Chemoenzymatic Synthesis of a Bifunctionalized Cellohexaoside as a Specific Substrate for the Sensitive Assay of Cellulase by Fluorescence Quenching**

Viviane Boyer,[a] Sébastien Fort,[a] Torben P. Frandsen,[b] Martin Schülein,[b] Sylvain Cottaz,[a] and Hugues Driguez*[a]

Abstract: A new bifunctionalized cellohexaose derivative was synthesized as a specific substrate for continuous assay of cellulases by resonance energy transfer. This cellohexaoside has a naphthalene moiety (EDANS) as a fluorescent energy donor at the reducing end and a 4-(4′-dimethylaminobenzeneazo)-benzene derivative as an acceptor chromophore at the non-reducing end. The key steps for the preparation of the target molecule involved transglycosylation reactions of cellobiosyl and cellotetraosyl fluoride donors onto cellobiosyl acceptors catalysed by the E197A mutant of cellulase Cel7B from *Humicola insolens*. Upon digestion with various cellulases, the energy transfer was disrupted and an increase of fluorescence was observed.

Keywords: carbohydrates · cellulase · enzymes · fluorescence resonance energy transfer · glycosynthase

Introduction

Cellulose, one of the most abundant polysaccharides on earth, is synergistically hydrolyzed by the cellulases usually classified as *endo*-1,4-β-D-glucan hydrolase [EG; EC 3.2.1.4] and 1,4-β-D-glucan cellobiohydrolase, known as *exo*-enzyme [CBH; EC 3.2.1.91].[2] Glycoside hydrolases can be grouped into different families according to a classification based on amino acid sequence similarities, and, quite unexpectedly, this classification does not coincide with the above biochemical division, since a given family can contain CBH and EG.[3] Heterogeneity of native cellulose has always impeded the use of the natural substrate for biochemical characterization of cellulases; soluble cellooligosaccharides labeled at their reducing end have proven useful for the specificity mapping of various cellulases. However, kinetic studies involved hplc monitoring of hydrolysis and therefore precluded a continuous assay.[4] These problems can be overcome by exploiting the fluorescence resonance energy transfer on bifunctionalized fluorogenic substrates.[5] We have described the synthesis of such fluorogenic penta- and tetrasaccharides, which proved to be excellent substrates for the study of α-amylases,[6] cellulases,[7] and chitinases.[8] In the 1,4-α-glucan series, such a substrate was useful for discrimination between the *exo* and *endo* types of action of starch-degrading enzymes.[6] In the 1,4-β-glucan series, the comparison of catalytic constants with those obtained on reduced cellodextrins showed that the aromatic groups at both ends of the fluorogenic tetrasaccharide did not hinder the binding in the catalytic sites of cellobiohydrolases, even so classified as *exo*-acting cellulases.[7] However, this bifunctionalized tetrasaccharide was no good as a subtrate for some *endo*-acting cellulases such as Cel45 (formerly EGV) from *Humicola insolens*,[9] or CelF from *Clostridium cellulolyticum*.[10] This was presumably related to the substrate specificity of these enzymes, which require a cellodextrin with at least five consecutive unmodified β-D-glucosyl units for binding and hydrolysis. Owing to the importance of these enzymes in the textile and detergent industries,[11] the synthesis of a longer oligosaccharide has been undertaken. In this paper, we report the preparation of a bifunctionalized cellohexaoside and its usefulness for sensitive and continuous assay of cellulases.

[a] Dr. H. Driguez, Dr. V. Boyer, Dr. S. Fort, Dr. S. Cottaz
Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS)
Affiliated with Université Joseph Fourier
B.P. 53, 38041 Grenoble cedex 9 (France)
Fax: (+33) 4-76-54-72-03
E-mail: hugues.driguez@cermav.cnrs.fr

[b] Dr. T. P. Frandsen, Dr. M. Schülein[+]
Novozymes
Smoermosevej 25, 2880 Bagsvaerd (Denmark)

[+] Deceased in July 2001.

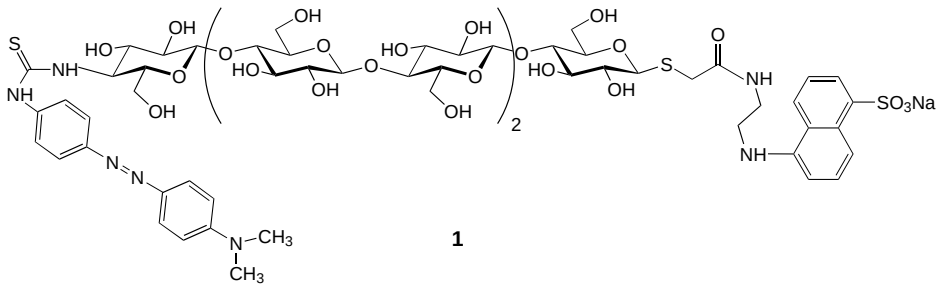
[**] Glycosynthase-Assisted Synthesis of Oligosaccharides, Part III. For Part II, see reference [1].

Results and Discussion

In earlier work,[7] the acceptor chromophore – donor fluorophore pair introduced at both ends of the β-(1 → 4) tetrasac-

  0947-6539/02/0806-1389 $ 17.50+.50/0

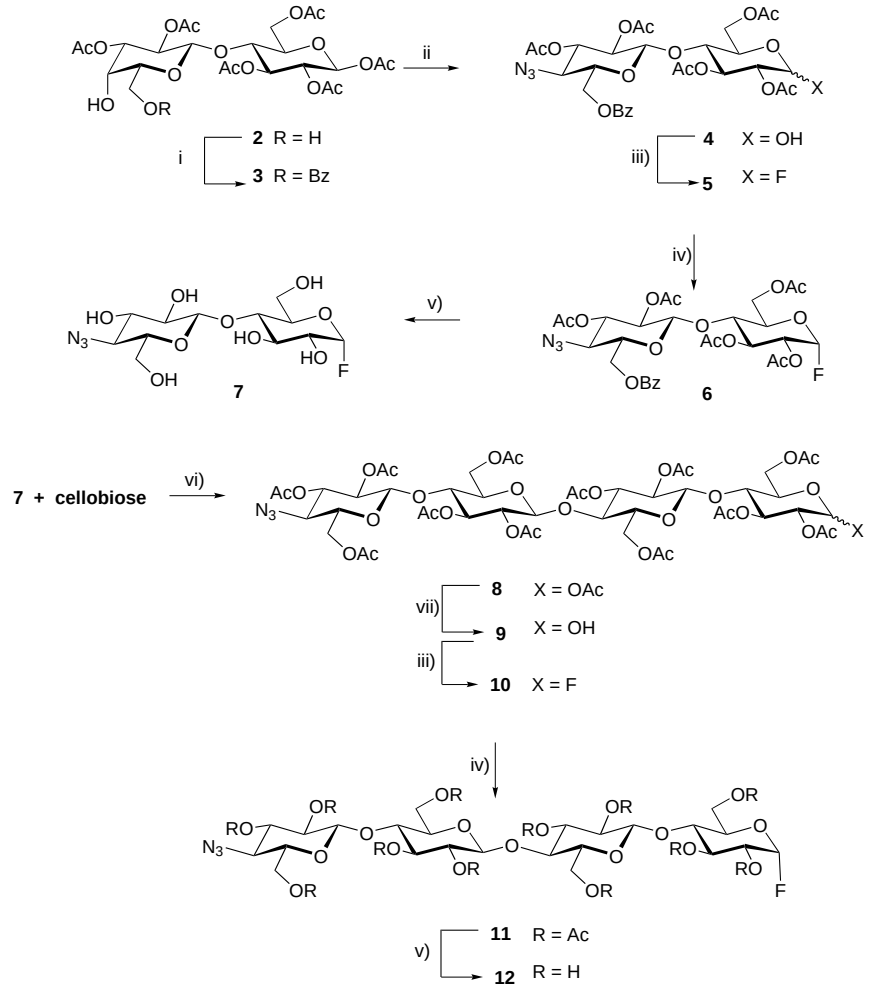
charide, indolethyl-EDANS, was mainly chosen for synthetic considerations. The efficiency of fluorescence resonance energy transfer (FRET) depends on the distance between the two groups, their spectral overlap, and the lifetime of the excited fluorophore. Since in the present work the length of the oligosaccharide was formally increased from 20 Å to 30 Å, we decided to prepare the conjugate (target compound **1**) with a most effective combination: a 4-(4′-dimethylaminobenzeneazo)benzene derivative as acceptor chromophore ($\lambda_{max} = 470-500$ nm) and (2′-aminoethyl)aminonaphthalene sulfonic acid (EDANS) as donor fluorophore (excitation $\lambda_{max} = 340$ nm, emission $\lambda_{max} = 490$ nm).[8] Furthermore, the relatively long EDANS lifetime (13 ns) and its ionic character should improve the quenching efficiency and solubility, respectively.[12]



1

The main task of the synthesis of **1** was the preparation of a cellohexaoside suitably functionalized on both ends, avoiding a cumbersome and low-yielding chemical approach. This was achieved by developing a new methodology based on protein engineering, retaining β-glycoside hydrolases in which the carboxylate nucleophile (a glutamic acid) was changed to an alanine.[1, 13, 14] As expected, they were unable to form the glycosyl enzyme intermediate, had no hydrolytic activity, yet were able to catalyse the quantitative transglycosylation of α-glucosyl fluorides (which mimic the glycosyl enzyme) on glucoside acceptors. This strategy of glycosylation of suitably activated donors (glycosyl fluoride) onto various acceptors catalysed by the Glu-197-Ala mutant of cellulase Cel7B from *H. insolens* was used as exemplified.

Hexa-*O*-acetyl lactose (**2**)[15] was treated with 1-(benzoyloxy)-benzotriazole in the presence of triethylamine in dichloromethane[16] to give the monoalcohol **3** in 72% yield (Scheme 1). Esterification of the remaining hydroxyl group with trifluoromethanesulfonic anhydride (triflic anhydride), followed by triflate displacement with sodium azide in the presence of 18-crown-6 in hexamethylphosphoramide (HMPA) gave rise to an azido derivative as a major compound. Unfortunately, anomeric de-*O*-acetylation had occurred during the reaction and compound **4** was isolated in 66% yield. This compound was subjected to DAST treatment, and as expected an anomeric mixture of fluorides **5** was obtained. Anomerization with commercially available pyridine–hydrogen fluoride reagent gave pure α-fluoride **6**,



Scheme 1. Syntheses of the disaccharide and the tetrasaccharide donors **7** and **12**. i) 1-(Benzoyloxy)-benzotriazole, TEA, CH_2Cl_2 (72%); ii) Tf_2O, pyridine, CH_2Cl_2, 0 °C to RT, then NaN_3, 18-crown-6, HMPA, 50 °C (66%); iii) DAST, CH_2Cl_2, −30 °C (70%); iv) HF/pyridine, −50 °C to −10 °C (88%); v) MeONa, MeOH, 0 °C (98%); vi) Cel7B Glu197Ala, phosphate buffer, 40 °C, then Ac_2O, pyridine, DMAP (66%); vii) NH_2NH_2-AcOH, DMF, 50 °C (91%); iii) DAST, CH_2Cl_2, −30 °C (85%); iv) HF/pyridine, −50 °C to −10 °C (84%); v) MeONa, MeOH, 0 °C (100%).

0947-6539/02/0806-1390 $ 17.50+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 6

which was isolated in 61% yield over the two steps. The 4[II]-azido-α-cellobiosyl fluoride (**7**) was then prepared in quantitative yield by catalytic de-*O*-acetylation of **6**. The condensation of **7** with an equimolecular amount of cellobiose gave the expected tetrasaccharide **8** in 66% yield after acetylation. This glycosylation reaction catalysed by the Glu-197-Ala mutant of Cel7B from *H. insolens* was conducted as already reported.[1] De-*O*-acetylation of the anomeric position of **8** with the mild hydrazine acetate procedure gave compound **9**, which was converted via the anomeric mixture of fluorides **10** into the pure α-fluoride **11** as already described for the preparation of **6** in 65% yield over the three steps.

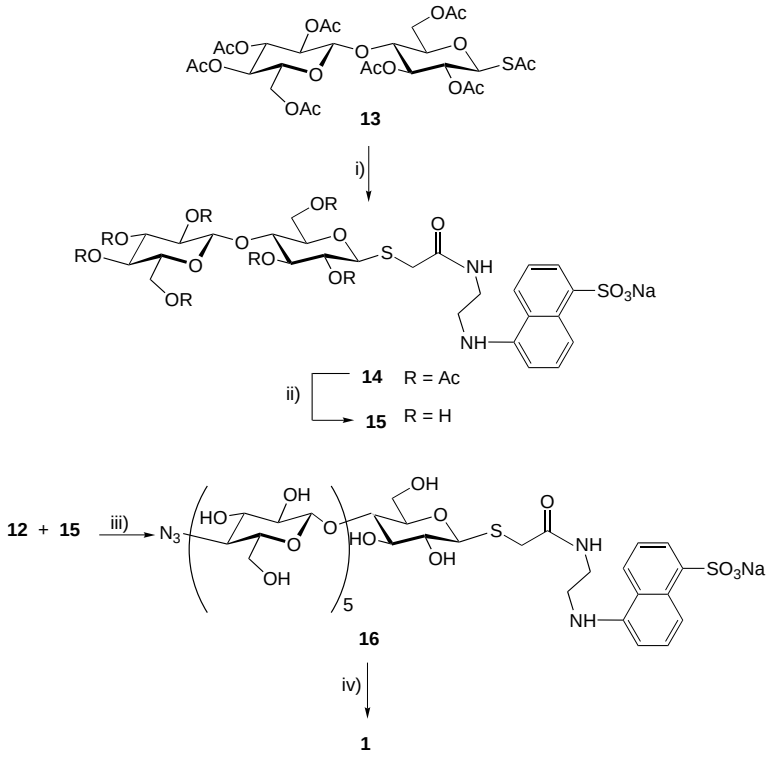
The acetylated precursor of the acceptor synthon **15** (Scheme 2) was prepared by selective de-*S*-acetylation and activation of fully acetylated 1-*S*-β-cellobiose (**13**),[17] using cysteamine in HMPA in the presence of 1,4-dithioerythritol (DTE),[18] and reaction with the commercially available *N*-iodoacetyl-EDANS. The expected fluorescent cellobioside **14** was obtained in 79% yield. Mild de-*O*-acetylation of both **11** and **14** led to **12** and **15**, respectively, which were incubated with the Glu-197-Ala mutant of Cel7B. The expected hexasaccharide **16** was isolated in 94% yield. Mild reduction of the azide was achieved by means of hydrogen sulfide,[19] and the resulting amine was coupled with the commercially available 4-dimethylaminophenylazophenyl isothiocyanate (DABITC). The target molecule **1** was obtained in 70% yield over the two steps.

Enzymatic hydrolysis of the heterobifunctionalized hexasaccharide substrate **1**, monitored by fluorescence energy transfer, was evaluated with four of the seven cellulases found in *H. insolens*, Cel6A, Cel6B, Cel7B, and Cel45A. Previously, no synthetic heterobifunctionalized tetrasaccharide compound was known as a substrate for Cel45A;[7] however, in the present study, compound **1** was found to be a good substrate for Cel45A with a K_m value of 28 μM. This is in good accordance with earlier studies showing an almost 1000-fold higher specificity for hydrolysis of reduced cellohexaose compared to reduced cellotetraose.[20] The relative rates for the hydrolysis of **1** with different cellulases are given in Table 1, and show this hexasaccharide to be a potent probe for the characterization of Cel45A.

Table 1. Relative rates for hydrolysis of **1** by cellulases Cel6A, Cel6B, Cel7B, and Cel45A.[a]

Cel6A	Cel6B	Cel7B	Cel45A
10	100	16	47

[a] Velocity relative to Cel6B (%), determined at 37 °C for substrate **1** (123 μM) in 0.1M MOPS at pH 7.5.



Scheme 2. Synthesis of the target molecule **1**. i) Sodium *N*-(iodoacetamidoethyl)-1-naphthylamine-5-sulfonate, DTE, cysteamine, HMPA (93%); ii) MeONa, MeOH (99%); iii) Cel7B Glu197Ala, phosphate buffer, 40 °C (94%); iv) H_2S, pyridine, H_2O, then 4-dimethylaminophenylazophenyl isothiocyanate, $NaHCO_3$, DMF, 40 °C (70%).

Experimental Section

General procedures: Roman numerals in ascending order are given to the residues from the reducing end. NMR spectra were recorded on a Bruker AC300, Bruker Avance 400 or Varian Unity 500. Proton chemical shifts (δ) are reported in ppm downfield from TMS. Coupling constants (J) are given in hertz (Hz) as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), multiplet (m), broad (b). Carbon chemical shifts (δ) are reported in ppm with the solvent as internal reference. High-resolution mass spectra (HRMS) were recorded on VG ZAB and low-resolution (MS) on a Nermag R-1010C spectrometers. Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Melting points (mp) were measured on a Büchi 535 apparatus. Microanalyses were performed by the Laboratoire Central d'Analyses du CNRS (Vernaison). Evolution of reactions was monitored by analytical thin-layer chromatography using silica gel 60 F254 precoated plates (E. Merck, Darmstadt). All reactions in organic medium were carried out under argon using freshly distilled solvents. After work-up, organic phases were dried over anhydrous Na_2SO_4.

2,3-Di-*O*-acetyl-6-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (3): A solution of 2,3-di-*O*-acetyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-acetyl-β-D-glucopyranose (**2**; 1 g, 1.7 mmol), 1-(benzoyloxy)-benzotriazole (453 mg, 2 mmol), and triethylamine (0.35 mL, 2.5 mmol) in dichloromethane (10 mL) was stirred for 12 h at room temperature. The resulting solution was diluted with dichloromethane, and washed successively with aq potassium hydrogensulfate, saturated aq sodium hydrogencarbonate, and brine, dried (Na_2SO_4), and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (1:1)] gave the

monohydroxy compound **3** (845 mg, 72%). $[\alpha]_D^{25} = +12$ ($c = 0.83$ in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.02$ (m, 2H; arom Hs), 7.49 (m, 3H; arom Hs), 5.65 (d, $^3J = 8.2$ Hz, 1H), 5.20 (m, 2H), 5.01 (dd, $^3J = 8.2$ and 9.6 Hz, 1H), 4.88 (dd, $^3J = 3.5$ and 10.3 Hz, 1H), 4.63 (dd, $^3J = 6.2$ and 11.3 Hz, 1H), 4.41 (m, 3H), 4.09 (m, 2H), 3.80 (m, 3H), 2.06–1.99 (m, 18H; $6 \times OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 169.4-168.8$ ($6 \times O\underline{C}OCH_3$ and $O\underline{C}OC_6H_5$), 133.5–128.6 (arom Cs), 101.1 (C-1^{II}), 91.7 (C-1^I), 75.8, 73.6, 73.3, 72.5, 72.4, 70.4, 69.5, 66.9 (C-$2^{I,II}$, C-$3^{I,II}$, C-$4^{I,II}$, C-$5^{I,II}$), 62.3–61.7 (C-$6^{I,II}$); 20,7–20,6 ($6 \times OCOCH_3$); elemental analysis calcd (%) for $C_{31}H_{38}O_{18}$: C 53.30, H 5.48%; found: C 53.07, H 5.47.

2,3-Di-*O*-acetyl-4-azido-6-*O*-benzoyl-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-D-glucopyranose (4): Trifluoromethylsulfonyl anhydride (0.3 mL, 2.8 mmol) was added dropwise to a stirred solution of the hydroxy derivative **3** (600 mg, 0.88 mmol) in anhydrous dichloromethane/pyridine (30 mL [10:1]) at 0°C. The mixture was stirred for 30 min while the reaction mixture was slowly reaching room temperature. The mixture was washed successively with aq potassium hydrogensulfate, saturated aq sodium hydrogencarbonate, and brine, dried (Na_2SO_4), and concentrated under reduced pressure. The triflate was obtained in quantitative yield and used in the subsequent step without further purification.

A mixture of this triflate derivative (730 mg, 0.88 mmol), sodium azide (286 mg, 4.4 mmol), and 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6; 5 mg) in HMPA (30 mL) was heated at 50°C for 12 h. The reaction mixture was diluted with diethyl ether, then was washed three times with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (3:7)] gave the azido derivative **4** (394 mg, 66%); IR (KBr): $\tilde{\nu} = 2113$ cm^{-1} (N_3); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.02$ (m, 2H; arom Hs), 7.50 (m, 3H; arom Hs), 5.45 (t, $^3J = 9.5$ Hz, 1H), 5.31 (d, $^3J = 3.7$ Hz, 1H), 5.16 (t, $^3J = 10.4$ Hz, 1H), 4.66 (m, 6H), 4.05 (m, 2H), 3.65 (m, 3H), 2.05–1.86 (m, 15H; $5 \times OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.2-169.3$ ($5 \times O\underline{C}OCH_3$), 165.9 ($O\underline{C}OC_6H_5$), 133.5–128.4 (arom Cs), 100.6 (C-1^{II}), 95.0 (C-$1^I\beta$), 89.8 (C-$1^I\alpha$), 73.9, 73.3, 72.9, 72.2, 71.8, 71.2, 69.1, 68.2, 63.1 (C-$2^{I,II}$, C-$3^{I,II}$, C-4^I, C-$5^{I,II}$), 61.8, 61.6 (C-$6^{I,II}$), 59.9 (C-4^{II}), 20.7–20.4 ($5 \times OCOCH_3$); FAB$^+$ MS: m/z: 682 [M^++H], 704 [M^++Na].

2,3-Di-*O*-acetyl-4-azido-6-*O*-benzoyl-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl fluoride (5): Diethylaminosulfur trifluoride (0.1 mL, 0.75 mmol) was added dropwise to a stirred solution of the azido derivative **4** (100 mg, 0.147 mmol) in anhydrous dichloromethane (5 mL) at −30°C. The solution was stirred for 2 h, then washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (2:1)] of the residue gave the fluoride **5** (70 mg, 70%) as a syrup containing the α and β anomers in the ratio 1:6. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.05$ (m, 2H; arom Hs), 7.50 (m, 3H; arom Hs), 5.57 (dd, 1H; $^3J_{1,2} = 3$ Hz, $^3J_{1,F} = 53$ Hz; H-$1^I\alpha$), 5.28 (dd, 1H; $^3J_{1,2} = 5.6$ Hz, $^3J_{1,F} = 53$ Hz; H-$1^I\beta$), 5.14 (m, 2H), 4.90 (m, 2H), 4.52 (m, 4H), 4.07 (m, 1H), 3.72 (m, 2H), 3.54 (m, 1H), 2.08–1.89 (m, 15H; $5 \times OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$), β anomer: $\delta = 170.1-169.1$ ($5 \times O\underline{C}OCH_3$), 165.9 ($O\underline{C}OC_6H_5$), 133.6–128.7 (arom Cs), 105.8 (d, $^3J_{1,F} = 217$ Hz; C-1^I), 100.6 (C-1^{II}), 75.4, 73.8, 72.6, 72.4, 71.7, 71.0, 70.7 (C-$2^{I,II}$, C-$3^{I,II}$, C-4^I, C-$5^{I,II}$), 63.0, 61.5 (C-$6^{I,II}$), 59.9 (C-4^{II}), 20.7–20.5 ($5 \times O\underline{C}OCH_3$); DCI$^+$ MS: m/z: 701 [M^++NH_3+H].

2,3-Di-*O*-acetyl-4-azido-6-*O*-benzoyl-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl fluoride (6): In a plastic vessel, a solution of the α/β-fluoride **5** (59 mg, 0.9 mmol) in hydrogen fluoride/pyridine (2 mL [7:3]) was stirred at −50°C for 15 min; then the temperature of the cooling bath was raised to −10°C for 2 h. The solution was diluted with dichloromethane (20 mL), then poured into a plastic beaker containing an ice-cooled solution of ammonia (25 mL, 3M). The organic layer was washed with saturated aq sodium hydrogencarbonate until neutralization, dried (Na_2SO_4), and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (2:1)] of the residue gave the α-fluoride **6** (52 mg, 88%). $[\alpha]_D^{25} = +42$ ($c = 1.7$, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): $\delta = 8.02$ (m, 2H; arom Hs), 7.57 (m, 1H; arom Hs), 7.47 (m, 2H; arom Hs), 5.61 (dd, $^3J_{1,2} = 1.8$ Hz, $^3J_{1,F} = 53$ Hz, 1H; H-1^I), 5.41 (t, $^3J = 9.7$ Hz, 1H), 5.13 (t, $^3J = 9.3$ Hz, 1H), 4.83 (m, 2H), 4.54 (m, 4H), 4.08 (m, 2H), 3.75 (m, 2H), 3.55 (m, 1H), 2.07–2.00 (m, 15H; $5 \times OCOCH_3$); FAB$^+$ MS: m/z: 694 [M^+ − F], 684 [M^++H], elemental anal. calcd (%) for $C_{27}H_{32}FN_3O_{15}$: C 46.38, H 5.19, F 3.06, N 6.76%; found: C 46.23, H 5.18, F 3.00, N 6.34.

4-Azido-4-deoxy-β-D-glucopyranosyl-(1 → 4)-α-D-glucopyranosyl fluoride (7): A solution of the fluoride **6** (355 mg, 0.52 mmol) in methanol (50 mL) was treated with sodium methoxide (0.2 mL, 1M in MeOH) at 0°C for 3 h. After neutralization with amberlite IRN 77 H^+ and evaporation to dryness, the residue was dissolved in cold deionized water (5 mL), then washed three times with diethyl ether. Freeze-drying of the aqueous phase gave the fluoride **7** (188 mg, 98%). The compound, which was pure by TLC, was only characterized by MS and was immediately used in the enzymatic experiment. FAB$^+$ MS: m/z: 392 [M^++Na].

2,3,4-Tri-*O*-acetyl-4-azido-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (8): Cel7B Glu197Ala glycosynthase (1 mg) was added to a solution of fluoride **7** (50 mg, 0.135 mmol) and cellobiose (46 mg, 0.135 mmol) in sodium phosphate buffer (1 mL, 0.1M, pH 7). The solution was placed in a rotative shaker for 12 h at 40°C and then freeze-dried, and the residue was treated with pyridine (5 mL) and acetic anhydride (5 mL) with a catalytic amount of 4-dimethylaminopyridine (1 mg) at room temperature for 12 h. Methanol (2.5 mL) was added to the reaction mixture at 0°C, the solution was concentrated under reduced pressure, and the residue was dissolved in dichloromethane and washed successively with aq potassium hydrogensulfate, saturated aq sodium hydrogencarbonate, and brine, then dried (Na_2SO_4) and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (1:2)] of the residue gave the tetrasaccharide **8** (110 mg, 66%). ^1H NMR (300 MHz, $CDCl_3$): $\delta = 6.20$ (d, $^3J_{1,2} = 3.8$ Hz, 1H; H-$1^I\alpha$), 5.61 (d, $^3J_{1,2} = 8.2$ Hz, 1H; H-$1^I\beta$), 5.37 (t, $^3J = 9.3$ Hz, 1H), 5.04 (m, 5H), 4.78 (m, 3H), 4.35 (m, 9H), 4.04 (m, 3H), 3.62 (m, 8H), 3.35 (m, 1H), 2.12–1.91 (m, 39H; $13 \times OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 169.8-168.8$ ($13 \times O\underline{C}OCH_3$), 101.0, 100.7, 100.3, 100.2 (C-$1^{II,III,IV}$), 91.5 (C-$1^I\beta$), 88.9 (C-$1^I\alpha$), 76.1, 76.0, 75.8, 73.8, 73.5, 72.7, 72.6, 72.5, 72.4, 72.2, 72.1, 71.9, 71.8, 71.7, 70.7, 70.5, 69.3 (C-$2^{I,II,III,IV}$, C-$3^{I,II,III,IV}$, C-$4^{I,II,III}$, C-$5^{I,II,III,IV}$), 62.6, 62.1, 62.0, 61.6 (C-$6^{I,II,III,IV}$), 59.7 (C-4^{IV}), 20.7–20.4 ($13 \times OCO\underline{C}H_3$); ES$^+$ HRMS: calcd for $C_{50}H_{67}N_3NaO_{33}$ ([M^++Na]) 1260.3555; found: 1260.3554.

2,3,4-Tri-*O*-acetyl-4-azido-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-D-glucopyranose (9): A mixture of tetrasaccharide **8** (231 mg, 0.19 mmol) and hydrazine acetate (21 mg, 0.22 mmol) in DMF (5 mL) was stirred at 50°C until complete dissolution of the hydrazinium salt, TLC analysis indicating the complete conversion of the starting product into a more polar compound. The solution was diluted with ethyl acetate, washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (1:2)] of the residue gave the tetrasaccharide **9** (204 mg, 91%); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.1-169.1$ ($12 \times O\underline{C}OCH_3$), 100.6, 100.4, 100.3 (C-$1^{II,III,IV}$), 95.1 (C-$1^I\beta$), 89.9 (C-$1^I\alpha$, 76.5, 76.2, 73.8, 72.7, 72.1, 71.9, 71.8, 71.7, 71.3, 69.3, 68.1 (C-$2^{I,II,III,IV}$, C-$3^{I,II,III,IV}$, C-$4^{I,II,III}$, C-$5^{I,II,III,IV}$), 62.6, 62.1, 61.7, 60.3 (C-$6^{I,II,III,IV}$), 59.7 (C-4^{IV}), 20.8–20.5 ($12 \times OCO\underline{C}H_3$); FAB$^+$ MS: m/z: 1218 [M^++Na].

2,3,4-Tri-*O*-acetyl-4-azido-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl fluoride (10): Diethylaminosulfur trifluoride (0.12 mL, 0.9 mmol) was added dropwise to a stirred solution of the hydroxy derivative **9** (204 mg, 0.17 mmol) in anhydrous dichloromethane (5 mL) at −30°C. The solution was stirred for 2 h, then washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. Flash column chromatography [petroleum ether/ethyl acetate (1:2)] of the residue gave the fluoride **10** (174 mg, 85%) as a foam containing the α and β anomers in the ratio 1:6. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 5.58$ (dd, $^3J_{1,2} = 3$ Hz, $^3J_{1,F} = 53$ Hz, 1H; H-$1^I\alpha$), 5.30 (dd, $^3J_{1,2} = 5.3$ Hz, $^3J_{1,F} = 53$ Hz, 1H; H-$1^I\beta$), 5.05 (m, 4H), 4.95 (m, 3H), 4.41 (m, 5H), 4.26 (m, 2H), 4.07 (m, 4H), 3.89 (t, $^3J = 9.3$ Hz, 1H), 3.73 (m, 2H), 3.59 (m, 3H), 3.35 (m, 1H), 2.09–1.92 (m, 36H; $12 \times OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$), β anomer: $\delta = 170.2-169.2$ ($12 \times O\underline{C}OCH_3$), 105.7 (d, $^3J_{1,F} = 217$ Hz; C-1^I), 100.6, 100.4, 98.6 (C-$1^{II,III,IV}$), 76.1, 76.0, 75.3, 73.8, 72.8, 72.7, 72.6, 72.4, 72.2, 71.8, 71.7, 71.6, 71.4, 70.9 (C-$2^{I,II,III,IV}$, C-$3^{I,II,III,IV}$, C-$4^{I,II,III}$, C-$5^{I,II,III,IV}$), 62.6, 62.0, 61.6, 60.3 (C-$6^{I,II,III,IV}$), 59.7 (C-4^{IV}), 20,7–20,4 ($12 \times OCO\underline{C}H_3$).

2,3,4-Tri-*O*-acetyl-4-azido-4-deoxy-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl fluoride (11): A solution

0947-6539/02/0806-1392 $ 17.50+.50/0 *Chem. Eur. J.* **2002**, *8*, No. 6

of the α/β-fluoride **12** (174 mg, 0.14 mmol) was treated as described for the synthesis of compound **6**. Flash column chromatography [petroleum ether/ethyl acetate (1:2)] gave the α-fluoride **11** (147 mg, 84%); $[\alpha]_D^{25} = +16$ ($c =$ 0.53, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 5.62$ (dd, $^3J_{1,2} = 2.6$ Hz, $^3J_{1,F} = 53$ Hz, 1H), 5.42 (t, $^3J = 9.9$ Hz, 1H), 5.05 (m, 3H), 4.79 (m, 4H), 4.42 (m, 5H), 4.26 (m, 1H), 4.07 (m, 5H), 3.62 (m, 6H), 3.36 (m, 1H), 2.13–1.92 (m, 36H; $12 \times OCOCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.0$–168.9 ($12 \times OCOCH_3$), 103.5 (d, $^3J_{1,F} = 218$ Hz; C-1), 100.4, 100.2, 99.9 (C-$1^{II,III,IV}$), 76.0, 75.2, 73.6, 72.6, 72.5, 72.4, 71.9, 71.7, 71.6, 70.5, 70.4, 70.1, 68.5 (C-$2^{I,II,III,IV}$, C-$3^{I,II,III,IV}$, C-$4^{I,II,III}$, C-$5^{I,II,III,IV}$), 62.5, 62.0, 61.9, 60.9 (C-$6^{I,II,III,IV}$), 59.5 (C-4^{IV}), 20.6–20.2 ($12 \times OCOCH_3$); FAB$^+$ MS: m/z: 1220 [M^++Na]; elemental anal. calcd (%) for $C_{48}H_{64}FO_{31}N_3$: C 48.12, H 5.38, F 1.59, N 3.51; found: C 47.81, H 5.39, F 1.57, N 3.31.

4-Azido-4-deoxy-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl fluoride (12): The peracetylated fluoride **11** (127 mg, 0.106 mmol) was treated in methanol (15 mL) with sodium methoxide (0.075 mL, 1M in MeOH) for 3 h at 0°C. The solution was neutralized with Amberlite IRN 77 H^+, then concentrated under reduced pressure, and the residue, dissolved in deionized water, was freeze-dried to give the fluoride **12**, which was pure by TLC, and used immediately in the next reaction. FAB$^+$ MS: m/z: 694 [M^++H], 716 [M^++Na].

Sodium *N*-[2-*N*[(*S*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-2-thioacetyl]aminoethyl]-1-naphthylamine-5-sulfonate (14): 1,4-Dithioerythritol (86 mg, 0.55 mmol) and then cysteamine (42 mg, 0.5 mmol) were added to a solution of *N*-(iodoacetamidoethyl)-1-naphthylamine-5-sulfonic acid sodium salt (*N*-iodoacetyl-EDANS, 90 mg, 0.207 mmol) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-1-*S*-acetyl-β-D-glucopyranose (**13**; 280 mg, 0.403 mmol) in HMPA (2 mL), and the mixture was stirred for 2 h at room temperature. Petroleum ether was added (100 mL) and the precipitate was filtered off; the solid was dissolved in dichloromethane and purified by flash column chromatography [dichloromethane to dichloromethane/methanol (1:1)] to give the EDANS conjugate **14** (184 mg, 93%); $[\alpha]_D^{25} = -19.5$ ($c = 0.73$, $CHCl_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 172.8$, 172.4, 172.3, 171.9, 171.7, 171.4, 171.3, 171.2 ($7 \times OCOCH_3$ and $1 \times$ OCONH), 145.4, 141.8, 131.4, 129.0, 126.9, 125.9, 125.7, 124.0, 116.2, 105.6 (arom Cs), 101.8 (C-1^{II}), 84.3 (C-1^{I}), 78.1, 77.9, 75.0, 74.5, 73.1, 72.7, 71.6, 69.3 (C-$2^{I,II}$, C-$3^{I,II}$, C-$4^{I,II}$, C-$5^{I,II}$), 63.7, 62.9 (C-$6^{I,II}$), 44.4 (SCH_2CONH), 39.7 and 39.6 (CH_2NH), 34.9 and 35.0 (CH_2NH), 21.2–20.8 ($7 \times COCH_3$); FAB$^+$ MS: m/z: 959 [M^++H], 981 [M^++Na], 1003 [M^++2Na].

Sodium *N*-[2-*N*[(*S*-(β-D-glucopyranosyl)-(1→4)-β-D-glucopyranosyl)-2-thioacetyl]aminoethyl]-1-naphthylamine-5-sulfonate (15): A solution of EDANS conjugate **14** (184 mg, 0.192 mmol) in methanol (30 mL) and sodium methoxide (0.3 mL, 1M in MeOH) was stirred for 12 h at room temperature. The resulting precipitate was redissolved by addition of deionized water (5 mL), and the solution was neutralized with Amberlite IRN 77 H^+, filtered, and concentrated under reduced pressure. The residue was freeze-dried to give compound **15** (131 mg, 99%). $[\alpha]_D^{25} = -15.4$ ($c =$ 0.23, H_2O); ^{1}H NMR (300 MHz, D_2O): $\delta = 7.95$ (m, 3H; arom Hs), 7.39 (m, 2H; arom Hs), 6.86 (m, 1H; arom H), 4.28 (d, $^3J_{1,2} = 9.5$ Hz, 1H; H-1^{I}), 4.17 (d, $^3J_{1,2} = 8.1$ Hz, 1H; H-1^{II}), 3.74 (dd, $^3J = 1.8$ and 12.4 Hz, 1H), 3.56 (m, 2H), 3.43–3.09 (m, 17H); ^{13}C NMR (75 MHz, D_2O): $\delta = 174.0$ (CONH), 139.8, 130.0, 128.7, 128.5, 128.4, 127.2, 125.5, 125.3, 119.7, 111.6 (arom Cs), 103.3 (C-1^{II}), 85.7 (C-1^{I}), 79.3, 76.7, 76.2, 73.9, 72.8, 70.3 (C-$2^{I,II}$, C-$3^{I,II}$, C-$4^{I,II}$, C-$5^{I,II}$), 61.4, 60.9 (C-$6^{I,II}$), 45.8 (SCH_2CONH), 38.7, 34.0 (CH_2NH); ES$^+$ HRMS calcd for $C_{26}H_{35}N_2Na_2O_{14}S_2$ ([M^++Na]): 709.1325; found: 709.1320; calcd for $C_{26}H_{35}KN_2NaO_{14}S_2$ ([M^++K]): 725.1065; found: 725.1066; calcd for $C_{26}H_{34}N_2Na_3O_{14}S_2$ ([$M^+ - H + 2Na$]): 731.1145, found: 731.1152.

Sodium *N*-[2-*N*[(*S*-(4-azido-4-deoxy-β-D-glucopyranosyl)-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl)-2-thioacetyl]aminoethyl]-1-naphthylamine-5-sulfonate (16): Cel7B Glu197Ala glycosynthase (1 mg) was added to a solution of fluoride **12** (37 mg, 0.053 mmol) and EDANS conjugate **15** (55 mg, 0.08 mmol) in sodium phosphate buffer (1 mL, 0.1M, pH 7). The solution was placed in a rotative shaker for 12 h at 40°C, then purified by loading onto a C-18 cartridge and eluting with water followed by water/methanol ([95:5]). The appropriate fractions were pooled, concentrated under reduced pressure, then freeze-dried to give the fluorescent hexasaccharide **16** (68 mg, 94%). $[\alpha]_D^{25} = -2.4$ ($c = 0.25$, H_2O); ^{1}H NMR (500 MHz, D_2O): $\delta = 8.01$ (m, 3H; arom Hs), 7.45 (m, 2H; arom Hs), 6.77 (d, 1H; arom H), 4.40 (m, 3H; H-1), 4.36 (d, $^3J_{1,2} =$ 8.0 Hz, 1H; H-1), 4.24 (d, $^3J = 9.0$ Hz, 1H; H-1^{I}), 4.08 (d, $^3J = 8.0$ Hz, 1H; H-1), 3.86–3.02 (m, 43H), 2.91 (t, $^3J = 8.5$ Hz, 1H); ^{13}C NMR (75 MHz, D_2O): $\delta = 173.6$ (CONH), 144.1, 138.8, 129.5, 128.8, 126.6, 125.4, 124.7, 123.9, 115.2, 106.5 (arom Cs), 102.7 (C-$1^{II\text{-}VI}$), 85.2 (C-1^{I}), 78.9, 78.7, 75.7, 75.1, 74.7, 74.3, 73.5, 73.3, 72.3 (C-$2^{I\text{-}VI}$, C-$3^{I\text{-}VI}$, C-$4^{I\text{-}V}$, C-$5^{I\text{-}VI}$), 61.9 (C-4^{VI}), 61.1, 60.4, 60.2 (C-$6^{I\text{-}VI}$), 43.0 (SCH_2CONH), 38.9, 33.8 (CH_2NH); ES$^+$ HRMS calcd for $C_{50}H_{74}N_5Na_2O_{33}S_2$ ([M^++Na]): 1382.3503; found: 1382.3492; calcd for $C_{50}H_{74}KN_5NaO_{33}S_2$ ([M^++K]): 1398.3242; found: 1398.3259; calcd for $C_{50}H_{73}N_5Na_3O_{33}S_2$ ([$M^+ - H + 2Na$]): 1404.3322; found: 1404.3341.

Sodium *N*-[2-*N*[(*S*-(4-deoxy-4-dimethylaminophenylazophenylthioureido-β-D-glucopyranosyl)-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl)-2-thioacetyl]aminoethyl]-1-naphthylamine-5-sulfonate (1): A solution of azidohexasaccharide **16** (40 mg, 29.4 μmol) in pyridine/H_2O (1:1, 7 mL) was saturated with H_2S, and the resulting mixture was stirred for 2 h at room temperature. After concentration under reduced pressure, the residue was redissolved in DMF (14 mL) and aq sodium hydrogencarbonate (3.5 mL, 0.35M). Then DABITC (13 mg, 46 μmol) was added, and the reaction mixture was stirred for 12 h at 40°C. Silica gel was added; then, after concentration under reduced pressure, the resulting solid was subjected to flash column chromatography [acetonitrile to acetonitrile/water (8:2)] to give the bifunctionalized hexasaccharide **1** (33.5 mg, 70%). ^{1}H NMR (400 MHz, [D_7]DMF): $\delta = 8.40$ (d, 1H; arom H), 8.34 (t, 1H; CH_2NH), 8.17 (d, 1H; arom H), 8.12 (d, 1H; arom H), 7.81 (m, 6H; arom Hs), 7.34 (t, 1H; arom H), 7.29 (t, 1H; arom H), 6.88 (m, 2H; arom Hs), 6.62 (d, 1H; arom H), 6.01 (t, 1H; CH_2NH), 5.59 (br s, OHs), 4.87 (br s, OHs), 4.52 (t, $^3J = 10.0$ Hz, 1H; H-4^{VI}),4.50 (m, 5H; H-1), 4.45 (d, $^3J = 8.0$ Hz, 1H; H-1), 3.09 (s, 6H; CH_3); ^{13}C NMR (75 MHz, [D_7]DMF): $\delta = 182.5$ (NHCSNH), 171.3 (CONH), 149.8, 145.3, 144.6, 143.9, 141.9, 131.4, 126.8, 125.3, 125.1, 124.9, 123.6, 123.3, 123.1, 122.9, 117.1, 112.2, 103.6 (arom Cs), 104.2 and 103.9 (C-$1^{II\text{-}VI}$), 85.8 (C-1^{I}), 80.8, 80.6, 80.2, 77.4, 76.1, 75.9, 75.8, 75.0, 74.2, 73.9 (C-$2^{I\text{-}VI}$, C-$3^{I\text{-}VI}$, C-$4^{I\text{-}V}$, C-$5^{I\text{-}VI}$), 61.7, 61.4, (C-$6^{I\text{-}VI}$), 57.7 (C-4^{VI}), 44.2 (SCH_2CONH), 40.17 (CH_3), 39.1, 33.4 (CH_2NH); ES$^+$ HRMS calcd for $C_{65}H_{90}N_7Na_2O_{33}S_3$ ([M^++Na]): 1638.1537; found: 1638.1533.

Expression and purification of the enzymes: The *Humicola insolens* cellulases were all cloned and expressed in *Aspergillus oryzae* essentially as described.[21] The cloned product was recovered after fermentation by separation of the extracellular fluid from the production organism by means of filtration through three layers of Whatman microfiber filters (1.6, 1.2, and 0.7 μm). Cel6A and Cel6B were subsequently purified by affinity chromatography with Avicel in 20 mM NaH_2PO_4, pH 7.5, essentially as described.[22] The culture supernatant was applied to Avicel (2.6×20 cm) in 20 mM NaH_2PO_4, pH 7.5 at a flow rate of 300 mL h^{-1}. The column was subsequently washed with 20 mM NaH_2PO_4, pH 7.5 ($5 \times$ column volume), 20 mM NaH_2PO_4, 0.5M NaCl, pH 7.5 ($5 \times$ column volume), 20 mM Tris, pH 8.0 ($5 \times$ column volume). The cellulases were finally eluted with 0.2M Tris/NaOH, pH 11.8. The Cel45 core was purified by ion-exchange chromatography with SP-Sepharose in 20 mM citrate, pH 3.5. The culture supernatant was applied to SP-Sepharose (2.6×20 cm) in 20 mM citrate, pH 3.5, at a flow rate of 300 mL h^{-1}. The column was subsequently washed with column buffer ($10 \times$ column volume), and proteins were then eluted by a linear gradient of 0–0.5M NaCl in the above buffer. Cel7B was purified by ion-exchange chromatography with SP-Sepharose in 20 mM sodium acetate, pH 5.0. The culture supernatant was applied to SP-Sepharose (2.6×20 cm) in 20 mM sodium acetate, pH 5.0, at a flow rate of 300 mL h^{-1}. The column was washed with column buffer ($10 \times$ column volume) and proteins were subsequently eluted by a linear gradient of 0–0.5M NaCl in the above buffer. The purified cellulases were evaluated by means of SDS-PAGE and found to be homogeneous. Protein concentrations were determined spectrophotometrically at 280 nm; we took $\varepsilon =$ 92600 M^{-1} cm^{-1} and a molecular weight of 65 kDa for Cel6A, $\varepsilon =$ 76020 M^{-1} cm^{-1} and a molecular weight of 45 kDa for Cel6B, $\varepsilon =$ 66300 M^{-1} cm^{-1} and a molecular weight of 50 kDa for Cel7B, and $\varepsilon =$ 42220 M^{-1} cm^{-1} and a molecular weight of 28 kDa for Cel45A core, respectively.[23]

Fluorimetric assays and determination of kinetic constants: The initial rate constants for enzymatic hydrolysis of the substrate **1** were determined at 37°C with 0.1M 3-(*N*-morpholino)propanesulfonic acid (MOPS) at pH 7.5.

All measurements were performed on a Perkin–Elmer LS 50B spectrofluorimeter at excitation wavelength $\lambda = 340$ nm and emission wavelength $\lambda = 470$ nm. Initial rate constants were determined at several substrate concentrations, ranging from 0.1 to 5 times the ultimately determined K_m value. K_m was obtained by fitting initial rate constants to the Michaelis–Menten equation by means of the program GraFit 4.0. The kinetic measurements were performed at an enzyme concentration of 2×10^{-4} mM for Cel45A. Substrate **1** at high substrate concentration (123 μM) was mixed with the different cellulases. Enzyme concentrations were 7.8×10^{-4} mM for Cel6A, 1×10^{-4} mM for Cel6B, 2×10^{-4} mM for Cel45A, and 5.7×10^{-4} mM for Cel7B.

Acknowledgements

This work was funded, in part, by CNRS and the European Union (under contract BIO4-CT97-2303).

[1] S. Fort, V. Boyer, L. Greffe, G. J. Davies, O. Moroz, L. Christiansen, M. Schülein, S. Cottaz, H. Driguez, *J. Am. Chem. Soc.* **2000**, *122*, 5429–5437.
[2] T. T. Teeri, *TIBTECH* **1997**, *15*, 160–167.
[3] B. Henrissat, A. Bairoch, *Biochem. J.* **1996**, *316*, 695–696.
[4] M. Claeyssens, B. Henrissat, *Protein Sci.* **1992**, *1*, 1293–1297.
[5] R. H. Fairclough, C. R. Cantor, *Methods Enzymol.* **1978**, *48*, 347–379.
[6] N. Payre, S. Cottaz, H. Driguez, *Angew. Chem.* **1995**, *107*, 1361–1364; *Angew. Chem. Int. Ed. Engl.* **1995**, *35*, 1239–1241.
[7] S. Armand, S. Drouillard, M. Schülein, B. Henrissat, H. Driguez, *J. Biol. Chem.* **1997**, *272*, 2709–2713.
[8] S. Cottaz, B. Brasme, H. Driguez, *Eur. J. Biochem.* **2000**, *267*, 5593–5600.
[9] G. J. Davies, S. P. Tolley, B. Henrissat, C. Hjort, M. Schülein, *Biochemistry* **1995**, *34*, 16210–16220.
[10] G. Parsiegla, M. Juy, C. Reverbel-Leroy, C. Tardif, J.-P. Belaïch, H. Driguez, R. Haser, *EMBO J.* **1998**, *17*, 5551–5562.
[11] "Enzyme Applications in Fiber Processing": M. Schülein, M. S. Kauppinen, L. Lange, S. F. Lassen, L. N. Andersen, S. Klysner, J. B. Nielsen, *ACS Symp. Ser.* **1998**, *687*, 66–74.
[12] E. D. Matayoshi, G. T. Wang, G. A. Kraft, J. Erickson, *Science* **1990**, *247*, 954–958.
[13] L. F. Mackenzie, Q. Wang, R. A. J. Warren, S. G. Withers, *J. Am. Chem. Soc.* **1998**, *120*, 5583–5584.
[14] C. Malet, A. Planas, *FEBS Lett.* **1998**, *440*, 208–212.
[15] H. H. Baer, S. A. Abbas, *Carbohydr. Res.* **1979**, *77*, 117–129.
[16] K. Sunggak, C. Heung, K. Wan Joo, *J. Org. Chem.* **1985**, *50*, 1751–1752.
[17] C. Reverbel-Leroy, G. Parsiegla, V. Moreau, M. Juy, C. Tardif, H. Driguez, J. P. Belaïch, R. Haser, *Acta Crystallogr. Sect. D* **1998**, *1*, 114–118.
[18] M. Blanc-Muesser, H. Driguez, *J. Chem. Soc. Perkin Trans. 1* **1988**, 3345–3351.
[19] T. Adashi, Y. Yamada, I. Inoue, *Synthesis* **1977**, 45–46.
[20] C. Schou, G. Rasmussen, M. B. Kaltoft, B. Henrissat, M. Schülein, *Eur. J. Biochem.* **1993**, *217*, 947–953.
[21] T. Christensen, H. Woeldike, E. Boel, S. B. Mortensen, K. Hjortshoej, L. Thim, M. T. Hansen, *Bio/Technology* **1988**, *6*, 1410–1422.
[22] C. Boisset, R. Borsali, M. Schülein, B. Henrissat, *FEBS lett.* **1995**, *376*, 49–52.
[23] M. Schülein, *J. Biotech.* **1997**, *57*, 71–81.

Received: October 8, 2001 [F3598]

  0947-6539/02/0806-1394 $ 17.50+.50/0