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Note

Chromogenic substrates for feruloyl esterases

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Abstract—Chromogenic mono- and diferuloyl-butanetriol analogs were prepared by chemical syntheses and their efficiency was evaluated as substrates for feruloyl esterases from *Aspergillus niger*.

Keywords: Feruloyl esterase; Chromogenic substrate

The enzymatic degradation of plant cell wall into fermentable sugars involves not only glycanases but also accessory enzymes like acetylxylan (AcXEs) and feruloyl esterases (FAE).The first set of enzymes is able to O-deacetylate xylans while the second one hydrolyzes phenolic groups that play a major role in the cross-linkage between hemicelluloses, pectin and lignin via ester bonds.[1,2] A growing interest in the search for new FAEs with enhanced enzymatic and physicochemical properties has recently emerged with the need to improve bioethanol production. Most of FAEs of eukaryotic and prokaryotic origins belong to family 1 of the carbohydrate esterase classification,[3] and were recently organized in four classes A–D, which take into account substrate specificities against synthetic methyl esters of hydroxycinnamic acids, growth substrate requirements of the microorganisms, and protein sequence identity. In practice, enzymatic assay of FAEs has been carried out using commercially available methyl and ethyl esters of hydroxycinnamic acids or using naturally feruloylated oligosaccharides isolated by controlled enzymatic digestion of arabinoxylan.[4] In the same context, Hatfield et al.[5] synthesized methyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside (FA Ara). In these examples ultraviolet spectrometric measurement was used to detect FAE activity since ferulic acid has a maximum absorption at 340 nm (pH 11) distinct from its esters at 375 nm. Recently, 4-nitrophenyl 2- and 5-*O*-*trans*-feruloyl-α-L-arabinofuranosides were found to be new chromogenic substrates for various feruloyl esterases.[6] The assay was based on tandem reaction involving the splitting of ester bond with almost concomitant release of 4-nitrophenol through addition of α-L-arabinofuranosidase in large quantity. However, all of these compounds suffer from major and notable drawbacks like the poor sensibility or stability under extreme pH and temperature. Furthermore, it is clear that new substrates for high-throughput screening of enzyme activities have to be designed and synthesized. It was shown that esters of 4-(nitrophenoxy)-1,2-butanediol provide excellent chromogenic substrates for the detection of lipase and esterase activities in microtiter plate setup.[7] The reaction product obtained after enzymatic treatment was subjected to periodate oxidation and β-elimination triggering *p*-nitrophenolate liberation (Chart 1).

Following an equivalent strategy, we decided to investigate the synthesis of mono- and diferuloyl chromogenic esters of (2-chloro-4-nitrophenyl)-1,2,4-butanetriol to

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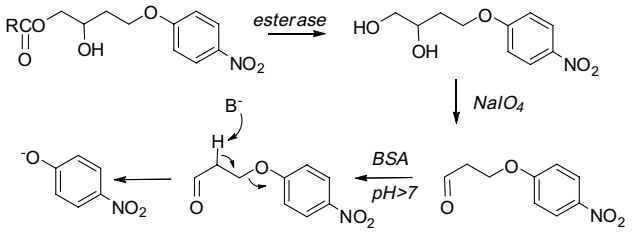


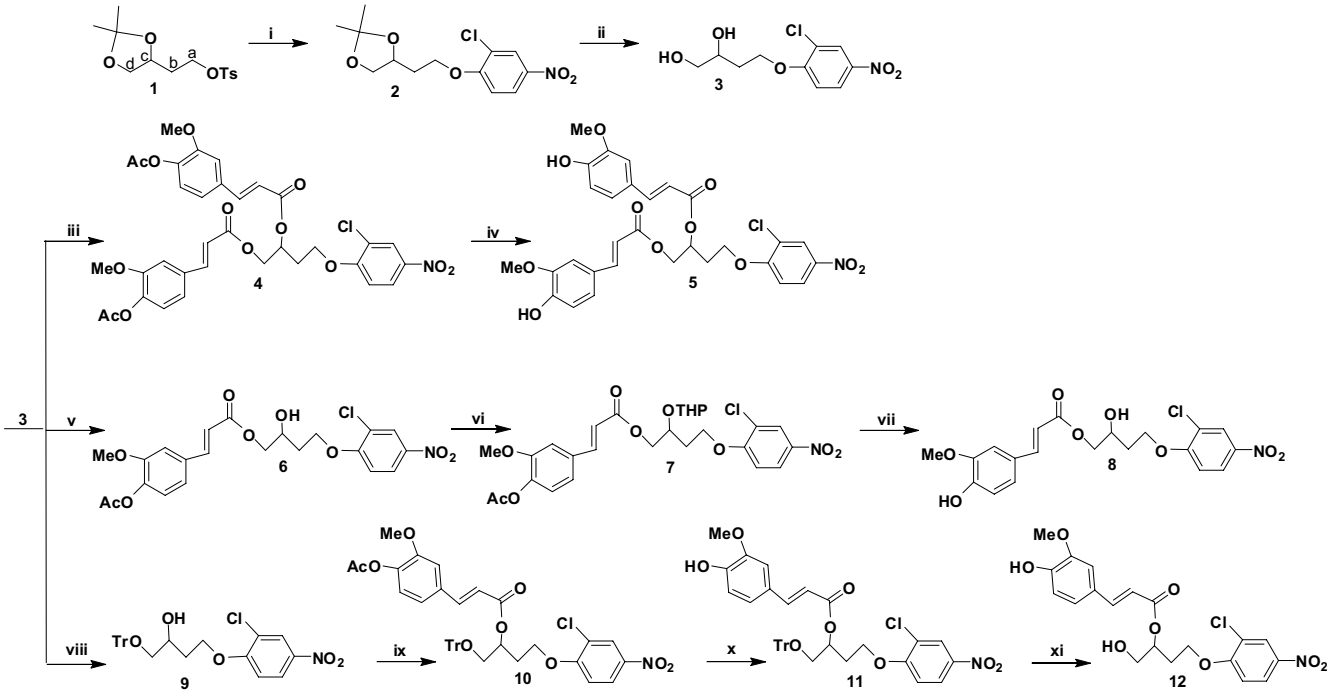
Chart 1. Release of nitrophenolate after action of the esterase or lipase on esters of 4-(nitrophenoxy)-1,2-butanediol with the presence of $NaIO_4$ and bovine serum albumin (BSA).

test them with feruloyl esterases from *Aspergillus niger* FAEA and FAEB.

As shown in Scheme 1, tosylate displacement of compound **1**[8] by 2-chloro-4-nitrophenol led to **2** in 86% yield. Removal of the isopropylidene group by acidic treatment and esterification of the key diol **3** with an excess of feruloyl chloride afforded the protected disubstituted compound **4** in 64% yield. O-Deacetylation gave target molecule **5** in 81% yield. Esterification of the primary hydroxyl of **3** under milder condition was achieved in 58% yield. However, direct O-deacetylation of **6** occurred with ester migration. To solve this problem, the secondary hydroxyl of **6** was temporarily blocked with a tetrahydropyranyl (THP) moiety, and then this intermediate was treated in basic conditions. THP removal led the expected compound **8** in 74% yield over the two steps. Selective protection of the primary hydroxyl group of **3** was achieved by tritylation. Compound **9** was obtained in 81% yield and was then esterified in 92% yield by treatment with feruloyl chloride to afford **10**. Removal of the acetyl group under basic conditions and then acidic hydrolysis of the trityl group gave target molecule **12**.

These three compounds **5**, **8** and **12** were tested as potential substrates for the feruloyl esterases FAEA and FAEB isolated from *A. niger*. On the opposite of what is described for lipases and esterases, a one-step procedure could not be performed, probably due to inhibition of feruloyl esterase activities at neutral or basic pH. Our assay required to be first carried out at pH 6 for optimal feruloylesterase activity before quantifying at pH 9 the chloro-nitrophenolate ion released upon periodate oxidation followed by β-elimination catalyzed by BSA. Although this two-step procedure precludes its use in a high-throughput manner, we analyzed the hydrolysis of compounds **5**, **8**, **12** by pure FAEA and FAEB enzymes from *A. niger* and demonstrated a linear relationship between the release of chloronitrophenol and the incubation time. Moreover, the rate of the released chloronitrophenol was proportional to enzyme concentration (Fig. 1).

Only FAEA showed detectable activity on all substrates with catalytic efficiencies in the order



Scheme 1. Reagents and conditions: (i) 2-chloro-4-nitrophenol, 18-Crown-6, K_2CO_3, acetone, 86%; (ii) AcOH aq, 94%; (iii) C_5H_5N, DMAP, 4-acetoxyferuloyl chloride, 64%; (iv) K_2CO_3, CH_2Cl_2–MeOH, 81%; (v) C_5H_5N, CH_3CN, 4-acetoxyferuloyl chloride, 58%; (vi) Dihydropyran, camphor-10-sulfonic acid, 98%; (vii) K_2CO_3, CH_2Cl_2–MeOH then, HCl, MeOH, 74%; (viii) TrCl, C_5H_5N, DMAP, 81%; (ix) C_5H_5N, 4-acetoxyferuloyl chloride, 92%; (x) K_2CO_3, CH_2Cl_2–MeOH then, HBF_4 35%, CH_3CN, 84%.

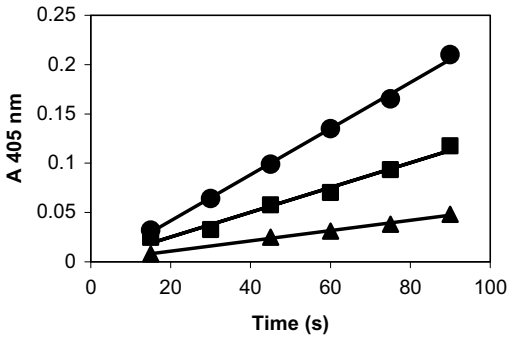


Figure 1. Time course of the liberation of 4-nitrophenol from compound **8** by *A. niger* FAEA. Three different enzyme concentrations were assayed: 5.6 nM (▲), 14 nM (■) and 28 nM (●).

Table 1. Kinetic parameters for the hydrolysis of the different synthesized substrates

	Catalytic efficiencies k_{cat}/K_m (M^{-1} s^{-1})	
	FAEA	FAEB
MFA	0.52×10^5	0.25×10^5
FA	2×10^6	2.1×10^5
8	0.5×10^5	ND
12	0.36×10^5	ND
5	0.0041×10^5	ND

MFA, methyl ferulate; FA, 5-*O*-*trans*-feruloyl-α-L-Ara*f*; ND: activity not detected.

8 > **12** ≫ **5** (Table 1). The preferential hydrolysis of ester of primary hydroxyl group found in this work has also recently been observed by Biely and co-workers. on 4-nitrophenyl 2-, 3- and 5-*O*-feruloyl-α-L-arabinofuranosides.[9]

On the other hand, FAEB showed no activity on **5** and a very weak activity on **8** and **12** when great amounts of enzyme were added, while ferulic acid methyl ester is hydrolyzed in a similar way by both enzymes.

In conclusion, we have described the synthesis of three new chromogenic substrates for feruloyl esterases based on a (2-chloro-4-nitrophenyl)-1,2,4-butanetriol scaffold. All the compounds act as moderate but specific substrate for FAEA with the same affinity as methyl ferulate while they remain unhydrolyzed by FAEB.

1. Experimental

1.1. General method

Reactions were monitored by TLC using Silica Gel 60 F254 precoated plates (E. Merck, Darmstadt) and detection by charring with sulfuric acid soln 3:45:45 H_2SO_4–MeOH–H_2O. For flash chromatography, E. Merck Silica Gel 60 was used. NMR spectra were recorded on Bruker AC 300 or Bruker Avance 400 at 298 K. Proton chemical shifts are reported in ppm relative to external $SiMe_4$ (0 ppm). Low-resolution mass spectra were recorded in the positive FAB mode on a R1010C quadripolar mass spectrometer (model 2000, Nermag, Reuil-Malmaison, France). MALDI-TOF measurements were performed on a Bruker Daltonics Autoflex apparatus. ESI experiments were performed on a Waters Micromass ZQ spectrometer. The spectrophotometric measurements were recorded on UVIKON XS spectrometer equipped with a thermostated cuvette holder.

1.2. (±)-1,2-*O*-Isopropylidene-4-*O*-(2-chloro-4-nitrophenyl)-1,2,4-butanetriol (2)

(±)-1,2-*O*-Isopropylidene-4-*O*-(4-tolylsulfonyl)-1,2,4-butanetriol **1**[10] (730 mg, 2.42 mmol) was dissolved in dry acetone (10 mL), 2-chloro-4-nitrophenol (405 mg, 2.90 mmol), 18-C-6 (44 mg, 0.17 mmol) and K_2CO_3 (670 mg, 4.84 mmol) were added to the soln. The reaction was heated under reflux for 24 h. Acetone was then removed under diminished pressure, the residue was dissolved in diethyl ether and washed with 0.1 M NaOH and brine, dried over Na_2SO_4. The crude product was purified by column chromatography (1:0→19:1 toluene–diethyl ether) to give title compound **2** as a syrup (633 mg, 86%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (1H, d, *J* 2.9 Hz, H–Ar), 8.11 (1H, dd, *J* 2.9 Hz, *J* 9.3 Hz, H–Ar), 6.97 (1H, d, *J* 9.3 Hz, H–Ar), 4.24 (4H, m, H-a/H-a′, H-c, Hd/H-d′), 3.68 (1H, t, *J* 8.3 Hz, H-a/H-a′), 2.12 (2H, m, H-b), 1.40 (3H, s, CH_3), 1.34 (3H, s, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 126.0, 123.9, 123.3, 111.7, 108.9 (*C*–CH_3, C–Ar), 72.9, 69.4, 66.6, 33.2 (C-a, C-b, C-c, C-d), 26.9, 25.5 (C–*C*H_3); DCIMS: *m*/*z* 302 $[M+H]^+$, 319 $[M+NH_4]^+$.

1.3. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-1,2,4-butanetriol (3)

Compound **2** (4.27 g, 14.1 mmol) was dissolved in acetic acid (30 mL) and water (2 mL) was added to the soln. The reaction mixture was stirred at room temperature overnight. Then the acid was removed under diminished pressure with several coevaporations with toluene. The residue was dissolved in EtOAc and precipitated with hexane to give the desired product **3** as a white solid (3.45 g, 94%); ^{1}H NMR (400 MHz, CD_3OD): δ 8.20 (1H, *J* 2.7 Hz, H–Ar) 8.13 (1H, dd, *J* 2.7 Hz, *J* 9.2 Hz, H–Ar), 7.19 (1H, d, *J* 9.2 Hz, H–Ar), 4.28 (2H, m, H-a), 3.85 (1H, m, H-c), 3.49 (2H, m, H-d), 2.05 (1H, m, H-b′), 1.82 (1H, m, H-b); ^{13}C NMR (100 MHz, CD_3OD): δ 126,4, 125.1, 113.5 (C–Ar), 69.7 (C-c), 67.7, 67.3 (C-a, C-d), 33.7 (C-b); ESIMS: *m*/*z* 284.2 $[M+Na]^+$; HRESIMS: calcd for $C_{10}H_{12}NO_5ClNa^+$, 284.0302; found *m*/*z* 284.0291.

1.4. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-di-1,2-*O*-(4-acetoxyferuloyl)-1,2,4-butanetriol (4)

Compound **3** (500 mg, 1.91 mmol) was dissolved in dry pyridine (25 mL) and DMAP (23 mg, 0.19 mmol) was added to the reaction. Then freshly prepared 4-acetoxyferuloyl chloride (1.9 g, 7.48 mmol) was added portionwise over 12 h. The reaction mixture was stirred at room temperature for 19 h. Then the reaction was concentrated and coevaporated with toluene (3 times). The residue was dissolved in CH_2Cl_2, and washed with HCl (1 M), a satd aq $NaHCO_3$, water and dried over Na_2SO_4. The crude product was purified by flash column chromatography (9:1 toluene–diethyl ether) to give title compound **4** as a white foam (855 mg, 64%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 (1H, d, J 2.7 Hz, H–Ar) 8.14 (1H, dd, J 2.7 Hz, J 9.0 Hz, H–Ar), 7.66 (2H, d, J 16 Hz, HC=CH), 6.98–7.26 (7H, m, H–Ar), 6.40 (2H, d, J 16 Hz, HC=CH), 5.56 (1H, m, H-c), 4.55 (1H, dd, $J_{cd'}$ 3.6 Hz, $J_{dd'}$, H-d′), 4.48 (1H, dd, J_{cd} 6.1 Hz, $J_{dd'}$ 12.1 Hz, H-d), 4.28 (2H, m, H-a), 3.85 (6H, s, 2 × OMe), 2.33–2.39 (8H, m, H-b, 2 × CH_3C=O); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.0–110.4 (C–Ar, C=O) 69.6 (C-c), 66.3 (C-a), 65.52 (C-d), 56.33 (2 × OMe), 31.06 (C-b), 21.05 (2 × CH$_3$C=O); ESIMS: m/z 720.24 $[M+Na]^+$; HRESIMS: calcd for $C_{34}H_{32}NO_{13}ClNa^+$, 720.1460; found m/z 720.1470.

1.5. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-di-1,2-*O*-(feruloyl)-1,2,4-butanetriol (5)

To a soln of **4** (300 mg, 0.43 mmol) in 1:1 CH_2Cl_2–MeOH, K_2CO_3 (75 mg, 0.54 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, then diluted with CH_2Cl_2, washed with NH_4Cl in water and dried over Na_2SO_4. The crude product was purified by flash column chromatography (9:1→4:1 toluene–acetone) to give title compound **5** as a yellowish foam (215 mg, 81%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.24 (1H, d, J 2.5 Hz, H–Ar), 8.10 (1H, dd, J 2.5 Hz, J 9.1 Hz, H–Ar), 7.62 (2 × 1H, d, J 15.8 Hz, HC=CH), 7.24–6.88 (6H, m, H–Ar), 6.29 (2 × 1H, d, J 15.6 Hz, HC=CH), 5.54 (1H, m, H-c), 4.46 (2H, m, H-d), 4.26 (2H, m, H-a), 3.88 (6H, s, 2 × OMe), 2.34 (2H, m, H-b); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.0, 166.8 (C=O), 159.4–109.5 (C–Ar, C=C), 69.1 (C-c), 66.1, 65.1 (C-a, C-d), 56.1 (2 × OMe), 30.8 (C-b); ESIMS: m/z 336.1 $[M+Na]^+$; HRESIMS: calcd for $C_{30}H_{28}NO_{11}ClNa^+$ 636.1248; found m/z 636.1245.

1.6. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-1-*O*-(4-acetoxyferuloyl)-1,2,4-butanetriol (6)

Compound **3** (400 mg, 1.53 mmol) was suspended in acetonitrile (7 mL) with pyridine (370 μL, 4.60 mmol), the reaction was ice cooled and freshly prepared 4-acetoxyferuloyl chloride (390 mg, 1.53 mmol) was added per portion to the reaction. Once the addition is complete the bath was removed. After 2 h acetoxyferuloyl chloride (80 mg, 0.30 mmol) and pyridine (370 μL, 4.60 mmol) were added once again to the mixture. Then 1 h later acetoxyferuloyl chloride (80 mg, 0.30 mmol) was added. The reaction mixture was stirred for 1 h, then diluted with CH_2Cl_2, washed with water and dried over Na_2SO_4. The crude product was purified by column chromatography (1:4→3:7, EtOAc–petroleum ether) and crystallized with EtOAc to give the desired compound **6** (430 mg, 58%); mp 145–146 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25 (1H, d, J 2.4 Hz, H–Ar) 8.11 (1H, dd, J 2.9 Hz, J 12.2 Hz, H–Ar), 7.64 (1H, d, HC=CH), 7.09–6.97 (4H, m, H–Ar), 6.37 (1H, d, J 16.2 Hz, HC=CH), 4.35–4.19 (4H, m, H-a, H-c, H-d), 3.82 (3H, s, OMe), 2.28 (3H, s, CH_3C=O), 2.00–2.12 (2H, m, H-b); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.9, 167.1 (C=O, CH_3C=O) 159.45–111.6 (C–Ar, C=C), 68.8, 67.5, 66.7 (C-a, C-c, C-d), 56.1 (OMe), 32.7 (C-b), 20.8 (CH$_3$C=O); HRESIMS: calcd for $C_{22}H_{22}NO_9ClNa^+$, 502.0881; found m/z 502.0871.

1.7. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-1-*O*-feruloyl-1,2,4-butanetriol (8)

1.7.1. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-1-*O*-(4-acetoxyferuloyl)-2-*O*-tetrahydropyranyl-1,2,4-butanetriol (7). Compound **6** (80 mg, 0.17 mmol) was suspended in dry CH_2Cl_2 (1 mL), dihydropyran (75 μL, 0.85 mmol) and a catalytic amount of camphorsulfonic acid were added to the mixture. The reaction mixture was stirred at room temperature for 2 h, then diluted with CH_2Cl_2, washed with satd aq $NaHCO_3$ and water and dried over Na_2SO_4. The crude product was purified by column chromatography (1:4→3:7 EtOAc–petroleum ether) to give title compound **7** as a yellow solid (92 mg, 98%); ESIMS: m/z 586.28 $[M+Na]^+$. The compound was used without any further characterization.

1.7.2. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-1-*O*-feruloyl-1,2,4-butanetriol (8). To a soln of **7** (82 mg, 0.14 mmol) in 1:1 CH_2Cl_2–MeOH (4 mL), K_2CO_3 (40 mg, 0.29 mmol) was added. The reaction mixture was stirred at room temperature for 5 h, then diluted with CH_2Cl_2, washed with water and dried over Na_2SO_4. Without any further purification and characterization, the resulting crude product was used in the next step. The compound was dissolved in MeOH (0.5 mL) and 1 M HCl (50 μL) was added to the soln, the reaction mixture was stirred at room temperature for 2.5 h, then neutralized with Et_3N and evaporated. The crude product was purified by flash column chromatography (3:7→1:1 EtOAc–petroleum ether) to give title compound **8** as a yellowish foam (47 mg, 74%); ^{1}H NMR (300 MHz, CD_3OD): δ 8.14 (1H, d J 3 Hz, H–Ar) 8.07 (1H, dd, J 3 Hz,

J 9 Hz, H–Ar), 7.53 (1H, d, *J* 12 Hz, HC=CH); 7.15 (1H, d, *J* 9 Hz, H–Ar), 7.05 (1H, d, *J* 1.5 Hz, H–Ar), 6.94 (1H, dd, *J* 1.5 Hz, *J* 8 Hz, H–Ar), 6.70 (1H, d, *J* 8 Hz, H–Ar), 6.26 (1H, d, *J* 16 Hz, HC=CH), 4.29–4.09 (5H, m, H-a, H-c, H-d), 3.79 (3H, s, OMe), 2.08 (1H, m, H-b′), 1.93 (1H, m, H-b); ^{13}C NMR (75 MHz, CD_3OD): δ 169.2 (C=O) 161.0–111.5 (C–Ar, C=C), 69.4, 67.6, 67.3 (C-a, C-c, C-d), 56.53 (OMe), 34.1 (C-b); ESIMS: *m*/*z* 460.10 $[M+Na]^+$; HRESIMS: calcd for $C_{20}H_{20}NO_8ClNa^+$, 460.0775; found *m*/*z* 460.0774.

1.8. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-1-*O*-(triphenylmethyl)-1,2,4-butanetriol (9)

Diol **3** (300 mg, 1.15 mmol) was dissolved in pyridine (5 mL), DMAP (28 mg, 0.23 mmol) and triphenyl methyl chloride (480 mg, 1.72 mmol) were added to the soln. Additional triphenylmethyl chloride (400 mg, 1.44 mmol) was added in portion over 12 h. Then the reaction was evaporated and coevaporated with toluene. The residue was dissolved in CH_2Cl_2, washed with water and dried over Na_2SO_4. The crude product was purified by flash column chromatography (3:7 EtOAc–toluene) to give title compound **9** as a white solid (470 mg, 81%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.3 (1H, d, *J* 2.9 Hz, H–Ar), 8.12 (1H, dd, *J* 2.5 Hz, *J* 8.8 Hz, H–Ar), 7.44–7.21 (18H, m, H–Ar), 6.94 (1H, d, *J* 9.3 Hz, H–Ar), 4.27–4.06 (4H, m, H-a, H-c), 3.25 (2H, m, H-d), 1.99 (2H, m, H-b); ^{13}C NMR (75 MHz, $CDCl_3$): δ 144.0–112.1 (C–Tri, C–Ar) 68.3, 67.8, 67.0 (C-a, C-c, C-d), 33.0 (C-b); ESIMS *m*/*z* 526.06 $[M+Na]^+$; HRESIMS: calcd for $C_{29}H_{26}NO_5ClNa^+$, 526.1397; found *m*/*z* 526.1391.

1.9. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-2-*O*-(4-acetoxyferuloyl)-1-*O*-(triphenylmethyl)-1,2,4-butanetriol (10)

Compound **9** (380 mg, 0.75 mmol) was dissolved in pyridine (10 mL). DMAP (18 mg, 0.15 mmol) and 4-acetoxyferuloyl chloride (380 mg, 1.50 mmol) were added to the soln. Then the reaction mixture was stirred overnight. The soln was evaporated and coevaporated with toluene. The crude product was purified by column chromatography (1:0→17:3 EtOAc–petroleum ether) to give title compound **10** as a white foam (500 mg, 92%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.26 (1H, d, *J* 2.7 Hz, H–Ar), 8.10 (1H, dd, *J* 2.7 Hz, *J* 9.1 Hz, H–Ar), 7.69 (1H, d, HC=CH), 7.48–7.07 (18H, m, H–Ar), 6.90 (1H, d, *J* 9.1 Hz, H–Ar), 6.45 (1H, d, *J* 16 Hz, HC=CH), 5.43 (1H, m, H-c), 4.15 (2H, m, H-a), 3.88 (3H, s, OMe), 3.43 (1H, dd, $J_{c,d'}$ 4.0 Hz, $J_{d,d'}$, H-d′), 3.31 (1H, dd, $J_{c,d}$ 4.9 Hz, $J_{d,d'}$ 10.2 Hz, H-d), 2.35 (5H, m, CH_3C=O, H-b); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.9–111.4 (C–Ar, C=O), 86.8 (C–Tri), 66.5 (C-c), 64.9 (C-a), 64.8 (C-d), 56.1 (OMe), 30.9 (C-b), 20.8 (*C*H_3C=O); FABMS *m*/*z* 745 $[M+Na]^+$; HRESIMS: calcd for $C_{41}H_{36}NO_9ClNa^+$, 744.1976; found *m*/*z* 744.1969.

1.10. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-2-*O*-feruloyl-1-*O*-(triphenylmethyl)-1,2,4-butanetriol (11)

To a soln of **10** (250 mg, 0.34 mmol) in 1:1 CH_2Cl_2–MeOH (4 mL), K_2CO_3 (96 mg, 0.68 mmol) was added. The reaction mixture was stirred at room temperature for 2 h, then diluted with CH_2Cl_2, washed with a NH_4Cl, water and dried over Na_2SO_4. The crude product was purified by flash column chromatography (9:1→7:3 petroleum ether–EtOAc) to give title compound **11** as a white foam (225 mg, 97%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (1H, d, *J* 2.8 Hz, H–Ar), 7.64 (1H, dd, *J* 2.9 Hz, *J* 8.8 Hz, H–Ar), 7.47–6.87 (20H, m, H–Ar), 6.32 (1H, d, *J* 16 Hz, HC=CH), 5.40 (1H, m, H-c), 4.13 (2H, m, H-a), 3.93 (3H, s, OMe), 3.39 (1H, dd, $J_{c,d'}$ 4.4 Hz, $J_{d,d'}$ 10.2 Hz, H-d′), 3.28 (1H, dd, $J_{c,d}$ 4.9 Hz, $J_{d,d'}$ 10.2 Hz, H-d), 2.32 (2H, m, H-b); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.8 (C=O) 159.55–109.6 (C–Ar, C=C), 86.9 (C–Tr), 65.0, 66.7, 70.3 (C-a, C-c, C-d), 56.2 (OMe), 31.0 (C-b); FABMS *m*/*z* 680 $[M+H]^+$; HRESIMS: calcd for $C_{39}H_{34}NO_8ClNa^+$, 702.1870; found *m*/*z* 702.1866.

1.11. (±)-4-*O*-(2-Chloro-4-nitrophenyl)-2-*O*-feruloyl-1,2,4-butanetriol (12)

Compound **11** (190 mg, 0.28 mmol) was dissolved in acetonitrile (4 mL), then 35% HBF_4 in water (60 μL) was added. The reaction mixture was stirred at room temperature for 1 h and neutralized with Et_3N. The crude product was purified by column chromatography (3:2→1:1 petroleum ether–EtOAc) to give title compound **12** as a yellowish solid (113 mg, 84%); ^{1}H NMR (300 MHz, CD_3OD): δ 8.17 (1H, d, *J* 2.4 Hz, H–Ar), 8.04 (1H, dd, *J* 2.4 Hz, *J* 8.8 Hz, H–Ar), 7.52 (1H, d, HC=CH), 7.05–7.05 (3H, m, H–Ar), 6.77 (1H, d, *J* 8.8 Hz, H–Ar), 6.20 (1H, d, *J* 15.6 Hz, HC=CH), 5.16 (1H, m, H-c), 4.17 (2H, m, H-a), 3.81 (3H, s, OMe), 3.71 (2H, m, H-d), 2.21 (2H, m, H-b); ^{13}C NMR (75 MHz, CD_3OD): δ 168.7 (C=O) 73.4 (C-a), 160.6–111.6 (C–Ar), 67.6, 64.5 (C-c, C-d), 56.4 (OMe), 31.4 (C-b); ESIMS *m*/*z* 460.03 $[M+Na]^+$; HRESIMS: calcd for $C_{20}H_{20}NO_8ClNa^+$, 460.07751; found *m*/*z* 460.0781.

1.12. Kinetic experiments

Pure recombinant feruloyl esterases FAEA and FAEB from *A. niger* were obtained according to the procedures already described.[11,12]

Methyl ferulate (MFA) was purchased from Apin Chemicals and the feruloylated arabinose FA, 5-*O*-

trans-feruloyl-α-L-Ara*f*, was purified as already described.[13] Esterase activities using MFA and FA were assayed by a continuous spectrophotometric method as previously described.[4]

Enzyme measurements using mono- and diferuloyl chromogenic esters of 2-chloro-4-nitrophenoxy-1,2-butanediol were adapted from Grognux et al.[7] The enzyme was incubated with the substrate (30 μM final concentration in 1 mL of 100 mM MOPS buffer, pH 6.0) during different times. For each time, the reaction was stopped by the addition of 100 μL 0.2 M borate buffer, pH 9.0. Then sodium periodate and BSA were added at a final concentration of, respectively, 7 mM and 2 mg/mL. The absorbance due to the release of 4-nitrophenol was followed at 405 nm.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2007.06.004.

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