Enzyme-Labile Protecting Groups in Peptide Synthesis: Development of Glucose- and Galactose-Derived Urethanes

Andrew G. Gum,[b] Thomas Kappes-Roth,[b] and Herbert Waldmann*[a]

Abstract: The development of the tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl (AGlOC) and tetra-*O*-acetyl-β-D-galactopyranosyloxycarbonyl (AGalOC) protecting groups, which are fully enzyme-labile, carbohydrate-derived urethanes, is described. The protected amino acids were easily synthesized and subsequently converted into a series of model dipeptides through classical peptide couplings. Cleavage of an α/β-anomeric mixture of a model AGlOC dipeptide was achieved with a "one-pot" procedure in good yield. To gain a better understanding of the enzymatic deprotection reaction, the AGalOC group was removed through a two step biotransformation (lipase catalyzed deacetylation, followed by β-galactosidase catalyzed glycosidic bond fragmentation). Under these very mild reaction conditions (aq. buffer pH 7.0, 37 °C), the desired N-terminal, unprotected dipeptide conjugates were obtained. The methodology was further utilized for the synthesis of an advanced tetrapeptide model system.

Keywords: biotransformations · enzyme-labile urethane · glycosidases · peptides · synthesis design

Introduction

Protein conjugates, such as glyco-, phospho-, and lipoproteins, in which the amino acid backbone is covalently linked to carbohydrates, phosphates, and lipids, play numerous important roles in many biological processes.[1] Peptide conjugates with the characteristic linkage between the peptide and the side chain modifications found in their parent proteins, have proven to be efficient and invaluable tools for the study of such biological phenomena. For instance, lipid-modified peptides were used in the study of membrane binding and plasma membrane targeting of the Ras proteins.[2] Due to the acid and base lability of the desired glyco-, phospho- and lipopeptide structures, standard preparations based on classical protecting group techniques, like N-terminal 9-fluorenylmethoxycarbonyl (Fmoc) and *tert*-butyloxycarbonyl (Boc) protected peptide building blocks, are not sufficient. Thus, methods that allow for the facile synthesis of the target compounds under the mildest conditions are in demand. The use of biotransformations, especially enzyme-labile protecting groups that are removed with a very high degree of selectivity and under desirable conditions (pH 6–8, aqueous solvents, ambient temperatures), offers an attractive alternative for the synthesis of peptides and related conjugates.[3]

When synthesizing peptides, it is necessary to protect the N-terminus as a urethane in order to prevent racemization of the amino acid upon activation. Unfortunately, to date, there are no known amidases, esterases, or lipases that are able to cleave the rather stable urethane bonds. We reasoned that these enzymes are not able to attack the carbonyl group of urethanes for electronic reasons (diminished reactivity of the carbonyl group due to two +M substituents). An alternative strategy would be to employ a biocatalyst that attacks a different bond, for example, an *O*-alkyl bond, and to use an urethane designed accordingly. It is well known that glycosidases hydrolyze their substrates by cleaving the glycosidic bond.[4] Therefore, a carbohydrate-derived urethane protecting group would provide the desired enzyme-lability (Figure 1). Additionally, such sugar derivatives have increased solubility in aqueous solutions, a necessary requirement for all biotransformations. This idea has already been employed for antibody-directed enzyme prodrug therapy (ADEPT)[5] as well as for the cleavage of glycosyl esters from amino acids.[6] Recently, our group reported a new enzyme-labile protecting group for peptide synthesis, a tetrabenzyl-glucose-derived urethane (BGlOC).[7] Removal of the BGlOC group requires hydrogenolytic cleavage of the benzyl ethers followed by glucosidase-mediated hydrolysis of the glycosidic bond. Benzyl-protected glycosides are acid-labile, thus, although the BGlOC-group demonstrates that the strategy delineated above can successfully be realized, it poses no significant

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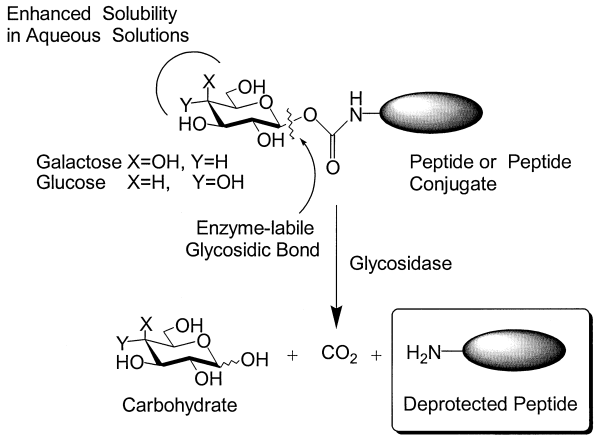


Figure 1. Principle of carbohydrate-derived urethane protecting groups.

advantages compared with classical hydrogenolytically removable protecting groups.

Herein, we wish to describe the development of the tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl (AGlOC) and tetra-*O*-acetyl-β-D-galactopyranosyloxycarbonyl (AGalOC) protecting groups; these are second generation enzyme-labile urethanes, which are readily attached to the desired amino acid and, after further synthetic steps, cleaved in two separate or sequential enzymatic steps.

Results and Discussion

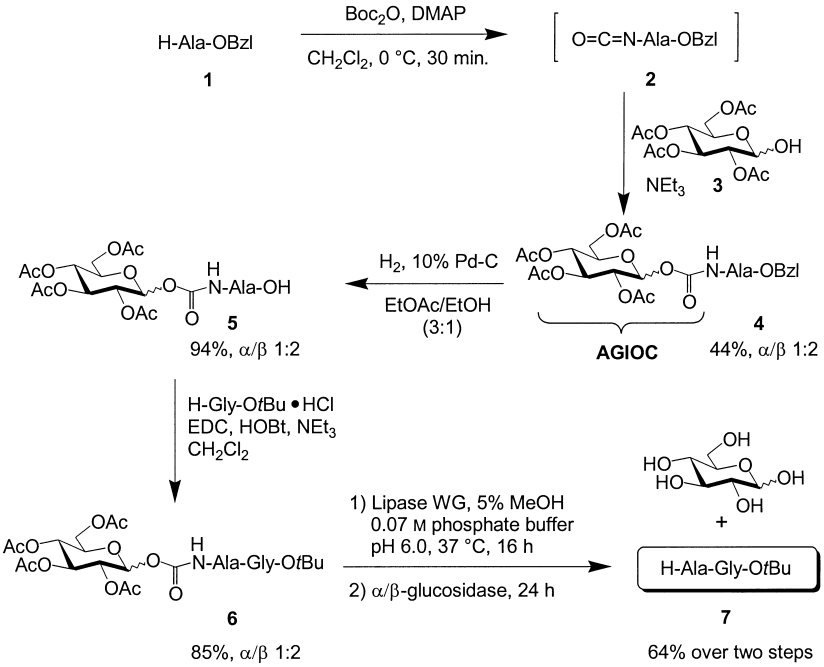
In the first extension of the previously reported BGlOC-derived urethane, the benzyl-protected glucose was replaced with the tetra-*O*-acetyl glucose derivative (AGlOC).[8] This exchange would allow for a completely enzyme-labile system (lipase deacetylation followed by glucosidase-catalyzed unmasking of the urethane), thus eliminating the need for any chemical deprotection steps. The protected amino acid **4** was synthesized through a "one-pot" procedure previously reported in the literature[9] (Scheme 1). When L-alanine benzyl ester **1**, 4-dimethylaminopyridine (DMAP) and Boc_2O were mixed, the intermediate isocyanate **2** was generated in situ and trapped, without being isolated, with tetra-*O*-acetyl glucose **3**[10] to give an anomeric mixture of the protected amino acid **4** (α/β 1:2). For this initial study, the anomeric mixture was not separated, but was utilized throughout the subsequent steps. After simple catalytic hydrogenation to afford amino acid **5**, the dipetide **6** was synthesized in high yield under standard peptide coupling conditions.

For the final enzymatic deprotection, a "one-pot" biotransformation was used. After overnight incubation of an aqueous buffered solution of dipeptide **6** in the presence of lipase WG and 5% methanol as co-solvent at 37 °C, thin layer chromatography (TLC) indicated complete cleavage of all acetate groups. Both α- and β-glucosidases were then added to the reaction and, after a further 24 hours of incubation, the desired unprotected dipeptide **7** was obtained in good yield over the two enzymatic steps. Only the desired dipeptide was isolated and no hydrolysis of either amide or ester bonds was observed. Unfortunately, attempts to utilize the AGlOC group for the synthesis of larger peptides were not successful, so an alternative group embodying the same advantageous properties was developed.

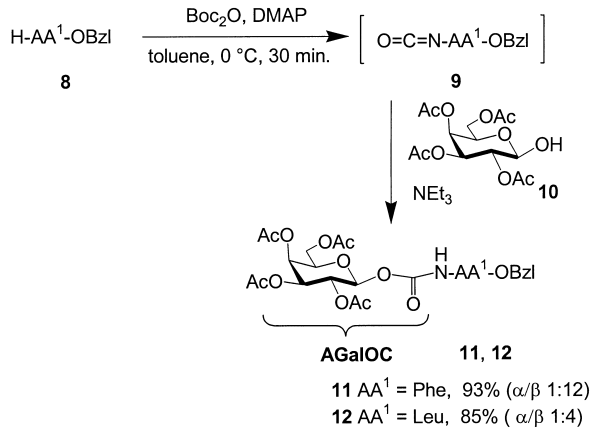
For the new group, a β-galactose-derived urethane was the initial choice, because a wider variety of commercial β-galactosidases is available, thus allowing for increased flexibility in the final deprotection step. Introduction of the protecting group was achieved under the previously described conditions by nucleophilic trapping of the appropriate amino acid isocyanate **9** with tetra-acetyl-β-galactose **10**[10] (Scheme 2). Although both protected amino acid benzyl esters **11** and **12** were obtained as anomeric mixtures, the α/β ratio was greatly improved when dry toluene was used as the solvent (from 1:2 up to 1:12).

The free acids **13** and **14** were obtained after hydrogenation of the benzyl ester by simply filtering the crude reaction mixture through celite; they did not require further purification (Scheme 3). After additional coupling reactions under various conditions with C-terminal ester-protected amino acids, a series of model AGalOC-protected dipeptides, **15**–**21**, was generated (Table 1).

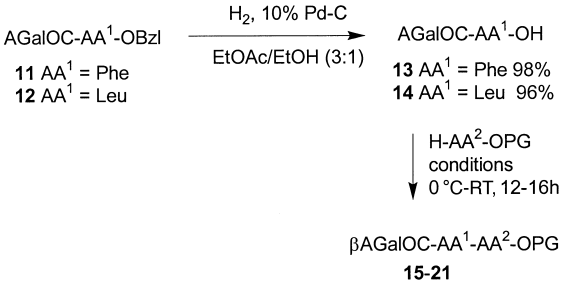
After the coupling, the anomers were separated through careful column chromatography on silica gel. The key characteristic for differentiating the anomers was apparent in the ^{13}C NMR shift differences (δ for $CH_{\alpha\text{-}C1}$ ~90 and for



Scheme 1. Cleavage of the α/β-mixed AGlOC-group.



Scheme 2. Introduction of the AGalOC-protecting group.



Scheme 3. Preparation of dipeptides.

Table 1. Summary of coupling reactions.

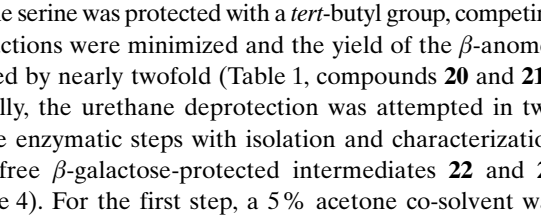
	AA^1 -AA^2 -OPG	Conditions	Yield of β-anomer
15	Phe-Val-O*t*Bu	EEDQ[a], CH_2Cl_2	55%
16	Phe-Ala-O*t*Bu	EEDQ, CH_2Cl_2	67%
17	Phe-Ser-O*t*Bu	EEDQ, CH_2Cl_2	40%
18	Leu-Ser-OBzl	EEDQ, CH_2Cl_2	40%
19	Leu-Ala-O*t*Bu	EDC[b], HOBt[c], DMF	52%
20	Leu-Ser-O*t*Bu	EDC, HOBt, DMF	40%
21	Leu-Ser(*t*Bu)-O*t*Bu	EDC, HOAt[d], CH_2Cl_2	74%

[a] EEDQ = *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. [b] EDC = *N*'-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride. [c] HOBt = 1-Hydroxybenzatriazole. [d] HOAt = 1-Hydroxyazabenzotriazole.

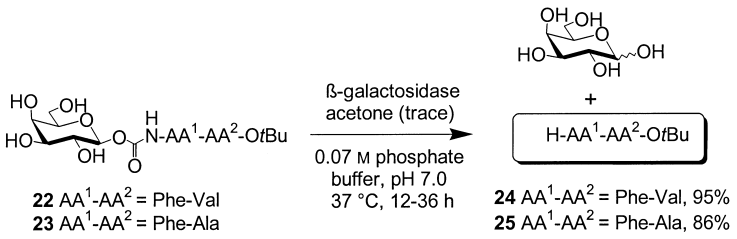
$CH_{\beta\text{-}C1}$ ~93). Although, in theory, the anomeric mixture could have been utilized for the study, in order to gain a better understanding of the enzymatic deprotection, only the β-anomer was used, allowing for easier product characterization.

It is also worth mentioning that, when the hydroxyl substituent of the serine was protected with a *tert*-butyl group, competing side reactions were minimized and the yield of the β-anomer increased by nearly twofold (Table 1, compounds **20** and **21**).

Initially, the urethane deprotection was attempted in two separate enzymatic steps with isolation and characterization of the free β-galactose-protected intermediates **22** and **23** (Scheme 4). For the first step, a 5% acetone co-solvent was used to enhance the solubility of the starting substrate. Protected dipeptides **15** and **16** were incubated for 48 to 72 h at 37 °C with lipase WG to remove the acetyl groups. After addition of a saturated sodium chloride solution and extraction into ethyl acetate, the dipeptides **22** and **23** were isolated in moderate yield. The intermediates were fully characterized to insure that all four of the acetates were cleaved in the first enzymatic step. In the second biotransformation, the intermediates **22** and **23** were stirred in the presence of β-galactosidase from *E. coli* for 12 to 36 h under conditions similar to those described above (Scheme 5). The known



Scheme 4. Initial enzymatic cleavage of acetyl groups.



Scheme 5. Liberation of N-terminal free dipeptides.

target N-terminal-unmasked dipeptides **24** and **25** were isolated in high yield by adjusting the pH of the aqueous phase to 11–12 and extracting with ethyl acetate. They have previously been reported in the literature.[11, 12]

After obtaining an understanding of the two separate enzymatic cleavage steps, the subsequent deprotection procedures were accomplished by following the previously described conditions in a two-step process, without isolation of the intermediates. AGalOC dipeptides **17** and **19** (Scheme 6) were successfully deprotected to afford the dipeptides **26** and **27**, which are known compounds,[13] in good yields over the two enzymatic steps. It was necessary to filter the reaction mixture after the initial biotransformation in order to remove all lipase residues, as these were found to adversely affect the overall isolated yields (see Experimental Section).

The developed technique was then used to synthesize a more complex AGalOC- protected tetrapeptide target, **30** (Scheme 7). The intermediate C-terminal-deprotected dipeptide **28** was obtained in excellent yield either by hydrogenation of benzyl ester **18** or, in a more efficient approach, by TFA catalyzed (TFA = trifluoroacetic acid) cleavage of the bis-*tert*-butyl-protected dipeptide, **21**. In the course of this acid-mediated transformation the glycosidic bond remained fully intact; this demonstrates the acid stability of the AGalOC group. The coupling of acid **28** with the known dipeptide **29**[14] proceeded in high yield, and the pure tetrapeptide **30** was obtained after washing the crude reaction mixture with 0.1N HCl and extraction into dichloromethane.

The final removal of the AGalOC protecting group was achieved by a two-step procedure without isolation of intermediate **31**. Although the initial lipase-catalyzed removal of the acetates to give the fully deacetylated intermediate β-galactose tetrapeptide **31** appeared

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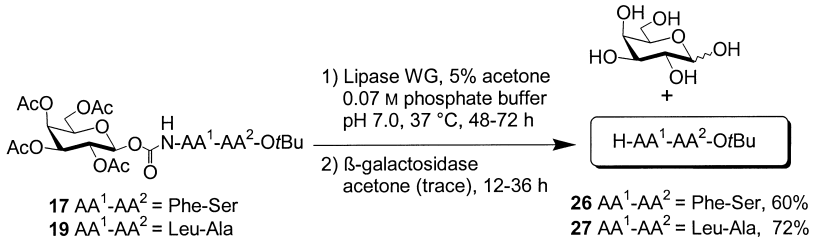
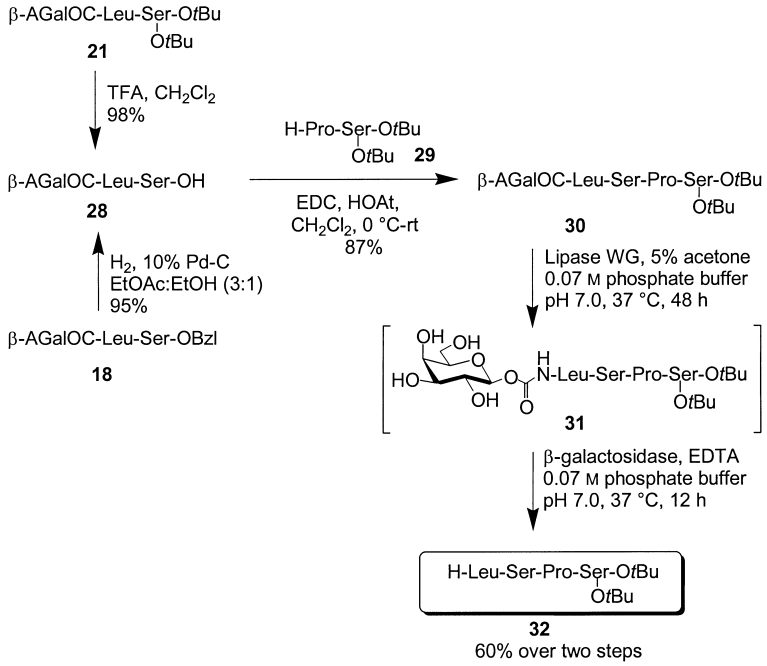


Scheme 6. Two step enzymatic deprotection.



Scheme 7. Model synthetic application.

to be complete according to TLC monitoring, a problem with repeated mass loss was encountered during the final deprotection step. It was determined that possible contamination with a metallo protease was causing the cleavage of the amide bonds in the peptide before any of the desired product could be isolated. This problem was alleviated by briefly stirring the commercial β-galactosidase in the presence of a catalytic amount of ethylenediaminetetra-acetate (EDTA) prior to the addition of the broth containing the crude intermediate **31**. Under these modified conditions, the desired tetrapeptide **32** was obtained in 60% yield over the two sequential enzymatic steps.

Conclusion

We have developed two new, fully enzyme-labile, carbohydrate-derived urethane protecting groups, which will be useful for the synthesis of complex polypeptides. The successful preparation of a series of dipetides and a tetrapeptide model system with this new strategy has been reported. The mildness of the reaction conditions (pH 7.0, 37 °C) and complete orthogonal stability due to the high selectivity of the enzyme, that is, no observable cleavage of peptide bonds, esters, or *t*Bu ethers, demonstrates the potential utility of this method for the preparation of sensitive, modified peptide conjugates. Furthermore, the deprotection was successful for both anomeric mixtures and pure substrates. Thus, in theory, tedious separation steps are not needed prior to the final enzymatic cleavage reaction. It is only necessary to use the appropriate combination of α- and β-glycosidases. Synthetic efforts to further employ this strategy for the preparation of additional polypeptide systems, which include phosphopeptides, are ongoing and will be reported in due course.

Experimental Section

General procedures: All ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC250, AM400, or DRX500 machines. Mass spectra were measured on a Finnigan MAT MS 70 spectrometer and for FAB-spectra a 3-nitrobenzyl alcohol (3-NBA) matrix was used. Specific rotations were obtained from a Perkin–Elmer 241 polarimeter. All column chromatography was performed on SDS 60 ACC silica gel and TLC on E. Merck Silica Gel 60 F_{254} plates. All dry solvents were either purchased or dried according to standard laboratory procedures. Lipase WG was purchased from Fluka, α- and β-glucosidases and β-galactosidase from Fluka or Sigma.

***N*-(2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl)-L-alanine benzyl ester (4)**: A solution of DMAP (162 mg, 1.33 mmol) in CH_2Cl_2 (5 mL), followed by a solution of amino acid ester **1** (238 mg, 1.33 mmol) in CH_2Cl_2 (5 mL) was added to a solution of Boc_2O (407 mg, 1.86 mmol) in dry CH_2Cl_2 (10 mL) at RT and under argon. After stirring the reaction for 30 min, a solution of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose (**3**) (648 mg, 1.86 mmol) in CH_2Cl_2 (10 mL) was added dropwise. The reaction was stirred overnight. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (ethyl acetate/hexane 2:3) to give protected amino acid **4** (329 mg, 44%, α/β 1:2) as a colorless wax. $R_f = 0.38$ (ethyl acetate/hexane 2:3); $[\alpha]_{20}^{D} = +30.7$ ($c = 1.2$ in $CHCl_3$);
β-anomer: ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.38-7.32$ (m, 5H), 5.66 (d, $J =$ 8.3 Hz, 1H), 5.54 (d, $J = 7.2$ Hz, 1H), 5.25 (t, $J = 9.5$ Hz, 1H), 5.20–5.10 (m, 4H), 4.38 (quint, 1H), 4.30 (dd, $J = 12.5$, 4.3 Hz, 1H), 4.11 (dd, $J = 12.5$, 2.0 Hz, 1H), 3.85–3.82 (m, 1H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 2.00 (s, 3H), 1.43 (d, $J = 7.2$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 172.2$, 170.6, 170.1, 169.6, 169.4, 153.6, 135.2, 128.7, 128.5, 128.2, 92.9, 72.8, 72.5, 70.1, 67.8, 67.3, 61.5, 49.8, 20.7, 20.6, 18.2; HRMS (FAB-3-NBA): $[M+H]^+$ calcd 554.1874; found 554.1806.

***N*-(2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl)-L-alanine (5)**: 10% Pd-C (30 mg) was added to a solution of AGlOC-protected **4** (265 mg, 0.48 mmol) in ethyl acetate/ethanol (30 mL:10 mL). The atmosphere was exchanged for hydrogen and the reaction was stirred for 6 h at RT. The crude mixture was filtered through celite and concentrated under reduced pressure to give the amino acid **5** (209 mg, 94%, α/β 1:2) as a colorless wax. $[\alpha]_{20}^{D} = +24.3$ ($c = 0.9$ in methanol); β-anomer: ^{1}H NMR

(500 MHz, $CDCl_3$): $\delta = 7.00$ (br s, 1H), 5.81 (d, $J = 7.2$ Hz, 1H), 5.68 (d, $J = 8.2$ Hz, 1H), 5.28 (t, $J = 9.5$ Hz, 1H), 5.15–5.10 (m, 2H), 4.33–4.29 (m, 2H), 4.13–4.10 (m, 1H), 3.88–3.86 (m, 1H), 2.08 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.45 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 176.6$, 170.8, 170.1, 169.9, 169.6, 153.3, 92.9, 72.7, 72.5, 70.3, 67.9, 61.5, 49.8, 20.7, 20.6, 18.0; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 464.1404; found 464.1460; elemental analysis calcd (%) for $C_{18}H_{25}NO_{13} \cdot H_2O$: C 44.91, H 5.65, N 2.91; found C 44.81, H 5.57, N 3.02.

***N*-(2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl)-L-alaninyl-L-glycine *tert*-butyl ester (6)**: A solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (EDC) (115 mg, 0.60 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a cooled (0 °C) solution of amino acid **5** (160 mg, 0.35 mmol), 1-Hydroxybenzatriazol (HOBt) (71 mg, 0.49 mmol), L-glycine *tert*-butyl ester hydrochloride (67 mg, 0.40 mmol) and triethylamine (68 μL, 0.49 mmol) in CH_2Cl_2 (20 mL). The reaction was warmed to RT and stirred overnight. The reaction was washed with 0.5 N HCl (3 × 50 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography (ethyl acetate/hexane 1:1) to give dipeptide **6** (170 mg, 85%, α/β 1:2) as a colorless wax. $R_f = 0.62$ (ethyl acetate/hexane 2:1); $[\alpha]_{20}^{D} = +18.9$ ($c = 1.0$ in chloroform); β-anomer: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 6.70$ (t, $J = 4.9$ Hz, 1H), 5.88 (d, $J = 7.6$ Hz, 1H), 5.72 (d, $J = 8.3$ Hz, 1H), 5.28 (t, $J = 9.5$ Hz, 1H), 5.17–5.06 (m, 2H), 4.37–4.26 (m, 2H) 4.14–4.07 (m, 1H), 3.97–3.87 (m, 3H), 2.08 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.47 (s, 9H), 1.42 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 171.8$, 170.6, 170.1, 169.6, 169.4, 168.7, 153.4, 92.9, 82.5, 72.8, 72.4, 70.1, 67.8, 61.5, 50.3, 42.0, 28.0, 20.7, 20.6, 18.6; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 577.2245; found 577.2383; elemental analysis calcd (%) for $C_{24}H_{36}N_2O_{14}$: C 50.00, H 6.29, N 4.86; found C 49.75, H 6.22, N 4.84.

L-Alanyl-L-glycine *tert*-butyl ester (7): A solution of dipeptide **6** (58 mg, 0.1 mmol) in methanol (2.5 mL) was added to a solution of lipase WG (1.6 units) in phosphate buffer (0.07 M, 47.5 mL, pH 6.0). The reaction mixture was incubated at 37 °C. After stirring for 16 h, α-glucosidase (3 mg, 12 units) and β-glucosidase (3 mg, 15 units) were added. After an additional 24 h, the entire reaction mixture was lyopholized, and the crude residue was purified by column chromatography (chloroform/methanol 7:1) to give the target dipetide **7** (13 mg, 64%) as a colorless oil, whose characteristics matched those previously reported. (see ref. [7])

General procedure for the synthesis of AGalOC amino bonzyl esters 11 and 12

*N-(2,3,4,6-Tetra-O-acetyl-D-galactopyranosyloxycarbonyl)-L-phenylalanine benzyl ester (**11**)*: A solution of L-phenylalanine benzyl ester (**8**, 306 mg, 1.20 mmol) in dry toluene (4 mL) was added dropwise to a cooled (0 °C) solution of Boc_2O (367 mg, 1.68 mmol) and DMAP (147 mg, 1.20 mmol) in dry toluene (10 mL). After stirring for 30 min at 0 °C, a solution of 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (**10**, 585 mg, 1.68 mmol) in dry toluene (10 mL) was added dropwise, followed by NEt_3 (145 mg, 200 μL, 1.44 mmol). The reaction was allowed to warm to RT and stirred overnight. The reaction mixture was then concentrated under reduced pressure, and the crude residue was purified by column chromatography (hexane/ethyl acetate 1:1 to 1:2) to afford the pure product **11** (699 mg, 93%, α/β 1:12) as a white foam. M.p. 52–54 °C; $R_f = 0.56$ (ethyl acetate/hexane 2:1); $[\alpha]_{22}^{D} = +24.2$ ($c = 1.0$ in $CHCl_3$); β-anomer: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 7.39–7.02$ (m, 10H), 5.64 (d, $J = 8.4$ Hz, 1H), 5.43 (d, $J = 3.2$ Hz, 1H), 5.38 (d, $J = 8.1$ Hz, 1H), 5.32 (t, $J = 8.4$ Hz, 1H), 5.15 (dd, $J = 12.1$, 10.3 Hz, 2H), 5.07 (dd, $J = 10.4$, 3.4 Hz, 1H), 4.65 (m, 1H), 4.16 (m, 1H), 4.06 (m, 1H), 3.15 (dd, $J = 13.9$, 6.0 Hz, 1H), 3.10 (dd, $J = 13.9$, 6.0 Hz, 1H), 2.17 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.8$, 170.4, 170.2, 170.0, 169.6, 153.3, 135.1, 134.9, 129.3, 128.8, 128.7, 128.6, 127.3, 93.5, 71.5, 70.9, 67.6, 67.4, 66.8, 61.0, 55.0, 37.7, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 630.2187; found 630.2267.

*N-(2,3,4,6-Tetra-O-acetyl-D-galactopyranosyloxycarbonyl)-L-leucine benzyl ester (**12**)*: The general procedure described above was used with: Boc_2O (1.13 g, 4.3 mmol), DMAP (550 mg, 5.2 mmol), L-leucyl benzyl ester (**8**, 984 mg, 4.3 mmol), 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (**10**, 1.65 g, 4.7 mmol) and NEt_3 (475 mg, 655 μL, 4.7 mmol). Compound **12** (2.19 g, 85%, α/β 1:4) was isolated as a white foam. M.p. 45–48 °C; $R_f = 0.38$ (ethyl acetate/hexane 1:1); $[\alpha]_{22}^{D} = +16.5$ ($c = 1.0$ in $CHCl_3$); β-anomer: ^1H NMR (250 MHz, $CDCl_3$): $\delta = 7.33$ (m, 5H), 5.62 (d, $J = 8.3$ Hz, 1H), 5.48 (br s, 1H), 5.42–5.40 (m, 1H), 5.34–5.29 (m, 1H), 5.17 (s, 2H), 5.06 (dd, $J = 10.4$, 3.4 Hz, 1H), 4.35–4.30 (m, 2H), 4.07–4.01 (m, 1H), 2.14 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.69–1.61 (m, 2H), 1.57–1.52 (m, 1H), 0.53–0.50 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 172.5$, 170.4, 170.3, 170.2, 169.8, 153.6, 135.2, 128.7, 128.5, 128.4, 128.2, 128.1, 93.5, 83.9, 71.5, 70.9, 68.7, 67.5, 67.4, 66.6, 61.3, 52.6, 27.6, 24.7, 22.8, 21.8, 20.6, 20.6; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 596.2343; found 596.2278; elemental analysis calcd (%) for $C_{28}H_{37}NO_{13}$: C 56.47, H 6.26, N 2.35; found C 56.08, H 6.20, N 2.03.

General procedure for the cleavage of the benzyl esters 13 and 14

*N-(2,3,4,6-Tetra-O-acetyl-D-galactopyranosyloxycarbonyl)-L-phenylalanine (**13**)*: 10% Pd-C (100 mg) was added to a solution of benzyl ester **11** (1.05 g, 1.67 mmol) in ethyl acetate/ethanol (30 mL:10 mL). The atmosphere was exchanged for hydrogen and the reaction was stirred at RT for 14 hours. The mixture was filtered through celite and concentrated under reduced pressure. The crude residue was purified by column chromatography (ethyl acetate with 1% acetic acid) to afford acid **13** (880 mg, 98%, α/β 1:12) as a white foam. M.p. 77–80 °C; $R_f = 0.07$ (ethyl acetate/hexane 2:1); $[\alpha]_{22}^{D} = +52.1$ ($c = 1.0$ in $CHCl_3$); β-anomer: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 7.32–7.26$ (m, 3H), 7.19–7.13 (m, 2H), 5.64 (d, $J = 8.3$ Hz, 1H), 5.46 (d, $J = 8.2$ Hz, 1H), 5.42 (dd, $J = 3.2$ Hz, 1H), 5.29 (dd, $J = 11.2$, 8.3 Hz, 2H), 5.07 (dd, $J = 10.5$, 3.3 Hz, 1H), 4.61 (dd, $J = 13.6$, 6.5 Hz, 1H), 4.14 (m, 2H), 4.06 (dd, $J = 13.7$, 6.7 Hz, 1H), 3.22 (dd, $J = 14.0$, 5.2 Hz, 1H), 3.08 (dd, $J = 14.0$, 6.5 Hz, 1H), 2.15 (s, 3H), 2.03 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.7$, 170.5, 170.4, 170.2, 170.1, 153.5, 135.2, 129.4, 129.3, 128.8, 128.7, 127.4, 93.5, 71.4, 70.8, 67.8, 66.8, 61.0, 54.8, 37.4, 20.7, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 540.1717; found 540.1796.

*N-(2,3,4,6-Tetra-O-acetyl-D-galactopyranosyloxycarbonyl)-L-leucine (**14**)*: The general procedure described above was used with: L-leucine benzyl ester **12** (1.62 g, 2.72 mmol) and 10% Pd-C (80 mg). Acid **14** (1.32 g, 96%, α/β 1:4) was isolated as a white foam. M.p. 67–70 °C (sublimed); $R_f = 0.07$ (ethyl acetate/hexane 1:1); $[\alpha]_{22}^{D} = +22.6$ ($c = 1.0$ in $CHCl_3$); β-anomer: ^1H NMR (500 MHz, $CDCl_3$): $\delta = 5.64$ (d, $J = 8.3$ Hz, 1H), 5.41 (d, $J = 3.4$ Hz, 1H), 5.38 (d, $J = 8.6$ Hz, 1H), 5.31 (dd, $J = 10.4$, 8.3 Hz, 1H), 5.08 (dd, $J = 10.5$, 3.4 Hz, 1H), 4.34 (dt, $J = 9.0$, 4.5 Hz, 1H), 4.15 (d, $J = 6.7$ Hz, 2H), 4.06 (t, $J = 6.4$ Hz, 1H), 2.16 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.74–1.66 (m, 2H), 1.59–1.53 (m, 1H), 0.95 (d, $J = 6.3$ Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 176.9$, 170.7, 170.4, 170.2, 170.1, 153.7, 93.5, 71.5, 70.8, 67.9, 66.8, 60.9, 52.4, 41.0, 27.8, 21.7, 21.6, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 506.1984; found 506.1984.

General procedure for dipeptide coupling to give compounds 15–21

*N-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyloxycarbonyl)-L-phenylalanyl-L-valine tert-butyl ester (**15**)*: A solution of AGalOC-phenylalanine acid **13** (78 mg, 0.14 mmol) in dry CH_2Cl_2 (5 mL) was added to a flask containing L-valine-*tert*-butyl ester hydrochloride (37 mg, 0.17 mmol). After cooling the solution to 0 °C for 15 min, NEt_3 (18 mg, 24 μL, 0.17 mmol) was added, followed by dropwise addition of a solution of the coupling reagent EEDQ (71 mg, 0.29 mmol) in dry CH_2Cl_2 (3 mL). The mixture was allowed to warm to RT and stirring was continued overnight. Additional CH_2Cl_2 (10 mL) was added, and the crude reaction mixture was washed with 0.1 N HCl (3 × 5 mL), saturated $NaHCO_3$ solution (3 × 5 mL), and water (3 × 5 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography (hexane/ethyl acetate 1:1 to 1:2) to give the β-dipeptide **15** (55 mg, 55%) as a white foam. M.p. 83–86 °C; $R_f = 0.57$ (ethyl acetate/hexane 2:1); $[\alpha]_{24}^{D} = +4.8$ ($c = 1.0$ in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 7.30–7.17$ (m, 5H), 6.16 (d, $J = 8.4$ Hz, 1H), 5.63 (d, $J = 8.4$ Hz, 1H), 5.54 (d, $J = 7.8$ Hz, 1H), 5.41 (d, $J = 3.0$ Hz, 1H), 5.30 (dd, $J = 8.4$ Hz, 1H), 5.06 (dd, $J = 10.4$, 3.2 Hz, 1H), 4.40 (q, $J = 6.7$ Hz, 1H), 4.33 (dd, $J = 8.4$, 4.0 Hz, 1H), 4.19–4.10 (m, 2H), 4.05 (d, $J = 7.1$ Hz, 1H), 3.08 (d, $J = 7.1$ Hz, 2H), 2.16 (s, 3H), 2.10 (m, 1H), 2.03 (s, 6H), 1.99 (s, 3H), 1.45 (s, 9H), 0.85 (t, $J = 6.6$ Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.4$, 170.2, 170.0, 169.8, 169.6, 153.4, 135.8, 129.3, 128.8, 127.3, 93.5, 82.2, 71.4, 70.9, 67.6, 66.8, 60.1, 57.6, 56.4, 38.3, 31.4, 28.0, 20.7, 20.6, 18.7, 17.7; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 695.3027, found 695.2971; elemental analysis calcd (%) for $C_{33}H_{46}N_2O_{14}$: C 57.05, H 6.67, N 4.03; found C 56.91, H 6.63, N 3.81.

*N-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyloxycarbonyl)-L-phenylalanyl-L-alanine tert-butyl ester (**16**)*: The general procedure for dipeptide

0947-6539/00/0620-3718 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 20

coupling was used with: L-alanine-*tert*-butyl ester hydrochloride (75 mg, 0.41 mmol), NEt_3 (41 mg, 57 μL, 0.41 mmol), AGalOC-phenylalanine acid **13** (183 mg, 0.34 mmol) in dry CH_2Cl_2 (15 mL), and EEDQ (168 mg, 0.68 mmol) in dry CH_2Cl_2 (3 mL). The β-dipeptide **16** (152 mg, 67%) was isolated as a white foam. M.p. 82–85 °C; $R_f = 0.43$ (ethyl acetate/hexane 2:1); $[\alpha]_{22}^{D} = +12.8$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta =$ 7.32–7.28 (m, 3H), 7.22–7.16 (m, 2H), 6.28 (m, 1H), 5.63 (d, $J = 8.3$ Hz, 1H), 5.53 (d, $J = 8.0$ Hz, 1H), 5.40 (d, $J = 3.1$ Hz, 1H), 5.30 (dd, $J = 10.3$, 8.4 Hz, 1H), 5.06 (dd, $J = 10.3$, 3.2 Hz, 1H), 4.35 (quint, $J = 7.1$ Hz, 1H), 4.18–4.13 (m, 2H), 4.10–4.05 (m, 2H), 3.13 (dd, $J = 13.8$, 6.2 Hz, 1H), 3.03 (dd, $J = 13.7$, 6.8 Hz, 1H), 2.15 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.97 (s,3H), 1.43 (s, 9H), 1.30 (d, $J = 7.1$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 171.5, 170.3, 170.1, 169.9, 169.7, 169.4, 153.3, 135.5, 129.3, 128.7, 127.2, 93.4, 82.1, 71.3, 70.8, 67.6, 66.7, 60.8, 56.1, 48.7, 38.4, 27.8, 20.7, 20.5, 18.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 667.2714; found 667.2551; elemental analysis calcd (%) for $C_{31}H_{42}N_2O_{14}$: C 55.85, H 6.35, N 4.20; found C 55.52, H 6.35, N 3.90.

*N-(2,3,4,6-Tetra-O-acetyl-*D*-galactopyranosyloxy-carbonyl)-*L*-phenylalanyl-*L*-serine tert-butyl ester (**17**)*: The general procedure for dipeptide coupling was used with: L-serine-*tert*-butyl ester (60 mg, 0.37 mmol), AGalOC-phenylalanine acid **13** (181 mg, 0.34 mmol) in dry CH_2Cl_2 (10 mL), and EEDQ (126 mg, 0.51 mmol) in dry CH_2Cl_2 (5 mL). The β-dipeptide **17** (92 mg,40%) was isolated as a white wax. $R_f = 0.33$ (ethyl acetate/hexane 2:1); $[\alpha]_{24}^{D} = +21.2$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta =$ 7.31–7.18 (m, 5H), 6.65 (d, $J = 6.8$ Hz, 1H), 5.62 (d, $J = 8.3$ Hz, 1H), 5.55 (d, $J = 7.6$ Hz, 1H), 5.40 (d, $J = 3.2$ Hz, 1H), 5.28 (dd, $J = 10.4$, 8.3 Hz, 1H), 5.07 (dd, $J = 10.4$, 3.4 Hz, 1H), 4.43–4.39 (m, 2H), 4.16–4.09 (m, 2H), 4.04 (d, $J = 6.8$ Hz, 1H), 3.91 (dd, $J = 11.4$, 3.1 Hz, 1H), 3.82 (dd, $J = 11.4$, 4.0 Hz, 1H), 3.10 (dd, $J = 6.7$, 3.8 Hz, 2H), 2.15 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 170.4, 170.3, 170.1, 169.9, 168.8, 153.5, 135.6, 129.2, 129.0, 128.9, 127.4, 93.6, 83.0, 71.4, 70.8, 67.7, 66.7, 63.3, 60.9, 56.4, 55.6, 38.1, 27.9, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 683.2663; found 683.2748.

*N-(2,3,4,6-Tetra-O-acetyl-β-*D*-galactopyranosyloxycarbonyl)-*L*-leucyl-*L*-serine benzyl ester (**18**)*:

The general procedure for dipeptide coupling was used with: L-serine-benzyl ester·TsOH (93 mg, 0.30 mmol), NEt_3 (30 mg, 42 μL, 0.30 mmol), AGalOC-leucine acid **14** (124 mg, 0.25 mmol) in dry CH_2Cl_2 (10 mL), and EEDQ (93 mg, 0.38 mmol) in dry CH_2Cl_2 (2 mL). The β-dipeptide **18** (69 mg, 40%) was isolated as a colorless oil. $R_f = 0.30$ (ethyl acetate/hexane 2:1); $[\alpha]_{22}^{D} = +2.2$ ($c = 1.35$ in $CHCl_3$); ^{1}H NMR (250 MHz, $CDCl_3$): $\delta =$ 7.28 (br s, 5H), 6.96 (d, $J = 7.9$ Hz, 1H), 5.57 (d, $J = 8.2$ Hz, 1H), 5.46 (d, $J =$ 7.9 Hz, 1H), 5.35 (d, $J = 3.2$ Hz, 1H), 5.23 (t, $J = 8.3$ Hz, 1H), 5.14 (s, 2H), 5.03 (dd, $J = 10.3$, 3.3 Hz, 1H), 4.65–4.61 (m, 1H), 4.22–4.16 (m, 1H), 4.09–3.99 (m, 3H), 3.94 (dd, $J = 11.5$, 3.2 Hz, 1H), 3.83 (dd, $J = 11.3$, 3.2 Hz, 1H), 2.80 (br s, 1H), 2.09 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.92 (s, 3H), 1.60–1.39 (m, 3H), 0.85 (d, $J = 5.6$ Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 171.9, 170.3, 170.2, 170.1, 170.0, 169.9, 153.9, 135.1, 128.7, 128.6, 128.2, 128.1, 93.6, 71.4, 70.7, 67.8, 67.6, 66.8, 62.8, 60.4, 54.8, 53.7, 41.3, 24.6, 22.9, 21.8, 20.7, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 683.2663; found 683.2549.

*N-(2,3,4,6-Tetra-O-acetyl-β-*D*-galactopyranosyloxycarbonyl)-*L*-leucyl-*L*-alanine tert-butyl ester (**19**)*: The general procedure for dipeptide coupling was used with: L-alanine-*tert*-butyl ester hydrochloride (62 mg, 0.34 mmol), NEt_3 (34 mg, 47 μL, 0.34 mmol); AGalOC-leucine acid **14** (156 mg, 0.31 mmol), EDC (73 mg, 0.37 mmol), and HOBt (72 mg, 0.47 mmol) were added to the reaction flask. The reaction was cooled (0 °C) and then DMF (10 mL) was added. After 14 h, the DMF was removed under reduced pressure. The crude residue was dissolved in ethyl acetate, extracted, and purified as described above. The β-dipeptide **19** (101 mg, 52%) was isolated as a colorless oil. $R_f = 0.23$ (ethyl acetate/hexane 1:1); $[\alpha]_{22}^{D} = +1.3$ ($c = 0.6$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 6.44$ (d, $J = 7.3$ Hz, 1H), 5.63 (d, $J = 8.4$ Hz, 1H), 5.41 (d, $J = 3.2$ Hz, 1H), 5.36 (d, $J = 8.4$ Hz, 1H), 5.30 (dd, $J = 10.4$, 8.3 Hz, 1H), 5.07 (dd, $J = 10.3$, 3.4 Hz, 1H), 4.40 (q, $J = 7.1$ Hz, 1H), 4.19–4.04 (m, 4H), 2.15 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.69–1.48 (m, 3H), 1.45 (s, 9H), 1.35 (d, $J = 7.1$ Hz, 3H), 0.93 (d, $J = 6.2$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 171.8, 170.8, 170.3, 170.2, 170.0, 169.7, 153.6, 93.5, 82.2, 71.4, 70.9, 67.7, 66.8, 60.9, 53.6, 48.7, 41.7, 27.9, 24.6, 22.9, 21.8, 20.7, 20.6, 18.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 633.2871; found 633.2938.

*N-(2,3,4,6-Tetra-O-acetyl-β-*D*-galactopyranosyloxycarbonyl)-*L*-leucyl-*L*-serine tert-butyl ester (**20**)*: The general procedure for dipeptide coupling was used with: L-serine-*tert*-butyl ester (60 mg, 0.37 mmol), AGalOC-leucine acid **14** (168 mg, 0.33 mmol), EDC (79 mg, 0.40 mmol), and HOBt (76 mg, 0.50 mmol) were added to the reaction flask, the mixture was cooled (0 °C); and DMF (10 mL) was added. The β-dipeptide **20** (89 mg, 40%) was isolated as a colorless oil. $R_f = 0.19$ (ethyl acetate/hexane 1:1); $[\alpha]_{24}^{D} = +5.3$ ($c = 1.5$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 6.83$ (d, $J = 7.3$ Hz, 1H), 5.62 (d, $J = 8.3$ Hz, 1H), 5.47 (d, $J = 7.8$ Hz, 1H), 5.42 (d, $J = 2.9$ Hz, 1H), 5.30 (dd, $J = 10.4$, 8.4 Hz, 1H), 5.08 (dd, $J = 10.4$, 3.3 Hz, 1H), 4.49 (dt, $J = 7.3$, 3.3 Hz, 1H), 4.20 (m, 1H), 4.18–4.10 (m, 2H), 4.0–4.04 (m, 1H), 3.91 (dd, $J = 11.3$, 3.2 Hz, 1H), 3.88 (dd, $J = 11.3$, 3.8 Hz, 1H), 2.88(br s, 1H), 2.15 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.69–1.49 (m, 3H), 1.47 (s, 9H), 0.95 (d, $J = 4.6$ Hz, 3H), 0.93 (d, $J = 4.6$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 171.8, 170.4, 170.1, 170.0, 169.9, 153.9, 93.6, 82.9, 71.4, 70.8, 67.8, 66.8, 63.3, 60.9, 55.4, 53.9, 41.3, 28.0, 24.6, 22.9, 21.8, 20.7, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 649.2820; found 649.2848.

*N-(2,3,4,6-Tetra-O-acetyl-β-*D*-galactopyranosyloxycarbonyl)-*L*-leucyl-O-(tert-butyl)-*L*-serine tert-butyl ester (**21**)*: The general procedure for dipeptide coupling was used with: *O*-(*tert*-butyl)-L-serine-*tert*-butyl ester (122 mg, 0.56 mmol), AGalOC-leucine **14** (228 mg, 0.45 mmol), 1-Hydroxy-azabenzotriazole (HOAt) (96 mg, 0.71 mmol) in dry CH_2Cl_2 (40 mL), and EDC (94 mg, 0.49 mmol) in dry CH_2Cl_2 (5 mL). The β-dipeptide **21** (235 mg, 74%) was isolated as a white foam. M.p. 79–82 °C; $R_f = 0.40$ (ethyl acetate/hexane 1:1); $[\alpha]_{22}^{D} = +16.0$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 6.53$ (d, $J = 8.1$ Hz, 1H), 5.60 (d, $J = 8.4$ Hz, 1H), 5.45 (d, $J = 8.3$ Hz, 1H), 5.41 (d, $J = 3.2$ Hz, 1H), 5.32 (dd, $J = 10.4$, 8.4 Hz, 1H), 5.06 (dd, $J = 10.4$, 3.4 Hz, 1H), 4.54 (dt, $J = 8.1$, 2.8 Hz, 1H), 4.20 (m, 1H), 4.15 (d, $J = 6.7$ Hz, 2H), 4.05 (t, $J = 7.2$ Hz, 1H), 3.77 (dd, $J = 8.8$, 2.8 Hz, 1H), 3.52 (dd, $J = 8.9$, 2.9 Hz, 1H), 2.16 (s, 3H), 2.06 (s, 3H), 1.98 (s, 3H), 1.69–1.64 (m, 2H), 1.58–1.50 (m,1H), 1.45 (s, 9H), 1.15 (s, 9H), 0.96 (d, $J = 6.3$ Hz, 3H), 0.94 (d, $J = 6.3$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta =$ 171.0, 170.3, 170.1, 170.0, 169.7, 169.0, 153.5, 93.4, 82.0, 73.2, 71.3, 70.9, 67.6, 66.8, 62.0, 60.8, 53.7, 53.2, 42.0, 28.0, 27.3, 24.6, 22.9, 21.8, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 705.3446; found 705.3424; elemental analysis calcd (%) for $C_{32}H_{52}N_2O_{15}$: C 54.54, H 7.44, N 3.97; found C 54.33, H 7.48, N 3.81.

General procedure for lipase WG catalyzed cleavage of acetate groups to give compounds 22 and 23

*N-(β-*D*-galactopyranosyloxycarbonyl)-*L*-phenylalanyl-*L*-valine tert-butyl ester (**22**)*: A solution of dipeptide **15** (55 mg, 0.08 mmol) in acetone (2.5 mL) was added to a solution of lipase WG (40 mg, 3.2 Units, Fluka) in Na-phosphate buffer (0.07 M, 47.5 mL, pH 7.0). The reaction was incubated at 37 °C and stirred for 48 h. A saturated sodium chloride solution (50 mL) was added, and the crude product was extracted into ethyl acetate (5 × 25 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give the pure, free galactose β-dipeptide **22** (29 mg, 69%) as an amorphous white wax. $R_f = 0.26$ (chloroform/methanol 9:1); $[\alpha]_{24}^{D} = +8.9$ ($c = 0.47$ in $CHCl_3$); ^{1}H NMR (500 MHz, CD_3OD): $\delta =$ 7.29–7.18 (m, 5H), 5.22 (d, $J = 8.1$ Hz, 1H), 4.50 (dd, $J = 9.0$, 5.3 Hz, 1H), 4.19 (d, $J = 5.9$ Hz, 1H), 3.85 (d, $J = 3.0$ Hz, 1H), 3.71–3.65 (m, 2H), 3.63 (dd, $J = 9.8$, 8.1 Hz, 1H), 3.58 (t, $J = 6.0$ Hz, 1H), 3.50 (dd, $J = 9.7$, 3.3 Hz, 1H), 3.31 (dd, $J = 14.0$, 5.3 Hz, 1H), 2.88 (dd, $J = 14.0$, 9.0 Hz, 1H), 2.13 (m, 1H), 1.47 (s, 9H), 0.95 (dd, $J = 6.8$, 2.1 Hz, 6H); ^{13}C NMR (125 MHz, CD_3OD): $\delta =$ 173.9, 171.9, 156.8, 138.2, 130.3, 129.5, 127.8, 97.5, 82.8, 77.3, 74.7, 71.3, 69.9, 62.2, 59.8, 57.3, 39.0, 31.9, 28.3, 19.5, 18.5; HRMS (FAB-3-NBA) $[M+Na]^+$: calcd 549.2424; found 549.2402.

*N-(β-*D*-Galactopyranosyloxycarbonyl)-*L*-phenylalanyl-*L*-alanine tert-butyl ester (**23**)*: The general procedure for lipase WG catalyzed cleavage was used with: lipase WG (110 mg, 8.8 Units, Fluka) in Na-phosphate buffer (0.07 M, 15 mL, pH 7.0), and dipeptide **16** (126 mg, 0.19 mmol) in acetone (0.75 mL). The reaction was incubated at 37 °C and for 48 h. Pure, free galactose β-dipeptide **23** (52 mg, 55%) was isolated as a white wax. $R_f =$ 0.40 (methanol/ethyl acetate 1:4); $[\alpha]_{22}^{D} = -10.1$ ($c = 1.0$ in CH_3OH); ^{1}H NMR (250 MHz, CD_3OD): $\delta =$ 7.30–7.19 (m, 5H), 5.21 (d, $J = 8.0$ Hz, 1H), 4.41 (dd, $J = 9.3$, 4.8 Hz, 1H), 4.27 (q, $J = 7.2$ Hz, 1H), 3.83 (d, $J =$ 3.0 Hz, 1H), 3.68–3.47 (m, 5H), 3.15 (dd, $J = 14.0$, 4.8 Hz, 1H), 2.86 (dd, $J = 14.0$, 9.3 Hz, 1H), 1.46 (s, 9H), 1.35 (d, $J = 7.2$ Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta =$ 173.6, 173.2, 156.8, 138.3, 130.4, 129.5, 127.8, 97.5,

82.7, 77.3, 74.8, 71.3, 70.0, 62.2, 57.3, 50.3, 39.1, 28.2, 17.5; HRMS (FAB-3-NBA) $[M+Na]^+$: calcd 521.2111; found 521.2055.

General procedure for sequential enzymatic cleavage to give compounds 26 and 27

L-*Phenylalanine*-L-*serine tert-butyl ester (**26**)*: A Na-phosphate buffer solution (0.07 M, 1.5 mL) followed by lipase WG (Fluka, 7 mg) was added to a solution of dipeptide **17** (12.5 mg, 0.012 mmol) in acetone (0.2 mL). The reaction was incubated at 37 °C for 72 h. After TLC indicated complete removal of the acetates, the reaction tube was centrifuged. The crude broth was separated from the lipase residue by pipette and was added to a solution of β-galactosidase (30 µL, 4.5 Units) in a Na-phosphate buffer solution (0.07 M, 0.3 mL). The reaction was incubated at 37 °C for an additional 24 h. A saturated NaCl solution (10 mL) was then added, and the crude product was extracted into ethyl acetate (3×2 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give the target N-terminal, free dipeptide **26** (2.2 mg, 60% over two steps) as a pale yellow oil which required no further purification. $R_f=0.49$ (chloroform/methanol/water 12:6:1); $[\alpha]_{22}^{D}=-83.3$ ($c=0.7$, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=8.09$ (d, $J=6.6$ Hz), 7.33–7.21 (m, 5H), 5.55 (br d, 2H), 4.50 (m, 1H), 3.90 (dd, $J=11.1$, 3.1 Hz, 1H), 3.85 (dd, $J=11.1$, 4.5 Hz, 1H), 3.67 (dd, $J=8.9$, 4.0 Hz, 1H), 3.20 (dd, $J=13.7$, 4.0 Hz, 1H), 2.99 (br s, 1H), 2.80 (dd, $J=13.7$, 8.9 Hz, 1H), 1.47 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=174.8$, 173.6, 169.6, 137.7, 129.2, 128.5, 126.7, 82.4, 62.9, 56.4, 55.2, 41.0, 27.8, 22.4; HRMS (EI, 70 eV, 110 °C) $[M]^+$: calcd 308.1736; found 308.1731.

Compound **27** was prepared according to the same procedure.[13]

***N*-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyloxycarbonyl)-L-leucyl-L-serine (28)**

*Synthesis by hydrogenation of benzyl ester **18***: 10% Pd-C (10 mg) was added to a solution of dipeptide benzyl ester **18** (70 mg, 0.10 mmol) in ethyl acetate and ethanol (6:18 mL). The atmosphere was exchanged for hydrogen, and the reaction was stirred at RT for 14 h. The mixture was filtered through celite and concentrated under reduced pressure. The crude residue was purified by column chromatography (ethyl acetate/acetic acid 99:1) to afford acid **28** (56 mg, 95%) as a white wax.

*Synthesis by TFA cleavage of bis tert-butyl dipeptide **21***: TFA (1 mL), followed by a catalytic amount of water, was added dropwise to a cooled (0 °C) solution of bis-*tert*-butyl dipeptide **21** (100 mg, 0.14 mmol) in CH_2Cl_2 (2 mL). The reaction mixture was stirred at 0 °C for 24 h. The mixture was then concentrated under reduced pressure and co-distilled with toluene several times to insure complete removal of TFA. The crude residue was dissolved in ethyl acetate (30 mL) and washed with a 5% $NaHCO_3$ solution (3×5 mL). The aqueous phase was adjusted to pH 2 with 6 M HCl and extracted with ethyl acetate (3×15 mL). The combined organic phases were dried over $MgSO_4$, filtered, and concentrated to give the pure dipeptide free acid **28** (81 mg, 98%) as a white wax. $R_f=0.07$ (ethyl acetate/hexane 1:1); $[\alpha]_{22}^{D}=+22.6$ ($c=1.0$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=5.64$ (d, $J=8.3$ Hz, 1H), 5.41 (d, $J=3.4$ Hz, 1H), 5.38 (d, $J=8.6$ Hz, 1H), 5.31 (dd, $J=10.4$, 8.3 Hz, 1H), 5.08 (dd, $J=10.5$, 3.4 Hz, 1H), 4.34 (dt, $J=9.0$, 4.5 Hz, 1H), 4.15 (d, $J=6.7$ Hz, 2H), 4.06 (t, $J=6.4$ Hz, 1H), 2.16 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.74–1.66 (m, 2H), 1.59–1.53 (m, 1H), 0.95 (d, $J=6.3$ Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=176.9$, 170.7, 170.4, 170.2, 170.1, 153.7, 93.5, 71.5, 70.8, 67.9, 66.8, 60.9, 52.4, 41.0, 27.8, 21.7, 21.6, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+Na]^+$: calcd 615.2013; found 615.1954.

***N*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyloxycarbonyl)-L-leucyl-L-seryl-L-prolyl-*O*-(*tert*-butyl)-L-serine *tert*-butyl ester (30)**: A solution of EDC (13 mg, 0.07 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a cooled (0 °C) solution of dipeptide acid **28** (39.6 mg, 0.07 mmol), HOAt (13.6 mg, 0.10 mmol) and dipeptide **29** (23.3 mg, 0.07 mmol) in CH_2Cl_2 (15 mL). The reaction was allowed to warm to RT and stirred for 14 h. The reaction mixture was concentrated under reduced pressure. The crude residue was dissolved in CH_2Cl_2 (20 mL) and extracted with 0.1 N HCl (3×10 mL) and water (1×10 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to give the tetrapeptide **30** (52 mg, 87%) as a white wax, which was pure and required no additional purification steps. $R_f=0.62$ (chloroform/methanol 9:1); $[\alpha]_{22}^{D}=-13.6$ ($c=0.9$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=6.93$ (d, $J=8.3$ Hz, 1H), 6.84 (d, $J=8.0$ Hz, 1H), 5.61 (d, $J=8.4$ Hz, 1H), 5.44 (d, $J=8.3$ Hz, 1H), 5.41 (d, $J=3.2$ Hz, 1H), 5.30 (dd, $J=10.3$, 8.4 Hz, 1H), 5.06 (dd, $J=10.4$, 3.4 Hz, 1H), 4.92 (m, 1H), 4.62 (m, 1H), 4.57 (m, 1H), 4.21–4.17 (m, 1H), 4.13 (d, $J=6.9$ Hz, 2H), 4.04 (t, $J=6.8$ Hz, 1H), 3.94 (dd, $J=10.7$, 4.6 Hz, 2H), 3.74 (dd, $J=8.3$, 2.8 Hz, 2H), 3.66 (dd, $J=17.4$, 7.5 Hz, 1H), 3.50 (dd, $J=8.8$, 2.8 Hz, 1H), 3.20 (br s, 1H), 2.20–2.16 (m, 3H), 2.15 (s, 3H), 2.08 (s, 3H), 2.04 (m, 1H), 2.02 (s,3H), 1.98 (s, 3H), 1.68–1.46 (m, 3H), 1.4 (s, 9H), 1.13 (s, 9H), 0.90 (dd, $J=6.3$, 2.3 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=171.4$, 171.1, 170.4, 170.2, 170.0, 169.8, 153.6, 93.5, 82.5, 73.4, 71.4, 70.9, 67.7, 66.8, 63.7, 62.2, 60.9, 60.5, 53.7, 53.3, 52.2, 47.6, 41.8, 29.1, 28.0, 27.3, 24.6, 23.0, 21.7, 20.7, 20.6, 20.5; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 889.4294; found 889.4196.

L-Leucyl-L-seryl-L-prolyl-*O*-(*tert*-butyl)-L-serine *tert*-butyl ester (32): A solution of the tetrapeptide **30** (10.8 mg, 0.011 mmol) in acetone (0.2 mL), followed by lipase WG (13 mg, Fluka), was added to a solution Na-phosphate buffer (0.07 M, 1.2 mL) in an Eppendorf tube. The reaction was incubated at 37 °C and stirred for 48 h, after which time TLC indicated complete removal of all acetate groups. In a second Eppendorf tube, a stock solution of β-galactosidase (50 µL, ~250 Units) was added to a Na-phosphate buffer (0.07 M, 1 mL). A few crystals of EDTA were added, and the mixture was briefly stirred. The mixture from the first enzymatic step was then centrifuged to allow for easy transfer of the reaction broth away from the undesired Lipase residue. The crude broth was transferred by pipette into the EDTA/β-galactosidase solution and further incubated at 37 °C for 12 h. Two drops of 1 N NaOH solution were added to the reaction mixture. The desired product was extracted into ethyl acetate, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give the N-terminal free tetrapeptide **32** (3.3 mg, 60% over two steps) as a colorless oil. $R_f=0.36$ (chloroform/methanol 3:1); $[\alpha]_{22}^{D}=-37.2$ ($c=1.1$ in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta=7.90$ (d, $J=8.2$ Hz, 1H), 6.99 (d, $J=8.4$ Hz, 1H), 4.94 (ddd, $J=12.8$, 8.0, 4.8 Hz, 1H), 4.63 (dd, $J=8.1$, 3.8 Hz, 1H), 4.58 (dt, $J=8.4$, 2.8 Hz, 1H), 3.97–3.93 (m, 1H), 3.91 (dd, $J=10.6$, 4.8 Hz, 1H), 3.79–3.70 (m, 3H), 3.51 (dd, $J=8.8$, 2.9 Hz, 1H), 3.38 (dd, $J=9.8$, 4.1 Hz, 1H), 2.20–2.10 (m, 2H), 2.02–1.96 (m, 2H), 1.75–1.62 (m, 2H), 1.45 (s, 9H), 1.39–1.33 (m, 1H), 1.13 (s, 9H), 0.94 (d, $J=6.4$ Hz, 3H), 0.91 (d, $J=6.4$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta=175.8$, 171.1, 170.7, 169.9, 82.3, 73.3, 64.1, 62.3, 60.4, 53.5, 53.3, 51.6, 47.6, 44.1, 29.1, 28.0, 27.3, 24.8, 24.6, 23.4, 21.3; HRMS (FAB-3-NBA) $[M+H]^+$: calcd 515.3445; found 515.3530.

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