Pergamon

Tetrahedron: Asymmetry Vol. 5, No. 12, pp. 2335-2338, 1994
Elsevier Science Ltd
Printed in Great Britain
0957-4166/94 $7.00+0.00

0957-4166(94)00314-9

Synthesis of Bi-Fluorescence-Labeled Lactoside: A Substrate for Continual Assay of Ceramide Glycanase

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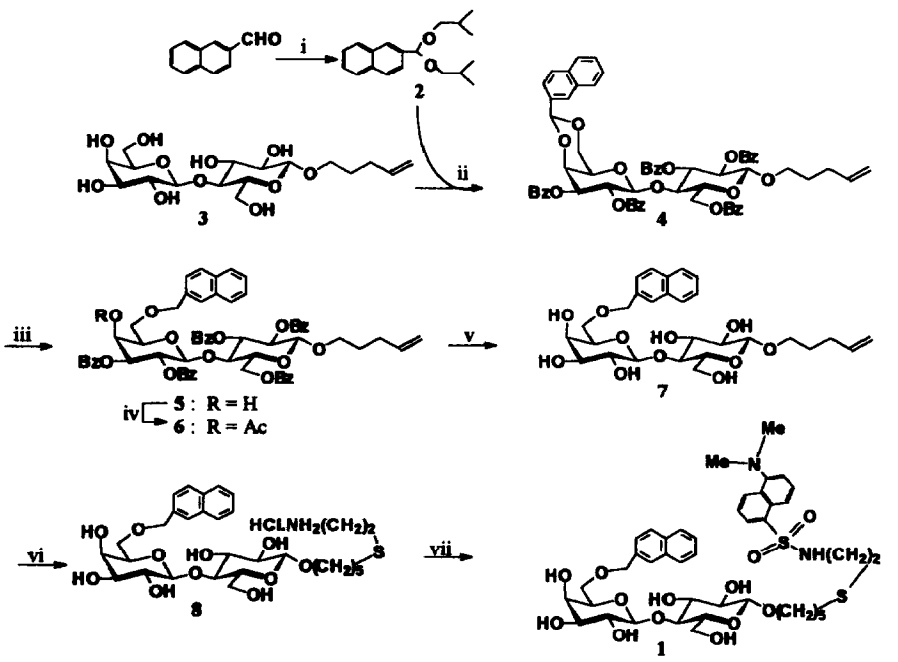
Abstract: A bi-fluorescence labeled derivative suitable for analysis of ceramide glycanase activity was constructed from 4-pentenyl lactoside. Selective modification of the galactosyl residue was attained by formation of 4',6'-naphthylmethylidene derivative, which was followed by regioselective reductive ring opening. The aglycon was extended by Michael addition of 2-aminoethanetiol, and dansylated at the terminal amino group. Excitation of the naphthyl group results in emission from the dansyl group, while the emission from the naphthyl group is quenched by the dansyl group. Upon digestion with ceramide glycanase, the energy transfer is severed and a decrease in the dansyl emission concommitant with an increase in the naphthyl emission was observed. This substrate was successfully used to analyze ceramide glycanase activity.

Fluorescence energy transfer has been utilized in determination of enzyme activities of endo-type proteases[1] and nucleases.[2] To the best of our knowledge, no similar application has been reported for endo-carbohydrases. In the past several years, we have successfully applied fluorescence energy transfer to determination of conformational structures of branched oligosaccharides derived from glycoproteins.[3] The principle used in these studies are immediately applicable for designing substrates for the endo-type carbohydrases. Ceramide glycanase (CGase) is an unique group of endo-type glycosidase which hydrolyze ceramide glycosides (glycosphingolipids) by cleaving the glycosidic linkage between oligosaccharide and ceramide. Such enzymes have been isolated from *Hirudo medicinalis* (European leech),[4] *Macrobdella decora* (American leech),[5] *Lumbricus terrestris* (earthworm),[6] as well as *Rhodococcus* sp.,[7,8] and *Corynebacterium* sp.[9] Some of these CGase can also perform transglycosylation, and is useful for synthesis of neoglycoconjugates.[10] In the past, determination of activity of these enzymes was done by a laborious and relatively insensitive tlc

method.[5] It would be useful to have a substrate which allows assays without separation of products and permits continual analysis. We have prepared such a substrate based on fluorescence energy transfer.



Scheme 1. Reagents and Conditions: i) i-Butanol, Drierite, CSA, rt, 2 d (90%); ii) CSA, reduced pressure, 55°C, 2h, then Bz-chloride, pyridine, 0°C to rt, 3 h (55%); iii) borane-trimethylamine complex, aluminum chloride, MS4Å, THF, rt (95%); iv) acetic anhydride, pyridine, rt, 11 h; v) sodium methoxide, methanol-THF, rt, 64 h (76%); vi) $HS(CH_2)_2NH_2$·HCl, methanol, uv irradiation, rt, 3 d (66%); vii) dnsyl chloride, triethylamine, methanol, rt, 30 min (91%).

In the design of such a substrate, some structural requirements reported for the leech CGase were adhered to. They were : 1) the glycoside must be larger than lactoside; and 2) the aglycon can be a single alkyl chain, provided it is longer than 6 carbons. Since we have used *n*-pentenyl β-lactoside (3) for a number of different syntheses, we chose to use this lactoside as starting material. The lactoside was first modified at the galactosyl residue with 4',6'-naphthylmethylidene group. The regioselective reductive ring opening successfully led to formation of 6'-nephthylmethyl ether. The aglycon was first extended with 2-aminoethanethiol by photocatalyzed Michael addition, and the terminal amino group was dansylated. After appropriate purification, an effective substrate for the leech CGase was obtained. The synthesis is outlined in Scheme 1.

In order to increase the reactivity of the aldehyde group of 2-naphthaldehyde, it was first converted into

the acetal **2** in good yield in *i*-butanol under catalysis of 10-camphorsulfonic acid in the presence of Drierite. 4-Pentenyl lactoside **3**[11] was transformed into its 4',6'-cyclic acetal by the acetal exchange reaction with **2**, and the cyclic acetal was benzoylated in pyridine at room temperature to give **4** in 55% overall yield (Successful regioselective reductive ring opening of a cyclic acetal of sugars requires a bulky neighboring group such as benzoyl, benzyl group.[12]). The cyclic acetal **4** was treated with 7 equivalent each of borane-trimethylamine complex and aluminum chloride in dry tetrahydrofuran at room temperature in the presence of powdered molecular sieves 4Å to give 6'-ether **5** in 95% yield. To confirm the structure of **5**, it was acetylated and the product **6** was examined by NMR.[13] Mono-acetylated derivative **6** showed down-field shifted H-4' signal and clear singlet of methyl group (in CH_3CO-) at 1.78 ppm in $CDCl_3$ thus confirming the identity of the product. Deprotection of **5** by the usual Zemplén method to provide the product **7**.

Michael addition of 2-aminoethanethiol (*via* its SH-group) to a double bond of **7** was catalyzed by uv irradiation to give ω-amino product **8**, and the dansylation of amino group of **8** was performed under standard

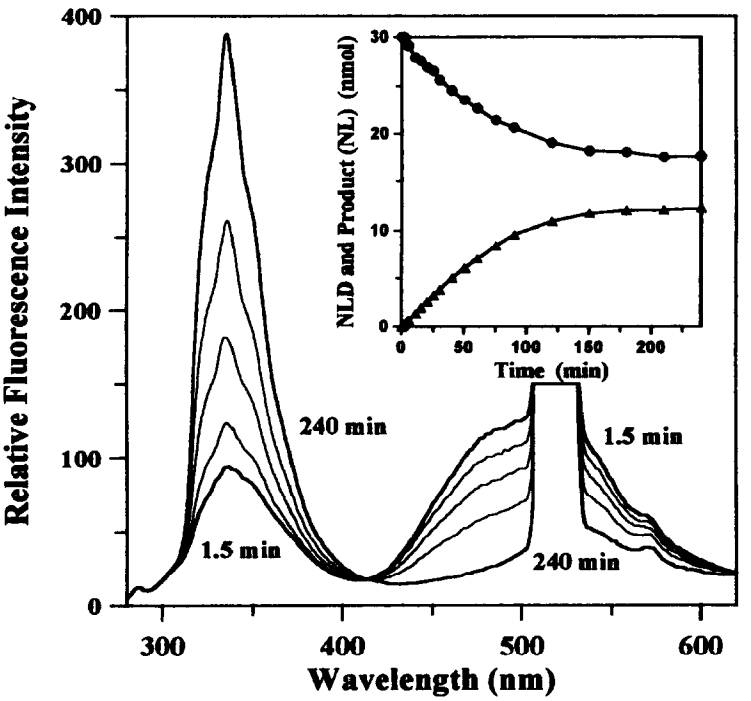


Figure 1. The time course of the fluorescence emission spectra excited at 260 nm of NLD during hydrolysis with CGase. The inset shows the result of the time course expressed as the concentrations of the product (NL) and the remaining NLD.

conditions to afford desired compound 1 (NLD). After appropriate purification, the bi-fluorescence-labeled compound was tested as substrate for CGase from leech (V-Lab, Covington, LA). As shown in Fig. 1, enzymatic hydrolysis of this substrate resulted in an increase of the naphthyl emission and a decrease in dansyl emission. K_M determined with this substrate, 7.7 μM, is in very good agreement with that determined with G_{M1}.[5]

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13 Selected spectroscopic data for key compounds: **6**, ^{1}H-NMR ($CDCl_3$), 1.78 (s, 3 H, Ac) 2.78 (t, 1 H, $J_{5',6'}$ 8.1 Hz and $J_{6'a,6'b}$ 9.1 Hz, H-6'a), 2.97 (dd, 1 H, $J_{5',6'b}$ 5.2 Hz, H-6'b), 3 61 (dd, 1 H, H-5'), 3.77 (m, 1 H, H-5), 4 18 (t, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 4.36 [m, 2 H, OCH_2-(2-naphthyl)], 4.38 (dd, 1 H, $J_{5,6a}$ 4.4 Hz and $J_{6a,6b}$ 12.1 Hz, H-6a), 4 56 (dd, 1 H, $J_{5,6b}$ <1 Hz, H-6b), 4.62 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.76 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 5.22 (dd, 1 H, $J_{3',4'}$ 3.3 Hz, H-3'), 5.38 (dd, 1 H, $J_{2,3}$ 9.6 Hz, H-2), 5.53 (1 H, $J_{4',5'}$ ~0 Hz, H-4'), 5.54 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.69 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 7.56 (m, 32 H, 5 Bz, 2-Naphthyl). **1**, ^{1}H-NMR (CD_3OD), 1.36 (m, 4 H, 2 CH_2), 1.55 (m, 2 H, OCH_2CH_2), 2.26 (m, 2 H, $CH_2SCH_2CH_2NH$), 2.39 (m, 2 H, SCH_2CH_2NH), 2.98 (m, 2 H, CH_2NH), 4.28 (d, 1 H, $J_{1',2'}$ 7 8 Hz, H-1'), 4.37 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.73 [m, 2 H, OCH_2-(2-naphthyl)], 7.91 (m, 13 H, dansyl, 2-naphthyl).

(*Received* 12 *September* 1994; *accepted* 24 *October* 1994)