NaCl) 729 (M + Na). Anal. $C_{31}H_{38}N_4O_{15}$ (C, H, N).

Digestion of Compound 12 by β-Glucuronidase. Compound **12** (1 mg, 1.4 μmol) was incubated with bovine liver β-glucuronidase (800 units) in phosphate buffer (0.02 M, pH 7.2, 1 mL) at 37 °C. The reaction was followed by HPLC (S_{10}, flow rate 0.6 mL/min). The starting material was consumed in 4 h with the formation of two major peaks at t_R 6.5 (Gly-Phe-Leu) and 14.0 (compound **8**).

H-Leu-OBn (13). Free base **13** was released from the commercially available tosylate using $NaHCO_3$ (pH 8.0) and extracted with ethyl acetate as a thick oil (5.1 g, 92%): R_f 0.6 (S_7); ^{1}H NMR δ 7.34 (bs, 5H), 5.07 (bs, 2H), 3.30 (bs, 1H), 1.71−1.66 (m, 3H), 1.40−1.29 (m, 2H), 0.82 (m, 6H); EI-MS 221 (M + H).

Boc-Thr(Bn)-Leu-OBn (14). HOBT (2.19 g, 16.16 mmol) and DCC (3.32 g, 16.16 mmol) were added with stirring to a solution of Boc-Thr(Bn)-OH (**15**, 5 g, 16.16 mmol) in THF (50 mL) at 0 °C. The stirring was continued for 30 min. The H-Leu-OBn (**13**, 4.46 g, 20.20 mmol) in DMF (30 mL) cooled to 0 °C was then added, and the mixture was stirred for 2 h and at room temperature for 16 h. The solvents were evaporated, the residue was dissolved in EtOAc (125 mL), and dicyclohexyl urea (DCU) was filtered off. The filtrate was washed twice with saturated aqueous $NaHCO_3$, 10% citric acid, and water (50 mL each); it was dried (Na_2SO_4) and evaporated. Chromatography using solvent S_8 as eluent gave dipeptide **14** (7.0 g, 84%): mp 84−85 °C, R_f 0.62 (S_8); ^{1}H NMR δ 8.24 (d, 1H) and 6.59 (d, 1H), 7.33−7.24 (m, 10H), 5.07 (bs, 2H) and 4.35 (m, 2H), 4.49 (m, 1H), 4.05 (m, 1H), 3.79 (t, 1H), 1.58−1.47 (m, 3H), 1.36 (s, 9H), 1.07 (d, 3H), 0.78 (dd, 6H); EI-MS 512 (M).

H-Thr(Bn)-Leu-OBn (16). A solution of dipeptide **14** (6.5 g, 12.69 mmol) in HCO_2H (97%, 50 mL) was stirred at room temperature for 6 h. The solvent was evaporated, residue was dissolved in water (150 mL), and solution was extracted with ether (2 × 50 mL). The pH of the aqueous phase was adjusted to 8.0 with aqueous $NaHCO_3$ and extracted with EtOAc (2 × 100 mL). The organic portion was washed with water (50 mL), and it was dried (Na_2SO_4) and evaporated to give dipeptide **16** as a thick oil (4.6 g, 87%): R_f 0.72 (S_2); ^{1}H NMR δ 8.56 (bs, 1H), 7.30 (2s, 10H), 5.09 (s, 2H), 4.50−4.35 (m, 4H), 3.81 (s, 1H), 1.58 (bs, 3H), 1.15−1.14 (d, 3H), 0.81 (dd, 6H); FAB-MS 413 (M + H).

Boc-Phe-Thr(Bn)-Leu-OBn (17). The reaction was performed as described for dipeptide **14** using Boc-Phe-OH (**18**, 3.66 g, 13.8 mmol), HOBT (1.87 g, 13.8 mmol), and DCC (2.84 g, 13.8 mmol) in THF (50 mL). Dipeptide **16** (3.8 g, 9.21 mmol) was then added in DMF solution (20 mL), and the mixture was stirred for 2 days at room temperature and worked-up as described for dipeptide **14**. Chromatography in CH_2Cl_2:MeOH (10:1) furnished tripeptide **17** (4.7 g, 77%): mp 150−152 °C; R_f 0.64 (S_1); ^{1}H NMR δ 8.24 (d, 1H), 7.82 (d, 1H) and 7.09 (d, 1H), 7.27 (m, 15H), 5.05 (dd, 2H), 4.48−4.35 (m, 4H), 4.23 (m, 1H), 3.90 (t, 1H), 2.95 (m, 1H), 2.71 (m, 1H), 1.54 (m, 3H), 1.25 (s, 9H), 1.08 (d, 3H), 0.82 (dd, 6H); FAB-MS (KCl) 698 (M + K).

H-Phe-Thr(Bn)-Leu-OBn (19). It was prepared as described for dipeptide **16** from tripeptide **17** (4 g, 6.06 mmol). The tripeptide **19** (2.8 g, 82%) was obtained as a thick oil: TLC, R_f 0.71 (S_2); HPLC (S_{12}), flow rate 0.6 mL/min, t_R 6.6 min, 96% purity); ^{1}H NMR δ 8.32 (d, 1H), 8.27 (d, 1H), 7.31−7.20 (m, 15H), 5.06 (dd, 2H), 4.47−4.36 (m, 5H), 3.90 (t, 1H), 3.69 (bs, 1H), 3.02 (m, 1H), 2.68 (m, 1H), 1.58−1.50 (m, 4H), 1.03 (d, 3H), 0.83 (dd, 6H); ESI-MS 560 (M + H).

H-Val-OBn (20). Free base was released from the corresponding hydrochloride as described for H-Leu-OBn (**13**) as a thick oil: R_f 0.7 (S_7); ^{1}H NMR δ 7.33 (m, 5H), 5.12 (dd, 2H), 3.13 (d, 1H), 1.83 (m, 1H), 1.69 (bs, 2H), 0.80 (dd, 6H); EI-MS 207 (M + H).

Boc-Phe-Val-OBn (21). The reaction was performed as described for dipeptide **14** with Boc-Phe-OH (**18**, 5.0 g, 18.84 mmol) and H-Val-OBn (**20**, 5.49 g, 26.35 mmol) to give dipeptide **21** (7 g, 81%): mp 68−69 °C; R_f 0.48 (S_8); ^{1}H NMR δ 8.17 (d, 1H), 7.38 (m, 10H), 6.95 (d, 1H), 5.11 (dd, 2H), 4.24 (t, 2H), 2.88 (m, 1H), 2.67 (t, 1H), 2.06 (m, 1H), 1.26 (s, 9H), 0.86 (m, 6H); EI-MS 454 (M).

Boc-Phe-Val-OH (22). Boc-Phe-Val-OBn (**21**, 4.0 g, 8.81 mmol) was hydrogenated in a Parr apparatus at 40 psi for 2 h in ethanol (50 mL) over Pd-C (10%, 0.42 g). The catalyst was filtered off, and the solvent was evaporated. The residue was partitioned between aqueous $NaHCO_3$ (100 mL) and ether (2 × 50 mL). The mixture (pH 3) was extracted with ethyl acetate (2 × 100 mL), and the organic phase was washed with water, dried (Na_2SO_4), and evaporated to give the dipeptide **22** (2.9 g, 90%); mp 77−78 °C; R_f 0.58 (S_7); HPLC (S_{12}, flow rate 0.6 mL/min, t_R 5.6 min, purity 97%); ^{1}H NMR δ 7.88 (d, 1H), 7.23 (m, 5H), 6.96 (d, 1H), 4.23−4.14 (m, 2H), 2.93 (m, 1H), 2.71 (q, 1H), 2.05 (m, 1H), 1.26 (s, 9H), 0.87 (m, 6H); FAB-MS (KCl) 403 (M + K).

Boc-Phe-Val-Phe-Thr(Bn)-Leu-OBn (23). The reaction of dipeptide **22** (2.5 g, 7.01 mmol) with tripeptide **19** (2.8 g, 5.00 mmol) was performed as described for dipeptide **14**. Chromatography in solvent (S_8) furnished pentapeptide **23** (4.4 g, 70%): mp 80−81 °C, R_f 0.66 (S_2), HPLC (S_{14}, flow rate 0.6 mL/min, t_R 11.4 min, purity 90%); ^{1}H NMR δ 8.19 (d, 1H), 8.12 (d, 1H, 8.02 (d, 1H) and 7.61 (d, 1H), 7.32−7.10 (m, 20H), 7.00 (d, 1H), 5.00 (dd, 2H), 4.70 (m, 1H), 4.47−4.36 (m, 6H), 4.21−4.11 (m, 2H), 3.89 (t, 1H), 3.00 (m, 1H), 2.87−2.63 (m, 2H), 1.90 (m, 1H), 1.53 (m, 2H), 1.25 (s, 9H), 1.05 (d, 3H), 0.82 (dd, 6H), 0.73 (m, 6H); ESI-MS (NaCl) 928 (M + Na).

H-Phe-Val-Phe-Thr(Bn)-Leu-OBn (24). Deprotection of pentapeptide **23** (0.5 g, 0.55 mmol) was performed as described for dipeptide **14** to afford free pentapeptide **24** (0.4 g, 90%): mp 147−148 °C; R_f 0.55 (S_2); HPLC (S_{14}, flow rate 0.6 mL/min, t_R 6.3 min, purity 96%); ^{1}H NMR δ 8.25 (d, 1H), 8.14 (d, 1H), 8.02 (d, 1H) and 7.89 (d, 1H), 7.30−7.17 (m, 20H), 5.05 (dd, 2H), 4.68 (m, 1H), 4.47−4.36 (m, 6H), 4.16 (m, 2H), 3.90 (t, 1H), 2.95 (m, 1H), 2.75 (m, 2H), 1.89 (m, 1H), 1.52 (m, 4H), 1.05 (d, 3H), 0.82 (dd, 6H), 0.75−0.63 (dd, 6H); ESI-MS 806 (M + H).

H-Gly-OBn (25). Free base was released from the corresponding hydrochloride as described in the workup procedure for dipeptide **16** as a thick oil: R_f 0.50 (S_1); ^{1}H NMR δ 7.38−7.30 (m, 5H), 5.11 (s, 2H), 3.87 (m, 2H), 1.89 (bs, 2H): ESI-MS 165 (M + H).

Boc-Leu-Gly-OBn (26). It was prepared as described for dipeptide **14** from Boc-Leu-OH (**27**, 5 g, 20.05 mmol) and Gly-OBn (**25**, 4.13 g, 25.06 mmol). Chromatography afforded dipeptide **26** as a thick oil (7.0 g, 87%): R_f 0.90 (S_8); ^{1}H NMR δ 8.26 (t, 1H), 7.32 (m, 5H), 6.89 (d, 1H), 5.09 (s, 2H), 3.91−3.82 (m, 3H), 1.60 (m, 1H), 1.34 (s, 11H), 0.81 (m, 6H); FAB-MS (KCl) 417 (M + K).

H-Leu-Gly-OBn (28). Deprotection of **26** (8 g, 21.16 mmol) was performed as described for dipeptide **14** to give **28** (5.88 g, 85%): R_f 0.51 (S_6); ^{1}H NMR δ 8.60 (bs, 1H), 7.34 (s, 5H), 5.10 (s, 2H), 3.91 (ddd, 2H), 3.40 (t, 1H), 1.69 (bs, 2H), 1.44 (m, 1H), 1.30 (m, 2H), 0.83 (m, 6H); ESI-MS 279 (M + H).

Boc-Ile-Leu-Gly-OBn (29). This tripeptide was prepared as described for dipeptide **14** from Boc-Ile-OH (**30**, 2.50 g, 10.80 mmol) and dipeptide **28** (2.5 g, 8.99 mmol). Chromatography gave tripeptide **29** (5.31 g, 77%): mp 50−51 °C; R_f 0.62 (S_8); ^{1}H NMR δ 8.40 (bs, 1H), 7.79 (d, 1H), 7.33 (s, 5H), 6.80 (d, 1H), 5.08 (s, 2H), 4.36 (m, 1H), 3.94−3.73 (m, 3H), 1.62 (bs, 2H), 1.34 (s, 9H), 1.04 (m, 2H), 0.83−0.77 (m, 14H); ESI-MS 492 (M + H).

Boc-Ile-Leu-Gly-OH (31). Tripeptide **29** (2.0 g, 4.07 mmol) was hydrogenated in ethanol (25 mL) over Pd−C (10%, 0.2 g) as described for dipeptide **22** to give tripeptide **31** (1.4 g, 85%): mp 108−109 °C; R_f 0.69 (S_1); HPLC (S_{13}, flow rate 0.8 mL/min, t_R 7.9, purity 98.5%); ^{1}H NMR δ 8.18 (bs, 1H), 7.79 (d, 1H), 6.82 (d, 1H), 4.36 (m, 1H), 3.77−3.62 (m, 3H), 1.63 (bs, 2H), 1.34 (s, 9H), 1.03 (m, 2H), 0.85−0.77 (m, 14H); ESI-MS 402 (M + H).

Boc-Ile-Leu-Gly-Phe-Val-Phe-Thr(Bn)-Leu-OBn (32). To a solution of tripeptide **31** (0.460 g, 1.02 mmol) in THF (20 mL) was added HOBT (0.14 g, 1.02 mmol) at 0 °C followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl, 0.196 g, 1.02 mmol). The reaction mixture was stirred for 30 min. A cooled solution of pentapeptide **24** (0.46 g, 0.57 mmol) in DMF (10 mL) was then added. The stirring was continued at 0 °C for 2 h and at room temperature for 3 days. The mixture was worked up as described for the dipeptide **14**. Chromatography of the crude product in solvent S_8 provided octapeptide **32** (0.45 g, 66%): mp 177−179 °C; R_f 0.57 (S_8); HPLC (S_{14}, flow rate 0.6 mL/min, t_R 9.20, 91% purity); ^{1}H NMR δ 8.11 (t, 2H), 8.03 (d, 1H), 7.98 (t, 1H), 7.92 (bs, 2H), 7.79 (d, 1H), 7.32−7.11 (m, 20H), 6.78 (d, 1H), 5.05 (dd, 2H), 4.70 (m, 1H), 4.55 (m, 1H), 4.47−4.36 (m, 4H), 4.27 (m, 1H), 4.13 (m, 1H), 3.89 (m, 2H), 3.59 (m, 2H), 2.80 (m, 4H), 1.34 (s, 9H), 1.56 (m, 3H), 1.05 (d, 3H), 0.83−0.77 (m, 26H); ESI-MS (NaCl) 1,211 (M + Na).

H-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (9). Octapeptide **32** (0.2 g, 4.07 mmol) was hydrogenated in AcOH (50 mL) over Pd−C (10%, 0.4 g) for 6 h. The catalyst was filtered off and the solvent evaporated. The residue was dissolved in TFA (10 mL), and the solution was stirred at room temperature for 1 h. The solvent was evaporated, and residue (0.14 g) was purified by HPLC using Phenomenex Aqua semipreparative column (10 × 250 mm, S_{16}, flow rate 2.4 mL/min, t_R 7.4 min) to give octapeptide **9** (95 mg, 62%): mp 124−126 °C; R_f 0.63 (S_7); HPLC (S_{15}, t_R 4.2 min, flow rate 0.8 mL/min, purity 92%); ^{1}H NMR δ 12.60 (bs, 1H), 8.43 (bs, 1H), 8.29 (d, 1H), 8.20 (bs, 1H), 8.07 (bs, 3H), 7.86 (bs, 1H), 7.20−7.14 (b, 10H), 4.79 (d, 1H), 4.61 (bs, 1H), 4.38 (m, 2H), 4.24−4.16 (m, 3H), 3.90 (bs, 1H), 3.76 (m, 1H), 3.61 (bs, 2H), 2.98 (m, 2H), 2.75 (m, 2H), 1.90 (m, 1H), 1.75 (m, 1H), 1.61−1.43 (m, 6H), 1.01 (bs, 3H), 0.86−0.72 (m, 26H); ESI-MS 909 (M + H).

Methyl *N*-[β-D-Glucopyranuronate-3-nitrobenzyloxycarbonyl]-Gly-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (11b). A solution of pentafluorophenylester **10** (136 mg, 0.22 mmol) in THF (10 mL) was added to a mixture of octapeptide **9** (100 mg, 0.11 mmol) and NEt_3 (15 μL, 0.11 mmol) in DMF (10 mL) with stirring at room temperature. The stirring was continued for 40 h, the solvents were evaporated, and residue was chromatographed (three times) using $CHCl_3$:MeOH (3:2) to obtain compound **11b** (68 mg, 46%) which was further purified by semipreparative HPLC (see octapeptide **9**, S_{16}, flow rate 3.4 mL/min) to give 52 mg (34%) of **11b**: HPLC (S_{16}, flow rate 1.0 mL/min, t_R 13.1, purity 86%); mp 140−142 °C; $[\alpha]^{26}_D$ 11° (*c* 0.1, MeOH); R_f 0.49 /$CHCl_3$:MeOH:NH_4OH (4:6:0.1)/; ^{1}H NMR δ 8.11 (bs, 4H), 8.03 (bs, 1H), 7.98 (d, 1H), 7.93 (d, 1H), 7.88 (d, 1H), 7.83 (bs, 1H), 7.61 (d, 1H),7.53 (bs, 1H), 7.41 (d, 1H), 7.26−7.11 (m, 10H), 5.54−5.50 (m, 2H), 5.30 (m, 2H), 4.99 (s, 2H), 4.66 and 4.52 (2m, 3H), 4.35 (bs, 1H), 4.25 (bs, 2H), 4.14−4.00 (m, 5H), 3.63 (s, 3H), 3.54 (d, 1H), 3.15 (d, 1H), 3.05 (m, 5H), 2.88−2.60 (m, 7H), 1.86 (m, 1H), 1.63 (m, 1H), 1.54 (m, 1H), 1.39 (m, 2H), 1.03 (m, 3H), 0.98 (d, 3H), 0.86−0.74 (m, 23H); Negative ESI-MS (NH_4OH) 1,349 (M − H).

***N*-[β-D-(Glucopyranuronic acid)-3-nitrobenzylcarbonyl]-Gly-Ile-Leu-Gly-Phe-Val-Phe-Thr-Leu-OH (2a).** A mixture of compound **11b** (20 mg, 0.014 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (4.6 mg, 0.014 mmol) in MeOH (10 mL) was stirred at room temperature for 3 h. Dowex 50 ($H^{(+)}$ form, 25 mg) was then added, and the mixture was filtered. The filtrate was evapo-

rated to give crude prodrug which was purified by semi-preparative HPLC (see octapeptide **9**, flow rate 3.4 mL/min) to give 9 mg (47%) of **2a**. Analytical HPLC (flow rate 1.0 mL/min, t_R 7.9 min, purity 76%): mp 180−181 °C, $[\alpha]_D^{26}$ 6.0° (*c* 0.1, MeOH); ^{1}H NMR (500 MHz) δ 8.07 (d, 1H), 8.01 (d, 1H), 7.96−7.91 (m, 4H), 7.87 (d, 1H), 7.84 (s, 1H), 7.81 (d, 1H), 7.61 (d, 1H), 7.51 (bs, 1H), 7.42 (d, 1H), 7.25−7.31 (m, 10H), 5.48 (bs, 1H), 5.40 (bs, 1H), 5.26 (d, 2H), 5.00 (s, 2H), 4.78 (bs, 1H), 4.67 and 4.55 (2m, 4H), 4.24−4.12 (m, 6H), 3.96−3.91 (m, 2H), 3.60 (m, 2H), 3.03 (m, 2H), 2.89 (m, 2H), 2.79 (m, 2H), 2.66 (m, 2H), 2.53 (bs, 5H), 1.90 (m, 1H), 1.66 (m, 2H), 1.41 (m, 2H), 1.22 (bs, 3H), 1.03 (d, 3H), 0.88−0.74 (m, 26H); Negative ESI-MS (NH_4OH) 1,335 (M − H).

Hydrolysis of Peptide Prodrug 2a by β-Glucuronidase. The hydrolysis of propeptide **2a** was perfomed as described for propeptide **12**. It was followed by HPLC (S_{16}, flow rate 1.0 mL/min). The starting material was consumed in 4 h with the formation of two peaks with t_R 3.6 and 4.5 min, corresponding to the authentic samples of 4-hydroxy-3-nitrobenzyl alcohol (**8**) and nonapeptide **2b**, respectively.

Binding of Nonapeptide 2b and Prodrug 2a to HLA-A2.1. T2 cells were maintained in AIM V medium (GIBCO BRL/Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum. A total of 3×10^5 T2 cells were incubated in the medium with varying concentrations of **2b** or **2a**. To activate the prodrug **2a**, β-glucuronidase from *E. coli* (200 and 300 units/mL) was added to the cell culture. The pH was maintained at 6.6 ± 0.2 in this medium. The mixtures were incubated at 37 °C for 1 h and then at 28 °C overnight. To measure the level of HLA-A2.1, cells were stained with mouse monoclonal antibody BB7.2 (American Type Culture Collection). Unbound antibody was removed by extensive washing, and bound antibody was visualized by staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunological Research Laboratory, West Grove, PA). Isotype matched monoclonal antibody was the negative control. Flow cytometric analysis was performed with a FACS Calibur (Beckton Dickinson, San Jose, CA), and the data are recorded as mean channel fluorescence. The results (average from three separate experiments) are given in Figure 1.

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