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Glycosylation of vanillin by amyloglucosidase in organic media

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Abstract—Glycosylation of vanillin using amyloglucosidase from a *Rhizopus* mold with D-glucose, D-galactose, D-mannose, maltose, sucrose and D-sorbitol in di-isopropyl ether yielded glycosides in the range 13–53%. NMR spectral data confirmed linking between the phenolic OH of vanillin and C1 and/or C6 of the carbohydrate moieties.

Vanillin **1** (4-hydroxy-3-methoxybenzaldehyde)[1] is used as an additive in food and beverages (60%), considerable amounts as flavour and fragrances (20–25%) and 5–10% as an intermediate for pharmaceuticals. It possesses a wide range of pharmacological activities such as antimicrobial,[2] anticarcinogenic,[3] antioxidant,[4] antifungal[5] and antimutagenic.[6] The solubility of vanillin in water varies from 3 g/l at 4.4 °C to 62.5 g/l at 80 °C.[7] Thus the solubility and bioavailability of **1** limits its pharmacological applications. Glycosylation is a useful tool to improve the water solubility and bioavailability[8,9] of vanillin.

The preparation of vanillin glycosides has been reported by cell suspension culture,[9] chemical[10] and plant cell tissue and organ culture methods.[11] However, preparations by enzymatic methods have not been previously reported. The present work describes an enzymatic method using amyloglucosidase from a *Rhizopus* mold for the preparation of glycosides with mono- and disaccharides in a non-polar solvent.

A typical synthesis involved reacting **1** (0.0005–0.0025 mol) and a mono- or disaccharide (**2**–**7**, 0.0005–0.0025 mol) at reflux with stirring in 100 ml of di-isopropyl ether in the presence of 10–80% (by weight of **2**–**7**) amyloglucosidase and 0.1–1.0 ml of 10 mM of pH 4.0–8.0 buffer for a period of 72 h. Refluxing di-isopropyl ether for 72 h did not produce any peroxides. Work-up involved distilling off the solvent and maintaining the reaction mixture at boiling water temperature for 5–10 min to denature the enzyme. The residue was repeatedly extracted with chloroform to remove unreacted **1** and the dried residue, consisting of vanillin glycoside and unreacted mono- or disaccharide, was subjected to HPLC analysis on an amino-propyl column (3.9 × 300 mm length), eluted with 80:20 (v/v) acetonitrile/water with a flow rate of 1 ml/min and monitoring with a RI detector. Conversion yields were determined from HPLC peak areas of the glycoside and free mono- or disaccharides and expressed with respect to mono- or disaccharide concentrations. Errors based on the HPLC measurements were of the order ±10%. Glycosides were isolated using a Sephadex G25 (100 cm × 1 cm) column, eluting with water and subjected to spectroscopic characterization.

Glycosylation of **1** was carried out with the following mono- and disaccharides: pentoses—D-ribose and D-arabinose; hexoses—D-glucose **2**, D-galactose **3** and D-mannose **4**; ketoses—D-fructose; and disaccharides—maltose **5**, lactose and sucrose **6**; carbohydrate alcohols—D-mannitol and D-sorbitol **7**. Amyloglucosidase exhibited maximum activity in non-polar solvents containing a certain minimum amount of water to stabilize its active conformation in a non-polar solvent compared to other enzymes.[12,13] Hence, glycosylation of **1** was always carried out in the presence of buffers of certain pH and concentration, worked out from a series of reactions conducted with different pH, buffer concentrations, substrate concentration, amyloglucosidase concentration and incubation period. Optimized reaction conditions for the preparation of vanillin glucoside at equimolar concentrations of **1** and **2** were found to be

Keywords: Amyloglucosidase; Disaccharides; Glycosylation; Monosaccharides; Organic media; Vanillin glycosides.
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Table 1. Synthesis of vanillin glycosides with mono- and disaccharides (**2**–**7**)[a]

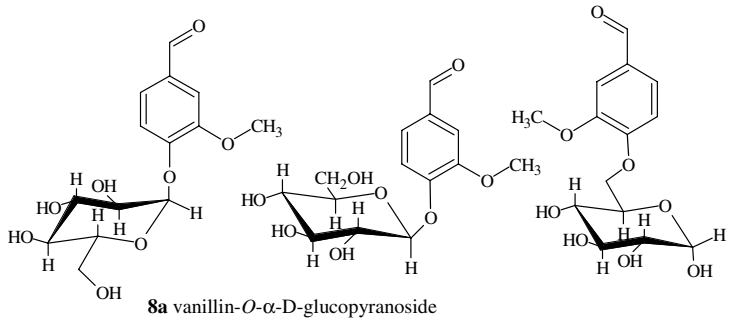
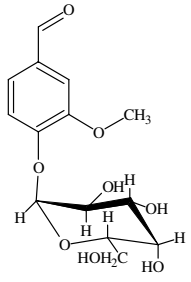
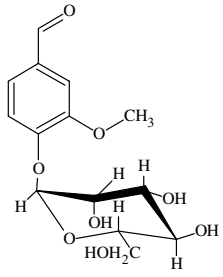
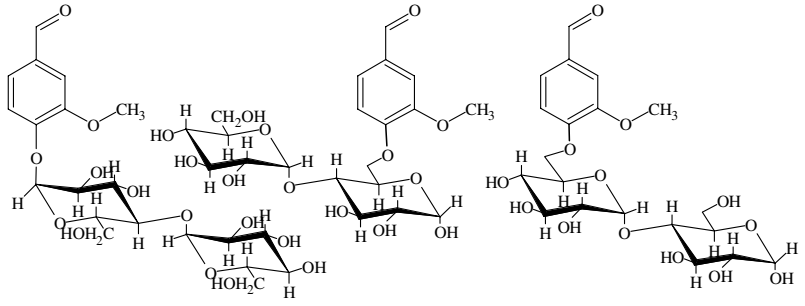
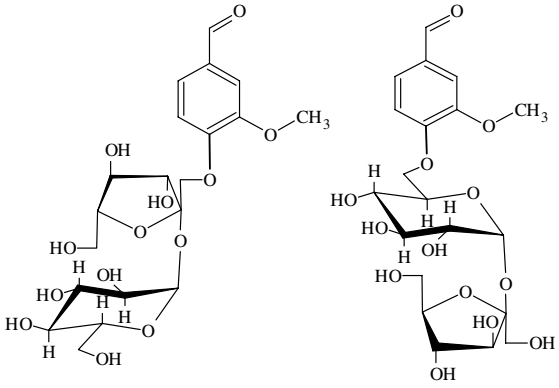
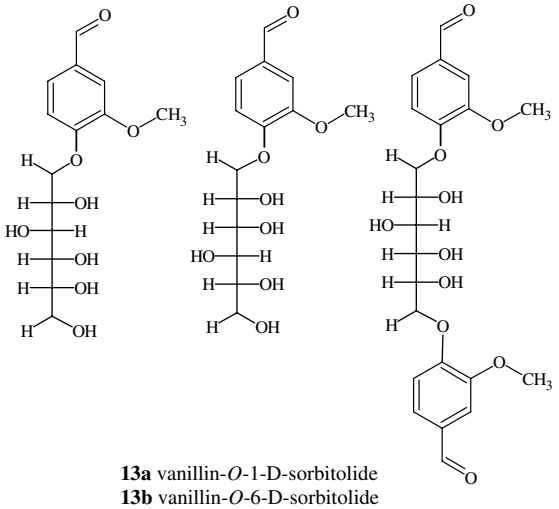
Glycosides	Glycosylation yield[b] (%)	Product (% proportion[c])
 **8a** vanillin-*O*-α-D-glucopyranoside **8b** vanillin-*O*-β-D-glucopyranoside **8c** vanillin-*O*-6-α-D-glucopyranoside	53	C1α (52), C1β (17) and C6α (31) mono-glucosides
 **9** vanillin-*O*-α-D-galactopyranoside	18	C1α galactoside
 **10** vanillin-*O*-α-D-mannopyranoside	13	C1α mannoside
 **11a** vanillin-*O*-α-maltopyranoside **11b** vanillin-*O*-6-α-maltopyranoside **11c** vanillin-*O*-6″-α-maltopyranoside	29	C1 (42), C6 (30) and C6″ (28) mono-maltosides

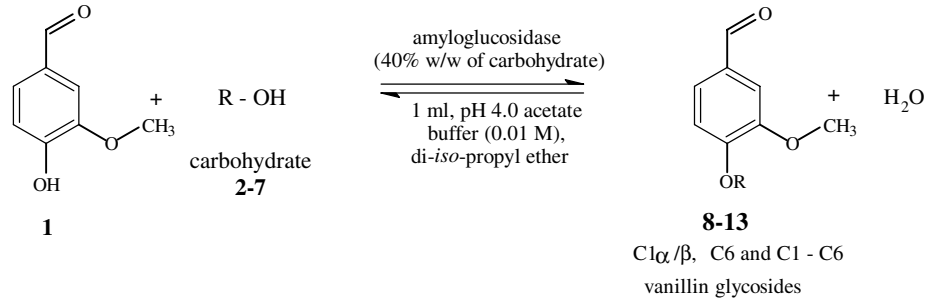
Table 1 (*continued*)

Glycosides	Glycosylation yield[b] (%)	Product (% proportion[c])
 **12a** vanillin-*O*-1-sucroside **12b** vanillin-*O*-6″-sucroside	23	C1 (39) and C6″ (61) mono-sucrosides
 **13a** vanillin-*O*-1-D-sorbitolide **13b** vanillin-*O*-6-D-sorbitolide **13c** vanillin-*O*-1,6-di-D-sorbitolide	13	C1 (13), C6 (25) mono- and C1–C6 (62) di-sorbitolides

[a] **1** and **2**–**7**—1 mmol each; enzyme concentration 40% w/w of **2**–**7**; incubation period—72 h; solvent—di-isopropyl ether; temperature—68 °C.
[b] Conversion yields were from HPLC with respect to free **2**–**7**. Error in yield measurements is ±10%.
[c] The product proportions were calculated from the area of respective carbon signals.

40% (w/w D-glucose **2**) of amyloglucosidase, 1.0 ml of 0.01 M pH 4.0 acetate buffer (corresponding to a concentration of 0.01 mM buffer in 100 ml solvent), and 72 h incubation period in di-isopropyl ether at 68 °C. A maximum yield of 53% was obtained for a mixture of three mono-glucosides (Table 1): vanillin-*O*-α-D-glucopyranoside **8a**, vanillin-*O*-β-D-glucopyranoside **8b** and vanillin-6-*O*-α-D-glucopyranoside **8c** (Scheme 1 and Table 1).

The vanillin glycosides (Table 1) were subjected to two-dimensional HSQCT NMR spectroscopy on a Bruker 500 MHz instrument. The glycosides were also tested for angiotensin converting enzyme inhibitory activities[14] and the IC_{50} values are also shown along with the spectral data.[15] Vanillin glycosides are surfactant molecules, which form micelles above certain critical micellar concentrations (CMC). Since the concentrations employed for NMR measurements are very much higher than their respective CMCs, the proton NMR signals were unusually broad such that, in spite of recording the spectra at 35 °C, the individual coupling constant values could not be determined precisely. Also, the carbon signals from tertiary carbon atoms could not be unambiguously assigned. In the case of vanillin-glucosides **8**, the downfield chemical shift value for C1α at 99.2 ppm, C1β at 101.5 ppm and C6 at 68.0 ppm and the corresponding protons at 4.65, 4.94 and 3.55 ppm indicated that vanillin formed three mono-glucosides with D-glucose at C1α, C1β and C6.[15] With D-galactose, only the α anomer reacted with vanillin, the ^{13}C chemical shift value for C1α in **9** being 95.8 ppm (^{1}H at 4.22 ppm). D-Mannose was also glycosylated (**10**) at C1α (100.8 ppm). Maltose, a disaccharide, was converted into three mono-glycosides **11** with signals at C1α (98.2 and 4.68 ppm), C6α (reducing end—67.2 and 3.54 ppm) and C6″α (non-reducing end—66.1 and 3.69 ppm). In the product from sucrose, the signals for the C1 (66.0



Scheme 1. Synthesis of vanillin glycosides.

and 3.49 ppm) position of the fructose moiety, and for the C6″ (66.1 and 3.72 ppm) position of the glucose moiety showed these to have been glycosylated in the two products **12**. NMR data also clearly showed that hydrolysis of sucrose to glucose and fructose had occurred, and the resulting glucose had glycosylated vanillin at C1β (101.5 and 4.94 ppm) and C6 (68.0 and 3.55 ppm). Maltose was not hydrolyzed. D-Sorbitol, the open chain sugar alcohol, gave **13**, comprising two mono-glycosides at C1 (67.2 and 3.65 ppm), C6 (66.2 and 3.58 ppm) and one di-glycoside at the C1 and C6 positions (66.5, 3.46–65.5, 3.65 ppm, respectively).

D-Ribose, D-arabinose, D-fructose, D-mannitol and lactose did not form glycosides perhaps because the required oxo-carbenium ion intermediate[16] was not formed during the catalytic conversion by amyloglucosidase. Although the D-glucose we employed was an α,β-anomeric mixture (40:60), the glycosides formed showed predominant proportions of the α anomer (>80%), indicating the potential for 'inverting' amyloglucosidase[16] (from *Rhizopus* mold) to convert the majority of β-D-glucose into its respective α-glycoside.

Acknowledgements

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15. The synthesized glycosides were characterized spectroscopically. Only resolvable signals are shown. Some assignments are interchangeable. Vanillin signals are primed; non-reducing end sugar units are double primed. **8a–c**: UV (H_2O, λ_{max}): 195.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{195.5}$—2241 M^{-1}), 279.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{279.5}$—291 M^{-1}), IR (stretching frequency): 3358 cm^{-1} (OH), 1260 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1030 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1408 cm^{-1} (C=C), 1636 cm^{-1} (CO), 2933 cm^{-1} (CH), optical rotation (*c* 1, H_2O): $[\alpha]_D$ at 25 °C = +62.8, ACE activity: IC_{50} value = 2.46 μmol, 2D-HSQCT (DMSO-d_6) C1α-glucoside (**8a**): ^1H NMR δ_{ppm}: (500.13 MHz): 4.65 (H-1α, 5.5 Hz), 3.23 (H-2α), 3.42 (H-3α), 3.78 (H-4α), 3.15 (H-5α), 3.60 (H-6α), ^{13}C NMR δ_{ppm} (125 MHz): 99.2 (C1α), 72.3 (C2α), 73.5 (C3α), 70.2 (C4α), 72.5 (C5α), 60.5 (C6α); vanillin (mono-glucoside **8a**): ^1H NMR δ_{ppm} 6.59 (H-2′), 6.20 (H-5′), 3.73 (OCH_3), ^{13}C NMR δ_{ppm}: 111.4 (C2′), 114.5 (C5′). C1β-glucoside (**8b**): ^1H NMR: 4.94 (H-1β, 5.3 Hz), 2.98 (H-2β), 3.22 (H-3β), 3.68 (H-6β). ^{13}C NMR: 101.5 (C1β), 74.6 (C2β), 76.1 (C3β), 60.8 (C6β), C6-glucoside (**8c**): ^1H NMR: 4.91 (H-1α), 3.23 (H-2α), 3.20 (H-3α), 3.62 (H-4α), 3.23 (H-5α), 3.55 (H-6α), ^{13}C NMR: 92.7 (C1α), 72.3 (C2α), 72.6 (C3α), 70.2 (C4α), 75.2 (C5α), 68.0 (C6α); **9**: UV (H_2O, λ_{max}): 198.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{198.0}$—2909.2 M^{-1}), 281.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{281.0}$—517.9 M^{-1}), IR (stretching frequency): 3271 cm^{-1} (OH), 1261 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1031 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1406 cm^{-1} (C=C), 1664 cm^{-1}(CO), optical rotation (*c* 1, H_2O), $[\alpha]_D$ at 25 °C = +8.82, ACE activity:IC_{50} value = 2.36 μmol, 2D-HSQCT (DMSO-d_6):C1α-glycoside:^1H NMR: 4.22 (H-1α), 3.69 (H-2α), 3.52 (H-3α), 3.48 (H-4α), 3.43 (H-5α), 3.35 (H-6α); ^{13}C NMR: 95.8 (C1α), 69.4 (C2α), 69.9 (C3α), 70.8 (C4α), 71.1 (C5α), 62.0 (C6α); vanillin (mono-galactoside):^1H NMR: 7.38 (H-2′), 6.90 (H-5′), 7.33 (H-6′), 3.86 (OCH_3), 9.74 (CHO), ^{13}C NMR: 129.2 (C1′),

111.3 (C2′), 148.6 (C3′), 153.5 (C4′), 115.9 (C5′), 126.4 (C6′), 56.1 (OCH_3), 191.4 (CHO); **10**:UV (H_2O, λ_{max}):198.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{198.5}$—3401.6 M^{-1}), 278.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{278.0}$—284.4 M^{-1}), IR (stretching frequency): 3365 cm^{-1} (OH), 1249 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1030 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1406 cm^{-1} (C=C), 1651 cm^{-1} (CO), 2940 cm^{-1} (CH), optical rotation (c 1, H_2O): $[\alpha]_D$ at 25 °C = −3.6, ACE activity:IC_{50} value = 2.31 μmol, C1α-glycoside:^{13}C NMR (DMSO-d_6):100.8 (C1α), 70.5 (C2α), 71.3 (C3α), 67.1 (C4α), 73.8 (C5α), 61.3 (C6α); vanillin (mono-mannoside):109.4 (C2′), 114.72 (C5′), 121.9 (C6′); **11a–c**:UV (H_2O, λ_{max}):194.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{194.5}$—4782.4 M^{-1}), 278.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{278.5}$—327.8 M^{-1}), IR (stretching frequency):3361 cm^{-1} (OH), 1265 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1024 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1412 cm^{-1} (C=C), 1651 cm^{-1} (CO), 2930 cm^{-1} (CH), 1205 cm^{-1} (OCH_3), optical rotation (c 1, H_2O): $[\alpha]_D$ at 25 °C = +92.0, ACE activity:IC_{50}value = 3.65 μmol, 2D-HSQCT (DMSO-d_6): C1α-glycoside (**11a**): ^{1}H NMR: 4.94 (H-1″α, 5.7 Hz), 4.68 (H-1α), 3.25 (H-2″α), 3.10 (H-2α), 2.88 (H-3″α), 3.20 (H-3α), 3.65 (H-4″α), 3.30 (H-4α), 3.72 (H-5α), 3.60 (H-6″α), 3.48 (H-6α); ^{13}C NMR: 100.3 (C1″α), 98.2 (C1α), 73.8 (C2″α), 70.1 (C2α), 74.5 (C3″α), 75.1 (C3α), 70.0 (C4″α), 79.1 (C4α), 69.8 (C5α), 60.8 (C6″α), 60.8 (C6α); vanillin (mono-maltoside,**11a**): ^{1}H NMR: 6.26 (H-2′), 6.62 (H-5′), 7.18 (H-6′), 3.73 (OCH_3), ^{13}C NMR: 130.0 (C1′), 109.5 (C2′), 114.2 (C5′), 126.8 (C6′); C6-glycoside (**11b**): ^{1}H NMR: 4.94 (H-1″α), 4.88 (H-1α), 3.54 (H-6α), ^{13}C NMR: 100.3 (C1″α), 92.4 (C1α), 67.2 (C6α); C6″-glycoside (**11c**): ^{1}H NMR: 4.94 (H-1″α), 4.88 (H-1α), 3.69 (H-6″α), ^{13}C NMR: 100.3 (C1″α), 92.4 (C1α), 66.1 (C6″α); **12a**,**b**: UV (H_2O, λ_{max}): 194.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{194.0}$—6820.8 M^{-1}), 278.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{278.5}$—423.4 M^{-1}), IR (stretching frequency): 3374 cm^{-1} (OH), 1254 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1026 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1412 cm^{-1} (C=C), 1650 cm^{-1} (CO), 2936 cm^{-1} (CH), 1211 cm^{-1} (OCH_3), optical rotation (c 1, H_2O): $[\alpha]_D$ at 25 °C = +48.6, ACE activity: IC_{50} value = 35.3 μmol, 2D-HSQCT (DMSO-d_6): C1-glycoside (**12a**): ^{1}H NMR: 4.72 (H-1″α), 3.68 (H-2″α), 3.46 (H-3″α), 3.62 (H-4″α), 3.65 (H-5″α), 3.59 (H-6″α), 3.49 (H-1), 3.88 (H-3), 3.89 (H-4), 3.86 (H-5), 3.4 (H-6); ^{13}C NMR: 98.5 (C1″α), 71.0 (C2″α), 72.2 (C3″α), 69.8 (C4″α), 72.1 (C5″α), 60.5 (C6″α), 66.0 (C1), 76.8 (C3), 80.9 (C4), 81.5 (C5), 62.2 (C6); vanillin (mono-sucroside **12a**): ^{1}H NMR: 7.22 (H-2′), 6.60 (H-5′), 8.35 (H-6′), ^{13}C NMR: 112.8 (C5′), 126.3 (C6′). C6″-Glycoside (**12b**): ^{1}H NMR: 4.63 (H-1″α), 3.08(H-2″α), 3.42 (H-3″α), 3.15 (H-4″α), 3.19 (H-5″α), 3.72 (H-6″α), 3.48 (H-1), 3.67 (H-3), 3.57 (H-5), 3.46 (H-6), ^{13}C NMR: 98.6 (C1″α), 69.9 (C2″α), 72.3 (C3″α), 69.8 (C4″α), 71.5 (C5″α), 66.1 (C6″α), 62.3 (C1), 76.5 (C3), 82.2 (C5), 60.5 (C6); **13a–c**: UV (H_2O, λ_{max}): 193.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{193.5}$—2939.9 M^{-1}), 273.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{273.0}$—289.8 M^{-1}), IR (stretching frequency): 3386 cm^{-1} (OH), 1260 cm^{-1} (glycosidic aryl alkyl C–O–C asymmetric), 1038 cm^{-1} (glycosidic aryl alkyl C–O–C symmetric), 1409 cm^{-1} (C=C), 2943 cm^{-1} (CH), optical rotation (c 1, H_2O): $[\alpha]_D$ at 25 °C = +13.9, ACE activity: IC_{50} value = 1.79 μmol, 2D-HSQCT (DMSO-d_6): C1-glycoside (**13a**): ^{1}H NMR: 3.65 (H-1), 3.37 (H-2), 3.48 (H-3), 3.57 (H-4), 3.54 (H-5), 3.58 (H-6); ^{13}C NMR: 67.2 (C1), 70.5 (C2), 74.1 (C3), 71.2 (C4), 69.0 (C5), 62.9 (C6); vanillin (mono-sorbitolide **13a**): ^{1}H NMR: 7.40 (H-2′), 7.20 (H-5′), 7.58 (H-6′), 3.81 (OCH_3), 9.75 (CHO), ^{13}C NMR: 130.5 (C1′), 111.2 (C2′), 153.8 (C4′), 111.1 (C5′), 124.5 (C6′), 55.9 (OCH_3), 191.5 (CHO). C6-Glycoside (**13b**): ^{1}H NMR: 3.55 (H-1), 3.54 (H-2), 3.44 (H-3), 3.68 (H-4), 3.46 (H-5), 3.58 (H-6); ^{13}C NMR: 63.2 (C1), 70.8 (C2), 72.5 (C3), 73.1 (C4), 68.2 (C5), 66.2 (C6); vanillin (mono-sorbitolide **13b**): ^{1}H NMR: 6.88 (H-2′), 6.85 (H-5′), 7.39 (H-6′), ^{13}C NMR: 129.1 (C1′), 110.8 (C2′), 153.8 (C4′), 111.4 (C5′), 126.1 (C6′). C1–C6 Di-glycoside (**13c**): ^{1}H NMR: 3.46 (H-1), 3.35 (H-2), 3.36 (H-3), 3.54 (H-4), 3.65 (H-6); ^{13}C NMR: 66.5 (C1), 67.0 (C2), 73.5 (C3), 76.1 (C4), 65.5 (C6); vanillin (di-sorbitolide **13c**): ^{1}H NMR: 6.65, 6.68 (H-5′), 6.69, 6.84 (H-6′), ^{13}C NMR: 129.1, 130.5 (C1′), 111.3, 111.7 (C2′), 153.5, 153.5 (C4′), 115.3, 115.9 (C5′), 119.8, 120.3 (C6′).

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