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A New Affinity Ligand for the Isolation of a Single 'Feruloyl Esterase' (FAE-III) from *Aspergillus niger*

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Abstract—8-Aminooctyl 5′-*S*-coniferyl-5′-deoxy-thio-α-L-arabinofuranoside has been synthesised and shown to be a selective affinity ligand for the feruloyl esterase **III** of *Aspergillus niger*. The hydrolyses of methyl 5-*O*-coumaroyl, feruloyl, or sinapoyl α-L-arabinofuranosides by this enzyme proceed at comparable rates.

Introduction

The molecular architecture of the cell walls of plants, and the enzymology of the degradation of the molecular structures found there, is a subject of great fundamental and practical importance. Although species such as pectic substances and xyloglucans play important roles, discussion of plant biomass can usefully be structured round the concept of three major components of lignocellulose—cellulose lignin, and the various alkali-soluble polysaccharides known for historical reasons as hemicellulose. There is increasing evidence that covalent linkages between these three components are important. Ester linkages between acids corresponding to the lignin alcohols and polysaccharides, and the enzymes which hydrolyse them therefore demand investigation. The structures of the lignin alcohols and the corresponding acids (**I**) are given below.

It is found that, in general, in the plant cell walls of monocots such as cereals and grasses the ferulic acid moiety occurs esterified to O-5 of the α-L-arabinofuranosyl moiety of the arabinoxylan, whereas in dicots it occurs esterified to the side chains of pectic substances, at O-2 of the arabinofuranosyl moiety and at O-6 of the galactopyranosyl moiety.[1,2]

As part of a programme into the lignocellulolytic enzymes of *Phanerochaete chrysosporium*,[3] we wished to isolate and characterise any ferulic esterase from this fungus, the best-understood organism which degrades all components of lignocellulose. In the event, we failed to find growth conditions under which this particular organism produced workable quantities of the enzyme. However, we produced readily-synthesised substrates and and an affinity ligand (**II**), which we validated with respect to the better-characterised ferulic esterase of *Aspergillus niger*. The ligand was designed to have a non-fissile linkage connecting the ferulic and arabinofuranosyl moieties, and also a spacer arm between the arabinofuranosyl moiety and the site of attachment to the support. The thioether, rather than ether linkage, less than optimal from the viewpoint of the ligand geometry, was necessitated by synthetic problems. Attempts to connect the two halves of the recognition function as an ether linkage via Williamson-type SN2 reactions with either O5 of a protected arabinofuranosyl unit or Oγ of a protected coniferyl alcohol as nucleophiles failed, we suspect because of competing electron-transfer reactions when the electrophile was derived from coniferyl alcohol, and competing elimination reactions when it was derived from aminooctyl arabinoside. Nonetheless, the ligand **II** when attached to cyanogen-bromide-activated Sepharose, selectively bound FAE-III, the *A. niger* enzyme with specificity for ferulic acid bound to O-5 of the α-L-arabinofuranosyl moiety, and not other ferulic acid esterases in the same culture filtrate.

As with most lignocellulolytic enzymes, the choice of substrate is problematical, with a three-way tension between ease of monitoring the enzymic reaction, ease of synthesis of the assay substrate, and fidelity to the structure of the natural substrate, lignocellulose. Recent

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studies have employed methyl[4,5] and ethyl esters,[6] as well as cell wall extracts released from 'cellulase-treated' plant cell walls.[7–9] Methyl and ethyl ferulate provide the proper phenolic residue but lack a realistic alcohol moiety. The procedures for the purification of the coumaryl and feruloyl arabinoxylan oligomers FAXX and PAXX (**III**) are low-yielding and tedious.[8] We elected to use the 5-*O*-feruloyl, coumaroyl, and sinapoyl esters of methyl α-L-arabinofuranoside (**IV**), which are chromogenic, resemble the natural substrate on both sides of the site of cleavage, and which have been previously described in terms of the coumaroyl (**IVa**)[10] and feruloyl (**IVb**)[11] compounds. We also synthesised the methyl 5-*O*-furylacryloyl arabinofuranoside in the hope that a highly sensitive fluorogenic assay substrate of the type that had been used for zinc proteinases[12] would be produced.

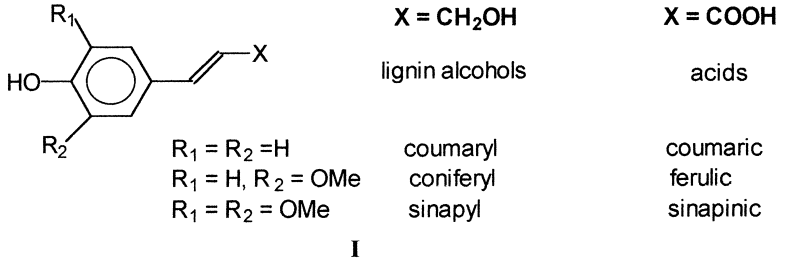
Results and Discussion

The synthesis of the affinity ligand (**II**) proved relatively straightforward; the sulfur atom was introduced for synthetic reasons since, attempts to construct an ether linkage with the ferulic moiety as either the electrophile or the nucleophile in SN2-type reactions failed. The synthetic pathway is set out in Scheme 1.

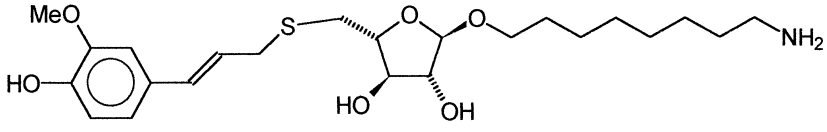
When attached to CNBr-activated Sepharose, the ligand proved successful in adsorbing the ferulic acid esterase. The Faulds and Williamson protocol for isolation of **FAE-III**[4] from *A. niger* culture filtrates involved ammonium sulfate fractionation, two hydrophobic chromatography steps, and an anion exchange chromatography. After one hydrophobic chromatography step, the affinity ligand proved capable of leading to pure protein. A purification table is given as Table 1.

A typical affinity chromatogram is given in Figure 1. Whatever the loading, some esterase activity was not adsorbed, and it is now clear that this is probably due to ferulic acid esterases with other leaving group specificities. The enzyme was eluted with a mixture of ferulic acid and arabinose. The absorbance of ferulic acid precluded the conventional use of A280 as a measure of protein concentration: fractions had to be assayed individually by the Bradford assay.

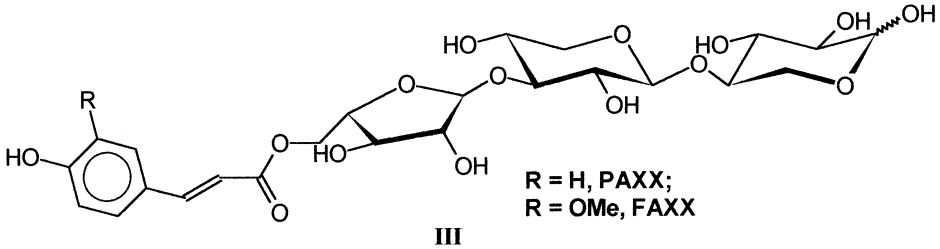
On SDS-PAGE, the feruloyl esterase preparation was electrophoretically homogenous and migrated as a



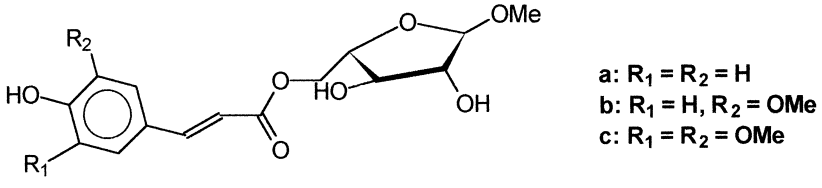
I



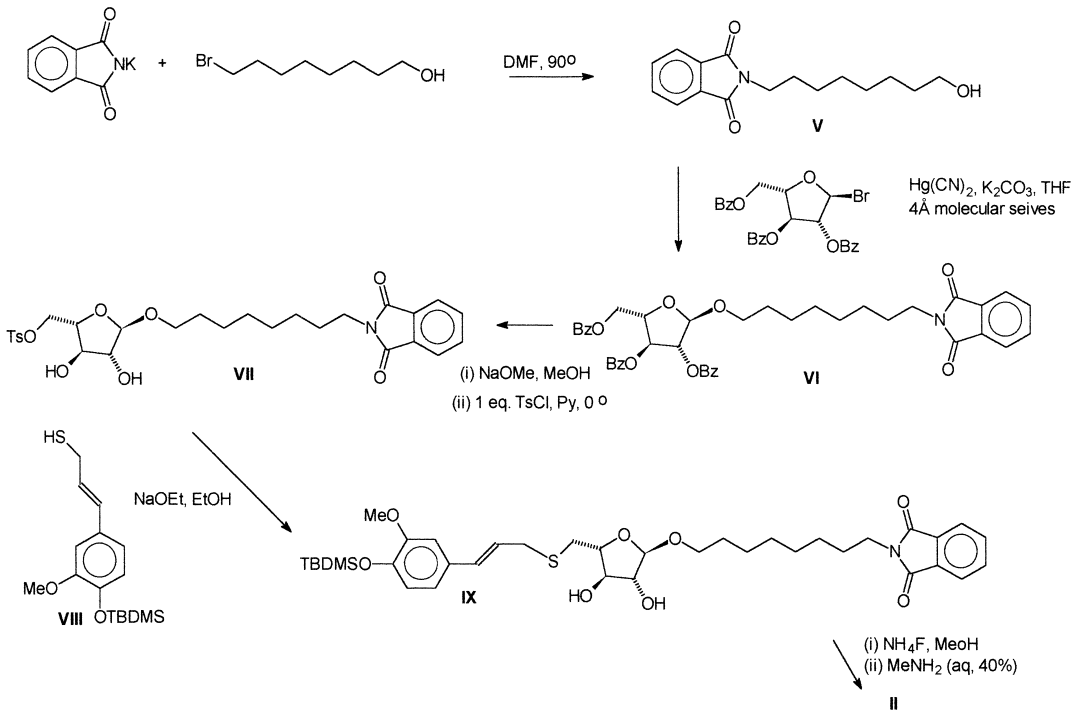
II



III



IV



Scheme 1.

Table 1. Purification of feruloyl esterase **FAE-III** from *A. niger*

Step	Vol. (mL)	Specific activity (U/mg protein)	Total activity (U)	Purification (*n*-fold)	Yield (%)
Crude supernatant	930	1.89	317	1	100
85% $(NH4)_2SO_4$	45	3.12	237	1.65	75
Hydrophobic chromatography	30[a]	23.4	212[a]	12.4	67[a]
Affinity chromatography	20[a]	53.7	81[a]	28.4	25[a]

[a]Value is derived from total of the feruloyl esterase protein and activity at each separation step.

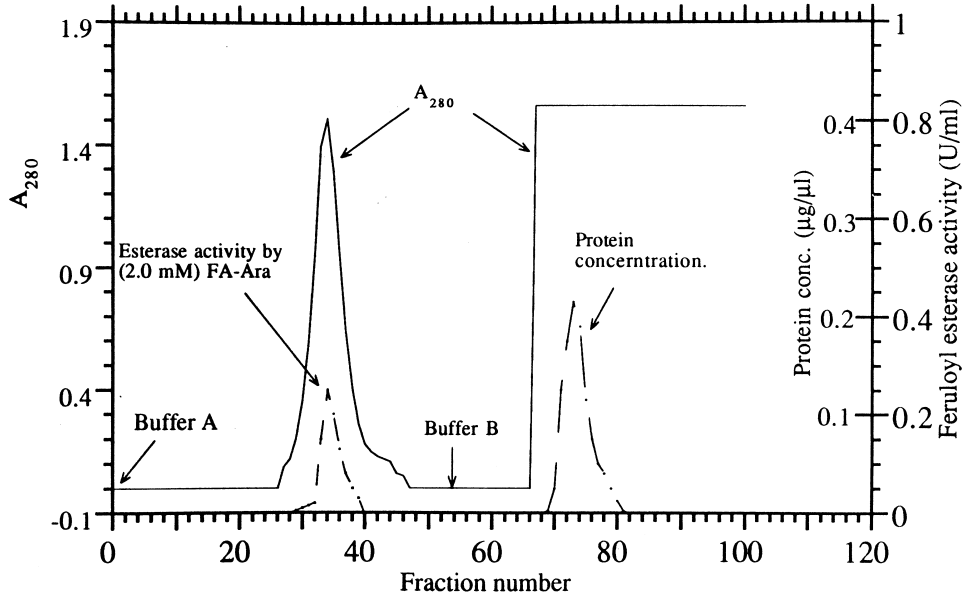


Figure 1. Affinity chromatogram of feruloyl esterase, using 50 mM potassium phosphate buffer, pH 6.0 (buffer A). Buffer B also contains 25 mM arabinose, 50 mM methyl α-L-arabinofuranoside, and 50 mM ferulic acid. FA-Ara is substrate **IVb**.

Table 2. Specificity of the hydrolyses of the hydroxycinnamyl substrates by the feruloyl esterase from *A. niger* at 30 °C, in 0.1 M potassium phosphate buffer, pH 6.0, containing 1.0 mM EDTA and 3 mM sodium azide

Substrates	Specific activity (U/(mg protein))	k_{cat} (s^{-1})	K_m (μM)
IVb (feruloyl)	53.7	33	20
IVc (sinapinoyl)	58.3	35	7.0
IVa (coumaroyl)	19.7	12	1230
Methyl ferulate	67.2[a]	ND	2080[a]
Methyl sinapinate	156[a]	ND	1440[a]
Methyl coumarate	0.07[a]	ND	ND

[a]Taken from ref 4.

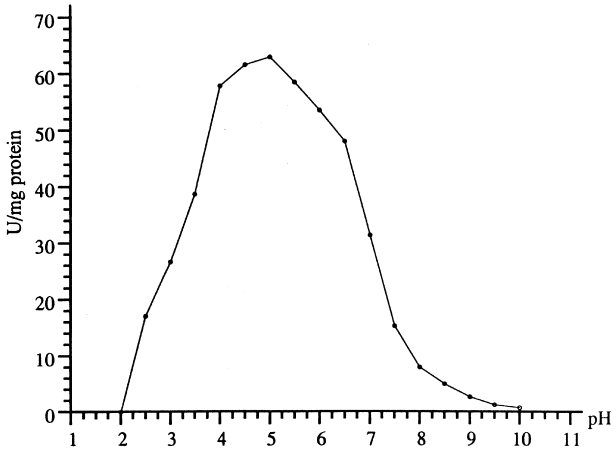


Figure 2. Variation of activity of **FAE-II** against substrate **IVb** ([S]≫K_m, at least at optimum pH).

single band corresponding to $M_r = 35.8$ kD. Gel permeation chromatography of the native enzyme indicated a single active peak correlating to $M_r = 36$–34.4 K. **FAE–III** was reported as a monomer of M_r 36 kD (SDS PAGE) and 28 kD (gel filtration).[4] The gene-sequence however indicates a protein M_r of 29 kD;[13] the protein is known to be *N*-glycosylated.

The purified feruloyl esterase exhibited Michaelis–Menten kinetics towards the three substrates **IV**. Michaelis–Menten parameters are given in Table 2, k_{cat} values being calculated on the assumption that the enzyme as first isolated were 100% active protein of $M_r = 36$ kD. The kinetic parameters for substrate **IVb** at 30 °C can be compared with those for 5-*O*-feruloyl arabinose, $k_{cat} = 63\ s^{-1}$, $K_m = 75$ μM at 37 °C, determined for conventionally isolated enzyme.[1] These data indicate the enzyme as isolated from the affinity column is largely active; the < 2-fold lower k_{cat} of substrate **IVb** with our preparation must reflect the lower assay temperature, the lower K_m a combination of the lower assay temperature and the freezing of the arabinose into the α-furanose form by the methyl glycoside.

The K_m values for substrates **IV** are, unsurprisingly, some 10^2 lower than those of the methyl esters, but, at least from the specific activities, k_{cat} values are also reduced; this decrease in k_{cat} with increasingly specific leaving groups was observed previously.[1] Although detailed mechanistic work requires an active site titration, at this stage the higher k_{cat} values that can be inferred for the methyl esters indicate that the hydrolysis of any acyl-enzyme intermediate does not limit the hydrolysis of substrates **IVb** and **IVc**.

The pH-activity profile for the enzyme against **IVb** is displayed in Figure 2: activity in this case means k_{cat}, at least at optimum pH, since the substrate concentration was $50 \times K_m$.

Detailed analysis of the present data is unwarranted, but from simple inspection it is clear the variation is not that of a classical 'bell-shaped' profile, since it is not symmetrical about the pH of maximal activity.

In the event methyl 5-*O*-(2′-furyl)acryloyl α-L-arabinofuranoside proved inert to the ferulic acid esterase we isolated, in accord with its identification of **FAE-III**. This particular esterase has a requirement for a phenolic hydroxy group on the acyl portion,[14] whereas the M_r 2×76 kD esterase (CinnAE)[15] does not. The furylacryloyl substrate may prove useful as a substrate for this enzyme.

Experimental

Substrates

5-*O*-Coumaroyl, feruloyl, sinapinoyl esters of methyl α-L-arabinofuranoside (IV). The coumaroyl and feruloyl esters **IVa** and **IVb** were prepared according to Helm et al.[10] and Hatfield et al.,[11] respectively, by selective 5-*O*-acylation of methyl arabinofuranoside by 4-*O*-acetyl feruloyl chloride, followed by selective removal of the aromatic 4-*O*-acetyl group with piperidine. The synthesis of substrate **IVc** proceeded exactly analogously, with the selective acylation of methyl arabinofuranoside proceeding in 49% yield, and the aromatic 4-*O*-deacetylation with piperidine in 79% yield.

Characterisation data for (**IVc**): mp 140–142.5 °C $[\alpha]_{25}^{D} = -39.8°$ (c 0.56, CH_3OH); ^1H NMR (CD_3OD) δ 7.60 (d, 1H, $J = 15.9$ Hz), 6.87 (s, 2H), 6.39 (d, 1H, $J = 15.9$ Hz), 4.75 (d, 1H, $J = 1.3$ Hz), 4.37 (dd, 1H, $J = 3.3$ Hz, 11.8 Hz), 4.23 (q, 1H), 4.05 (m, 1H), 3.95 (m, 1H); 3.85 (m, 1H); 3.83 (s, 6H), 3.34 (s, 3H); ^{13}C NMR (CD_3OD) δ 169.0, 149.6, 139.7, 126.7, 115.7, 110.8, 107, 83.5, 82.8, 79.4, 65.2, 56.9 (2C), 55.6; GC–MS 370 (M+), 338, 310, 266, 224, 207, 175, 176, 147, 121, 105, 91, 73, 61, 45; FT–IR (KBr) 3630–3100 (s), 2939, 2842 (w), 1700 (s), 1632, 1604, 1516, 1460, 1426, 1339, 1286, 1180, 1114, 1013, 951, 825, 626 cm^{-1}. Anal. calcd for $C_{17}H_{22}O_9$: C, 55.13; H, 5.99. Found: C, 54.83; H, 5.96.

Methyl 5-*O*-(E-2′ furylacryloyl)-α-L-arabinofuranoside. This was prepared by selective 5-*O*-acylation of methyl arabinofuranoside with the acyl chloride in a similar way to substrates **IV**. Yield 79% mp 69–71 °C;

$[\alpha]_{25}^{D} = -71.1°$ (c 1.0, CH_3OH); R_f 0.51 (EtOAc); ^1H NMR ($(CD_3)_2CO$) δ 7.72 (d, 1H, $J = 1.7$ Hz), 7.50 (d, 1H, $J = 15.6$ Hz), 6.88 (d, 1H, $J = 3.4$ Hz), 6.60 (dd, 1H, $J = 1.7$ Hz, 3.5 Hz), 6.28 (d, 1H, $J = 15.8$ Hz), 4.76 (d, 1H, $J = 1.6$ Hz), 4.54 (m, 1H), 4.39 (dd, 1H, $J = 3.7$ Hz, 11.8 Hz), 4.24 (dd, 1H, $J = 5.9$ Hz, 11.7 Hz), 4.09 (m, 1H), 3.99 (m, 1H), 3.90 (m, 1H), 3.30 (s, 3H); ^{13}C NMR δ 146.4, 132.2, 116.3, 116.0, 113.4, 110.5, 83.4, 82.4, 79.4, 65.0, 55.0; MS 284 (M+), 253, 224, 213, 206, 181, 138, 121, 110, 94, 87, 73, 65, 57; IR (KBr), 3500–3200 (s), 2969, 2916, 1716 (s), 1643, 1559, 1481, 1302, 1211, 1170, 1095, 1003, 936, 860, 758, 595 cm^{-1}. Anal. calcd for $C_{13}H_{16}O_7$: C, 54.93; H, 5.67. Found: C, 54.89; H, 5.66.

Synthesis of affinity ligand II

***N*-8′-Hydroxyoctyl phthalimide (V).** Potassium phthalimide (4.80 g, 25.4 mmol) was added to a solution of 8-bromo-1-octanol (4.90 g, 22.2 mmol) in dry DMF (25 mL). The reaction was heated to 90 °C for 30 min, then kept at room temperature overnight. After the addition of chloroform (50 mL), the mixture was poured into water (100 mL). The aqueous phase was separated and extracted with two 15 mL portions of chloroform. The combined chloroform extract was washed with 0.2 M NaOH (25 m1), to remove unreacted phthalimide, and water (25 mL). The chloroform was dried and evaporated and the residue was recrystallised from ethyl acetate/hexane to afford 5.6 g (91%) of the title product; mp 66–68 °C; ^1H NMR ($CDCl_3$) δ 7.83 (dd, 2H, $J = 3.1$ Hz, 5.5 Hz), 7.69 (dd, 2H, $J = 3.1$ Hz, 5.5 Hz), 3.70–3.58 (q, 4H), 1.68–1.29 (m, 12H); IR 3510 (s), 2946, 2923, 2853, 1773, 1707 (s), 1469, 1406, 1376, 1342, 1190, 1063, 940, 721 cm^{-1}.

8′-Phthalimidooctyl 2, 3, 5-tri-*O*-benzoyl-α-L-arabinofuranoside (VI). To a mixture of *N*-8′-hydroxyoctyl phthalimide (6.07 g, 22.1 mmol), $Hg(CN)_2$ (4.0 g) and Na_2CO_3 (2.0 g) in dry THF (60 mL), stirred under nitrogen in the presence of 4 Å molecular sieve, was added 14.0 g (26.6 mmol) of 2, 3, 5-tri-*O*-benzoyl-α-L-arabinofuranosyl bromide.[16] The reaction was allowed to stir 24 h at ambient temperature. The reaction was filtered by suction and the solvent was removed in vacuo. The crude product was subjected to flash chromatography on silica gel using EtOAc:$CHCl_3$ (1:20) as eluant. Concentration of the appropriate fractions and recrystallization from dry benzene afforded 9.62 g (60.6%) of the title product. Mp 90–92 °C; ^1H NMR ($CDCl_3$) δ 8.09–7.98 (m, 6H), 7.82 (dd, 2H, $J = 3.0$ Hz, 5.6 Hz), 7.67 (dd, 2H, $J = 3.1$ Hz, 5.5 Hz), 7.59–7.25 (m, 9H), 5.64 (d, 1H, $J = 5.1$ Hz, H-3), 5.50 (d, 1H, $J = 0.8$ Hz, H-2), 5.27 (d, 1H, $J = 0.5$ Hz, H-1), 4.83–4.54 (m, 3H), 3.85–3.58 (m, 4H), 3.48 (m, 1H), 1.70–1.21 (m, 12H).

8′-Phthalimidooctyl α-L-arabinofuranoside. Removal of benzoyl groups was achieved under standard Zemplén conditions. The crude product was purified by silica gel chromatography (CH_3OH:$CHCl_3$ 1:13 as eluent) affording 2.90 g (81%) of the title compound. The product was co-evaporated several times with dry toluene, dry ether and recrystallized from dry ethanol/pentane to form white solid. Mp 79–81 °C; ^1H NMR ($CDCl_3$) δ 7.82 (dd, 2H, $J = 3.1$ Hz, 5.4 Hz), 7.69 (dd, 2H, $J = 3.0$ Hz, 5.4 Hz), 4.98 (s, 1H), 4.13 (d, 1H, $J = 2.1$ Hz), 3.99 (s, 2H), 3.81 (m, 2H), 3.71–3.60 (m, 4H), 3.43 (m, 1H), 3.11 (m, 1H), 1.64–1.29 (m, 12H).

8′-Phthalimidooctyl 5-*O*-(*p*-toluenesulfonyl)-α-L-arabinofuranoside (VII). To a stirred solution of 8′-phthalimidooctyl α-L-arabinofuranoside (1.02 g, 2.50 mmol) in dry pyridine (10 mL) with 4 Å molecular sieve under N_2 at 0 °C, was added *p*-toluenesulfonyl chloride (0.53 g, 2.75 mmol). The mixture was stirred at 0 °C for 4 h, at 4–7 °C for 24 h and at room temperature 5 h. The reaction mixture was filtered by suction and the solvent was removed in vacuo. The crude product was purified by silica gel chromatography (CH_3OH:$CHCl_3$ 1:35). Yield 1.08 g (77.7%) of the title product. ^1H NMR ($CDCl_3$) δ 7.82–7.66 (m, 6H), 7.32 (d, 2H, $J = 8.2$ Hz), 4.91 (s, 1H), 4.21–4.04 (m, 4H), 3.87 (s, 1H), 3.67–3.59 (m, 3H), 3.34 (m, 1H), 3.15 (s, 1H), 2.41 (s, 3H), 1.67–1.47 (m, 4H), 1.27 (m, 8H); ^{13}C NMR δ 168.5, 145.1, 133.9, 132.2, 132.0, 129.9, 128.0, 123.1, 107.6, 83.1, 80.0, 69.1, 67.8, 37.9, 29.2, 28.9, 28.4, 26.6, 25.8, 21.6; IR 3570–3350 (s), 1771 (m), 1711 (s), 1610 (w), 1438 (m), 1398 (s), 1357 (s), 1176 (s), 1095 (s), 971 (s), 722 (s), 666 (m) cm^{-1}.

E-4-*O*-(*tert*-Butyldimethylsilyl)-3-methoxycinnamaldehyde. To a stirred solution of E-4-hydroxy-3-methoxycinnamaldehyde (9.0 g, 50.5 mmol) in dry pyridine (80 mL) under N_2, was added *tert*-butyldimethylsilyl chloride (9.0 g, 59.8 mmol). The reaction was allowed to stir overnight at room temperature. After removal of the solvent in vacuo, the crude product was re-dissolved in ethyl acetate:hexane (1:1), filtered, evaporated and crystallized from ethyl acetate:pentane. Yield 10.5 g (72%) of the title product, mp 131–134 °C: ^1H NMR ($CDC1_3$) δ 9.69 (d, 1H, $J = 7.5$ Hz), 7.70 (d, 1H, $J = 15.8$ Hz), 7.00 (d, 2H, $J = 6.5$ Hz), 6.85 (d, 1H, $J = 8.7$ Hz), 6.31 (d,1H, $J = 15.8$ Hz), 3.85 (s, 3H), 1.01 (s, 9H), 0.18 (s, 6H).

E-4-*O*-(*tert*-Butyldimethylsilyl)-3-methoxycinnamyl alcohol. To a stirred solution of E-4-*O*-(*tert*-butyldimethylsilyl)-3-methoxycinnamaldehyde (6.13 g, 21.0 mmol) and $CeCl_3$ (6.0 g) in 80 mL dry THF under N_2, was added 1.3 g (34.4 mmol) of $NaBH_4$. The mixture was stirred overnight, filtered with filter agent, evaporated and purified with silica gel chromatography (ethyl acetate:hexane 1:1). Yield 5.0 g (81%). ^1H NMR ($CDCl_3$) δ 6.90–6.78 (m, 3H), 6.53 (d, 1H, $J = 15.8$ Hz), 6.24 (ddd, 1H), 4.28 (dd, 1H, $J = 0.85$ Hz, 5.9 Hz), 3.81 (s, 3H), 0.99 (s, 9H), 0.15 (s, 6H); ^{13}C NMR 150.9, 145.0, 131.3, 130.5, 126.4, 120.8, 119.5, 109.7, 63.8 (OCH_2), 55.3 (OCH_3), 25.6, 18.4.

E-4-*O*-(*tert*-Butyldimethylsilyl)-3-methoxycinnamyl thiolacetate. To an efficiently stirred solution of triphenylphosphine (7.0 g, 26.6 mmol) in dry THF (60 mL) at 0 °C was added diisopropyl azodicarboxylate (5.3 g, 26.2 mmol) under N_2. The mixture was stirred at 0 °C for 30 min. A white precipitate resulted. To the mixture was added dropwise over 10 min a mixture of thioacetic acid (Aldrich, 2.0 mL, 25.3 mmol) and E-4-*O*-(*tert*-

butyldimethylsilyl)-3-methoxycinnamyl alcohol (3.9 g, 13.3 mmol) in dry THF (25 mL) over 10 min. The reaction was kept at 0 °C for 1 h and room temperature for 1 h. After removal of the solvent in vacuo, the red crude product was re-dissolved in hexane, filtered, evaporated and purified by silica gel chromatography (CH_2Cl_2: hexane 1:2). Yield 4.40 g (95%) of the title product as red oil. ^{1}H NMR ($CDCl_3$) δ 6.85 (d, 1H, J = 1.7 Hz), 6.78 (m, 2H), 6.49 (d, 1H, J = 15.7 Hz), 6.02 (q, 1H, J = 7.7 Hz, 8.0 Hz, 15.7 Hz), 3.80 (s, 3H), 3.68 (m, 2H), 2.35 (s, 3H), 0.98 (s, 9H), 0.14 (s, 6H); ^{13}C NMR δ 195.2, 150.9, 144.9, 132.9, 130.4, 127.6, 122.2, 120.8, 119.4, 109.6, 55.3, 31.8, 31.5, 30.4, 25.6, 18.4, 14.0, −4.7.

E-4-*O*-(*tert*-Butyldimethylsilyl)-3-methoxycinnamyl thiol (VIII). To a stirred slurry of $LiAlH_4$ (1.0 g, 26.3 mmol) and dry cesium chloride (1.5 g, 6.08 mmol) in dry ether (25 mL) was added dropwise E-4-*O*-(*tert*-butyldimethylsilyl)-3-methoxycinnamyl thiolacetate (4.4 g, 12.5 mmol) in ether (25 mL). After addition, the reaction was kept overnight at ambient temperature. The excess lithium aluminum hydride was destroyed by the careful addition of water (10 mL). 0.1 M HOAc was added carefully until the pH of the solution became 7.0. The ether layer was separated, dried over $MgSO_4$, filtered and evaporated. The crude product was purified by silica gel chromatography (THF:hexane 1:25). Yield 3.6 g (93%) of the title compound as clear oil; ^{1}H NMR ($CDCl_3$) δ 6.88 (d, 1H, J = 1.5 Hz), 6.80 (m, 2H), 6.41 (d, 1H, J = 15.5 Hz), 6.17 (ddd, 1H), 3.82 (s, 3H), 3.33 (t, 2H), 1.50 (t, 1H, J = 7.6 Hz, 15.2 Hz), 0.99 (s, 9H), 0.15 (s, 6H); ^{13}C NMR 153.4, 151.0, 130.7, 130.5, 126.7, 120.9, 119.5, 109.6, 55.4, 27.3, 25.6, 18.4, −4.7; IR 3027, 2997, 2895, 1600, 1512 (s), 1415, 1307, 1281, 1159, 1037, 904, 839, 782 cm^{-1}.

8′-Phthalimidooctyl 5-deoxy-5-(E-4″-*O*-(*tert*-butyldimethylsilyl)-3″-methoxycinnamyl)-thio-α-L-arabinofuranoside (IX). Sodium (49 mg) was dissolved in dry ethanol (35 mL) under a blanket of helium. To this mixture was added E-4-*O*-(*tert*-butyldimethylsilyl)-3-methoxycinnamyl thiol (**VIII**) (0.67 g, 2.16 mmol) also under helium. The mixture was kept to stir for 2 h. To this mixture was added 8′-phthalimidooctyl 5-*O*-(*p*-toluenesulfonyl)-α-L-arabinofuranoside (**VII**) (1.21 g, 2.15 mmol). The reaction was refluxed under He for 18 h, evaporated and purified with silica gel chromatography (chloroform: methanol 1:40). Yield 0.41 g (27%). ^{1}H NMR ($CDCl_3$) δ 7.82 (dd, 2H, J = 3.2 Hz, 5.5 Hz), 7.68 (dd, 2H, J = 3.1 Hz, 5.5 Hz), 6.88 (d, 1H, J = 1.5 Hz), 6.79 (m, 2H), 6.38 (d, 1H, J = 15.7 Hz), 6.00 (ddd, 1H, J = 7.7 Hz, 8.0 Hz, 15.7 Hz), 4.97 (s, 1H), 4.21 (m, 1H), 4.04 (s, 1H), 3.87 (s, 1H), 3.80 (s, 3H), 3.77–3.61 (m, 5H), 3.41–3.33 (m, 3H), 2.85–2.79 (2H), 1.69–1.50 (m, 4H), 1.29 (m, 8H), 0.97 (s, 9H), 0.13 (s, 6H); ^{13}C NMR δ 168.4, 150.9, 144.9, 133.8, 132.9, 132.0, 130.4, 127.1, 123.1, 123.0, 120.8, 119.5, 109.6, 107.7, 85.5, 80.3, 80.2, 67.5, 55.4, 37.9, 35.6, 32.9, 29.3, 28.4, 26.6, 25.9, 25.6, 18.4, −4.71; IR 3500–3300 (s), 2930, 1714 (s), 1684 (w), 1508 (m), 1397 (m), 1281 (m), 1035 (m), 720 (m) cm^{-1}.

8′-Phthalimidooctyl 5-deoxy-5-thio-(E-4″-hydroxy-3″-methoxycinnamyl)-α-L-arabinofuranoside. To a stirred solution of 8′-phthalimidooctyl 5-deoxy-5-thio-[E-4″-*O*-(*tert* butyldimethylsilyl)-3″-methoxycinnamyl)-α-L-arabinofuranoside (0.30 g, 0.43 mmol) in methanol (15 mL) was added ammonium fluoride (30 mg, 0.81 mmol). The mixture was stirred in an oil bath at 60 °C for 3 h. To the mixture was added silica gel (~0.5 g). After removal of the solvent in vacuo, the dry adsorbed silica gel was added to the top of a silica column. The column was eluted by chloroform:methanol (20:1). Yield 0.24 g (95%). ^{1}H NMR ($CDCl_3$) δ 7.83 (dd, 2H, J = 3.0 Hz, 5.7 Hz), 7.70 (dd, 2H, J = 3.1 Hz, 5.7 Hz), 6.89 (s, 1H), 6.83 (t, 2H), 6.38 (d, 1H, J = 15.7 Hz), 6.00 (p, 1H), 4.97 (s, 1H), 4.22 (m, 1H), 4.03 (d, 1H, J = 1.2 Hz), 3.89 (s, 3H), 3.86 (s, 1H), 3.72–3.62 (m, 3H), 3.39 (m, 3H), 2.82 (t, 2H), 1.70–1.51 (m, 4H), 1.32–1.25 (m, 8H); ^{13}C NMR δ 168.5, 144.8, 133.8, 132.9, 127.2, 123.2, 122.6, 120.2, 114.3, 108.0, 107.7, 85.8, 80.3, 80.0, 67.5, 55.8, 38.1, 35.7, 32.5, 29.0, 28.9, 26.6, 26.0, 24.8.

8′-Aminoooctyl 5-deoxy-5-thio-(E-4″-hydroxy-3″-methoxycinnamyl)-α-L-arabinofuranoside (II). To a stirred solution of 8′-phthalimidooctyl 5-deoxy-5-thio-(4″-hydroxy-3″-methoxycinnamyl)-α-L-arabinofuranoside (50 mg, 0.090 mmol) in methanol (2 mL) was added methylamine (2 mL) under N_2. The mixture was stirred for 15 min under nitrogen. The mixture was evaporated and redissolved in 0.1 M disodium hydrogen phosphate (pH 8.5) and evaporated under oil pump vacuum, and purified with silica gel chromatography (the silica gel was washed thoroughly with Et_3N first) using THF: methanol:triethylamine (10:10:1) as eluent. Yield 37 mg (87%) of the title product $[\alpha]_{25}^{D} = -36.0°$ (c 1.1, CH_3OH); ^{1}H NMR (CD_3OD) δ 6.98 (d, 1H, J = 1.8 Hz), 6.82 (dd, 1H, J = 1.8, 8.1 Hz), 6.71 (d, 1H, J = 8.1 Hz), 6.39 (d, 1H, J = 15.7 Hz), 6.03 (p, 1H), 4.82 (d, 1H, J = 1.7 Hz), 4.01–3.91 (m, 2H), 3.85 (s, 3H), 3.81–3.64 (m, 3H), 3.44–3.35 (m, 3H), 2.83–2.69 (m, 4H), 1.89 (s, 1H), 1.69–1.30 (m, 12H); ^{13}C NMR δ 149.1, 133.7, 130.4, 128.3, 124.1, 120.9, 116.2, 110.3, 109.3, 84.2, 83.8, 81.8, 68.8, 56.3, 41.4, 35.9, 33.9, 30.4, 30.3, 27.6, 27.1. Anal. calcd for $C_{23}H_{37}O_6NS$: C, 60.63; H, 8.19; N, 3.07; S, 7.04. Found: C, 60.37; H, 8.02; N, 2.98; S, 6.87.

Enzyme and protein assays during purification

Spectrophotometric activity assays. The assay solution consisted of 100–200 μL (10.0 mM or 5.0 mM) of compound **IVb**, 10–50 μL of enzyme solution (colourless) and buffer to a final volume of 1.0 mL. The buffers were used pH 5.0 (0.1 M sodium acetate), or pH 6.0 or 7.0 (0.1 M potassium phosphate) or universal buffer (pH = 2–12 see pH optimum). Enzyme activity values were obtained by continuously monitoring the absorbance decrease with Perkin–Elmer Lambda 6 or 3 spectrometer over 20–300 min, and the absorbances were read at 350–390 nm. Blank controls consisted of the above mixtures without enzyme additions or with boiled enzyme preparations.

Chromatographic assay. The dark colour and consequent high absorbances of some enzyme preparations made the spectrophotometric assay impracticable. HPLC separation of the esterase substrate **IVb** and

its hydrolysis product was carried out on a ternary HPLC pump system (Spectra-Physics, SP 8800) using a Econosphere Si 5 U column (Alltech, 4.6×150 mm) with a matching guard column (All-guard Cartridge System, Econosphere Silica 5 U, Alltech). The mobile phase was CH_2Cl_2:CH_3CN:AcOH (80:50:1) and the flow rate was 0.5 mL/min. The substrates and the hydrolytic products were detected with a UV detector (Spectra 100 UV Detector, Spectra-Physics) set at 320 nm. After substrate and enzyme mixtures as for the UV assay were incubated for 3–1800 min, the reactions were stopped by adding 1 equivalent volume of methanol.

One unit of activity is defined as the amount of enzyme releasing 1 μmol of ferulic acid per min at 30 °C. All assays were performed in duplicate, with blanks to correct for background reactions in enzyme and substrate samples.

Proteins. These were estimated using the Bradford method or Lowry method.[17]

Growth of *Aspergillus niger* and isolation of enzyme

The growth of the organism and initial stages of the purification followed Faulds and Williamson.[4]

Growth of *A. niger*. Strain CBS 120.49 (American Type Culture Collection) was grown from spores. After a 4-day growth in shake-flask culture, solids were removed by centrifugation at 8000 g for 15 min. The protein solution was filtered with a glass microfibre filter (Whatman) and stored at 4 °C for further purification.

Preparation of the affinity column. (1) Activation of Sepharose. Sepharose 4B (100 mL) was filtered from the liquid in which it was supplied, rinsed extensively with ice–water, and then placed into gently stirred 100 mL of 2 M sodium carbonate solution at 0 °C. Cyanogen bromide (10 g) in acetonitrile (20 mL) was added to this stirred solution. After 15 min reaction at room temperature, the activated material was filtered off and rinsed extensively with ice–water using suction.

(II) Coupling. To a completely dissolved solution of ligand **II** (30 mg), was added the activated Sepharose. The slurry was stirred gently overnight and the next day it was poured in a column (1.6×20 cm), rinsed extensively with a solution of sodium bicarbonate (0.1 M) and then solution of glycine (0.2 M).

1. Ammonium sulfate precipitation. The proteins from *A. niger* culture supernatant were precipitated by adding $(NH_4)_2SO_4$ powder to 85% (608 g/L) saturation at 0–4 °C centrifuged at 4000 g for 20 min. The supernatant was decanted, the precipitate was resuspended in 1/100 of the original volume of buffer (25 mM KH_2PO_4, 1.0 mM EDTA and 0.02% NaN_3 pH 7.0), filtered with glass fiber filter papers and stored at 4 °C.

2. Hydrophobic interaction chromatography. The 85% pellet was fractionated by hydrophobic interaction chromatography: the column (2.6×25 cm) contained phenyl Sepharose (Pharmacia), and the medium pressure protein purification system used was Waters 650. An ammonium sulfate gradient (0–2.0 M) in 50 mM sodium phosphate (pH 7.0) with 1.0 mM EDTA and 0.02% NaN_3 was applied at 3.0 mL/min. Fractions of 3.0 mL were collected.

3. Affinity chromatography. The pooled and concentrated feruloyl esterase fractions from the hydrophobic interaction chromatography step were passed down the affinity column equilibrated with 50 mM of KH_2PO_4, pH 6.0 containing 2 mM arabinose (flow rate 0.5 mL/min). After some protein eluted, the affinity column was washed with 50 mM of KH_2PO_4, pH 6.0, containing 25 mM L-arabinose, 50 mM ferulic acid, 50 mM methyl arabinofuranoside, 1 mM EDTA at 0.75 mL/min. The desorbed protein fractions were checked using Bradford reagent because ferulic acid has strong absorption around 280 nm and it screens detection of the absorbance around this wavelength. After desorption, the protein was concentrated and dialyzed against 20 mM KH_2PO_4, pH 6.0 containing 1.0 mM EDTA and 0.02% NaN_3.

4. Concentration. Enzyme solutions were normally concentrated using a Minitan (Millipore) ultrafiltration unit or Amicon unit with a 10 KDa cut-off membrane. The filtrate was assayed for residual protein to check for the correct membrane fraction.

Enzyme kinetics

The substrates **IVa–c** were preincubated in a cuvette in the cell-block of a Perkin–Elmer Lambda 6 for 5 min. After addition of enzyme, the UV absorbance was continuously monitored between 350 and 390 nm, the wavelength being chosen for a convenient initial absorbance and total rates. Extinction coefficient differences for the three substrates as a function of wavelength and pH are given in Table 3.

Michaelis–Menten parameters were estimated by direct fitting of initial rates to a rectangular hyperbola using KaleidaGraph (Synergy, Inc, Philadelphia, PA).

Buffers for kinetic studies. The pH optimum for the hydrolysis of substrate **IVb** (1.0 mM) was determined in universal buffer: citric acid (0.0286 M), boric acid (0.0286 M), and 'bis-tris propane' (Sigma) (0.0286 M). The mixture was titrated with 0.2 M KOH or 0.2 M HCl to give the required pH. This universal buffer is a Britton–Robinson type,[18] except that 'bis-tris propane' replaces diethyl barbituric acid.

Other kinetic parameters were determined in 0.1 M potassium phosphate buffer, pH 6, 1.0 mM in EDTA.

Molecular weight determination

SDS-PAGE was performed using a Pharmacia Phast-System, using a 8–25% gradient PhastGel (Pharmacia): protein bands were determined by Coomassie staining. M_r values were estimated from a plot of log M_r versus

Table 3. Extinction coefficient differences ($\Delta\epsilon/10$, i.e., M^{-1} mm^{-1}, or 10^{-1} M^{-1} cm^{-1}) at 30 °C between substrates (**IV**) and the product acid, measured in 0.1 M buffer (sodium acetate pH 5.0 and potassium phosphate pH 6.0 and 7.0)[a]

Substrate	IVa	IVa	IVa	IVb	IVb	IVb	IVc	IVc	IVc
λ/(pH)	5.0	6.0	7.0	5.0	6.0	7.0	5.0	6.0	7.0
310	480	688	673	19.3	−112	−120	−152	−81	1.5
315	716	976	969	183	72	67	54	169	247
320	856	1141	1136	421	348	346	303	461	533
325	851	1117	1114	672	637	636	558	759	822
330	726	943	947	868	879	880	776	1001	1053
335	551	714	724	945	989	992	896	1128	1167
340	390	500	520	904	957	970	922	1139	1167
345	249	322	352	784	830	848	869	1055	1074
350	130	189	218	606	634	651	742	884	898
355	65	97	133	408	431	456	566	668	684
360	245	474	865	251	268	302	406	472	493
365	12.3	31	65	140	160	195	271	313	335
370	5.8	16.5	50	79	95	134	167	195	221
λ_{max}/(nm)	322	322	322	336	336	336	339	338	337
$\Delta\epsilon_{max}$	871	1153	1146	946	992	999	926	1147	1177

[a]A positive value corresponds to a decrease in absorbance on hydrolysis.

mobility using commercially available protein molecular weight standards (MW-SDS-70L, Sigma).

Gel permeation chromatography determination of molecular weight. Pooled feruloyl esterase active fractions from the affinity chromatography were fractionated on a Waters 650 rapid protein purification system, using a gel filtration column (0.8×30 cm, Protein PAK 300SW, Waters) previously equilibrated with 50 mM KH_2PO_4, 1.0 mM EDTA and 0.02% NaN_3 pH 6.0, and calibrated with MW-GF-70 and MW-GF-200 protein molecular weight standard kits (Sigma). Fractions of 0.2 mL were collected.

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