New chromogenic substrates for feruloyl esterases

Laurence Marmuse,[a] Michèle Asther,[b] Emeline Fabre,[c] David Navarro,[b] Laurence Lesage-Meessen,[b] Marcel Asther,[b] Michael O'Donohue,[c] Sébastien Fort[a] and Hugues Driguez*[a]

Received 16th November 2007, Accepted 5th February 2008
First published as an Advance Article on the web 29th February 2008
DOI: 10.1039/b717742a

Indolyl and nitrophenyl 5-*O*-hydroxycinnamoyl-α-L-arabinofuranosides were prepared by chemo-enzymatic syntheses. These probes were designed as substrates to be used in assays of feruloyl esterase activity (EC 3.1.1.77). Color development in the assays only occurs when feruloyl esterase activity releases an intermediate chromogenic arabinoside that is a suitable substrate for α-L-arabinofuranosidase (EC 3.2.1.55), which in turn releases the free chromogenic group. The usefulness of these compounds was evaluated in both qualitative solid media-based assays and quantitative liquid assays that can be performed in microtiter plates using feruloyl esterases and arabinofuranosidases from various origins.

Introduction

Plant cell walls are mainly composed of cellulose microfibrils embedded in a matrix of hemicelluloses, pectins and lignins. Xylans are the most abundant hemicelluloses in cereal and hardwood, accounting for as much as 35% of the dry weight of higher plants. Their repeating xylosyl units are generally linked through β(1→4) glycosidic bonds and can be mainly substituted at O-2 or O-3 positions by esters such as acetyl, feruloyl, *p*-coumaroyl and D-galactosyl, L-arabinosyl or D-glucuronic acid residues.[1] In arabinoxylans, some L-arabinofuranosyl residues can be esterified at O-5 by phenolic acids.[2,3] Among these, *trans*-ferulic acid is particularly important because it participates in cross-linking between hemicelluloses, pectins and lignins *via* ester and ether bonds.[4] The resulting network enhances the mechanical strength of cell walls and increases their resistance towards cellulases and xylanases. To counter this cross-linking, many lignocellulolytic microorganisms produce feruloyl esterases (FAE) that catalyze the cleavage of ester linkages between ferulic acid and sugars. Likewise, FAE act in synergy with other debranching enzymes and depolymerizing cellulases and xylanases in order to bring about complete degradation of biomass.[5] Presently, the growing interest in the development of industrial bioprocessing strategies using engineered lignocellulolytic microorganisms is providing an impetus to research aimed at improving the intrinsic properties of FAE. Most FAE from eukaryotic and prokaryotic sources belong to family 1 of the carbohydrate esterase classification.[6] Recently, on the basis of substrate specificities and growth requirements of the producer microorganisms, this family has been divided into four functional classes A–D. So far, FAE activity has been monitored using a variety of natural and synthetic compounds.[7] Recently, the synthesis of 4-nitrophenyl 2- and 5-*O*-*trans*-feruloyl-α-L-arabinofuranosides was reported.[8] The use of this substrate relies on a tandem reaction involving the splitting of the ester bond and almost concomitant release of 4-nitrophenol through addition of α-L-arabinofuranosidase, which is added in large quantity. However, for routine use the addition of a detergent is required to improve solubility and assays are necessarily discontinuous, because basic pH conditions are required for accurate measurement of 4-nitrophenolate absorbance. Therefore, 4-nitrophenyl 2- and 5-*O*-*trans*-feruloyl-α-L-arabinofuranosides are not particularly convenient substrates for high-throughput screening assays, where it is desirable to minimize the number of steps.

In the 1990s, numerous chromogenic and fluorogenic compounds were synthesized for the detection of hydrolytic activities of enzymes acting in large range of pH. For instance, Claeyssens *et al.* demonstrated that 2-chloro-4-nitrophenyl and 4-methylumbelliferyl β-cello-oligosaccharides offer good sensitivity and stability for continuous assays of various cellulases.[9] Similarly, 2-chloro-4-nitrophenyl β-maltoheptaoside was prepared and included in a commercial kit for α-amylase determination.[10] The advantage of these substrates resides in the fact that, unlike 4-nitrophenol whose molar extinction coefficient is halved at pH 7 compared to that at pH 9, the molar extinction coefficient of 2-chloro-4-nitrophenol is almost pH-independent. Consequently, the sensitivity of absorbance measurements at neutral pH is increased and continuous absorbance measurements are possible. Another important step was the development of 5-bromo-4-chloro-3-indolyl glycosides for assays in solid media. The well known 5-bromo-4-chloro-3-indolyl β-galactoside (X-Gal) has proved to be an essential tool in molecular biology for the detection of β-gal$^+$ microrganisms grown on solid agar media. This glycoside is colorless, but the free aglycon is rapidly air-oxidized to form an indigo blue colored dimer. Likewise, microorganisms expressing β-galactosidase grow as indigo-blue-colored colonies on agar plates.[11]

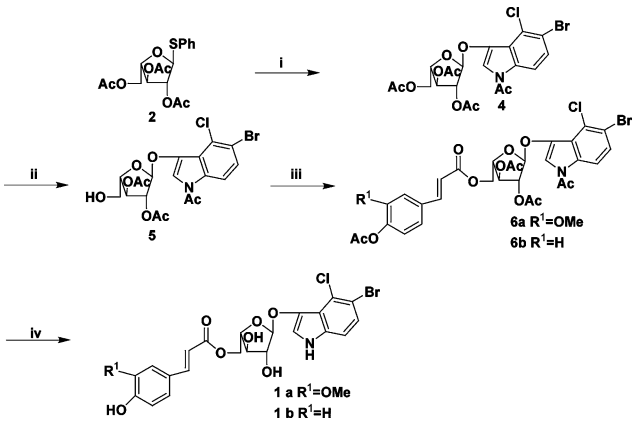
In the present work, we have designed new chromogenic substrates that provide assays for feruloyl esterase activity either on solid agar plate media or in continuous liquid-based reactions. Because fluorogenic substrates require equipment that is not always available in laboratories, especially on high-throughput

[a]*Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), BP53, 38041, Grenoble cedex 9, France. E-mail: Hugues.Driguez@cermav.cnrs.fr; Fax: +33 476 547 203; Tel: +33 476 037 664*
[b]*UMR-1163 INRA de Biotechnologie des Champignons Filamenteux, IFR86-BAIM. Universités de Provence et de la Méditerranée, ESIL, 163 avenue de Luminy, CP 965, 13288, Marseille Cedex 09, France*
[c]*INSA/INRA UMR 792, 135 avenue de Rangueil, 31077, Toulouse cedex 04, France*

platforms, we have concentrated on more universally applicable substrates based on indolyl and 2-chloro-4-nitrophenyl moieties.

Results and discussion

Our first target molecules were the 5-bromo-4-chloro-3-indolyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside **1a** and the 5-bromo-4-chloro-3-indolyl 5-*O*-*trans*-coumaroyl-α-L-arabinofuranoside **1b** (Scheme 1)



Scheme 1 *Reagents and conditions*: i) 1-acetyl-5-bromo-4-chloroindoxyl-3-ol **3**, NIS, $Sn(OTf)_2$, CH_2Cl_2, **4** 58%; ii) lipase Novozym 435, *i*-PrOH, **5** 75%; iii) pyridine, DMAP, 4-acetoxyferuloyl chloride, **6a** 82%, 4-acetoxy-coumaroyl chloride, **6b** 80%; iv) NaOMe, MeOH, **1a** 74%, **1b** 86%.

The synthesis of 5-bromo-3-indolyl α-L-arabinofuranoside in 48% yield was previously described by Berlin and Sauer starting from benzoylated arabinosyl bromide as donor.[12] Due to the known instability of furanosyl halides, we decided to investigate the glycosyl donor property of the acetylated thiophenyl arabinofuranoside **2**, a compound recently described as an efficient glycoside donor[13] (Scheme 1).

Coupling of the thiosugar **2** with 1-acetyl-5-bromo-4-chloroindoxyl-3-ol **3** was achieved in the presence of thiophilic promoter system NIS/$Sn(OTf)_2$ in DCM at −10 °C. The expected compound **4** was obtained in 58% yield. Compound **5**, prepared in high yield from **4** by selective *O*-deacetylation of its primary hydroxyl group using the *Candida antarctica* lipase Novozym 435 was esterified with 4-acetoxyferuloyl chloride[14] or acetoxycoumaroyl chloride[15] to give **6a** or **6b** in 82 and 80% yield respectively. Catalytic *O*-deacetylation afforded the target compounds **1a** and **1b**, which are potentially useful substrates for assaying FAE and, perhaps, for differentiating between enzymes that are more specific towards methoxylated or hydroxylated hydroxycinnamic acids.

Compounds **1a** and **1b** were evaluated as substrates for FAEA and FAEB from *Aspergillus niger*. First, a spectrophotometric qualitative assay was developed using commercially available α-L-arabinofuranosidase from *Aspergillus niger* (AN-ABF) and α-L-arabinofuranosidase from *Bifidobacterium longum* (BL-ABF) (Fig. 1). Both substrates were readily soluble in methanol or DMSO and were stable for several hours in the assay conditions. Moreover, controls indicated that in the absence of FAE, α-L-arabinofuranosidase was not able to cleave **1a** or **1b**, although a yellow coloration was observed (Fig. 1, well 2). This artifact was caused by the pH of the reaction medium used for the α-L-arabinofuranosidases, which was fixed at a value of 5.5. Raising the pH to 6 (pH of buffer for FAEA, Fig. 1, well 3) diminished this effect. Nevertheless, a very clear positive blue/indigo coloration was obtained when substrates **1a** or **1b** were incubated with FAE and AN-ABF or BL-ABF (Fig. 1, well 4). This visual change was correlated with a substantial increase in absorbance at 620 nm, an effect that was totally absent when only FAEA was deployed (Fig. 1, well 3).

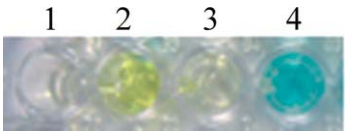


Fig. 1 Detection of combined action of α-L-arabinofuranosidase and FAEA activities in the presence of substrate. Only reactions using BL-ABF are shown. **1a**. Well 1, substrate **1a** alone; Well 2, substrate **1a** +BL-ABF; Well 3, substrate **1a** + FAEA; Well 4, substrate **1a** + BL-ABF + FAEA.

In a further test, the suitability of **1a** for use in solid media for the detection $FAEA^+$ microorganisms was studied. Fig. 2 shows that when BL-ABF is applied to solid LB agar medium containing substrate **1a**, upon which *Escherichia coli* $FAEA^+$ cells have been grown, a blue color is developed in the vicinity of the application zone. This blue coloration is the result of three successive reactions: i) the FAEA-catalyzed cleavage of the ester bond and the liberation of the 5-bromo-4-chloroindoxyl α-L-arabinofuranoside, ii) the BL-ABF-catalyzed cleavage of the pseudo-glycosidic bond and the release of 5-bromo-4-chloroindoxyl-3-ol, and iii) the spontaneous oxidation of the 5-bromo-4-chloroindoxyl-3-ol. It is important to note that in Fig. 2 the blue color is quite diffuse. This is because BL-ABF was applied as a liquid sample after bacterial growth and expression of FAEA. In order to obtain single colony coloration it would probably be necessary to create a tandem expression system that would provide simultaneous intracellular production of both α-L-arabinofuranosidase and FAEA or more simply to use a sterile top agar containing α-L-arabinofuranosidase.

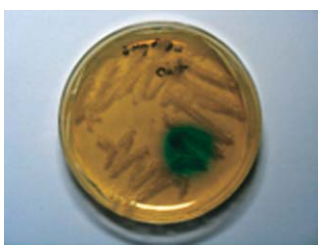
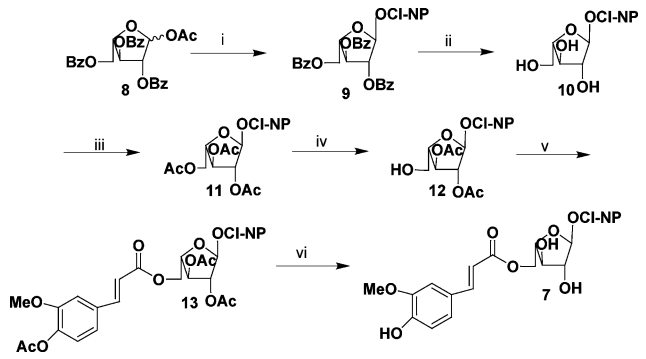


Fig. 2 Detection of FAEA activity on solid LB agar medium using the chromogenic substrate **1a**. The FAE^+ microorganism was *Escherichia coli* bearing a plasmid that expresses a thioredoxin–FAEA fusion protein.

In a second series of experiments, a structural analogue of **1a**/**1b** in which the indolyl aglycon was replaced by 2-chloro-4-nitrophenol was prepared. Unfortunately, we were unable to condense the thioglycoside **2** with 2-chloro-4-nitrophenol, despite the use of several promoters. Therefore, the synthesis of **7** (Scheme 2) was carried out essentially as recently described for the preparation of 4-nitrophenyl α-L-arabinofuranoside.[16]

Glycosylation of acylated furanose **8** with 2-chloro-4-nitrophenol was catalyzed by Lewis acid and gave benzoylated glycoside **9** in 48% yield. *O*-Debenzoylation with 1 M NaOMe



Scheme 2 *Reagents and conditions*: i) $BF_3{\cdot}Et_2O$, CH_2Cl_2, 2-chloro-4-nitrophenol, 61%; ii) NaOMe, MeOH, 72%; iii) Ac_2O, pyridine, 93%; iv) lipase Novozym 435, *i*-PrOH, 96%; v) Et_3N, DMAP, 4-acetylferuloyl chloride, CH_2Cl_2, 66%; vi) K_2CO_3, DCM–MeOH, 64%.

in MeOH afforded the expected compound **10** in 72% yield after column chromatography on silica. *O*-Acetylation gave the fully acetylated derivative **11**, which was treated by the *Candida antarctica* lipase to selectively remove the primary acetate. The deprotected derivative **12** was obtained in 89% overall yield. Esterification of the primary hydroxyl using 4-acetoxyferuloyl chloride was achieved in 66% yield. De-*O*-acetylation of **13** was a delicate issue, and several attempts were needed before the isolation of the target molecule **7** in reasonably good yield (64%).

Compound **7** proved to be a suitable substrate for continuous monitoring of FAE activity. This procedure involved a two-step enzymatic process: first the enzymatic splitting of the ester function by FAE, followed by the liberation of the 2-chloro-4-nitrophenol through the action of an α-L-arabinofuranosidase. To ascertain the usefulness of **7**, the combined hydrolytic activity of FAE and AN-ABF were evaluated by monitoring the evolution of the substrate's absorption spectra over time. At the end of the reaction period, the spectra clearly indicated characteristic adsorption peaks for free ferulic acid at 288 nm and 310 nm[7] and a peak at 405 nm, corresponding to free 2-chloro-4-nitrophenol (Fig. 3). Importantly, because the absorbance of 2-chloro-4-nitrophenol at 405 nm is sufficiently high at neutral pH, direct

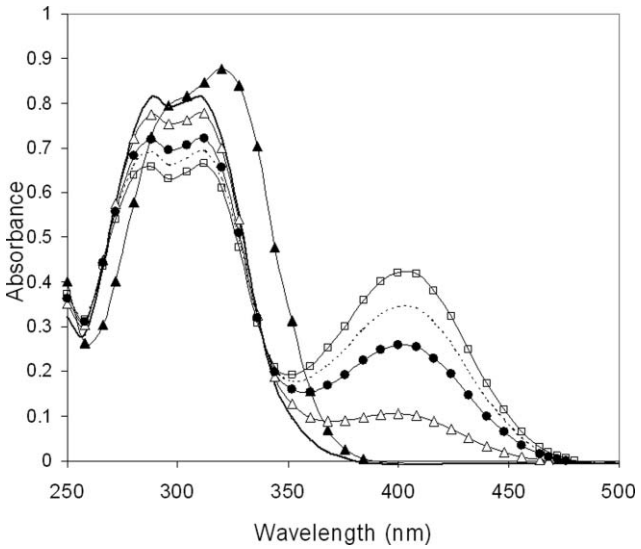


Fig. 3 Changes in absorption spectra during hydrolysis of compound **7** by FAEA from *A. niger* according to time. FAEA (33 nM) 32 mU ml^{-1} (using MFA as substrate) was incubated with compound **7** (41.5 μM) in 100 mM MOPS Buffer pH 6.0 and AN-ABF (250 mU ml^{-1}). Spectra were recorded according to time every 2 minutes. ▲–▲ 0 min; —— 2 min; △–△ 4 min; ●–● 6 min; --- 8 min; □–□ 10 min.

measurement without alkalinization of the medium is possible. Therefore **7** presents at least two clear advantages: i) it can be employed in a continuous high-throughput assay using routine equipment, and ii) it avoids interference with the spectrum of ferulic acid that absorbs strongly at alkaline pH. In addition, only reasonable amounts of L-arabinofuranosidase are required since the release of the 2-chloro-4-nitrophenol by this enzyme should not be rate-limiting. Using an excess of L-arabinofuranosidase, a linear relationship could be obtained between the rate of 2-chloro-4-nitrophenol release and the amount of FAE (Fig. 4).

Catalytic parameters for FAEA and FAEB have been determined using **7**, **14** (methyl ferulate) and **15** 5-*O*-*trans*-feruloyl-α-L-Ara*f* as substrates. Both enzymes display higher catalytic efficiencies on the natural substrate **15** and the synthetic one **7** than on

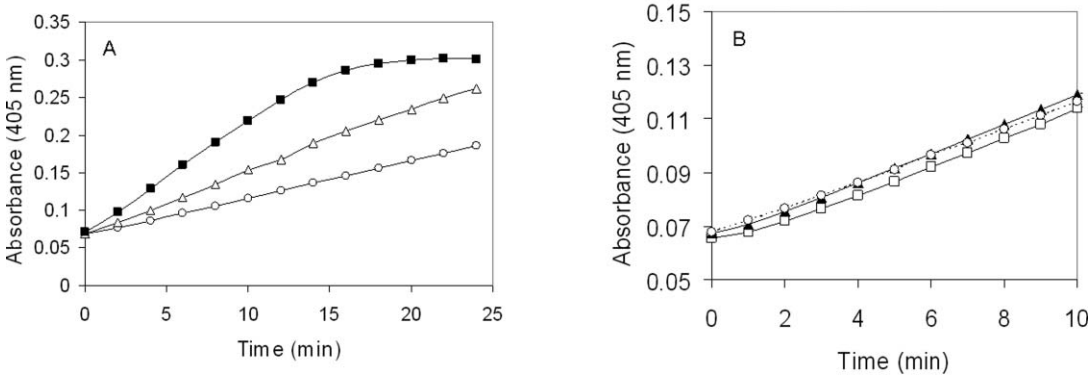
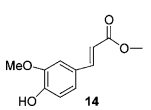


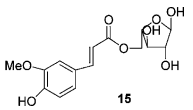
Fig. 4 Time course of hydrolysis of compound **7** by FAEA followed by the release of chloronitrophenol at 405 nm. A: Kinetics were followed at different FAEA concentrations: ○–○ (0.1 mU ml^{-1}), △–△ (0.2 mU ml^{-1}); ■–■ (0.4 mU ml^{-1}) in MOPS buffer pH 6.0 with 20 μM substrate and AN-ABF 250 mU ml^{-1} at 37 °C. B: Kinetics were followed in the same conditions but with FAEA (0.1 nM) using AN-ABF at different concentrations: □–□ (250 mU ml^{-1}); ▲–▲ (500 mU ml^{-1}); ○–○ (1 U ml^{-1}).

Table 1 Kinetic parameters for the hydrolysis of the synthesized substrate **7** (2-chloro-4-nitrophenyl 5-*O*-feruloyl-α-L-arabinofuranoside) compared to **14** (methyl ferulate) and **15** (5-*O*-*trans*-feruloyl-α-L-Ara*f*) by the two feruloyl esterases FAEA and FAEB from *A. niger*

Substrate	Catalytic efficiencies (k_{cat}/K_m)/M^{-1} s^{-1}	
	FAEA	FAEB
14	0.52×10^5	0.25×10^5
15	2×10^6	2.1×10^5
7	5×10^6	3.5×10^5



14



15

the methyl ester derivative **14**, thus confirming the importance of the L-arabinofuranosyl moiety substrate recognition.[17] Moreover, no significant differences could be observed between **15** and **7**, indicating that the presence of the nitrophenyl aglycon on the sugar does not have an inhibitory effect (Table 1). These results are coherent with structural data that indicate that the xylosyl moieties of natural arabinoxylans do not interact with active site elements of FAEs.

Various strains of *Aspergillus niger* belonging to the CIRM-BRFM Collection (Centre International de Ressources Microbiennes, Banque de Ressources Fongiques de Marseille, France) chosen for their ability to produce different types of feruloyl esterases (FAEA and FAEB) were cultivated in previously described conditions.[18] The culture medium was analyzed using a TECAN Freedom Evo automated platform. Both substrates **1a** and **7** were checked in these conditions and it was shown that they were completely adaptable in microplate screening. Positives responses were obtained with FAE-producing strains with both substrates. Moreover, using compound **7** kinetics could be monitored in order to deduce relative activity levels (results not shown).

Conclusions

Over the last ten years, the areas of enzyme discovery and enzyme engineering using molecular evolution technology have strongly been developed. Likewise, high-throughput methods have been vitally important for the screening of vast natural and artificially generated biodiversity. Therefore, it is increasingly important to devise enzyme assays that are both specific and simple to implement. In this regard, soluble and stable chromogenic substrates are highly desirable, because they are generally reliable and quite simple to use.

Indolyl and 2-chloro-4-nitrophenyl are already present as the aglycon group in several valuable substrates that have been widely used in enzyme assays and various screens. In this work, we have used these two familiar aglycons in order to design new chromogenic compounds with great potential for the screening of FAE activities. The syntheses that are described are quite straightforward and employ enzymatic de-*O*-acetylation catalyzed by *Candida antarctica* lipase. These probes are more water-soluble than the corresponding 4-nitrophenyl derivatives, allowing their use in a microtiter plate format without any use of detergent. According to our evaluations, the indolyl-based compounds are unsuitable for comparative activity assays, but are actually very useful for assays in solid medium where only the endpoint of the reaction is observed. With regard to the 2-chloro-4-nitrophenyl based substrates, these are particularly useful for one-step liquid assays that can provide quite accurate data concerning reaction rates. Together, these substrates will be valuable tools for a variety of high throughput screening applications, for example for a two-stage screening strategy involving initial agar plate screening to eliminate inactive clones, followed by liquid screening in 96-well microplate format to detect improved activity.

Experimental

General methods

Reactions were monitored by TLC using silica gel 60 F254 precoated plates (E. Merck, Darmstadt) and detection by charring with sulfuric acid solution (H_2SO_4/MeOH/H_2O 3:45:45). For flash chromatography, 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 internal $SiMe_4$ (0 ppm). Low-resolution FAB mass spectra were recorded in the positive mode of an R1010C quadripolar mass spectrometer (model 2000, Nermag, Reuil-Malmaison, France). MALDI-TOF measurements were performed on a Bruker Daltonics Autoflex apparatus. ES experiments were performed on a Waters Micromass ZQ spectrometer. The spectrophotometric measurements were recorded on UVIKON XS spectrometer equipped with a thermostated cuvette holder.

Enzymes and standard assays

Pure feruloyl esterases FAEA and FAEB from *Aspergillus niger* were obtained according to the procedures described respectively by Record *et al.*[19] and Levasseur *et al.*[20] Methyl ferulate (MFA) was purchased from Apin Chemicals and the feruloylated oligosaccharide FA, 5-*O*-*trans*-feruloyl-α-L-Ara*f* was purified according to a known procedure.[21] Esterase activities using MFA and FA were assayed by a continuous spectrophotometric method as previously described.[7] The α-arabinofuranosidase from *Aspergillus niger* was obtained from Megazyme (Bray, Ireland), whereas the α-arabinofuranosidase from *Bifidobacterium longum* NCC2705 (BL-ABF) is a recombinant His-tagged enzyme produced in-house. The sequence encoding this enzyme was fused to the 6 × His tag in pET28a. The resulting plasmid was used to transform *Escherichia coli* BL21 DE3 cells and protein expression *via* the pT7 promoter was performed according to established procedures. Likewise, His-tagged BL-ABF was purified using His-trap chromatography (GE Healthcare) following the manufacturer's recommendations. Activities of BL-ABF and AN-ABF were determined using 5 mM 4-nitrophenyl arabinofuranoside and 50 mM acetate buffer (pH 5.8) at 37 °C.[22] Activities were expressed in units (U), where one unit is the amount of enzyme needed to liberate one μmol of pNP per minute.

Detection of FAE activity using synthetic substrates

For the evaluation of the usefulness of substrate **1a** for the detection of FAE activity on solid media, *Escherichia coli* TOP10 cells bearing a plasmid derived from pBAD-Thio (Invitrogen, NL) were used. In this plasmid, the FAEA coding sequence was fused

to that of thioredoxin. After plating on LB agar plates containing ampicillin (100 μg ml^{-1}) and substrate **1a** (60 μg ml^{-1}) bacterial colonies were allowed to develop overnight at 30 °C. After, a 10 μL aliquot (3 U) of BL-ABF was carefully applied to the agar plate, which was incubated at 37 °C for 1 h in order to allow color development.

For 2-chloro-4-nitrophenyl 5-*O*-feruloyl-α-L-arabinofuranoside, a stock solution of the substrate (20 mM) was prepared in methanol. The incubation mixture contained 100 mM MOPS buffer pH 6.0, 0.25 U α-L-arabinofuranosidase, substrate concentrations ranging from 1 to 50 μM and appropriate amounts of FAEA or FAEB. Activities were followed at 37 °C by the release of 2-chloro-4-nitrophenol at 405 nm according to time.

Assays using a high-throughput screening platform were performed in 96-well microtiter plates using experimental conditions identical to those previously described, but using a 200 μL final volume. Additionally, culture supernatants were used as the enzyme source for screening in the place of pure FAE.

Phenyl 2,3,5-tri-*O*-acetyl-1-thio-α-L-arabinofuranoside 2

The title compound was prepared as recently described.[13]

***N*-Acetyl-5-bromo-4-chloro-3-indolyl 2,3,5-tri-*O*-acetyl-α-L-arabinofuranoside 4**

The compounds **2** (225 mg, 0.60 mmol) and **3** (210 mg, 0.73 mmol) were stirred in dry CH_2Cl_2 (4 mL) under Ar at −10 °C in presence of activated 4 Å molecular sieves (200 mg) for 10 min. Then *N*-iodosuccinimide (165 mg, 0.73 mmol) and $Sn(OTf)_2$ (50 mg, 0.122 mmol) were successively added to the reaction. After 20 min the reaction was neutralized with Et_3N. The mixture was filtered through Celite and the filtrate was diluted with CH_2Cl_2 and washed successively with a $Na_2S_2O_3$ solution, then with water, the organic phase was dried over Na_2SO_4. The crude product was purified by flash column chromatography (toluene–ethyl acetate, 9:1→8:2 v/v) to give the title compound **4** as an amorphous solid (204 mg 58%); m.p. 158–160 °C (MeOH); $[\alpha]_D$ −49 (*c* 0.5 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.17–8.29 (3H, m, H-indoxyl), 5.65 (1H, s, $J_{1,2}$ <1 Hz, H-1), 5.44 (1H, d, $J_{2,3}$ 0.7 Hz, H-2), 5.11 (1H, d, $J_{3,4}$ 4 Hz, H-3), 4.31–4.50 (3H, m, H-4, H-5a, H-5b), 2.59 (3H, s, N–CH_3C=O), 2.13, 2.15, 2.17 (9H, 3 × s, CH_3C=O); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.70, 170.4, 169.7, 168.4 (CH_3C=O), 139.7–109.2 (C-indolyl), 105.5 (C-1), 82.7, 81.0, 76.8 (C-2, C-3, C-4), 63.2 (C-5), 24.1 (N–CH_3C=O), 20.9 (3 × CH_3C=O); *m/z* (FAB) 568 [M + Na]$^+$; HRMS calcd for $C_{21}H_{21}NO_9ClBrNa^+$ 567.9985; found 567.9972.

***N*-Acetyl-5-bromo-4-chloro-3-indolyl 2,3-di-*O*-acetyl-α-L-arabinofuranoside 5**

To compound **4** (150 mg, 0.27 mmol) suspended in isopropanol (10 mL) was added lipase Novozym 435 (1.5 g). The reaction was slowly agitated at 40 °C for 8 h. The mixture was filtered, the solution was evaporated and the crude product was purified by column chromatography (petroleum ether–ethyl acetate, 1:1→3:7 v/v) to give the title compound **6** as a white foam (134 mg, 98%); $[\alpha]_D$ −51 (*c* 0.45 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.25–7,16 (3 H, m, H-indolyl), 5.64 (1H, s, $J_{1,2}$ <1 Hz, H-1), 5.47 (1H, d, $J_{2,3}$ <1 Hz, H-2), 5.15 (1H, d, $J_{3,4}$ 4 Hz, H-3), 4.37–3.86 (3H, m, H-4, H-5a, H-5b), 2.58 (3H, s, N–CH_3C=O), 2.15, 2.14 (6H, 2 × s, CH_3C=O); ^{13} C NMR (75 MHz, $CDCl_3$): δ 170.9, 169.8, 168.4, (CH_3C=O), 139.6–109.2–139 (C-indoxyl), 105.4 (C-1), 85.2, 81.0, 76.8 (C-2, C-3, C-4), 62.0 (C-5), 24.0 (N-CH_3C=O), 20.95, 20.9, (2 × CH_3C=O); *m/z* (ES) 521 [M + NH_4]$^+$, 526 [M + Na]$^+$; HRMS calcd for $C_{19}H_{19}NO_8ClBrNa^+$ 525.9880; found 525.9874.

***N*-Acetyl-5-bromo-4-chloro-3-indolyl 5-*O*-(4-acetoxyferuloyl)-2,3-di-*O*-acetyl-α-L-arabinofuranoside 6a**

To a solution of **5** (108 mg, 0.21 mmol) in pyridine (5 mL) were added DMAP (5 mg, 0.04 mmol) and 4-acetoxyferuloyl chloride (216 mg, 0.85 mmol). The reaction was stirred for 4 h at room temperature and diluted with CH_2Cl_2, washed with H_2O and the organic phase was dried over Na_2SO_4. The crude product was purified by flash column chromatography (toluene–ethyl acetate, 9:1→85:15 v/v) to give the title compound **6a** as a white foam (102 mg, 67%); $[\alpha]_D$ −23 (*c* 0.5 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.25 (1H, d, *J* 8 Hz, H-Ar), 7.68 (1H, d, *J* 16 Hz, HC=CH), 7.02–7.55 (5H, m, H-Ar), 6.40 (1H, d, *J* 14 Hz, HC=CH), 5.65 (1H, s, $J_{1,2}$ <1 Hz, H-1), 5.43 (1H, $J_{2,3}$ <1 Hz, H-2), 5.16 (1H, d, $J_{4,3}$ 4 Hz, H-3), 4.58 (1H, dd, $J_{4,5b}$ 3.5 Hz, $J_{5a,5b}$ 11 Hz, H-5b), 4.53 (1H, m, H-4), 4.51 (1H, dd, $J_{4,5a}$ 5.3 Hz, $J_{5a,5b}$ 11 Hz, H-5a), 3.84 (3H, OMe), 2.55 (3H, s, CH_3C=O), 2.30 (3H, s, N-CH_3C=O), 2.13, 2.12 (6H, 2 × s, CH_3C=O); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.4, 169.6, 168.8, 168.3, 166.4 (C=O), 151.6–109.2 (C-ferulate, C-indolyl), 105.4 (C-1), 82.7, 80.7, 76.9 (C-2, C-3, C-4), 63.3 (C-5), 56.0 (OMe), 24.0 (N–CH_3C=O), 20.9 (2x), 20.8, (CH_3C=O); *m/z* (MALDI) 744.02 [M + Na]$^+$; HRMS calcd for $C_{31}H_{29}NO_{12}ClBrNa^+$ 744.0459; found 744.0476.

***N*-Acetyl-5-bromo-4-chloro-3-indolyl 5-*O*-(4-acetoxycoumaroyl)-2,3-di-*O*-acetyl-α-L-arabinofuranoside 6b**

To a solution of **5** (115 mg, 0.23 mmol) and DMAP (5 mg, 0.04 mmol) in pyridine (5 mL), 4-acetoxy-coumaryl chloride (160 mg, 0.69 mmol) was added portionwise. The reaction was stirred for 2.5 h, diluted with CH_2Cl_2, washed with sat. aq. $NaHCO_3$ solution and H_2O, and dried over Na_2SO_4. The crude product was purified by flash column chromatography (toluene–ethyl acetate, 9:1 v/v) to give the title compound **6b** as a white foam (130 mg, 80%); $[\alpha]_D$ −28 (*c* 0.53 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.24 (1H, d, *J* 8.8 Hz, H-Ar), 7.72 (1H, d, *J* 16 Hz, HC=CH), 7.54–7.11 (6H, m, H-Ar), 6.42 (1H, d, *J* 16 Hz, HC=CH), 5.67 (1H, s, $J_{1,2}$ <1 Hz, H-1), 5.45 (1H, s, $J_{2,3}$ <1 Hz, H-2), 5.19 (1H, d, $J_{4,3}$ 4 Hz H-3), 4.49–4.63 (3H, m, H-4, H-5a, H-5b), 2.56 (3H, s, N-CH_3C=O), 2.30, 2.15 (6H, 2 × s, CH_3C=O); ^{13} C NMR (75 MHz, $CDCl_3$): δ 170.3, 169.6, 169.1, 168.3, 166.3 (C=O), 152.4–109.2 (C-ferulate, C-indolyl), 105.4 (C-1), 82.7, 80.7, 76.8 (C-2, C-3, C-4), 63.2 (C-5), 24.0 (N-CH_3C=O), 21.2, 20.9 (×2) (CH_3C=O); *m/z* (ESI): 714.1 [M + Na$^+$], 730.0 [M + K$^+$]; HRMS calcd for $C_{30}H_{27}NO_{11}ClBrNa^+$ 714.0353; found 714.0370.

5-Bromo-4-chloro-3-indolyl 5-*O*-feruloyl α-L-arabinofuranoside 1a

The compound **6a** (100 mg, 0.14 mmol) was suspended in dry MeOH (2 mL). The mixture was cooled with an ice-water bath and 1 M NaOMe (150 μL) was added. After 2 h, the reaction was neutralized with Amberlite H^+ and filtered. The crude product was purified by flash column chromatography (CH_2Cl_2–MeOH,

95:5 v/v) to give the title compound **1a** as a yellowish foam (57 mg, 74%); $[\alpha]_D$ −85 (*c* 0.54 in CH_3OH); ^1H NMR (400 MHz, CD_3OD): δ 7.65 (1H, d, *J* 16 Hz, HC=CH), 7.28 (1H, d, *J* 8 Hz, H-Ar), 7.17–7.05 (4H, m, H-Ar), 6.80 (1H, d, *J* 8 Hz, H-Ar), 6.40 (1H, d, *J* 16 Hz, HC=CH), 5.37 (1H, s, $J_{1,2}$ <1 Hz, H-1), 4.48 (1H, dd, $J_{4,5a}$ 2.8 Hz, $J_{5a,5b}$ 11.4 Hz, H-5b), 4.41–4.32 (3H, m, H-2, H-4, H-5), 4.03 (1H, dd, *J* 3.7 Hz, *J* 6.2 Hz, H-3), 3.87 (3H, s, OMe); ^{13} C NMR (75 MHz, CD_3OD): δ 169.0 (C=O), 150.5–111.4 (C-1, C-ferulate, C-indolyl), 83.6, 83.3, 79.5, 64.9 (C-2, C-3, C-4, C-5), 56.4 (OMe); *m/z* (ESI): 576.0 (M + Na^+); HRMS calcd for $C_{23}H_{21}NO_8ClBrNa^+$ 576.0036; found 576.0036.

5-Bromo-4-chloro-3-indolyl 5-*O*-coumaroyl α-L-arabinofuranoside 1b

The compound **6b** (110 mg, 0.16 mmol) was suspended in MeOH (3 mL). The mixture was cooled with an ice-water bath and 1 M NaOMe (170 μL) was added. After 90 min, the reaction was neutralized with Amberlite H^+ and filtered. The crude product was purified by flash column chromatography (CH_2Cl_2–MeOH, 95:5 v/v) to give the title compound **1b** as a yellowish foam (72 mg, 86%); ^1H NMR (300 MHz, CD_3OD): δ 7.58 (1H, d, *J* 16 Hz, HC=CH), 6.73–7.37 (7H, m, H-Ar), 6.28 (1H, d, *J* 16 Hz, HC=CH), 5.32 (1H, s, $J_{1,2}$ <1 Hz, H-1), 4.44–4.25 (4H, m, H-2, H-4, H-5a, H-5b), 3.97 (1H, dd, *J* 3.4 Hz, *J* 5.8 Hz, H-3), ^{13}C NMR (75 MHz, CD_3OD): δ 169.0 (C=O), 161.3–111.4 (C-1, C-ferulate, C-indolyl), 83.7, 83.4, 79.6, 65.04 (C-2, C-3, C-4, C-5); *m/z* (ESI): 546.0 [M + Na^+], 562.0 [M + K^+]; HRMS calcd for $C_{22}H_{19}ClBrNa^+$ 545.9931; found 545.9928.

2-Chloro-4-nitrophenyl 2,3,5-tri-*O*-benzoyl-α-L-arabinofuranoside 9

To a solution of **8** (2.6 g, 5.17 mmol) in dry CH_2Cl_2 (35 mL) containing activated 4 Å molecular sieves (2.5 g) was added 2-chloro-4-nitrophenol (1.80 g, 5.17 mmol). The reaction was then cooled to −5 °C and $BF_3{\cdot}Et_2O$ (3.2 mL, 25.85 mmol) was added dropwise to the mixture. After 45 min the reaction was warmed up to room temperature and stirred for 6 h. The mixture was filtered through Celite, and the solution was diluted with CH_2Cl_2, washed successively with sat. aq. $NaHCO_3$ solution and H_2O, and dried with Na_2SO_4. The crude product was purified by flash column chromatography (petroleum ether–ethyl acetate, 8:2 v/v) to give the title compound **9** as a white foam (1.56 g, 49%); $[\alpha]_D$ −46 (*c* 0.5 in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 7.58–8.64 (18H, m, H-Ar), 6.56 (1H, s, H-1), 6.22 (1H, s, H-2), 6.06 (1H, d, $J_{3,4}$ 2.9 Hz, H-3), 5.18–5.04 (3H, m, H-5, H-5′, H-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ 166.1, 165.6, 165.3 (*C*=O), 156.5–115.3 (C-Ar), 104.3 (C-1), 83.7 (C-4), 81.6 (C-2), 77.1 (C-3), 63.3 (C-5); *m/z* (DCI) 635 [M + NH_4]$^+$; HRMS calcd for $C_{32}H_{24}NO_{10}ClNa^+$ 640.0986; found 640.0993.

2-Chloro-4-nitrophenyl α-L-arabinofuranoside 10

Compound **9** (1.0 g, 1.62 mmol) was suspended in dry MeOH (25 mL), the reaction was cooled with an ice-water bath and 1 M NaOMe (350 μL) was added to the mixture. After 2 h, the reaction was complete and was neutralized with silica. The crude product was evaporated and purified by flash column chromatography (CH_2Cl_2–MeOH, 10:0→98:2 v/v) to give the title compound **10** as a yellowish solid (360 g, 72%); $[\alpha]_D$ −201 (*c* 0.2 in CH_3OH); ^1H NMR (300 MHz, CD_3OD): δ 8.23 (1H, d, *J* 2.9 Hz, H-Ar), 8.12 (1H, dd, *J* 9.3 Hz, *J* 2.9 Hz, H-Ar), 7.4 (1 H, d, *J* 9.3 Hz, H-Ar), 4.39 (1H, dd, $J_{1,2}$ 1.5 Hz, $J_{2,3}$ 3.9 Hz, H-2), 5.70 (1 H, d, $J_{1,2}$, H-1), 4.01–4.12 (2H, m, H-3, H-4), 3.79 (1H, dd, $J_{5,4}$ 2.9 Hz, H-5b), 3.67 (1H, dd, $J_{5,4}$ 4.8 Hz, $J_{5a,5b}$ 12.2 Hz, H-5a); ^{13}C NMR (75 MHz, CD_3OD): δ 117.1–159.2 (C-Ar), 108.8 (C-1), 87.0, 83.8, 78.2 (C-2, C-3, C-4), 62.6 (C-5); *m/z* (ES) 328 [M + Na]$^+$; HRMS calcd for $C_{11}H_{12}NO_7ClNa^+$ 328.0200; found 328.0106.

2-Chloro-4-nitrophenyl 2,3,5-tri-*O*-acetyl-α-L-arabinofuranoside 11

2-Chloro-4-nitrophenyl α-L-arabinofuranoside **10** (360 mg, 1.18 mmol) was dissolved in pyridine (5 mL). The reaction was ice-cooled and acetic anhydride (1 mL) was added to the mixture. After addition, the reaction was stirred for 3 h at room temperature. After concentration *in vacuo* and coevaporation with toluene, the residue was purified by silica gel chromatography (petroleum ether–ethyl acetate, 8:2 v/v) to afford the title compound **11** as a syrup (470 mg, 93%); $[\alpha]_D$ −121 (*c* 0.5 in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 8.24 (1H, d, *J* 2.4 Hz, H-Ar), 8.08 (1H, dd, *J* 9.3 Hz, *J* 2.4 Hz, H-Ar), 7.23 (1H, d, *J* 9.3 Hz, H-Ar), 5.83 (1H, s, H-1), 5.41 (1H, d, $J_{2,3}$, H-2), 5.07 (1H, dd, $J_{2,3}$ 1 Hz, $J_{3,4}$ 3.8 Hz, H-3), 4.37–4.40 (2H, m, H-4, H-5b), 4.23 (1H, dd, $J_{4,5}$ 6.8 Hz, $J_{5a,5b}$ 12.1 Hz, H-5a), 2.12, 2.11, 2.06 (12 H, 3 × s, 3 × CH_3C=O); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 170.0, 169.4 (3 × CH_3C=O), 156.4–115.2 (C-Ar), 103.9 (C-1), 82.8, 80.5, 76.4 (C-2, C-3, C-4), 62.7 (C-5), 20.56 (×3) (*C*H_3C=O); *m/z* (ES) 453.9 [M + Na]$^+$; HRMS calcd for $C_{17}H_{18}NO_{10}ClNa^+$ 454.0517; found 454.0524.

2-Chloro-4-nitrophenyl 2,3-di-*O*-acetyl-α-L-arabinofuranoside 12

The compound **11** (470 mg, 1.09 mmol) was dissolved in isopropanol (25 mL) and lipase Novozym 435 (4.7 g) was added. The reaction mixture was agitated at 120 rpm and at 37 °C for 48 h. The crude product was purified by column chromatography (petroleum ether–ethyl acetate, 3:2 v/v) to give the title compound **12** as a syrup (410 mg, 96%); $[\alpha]_D$ −77 (*c* 0.53 in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 8.24 (1H, d, *J* 2.4 Hz, H-Ar), 8.07 (1H, dd, *J* 9.3 Hz, *J* 2.4 Hz, H-Ar), 7.23 (1H, d, *J′* 9.3 Hz, H-Ar), 5.82 (1H, s, H-1), 5.44 (1H, d, $J_{2,3}$, H-2), 5.12 (1H, dd, $J_{2,3}$ 1.5 Hz, $J_{3,4}$, H-3), 4.25 (1H, dd, $J_{3,4}$ 4.4 Hz, $J_{4,5}$ 8.3 Hz, H-4), 3.77–3.89 (2H, m, H-5a, H-5b), 2.11 (6H, s, CH_3C=O); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 169.5 (2 × CH_3C=O), 156.5–115.3 (C-Ar), 103.9 (C-1), 85.3, 81.0, 76.3 (C-2, C-3, C-4), 61.6 (C-5), 20.6, 20.5 (2 × *C*H_3C=O); *m/z* (ES) 411.9 [M + Na]$^+$; HRMS calcd for $C_{15}H_{16}NO_9ClNa^+$ 412.0411; found 412.0409.

2-Chloro-4-nitrophenyl 5-*O*-(4-acetoxyferuloyl)-2,3-di-*O*-acetyl-α-L-arabinofuranoside 13

To a solution of **12** (395 mg, 1.01 mmol) and DMAP (14 mg, 0.12 mmol) in pyridine (5 mL), 4-acetoxyferuloyl chloride[23] (200 mg, 0.77 mmol) was added portionwise. The reaction was stirred at room temperature. for 3.5 h then coevaporated with toluene. Then the residue was diluted with CH_2Cl_2 (100 mL) and washed successively with HCl (1M), sat. aq. $NaHCO_3$ solution, H_2O and dried with $NaSO_4$. The crude product was purified by column chromatography (petroleum ether– ethyl acetate, 6:4 v/v)

to give the title compound **13** as a white foam (240 mg, 66%); $[\alpha]_D$ −42 (*c* 0.52 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.27 (1H, d, *J* 2.4 Hz, H-Ar), 8.10 (1H, dd, *J* 9.3 Hz, *J* 2.4 Hz, H-Ar), 2.11, 2.13, 2.28 (9 H, 3 × s, 3 × CH_3C=O), 3.82 (3H, s, OMe), 4.37–4.56 (3H, m, H-4, H-5a, H-5b), 5.17 (1H, d, $J_{3,4}$ 3.4 Hz, H-3), 7.65 (1H, d, *J* 16 Hz, HC=CH), 7.25 (1H, d, *J* 9.3 Hz, H-Ar), 7.00–7.01 (3H, m, H-Ar), 6.36 (1H, d, *J* 16 Hz, HC=CH), 5.85 (1H, s, H-1), 5.44 (1H, d, $J_{2,3}$ 1 Hz, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.1, 169.4, 168.7, 166.2 (3 × CH_3C=O, C=O), 156.5–111.3 (C-Ar, 2 × HC=CH), 104.0 (C-1), 82.98, 80.65, 77.4 (C-2, C-3, C-4), 62.9 (C-5), 55.9 (OMe), 29.7, 20.7, 20.6 (CH_3C=O); *m*/*z* (ES) 630.1 $[M + Na]^+$. HRMS calcd for $C_{27}H_{26}NO_{13}ClNa^+$ 630.0990; found 630.0993.

2-Chloro-4-nitrophenyl 5-*O*-feruloyl-α-L-arabinofuranoside 7

Compound **13** (160 mg, 0.26 mmol) was dissolved in CH_2Cl_2–MeOH (4 mL) (1:1, v:v). The reaction was ice-cooled and K_2CO_3 (75 mg, 0.54 mmol) was added to the mixture. The reaction was allowed to warm up to room temperature. After 6.5 h the reaction was complete and neutralized with silica. The crude product was purified by two flash columns chromatography (hexane–acetone, 8:2→7:3 v/v) and (CH_2Cl_2–MeOH, 98:2 v/v) to give the title compound **7** as a yellowish solid (80 mg, 64%); $[\alpha]_D$ −55 (*c* 1.2 in MeOH); ^{1}H NMR (300 MHz, CD_3OD): δ), 8.23 (1H, d, *J* 2.9, H-Ar), 8.1 (1H, dd, *J* 9.3 Hz, *J* 2.9 Hz, H-Ar), 7.70 (1H, d, *J* 16 Hz, HC=CH), 7.36 (1H, d, *J* 9.3, H-Ar), 7.11 (1H, d, *J* 2 Hz, H-Ar), 6.99 (1H, dd, *J* 2 Hz, *J*′ 8 Hz, H-Ar), 6.75 (1H, d, *J* 8 Hz, H-Ar), 6.32 (1H, d, *J* 16 Hz, HC=CH), 5.72 (1H, d, $J_{1,2}$ 1.3 Hz, H-1), 4.43–4.03 (5 H, m, H-2, H-3, H-4, H-5a, H-5b), 3.83 (3H, s, OMe); ^{13}C NMR (75 MHz, CD_3OD): δ 169.1 (C=O), 159.2–108.9 (C-1, C-Ar, HC=CH), 84.3, 84.0, 79.0 (C-2, C-3, C-4), 64.9 (C-5), 56.7 (OMe); *m*/*z* (ES) 504.08 $[M + Na]^+$. HRMS calcd for $C_{21}H_{20}NO_{10}ClNa^+$ 504.0673; found 504.0674.

Acknowledgements

Ms Patricia Chaud and Stéphanie Boullanger are thanked for technical assistance. This work was supported by CNRS, INRA and in part, by Grants 0401C0042 from ADEME and PNRB2005-11 from ANR.

References

1 K. Mazeau, C. Moine, P. Krausz and V. Gloaguen, *Carbohydr. Res.*, 2005, **340**, 2752–2760.
2 L. Saulnier and J.-F. Thibault, *J. Sci. Food Agric.*, 1999, **79**, 396–402.
3 T. Ishii and T. Hiroi, *Carbohydr. Res.*, 1990, **196**, 175–183.
4 T. Ishii, *Plant Sci.*, 1997, **127**, 111–127.
5 M. G. Tabka, I. Herpoel-Gimbert, F. Monod, M. Asther and J. C. Sigoillot, *Enzyme Microb. Technol.*, 2006, **39**, 897–902.
6 P. Couthino and H. Henrissat, 1999, http://afmb.cnrs-mrs.fr/CAZY/.
7 M.-C. Ralet, C. B. Faulds, G. Williamson and J.-F. Thibault, *Carbohydr. Res.*, 1994, **263**, 257–269.
8 V. Puchart, M. Vrsanska, M. Mastihubova, E. Topakas, C. Vafiadi, C. B. Faulds, M. Tenkanen, P. Christakopoulos and P. Biely, *J. Biotechnol.*, 2007, **127**, 235–243.
9 M. Claeyssens and G. Aerts, *Bioresour. Technol.*, 1992, **39**, 143–146.
10 E. Farkas, L. Janossy, J. Harangi, L. Kandra and A. Liptak, *Carbohydr. Res.*, 1997, **303**, 407–415.
11 S. Maas, *Bio Techniques*, 1999, **27**, 1126–1128.
12 W. Berlin and B. Sauer, *Anal. Biochem.*, 1996, **243**, 171–175.
13 X. Zhu, S. Kawatkar, Y. Rao and G.-J. Boons, *J. Am. Chem. Soc.*, 2006, **128**, 11948–11957.
14 M. Mastihubova, V. Mastihuba, L. Kremnicky, J. L. Willet and G. L. Côté, *Synlett*, 2001, **10**, 1559–1560.
15 M. Gao, B. H. Mock, G. D. Hutchins and Q.-H. Zheng, *Bioorg. Med. Chem.*, 2005, **13**, 6233–6243.
16 C. Rémond, M. Frechichi, N. Aubry, R. Plantier-Royon, C. Portella and M. O'Donohue, *Tetrahedron Lett.*, 2002, **43**, 9653–9655.
17 C. Williamson, C. B. Faulds and P. A. Kroon,, *Biochem. Soc. Trans.*, 1998, **26**, 205–209.
18 M. Asther, M. Haon, S. Roussos, E. Record, M. Delattre, L. Lesage-Meessen, M. Labat and M. Asther, *Proc. Biochem.*, 2002, **38**, 685–691.
19 E. Record, M. Asther, C. Sigoillot, S. Pagès, P. J. Punt, M. Delattre, M. Haon, C. A. M. J. J. Van Den Hontel, J. C. Sigoillot, L. Lesage-Meessen and M. Asther, *Appl. Microbiol. Biotechnol.*, 2003, **62**, 349–355.
20 A. Levasseur, I. Benoit, M. Asther, M. Asther and E. Record, *Protein Expr. Purif.*, 2004, **37**, 126–133.
21 L. Saulnier, C. Marot, M. Elgorriaga, E. Bonnin and J.-F. Thibault, *Carbohydr. Polym.*, 2001, **45**, 269–275.
22 T. Debeche, N. Cummings, I. Connerton, P. Debeire and M. J. O'Donohue, *Appl. Environ. Microbiol.*, 2000, **66**, 1734–1736.
23 R. D. Hatfield, R. F. Helm and J. Ralph, *Anal. Biochem.*, 1991, **194**, 25–33.

