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Regioselective esterase-catalyzed feruloylation of L-arabinobiose

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Abstract—The regioselective chemoenzymatic synthesis of *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→5)-L-arabinofuranose has been achieved. The reaction parameters affecting the feruloylation rate and conversion of the enzymatic synthesis, such as the composition of the reaction medium, substrate and enzyme concentration, have been investigated.

Keywords: Feruloyl esterase; Enzymatic transesterification; Regioselectivity; Feruloyl oligosaccharide; Detergentless microemulsions

1. Introduction

Ferulic acid esterases (FAE, E.C. 3.1.1.73) are a subclass of carboxylic acid esterases that catalyze the hydrolysis of the ester bond between hydroxycinnamic acids and sugars in the plant cell walls. Most of the FAEs have been purified and partially characterized from fungi,[1–6] a small number from bacteria[7,8] and recently, for the first time, from human intestinal bacteria.[9] Cereal grains have also been shown to contain feruloyl esterase activity, which decreases upon germination.[10]

Feruloyl esterases have been classified into four types (A–D) based on their specificity towards mono- and diferulates, for substitutions on the phenolic ring, and on their amino acid sequence identity.[11] The nomenclature of feruloyl esterases follows both the source of the enzyme and the type of feruloyl esterases (e.g., the type-C feruloyl esterase from *Sporotrichum thermophile* is termed StFaeC).

In spite of numerous reports on esterases and lipases acting as biosynthetic catalysts, enzymatic esterification of phenolic acids has been rarely reported, although in general, enzymatic esterification offers an alternative to the poor selectivity of the chemical synthesis.[12] Recently, the potential use of ternary water–organic solvent mixtures,[3–6,13] or oil-in-water microemulsions[14] as a reaction system for the esterification or transesterification of various cinnamic acids catalyzed by feruloyl esterases has been reported. Organic–water mixtures called surfactantless microemulsions, formed in ternary systems consisting of a hydrocarbon, short chain alcohol and water, represent thermodynamically stable and optically transparent dispersions of aqueous microdroplets in the hydrocarbon solvent and have been already shown to serve as appropriate media for enzymatic catalysis.[15,16] An important advantage of these mixtures as reaction media is that they allow the problem of separation of reaction products and enzyme reuse to be solved easily,[15] while the solubility of relatively polar phenolic acids is high due to the presence of large amount of polar alcohol.

Several authors demonstrated that the lipase-inhibiting effect of electron-donating substituents, conjugated to the carboxylic groups in hydroxylated derivatives of cinnamic acids, is strong.[17] For example, the direct esterification of natural phenolic acids including various hydroxycinnamic acids (ferulic, *p*-coumaric, caffeic, sinapinic) with aliphatic alcohols catalyzed by various lipases in organic media has been reported, but the reaction rates and yields were low.[18] Esterification was shown to take place only if the aromatic cycle is not *p*-hydroxylated and the lateral chain is saturated. Therefore, enzymatic esterification of cinnamoyl substrates can be

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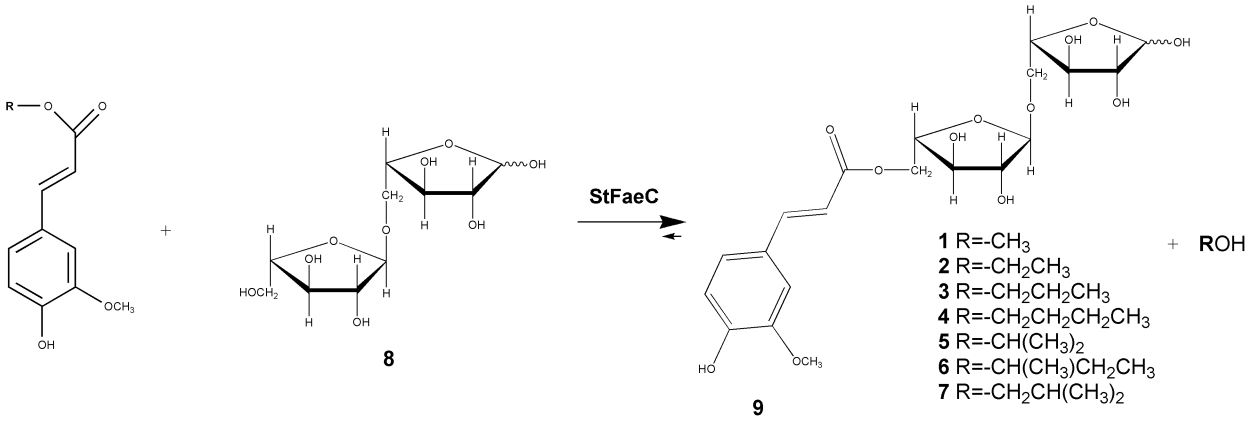

Scheme 1. Transesterification of alkyl ferulates **1**–**7** with L-arabinobiose (**8**).

reached using only feruloyl esterases. Recently, commercial enzyme preparations from *Humicola insolens*, *Thermomyces lanuginosus* and *Aspergillus niger* showing feruloyl esterase activity were tested for transesterifications of ferulate esters to various glycosides at their primary hydroxyl group.[19] The Lipolase 100T preparation from *T. lanuginosus* in polar aprotic solvents, such as acetonitrile and methyl isobutyl ketone, was found to be the best enzyme catalyst for feruloylation of alkyl and aryl hexopyranosides.

It was observed that the synthetic activity pattern of feruloyl esterases was parallel to their hydrolytic action toward various methyl esters of cinnamic acids.[20] We have previously reported that the type-C feruloyl esterase from *S. thermophile* (StFaeC) demonstrated a maximum hydrolytic activity against methyl ferulate among 26 model substrates tested, indicating that it may be the most promising type of biocatalyst for the enzymatic feruloylation of aliphatic alcohols, oligo- and polysaccharides.[20] Indeed, this enzyme proved to be able to catalyze the transfer of the feruloyl group to L-arabinose, resulting in the first example of enzymatic feruloylation of a carbohydrate.[6] Phenolic acid sugar esters have demonstrated antitumour activity and have the potential to be used in the formulation of antimicrobial, antiviral and/or antiinflammatory agents.[21–23] As esters based on unsaturated arylaliphatic acids, like cinnamic acid and its derivatives, are known to display anticancer activity,[24] specific feruloyl esterases could be employed in the tailored synthesis of such pharmaceuticals. Furthermore, it was reported that oligosaccharides containing C-5 modified D-arabinofuranosyl residues, such as D-feruloylated arabinose, are of interest as potential inhibitors of the α-(1→5)-arabinosyltransferase involved in the assembly of mycobacterial cell-wall arabinan.[25,26] Mycobacterial infections and most notably tuberculosis have long been known as a cause of morbidity and mortality worldwide.

In the present study, reaction parameters including the composition of the reaction media, the substrate concentration and the type of alkyl ferulates used as feruloyl donors were investigated to evaluate their effect on initial rate and conversion of feruloyl L-arabinobiose (Scheme 1).

2. Results and discussion

At first, we examined the enzyme synthetic activity with various ratios of the *n*-hexane–2-methyl-2-propanol–water ternary system by monitoring the transesterification of ferulic acid methyl ester **1** with L-arabinobiose **8** (Table 1). The respective ratio 47.2:50.8:2.0 (v/v/v), which involves the lower water content, was found to be the best and the transesterification reached 15% using 50 mM **1**, 30 mM **8** and 0.037 nM enzyme for 4 days. In agreement with the results reported for the transesterification of L-arabinose by StFaeC using the same sampling of alkyl ferulates (compounds **1**–**7**),[13] the feruloyl esterase shows strong preference for short chain length alkyl ferulates and especially for **1** (Table 2).

Kinetic studies on the hydrolytic activity of StFaeC against these alkyl ferulates have shown that the enzyme hydrolyzed 1-propyl ferulate **3** faster and 2-propyl ferulate **5** more efficiently.[13] In agreement with our previous study, there is no correlation between hydrolytic and synthetic activities against alkyl ferulates. Lengthening the alkyl chain of the ferulate substrates increased the hydrophobicity, resulting in less compatibility of the

Table 1. Effect of microemulsion composition on the reaction initial rate and conversion yield for the transesterification of ferulic acid methyl ester **1** with L-arabinobiose **8** under catalysis of StFaeC

n-Hexane–*t*-butanol–water (v/v/v)	Initial rate (mmol L^{-1} h^{-1} mg^{-1})	Conversion (%)
37.8:57.2:5.0	7.92	14
53.4:43.4:3.2	6.14	12
47.2:50.8:2.0	7.92	15
19.8:74.7:5.5	2.02	10

Table 2. Effect of the feruloyl donor on the reaction initial rate and conversion yield for the transesterification of L-arabinobiose **8** under catalysis of StFaeC

Feruloyl donor	Initial rate (mmol L^{-1} h^{-1} mg^{-1})	Conversion (%)
1	8.46	18
2	2.06	3
3	2.96	4
4	—	—
5	0.04	0.2
6	0.02	0.2
7	0.08	0.1

substrates in the relative polar reaction medium of the detergentless microemulsion system. However, the use of a more polar medium is necessary for the solubility of the disaccharide **8** in the synthetic reaction.

Under the reaction conditions (35 °C, esterase 0.037 nM; **1** as feruloyl donor), the effect of **1** and **8** concentration on the L-arabinobiose feruloylation rate was studied. The dependence of the initial rates (Fig. 1) as a function of the substrate concentrations was used to calculate apparent K_m values for both substrates.

As can be seen from Figure 1, the feruloylation reaction follows Michaelis–Menten kinetic. For the determination of kinetic parameters, the two reaction components **1** and **8** were used in a concentration range 10–120 and 5–30 mM, respectively, the latter concentration being the solubility limit of these substrates in the microemulsion system 47.2:50.8:2.0 (v/v/v) *n*-hexane–2-methyl-2-propanol–water at 35 °C. The calculated apparent K_m and V_{max} values for **1** and **8** are as follows $K_{m\,(L\text{-arabinobiose})}$ 178 mM, $K_{m\,(methyl\ ferulate)}$ 74 mM, $k_{cat\,(L\text{-arabinobiose})}$ 2328 min^{-1}, $k_{cat\,(methyl\ ferulate)}$ 510 min^{-1}. However, the lines obtained from the Lineweaver–Burk plots, especially in the case of different concentrations of **8** (Fig. 1b), were crossing both axes very close to the coordinates origin leading to low accuracy of the kinetic parameters.

The structure of the feruloylated arabinobiose **9** was first analyzed by ^1H NMR spectroscopy. As can be seen from the chemical shifts, apart from the resonances corresponding to H-1 protons, we did not detect any resonances in the region 5.0–5.1 suggesting that no esterification of the hydroxyl group of the arabinofuranose ring took place.[27] If the feruloyl group had been located on the primary hydroxyl group of the nonreducing arabinofuranose ring, then H-5 should experience a considerable downfield shift from approximately 3.8 ppm (free $-CH_2OH$ group) to ~4.4 ppm.[28] Due to the complexity of the resonances in this area (4.37–3.67 ppm), we were not able to confirm esterification at this site by considering only the proton NMR data.

In order to confirm the position of the feruloyl group on the arabinobiose molecule and thereupon the specificity of the feruloyl esterase StFaeC, we turned to mass spectrometry. In the positive ion mode, the first-order spectrum of **9** showed mainly the singly charged $[M+Na]^+$ ion (figure not shown). Tandem mass spectra of the singly charged $[M+Na]^+$ are presented in Figure 2a. Similar to the fragmentation analysis of other cationized oligosaccharides,[29–32] the most prominent fragment ion observed (*m*/*z* 421) appeared to be the $^{0,2}A_2$ cross-ring cleavage ions resulting from $C_2H_4O_2$ (60 Da) loss. The ion at *m*/*z* 463 corresponds to the loss of one water molecule, and ions at *m*/*z* 451 and 391 correspond to $^{0,1}A_2$ (30 Da loss) and $^{0,3}A_2$ (90 Da loss) cross-ring cleavages of the reducing arabinofuranose moiety, respectively. These cross-ring fragments reveal that the reducing arabinofuranose residue is not esterified and is linked through O-5. Ions below *m*/*z* 391 were not assigned because they do not give any information about the nonreducing arabinofuranose ring. Therefore, we investigated the negative ionization mode as it is generally considered simpler, more sensitive and structurally more informative than the positive ion mode.[32]

In the negative ion mode, the first-order spectrum of **9** consisted mainly of the deprotonated $[M-H]^-$ pseudomolecular ion at *m*/*z* 457 (figure not shown). CID of this ion (Fig. 2b) showed a main C-1 glycosidic cleavage ion

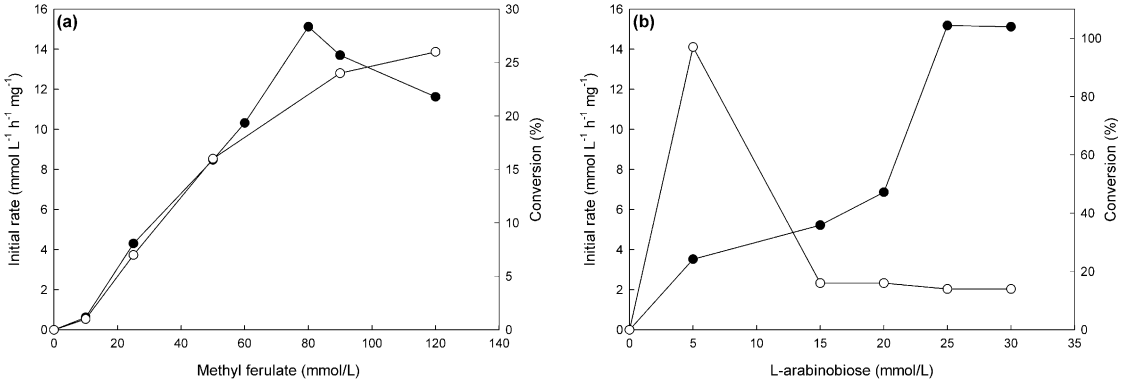

Figure 1. Effect of substrate concentration on initial rate (●) and conversion (○). The reactions were performed in 47.2:50.8:2.0 *n*-hexane–2-methyl-2-butanol–water using (a) 30 mM of **8** or (b) 50 mM of **1** and 0.037 nM enzyme at 35 °C for 4 days.

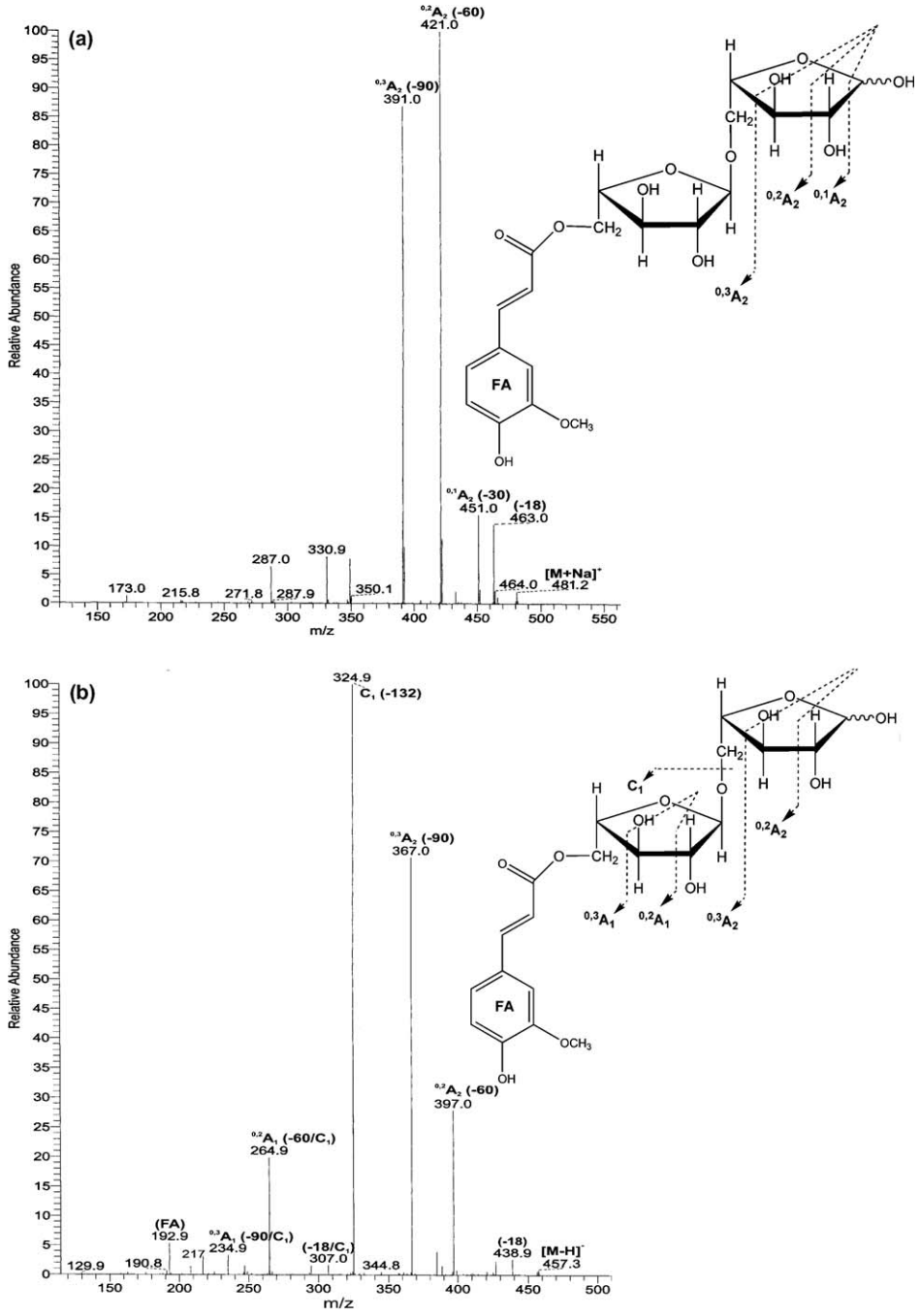

Figure 2. Structure and CID mass spectra of **9** produced by the synthetic reaction catalyzed by StFaeC in nonconventional media. (a) Complete MS/MS in positive ion mode of the singly charged $[M+Na]^+$ pseudomolecular ion at *m/z* 481; (b) complete MS/MS of the negative ion mode of the $[M-H]^-$ pseudomolecular ion at *m/z* 457. Fragments ions are identified according to the Domon and Costello nomenclature.[29]

at *m/z* 325 resulting from the loss of one anhydroarabinose residue (132 Da loss). This fragment ion is in agreement with the data in positive mode suggesting that the reducing arabinose unit is not substituted by ferulic acid. As a consequence, the prominent ions at *m/z* 397 and 367 may be assigned to $^{0,2}A_2$ and $^{0,3}A_2$ cross-ring cleavage ions, respectively. The high relative abundance of these cross-ring cleavage ions contrasts with the low relative abundance of the ion at *m/z* 439 resulting from water loss. In contrast to the positive ion spectra, no $^{0,1}A_2$ cross-ring cleavage was detected. This fragmentation pattern is consistent with the linkage of the second arabinofuranose ring to O-5 of the reducing one. The above results are in agreement with those previously reported for *O*-[2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→5)-L-arabinofuranose.[32] The ion at *m/z* 307 is formed from the loss of a water molecule. Ions at *m/z* 265 and 235 correspond to $^{0,2}A_1$ and $^{0,3}A_1$ cross-ring

cleavage fragments, respectively. The high relative abundance of the $^{0,2}A_1$ compared to the $^{0,3}A_1$ fragment ions (Fig. 2b) is consistent with esterification at O-5 of the nonreducing arabinofuranose unit with ferulic acid, in agreement with the results of Quemener and Ralet.[32] The ion at *m*/*z* 193 is due to the ferulic acid unit.

Considering the ^{1}H NMR and IT-ESIMS spectral data, the structure of the reaction product is *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→5)-L-arabinofuranose.

In conclusion, 0.037 nM of StFaeC entrapped into the detergentless microemulsion medium consisting of *n*-hexane–2-methyl-2-propanol–water 47.2:50.8:2.0 (v/v/v), with substrates **1** and **8** in concentrations of 90 and 30 mM, respectively, at 35 °C, converted 24% of the disaccharide to **9**. The conversion yield is comparable for the production of feruloylated L-arabinose catalyzed by StFaeC in a slightly different composition of the detergentless microemulsion system (53.4:43.4:3.2, v/v/v).[6] For the first time, we have carried out a simple regioselective chemoenzymatic synthesis of a feruloyl oligosaccharide of interest in medicinal chemistry.

3. Experimental

3.1. General methods

The esterase was purified to homogeneity from a culture supernatant of *S. thermophile* grown on wheat straw, as described previously.[6] All enzymatic reactions were performed with 0.037 nM StFaeC.

The esterase activity was assayed as described previously.[6] Kinetic constants (K_{cat}, K_m) were determined from the Michaelis–Menten equation, using the Lineweaver–Burk double reciprocal plots and the values were estimated using a nonlinear regression model (GraFit).[33]

Alkyl ferulates **2**–**7** were prepared as previously described.[13] The methyl ester of ferulic acid **1** was purchased from Apin Chemicals Ltd (Abingdon, UK). The surfactantless microemulsions were prepared as described previously.[13]

Qualitative analysis of the samples was made by TLC. Aliquots (5 μL) of reaction mixtures were spotted on aluminium sheets coated with Silicagel 60 (E. Merck, Germany). The solvent system used for the resolution of the feruloylated product formed by the transesterification of purified StFaeC was 65:15:2 $CHCl_3$–MeOH–water. TLC plates were visualized under a UV lamp. Sugar components were detected on dried chromatograms by the aniline–hydrogen phthalate reagent.[34]

Quantitative analysis of samples was made by HPLC on a C_{18} Nucleosil column (250 mm × 4.6 mm) (Macherey Nagel, Dóren, Germany). Detection was achieved by a Jasco UV-975 detector set at 300 nm based on the calibration curve prepared using standard solutions of the product in 1:1 water–MeOH. Elution was conducted with the following gradient system: 100% solvent A (1.5:7:1 MeCN–water–formic acid), 0–10 min and 100% solvent B (7:1.5:1 MeCN–water–formic acid), 10–30 min at a flow rate of 1.0 mL min^{-1} and at ambient temperature. Yields for the synthesis of the phenolic sugar ester were calculated from the amount of **8** having reacted compared to the initial quantity of the sugar. No consumption of **8** was observed in the absence of esterase preparation.

The ^{1}H NMR spectra were recorded on a Bruker DRX-400 400 MHz instrument in D_2O. For MS/MS experiments, the various parameters (collision energy, *qz* activation value and activation time) were adjusted in order to optimize the signal and obtain maximal structural information from the ion of interest. The Domon and Costello nomenclature[35] is used for the fragment ions. Fragments containing the nonreducing end are labeled $^{k,l}A_i$ and C_i, where *i* corresponds to the number of glycosidic bond broken from the nonreducing end, and *k* and *l* indicate the cleavages within the carbohydrate ring (Fig. 2).

3.2. *O*-[5-*O*-(*trans*-Feruloyl)-α-L-arabinofuranosyl]-(1→5)-L-arabinofuranose

In a preparative scale reaction, the transesterification in the detergentless microemulsion medium (10 mL) consisting of 47.2:50.8:2.0 *n*-hexane–2-methyl-2-propanol–water was carried out in a sealed flask at 35 °C without stirring. The feruloyl donor **1** (187 mg, 0.9 mmol) was diluted in the mixture of *n*-hexane–2-methyl-2-propanol, while StFaeC (0.37 pmol) and disaccharide **8** (85 mg, 0.3 mmol) were introduced in the form of concentrated stock soln in the buffer 20 mM piperazine–HCl, pH 6.0, followed by vigorous shaking for several seconds until a stable transparent soln was obtained. Isolation of the feruloylated sugar **9** was made by preparative HPLC on a C_{18} Luna 5 μm column (250 mm × 21.2 mm) (Phenomenex). Detection was achieved by a Jasco UV-975 detector set at 300 nm. The reaction mixture, after evaporation under diminished pressure, was eluted several times with *n*-hexane for partially removing **1** and ferulic acid, and then diluted in MeOH (1 mL) before analysis. Elution was conducted with the following gradient system as a mobile phase: 100% solvent A 0–20 min and 100% solvent B 20–100 min at a flow rate of 8.0 mL min^{-1} and at ambient temperature. Fractions containing the feruloylated disaccharide **9** were pooled and evaporated under diminished pressure (33 mg, 24%).

^{1}H NMR (D_2O, 400 MHz): δ FA, 3.77 (3H, s, OC*H*$_3$), 6.33 (1H, d, *J* 15.9 Hz, CHC*H*COO), 6.81–7.17 (3H, m, aromatic), 7.59 (1H, d, *J* 15.9 Hz, C*H*CHCOO); L-Ara*f*, 5.17 (1H, d, H-1), 3.67–4.37 (4H, m, sugar protons); L-Ara*f*, 5.13 (1H, d, H-1), 3.67–4.37 (4H, m, sugar protons).

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