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Synthesis and reactivity of the monosaccharide esters of amino acids as models of teichoic acid fragment

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The increasing prevalence of sepsis from Gram-positive bacterial pathogens necessitates further evaluation of the basic assumptions about the molecular pathogenesis of septic shock. Since diverse physiological functions of Gram-positive bacteria are controlled by the degree of esterification of teichoic acids with D-alanine, we examined the reactivity of monosaccharide esters in which anomerically free or protected D-glucose is linked through its C-6 hydroxy group to either phenylalanyl or tyrosyl residues as models for teichoic acid fragment. We show that the attached sugar moiety induces activation of the amino acid residue. Due to the enhanced reactivity of the NH_2 group in the monosaccharide esters studied, the formation of products generated by intramolecular and intermolecular glycation reactions is accelerated resulting in heterogeneous mixture of compounds. These findings suggest that, if similar adducts are formed by glycation of D-alanine in teichoic acid of Gram-positive bacteria, they should be examined as potential bioactive ligands or chemical message for infection.

Keywords: **monosaccharide, amino acid, ester, glycation, teichoic acid, Gram-positive bacteria**

Introduction

The life-threatening complications of sepsis in humans are elicited by Gram-negative as well as Gram-positive bacteria. Yet, there is increasing experimental evidence that fundamental differences exists in the host response to Gram-positive bacterial pathogens compared to Gram-negative organisms [1]. Gram-positive bacteria are not protected by an outer membrane, instead, the cell-wall is formed by a thick peptidoglycan fabric (PGA) and by polymers of alternating phosphate and alditol groups called teichoic acids (TA). These polymer chains are either covalently connected to the peptidoglycan or to membrane glycolipids (lipoteichoic acids, LTA) [2]. PGA and LTA can elicit most of the clinical manifestations of bacterial infection, inducing the excessive release of pro-inflammatory cytokines, such as the tumor necrosis factors, IL-1 and IL-6, and other inflammatory mediators, including macrophages, which ultimately cause clinical symptoms [3].

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Teichoic acids of the various Gram-positive species are highly variable with regard to the alditol moieties (glycerol or ribitol), and modifications of the alditol hydroxy groups with glycosyl residues and with D-alanine esters. Altering the process of D-alanylation usually has a dramatic influence on the functionality of the bacterial cell-wall [4]. D-Alanine esterified to TA and LTA brings its positive charge as a counterion to those of phosphate or carboxyl. In addition, the degree of esterification of TA with D-alanine controls autolysis and viability of bacteria, and the absence of this amino acid leads to increased sensitivity toward cationic antimicrobial peptides [5].

It occured to us, that the ability of Gram-positive strains to stimulate the production of pro-inflammatory cytokines, which may be important in the pathogenesis of shock caused by these bacteria, reveals some striking similarities with the effects caused by advanced glycation end products (AGEs) [6]. Recent studies demonstrated that AGE products, in particular *N*-carboxymethyl (CML) modifications of proteins, engage cellular receptor for AGEs (RAGE), thereby activating cell signalling pathways, such as NF-κB, and modulating gene expression [7]. Thus, CML-RAGE interaction triggers

processes intimately linked to accelerated vascular and inflammatory complications that typify disorders in which inflammation is an established component. Interestingly, *N*-carboxymethyl- and *N*-carboxyethyl-derivatives of amino acids (called *opines*) have been isolated from diverse sources including plant tumors (crown gall), induced by strains of *Agrobacterium tumefaciens*, eucaryotic cells, bacteria, and muscle tissue of marine invertebrates [8]. Opines produced by plant tumor cells provide, together with another metabolite acetosyringone, a chemical "message" to the phytopathogen indicating a host suitable for colonization, whereas the function(s) of opines in bacteria are presently unknown. Opines are regarded as products of α-keto acids [8] and/or metabolites derived from the condensation of reducing sugars with the NH_2 groups of amino acids [9]. On the other hand, AGEs are products of a similar reaction in which the initial step is the condensation of reducing sugars with N-terminal amino groups of proteins [10].

In our opinion, these findings raise the question whether, in Gram-positive pathogens, the free α-NH_2 group of D-alanine in esterified teichoic acids could form specific AGE-like products which would engage cellular receptors thus redirecting, or have direct impact on, inflammatory processes.

In this study we demonstrate on model compounds in which an anomerically free or protected carbohydrate moiety is linked to an amino acid (tyrosine, phenylalanine) through an ester bond that, due to the enhanced reactivity of the α-NH_2 group in monosaccharide esters, these compounds readily undergo intermolecular and intermolecular glycation reactions leading to a heterogeneous mixture of compounds.

Materials and methods

Melting points were determined on a Tottoli (Büchi) apparatus and are uncorrected. Optical rotations were measured at room temperature using an Optical Activity LTD automatic AA-10 Polarimeter. Reactions were monitored by TLC on Silica Gel 60 F_{254} plates (Merck; Darmstadt, Germany) using detection with ninhydrin, the chlorine-iodine reagent, or heating with H_2SO_4. The optical purity of the amino acids was examined on Chiral Plates (Aldrich). RP HPLC was performed on a Varian 9010 HPLC system with a Eurospher 100 reversed-phase C-18 semipreparative column (250 × 8 mm I.D., 5 μm) under isocratic conditions by using different concentrations of MeOH in 0.1% aqueous trifluoroacetic acid (TFA), flow rate 1.1 ml min^{-1}. UV detection (Varian Model 9050 variable-wavelength UV-Vis detector) was performed at 254 and 280 nm. NMR spectra were recorded on a Varian Gemini spectrometer, operating at 300.1 MHz for ^1H and 75.5 MHz for ^{13}C nuclei. Samples were measured at 25°C in 5 mm NMR tubes. Chemical shifts, in ppm, are referred to TMS. Elemental analyses were carried out at the Microanalytical Laboratory, Ruđer Bošković Institute.

Methyl α-D-*glucopyranoside* was purchased from Fluka. N-*(1-Deoxy*-D-*fructos-1-yl)*-L-*tyrosine* (**11**) was obtained under the conditions described by Jakas and Horvat [11]. Compounds **12**–**15** were purchased from Aldrich.

6-O-*(*N-tert-*Butyloxycarbonyl*-L-phenylalanyl)-D-glucopyranose (**1**)

D-Glucose (1620 mg, 9 mmol) was dissolved in dry pyridine (60 ml) and allowed to react with *N-tert*-butyloxycarbonyl-L-phenylalanine pentachlorophenyl ester (Boc-Phe-OPCP) (1530 mg, 3 mmol) in the presence of imidazole (1020 mg, 15 mmol). The reaction mixture was stirred overnight at room temperature and the solved was evaporated. To the residue 10% citric acid (50 ml) and ethyl acetate (50 ml) were added. The organic layer was separated and the water layer extracted with ethyl acetate (3 × 20 ml). The combined organic extracts were dried (Na_2SO_4), the solvent was evaporated and the residue was purified by flash chromatography on silica gel with ethyl acetate–acetic acid–water (70:2:2) as the eluent to yield ester **1** (540 mg, 42%) which was recrystallized from CH_2Cl_2/hexane; mp 84–87°C; $[\alpha]_D^{22} + 29°$ (c = 1.0, MeOH). Anal calcd. for $C_{20}H_{29}NO_9$: C, 56.20; H, 6.84; N, 3.27. Found: C, 56.39; H, 7.07; N, 3.34.

6-O-*(*N,O-*Di*-tert-*butyloxycarbonyl*-L-*tyrosyl)*-D-*glucopyranose* (**2**)

D-Glucose (2160 mg, 12 mmol) was treated with *N,O*-di-*tert*-butyloxycarbonyl-L-tyrosine pentachlorophenyl ester (2520 mg, 4 mmol) and imidazole (1360 mg, 20 mmol) in the same way as described for compound **1** to give pure **2** (980 mg, 45%) which was recrystallized from ether/hexane; mp 103–106°C; $[\alpha]_D^{22} + 50°$ (c = 1.0, MeOH). Anal calcd. for $C_{25}H_{37}NO_{12}$: C, 55.24; H, 6.88; N, 2.58. Found: C, 55.34; H, 6.75; N, 2.70.

6-O-(L-*Phenylalanyl)*-D-*glucopyranose* (**3**)

Compound **1** (100 mg, 0.23 mmol) was treated with TFA–water (9:1, 2 ml) in the presence of anisole (0.4 ml) for 1 h at room temperature. After addition of diisopropyl ether (80 ml) at 0°C, the precipitate was collected by centrifugation and triturated several times with diisopropyl ether. The obtained residue was purified by semipreparative RP HPLC by using 40% MeOH/0.1% TFA as the eluent (retention time of **3**: 7.95 min) and lyophilized to yield, after crystallization from ethanol/diisopropyl ether, pure compound **3** (81 mg, 80%); mp 89–95°C (decomp.); $[\alpha]_D^{22} + 23°$ (c = 1.0, MeOH). For ^1H and ^{13}C NMR data see Tables 1 and 2. Anal calcd. for $C_{15}H_{21}NO_7 \cdot CF_3COOH$: C, 46.26; H, 5.02; N, 3.17. Found: C, 46.24, H, 5.21; N, 3.25. Desalting on a short (10 × 0.8 cm). Dowex 1X2, 200 (Ac) column and lyophilization gave ester **3** which was used in the subsequent experiments.

6-O-*(*L-*Tyrosyl)*-D-*glucopyranose* (**4**)

Compound **2** (500 mg, 0.92 mmol) was treated in the same way as described for compound **3** to give after semipreparative RP HPLC

Table 1. ^{1}H NMR chemical shift data (δ, ppm) of compounds **3–8**.[a]

		3[b]		***4***[c]					
Residue	*Proton*	*α-anomer*	*β-anomer*	*α-anomer*	*β-anomer*	***5***	***6***	***7***	***8***
Carbohydrate moiety	1	4.92	4.33	4.93	4.28	4.54	4.59	4.71	4.54
	2	3.15	2.94	3.14	3.02	3.24	3.43	4.50	3.22
	3	3.45	3.15	3.38	3.16	3.41	4.98	3.66	3.40
	4	3.10	3.10	3.16	3.16	3.09	3.32	3.23	3.13
	5	3.83	3.32	3.85	3.38	3.58	3.41	3.38	3.60
	6/6′	4.15/4.39	4.15/4.39	4.20/4.38	4.20/4.38	4.09/4.33	3.46/3.62	3.51/3.68	4.20/4.38
	CH_3					3.28	3.32	3.29	3.25
Phe/Tyr[d]	α	4.35	4.35	4.28	4.28	4.21	4.28	4.21	4.38
	β/β′	3.12	3.12	3.02/3.16	3.02/3.16	2.85/3.07	2.72/3.12	2.84/3.15	3.12
	δ	7.28	7.28	7.05	7.05	7.31	7.26	7.28	7.27
	ε	7.34	7.34	6.72	6.72	7.31	7.21	7.30	7.33
	ζ	7.32	7.32			7.25	7.16	7.23	7.27
	NH	8.44	8.44	8.40	8.40	7.20	7.16	7.23	–[e]
Boc	CH_3					1.31	1.31	1.32	

[a]In DMSO-d_6 at 25°C.
[b]α:β = 46 : 54.
[c]α:β = 55 : 45.
[d]Phe residue for compounds **3**, **5**, **6**, **7**, **8** and Tyr residue for compound **4**.
[e]Not observed.

Table 2. ^{13}C NMR chemical shift data (δ, ppm) of compounds **3–8**.[a]

		3		***4***					
Residue	*Carbon atom*	*α-anomer*	*β-anomer*	*α-anomer*	*β-anomer*	***5***	***6***	***7***	***8***
Carbohydrate moiety	1	92.65	97.25	92.67	97.28	99.91	99.68	96.60	100.10
	2	72.40	74.94	72.35	74.89	71.94	69.70	74.29	71.86
	3	73.14	76.69	73.09	76.64	73.37	76.74	70.62	73.28
	4	70.58	70.26	70.57	70.22	70.59	67.79	70.32	70.32
	5	69.19	73.52	69.16	73.48	69.69	72.65	72.97	69.45
	6	65.79	65.79	65.74	65.74	64.68	60.58	60.74	65.68
	CH_3					54.47	54.56	54.55	54.76
Phe/Tyr[b]	α	53.41	53.41	53.49	53.49	55.24	55.07	55.21	53.20
	β	36.06	36.06	35.20	35.20	36.51	36.90	36.45	35.94
	γ	134.80	134.80	124.35	124.35	137.99	138.27	138.02	134.62
	δ	129.99	129.99	130.99	130.99	129.30	129.42	129.36	129.76
	ε	129.07	129.07	115.77	115.77	128.48	128.37	128.47	128.97
	ζ	127.71	127.71	157.10	157.10	126.70	126.51	126.67	127.62
	CO	169.48	169.48	169.51	169.51	172.40	171.86	172.23	169.40
Boc	CH_3					28.20	28.20	28.22	
	C					78.40	78.34	78.47	
	CO					155.69	155.67	155.65	

[a]In DMSO-d_6 at 25°C.
[b]Phe residue for compounds **3**, **5**, **6**, **7**, **8** and Tyr residue for compound **4**.

purification with 16.5% MeOH/0.1% TFA as the eluent (retention times of **4**: β-anomer 11.9 min; α-anomer 13.1 min) pure compound **4** which was recrystallized from ethanol/diisopropyl ether (375 mg, 90%); mp 95–102°C (decomp.); $[\alpha]_D^{22} + 23°$ (c = 1.0, MeOH). For ^{1}H and ^{13}C NMR data see Tables 1 and 2. Anal calcd. for $C_{15}H_{21}NO_8 \cdot CF_3COOH$: C, 44.65; H, 4.86; N, 3.06. Found: C, 44.52; H, 4.77; N, 3.06. Desalting was performed as described for ester **3**.

*Methyl 6-*O*-(*N*-tert-butyloxycarbonyl-*L*-phenylalanyl)-α-*D*-glucopyranoside* (**5**), *methyl 3-*O*-(*N*-tert-butyloxycarbonyl-*L*-phenylalanyl)-α-*D*-glucopyranoside* (**6**) *and methyl 2-*O*-(*N*-tert-butyloxycarbonyl-*L*-phenylalanyl)-α-*D*-glucopyranoside* (**7**)

To a solution of methyl α-D-glucopyranoside (1747 mg, 9 mmol) in dry pyridine (60 ml), Boc-Phe-OPCP (1540 mg, 3 mmol) and imidazole (1020 mg, 15 mmol) were added. The reaction mixture was stirred overnight at room temperature and worked up using 10% citric acid and ethyl acetate as described for compound **1**. After evaporation of the solvent the residue was first subjected to flash chromatography on silica gel with ethyl acetate–acetic acid–water (70:2:2) (eluent A), followed by column chromatography with A-toluene (9:2) (eluent B) to give pure esters **5** (R_f in B: 0.2), **6** (R_f in B: 0.3) and **7** (R_f in B: 0.4). Compound **5** (recrystallized from CH_2Cl_2/hexane) (366 mg, 28%); mp 62–68°C; $[\alpha]_D^{22} + 81°$ (c = 1.0, MeOH). For ^{1}H and ^{13}C NMR data see Tables 1 and 2. Anal calcd. for $C_{21}H_{31}NO_9$: C, 57.13; H, 7.08; N, 3.17. Found: C, 57.12; H, 6.86; N, 3.30. Compound **6** (recrystallized from ether/hexane) (26 mg, 2%); mp 37–75°C (decomp.); $[\alpha]_D^{22} + 83°$ (c = 1.0, MeOH). For ^{1}H and ^{13}C NMR data see Tables 1 and 2. Anal calcd. for $C_{21}H_{31}NO_9$: C, 57.13; H, 7.08; N, 3.17. Found: C, 57.21; H, 7.31; N, 2.96. Compound **7** (recrystallized from ether/hexane) (134 mg, 10%); mp 39–73°C (decomp.); $[\alpha]_D^{22} + 75°$ (c = 1.0, MeOH). For ^{1}H and ^{13}C NMR data see Tables 1 and 2. Anal calcd. for $C_{21}H_{31}NO_9 \cdot C$, 57.13; H, 7.08; N, 3.17. Found: C, 57.05; H, 7.03; N, 2.97.

*Methyl 6-*O*-(*L*-phenylalanyl)-α-*D*-glucopyranoside* (**8**)

Compound **5** (100 mg, 0.22 mmol) was treated in the same way as described for ester **3** to yield, after semipreparative RP HPLC purification with 30% MeOH/0.1% TFA, compound **8** (retention time: 13.4 min) which was crystallized from ethanol/diisopropyl ether (85 mg, 85%); mp 89–95°C; $[\alpha]_D^{22} + 77°$ (c = 1.0, MeOH). For ^{1}H and ^{13}C NMR data see Tables 1 and 2. Anal calcd. for $C_{16}H_{23}NO_7 \cdot CF_3COOH$: C, 47.48; H, 5.31; N, 3.08. Found: C, 47.37; H, 5.52; N, 3.12. Desalting was performed as described for ester **3**.

2-(5'-Hydroxymethyl-2'-formylpyrrol-1'-yl)-3-phenyl-propionic acid lactone (**9**)

Monosaccharide ester **3** (180 mg, 0.55 mmol) was dissolved in pyridine–acetic acid (1:1) (180 ml) and the solution was kept in a well-closed round bottom flask for 24 h at 50°C. The solvent was evaporated and the residue was purified by semipreparative RP HPLC using 50.5% MeOH/0.1% TFA as the eluent to give fractions enriched in **9**. Repeated RP HPLC purification with 54% MeOH/0.1% TFA as the eluent gave pure lactone **9** (retention time: 21.6 min) (15 mg, 11%). For ^{1}H and ^{13}C NMR data see Table 3.

*Incubation of 6-*O*-(*L*-tyrosyl)-*D*-glucopyranose* (**4**) *in pyridine-acetic acid – Isolation and identification of compounds* **10**–**15**

Compound **4** (100 mg, 0.29 mmol) was dissolved in pyridine–acetic acid (1:1) (100 ml) and the solution kept in a well-closed round bottom flask for 24 h at 50°C. The solvent was evaporated and the residue was purified several times by semipreparative RP HPLC with 40% MeOH/0.1% TFA to give pure 2-(5'-hydroxymethyl-2'-formylpyrrol-1'-yl)-3-(4-hydroxyphenyl)-propionic acid lactone (**10**) (retention time: 22.8 min) (4 mg, 5%) and compounds **11**–**15** in less than 1% yield

Table 3. ^{1}H and ^{13}C NMR chemical shift data (δ, ppm) of compounds **9** and **10**.[a]

	9		***10***	
Atom[b]	δ_H	δ_C	δ_H	δ_C
a	7.30 m	127.96	–	157.32
b	7.26 m	128.88	6.61 q	115.63
c	6.85 q	129.62	6.57 q	130.57
d	–	134.91	–	132.28
e	3.32 m	39.18	3.20 m	40.00
f	5.85 t	58.71	5.79 t	58.88
g	–	130.48[c]	–	130.36[c]
h	9.52 s	179.80	9.52 s	179.72
i	7.20 d (*J* 4.0 Hz)	125.18	7.20 d (*J* 4.0 Hz)	125.18
j	6.17 d (*J* 4.0 Hz)	106.12	6.17 d (*J* 3.7 Hz)	105.95
k	–	132.16[c]	–	124.61[c]
l	4.05 d (1H, *J* 15.2 Hz) 5.25 d (1H, *J* 15.2 Hz)	63.27	3.92 d (1H, *J* 14.9 Hz) 5.24 d (1H, *J* 14.9 Hz)	63.30
m	–	167.09	–	167.36
a-OH			9.46 s	

[a]In DMSO-d_6 at 25 °C.
[b]Designations of particular atoms are given in Figure 1.
[c]Assignments of signals can be interchangeable.

each, respectively. For ^{1}H and ^{13}C NMR data of lactone **10** see Table 3. For *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosine (**11**) (retention time 7.5 min), ^{1}H NMR data were identical with those reported for the authentic compound [11]. Compounds **11**–**15** were identical with authentic compounds. 4-Hydroxybenzaldehyde (**12**) (retention time: 14.5 min); ^{1}H NMR (DMSO-d_6): δ 6.94 d (2H, J 8.4 Hz, H-3, H-5), 7.76 d (2H, J 8.4 Hz, H-2, H-6), 9.79 s (1H, OH), 10.63 s (1H, CHO). ^{13}C NMR (DMSO-d_6): δ 116.09 (C-3, C-5), 128.70 (C-1), 132.39 (C-2, C-6), 163.64 (C-4), 191.36 (CHO). 4-Hydroxybenzoic acid (**13**) (retention time: 12.2 min); ^{1}H NMR (DMSO-d_6): δ 6.82 d (2H, J 8.7 Hz, H-3, H-5), 7.80 d (2H, J 8.7 Hz, H-2, H-6), 10.24 s (1H, OH), 12.43 bs (1H, COOH). 4-Hydroxyphenylacetic acid (**14**) (retention time: 11.6 min); ^{1}H NMR (DMSO-d_6): δ 3.42 s (2H, CH_2), 6.69 d (2H, J 8.4 Hz, H-3, H-5), 7.04 d (2H, J 8.4 Hz, H-2, H-6), 9.28 s (1H, OH), 12.19 bs (1H, COOH). *N*-Acetyl-L-tyrosine (**15**) (retention time: 9.8 min); ^{1}H NMR (DMSO-d_6): δ 1.79 s (3H, CH_3CO), 2.70 d (1H, Tyr CH_2), 2.90 d (1H, Tyr CH_2), 4.30 m (1H, Tyr CH), 6.65 d (2H, J 8.4 Hz, Tyr arom H-3, H-5), 7.01 d (2H, J 8.4 Hz, Tyr arom H-2, H-6), 8.13 d (1H, J 7.8 Hz, Tyr NH).

*RP HPLC analysis of the incubation mixtures of compound **4** and of D-glucose with L-tyrosine in pyridine-acetic acid*

Monosaccharide ester **4** (2 mg, 5.8 µmol) dissolved in pyridine–acetic acid (1:1) (2 ml) was incubated at 50°C. For comparison, D-glucose (1 mg, 5.8 µmol) was incubated in pyridine–acetic acid (1:1) (2 ml) with L-tyrosine (1 mg, 5.8 µmol) at 50°C. The progress of the reaction was monitored withdrawing aliquots from the reaction mixtures every 24 h, over the period of 3 days. The respective samples were immediately frozen and, after lyophilization, were directly analysed by RP HPLC using 40% MeOH/0.1% TFA as the eluent. The relative amounts of compounds **10**–**15** formed after 24 h are presented in Figure 2.

*Methyl 6-*O*-[N-(1-deoxy-*D*-fructos-1-yl)-*L*-phenylalanyl]-α-*D*-glucopyranoside* (**16**)

D-Glucose (675 mg, 3.7 mmol) was suspended in pyridine–acetic acid (1:1) (75 ml), the suspension was ultrasonicated for 10 min, and then monosaccharide ester **8** (126 mg, 0.37 mmol) was added. The solution was kept in a well-closed round-bottom flask for 24 h at 37°C. After removal of the solvent, the residue was purified by semipreparative RP HPLC using 30% MeOH/0.1% TFA as the eluent (retention time of **16**: 13.4 min) to give pure **16** which was recrystallized from ethanol/diisopropyl ether (100 mg, 44%); mp 75–85°C (decomp.); $[\alpha]_D^{22} + 25°$ (c = 1.0, H_2O). ^{13}C NMR (D_2O) [mixture of β-pyranose (**16** β-*p*), α-furanose (**16** α-*f*) and β-furanose (**16** β-*f*) tautomers]; **16** β-*p*: δ 35.63 (Phe β-C), 53.05 (dFru C-1), 56.09 (MeGlc OCH_3), 61.97 (Phe α-C), 64.77 (dFru C-6), 65.80 (MeGlc C-6), 69.69 (dFru, MeGlc C-5), 70.11 (dFru, MeGlc C-4), 70.84 (dFru C-3), 71.86 (MeGlc C-2), 73.77 (MeGlc C-3), 96.16 (dFru C-2), 100.26 (MeGlc C-1), 129.14 (Phe ζ-C), 130.18 (Phe ε-C), 130.25 (Phe δ-C), 134.31 (Phe γ-C), 169.65 (Phe CO). **16** α-*f*: δ 35.63 (Phe β-C), 50.90 (dFru C-1), 78.89 (dFru C-4), 83.30 (dFru C-5), 83.52 (dFru C-3), 102.26 (dFru C-2) (the chemical shifts of other carbon atoms are identical with those of **16** β-*p*). **16** β-*f*: δ 35.63 (Phe β-C), 52.01 (dFru C-1), 62.39 (dFru C-6) 74.81 (dFru C-4), 76.67 (dFru C-3), 81.90 (dFru C-5), 99.80 (dFru C-2) (the chemical shifts of other carbon atoms are identical with those of **16** β-*p*). Anal calcd. for $C_{22}H_{33}NO_{12} \cdot CF_3COOH$: C, 46.68; H, 5.55; N, 2.27. Found: C, 46.54; H, 5.77; N, 2.48.

*Incubation of methyl 6-*O*-(L-phenylalanyl)-α-*D*-glucopyranoside* (**8**) *in pyridine-acetic acid*

Ester **8** (370 mg, 1.08 mmol) was dissolved in pyridine–acetic acid (1:1) (100 ml) and the solution was kept in a well-closed round bottom flask for 5 days at 37°C. The solvent was evaporated and the residue purified by flash chromatography on silica gel with ethyl acetate–acetone–acetic acid (5:20:0.5) as the eluent to yield methyl 6-*O*-(DL-phenylalanyl)-α-D-glucopyranoside (DL-**8**) (R_f 0.08) (140 mg, 38%), methyl α-D-glucopyranoside (R_f 0.33) (14 mg, 7%) and methyl 6-*O*-(*N*-acetyl-DL-phenylalanyl)-α-D-glucopyranoside (**17**) (R_f 0.51) (63 mg, 15%). For **17**, ^{13}C NMR (DMSO-d_6): δ 22.30 ($C H_3CO$), 36.75, 36.96 (Phe β-C), 54.55 (MeGlc OCH_3), 55.55, 55.76 (Phe α-C), 64.11, 64.25 (MeGlc C-6), 69.71, 69.77 (MeGlc C-5), 70.55, 70.62 (MeGlc C-4), 71.97 (MeGlc C-2), 73.32, 73.38 (MeGlc C-3), 99.96 (MeGlc C-1), 126.59 (Phe ζ-C), 128.42 (Phe ε-C), 129.51 (Phe δ-C), 137.96, 138.08 (Phe γ-C), 172.60, 174.86 (Phe, N-Ac CO). The structure of compound **17** was confirmed by N-acetylation of DL-**8** (10 mg) in water–acetone–acetic anhydride (1:1:0.5) (2.5 ml) for 24 h. Removal of the solvent and purification by flash chromatography with ethyl acetate–acetone–acetic acid (5:20:0.5) as the eluent gave an *N*-acetyl derivative (6 mg, 60%) identical with **17**.

Determination of the optical purity

Samples (1 mg) were hydrolyzed in 6 M HCl (1 ml) at 100°C in an evacuated vial for 24 h. The solvent was removed and the residue dissolved in MeOH–water (1:1) (1 ml). The optical purity of the corresponding amino acid was determined on Chiral Plates (Aldrich) developed with MeOH–water–acetonitrile (1:1:4) and sprayed with ninhydrin.

Results and discussion

Monosaccharide esters **1**–**2**, in which either *N-tert*-butyloxycarbonyl-L-phenylalanine (**1**) or *N*,*O*-di-*tert*-butyloxycarbonyl-L-tyrosine (**2**) was coupled through an ester linkage to the C-6 hydroxy group of the sugar moiety, were obtained by treating D-glucose with amino acid pentachlorophenyl ester in the presence of imidazole as a promoting agent for the transfer of the acyl group to the sugar component [12]. Removal of the N-protecting group from the amino acid residue in esters **1** and

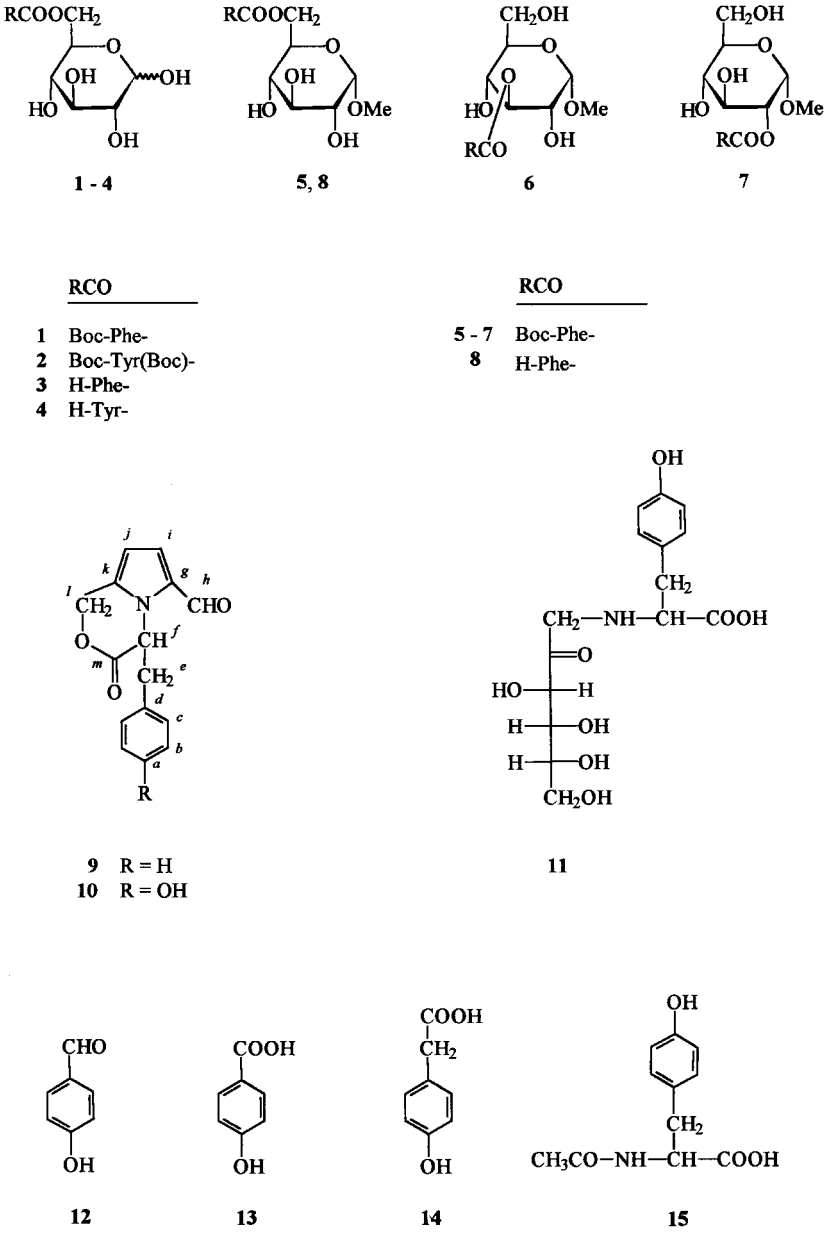


Figure 1. Structures of the monosaccharide esters **1–8** and of the islated compounds **9–15**.

2 resulted in the corresponding 6-*O*-(L-phenylalanyl)- (**3**) and 6-*O*-(L-tyrosyl)-D-glucopyranose (**4**) (Figure 1). The structures of products **1**–**4** were confirmed by NMR analysis. The ^{1}H and ^{13}C chemical shifts data of the unprotected esters **3** and **4** are given in Tables 1 and 2.

In contrast to the regioselectivity observed in the reaction with D-glucose, the esterification of Boc-Phe-OPCP with methyl α-D-glucopyranoside under identical reaction condition afforded, as evidenced by NMR analysis, ester derivatives **5**, **6** and **7** (Figure 1) in a 61:14:25 ratio. After purification by column chromatography the major product, 6-*O*-ester **5**, was isolated in a yield of 28%, whereas the minor components, 3-*O*-ester **6** and 2-*O*-ester **7**, were obtained in 2% and 10% yield, respectively. Removal of the Boc-protecting group from the phenylalanine residue in monosaccharide ester **5** resulted in the desired methyl 6-*O*-(L-phenylalanyl)-α-D-glucopyranoside (**8**). The identity of the three isomeric esters **5**–**7** as well as of unprotected compound **8** was confirmed by NMR. The chemical shift data in DMSO-d_6 solution are summarized in Tables 1 and 2. Comparison of ^{1}H chemical shifts for **5**–**7** (Table 1) revealed a significant downfield shift (~0.5 ppm) for the proton(s) attached to the esterified carbon atom, accompanied by smaller downfield shifts (~0.2 ppm) for the neighbouring protons. In the ^{13}C NMR spectra (Table 2), the resonances of either C-6, C-3 or C-2 in esters **5**–**7** displayed characteristic downfield shifts (5–6 ppm), owing to the

deshielding effect of the aminoacyl residue, while the neighbouring carbon atoms were shifted upfield.

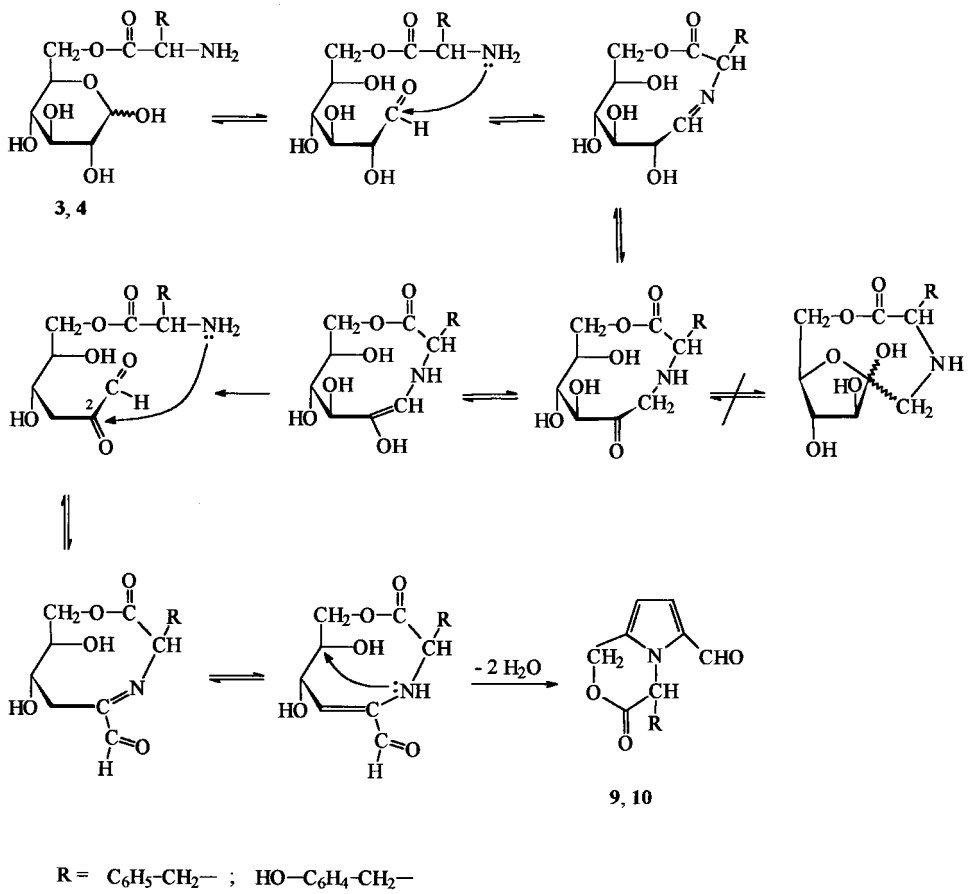
The sequence of non-enzymatic glycation involves the reaction of the open-chain form of a reducing sugar with free amino groups of proteins (Maillard reaction) leading initially to the formation of labile Schiff bases. With the subsequent complex acid-base catalysed Amadori rearrangement [for review see Ref. 13], the aminoketoses formed undergo a variety of reactions producing reactive carbonyl compounds which continue to react with free amino groups, leading to cross-linking of proteins *via* AGE products [14]. In previous publications [15,16] we found that pyridine-acetic acid appears to be an ideal solvent for the Amadori rearrangement, catalysing all transformation steps to the keto sugar derivatives. Thus, monosaccharide esters **3** and **4** were incubated in pyridine–acetic acid (1:1) at 50°C to assess the reactivity of the free NH_2 group in either the phenylalanyl or the tyrosyl residue. As evidenced by RP HPLC, after 24 h of incubation, the abundance of degradation products exceeded 90% in both **3** and **4**, indicating a high reactivity for the amino acid esters studied.

From the numerous reaction products formed from **3**, it was possible using RP HPLC semipreparative chromatography, to isolate pure 2-(5'-hydroxymethyl-2'-formylpyrrol-1'-yl)-3-phenyl-propionic acid lactone (**9**) (Fig. 1) as a major product in 11% yield. Incubation of ester **4** at 50°C for 24 h gave also, after RP HPLC isolation, lactone **10** (5%) as a major rearrangement product. The lactones **9** and **10** were characterized by NMR spectroscopy. The assignment of the signals was made by comparison with those reported for lactone **9** in $CDCl_3$ [17] as well as by using COSY and HETCOR experiments. The ^1H and ^{13}C chemical shift values of compounds **9** and **10** are summarized in Table 3. Dickerson *et al.* [18] first identified pyrrololactone **9**, isolated in 1% yield from a roasted mixture of phenylalanine and D-glucose. Although two reaction pathways are possible for such pyrrololactones, it was suggested [17] that compound **9** is formed by reaction of phenylalanine with 3-deoxyhexosone, a reactive carbonyl compound formed by degradation of Amadori products, and not with hydroxymethylfurfural. Considering the formation of lactones **9** and **10** from monosaccharide esters **3** and **4**, the question arises whether the ester bond is hydrolysed before or after the intramolecular reaction leading to the Amadori product, or remains intact during all rearrangement steps to lactones **9** and **10**. Since, under identical reaction conditions, hydrolysis of the anomerically protected monosaccharide ester **8** did not exceed 10%, and the formation of **10** in a model reaction of L-tyrosine with D-glucose (see discussion below) is less favored as compared to **4**, we assume that hydrolysis of the ester bond in the course of the reactions leading to lactones **9** and **10** is not of major significance. A possible mechanism is proposed in presented Scheme 1. Initially, the aldehyde group of the open-chain of the monosaccharide ester is intramolecularly attacked by the free amino terminus of the amino acid moiety and the Schiff base formed rearranges to the corresponding keto sugar or Amadori product. In contrast to the intramolecular rearrangements of the monosaccharide esters of the leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) peptide yielding the stable Amadori products under identical reaction conditions [15], the unstable cyclic keto sugar rearranges by enolization and β-elimination further to the reactive 3-deoxyhexosone amino acid ester. For steric reasons, the nucleophilic attack of the free NH_2 group of the esterified amino acid residue at the 2-carbonyl group of the sugar residue is facilitated resulting, after dehydration, in the corresponding lactones **9** and **10**.

In addition to lactone **10**, obtained from the reaction mixture by incubation of the tyrosine-related monosaccharide ester **4** (RP HPLC chromatogram presented in Fig. 2), it was possible to isolate, after repeated HPLC chromatography, in less than 1% yield each, *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosine (**11**), 4-hydroxybenzaldehyde (**12**), 4-hydroxybenzoic acid (**13**), 4-hydroxyphenylacetic acid (**14**) and *N*-acetyl-tyrosine (**15**) (Fig. 1). The structures of compounds **11**–**15** were established by NMR spectroscopy, by chemical synthesis and/or by comparison with commercially available compounds. The isolation of Amadori product **11** indicates ring opening in **4** and intramolecular attack of the free NH_2 group, followed by rearrangement to the keto sugar and ester bond hydrolysis. Acid **14** is the oxydation product of 4-hydroxyphenylacetaldehyde, formed by Strecker degradation of the tyrosine adduct with α-dicarbonyl compounds.

4-Hydroxybenzaldehyde **12**, and the corresponding acid **13**, presumably also arose from the reactive 4-hydroxyphenylacetaldehyde. It should be noted that benzaldehyde was also found in low yield in the specific products obtained after boiling phenylalanine with D-glucose [17]. The mechanism of the formation of *N*-acetyl derivative **15** from ester **4** is not obvious. The detection of **15** in the tyrosine/D-glucose incubation mixture (see below) suggests a process that initially involves condensation of the amino acid with the 2-carbonyl group in the 1-deoxy-2,3-hexodiulose formed by rearrangement of the Amadori compound. Another reasonable mechanism which might be considered for **4** → **15** conversion is the possibility of nucleophilic attack by the acetate ion, present in the incubation solvent, on the tyrosine carbonyl carbon atom, activated by esterification with the monosaccharide moiety. The mixed anhydride formed produces, as a by-product, the *N*-acetyl derivative of the present amino component as previously postulated by Bodanszky [19].

In order to gain better understanding of the effect imposed by esterification of the amino acid with a carbohydrate moiety on the reactivity of the amino group, ester **4** as well as L-tyrosine and D-glucose were incubated separately in pyridine–acetic acid at 50°C for 24 h. The relative intensities of the HPLC peaks clearly show (Fig. 3) that the formation of lactone **10**, compounds **12**–**14** as well as *N*-acetyl-tyrosine **15** from ester **4** is favored in comparison with the formation of these products from D-glucose and tyrosine. Even longer incubation time (48 h) did not change the amounts of products formed in the Tyr/Glc model system.



Scheme 1.

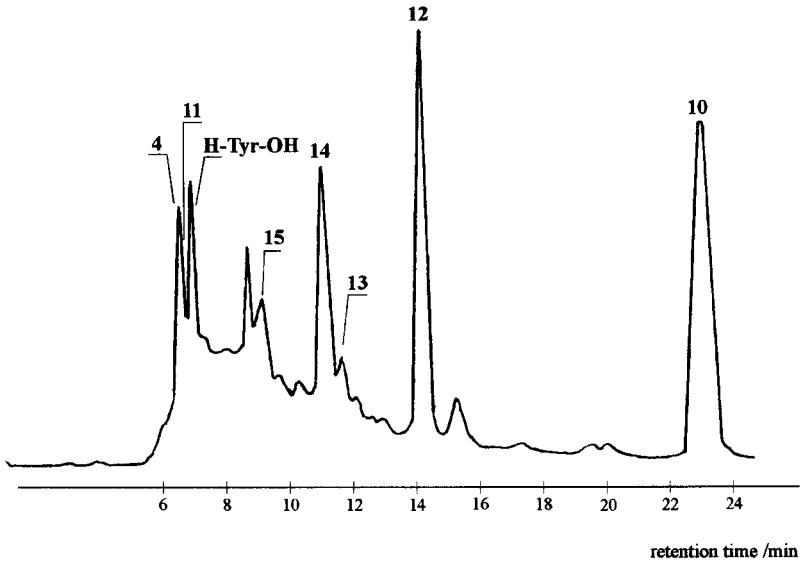


Figure 2. RP HPLC elution profile of the products formed by incubation of ester **4** in pyridine-acetic acid for 24 h at 50°C (for RP HPLC conditions see Materials and methods).

We next examined the reactivity of L-phenylalanine-related monosaccharide ester **8**. Owing to the fact that the anomeric position of the monosaccharide moiety in **8** is protected, an intramolecular nucleophilic attack of the amino group on C-1 is not possible. Thus, the reactivity of ester **8** was tested in the presence of D-glucose in pyridine-acetic acid. The temperature dependence of the reaction between **8** and reducing sugar was checked at 4, 37 and 50°C for 24 h. As evidenced by RP HPLC,

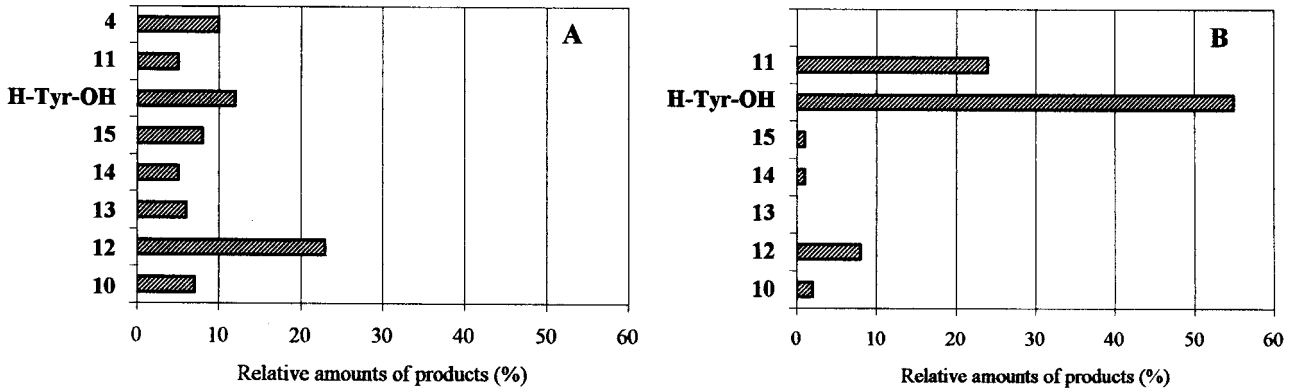


Figure 3. Relative amounts of products formed by incubation of compound **4** (A) and D-glucose with L-tyrosine (B) in pyridine-acetic acid for 24 h at 50°C. The relative concentrations were determined by RP HPLC (see Materials and methods).

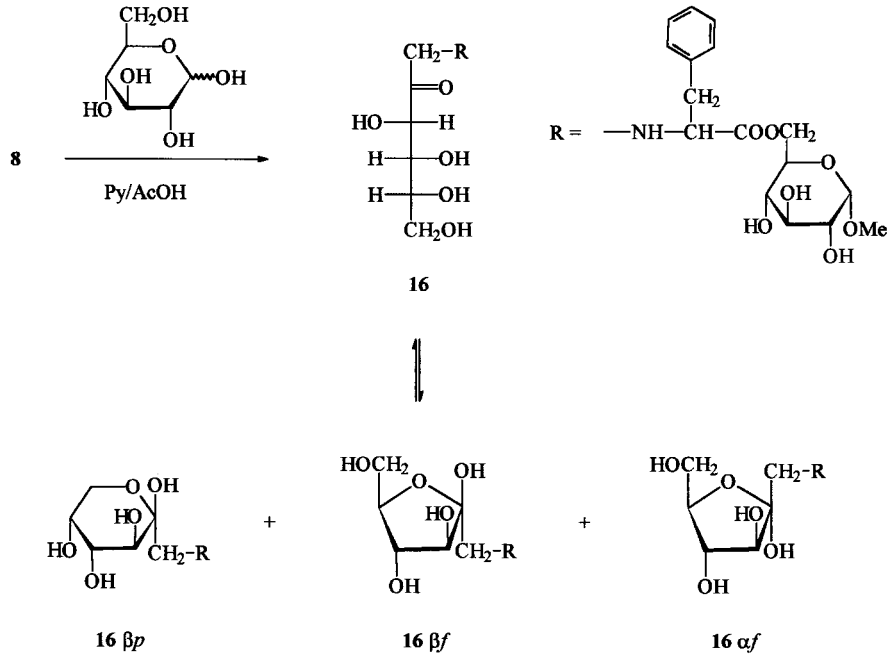


Figure 4. Synthesis of Amadori compound **16** from methyl 6-*O*-(L-phenylalanyl)-α-D-glucopyranoside (**8**).

at 4°C only starting ester **8** could be detected, whereas at 37°C product **16** was formed. Incubation at 50°C gave, in addition to **16**, a heterogenous mixture of products. Treatment of **8** with ten equivalents of D-glucose in pyridine-acetic acid at 37°C for 24 h gave, after isolation by semipreparative RP HPLC, product **16** in 44% yield. By using NMR spectroscopy compound **16** was identified as methyl 6-*O*-[*N*-(1-deoxy-D-fructos-1-yl)-L-phenylalanyl]-α-D-glucopyranoside (Fig. 4). It is postulated that **16** results form condensation of the phenylalanine amino group in **8** with the open-chain form of D-glucose and subsequent Amadori rearrangement to a keto sugar derivative. The ^{13}C NMR spectrum of a solution of compound **16** in D_2O revealed that, with regard to the 1-deoxy-D-fructosyl moiety, the β-pyranose form is the major tautomer (69%), whereas the α-furanose and β-furanose forms (Figure 4) account for 16 and 15%, respectively. No signals were observed which could be attributed to either the α-pyranose or to the open-chain form of the keto sugar residue in **16**.

Interestingly, heating of monosaccharide ester **8** in pyridine-acetic acid at 37°C for 5 days, in the absence of D-glucose, resulted in extensive racemization of the phenylalanyl residue. Chromatography of the reaction mixture gave DL-**8** in 38% isolated yield, methyl 6-*O*-(*N*-acetyl-DL-phenylalanyl)-α-D-glucopyranoside (**17**) in 15%, as well as methyl α-D-glucopyranoside in 7% yield. The structure of *N*-acetyl derivative **17** was proven by NMR spectroscopy and by chemical synthesis from ester **8**. Formation of the *N*-acetyl product **17** indicates that esterification of the amino acid carboxyl group led to activation

and rendered the carbonyl group susceptible to N-acetylation through a mixed anhydride intermediate.

In conclusion, the model studies in this paper are the first to describe the reactivity of the compounds in which anomerically free or protected D-glucose is linked *via* its C-6 hydroxy group to an amino acid moiety through an ester bond, as a model for D-alanine esterified teichoic acid fragment. We demonstrated an activating effect of the sugar substituent on the amino acid residue resulting in complex mixtures of products generated by intra- and intermolecular nucleophilic attacks of the amino group on the available carbonyl compounds. Taken together, our findings suggest that the D-alanine present in teichoic acids of Gram-positive bacteria could be considered as the participant in molecular recognition processes by producing bioactive species (chemical messengers) capable of altering the properties of the host cells.

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