Protease-Catalyzed Regioselective Esterification of Sugars and Related Compounds in Anhydrous Dimethylformamide

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Abstract: The proteolytic enzyme subtilisin has been found to be catalytically active in anhydrous dimethylformamide. By taking advantage of the unique dissolving potency of the latter and a broad substrate specificity of subtilisin, a number of carbohydrates and other sugar-containing compounds have been regioselectively acylated via enzymatic transesterification. Monobutyryl esters of the disaccharides maltose, cellobiose, lactose, and sucrose have been readily prepared on a gram scale. The presence of a bulky aglycon moiety does not substantially reduce the catalytic efficiency of subtilisin in dimethylformamide, thus permitting preparative enzymatic esterification of natural compounds such as riboflavin, salicin, and the nucleosides adenosine and uridine. The reactivity of subtilisin only modestly drops upon an increase in the size of the carbohydrate substrate from maltose to maltoheptaose. In addition to the butyryl group, various *N*-acetylamino acid residues have been enzymatically introduced into sugars. Both highly purified and crude preparations of subtilisin have been successfully employed as practical catalysts of transesterifications in dry dimethylformamide.

Enzymes possess a proven record as selective catalysts in organic chemistry.[1] The recent realization that they can function not only in aqueous solutions but also in dry organic solvents has given the synthetic potential of enzymes a powerful boost, for many processes, while difficult in water, readily take place in nonaqueous media.[2] For example, lipases, the most popular group of enzymes to be used as catalysts in anhydrous organic solvents to date, have been successfully utilized for a wide range of stereoselective and regioselective transformations.[3-5]

Carbohydrate molecules represent a particularly challenging target for regioselective modifications due to their multiple hydroxyl groups.[6] We have recently demonstrated[4] that porcine pancreatic lipase regioselectively acylates the primary hydroxyl group of monosaccharides in pyridine. Sugars are reasonably soluble in only a few, very hydrophilic organic solvents such as pyridine and dimethylformamide; unfortunately, most enzymes we have tested are catalytically inactive in these solvents.[2] For instance, out of the dozen commercially available lipases, only those from porcine pancreas and, to a small extent, *Chromobacterium viscosum* were active in pyridine,[4,5] and none exhibited appreciable activity in dimethylformamide.[7] Moreover, porcine pancreatic lipase was found to be virtually unreactive with di- and oligosaccharides,[4] thereby severely restricting the scope of its potential applications.

In the present study, we have endeavored to extend our regioselective acylation methodology to sugars larger than monosaccharides by using *non-lipase* hydrolases. Proteolytic enzymes, specifically serine proteases, appeared a sensible choice because they share with lipases a common active center head nucleophile[8] that is mechanistically essential for a transesterification reaction.[7] Undeterred by recent claims that "proteases are generally not stable in anhydrous environments",[9] we have proceeded to find that *Bacillus subtilis* protease (subtilisin), a commercially available enzyme, is both stable and active in numerous anhydrous organic solvents including pyridine and dimethylformamide. Furthermore, subtilisin has been found to readily and regioselectively acylate di- and oligosaccharides, as well as nucleosides and related large molecules, in the latter solvent. Consequently, a number of interesting and difficult to prepare monoesters have been enzymatically synthesized in one step on a preparative scale.

Results and Discussion

The strategy previously employed by us[4] for lipase-catalyzed regioselective acylation of monosaccharides was enzymatic transesterification, where R is an aliphatic moiety and R'OH is 2-chloroethanol or 2,2,2-trichloroethanol.[11] This process requires

$$\text{RCOOR}' + \text{sugar} \rightarrow \text{sugar monoester} + \text{R}'\text{OH} \quad (1)$$

organic solvents as the reaction medium because in aqueous solutions water will replace sugar as a nucleophile, thus leading to hydrolysis instead of transesterification. Moreover, anhydrous organic solvents have to be used[4,5] to prevent hydrolysis of the activated ester RCOOR'.

In an attempt to overcome the limitations of lipases mentioned in the introduction, we explored the protease subtilisin (known for its high conformational stability)[12] as a catalyst of reaction 1 under the same conditions as lipase. We dissolved 11 mmol of

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Table I. Transesterification Reactions between Various Disaccharides and Trichloroethyl Butyrate Catalyzed by Subtilisin in Anhydrous Dimethylformamide[a]

disaccharide	reactn time, days	conv,[b] %	isol yield, g (% theor)	product compn[c]
2	7	83	4.6 (45)	>95% 6′-*O*-monobutyrylmaltose
3	3.5	94	0.7 (47)	95% 6′-*O*-monobutyrylcellobiose
4	2	96	1.6 (51)	75% 6′-*O*-monobutyryllactose 10% 3′-*O*-monobutyryllactose 10% 4′-*O*-monobutyryllactose
5	2.5	85	2.3 (57)	90% 1′-*O*-monobutyrylsucrose

[a] Conditions: disaccharides, 25, 3.75, 7.5, and 10 mmol for **2**–**5**, respectively; molar excess of the ester TCEB, 2-fold in all instances, except for **5** where it was 20%; solvent dimethylformamide, 25 mL for **2** and **5**, 50 mL for **3** and **4**; 30 mg/mL subtilisin prepared as described in the Experimental Section; 45 °C; shaking at 250 rpm. After the periods of time indicated in the second column of the table, the enzyme was removed by filtration, the solvent evaporated under vacuum, and the remaining residue subjected to silica gel chromatography with a mixture of ethyl acetate, methanol, and water (17:3:1) as the solvent. [b] Determined by gas chromatography on the basis of disappearance of the substrate disaccharide. In no case was any appreciable conversion detected without the enzyme. [c] In the case of all four disaccharides, the isolated product came out of the silica gel column as a single peak and was pure by TLC. It was subsequently analyzed by ^{13}C NMR (see Experimental Section), which revealed the composition depicted in the last column of the table.

glucose (**1**) and 33 mmol of 2,2,2-trichloroethyl butyrate (TCEB) in 25 mL of anhydrous pyridine. Then 0.75 g of solid subtilisin was added, and the suspension[13] was shaken at 45 °C. After 18 h no free **1** was left (as judged by gas chromatography). The enzyme was removed by filtration and the product isolated and purified by silica gel chromatography. As a result, 1.78 g (64% isolated yield) of pure 6-*O*-butyrylglucose was obtained. Thus, subtilisin, whose natural function is to hydrolyze proteins,[12] is also able to regioselectively acylate **1** in anhydrous pyridine (no appreciable reaction was observed in the absence of the enzyme).

Dimethylformamide is a much better solvent for carbohydrates than pyridine.[14] Although all lipases[7] and other enzymes examined to date[2] have been completely inactive in dimethylformamide, we tested subtilisin in that solvent. When the glucose acylation experiment described above was carried out in anhydrous dimethylformamide, 77% conversion was achieved after 5 days and consequently 1.66 g (60% isolated yield) of pure 6-*O*-butyrylglucose was obtained. (No reaction was observed in the absence of the enzyme or in the presence of subtilisin preinactivated by the active-site-directed inhibitor phenylmethanesulfonyl fluoride.[15]) Hence, subtilisin is the first known example of an enzyme expressing significant catalytic activity in anhydrous dimethylformamide;[16] the general preparative importance of this fact is the high dissolving power of this solvent, appropriately termed "the universal organic solvent".[18]

(13) Subtilisin is insoluble in pyridine, dimethylformamide, and other organic solvents.

(14) E.g., at 30 °C the solubility of sucrose in dimethylformamide is almost 5 times greater than in pyridine; in fact, the former is considered to be "the best known of the dipolar, aprotic solvents for carbohydrates": Moye, C. J. *Adv. Carbohydr. Chem. Biochem.* **1972**, *27*, 85–125.

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(16) The unsuitability of dimethylformamide as the reaction medium for enzymatic processes has been ascribed[2] to its ability to dissolve most proteins;[17] subtilisin is a rare exception in that regard.

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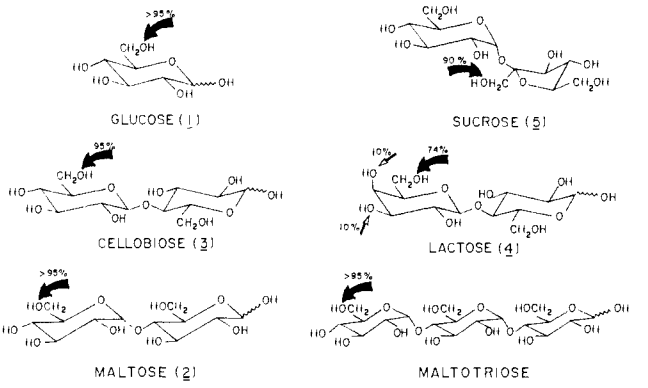

Figure 1. Structures of carbohydrates used as targets for subtilisin-catalyzed acylations in dimethylformamide. Arrows indicate the position of acylation: large, completely filled arrows denote the main direction of acylation; smaller, half-filled arrows correspond to minor routes of acylation. Percentages above the arrows are the fraction of a given acylation compared with the total. Assignment of greater than 95% means that no other acylations were detected by ^{13}C NMR.

Table II. Initial Rates of the Reaction between Maltose Oligosaccharides and Trichloroethyl Butyrate Catalyzed by Subtilisin in Anhydrous Dimethylformamide[a]

oligosaccharide	init reactn rate,[b] μmol/h	oligosaccharide	init reactn rate,[b] μmol/h
maltose	2.04	maltohexaose	0.87
maltotriose	1.78	maltoheptaose	0.81
maltotetraose	1.21	α-cyclodextrin	0
maltopentaose	0.91		

[a] Conditions: 0.15 M oligosaccharides, 0.19 M TCEB, 10 mg/mL subtilisin, 45 °C, shaking at 250 rpm. [b] Determined by gas chromatography following production of the product trichloroethanol. No appreciable release of the alcohol was observed when either the enzyme or the sugar was absent.

The next step was to ascertain whether subtilisin could acylate *disaccharides* in dimethylformamide (something that lipases had failed to do even in pyridine[4]). To that end, the enzymatic transesterification experiments similar to that described above for **1** were carried out for maltose (**2**), cellobiose (**3**), lactose (**4**), and sucrose (**5**). In all cases the reaction readily took place. Furthermore, the efficiency of subtilisin-catalyzed acylations for all four disaccharides was sufficient to prepare gram quantities of the corresponding sugar monoesters with the isolated yield of around 50% (Table I).

The positions of enzymatic acylation in all products, determined by ^{13}C NMR, are depicted by arrows in Figure 1. In the case of **2**, acylation occurs exclusively at the C6′ position. Transition from α- to β-glycosidic linkage (replacement of **2** with **3**) does not alter the direction of acylation or its regioselectivity. It is worth mentioning that in the chemical acylation of **2** the C6′ hydroxyl group is also the most reactive one,[19] although the regioselectivity is not nearly as great as that observed for the subtilisin-catalyzed transesterification. In the case of **4**, one can see that the enzyme is less discriminating: about three-quarters of the acylation occurs in the C6′ position and 10% each at C3′ and C4′ (note that subtilisin reacts only with the glucose moiety of **4**). Similar data have been reported for chemical acylations of **4**.[20]

Surprisingly, in the case of **5** subtilisin acylates the C1′ hydroxyl group (in the fructose moiety), whereas in the chemical acylation[21] the most reactive OH is in the C6 position followed by C6′. Thus, interestingly, while for **2** and **4** there is a correlation between enzymatic and chemical acylations, in the case of **5** the two alternative methods afford strikingly different products. This may lead to new sucrose esters with useful properties.[22]

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(20) Thelwall, L. A. W. *J. Dairy Res.* **1982**, *49*, 713–724.

(21) Khan, R. *Pure Appl. Chem.* **1984**, *56*, 833–844.

(22) A number of applications for sucrose esters have been proposed: *Sugar Esters*; Noyes Development: Park Ridge, NJ, 1968.

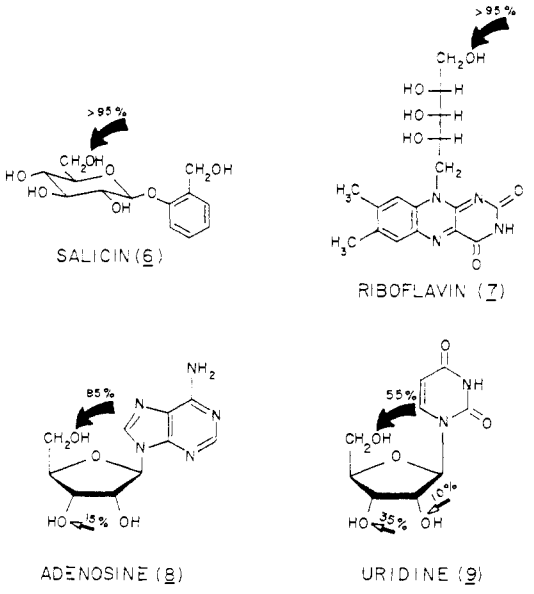

Figure 2. Structures of compounds containing both sugar and aglycon moieties used as targets for subtilisin-catalyzed acylations in dimethylformamide. For symbols, see the legend to Figure 1.

In all the experiments described thus far, highly purified subtilisin was employed as a catalyst. The relatively high cost of this enzyme preparation[23] could be an obstacle for practical transformations. To address this issue, we examined the use of a crude, inexpensive preparation[23] of subtilisin for a large-scale synthesis. We dissolved 13.7 g of **5** and 4 mol equiv of TCEB in 100 mL of dimethylformamide, followed by addition of 6 g of crude subtilisin. The suspension[13] was shaken at 45 °C for 6 days, and the product was then isolated as previously described with the purified enzyme. As a result, 10.1 g (61% isolated yield) of the monoester identical with that listed in the last line of Table I was obtained. Therefore, the purity of subtilisin is not essential for either the yield or regioselectivity of the enzymatic acylation.

It was important to establish whether subtilisin could esterify carbohydrates larger than disaccharides. To that end, we determined the rates of the reactions of various maltose oligomers with TCEB catalyzed by subtilisin in dimethylformamide. The data obtained, presented in Table II, indicate that the enzyme is quite tolerant to the length of the glucose chain: the reactivity of maltoheptaose is as high as 40% of that of **2**. At the same time, α-cyclodextrin, a cyclic analogue of maltohexaose, was completely unreactive toward subtilisin, probably due to the bulkiness of this substrate. The enzymatic acylations of maltotriose were carried out on a preparative scale; characterization of the product(s) by ^{13}C NMR revealed that, as in the case of **2**, subtilisin acylates exclusively the C6 hydroxyl group of the tail (terminal nonreducing) glucose moiety (Figure 1). This fact suggests that this moiety binds to the enzyme's active center in a fixed way. It is noteworthy that enzymatic acylation of maltose and its oligomers has been also observed in vivo, apparently as a part of a detoxification mechanism.[24]

Encouraged by the transpired versatility of subtilisin, we extended the repertoire of target molecules to conjugates of sugars with aglycons, including several biologically important compounds. Salicin (**6**) represents an interesting challenge for a selective conversion, as this wood component contains two primary hydroxyl groups—one in the glucose moiety and one attached to the benzyl moiety (Figure 2). Not only was subtilisin reactive with salicin, but the enzyme exhibited absolute regioselectivity with respect to the sugar primary hydroxyl (Table III). In the case of riboflavin (vitamin B_2, **7**), subtilisin acylates only the C5′ hydroxyl group (Table III). The resultant riboflavin 5′-monobutyrate has numerous pharmacological activities.[25] While it is prepared by a chemical synthesis with a 10% yield,[26] the nonoptimized enzymatic conversion afforded a 25% isolated yield (Table III). As seen in the table, subtilisin also readily reacts with two nucleosides, adenosine (**8**) and uridine (**9**), although the regioselectivity of the enzymatic acylation here is not as overwhelming as in the other cases. Importantly, comparison of the data in Tables I and III reveals similar efficiencies of enzymatic reactions (per given amount of the biocatalyst used). Hence, the presence of a large aglycon moiety does not significantly reduce the reactivity of the substrate. This observation suggests the feasibility of subtilisin-catalyzed acylation of even larger molecules of diverse structures.

Table III. Transesterification Reactions between Various Naturally Occurring Sugar-Containing Compounds and Trichloroethyl Butyrate Catalyzed by Subtilisin in Anhydrous Dimethylformamide[a]

compd	reactn time, days	conv,[b] %	isol yield, g (% theor)	product compn[c]
6	6	57	1.5 (34)	>95% 6′-*O*-monobutyrylsalicin
7	6	58	0.2 (25)	>95% riboflavin 5′-monobutyrate
8	7	68	1.1 (35)	85% adenosine 5′-monobutyrate 15% adenosine 3′-butyrate
9	4	85	2.0 (43)	55% uridine 5′-monobutyrate 35% uridine 3′-monobutyrate 10% uridine 2′-monobutyrate

[a] Conditions: sugar-containing compounds, 12.5, 1.9, 10, and 15 mmol for **6**–**9**, respectively; molar excess of TCEB, 20% in all instances; solvent dimethylformamide, 25 mL for 6 and **9**, 100 mL for **7**, 50 mL for **8**; subtilisin, 30 mg/mL for 6, 10 mg/mL for **7**, 20 mg/mL for **8** and **9**; 45 °C; shaking at 250 rpm. After the periods of time indicated in the second column of the table, the enzyme was removed by filtration, the solvent evaporated under vacuum, and the remaining residue subjected to silica gel chromatography with a mixture of ethyl acetate, methanol, and water (200:10:1, 100:10:1, 100:10:1, and 98:2:0 for **6**–**9**, respectively) as the solvent. [b] Determined by gas chromatography on the basis of accumulation of the product trichloroethanol. In no case was any appreciable conversion detected without the enzyme. [c] In all cases (except for **8**), the isolated product came out of the silica gel column as a single peak and was pure by TLC. It was subsequently analyzed by ^{13}C NMR (see the Experimental Section), which revealed the composition shown in the last column of the table. In the case of **8**, the silica gel column chromatography afforded preparative separation of the two positional isomers depicted in the table.

In all the examples discussed thus far, subtilisin was used to introduce a model aliphatic acyl (butyryl) moiety into carbohydrates. It would be of interest to use the enzyme to couple sugars to amino acid derivatives, for such conjugates of these two basic classes of natural compounds possess various biological activities.[27] The approach selected by us was analogous to that outlined previously, except that RCOOR′ in reaction 1 was an *N*-acetylamino acid 2-chloroethyl ester and monosaccharides were used as nucleophiles. In the initial experiment, 15 mmol of **1** and 6 mmol of *N*-acetyl-L-phenylalanine chloroethyl ester (**10**) were dissolved in 30 mL of anhydrous dimethylformamide, followed by addition of 0.6 g of subtilisin. The suspension[13] was shaken at 45 °C and 250 rpm for 16 h, after which time almost two-thirds of the ester had reacted. The enzyme was removed by filtration, and the standard isolation procedure resulted in 0.95 g (43% isolated yield) of product. ^{13}C NMR revealed that the latter consisted of three positional isomers of the monoester *N*-acetyl-L-phenylalanylglucose: 6-*O* (80%), 3-*O* (15%), and 2-*O* (5%). To make this synthesis more practical, we employed the crude

(23) The highly purified preparation of subtilisin obtained from Sigma costs $90/g. The crude enzyme was purchased from Amano at 20 cents/g.
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(25) Ogura, A. *Chem. Abstr.* **1983**, *98*, 191375g. Yamamoto, Y.; Okada, T.; Morioka, S.; Kaneko, T. *Chem. Abstr.* **1984**, *100*, 73977s. Zenyaku Kogyo Co. *Chem. Abstr.* **1984**, *101*, 43614z. Otaki, M.; Iijima, T. *Chem. Abstr.* **1986**, *106*, 85221g.
(26) Okuda, J.; Horiguchi, N. *Chem. Pharm. Bull.* **1980**, *28*, 181–188.
(27) Hunt, S. In *Chemistry and Biochemistry of the Amino Acids*; Barrett, G. C., Ed.; Chapman and Hall: London, 198; Chapter 4. Davies, J. S., Ed. *Amino Acids and Peptides*; Chapman and Hall: London, 1985.

Table IV. Transesterification Reactions between Various Monosaccharides and Monochloroethyl Esters of *N*-Acetylamino Acids Catalyzed by Subtilisin in Anhydrous Dimethylformamide[a]

monosaccharide	amino acid	reactn time, h	conv,[b] %	isol yield, g (% theor)	product compn[c]
D-glucose	L-phenylalanine	60	73	0.90 (40)	80% 6-*O*-monoester 15% 3-*O*-monoester 5% 2-*O*-monoester
D-fructose	L-phenylalanine	60	61	0.91 (41)	50% 1-*O*-monoester 40% 6-*O*-monoester
D-sorbitol	L-phenylalanine	60	73	0.92 (41)	50% 6-*O*-monoester 30% 1-*O*-monoester 10% 2-*O*-monoester
methyl-β-D-galactopyranoside	L-phenylalanine	60	66	0.84 (36)	95% 6-*O*-monoester
methyl-β-D-galactopyranoside	L-methionine	60	70	1.19 (54)	87% 6-*O*-monoester 8% 3-*O*-monoester
methyl-β-D-galactopyranoside	L-alanine	20	70	0.91 (49)	84% 6-*O*-monoester 8% 4-*O*-monoester
methyl-β-D-galactopyranoside	D-alanine	72	35	0.22 (12)	68% 6-*O*-monoester 10% 2-*O*-monoester 10% 3-*O*-monoester 10% 4-*O*-monoester

[a] Conditions: monosaccharides and *N*-acylamino acid chloroethyl esters, in all cases 15 mmol and 6 mmol, respectively; solvent dimethylformamide, 30 mL; in all cases (except for the D-alanine derivative) 1.8 g of crude subtilisin was used as a catalyst; in the D-alanine reaction shown in the last line of the table, 0.6 g of purified subtilisin was employed; 45 °C; shaking at 250 rpm. After the periods of time indicated in the third column of the table, the products were isolated by silica gel chromatogrlaphy with mixtures of chloroform, methanol, and water as solvents. [b] Estimated by gas chromatography as a ratio of the area of the peak corresponding to the product ester to the sum of this parameter and the area of the peak corresponding to the substrate ester. In no case was any appreciable conversion detected without the enzyme. [c] See footnote *c* to Table I, except that some of the isomers were partially resolved by TLC.

enzyme as a catalyst instead of its highly purified counterpart. As the data in the first line of Table IV indicate, the regioselectivity of the enzymatic acylation was unaffected by that transition.

In addition to **1**, several other monosaccharides were used as nucleophiles in the transesterification reaction with **10** catalyzed by crude subtilisin in dimethylformamide. As a result, monoesters of fructose, sorbitol, and methyl-β-galactopyranoside were readily prepared (Table IV). The enzyme was capable of monoacylating sugars with other amino acids such as methionine and alanine; interestingly, in the latter both L and D isomers could be enzymatically incorporated (Table IV). Thus, subtilisin can be used as a catalyst of facile coupling of sugars and amino acids.

To summarize, the protease subtilisin has been employed in the present study for regioselective acylation of carbohydrates and related compounds in dimethylformamide. This work leads to a straightforward synthetic methodology for the preparative production of monoesters of diverse sugar-related substances. From the conceptual standpoint, this investigation achieves the ultimate evolution of the reaction medium in preparative enzymatic catalysis—from water to aqueous–organic biphasic mixtures,[28] to reversed micelles,[29] to monophasic, water-immiscible organic solvents saturated with water,[30] to anhydrous hydrophobic organic solvents,[3,31] to anhydrous water-miscible organic solvents,[4,5,31] and finally to the universal organic solvent,[18] anhydrous dimethylformamide.

Experimental Section

Materials. Two different preparations of subtilisin (EC 3.4.21.14, protease from *B. subtilis*) were used in this work: the highly purified (54% purity, as determined by us by means of spectrophotometric titration with *N-trans*-cinnamoylimidazole[32]), crystalline enzyme (type VIII) from Sigma Chemical Co. (St. Louis, MO) and the crude enzyme (protease N) obtained from Amano International Enzyme Co. (Troy, VA). Unless stated otherwise, the purified sample of subtilisin was employed as a catalyst. Prior to use, both enzyme preparations were dissolved in water containing 0.1 M phosphate, the solution was adjusted to pH 7.8, and it was freeze-dried. This "pH adjustment"[2] markedly increases catalytic activity of subtilisin in organic solvents.[33]

N,N-Dimethylformamide (analytical grade) was used without further purification apart from drying by shaking with 3-Å molecular sieves (Linde). Commercially obtained pyridine was purified and dried as outlined before.[4] The solvents thus prepared contained no detectable water (see below).

Trichloroethyl butyrate was synthesized from butyryl chloride and 2,2,2-trichloroethanol following a general methodology[34] and had the same characteristics as previously described.[5] Monochloroethyl esters of *N*-acetylamino acids were prepared from the corresponding *N*-acetylamino acids (obtained commercially) and 2-chloroethanol by the general methodology.[35] The properties of the resultant esters were as follows. For **10**: mp 99–100 °C; $[\alpha]^{30}_D$ +4.5° (*c* 1, MeOH); ^{1}H NMR ($CDCl_3$, TMS as the internal reference) δ 7.31 (3 H, m), 7.12 (2 H, m), 5.92 (1 H, br d, J = 7.5 Hz), 4.94 (1 H, m), 4.37 (2 H, t, J = 5.5 Hz), 3.64 (2 H, t, J = 5.5 Hz), 3.16 (2 H, dd, J_1 = 5.8 Hz, J_2 = 2.3 Hz), 1.99 (3 H, s). Anal. Calcd for $C_{13}H_{16}ClNO_3$: C, 57.94; H, 5.98; Cl, 13.15; N, 5.19; O, 17.81. Found: C, 57.71; H, 5.88; Cl, 13.05; N, 4.92; O, 17.67. For *N*-acetyl-L-methionine chloroethyl ester: oil; $[\alpha]^{30}_D$ −24.8° (*c* 1, MeOH); ^{1}H NMR ($CDCl_3$) δ 6.35 (1 H, br d, J = 7.5 Hz), 4.77 (1 H, dt, J_1 = 7.4 Hz, J_2 = 5.0 Hz), 4.40 (2 H, m), 3.71 (2 H, t, J = 5.5 Hz), 2.56 (2 H, t, J = 7.4 Hz), 2.11 (3 H, s), 2.10 (2 H, m), 2.05 (3 H, s). Anal. Calcd for $C_9H_{16}ClNO_3S$: C, 42.64; H, 6.36; Cl, 13.98; N, 5.52; O, 18.93; S, 12.65. Found: C, 42.41; H, 6.45; Cl, 13.83; N, 5.32; O, 18.71; S, 12.42. For *N*-acetyl-L-alanine chloroethyl ester: mp 70–71 °C; $[\alpha]^{30}_D$ −39.5° (*c* 0.2, acetone); ^{1}H NMR ($CDCl_3$) δ 6.10 (1 H, br s), 4.64 (1 H, m, J = 7.3 Hz), 4.40 (2 H, m), 3.70 (2 H, t, J = 5.6 Hz), 2.03 (3 H, s), 1.44 (3 H, d, J = 7.3 Hz). Anal. Calcd for $C_7H_{12}ClNO_3$: C, 43.45; H, 6.25; Cl, 18.32; N, 7.24; O, 24.80. Found: C, 43.12; H, 5.95; Cl, 18.24; N, 6.94; O, 24.88. For *N*-acetyl-D-alanine chloroethyl ester: mp 72–73 °C; $[\alpha]^{30}_D$ +39.4° (*c* 0.2, acetone). Anal. Calcd for $C_7H_{12}ClNO_3$: C, 43.45; H, 6.25; Cl, 18.32; N, 7.24; O, 24.80. Found: C, 43.48; H, 6.28; Cl, 18.16; N, 7.11; O, 25.03.

α-D-(+)-Glucose, β-D-(−)-fructose, D-sorbitol, α-lactose, sucrose, maltose, D-(+)-cellobiose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were purchased from Sigma. Methyl-β-D-galactopyranoside, α-cyclodextrin, salicin, (−)-riboflavin, (−)-adenosine, and uridine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Some of the aforementioned carbohydrates were

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commercially available only as hydrates. In those cases the compounds were dissolved in dimethylformamide, and the solutions were shaken with 3-Å molecular sieves (usually overnight) until no more water could be measured in the solvent (see below).

Assays. All sugar-based compounds and their derivatives, as well as 2,2,2-trichloroethanol and 2-chloroethanol, used in this work were detected by gas chromatography (GC) with either a 5-m HP1 capillary silica gel column coated with methylsilicon gum (Hewlett-Packard) or a 2-m steel column packed with Supelco OV 17 resin (in the case of all amino acid derivatives) (in both cases N_2 carrier gas, 30 mL/min, detector and injector port temperature 300 °C). All reaction mixtures were subjected to precolumn derivatization with 1,1,1,3,3,3-hexamethyldisilazane.[36]

In addition to GC, the purity of all products was tested by TLC with precoated silica gel IB-F sheets (Baker) and appropriate mixtures of ethyl acetate–methanol–water or chloroform–methanol–water as solvents. The spots were developed by spraying with concentrated H_2SO_4, followed by heating.

Optical rotations were measured at 589 nm (sodium line) at 30 °C in a Perkin-Elmer 253 B polarimeter.

The water content in the solvents was measured by the optimized titrimetric Fischer method.[37] The sensitivity limit of this method under our conditions is approximately 0.02% (v/v) H_2O.[7] The term "anhydrous organic solvent" is used when no water is detected by the Fischer method.

Structure Determinations. The positions of acylation in all enzymatically prepared compounds were estabilished by ^{13}C NMR (Brucker WM 270 spectrometer) following the general strategy developed by Yoshimoto et al.[38] The specific approach used was the same as previously described[5] with the following additions. In the case of the furanose ring-containing sugars fructose and **5**, O-acylation at C1 results in a downfield shift of the peak at C1 and an upfield shift of peak at C2. In the case of **7–9**, acylation of a hydroxyl group at C2, C3, and C5 positions of the pentose moiety results in a downfield shift (1–3 ppm) of the peak corresponding to the O-acylated carbon and a similar upfield shift of the peak(s) corresponding to the neighboring carbon(s). Importantly, in the case of the compounds containing aglycon moieties (**6–9**), no changes were observed in the chemical shifts of the carbon atoms of the latter, thus ruling out acylation of that part of the molecule.

Enzymatic Synthesis of 6-*O*-Butyrylglucose. This monoester was prepared both in anhydrous pyridine and in anhydrous dimethylformamide as described in the text. In the former solvent, the product, crystallized from ethyl acetate, was pure as determined by TLC and GC: mp 111–113 °C; ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 95.0, 91.1, 74.6, 73.1, 72.4, 71.6, 70.4, 68.6, 68.2, 62.3 (a mixture of α and β anomers) (C1, β), (C1, α), (C3, β), (C2, β), (C5, β), (C3, α), (C2, α), (C4, α, β), (C5, α), (C6, α, β); for the butyryl moiety ^{13}C NMR δ 175.6, 34.6, 16.9, 11.8. Anal. Calcd for $C_{10}H_{18}O_7$: C, 48.00; H, 7.20. Found: C, 47.86; H, 7.42.

6-*O*-Butyrylglucose, which was enzymatically prepared in dimethylformamide, was also pure as determined by TLC and GC: mp 112–114 °C; ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 95.1, 91.2, 74.6, 73.1, 72.5, 71.7, 70.5, 68.7, 68.3, 62.3 (a mixture of α and β anomers) (C1, β), (C1, α), (C3, β), (C2, β), (C5, β), (C3, α), (C2, α), (C4, α, β), (C5, α), (C6, α, β); for the butyryl moiety ^{13}C NMR δ 175.7, 34.7, 17.0, 11.8. Thus, the two products are identical.

Enzymatic Synthesis of Monobutyrylmaltose. The experimental protocol and product composition are described in Table I. The 6-*O* monoester was an amorphous solid: $[\alpha]^{30}_D$ +103.3° (*c* 1, H_2O, equilibrium); ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 99.3, 95.1, 91.1, 77.7, 77.3, 75.3, 73.9, 73.2, 72.3, 72.1, 71.1, 70.6, 69.7, 69.4, 69.0, 62.7, 60.1, 60.0 (a mixture of α and β anomers) (C1′), (C1, β), (C1, α), (C4, α), (C4, β), (C3, β), (C5, β), (C2, β), (C3, α), (C3′), (C2′), (C2, α), (C5′), (C5, α), (C4′), (C6′), (C6, β), (C6, α); for the butyryl moiety ^{13}C NMR δ 175.3, 34.8, 17.0, 12.0. These NMR data were obtained at 60 °C to permit direct peak assignment from the literature data[39] for **2**. Anal. Calcd for $C_{16}H_{28}O_{12}$: C, 46.60; H, 6.80. Found: C, 46.59; H, 7.09.

Enzymatic Synthesis of Monobutyrylcellobiose. The experimental protocol and product composition are described in Table I. The 6′-*O* monoester obtained was an amorphous solid: $[\alpha]^{30}_D$ +23.5° (*c* 0.4, H_2O, equilibrium); ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 101.8, 95.1, 91.1, 78.7, 74.8, 73.9, 73.6, 73.3, 72.9, 72.4, 70.6, 69.2, 68.9, 62.3, 59.6 (a mixture of α and β anomers) (C1′), (C1, β), (C1, α), (C4, α, β), (C3′), (C5, β), (C3, β), (C2, β), (C5′), (C2′), (C2, α; C3, α), (C5, α), (C4′), (C6′), (C6, α, β); for the butyryl moiety ^{13}C NMR δ 175.4, 34.8, 17.1, 12.0. These NMR data were obtained at 60 °C to permit direct peak assignment from the literature data[39] for **3**. Anal. Calcd for $C_{16}H_{28}O_{12}$: C, 46.60; H, 6.80. Found: C, 46.48; H, 6.75.

Enzymatic Synthesis of Monobutyryllactose. The experimental protocol and product composition are described in Table I. The monoester obtained was an amorphous solid. For the 6′-isomer: ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 102.3, 95.0, 91.0, 78.6, 78.4, 73.8, 73.6, 73.0, 71.8, 71.6, 70.6, 70.4, 69.8, 69.0, 67.6, 62.7, 59.2 (a mixture of α and β anomers) (C1′), (C1, β), (C1, α), (C4, α), (C4, β), (C5, β), (C3, β), (C2, β), (C5′), (C3′), (C5, α), (C3, α), (C2′), (C2, α), (C4′), (C6′), (C6, α, β); for the butyryl moiety δ 175.4, 34.7, 17.0, 12.0. For the 3′-isomer (resolved peaks): ^{13}C NMR δ 102.3, 74.3, 73.2, 67.9, 65.6, 59.6 (C1′), (C5′), (C3′), (C2′), (C4′), (C6′). For the 4′-isomer (resolved peak): ^{13}C NMR δ 73.2 (C3′). The peak assignment was accomplished by using the literature data[40] for **4**. Anal. Calcd for $C_{16}H_{28}O_{12}$: C, 46.60; H, 6.80. Found: C, 46.32; H, 7.07.

Enzymatic Synthesis of Monobutyrylsucrose. The experimental protocol and product composition are described in Table I. The 1′-*O* monoester obtained was an amorphous solid: ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 101.5, 91.8, 80.7, 75.9, 72.6, 71.7, 70.1, 68.4, 61.6, 61.1, 59.4 (C2′), C1), (C5′), (C3′), (C4′), (C5, C3), (C2), (C4), (C1′), (C6′), (C6); for the butyryl moiety ^{13}C NMR δ 174.8, 34.7, 17.0, 12.0. The carbon atoms bonded to the primary hydroxyl groups were identified by using the literature data.[41] In order to positively confirm the structure of the enzymatically formed monobutyrylsucrose, the glycosidic linkage of the product (1 g/10 mL) was hydrolyzed with yeast α-glucosidase (23 mg/10 mL, Sigma type I) in 20 mM phosphate buffer (pH 6.0) for 24 h. Then the solution was freeze-dried, and the residue was subjected to silica gel chromatography with ethyl acetate–methanol–water (190:10:1) as the solvent. The product obtained was identified by ^{13}C NMR as 1-*O*-butyrylfructose by comparison with 1-*O*-acetylfructose prepared[4] with porcine pancreatic lipase as a catalyst in pyridine, thus proving our original structure assignment. Anal. Calcd for $C_{16}H_{28}O_{12}$: 46.60; H, 6.80. Found: C, 46.58; H, 6.91.

When crude subtilisin was used for the acylation of **5** as outlined in the text, the product obtained was identical with the one prepared by using the highly purified enzyme (see above).

Enzymatic Synthesis of Monobutyrylmaltotriose. We dissolved 1 g of maltotriose and 0.87 g of TCEB in 10 mL of anhydrous dimethylformamide. Following addition of 300 mg of subtilisin, the suspension[13] was shaken at 45 °C and 250 rpm for 4 days. Then the enzyme was removed by filtration, the solvent evaporated, and the residue subjected to silica gel chromatography with ethyl acetate–methanol–water (16:4:1) as the solvent. As a result, 0.33 g (29% isolated yield) of an amorphous solid with $[\alpha]^{30}_D$ +115.8° (*c* 1.2, H_2O, eq), which was pure by TLC and NMR, was obtained: ^{13}C NMR (67.9 MHz, D_2O, 1.3% pyridine as the internal reference) δ 99.0, 98.6, 94.8, 90.9, 76.6, 76.3, 75.1, 73.6, 73.0, 72.3, 72.1, 71.7, 70.7, 70.5, 70.3, 69.5, 69.0, 68.6, 62.5, 59.7, 59.6 (a mixture of α and β anomers) (C1″), (C1′), (C1, β), (C1, α), (C4′; C4, α), (C4, β), (C3, β), (C5, β), (C2, β), (C3′), (C3, α), (C3″), (C2″), (C2′), (C5′; C2, α), (C5″), (C5, α), (C4″), (C6″), (C6, β), (C6′; C6, α); for the butyryl moiety ^{13}C NMR δ 175.4, 34.7, 17.0, 12.0. The peak assignment was accomplished by using the literature data[42,43] for maltotriose. Anal. Calcd for $C_{22}H_{38}O_{17}$: C, 45.99; H, 6.62. Found: C, 45.87; H, 6.94.

Enzymatic Synthesis of Monobutyrylsalicin. The experimental protocol and product compodition are described in Table III. The 6′-*O* monoester obtained was a crystalline solid: mp 128–130 °C; $[\alpha]^{30}_D$ −55.0° (*c* 1.5, H_2O); ^{13}C NMR (67.9 MHz, DMSO) δ 154.6, 131.7, 127.6, 127.4, 122.0, 114.8, 101.3, 76.3, 73.8, 73.4, 70.1, 63.4, 58.3 (the first six peaks correspond to the aromatic carbon atoms), (C1′), (C3′), (C5′), (C2′), (C4′), (C6′), (C7); for the butyryl moiety ^{13}C NMR δ 172.6, 35.5, 18.0, 13.5. Anal. Calcd for $C_{16}H_{12}O_8$: C, 57.30; H, 6.74. Found: C, 57.10; H, 6.91.

Enzymatic Synthesis of Riboflavin Monobutyrate. The experimental protocol and product composition are described in Table III. The 5′-*O* monoester obtained was a yellow crystalline solid: mp 240–242 °C (lit.[26] mp 244–245 °C); UV λ_{max} (EtOH, 25 °C) 223, 270, 360, 445 nm (lit.[26]

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223, 270, 352, 445 nm); ^{13}C NMR (67.9 MHz, DMSO) δ 159.8, 155.4, 150.7, 146.0, 136.9, 135.7, 133.9, 132.0, 130.6, 177.4, 73.6, 69.7, 68.6, 66.0, 47.5, 20.7, 18.7 (the first ten and the last two peaks correspond to the carbon atoms of the isoalloxazine moiety), (C2′), (C4′), (C3′), (C5′), (C1′); for the butyryl moiety ^{13}C NMR δ 173.0, 35.4, 17.9, 13.5. Anal. Calcd for $C_{21}H_{26}N_4O_7$: C, 56.50; H, 5.83; N, 12.56. Found: C, 56.16; H, 5.72; N, 12.47.

Enzymatic Synthesis of Adenosine Monobutyryate. The experimental protocol and product composition are described in Table III. The 5′-*O* monoester was a crystalline solid with mp 121–123 °C and $[\alpha]^{30}_D$ −19.4° (*c* 0.5, MeOH), and it was pure by TLC and NMR: ^{13}C NMR (67.9, MHz, DMSO) δ 156.1, 152.7, 149.3, 139.7, 119.2, 87.8, 81.5, 72.9, 70.3, 63.6 (the first five peaks correspond to the carbon atoms of the adenine ring), (C1′), (C4′), (C2′), (C3′), (C5′); for the butyryl moiety ^{13}C NMR δ 172.1, 35.4, 17.9, 13.4. Anal. Calcd for $C_{14}H_{19}N_5O_5$: C, 49.85; H, 5.64; N, 20.77. Found: C 49.60; H, 5.94; N, 20.49. The 3′-*O* monoester was an amorphous solid pure by TLC: ^{13}C NMR (67.9 MHz, DMSO) δ 156.3, 152.5, 149.1, 139.9, 119.4, 87.8, 83.7, 73.2, 71.7, 61.6 (the first five peaks correspond to the carbon atoms of the adenine ring), (C1′), (C4′), (C3′), (C2′), (C5′); for the butyryl moiety ^{13}C NMR δ 172.1, 35.4, 17.9, 13.4. Anal. Calcd for $C_{14}H_{19}N_5O_5$: C, 49.85; H, 5.64; N, 20.77. Found: C, 49.56; H, 5.88; N, 20.40. The NMR peak assignment was accomplished by using the literature data[44] for **8**.

Enzymatic Synthesis of Uridine Monobutyrate. The experimental protocol and product composition are described in Table III. The monoester obtained was a crystalline compound. For uridine 5′-monobutyrate: ^{13}C NMR (67.9 MHz, DMSO) δ 163.1, 150.6, 140.7, 102.0, 88.8, 81.1, 72.7, 69.8, 63.6 (the first four peaks correspond to the carbon atoms of the uracil ring), (C1′), (C4′), (C2′), (C3′), (C5′); for the butyryl moiety ^{13}C NMR δ 172.6, 35.2, 17.9, 13.4. For uridine 3′-monobutyrate: ^{13}C NMR (67.9 MHz, DMSO) δ 163.1, 150.9, 140.4, 102.3, 87.2, 82.8, 72.6, 71.7, 61.0 (the first four peaks correspond to the carbon atoms of the uracil ring), (C1′), (C4′), (C3′), (C2′), (C5′); for the butyryl moiety ^{13}C NMR δ 172.2, 35.3, 17.9, 13.4. For uridine 2′-monobutyrate: ^{13}C NMR (67.9 MHz, DMSO) δ 163.1, 150.9, 140.4, 102.2, 85.6, 74.9, 68.6, 60.7 (the first four peaks correspond to the carbon atoms of the uracil ring), (C1′, C4′), (C2′), (C3′), (C5′); for the butyryl moiety ^{13}C NMR δ 172.0, 35.3, 17.9, 13.4. Anal. Calcd for $C_9H_{11}N_2O_6$: C, 49.68; H, 5.73; N, 8.92. Found: C, 49.36; H, 5.93; N, 8.81. The NMR peak assignment was accomplished by using the literature data[45] for **9**.

Enzymatic Synthesis of *N*-Acetyl-L-phenylalanylglucose. The experimental protocol and product composition are described in Table IV. The monoester obtained was an amorphous solid. For the main product 6-*O* isomer: ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 98.5, 93.9, 78.1, 76.3, 74.9, 73.9, 71.9, 71.5, 70.5, 65.8, 65.7 (a mixture of α and β anomers) (C1, β), (C1, α), (C3, β), (C2, β), (C5, β; C3, α), (C2, α), (C4, α), (C4, β), (C5, α), (C6, α), (C6, β); for the aliphatic part of the amino acid moiety ^{13}C NMR δ 54.4, 37.8, 22.5. For the 3-*O* isomer (resolved peaks): ^{13}C NMR δ 80.6, 69.6, 69.4 (C3, β), (C4, α), (C4, β). For the 2-*O* isomer (resolved peaks): ^{13}C NMR δ 96.0, 90.7, (C1, β), (C1, α). Anal. Calcd for $C_{17}H_{23}NO_8$: C, 55.28; H, 6.28; N, 3.79. Found: C, 55.01; H, 6.37; N, 3.65.

Enzymatic Synthesis of *N*-Acetyl-L-phenylalanylfructose. The experimental protocol and product composition are described in Table IV. The monoester obtained was an amorphous solid. For the 1-*O* positional isomer: ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 104.8, 101.5, 98.2, 84.3, 83.7, 78.8, 78.2, 71.3, 70.4, 69.7, 69.4, 67.2, 66.0, 65.2, 64.8, 64.5 [a mixture of a β-pyranose (p) and α- and β-furanose (f) isomers] (C2, α-f), (C2, β-f), (C2, p), (C5, α-f), (C5, β-f; C3, α-f), (C3, β-f; C4, α-f), (C4, β-f), (C4, p), (C5, p), (C3, p), (C1, p), (C1, β-f), (C6, α-f), (C1, α-f), (C6, p), (C6, β-f); for the aliphatic part of the amino acid moiety δ 54.5, 37.7, 22.5. For the 6-*O* positional isomer: ^{13}C NMR δ 106.3, 104.0, 83.7, 80.0, 76.5, 73.3, 68.2, 66.9, 64.8, 62.7 (a mixture of α and β furanose anomers) (C2, α), (C2, β), (C3, α), (C5, α, β), (C4, β), (C4, α; C3, β), (C6, α), (C6, β), (C1, β), (C1, α); for the aliphatic part of the amino acid moiety ^{13}C NMR δ 54.5, 37.7, 22.5. Anal. Calcd for $C_{17}H_{23}NO_8$: C, 55.28; H, 6.28; N, 3.79. Found: C, 54.99; H, 6.43; N, 3.54.

Enzymatic Synthesis of *N*-Acetyl-L-phenylalanylsorbitol. The experimental protocol and product composition are described in Table IV. The monoester obtained was an oil. For the 6-*O* positional isomer: ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 73.9, 72.5, 69.7, 69.3, 67.7, 63.2 (C2), (C4), (C3), (C5), (C6), (C1); for the aliphatic part of the amino acid moiety δ 53.9, 37.0, 21.7. For the 1-*O* positional isomer: ^{13}C NMR δ 72.5, 72.1, 71.1, 70.0, 66.7, 63.8 (C4), (C2), (C5), (C3), (C1), (C6); for the aliphatic part of the amino acid moiety ^{13}C NMR δ 53.9, 37.0, 21.7. For the 2-*O* positional isomer (resolved peak): ^{13}C NMR δ 60.2 (C1). Anal. Calcd for $C_{17}H_{25}NO_8$: C, 54.98; H, 6.79; N, 3.77. Found: C, 54.79; H, 7.00; N, 3.61.

Enzymatic Synthesis of *N*-Acetyl-L-phenylalanyl Methyl-β-D-Galactopyranoside. The experimental protocol and product composition are described in Table IV. The monoester obtained was an amorphous solid: mp 175–177 °C; $[\alpha]^{30}_D$ +15.6° (*c* 2.5, H_2O); ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 105.7, 74.5, 73.3, 71.8, 69.5, 65.0, 56.6 (C1), (C3), (C5), (C2), (C4), (C6), (*C*-methyl); for the aliphatic part of the amino acid moiety ^{13}C NMR δ 54.5, 37.9, 22.5. Anal. Calcd for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65. Found: C, 56.23; H, 6.68; N, 3.48.

Enzymatic Synthesis of *N*-Acetyl-L-methionyl Methyl-β-D-galactopyranoside. The experimental protocol and product composition are described in Table IV. The monoester obtained was an amorphous solid. For the 6-*O* isomer: ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 105.4, 74.4, 73.4, 71.7, 69.7, 65.3, 56.8 (C1), (C3), (C5), (C2), (C4), (C6), (C methyl); for the amino acid moiety ^{13}C NMR δ 52.4, 31.6, 30.6, 22.5, 15.1. For the 3-*O* isomer (resolved peaks): ^{13}C NMR δ 78.3, 69.2, 67.3, (C3), (C2), (C4). Anal. Calcd for $C_{14}H_{25}NO_8S$: C, 45.77; H, 6.86; N, 3.81; S, 8.71. Found: C, 45.69; H, 6.97; N, 3.62; S, 8.73.

Enzymatic Synthesis of *N*-Acetyl-L-alanyl Methyl-β-D-galactopyranoside. The experimental protocol and product composition are described in Table IV. The monoester obtained was an amorphous solid. For the 6-*O* isomer: ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 105.7, 74.6, 73.4, 71.9, 69.7, 65.0, 56.6 (C1), (C3), (C5), (C2), (C4), (C6), (C methyl); for the amino acid moiety ^{13}C NMR δ 48.8, 22.5, 17.5. For the 4-*O* isomer (resolved peaks): ^{13}C NMR δ 75.2, 73.4, 73.1 (C5), (C4), (C3). Anal. Calcd for $C_{12}H_{21}NO_8$: C, 46.90; H, 6.89; N, 4.56. Found: C, 46.67; H, 7.02, N, 4.39.

Enzymatic Synthesis of *N*-Acetyl-D-alanyl Methyl-β-D-galactopyranoside. The experimental protocol and product composition are described in Table IV. The monoester obtained was an amorphous solid. For the 6-*O* isomer: ^{13}C NMR (67.9 MHz, pyridine, TMS as the internal reference) δ 105.7, 74.6, 73.3, 71.8, 69.6, 64.8, 56.7 (C1), (C3), (C5), (C2), (C4), (C6), (C methyl); for the amino acid moiety ^{13}C NMR δ 48.9, 22.5, 17.4. For the 2-*O* and 3-*O* isomers (resolved peaks): ^{13}C NMR δ 103.0 (C1) and δ 67.2 (C4), respectively. Anal. Calcd for $C_{12}H_{21}NO_8$: C, 46.90; H, 6.89; N, 4.56. Found: C, 46.76; H, 7.16; N, 4.49.

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