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Simple and conveniently accessible bi-fluorescence-labeled substrates for amylases

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ABSTRACT

Synthesis of bi-fluorescence-labeled maltooligosaccharides for amylase assay was accomplished. Preliminary biological evaluation of both bi-fluorescence-labeled maltohexasaccharide and maltose using α-amylase was carried out, and the hexaosyl derivative showed unique variation on the basis of fluorescence resonance energy transfer (FRET).

α-Amylase (EC.3.2.1.1), an endo-type enzyme, hydrolyzes α-glycosidic bonds in maltopolysaccharides such as starch and glycogen.[1] Human α-amylases are present mainly in the salivary gland and pancreas. In the clinical field, amylase level has been widely measured by means of a clinical blood test because hyperamylasemia is caused by various inflammatory diseases such as pancreatitis and parotitis.[2] Recently, various α-amylase-isozymes have been widely investigated. For example, a highly sialylated α-amylase was isolated from myelomatous cells[3] as a unique α-amylase, and the relationship between α-amylase and diabetes[4] has been studied in order to elucidate the role of the amylase. In the forensic scientific field, amylase levels and isozymic analyses are of considerable importance for evidence of saliva, feces, and vaginal secretion.[5] Obtaining evidence of salivary stain is also important for criminal investigations. Salivary stains indicate the relationship between the suspect and the crime, and they are often found on a cigarette left behind at the crime scene and on the victim's clothes in a sexual assault. Levels of amylase activity in those materials are usually very low because the materials have been exposed to sunlight, dryness, and putrefaction. Amylase levels can be measured for a clinical blood test by using starch-iodine,[6] starch-blue dye,[7] and *p*-nitrophenyl maltoheptaoside,[8] which are all commercially available, and fluorophore-modified cyclodextrin,[9] fluorescence-labeled maltoheptaoside,[10] and dinitrophenyl maltotrioside[11] have also been synthesized for measurement of amylase levels. Although various methods are currently used for the detection of amylases, development of a method with higher detection sensitivity and specificity is needed for the forensic scientific field. In addition to improvement of detection sensitivity, various amylase substances are also required for the study of myeloma and diabetes. In this paper, we report a novel synthetic route for construction of bi-fluorescence-labeled maltooligosaccharides for α-amylase assay based on fluorescence resonance energy transfer (FRET) and results of preliminary biological evaluations.

Maltose was chosen as the simplest maltooligosaccharide having one α-1,4-linkage in order to establish the synthetic scheme for construction of the bi-fluorescence-labeled substrate. A naphthymethyl residue and a dansyl residue are feasibly selected as a fluorescent donor and an acceptor, respectively, because that pair has suitable efficiency on FRET,[12] stability in aqueous media and low cost for chemical synthesis. In Scheme 1, we selected a readily available *S*-pentenyl derivative **4** as a key intermediate, which has an expansible double bond at the terminal of the sugar aglycon. Acetylation of maltose and subsequent anomeric bromination gave α-bromomaltoside **1**. Walden inversion of **1** was performed by means of potassium thioacetate to give the corresponding anomeric thioacetate **2** as a β anomer. A one-pot procedure involving anomeric de-*S*-acetylation of **2** with diethylamine and subsequent coupling reaction with an *n*-pentenyl bromide afforded the *n*-pentenyl thiomaltoside **3**[†] in 90.9% yield, $[\alpha]_D^{25}$ +44.1° (*c* 0.642, $CHCl_3$), ^1H NMR ($CDCl_3$) δ 5.77 (m, 1H,–CH_2CH=CH_2), 5.41 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 4.53 (d, 1H, $J_{1,2}$ = 10.1 Hz, H-1). Ester exchange reaction of **3**

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† All new compounds with specific rotation data gave satisfactory results of elemental analyses or high-resolution mass spectra.

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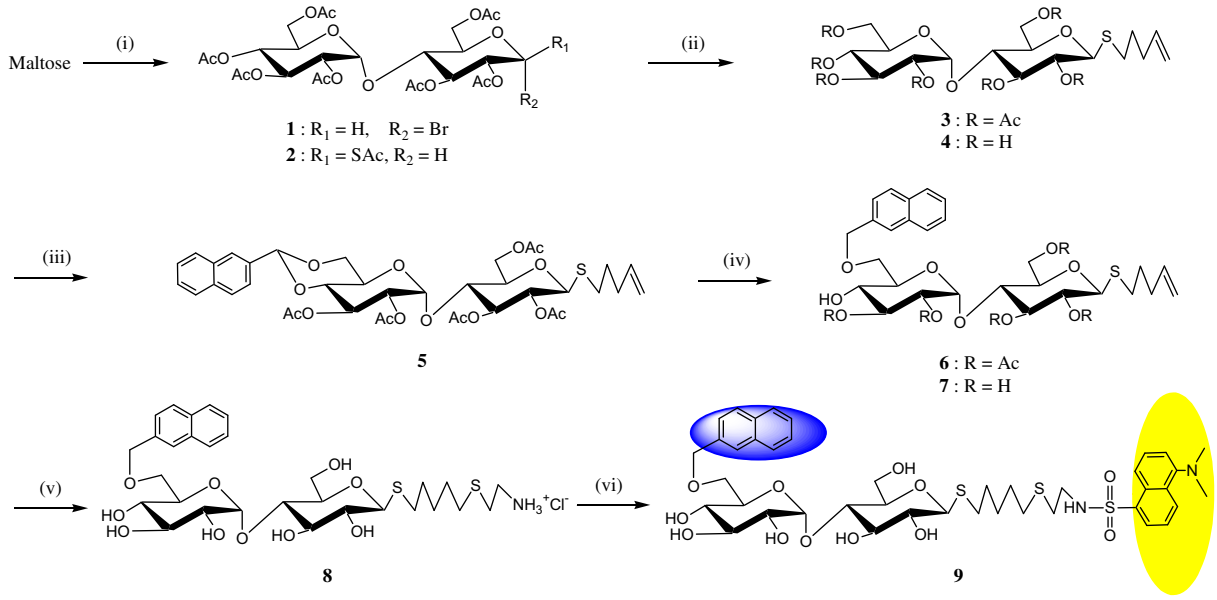
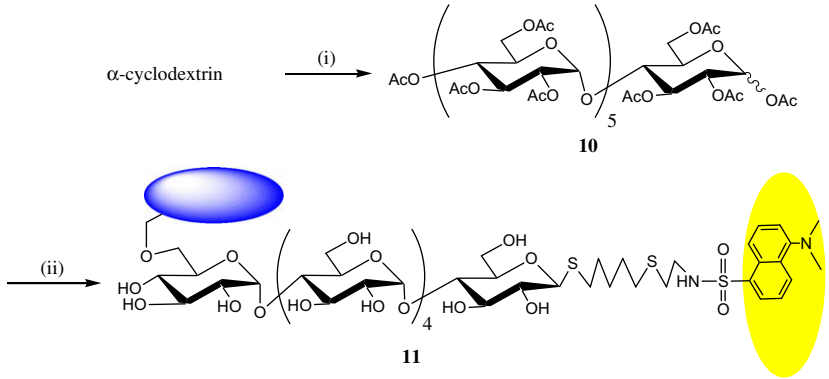


Scheme 1. Reagents and conditions: (i) HBr/AcOH, Ac_2O, AcOH, rt, then AcSK, DMF; (ii) (a) 5-bromopent-1-ene, DMF, diethylamine, −15 °C; (b) NaOMe, MeOH, rt; (iii) 2-naphthaldehyde di-*i*-butyl acetal, CSA, reduced pressure, 50 °C, then Ac_2O-pyr, rt; (iv) (a) borane–trimethylamine complex, $AlCl_3$, MS 4 Å, THF, rt; (b) NaOMe, MeOH, rt; (v) $HS(CH_2)_2NH_2HCl$, MeOH–H_2O, UV irradiation, rt; and (vi) dansyl chloride, Et_3N, MeOH, rt.

using Zemplén's transesterification quantitatively yielded unprotected pentenyl thiomaltoside **4**. 4′,6′-*O*-Naphthylmethylidene acetal **5** was synthesized through acetal exchange reaction between 2-naphtoaldehyde di-*i*-butylacetal[12] and **4**, followed by subsequent 2,2′,3,3′,6-*O*-acethylation in moderate yield (two steps). Direct formation of the acetal **4** using 2-naphtoaldehyde, instead of 2-naphtoaldehyde di-*i*-butylacetal, was scarcely detected. Regioselective reductive ring opening of **5** in the presence of BH_3–NMe_3 and $AlCl_3$ in THF successfully gave a stable 6′-naphthylmethyl ether **6** in 74.0% yield, $[\alpha]_D^{23}$ +39.9° (*c* 0.688, $CHCl_3$), ^{1}H NMR ($CDCl_3$) δ 7.86–7.45 (2 m, 7H, naphthyl), 5.76 (m, 1H, –*CH*=CH_2), 5.37 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 5.00 (m, 2H, –CH=*CH*$_2$). All ester protections on **6** were readily de-*O*-acetylated using Zemplén's transesterification conditions to give **7** in quantitative yield. In order to introduce a dansyl group at the terminal end of the sugar aglycon, radical addition of aminoethane thiol into the terminal C=C double bond of **7** was carried out and the reaction proceeded smoothly to yield amine **8**. Coupling reaction between dansyl chloride and **8** was performed quantitatively to give bi-fluorescence-labeled maltoside **9** after size exclusion chromatographic purification, ^{1}H NMR (CD_3,OD) δ 8.47–7.27 (m, 13H, naphthyl and dansyl), 5.01 (d, 1H, $J_{1',2'}$ = 3.7 Hz, H-1′), 4.57 (s, 2H, naphthyl-*CH*$_2$O), 4.18 (d, 1H, $J_{1,2}$ = 9.7 Hz, H-1), 3.53 (dd, 1H, J = 6.4 Hz, J = 10.7 Hz), 3.01 (s, 6H, dansyl-N*Me*$_2$), 2.87 (t, 2H, J = 7.2 Hz, SCH_2CH_2N), 2.50 (m, 2H, G-*SCH*$_2$–), 2.25 (t, 2H, J = 7.2 Hz, *SCH*$_2$$CH_2N$), 2.12 (t, 2H, J = 6.5 Hz, –*CH*$_2$$SCH_2CH_2N$), 1.39 (m, 2H, Glc-$SCH_2$*CH*$_2$–), 1.22 (m, 4H, Glc-$SCH_2CH_2$*CH*$_2$*CH*$_2$$CH_2S$), MALDI-TOF MS calcd for $[M+Na]^+$: 899.289; found *m*/*z*: 899.241.

Since a synthetic route for construction of bi-fluorescence-labeled maltoside was successfully established, we applied this method to the synthesis of maltooligosaccharide having different saccharidic chain lengths from maltoside. Maltohexasaccharide consisting of six glucose units was secondly selected as the synthetic target since many maltohexose derivatives have been widely applied to amylase assay.[13] Maltohexasaccharide **10** was prepared from α-cyclodextrin through ring opening acetolysis reaction[14] as shown in Scheme 2. Preparation of bi-fluorescence-labeled maltohexaoside **11** from **10** was carried out in the same manner as that described for the bi-fluorescence-labeled maltoside **9** in 8.2% (10 steps), $[\alpha]_D^{29}$ +76.9° (*c* 0.534, MeOH), ^{1}H NMR (CD_3OD) δ 8.39–7.19 (m, 13H, naphthyl and dansyl), 4.56 (s, 2H, naphthyl-*CH*$_2$O), 4.18



Scheme 2. Reagents and conditions: (i) Ref. 13; (ii) (a) HBr/AcOH, Ac_2O, AcOH, rt, then AcSK, DMF; (b) 5-bromopent-1-ene, DMF, diethylamine, −15 °C; (c) NaOMe, MeOH, rt; (d) 2-naphthaldehyde di-*i*-butyl acetal, CSA, reduced pressure, 50 °C, then Ac_2O-pyr, rt; (e) borane–trimethylamine complex, $AlCl_3$, MS 4 Å, THF, rt; (f) NaOMe, MeOH, rt; (g) $HS(CH_2)_2NH_2HCl$, MeOH–H_2O, UV irradiation, rt; and (h) dansyl chloride, Et_3N, MeOH, rt.

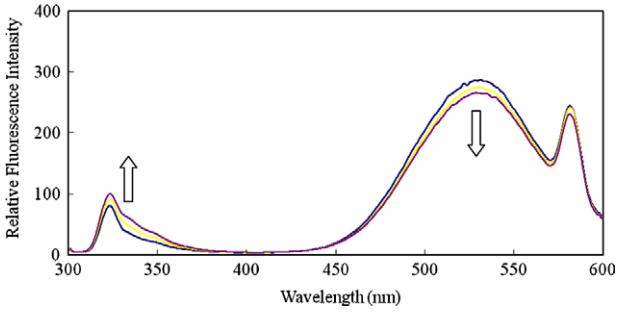


Figure 1. Variation of fluorescent intensity with bi-fluorescence-labeled maltohexaoside **11** in the presence of α-amylase.

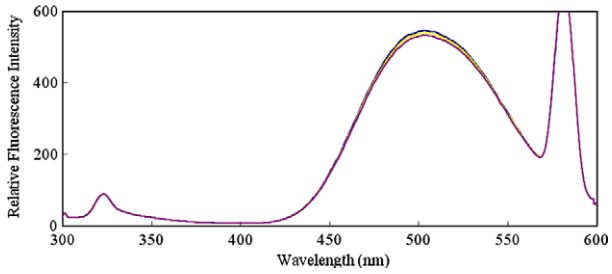


Figure 2. Variation of fluorescent intensity with bi-fluorescence-labeled maltoside **9** in the presence of α-amylase.

(d, 1H, $J_{1,2}$ = 9.7 Hz, H-1), 2.82 (t, 2H, J = 7.3 Hz, SCH_2CH_2N), 2.77 (s, 6H, naphthyl-NMe_2), 2.49 (m, 2H, Glc-SCH_2–), 2.22 (t, 2H, J = 7.3 Hz, SCH_2CH_2N), 2.07 (t, 2H, J = 6.6 Hz, $–CH_2SCH_2CH_2N$), 1.38 (t, 2H, J = 6.8 Hz, Glc-SCH_2CH_2–), 1.19 (m, 4H, Glc-$SCH_2CH_2CH_2CH_2CH_2S$), MALDI-TOF MS calcd for $[M+Na]^+$: 1547.500; found m/z: 1547.557.

Biological evaluation of bi-fluorescence-labeled maltoside and maltohexaoside was preliminarily carried out using human saliva α-amylase.[15] When a naphthymethyl residue is excited at 290 nm, emission of the fluorophore is observed around at 333 nm. Since this fluorescence field (333 nm) covers an excitation wavelength of a dansyl fluorophore, the bi-fluorescence-labeled maltooligosaccharides give the emission at 530 nm. Both a double scattering peak (580 nm) and a scattering peak (324 nm) of excitation wavelength were observed even when measurement of blank water was used as the solvent. As shown in Figure 1, reaction monitoring of bi-fluorescence-labeled maltohexaoside **11** with α-amylase was indicated, and decrease in fluorescent intensity at 530 nm and increase in fluorescent intensity at 333 nm were simultaneously observed. The results indicated that variation of FRET was caused by changes in distance between the naphthymethyl residue and dansyl residue. This phenomenon indicates human saliva α-amylase hydrolyzes the maltohexaoside **11**. On the other hand, with bi-fluorescence-labeled maltoside **9**, no variation of fluorescent intensity was observed as shown Figure 2. This means that the maltosyl derivative **9** was unsuitable as the substrate for human saliva α-amylase because of a shorter saccharidic chain length than the appropriate length for the active site of the amylase. Furthermore, fluorescent maximum wavelength of dansyl residue of the maltoside **9** was at 503 nm, which is different from that of the maltohexaoside **11** (530 nm). The time course in variation of fluorescent intensity with 8.3 μM maltohexaoside **11** is shown in Figure 3. With human saliva α-amylase, fluorescent intensity at 333 nm was increased gradually. Without human saliva α-amylase, variation of fluorescent intensity was not observed. This result indicates that the synthesized maltohexaoside was comparatively stable in an aqueous buffer.

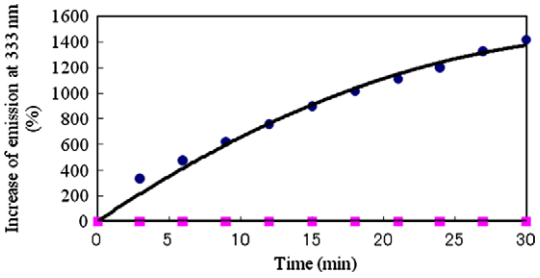


Figure 3. Time course of variation in fluorescent intensity of bi-fluorescence-labeled maltohexaoside **11** with (●)/without (■) α-amylase.

In conclusion, we have established a synthetic route for efficient construction of bi-fluorescence-labeled maltooligosaccharides. Construction of a library of bi-fluorescence-labeled maltooligosaccharides having various saccharidic chain lengths is now underway. In addition to the chemical synthesis of a series of bi-fluorescence-labeled maltooligosaccharides, the structure–activity relationship (SAR) between maltooligosaccharides and amylase-isozyme will also be reported elsewhere in the near future.

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15. *Assay method*: α-amylase from human saliva was purchased from Cosmo Bio Japan. 10 mM HEPES-NaOH (pH 7.2), 50 mM NaCl, 10 mM $CaCl_2$, 0.01% NaN_3, 4.2 μM synthesized bi-fluorescence-labeled maltooligosaccharide and α-amylase (0.03 U (caraway method)/vial) were incubated at 37 °C (total 3 mL).