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Synthesis of 4-Methylcoumarin-7-yloxy Tetra-*N*-acetyl-β-chitotetraoside, a Novel Synthetic Substrate for the Fluorometric Assay of Lysozyme

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4-Methylcoumarin-7-yloxy tetra-*N*-acetyl-β-chitotetraoside (**10**) was synthesized from chitin in pure form by a novel procedure.

After acetolysis of chitin, chitotetraose tetradecaacetate (**4**) was isolated by Sephadex LH-20 column chromatography. Compound **4** was chlorinated with dry hydrogen chloride to produce tridecaacetyl chitotetraosyl chloride (**6**). 7-Hydroxy-4-methylcoumarin sodium salt was condensed with **6** under Koenigs–Knorr reaction conditions, and the final product (**10**) was obtained by de-*O*-acetylation of condensation product (**8**) with sodium methoxide. 4-Methylcoumarin-7-yloxy tri-*N*-acetyl-β-chitotrioside (**9**) was also synthesized through chitotriose undecaacetate (**3**) isolated together with **4** in the same chromatography. Compound **10** was used in a fluorometric assay of lysozyme in comparison with **9**. The high sensitivity of fluorometric determination of 7-hydroxy-4-methylcoumarin (**12**) made it possible to determine lysozyme concentration in the microgram range by using this substrate (**10**). Unlike the assay using *Micrococcus lysodeikticus* cell powder, lysozyme assay with this synthetic substrate (**10**) could be performed directly in biological materials.

Keywords——synthesis; lysozyme; enzyme assay; fluorometry; substrate; Sephadex LH-20; 7-hydroxy-4-methylcoumarin; 4-methylcoumarin-7-yloxy tetra-*N*-acetyl-β-chitotetraoside; 4-methylcoumarin-7-yloxy tri-*N*-acetyl-β-chitotrioside

Determination of lysozyme in biological materials is troublesome because of the difficulty of obtaining a suitable substrate for the assay. The most convenient substrate for lysozyme at present is a spray-dried cell powder of *Micrococcus lysodeikticus*,[1,2] and this is still used in the fields of both research and clinical medicine. However, the bacterial cell powder is not always uniform, and may be contaminated with live cells. Further, lysozyme is not only a hydrolytic enzyme, but it also has sugar-transferring activity. Hence, oligosaccharides liberated by lysozyme from polysaccharide of the cell wall may again be transferred to another polysaccharide chain. This phenomenon may delay termination of the hydrolytic reaction of lysozyme. Finally, lysozyme assay using the cell powder as a substrate is remarkably influenced by the ionic strength of the medium.[3–5] Thus, careful adjustment of the ionic strength is required before the assay of biological materials.

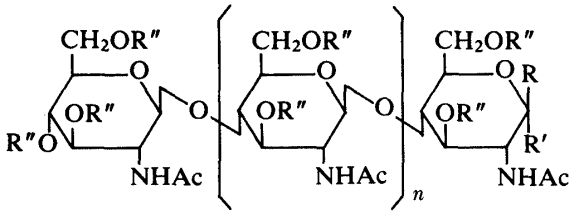
Therefore, a synthetic substrate having a definite and uniform structure is desirable for the accurate determination of lysozyme, and so we synthesized 4-methylcoumarin-7-yloxy tetra-*N*-acetyl-β-chitotetraoside (**10**) as a candidate substrate. This paper deals with the synthesis and application of this substrate.

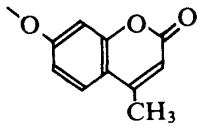
Previously, Osawa *et al.*[6,7] synthesized *p*-nitrophenyl tri-*N*-acetyl-β-chitobioside (**1**) from chitin through **3**, and determined the lysozyme activity from the absorption of *p*-nitrophenol liberated from **1** by the enzyme, but the accuracy of the assay was insufficient. Later, Delmotte *et al.*[8] synthesized 4-methylcoumarin-7-yloxy tri-*N*-acetyl-β-chitotrioside (**9**) and performed the lysozyme assay with this substrate (**9**) by determining the fluorescence due to 7-hydroxy-4-

methylcoumarin (**12**) liberated. As this assay is fluorometric, the accuracy and sensitivity was greatly superior to those with **1**. Recently, Ballardie *et al.*[9] synthesized 3,4-dinitrophenyl tetra-*N*-acetyl-β-chitotetraoside from chitin through **4**. Yang *et al.*[10] reported lysozyme-catalyzed hydrolysis of **10** synthesized according to the method of Ballardie *et al.*,[9] but the detailed procedure and the characteristics of **10** were not described.

As the *endo*-β-*N*-acetylglucosaminidase activity of lysozyme is well-known,[11] it was naturally expected that the rate of hydrolysis catalyzed by lysozyme would be larger for a substrate with longer sugar chains. However, in view of practical considerations of solubility and synthetic complexity, chitotetraose was thought to be the largest likely candidate.

From this point of view, we attempted to develop an effective procedure to synthesize 4-methylcoumarin-7-yloxy tetra-*N*-acetyl-β-chitotetraoside (**10**) from chitin.





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1 : $n=1$, R = $OC_6H_4NO_2(p)$, R′ = R″ = H

2 : $n=0$, R = H, R′ = OAc, R″ = Ac
3 : $n=1$, R = H, R′ = OAc, R″ = Ac
4 : $n=2$, R = H, R′ = OAc, R″ = Ac
5 : $n=1$, R = H, R′ = Cl, R″ = Ac
6 : $n=2$, R = H, R′ = Cl, R″ = Ac
7 : $n=1$, R = **11**, R′ = H, R″ = Ac
8 : $n=2$, R = **11**, R′ = H, R″ = Ac
9 : $n=1$, R = **11**, R′ = R″ = H
10 : $n=2$, R = **11**, R′ = R″ = H

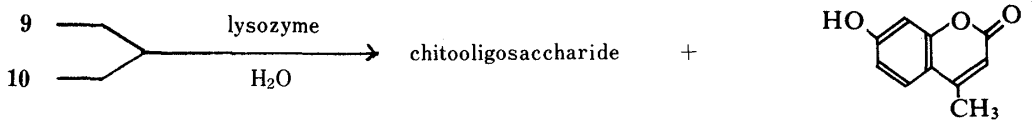

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Chart 1

Earlier researchers[7,8] prepared the key intermediates **3** and **4** through the following sequence: (1) partial hydrolysis of chitin, (2) carbon-celite chromatographic separation of chitooligosaccharides, (3) acetylation of isolated chitooligosaccharides. However, the separation procedure in step (2) was complicated and the yield was very low, as confirmed in our experiments. Therefore, we investigated the following alternative sequence: (1) acetolysis of chitin, (2) Sephadex LH-20 chromatographic separation of chitooligosaccharide acetates. This method provided a direct route to the key intermediates, **3** and **4**, with high purity. Through **3** and **4**, further intermediates, that is decaacetyl chitotriosyl chloride (**5**), tridecaacetyl chitotetraosyl chloride (**6**), 4-methylcoumarin-7-yloxy decaacetyl-β-chitotrioside (**7**), 4-methylcoumarin-7-yloxy tridecaacetyl-β-chitotetraoside (**8**), and finally **9** and **10** were synthesized in pure form. Details of our procedure are given below.

We found that the important point in the first step was the purification and trituration of

chitin. Commercial chitin was difficult to powder effectively, so it was dissolved in conc. hydrochloric acid, then the solution was kept at room temperature overnight, and poured into water. The resulting precipitates were collected, washed with water, dried with ethanol and diethyl ether, and crushed and triturated in an auto-mortar.

Next, we examined the acetolysis of chitin, and the relationship between the reaction time at 55 °C and the yields of the products is shown in Fig. 1. The reaction time for the best yield of **4** was 3 h. After acetolysis, the mixture of chitooligosaccharide acetates was extracted with chloroform. The residue from the chloroform extract was washed with ethyl acetate. The ethyl acetate-insoluble residue was presumed to consist mainly of **2**, **3**, and **4** based on the result of thin layer chromatography (TLC, Fig. 2). The residue was dissolved in chloroform–methanol (1 : 1) mixture, and separated on a Sephadex LH-20 column into three fractions, I, II, and III, in order of elution. Recrystallization of fraction I (higher molecular weight), fraction II (medium molecular weight), and fraction III (lower molecular weight) gave **4**, **3**, and chitobiose octaacetate (**2**) as crystals or crystalline powder. Analytical values showed good agreement with the chemical formulae, and spectral data were consistent with the expected structure. From the ethyl acetate-soluble fraction, a small amount of **2** was recovered by direct crystallization.

The subsequent reactions, namely chlorination (**3**→**5**, **4**→**6**), condensation with 7-hydroxy-4-methylcoumarin (**5**→**7**, **6**→**8**), and de-*O*-acetylation (**7**→**9**, **8**→**10**) were performed under carefully controlled conditions, and intermediates (**5**, **6**, **7**, **8**) and fianl products (**9**, **10**) with high purity were obtained. All analytical values and spectral data for those compounds were consistent with the expected chemical structures.

Lysozyme assay with synthetic substrate (**9** and **10**) is based on the fluorometric determination of 7-hydroxy-4-methylcoumarin (**12**) liberated from the substrate by the enzyme, so we determined the fluorescence spectra of substrates **10** and **12**; the results are

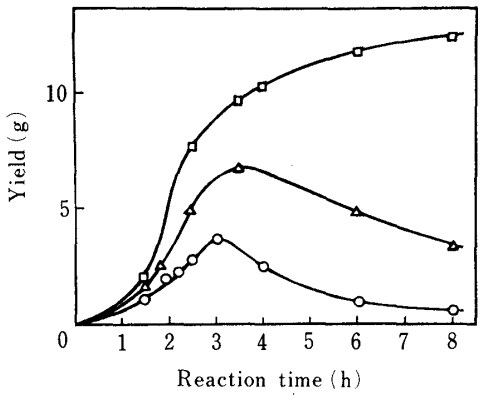

Fig. 1. Formation of Chitosaccharide Acetates by Means of Chitin Acetolysis

Yields of chitooligosaccharide acetates obtained after acetolysis of purified chitin (100 g), washing of the product with AcOEt, and fractionation on a Sephadex LH-20 column.

□—□, compound **2**; △—△, compound **3**; ○—○, compound **4**.

Fig. 2. Thin Layer Chromatography of the Products after Chitin Acetolysis

Solvent system A.

(1) Compound **2** prepared by Osawa's method[6] (standard).
(2) Crude product extracted with $CHCl_3$ from the reaction solution after chitin acetolysis.
(3) AcOEt-insoluble fraction of the crude product (2).
(4) AcOEt-soluble fraction of the crude product (2).
(5) Crystals separated by direct crystallization of the AcOEt-soluble fraction (4).
(6) Crystals separated from the AcOEt-insoluble fraction (3) by chromatography on a Sephadex LH-20 column (identical with **4**).

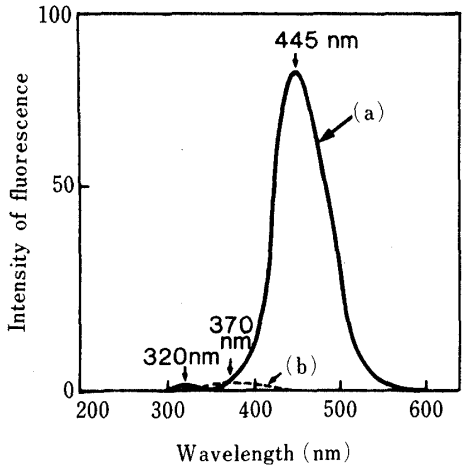

Fig. 3. Fluorescence Spectra of 7-Hydroxy-4-methylcoumarin (**12**) and Compound **10**

(a) 7-Hydroxy-4-methylcoumarin (**12**) (concentration: 0.001 mM).
(b) Compound **10** (concentration: 5.61 mM) in 0.05 M sodium citrate buffer (pH 4.8). The wavelength of excitation was 320 nm.

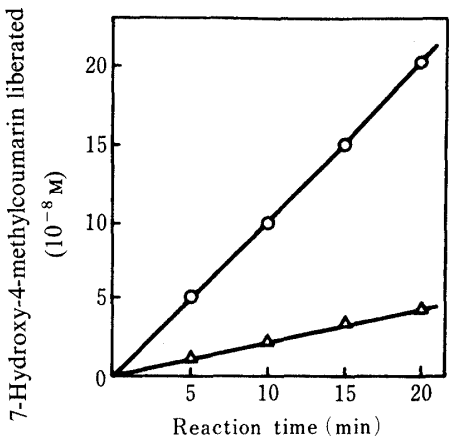

Fig. 4. Hydrolysis of 4-Methylcoumarin-7-yloxy Glycosides Catalyzed by Human Lysozyme

The lysozyme-catalyzed hydrolysis of 4-methylcoumarin-7-yloxy glycoside (**9** or **10**) was performed at 37 °C in a solution containing 0.028 mM glycoside and 250 μg/ml of lysozyme in 0.05 M sodium citrate buffer at pH 4.8.

○—○, compound **10**; △—△, compound **9**. 7-Hydroxy-4-methylcoumarin (**12**) liberated was determined by fluorometry as described in the text.

shown in Fig. 3. The fluorescence maximum of **10** (5.61 mM) was located at 370 nm and that of **12** (0.001 mM) was located at 445 nm, and intensity of fluorescence of **10** at 445 nm was negligible compared with that of **12**. As the lysozyme assay was performed with a much lower concentration of the substrate (*e.g.* 0.028 mM), **12** liberated after hydrolysis was measured directly in the presence of intact substrate.

By means of this fluorometric assay, we estimated the lysozyme activity towards **10** compared with that towards **9** which has one less sugar unit; the result is shown in Fig. 4. The velocity of hydrolysis of **10** was 6.30×10^{-7} M/h at the concentration of 250 μg/ml of human lysozyme at 37 °C, and that of **9** was 1.32×10^{-7} M/h at the same temperature. These data indicated that the rate of hydrolysis of **10** catalyzed by human lysozyme at microgram levels was much larger than that of **9**. This is in accordance with the result of Yang *et al.*[10] These data confirmed the *endo*-β-*N*-acetylglucosaminidase activity of lysozyme.[11]

As described at the beginning of this paper, the commonly used substrate for lysozyme assay has several deficiencies. In contrast, the synthetic substrate **10** exhibited superior characteristics, such as well-defined structure, uniformity, and stability over a wide pH range (pH 3—11). Furthermore, the fluorometric assay of lysozyme using **10** showed excellent sensitivity and reproducibility. Thus, 4-methylcoumarin-7-yloxy tetra-*N*-acetyl-β-chitotetraoside (**10**), synthesized in pure state by us, is considered to be a most suitable substrate for microassay of lysozyme in biological materials.

Experimental

All melting points were determined on a Mitamura Riken micro melting point apparatus (a hot stage type) and are uncorrected. The infrared (IR) spectra were obtained with a Shimadzu IR-430 spectrometer, and ultraviolet (UV) spectra with a JASCO UVIDEC-505 recording digital spectrometer. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded with a Varian HA-100 spectrometer at 100 MHz, with tetramethylsilane as an internal standard. The specific rotations were measured with a JASCO DIP-181 automatic digital polarimeter. Sephadex LH-20 (Pharmacia Fine Chemicals) was used for column chromatography. TLC was carried out on plates prepared in our

laboratories with Silica gel GF_{254} (type 60, Merck). The solvent combinations (v/v) on TLC were as follows: (A), $CHCl_3$–MeOH (50 : 7); (B), MeOH–AcOH–H_2O (4 : 1 : 5). Detection was done by spraying 50% H_2SO_4 and heating the plates at 250 °C.

Acetolysis of Chitin——Dried, purified and triturated chitin (100 g) was added to a cooled mixture of Ac_2O (500 ml) and conc. H_2SO_4 (65 ml). The mixture was kept at room temperature overnight, and then heated at 55 °C for 3 h. After the reaction, the mixture was poured into a cooled solution of CH_3COONa (400 g) in water (2600 ml) under stirring, and after filtration, the filtrate was extracted with $CHCl_3$ (1000 ml × 3). The combined extracts were washed with water, ice-cold saturated $NaHCO_3$ solution, and water successively. The $CHCl_3$ solution was dried over Na_2SO_4, and evaporated. The residue was dried over P_2O_5 *in vacuo* to give a yellow crystalline material (65.5 g). This material was suspended in AcOEt. The suspension was filtered after being stirred at room temperature for 1 h, and insoluble product (33.8 g) consisting of three main components was obtained. This product (in 6 g portions) was dissolved in a mixture of $CHCl_3$–MeOH (1 : 1) (30 ml) and separated on a Sephadex LH-20 column (size: 10 × 86 cm) with the same solvent into three main fractions (fractions, I, II, and III in order of elution). Recrystallization of the AcOEt-soluble fraction (20.5 g) from MeOH–Et_2O gave 1.5 g of **2**.

***O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (Chitobiose Octaacetate) (2)**——Fraction III (total 7.2 g) was recrystallized from MeOH–Et_2O to give 6.1 g (6.1% based on chitin 100 g) of **2**, as white needles, mp 304—305 °C (dec.), $[\alpha]_D^{28}$ +54.8° (c=0.75, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 2930 (C–H), 1720 (OAc), 1662 (amide I), 1528 (amide II), 905 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.72—1.86 (6H, m, NAc × 2), 1.92—2.22 (18H, m, OAc × 6), 7.90—8.20 (2H, m, NH × 2). TLC: *Rf* 0.57 (solvent A). *Anal.* Calcd for $C_{28}H_{41}N_2O_{17}$: C, 49.43; H, 6.10; N, 4.13. Found: C, 49.55; H, 5.96; N, 4.19.

***O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (Chitotriose Undecaacetate) (3)**——Fraction II (total 7.4 g) was recrystallized from $CHCl_3$–MeOH–Et_2O to give 5.4 g (5.4%) of **3** as white needles, mp >350 °C, $[\alpha]_D^{28}$ +37.5° (c=0.85, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3350 (NH), 2940 (C–H). 1742 (OAc), 1660 (amide I), 1534 (amide II), 908 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.70—1.83 (9H, m, NAc × 3), 1.85—2.18 (24H, m, OAc × 8), 7.80—8.04 (3H, m, NH × 3). TLC: *Rf* 0.45 (solvent A). *Anal.* Calcd for $C_{40}H_{57}N_3O_{24}$: C, 49.84; H, 5.96; N, 4.36. Found: C, 50.05; H, 6.16; N, 4.56.

***O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (Chitotetraose Tetradecaacetate) (4)**——Fraction I (total 4.8 g) was recrystallized from $CHCl_3$–MeOH–Et_2O to give 3.7 g (3.7%) of **4** as a white powder, mp >350 °C, $[\alpha]_D^{28}$ +32.2° (c=0.60, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 2950 (C–H), 1745 (OAc), 1666 (amide I), 1538 (amide II), 910 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.68—1.80 (12H, m, NAc × 4), 1.85—2.12 (30H, m, OAc × 10), 7.75—7.92 (4H, m, NH × 4). TLC: *Rf* 0.33 (solvent A). *Anal.* Calcd for $C_{52}H_{74}N_4O_{31}\cdot 2H_2O$: C, 48.52; H, 6.12; N, 4.35. Found: C, 48.50; H, 6.01; N, 4.30.

***O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl Chloride (Decaacetyl Chitotriosyl Chloride) (5)**——A solution of **3** (10 g) in a mixture of AcOH (120 ml) and Ac_2O (1 ml) saturated with dry hydrogen chloride at 0 °C was kept at room temperature for 48 h. $CHCl_3$ (2000 ml) was poured into this solution, and the whole was washed with water, twice with ice-cold saturated $NaHCO_3$ solution, and again with water. The solution was dried over Na_2SO_4, and evaporated. The crystalline residue was dried over P_2O_5 *in vacuo*, then recrystallized from $CHCl_3$–Et_2O to give 4.2 g (43.4%) of **5** as white needles, mp 238—240 °C (dec.) after sintering at 216 °C, $[\alpha]_D^{25}$ +18.2 (c=0.68, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3440 (NH), 2950 (C–H), 1744 (OAc), 1660 (amide I), 1530 (amide II), 900 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.63—1.81 (9H, m, NAc × 3), 1.85—2.13 (21H, m, OAc × 7), 7.77—8.09 (3H, m, NH × 3). TLC: *Rf* 0.62 (solvent A). *Anal.* Calcd for $C_{38}H_{54}ClN_3O_{32}$: C, 48.54; H, 5.97; N, 4.47. Found: C, 48.32; H, 5.83; N, 4.50.

***O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl Chloride (Tridecaacetyl Chitotetraosyl Chloride) (6)**——A mixture of AcOH (102 ml) and Ac_2O (1 ml) containing **4** (8.5 g) was saturated with dry hydrogen chloride at 0 °C. The reaction mixture was kept at room temperature for 48 h, then $CHCl_3$ (1700 ml) was added. The whole was washed with water, twice with ice-cold saturated $NaHCO_3$ solution, and again with water. The solution was dried over Na_2SO_4, and evaporated. The crystalline residue was dried over P_2O_5 *in vacuo*, and recrystallized from $CHCl_3$–Et_2O to give 2.7 g (32.1%) of **6** as a white powder, mp >350 °C, $[\alpha]_D^{28}$ +15.5° (c=0.58, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 2950 (C–H), 1745 (OAc), 1668 (amide I), 1538 (amide II), 910 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.68—1.76 (12H, m, NAc × 4), 1.84—2.14 (27H, m, OAc × 9), 7.76—8.08 (4H, m, NH × 4). TLC: *Rf* 0.36 (solvent A). *Anal.* Calcd for $C_{50}H_{71}ClN_4O_{29}\cdot 3H_2O$: C, 46.86; H, 6.06; N, 4.37. Found: C, 46.67; H, 5.65; N, 3.89.

4-Methylcoumarin-7-yloxy *O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (4-Methylcoumarin-7-yloxy Decaacetyl-β-chitotrioside) (7)——The chloride **5** (2.06 g, 2.2 mmol) was

gradually added under stirring to a suspension of 7-hydroxy-4-methylcoumarin sodium salt (0.67 g, 3.4 mmol) (prepared by the procedure of Delmotte *et al.*[8]) in *N,N*-dimethylformamide (20 ml). The reaction mixture was kept at 5 °C for 16 h, then poured into ice water (200 ml). The heavy gel that rapidly formed was centrifuged for 15 min at 5000 *g*. The residue was suspended in water and centrifuged repeatedly under the same conditions, until the supernatant was colorless. The colorless residue, after being dried over P_2O_5 *in vacuo*, was recrystallized from $CHCl_3$–MeOH–Et_2O to give **7** as white needles, yield 1.94 g (81.7%), mp 306—308 °C (dec.), $[\alpha]_D^{25}$ −39.8° (*c* = 0.49, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3420 (NH), 2950 (C–H), 1742 (OAc, δ-lactone), 1660 (amide I), 1620 (C=C, ph), 1540 (amide II), 900 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.70—1.81 (9H, m, NAc × 3), 1.86—2.15 (21H, m, OAc × 7), 2.39 (3H, s, CH_3), 6.23, 6.99, 7.62, 7.71 (4H, all s, aromatic protons), 7.79—8.01 (3H, m, NH × 3). TLC: *Rf* 0.57 (solvent A). *Anal.* Calcd for $C_{48}H_{61}N_3O_{25}$: C, 53.38; H, 5.69; N, 3.89. Found: C, 53.35; H, 5.81; N, 3.97. UV λ_{max}^{DMSO} nm (ε): 288 (10270), 316 (15350).

4-Methylcoumarin-7-yloxy *O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (4-Methylcoumarin-7-yloxy Tridecaacetyl-β-chitotetraoside (8)——The chloride **6** (2.70 g, 2.2 mmol) was added to a suspension of 7-hydroxy-4-methylcoumarin sodium salt (0.87 g, 4.4 mmol) in *N,N*-dimethylformamide (27 ml). The reaction was continued under stirring for 16 h at 5 °C. The reaction mixture was then poured into ice water (270 ml). The white gel that formed was centrifuged according to the procedure described for **7**. The residue, after being dried over P_2O_5 *in vacuo*, was recrystallized from $CHCl_3$–MeOH–Et_2O to give **8** as a white powder, yield 2.00 g (67.9%), mp 301—303 °C (dec.), $[\alpha]_D^{28}$ −9.7° (*c* = 0.75, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 3090 (C=C, ph), 2940 (C–H), 1740 (OAc, δ-lactone), 1665 (amide I), 1618 (C=C, ph), 1540 (amide II), 905 (β-glycoside). ^{1}H-NMR (DMSO-d_6) δ: 1.70—1.80 (12H, m, NAc × 4), 1.86—2.12 (27H, m, OAc × 9), 2.40 (3H, s, CH_3), 6.22, 7.00, 7.62, 7.72 (4H, all s, aromatic protons), 7.76—8.20 (4H, m, NH × 4). TLC: *Rf* 0.34 (solvent A). *Anal.* Calcd for $C_{60}H_{78}N_4O_{32}$: C, 52.71; H, 5.75; N, 4.10. Found: C, 52.71; H, 5.80; N, 4.08. UV λ_{max}^{DMSO} nm (ε): 289 (10590), 316 (15750).

4-Methylcoumarin-7-yloxy *O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (4-Methylcoumarin-7-yloxy Tri-*N*-acetyl-β-chitotrioside) (9)——A suspension of **7** (0.50 g) in a mixture of $CHCl_3$–MeOH (1 : 1) (70 ml) was treated with 1.0 M sodium methoxide (2.7 ml) under stirring at 0 °C. Compound **7** soon dissolved. The precipitation of the de-*O*-acetylated product began after 10 min, and the mixture was further stirred for 16 h at 5 °C. The precipitates were collected by filtration, washed with ice-cold MeOH, and dried over P_2O_5 *in vacuo*. Recrystallization of the product was performed from aqueous MeOH to give **9** as a white crystalline powder, yield 0.29 g (80.0%), mp 248—249 °C (dec.), $[\alpha]_D^{25}$ −30.7° (*c* = 0.55, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 3100 (C–H, ph), 2950, 2900 (C–H), 1730 (C=O, δ-lactone), 1660 (amide I), 1620 (C=C, ph), 1550 (amide II), 900 (β-glycoside). TLC: *Rf* 0.56 (solvent B). *Anal.* Calcd for $C_{34}H_{47}N_3O_{18} \cdot 3/2H_2O$: C, 50.25; H, 6.21; N, 5.17. Found: C, 50.26; H, 6.13; N, 5.06. UV λ_{max}^{DMSO} nm (ε): 288 (8270), 318 (12620).

4-Methylcoumarin-7-yloxy *O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (4-Methylcoumarin-7-yloxy Tetra-*N*-acetyl-β-chitotetraoside) (10)——A suspension of **8** (2.00 g) in a mixture of $CHCl_3$–MeOH (1 : 1) (300 ml), was treated with 1.0 M sodium methoxide (11.5 ml) under stirring. Compound **8** soon dissolved completely, and after 10 min, the de-*O*-acetylated derivative precipitated gradually. The white crystals of **10** thus produced were filtered off, washed with cold MeOH, and dried. Recrystallization was performed from aqueous MeOH to give **10** as a white crystalline powder, yield 1.30 g (87.4%), mp 288—289 °C (dec.), $[\alpha]_D^{28}$ −17.4° (*c* = 0.78, DMSO). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 3300 (NH), 3090 (C–H, ph), 2940. 2870 (C–H), 1730 (C=O, δ-lactone), 1660 (amide I), 1620 (C=C, ph), 1550 (amide II), 900 (β-glycoside). TLC: *Rf* 0.62 (solvent B). *Anal.* Calcd for $C_{42}H_{60}N_4O_{23} \cdot 1/2H_2O$: C, 50.55; H, 6.16; N, 5.61. Found: C, 50.52; H, 6.26; N, 5.65. UV λ_{max}^{DMSO} nm (ε): 289 (8700), 314 (13140).

Lysozyme Assay——Fluorometric assay: 0.028 mM 4-methylcoumarin-7-yloxy glycoside (**9** or **10**) (5 ml) in 0.05 M sodium citrate buffer (pH 4.8), containing 250 μg/ml of lysozyme, was incubated at 37 °C. After an appropriate time, 7-hydroxy-4-methylcoumarin (**12**) liberated was measured at 445 nm with excitation at 320 nm.

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