4-Methylumbelliferyl glycosides of N-acetyl 4-thiochito-oligosaccharides as fluorogenic substrates for chitodextrinase from Vibrio furnissii

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The degradation of chitin involves a diverse array of enzymes, some with overlapping substrate specificities. In order to distinguish between different types of enzymes, specific substrates are needed. Toward this end, two new fluorogenic substrates containing thio-glycosidic linkages, 4-methylumbelliferyl N,N'-diacetyl-4-thio-β-chitobioside (Mu-TCB) and N,N',N'-triacetyl-4,4'-dithio-β-chitotrioside (Mu-TCT) are described. The substitution of the glycosidic oxygens (except the one that links oligosaccharide with the fluorogenic aglycon) with a sulfur atom resulted in resistance of these compounds to N-acetyl-β-hexosaminidases while they were specific substrates for the newly discovered chitodextrinase from Vibrio furnissii (Keyhani,N.O. and Roseman S. (1996) J. Biol. Chem., 271, 33414-33424) and some bacterial chitinases. The enzyme kinetics of these 4-S-linked substrates, Mu-TCB and Mu-TCT, as well as the O-linked 4-methylumbelliferyl N,N'-diacetyl-β-chitobioside (Mu-CB) and N,N',N'-triacetyl-β-chitotrioside (Mu-CT) with the chitodextrinase were studied and compared. The usefulness of the substrates for screening for chitodextrinase and/or chitinase activity was demonstrated.

Key words: chitodextrinase/chitinase/fluorometric assay/4-methylumbelliferyl glycoside/thioclothio oligosaccharide

Results and discussion

Synthesis of the substrates

We have previously described the synthesis of peracetylated 4-thiochitobiose and 4,4’-dithiochitobiote (Wang and Lee, 1996), as well as their usefulness in characterizing transport processes (Keyhani et al., 1996). These compounds are the starting materials for the preparation of the designed fluorogenic substrates described here. We found that catalyzed phase-transfer reaction (Roy and Tropper, 1991) was very effective for the synthesis of the 4-methylumbelliferyl β-glycosides from the corresponding glycosyl chlorides, which were prepared from the peracetylated 4-thiochitoligosaccharides. De-O-acetylation of the respective coupling products resulted in Mu-TCB and Mu-TCT. Starting from the corresponding peracetylated 4-thiochitoligosaccharides, in three steps, the overall yields of Mu-TCB and Mu-TCT were 71% and 65%, respectively. The structures of Mu-TCB and Mu-TCT were confirmed by 1H-NMR as well as elemental analysis. In the 1H-NMR spectrum of Mu-TCB, H-1 appears as a doublet at δ 5.137 with a relatively large coupling constant (J = 8.2 Hz), indicating the expected β-O-glycosidic linkage. Similarly, the data for H-1, δ 5.110 (d, J 8.1 Hz), reveal that the newly constructed glycosidic linkage is in β-D-configuration. Other NMR features of the products are in agreement with the structures.
Analysis of the products from enzymatic hydrolysis of the fluorogenic substrate analogs

The products of the enzymatic hydrolysis of the fluorogenic substrates by the purified V. furnissii chitodextrinase were analyzed by thin layer chromatography (TLC). The purified chitodextrinase cleaved Mu-TCB at the aglyconic bond releasing Mu and TCB (Figure 2, lane 2). The enzyme also hydrolyzed Mu-TCT into Mu and TCT (Figure 2, lane 5) (Mu released by the enzymatic reaction could be visualized under a long-wavelength UV lamp before spraying with sulfuric acid). No evidence of cleavage at the thioglycosidic linkage was detected, indicating that the thioglycosidic bond is resistant to hydrolysis by the chitodextrinase. For comparison, the hydrolysis of the corresponding O-linked substrates: Mu-CB and Mu-CT by the chitodextrinase was also examined. As shown in Figure 2, Mu-CB was hydrolyzed by the chitodextrinase yielding Mu and CB (lane 8), whereas Mu-CT was cleaved mostly into Mu-GlcNAc and CB, together with trace amounts of Mu and CT (lane 11). The results with the Mu-CB and Mu-TCB substrates are worthy of note since the chitodextrinase apparently does not cleave the natural N-acetyl-chitotriose (CT) (Keyhani and Roseman, 1996b). Furthermore, hydrolysis of the aglyconic bond of Mu-TCT (the only possible cleavage site) by the chitodextrinase indicates that the enzyme possesses some flexibility at the active site, although the predominant products of (GlcNAc)₄ hydrolysis are N-acetyl-chitobiose (CB) with no GlcNAc/CT detectable (Keyhani and Roseman, 1996b). A possible explanation is that the chitodextrinase possesses a hydrophobic binding site that, upon binding with the Mu-group, is capable of orienting the substrate properly at the active site, allowing hydrolysis to occur.

Enzyme kinetics

The V. furnissii chitodextrinase displayed a pH optimum around 6.5 with the 4-methylumbelliferyl derivatives including the O-linked (Mu-CB and Mu-CT) and the 4,S-linked oligosaccharides (Mu-TCB and Mu-TCT) (data not shown). This optimum is similar to that described for the natural oligosaccharides (Keyhani and Roseman, 1996b). The time courses of the enzymatic hydrolysis of the fluorogenic substrates are shown in Figure 3A (for Mu-TCB and Mu-TCT) and Figure 3B (for Mu-CB and Mu-CT). Figure 4A through Figure 4D showed the Michaelis–Menten curves that were constructed with the observed enzyme activities for different substrate concentrations. Because of solubility problems, the maximum concentration used was 300 µM. It should be noted that Mu-CT has three possible cleavage sites yielding either (1) Mu and CT; (2) Mu-GlcNAc and CB; or (3) Mu-CB and GlcNAc. Thus, in order to measure the total activity, a coupled enzyme assay was used. It was found that jack bean β-N-acetyl-glucosaminidase did not hydrolyze Mu-CB and Mu-CT but could hydrolyze Mu-GlcNAc into Mu and GlcNAc at a reasonable rate at pH 6.5 (data not shown). Therefore, the jack bean enzyme can be used for a coupled assay. Thus, after digestion of Mu-CT with the chitodextrinase, the reaction was stopped by boiling, and then jack bean β-N-acetyl-glucosaminidase was directly added to the reaction mixture for the purpose of hydrolyzing the intermediate Mu-GlcNAc. The total amount of Mu produced was then measured. Mu-TCB, Mu-TCT, and Mu-CT exhibit apparent Michaelis–Menten kinetics in the range of 0–200 µM substrate concentrations, but there is an apparent substrate inhibition with Mu-CT above a concentration of 20 µM (see Figure 4D). The enzyme also shows substrate inhibition with the natural chitin oligosaccharides (Keyhani and Roseman, 1996b). The apparent kinetic parameters (Kₘ and Vₜₐₙₐₚₐₓ) for the substrates were obtained by Woolf–Augustinsson plot (V vs. V/[S] plot) using the linear portions of the data and are listed in Table I.

As shown in Figure 3A, the chitodextrinase hydrolyzes Mu-TCB much faster than Mu-TCT. The initial rate of Mu-TCB hydrolysis is about 14 times higher than that of Mu-TCT under the given conditions. These results indicate that Mu-TCB is a more sensitive substrate than Mu-TCT for assaying chitodextrinase activity. Likewise, comparison of the initial rates between Mu-CB and Mu-CT reveals that the chitodextrinase hydrolyzes Mu-CB about 11 times faster than Mu-CT (Figure 3B). It should be mentioned that the hydrolysis of Mu-CT at the aglyconic bond is very slow as indicated by the fact that, without subsequent action of jack bean N-acetyl-β-
An enzymatic hydrolysis of the fluorogenic substrates was used to study the activity of the chitodextrinase. The fluoroenic substrates X-O Mu-CB, X-S Mu-TCB, X-O Mu-CT, and X-S Mu-TCT were incubated with the enzyme at pH 6.5. The hydrolysis was monitored by the decrease in fluorescence intensity of the substrate. The hydrolysis of Mu-TCB was much faster than that of Mu-TCT, with an initial rate of 11 times higher. The hydrolysis of Mu-CB and Mu-CT was also monitored, with the hydrolysis of Mu-CB being about 11 times faster than that of Mu-CT. The hydrolysis of Mu-GlcNAc into Mu and GlcNAc was monitored at a reasonable rate at pH 6.5. The rate of hydrolysis was found to be dependent on the substrate concentration, with an apparent Michaelis-Menton kinetics in the range of 0-200 μM substrate concentrations. The hydrolysis of Mu-TCB was also examined, and it was found to be much faster than that of Mu-TCT. The products of the hydrolysis were monitored by thin layer chromatography (TLC) and visualized by spraying with sulfuric acid. The products were identified as N-acetyl-chitobiose and N-acetyl-chitotriose. The hydrolysis of Mu-CB and Mu-CT was also monitored, with the hydrolysis of Mu-CB being about 11 times faster than that of Mu-CT. The hydrolysis of the fluorogenic substrates was also monitored by the decrease in fluorescence intensity of the substrate.
Fluorogenic substrates for chitodextrinase

Fig. 3. Enzymatic release of Mu from the fluorogenic substrates. (A) hydrolysis of Mu-TCB (circle) and Mu-TCT (triangle); (B) hydrolysis of Mu-CB (circle) and Mu-CT (triangle or diamond). A solution of 0.25 mM respective substrate in 240 μL of 50 mM phosphate buffer (pH 6.5) was incubated with a suitable amount of chitodextrinase (0.25 mU for Mu-TCB, Mu-CB and Mu-CT, and 1.2 mU for Mu-TCT) for a predetermined length of time at 37°C. The reaction was stopped by boiling at 100°C for 3 min and the fluorescence was determined as described in Materials and methods. In the case of Mu-CT, the fluorescence was determined either before or after subsequent incubation of the first enzymatic reaction mixture with 150 mU of jack bean N-acetyl-β-D-glucosaminidase for 30 min at 37°C. (triangle) Mu-CT with subsequent action of jack bean N-acetyl-β-D-glucosaminidase; (diamond) Mu-CT without subsequent action of jack bean N-acetyl-β-D-glucosaminidase (B).

Table I shows that the K_m value for the trisaccharide derivative is much lower than the disaccharide derivative (Mu-TCB vs. Mu-CB, Mu-TCT vs. Mu-CT), and the K_m for 4-S-linked substrates is about three times higher than the corresponding O-linked substrates (Mu-TCB vs. Mu-CB, and Mu-TCT vs. Mu-CT). If K_m values are used as a rough estimate of substrate binding ability to the chitodextrinase active sites, the results suggest that the chitodextrinase has a higher "affinity" toward higher oligomer substrates, whereas the substitution of O-glycosidic linkage with S-glycosidic bond leads to a decrease in the affinity of the substrates toward the enzyme.

Although the difference between Mu-TCB and Mu-CB is a substitution of one atom (S vs. O), this replacement of the inter-O-glycosidic linkage in Mu-CB with the thio-glycosidic linkage results in a dramatic decrease in both its hydrolysis rate and its "binding affinity" with regards to the enzyme. These results imply that the glycosidic oxygen participates in recognition by the enzyme, perhaps through hydrogen-bonding interactions since sulfur is a less effective hydrogen-bond acceptor than oxygen. Another explanation may be that the substitution of the glycosidic oxygen with a sulfur atom results in a change of the conformation of the molecule. The carbon–sulfur bond (≈1.8 Å) is longer than the carbon–oxygen bond (≈1.4 Å) (Lide, 1990–1991). Additionally, the C-S-C bond angle (≈105°) is slightly smaller than that of C-O-C (≈110°) (Lide, 1990–1991). Therefore, the relative spatial orientation of the two GlcNAc residues as well as the aglycon would be slightly different between Mu-TCB and Mu-CB, resulting in the observed differences in behaviors towards the enzyme. Very recently, a report showed that the replacement of a glycosidic oxygen with a sulfur atom in galabioside resulted in a 30-fold reduction of its binding strength to its receptor protein, and the difference was attributed to the lack of a single hydrogen bond because of the resultant conformational change (Nilsson et al., 1996).

Routine assay of chitodextrinase activity using Mu-TCB

Although Mu-CB and Mu-CT are good substrates for the chitodextrinase, they are not suitable for specific assay of the enzyme in vivo or in crude biological preparations. This is because contaminating bacterial exo-β-N-acetyl-glucosaminidases can also hydrolyze Mu-CB and Mu-CT at the nonreducing termini and eventually release Mu, thus interfering with the assay results (data not shown). In contrast, Mu-TCB and Mu-TCT are completely resistant to exo-enzymes because of the thio-glycosidic linkage between the GlcNAc residues. They are susceptible to the chitodextrinase exclusively at the aglyconic bond, releasing the fluorophore (Mu) for easy detection and quantification. They are, therefore, useful fluorogenic substrates for the routine assay of chitodextrinase. Because Mu-TCB is much more sensitive than Mu-TCT (Figure 3), Mu-TCB is a superior substrate for the assay. A linear relationship was observed over the indicated time course between enzyme concentration and the amount of Mu released from Mu-TCB under the assay conditions (Figure 5).

Some bacterial chitinases such as those from Streptomyces griseus and Serratia marcescens (Sigma) can also hydrolyze Mu-TCB and Mu-TCT giving Mu, showing the potential usefulness of these substrates for assaying these chitinases (data not shown). On the other hand, chitinase and chitodextrinase can be distinguished from each other through the use of a conventional chitinase assay (Boiler and Mauch, 1988; Cabib, 1988; Ohtakara, 1988). Briefly, chitinases hydrolyze insoluble
Fig. 4. Reaction rate of enzymatic hydrolysis as a function of substrate concentration. (A) Mu-TCB; (B) Mu-TCT; (C) Mu-CB; (D) Mu-CT. A solution of the respective substrate in 240 μl of 50 mM phosphate buffer (pH 6.5) at different concentrations (0–300 μM) was incubated with a suitable amount of chitodextrinase (2 mU for Mu-TCB; 5 mU for Mu-TCT; and 0.5 mU for Mu-CB and Mu-CT) for 5 min at 37°C. The reaction was stopped by boiling at 100°C for 3 min and the fluorescence was determined as described in Materials and methods. In the case of Mu-CT, the intermediate Mu-GlcNAc was subsequently hydrolyzed by incubation with 150 mU of jack bean N-acetyl-β-D-glucosaminidase for 30 min at 37°C, and the total amount of Mu released was fluorometrically measured.

and/or soluble chitin whereas the chitodextrinase does not hydrolyze chitin.

Use of fluorogenic substrates as a specific screen for different enzymes

Mu-TCB and Mu-TCT were tested for their usefulness as specific screens for isolating endolytic enzymes. A V.fumissii genomic cosmid library was screened in E.coli using the conventional O-linked fluorescent substrates (Mu-CB and Mu-CT) as well as the 4-S-linked derivatives described in this report (Materials and methods). Briefly, E.coli transformant colonies releasing Mu were bright blue. Positive transformants can be isolated using all the substrates tested. Each positive transformant originally isolated using a particular fluorogenic substrate was then tested for its ability to hydrolyze the other substrates, and the data are summarized in Table II. These data show that (1) of the seven colonies isolated using Mu-CB, only two were endolytic enzymes hydrolyzing Mu-TCB and Mu-TCT, while the other five are probably β-N-acetyl-hexosaminidases (i.e., false positives); (2) of the nine colonies isolated using Mu-TCB, none were β-N-acetylhexosaminidases; and (3) of the three colonies isolated using Mu-TCT, none hydrolyzed Mu-GlcNAc. Thus, the thio-derivatives can be used as a specific screen for endolytic enzymes, i.e., chitodextrinase and/or chitinase activity.

<table>
<thead>
<tr>
<th>Table I. The kinetic data for the fluorogenic substrates</th>
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<tr>
<td>Substrate</td>
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<td></td>
</tr>
<tr>
<td>Mu-TCB</td>
</tr>
<tr>
<td>Mu-TCT</td>
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<tr>
<td>Mu-CB</td>
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<td>Mu-CT</td>
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In summary, the substitution of a sulfur atom for the oxygen at the glycosidic bond between GlcNAc moieties resulted in two new compounds, Mu-TCB and Mu-TCT. Hydrolysis of these compounds at the aglyconic bond results in the formation of Mu, which can be easily quantitated. The thiglycosidic bond is resistant to the cleavage and thus most exo-B-N-acetylhexosaminidases should not be able to hydrolyze the synthetic analogs. Indeed, neither jack bean hecosaminidase nor the V.furnissii hecosaminidase can cleave either Mu-TCB or Mu-TCT (data not shown). Thus, these analogs can serve two useful purposes: (1) accurate measurement of chitodextrinase and/or chitinase activity in crude extracts and (2) as a specific substrate in screening for chitodextrinase/chitinase (s). In addition, the kinetics of the hydrolysis of the specific substrates may also yield useful information about the mechanism of enzyme action.

Table II. Characterization of transformed E. coli colonies screened with various Mu-substrates

<table>
<thead>
<tr>
<th>Colonies screened</th>
<th>Substrates used</th>
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</thead>
<tbody>
<tr>
<td>Mu-GlcNac</td>
<td>Mu-CB</td>
</tr>
<tr>
<td>(1) Total number</td>
<td>-2000</td>
</tr>
<tr>
<td>(2) Number positive</td>
<td>11</td>
</tr>
<tr>
<td>(3) Number in (2) positive on:</td>
<td></td>
</tr>
<tr>
<td>Mu-GlcNac</td>
<td>-</td>
</tr>
<tr>
<td>Mu-CB</td>
<td>11</td>
</tr>
<tr>
<td>Mu-TCB</td>
<td>0</td>
</tr>
<tr>
<td>Mu-TCT</td>
<td>0</td>
</tr>
</tbody>
</table>

*Same colonies.
*Not tested.

Materials and methods

Buffers and reagents were purchased from standard commercial sources. Reagents for bacterial media were purchased from Difco Laboratories (Detroit, MI). Reagents for molecular biology were obtained from New England Biolabs (Beverly, MA) and Stratagene (La Jolla, CA). 4-Methylumbelliflorone (Mu) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and crystallized from acetone before use. Mu-CB and Mu-CT were purchased from Sigma Chemical Co. (St. Louis, MO). The Vibrio furnissii chitodextrinase was cloned and purified to homogeneity from an E.coli recombinant (Keyhani and Roseman, 1996b).

Enzymatic reactions were performed at 37°C over the indicated lengths of time and the reactions were stopped by boiling the mixture for 3 min. For direct quantification of 4-methylumbelliflorone (Mu) released by enzymatic hydrolysis, the reaction mixture was diluted with 0.15 M glycine–NaOH buffer (pH 10.5) to both terminate the reaction and enhance the fluorescence quantum yield of Mu, and fluorescence was measured using a Perkin-Elmer luminescence spectrometer LS50B (Perkin-Elmer Corp., Rockville, MD). A standard curve was prepared by dissolving an appropriate amount of Mu in minimum acetonitrile and then diluting to different concentrations with 0.15 M glycine–NaOH buffer (pH 10.5). The fluorescence was determined at 445 nm (emission slit width, 5 nm) by excitation at 360 nm (excitation slit width, 5 nm). There was a linear relationship in the range of 0-0.35 nmol/ml. When Mu-CT was used as substrate, jack bean N-acetyl-B-glucosaminidase was used in a coupled assay in order to hydrolyze the product of chitodextrinase digestion, Mu-GlcNAc, into GlcNAc and Mu, which was determined. One unit of chitodextrinase activity was defined as the amount of enzyme that releases 1 μmol p-nitrophenol per min from p-nitrophenyl N-acetyl-B-D-glucosaminide at pH 5 at 37°C.

Enzymatic reactions were performed at 37°C over the indicated lengths of time and the reactions were stopped by boiling the mixture for 3 min. For direct quantification of 4-methylumbelliflorone (Mu) released by enzymatic hydrolysis, the reaction mixture was diluted with 0.15 M glycine–NaOH buffer (pH 10.5) to both terminate the reaction and enhance the fluorescence quantum yield of Mu, and fluorescence was measured using a Perkin-Elmer luminescence spectrometer LS50B (Perkin-Elmer Corp., Rockville, MD). A standard curve was prepared by dissolving an appropriate amount of Mu in minimum acetonitrile and then diluting to different concentrations with 0.15 M glycine–NaOH buffer (pH 10.5). The fluorescence was determined at 445 nm (emission slit width, 5 nm) by excitation at 360 nm (excitation slit width, 5 nm). There was a linear relationship in the range of 0-0.35 nmol/ml. When Mu-CT was used as substrate, jack bean N-acetyl-B-glucosaminidase was used in a coupled assay in order to hydrolyze the product of chitodextrinase digestion, Mu-GlcNAc, into GlcNAc and Mu, which was determined. One unit of chitodextrinase activity was defined as the amount of enzyme that releases 1 μmol Mu per minute when incubated with 0.25 mM Mu-CB in a sodium phosphate buffer (50 mM, pH 6.5) at 37°C.

H-NMR spectra were recorded with a Bruker AMX-300 NMR spectrometer at 25°C in CDC13 or DMSO-d6, as specified. The chemical shifts (δ) are expressed in parts per million (ppm) relative to internal standard tetramethylsilane (TMS, 0 ppm). Multiplicities of signals are abbreviated as follows: s, singlet; d, doublet; t, triplet; and m, multiplet.

Preparation of V.furnissii genomic cosmid library

Total V.furnissii genomic DNA was prepared by standard techniques (Ausubel et al., 1996). The genomic DNA was partially digested with restriction enzyme Sau3AI and ligated into Supercos1 cosmid vector (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The ligation mixture was then packaged using Gigapack III Gold Packaging extract (Stratagene, La Jolla, CA) and transfected into E.coli strain XL1-Blue MR (Stratagene, La Jolla, CA). The transformants were plated onto Luria broth agar (10 g bacto-tryptone, 10 g NaCl, 5 g yeast extract, 15 g bactoagar/per liter) plates supplemented with 75 μg/ml ampicillin.

Screening of E.coli recombinant V.furnissii library with fluorogenic substrates

E.coli transformants harboring the V.furnissii cosmid library were plated onto LB/ampicillin plates as described above, to a density of 200-300 transformants per plate (100 x 15 mm petri dishes). The colonies were lifted onto Whatman 1 filter paper (9 cm diameter) and the filters were sprayed with 0.5 mM solution containing the fluorogenic substrate in H2O or 10 mM phosphate buffer (pH 7.0). The filters were incubated at 37°C for 15-45 min and then sprayed with saturated sodium bicarbonate (to enhance the fluorescent quantum yield of MU). Positive transformants (i.e., those capable of cleaving the fluorescent substrates) were identified by UV illumination of the filter papers.

Synthesis of the fluorogenic substrates: Mu-TCB and Mu-TCT

4-Methylumbelliferyl N,N'-diacetyl-4-thio-B-chitobioside (Mu-TCB). A suspension of N,N'-diacetyl-hexa-O-acetyl-4-thio-a-chitobioside (44 mg, 63.5 μmol) in acetyl chloride (5 ml) was saturated with hydrogen chloride gas at 0°C. The suspension became a clear solution when HCl gas was introduced. The reaction flask was then sealed and kept at room temperature for 40 h. Acetyl chloride was evaporated and the residue was coevaporated with benzene (2 x 5 ml) to give the peracetylated 4-thio-a-chitobiosyl chloride (45 mg) as a white powder, which was then immediately used for the glycosylation reaction without purification.

The glycosyl chloride was dissolved in CHCl3 (3 ml), and mixed with a solution of aqueous Na2CO3 (3 ml) containing 4-methylumbelliflorone (sodium
Atmospheric Administration to Maryland Sea Grant. This work was supported by NIH Grants DK09970 (to Y.C.L.) and GM51215 (to S.R.), and Grant NA46RG0091 (to S.R.) from the National Oceanic and Atmospheric Administration to Maryland Sea Grant.

De-O-acetylation of the peracylated 4-methylumbelliferyl 4-thio-
-biothiocibiose (28 mg. 34.6 mmol) with MeONa-MeOH (6 ml, 10 mM) was evaporated to give 4-methylumbelliferyl N,N'-diacetyl-3,6',3',4',6'-penta-O-
-acyetyl-4-thio-beta-chitobiose (43 mg, 83%) as a white solid, which was crystallized from EtOH. m.p. > 297°C; 1H-NMR (300 MHz, CDCl3): 8 7.486 (d, 1 H, H-1), 7.477 (d, 1 H, H-3 in coumarin), 7.467 (d, 1 H, H-1), 5.740 (t, 1 H, J = 9.5 Hz, H-3), 5.570 (d, 1 H, J = 8.2 Hz, H-1), 5.117 (t, 1 H, J = 9.7 Hz, H-3'), 5.051 (t, 1 H, J = 9.7 Hz, H-4'), 4.803 (d, 1 H, J = 10.6 Hz, H-1'), 4.627 (dd, 1 H, J = 1.8 and 12.5 Hz, H-6a), 4.560 (dd, 1 H, J = 4.8 and 12.5 Hz, H-6'), 4.210-4.800 (m, 3 H, H-2,5,6'b), 3.850-3.730 (m, 2 H, H-2',5'), 2.897 (t, 1 H, J = 10.8 Hz, H-4), 2.897 (t, 3 H, CH3 in coumarin), 2.143, 2.128, 2.094, 2.024, 2.023, 2.005, and 1.937 (each s, each 3 H, 7 Ac).

De-O-acetylation of the peracetylated 4-methylumbelliferyl 4-thio-
-biothiocibiose (5 mg, 8.2 mmol) with MeONa-MeOH (6 ml, 10 mM) was carried out at room temperature for 16 h. The white precipitate formed was collected by filtration and washed with cold MeOH (2 x 2 ml), yielding Mu-TCT (18 mg, 86%), m.p. 227-228°C; 1H-NMR (300 MHz, DMSO-d6 + 1% D2O): 8 7.712 (d, 1 H, J = 9.4 Hz, H-5 in coumarin), 6.978-6.935 (m, 2 H, H-6,8 in coumarin), 5.256 (s, 1 H, H-3 in coumarin), 3.127 (d, 1 H, J = 8.3 Hz, H-1'), 4.572 (d, 1 H, J = 10.3 Hz, H-1'), 3.910-3.100 (m, 11 sugar residues, 2 NAc), 1.853 (s, 3 H, CH3 in coumarin).


Received on January 9, 1997; revised on March 7, 1997; accepted on March 16, 1997.