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Design, synthesis, and enzymatic property of a sulfur-substituted analogue of trigalacturonic acid

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Abstract—A sulfur-substituted analogue of trigalacturonic acid (**3**) was synthesized. The synthesis features the application of 3-cyano-3-(*tert*-butyldimethylsilyl)oxypropylthioether (CSP) as a novel protective group for thiols. This analogue was designed with the expectation that it would be a stable analogous substrate for *endo*-polygalacturonase isolated from *Stereum purpureum* based on computer modeling experiments. Surface plasmon resonance experiments revealed that **3** forms a stable complex with the target enzyme.

Endo-polygalacturonase (*endo*-PGs) hydrolyzes the internal glycosidyl linkages of polygalacturonic acids (pectic acids) into oligomers. Because oligogalacturonic acids (low-molecular-sized pectic acids) have recently attracted attention as functional supplemental foods,[1] such as Ca^{2+} carriers,[2] dietary fiber,[3] and so on, investigating in detail that the mechanism of *endo*-PG has gained importance in the last decade. However, elucidation of the details of the reaction for *endo*-type glycosidases including *endo*-PGs generally lags behind those of *exo*-glycosidases due to difficulty in designing suitable molecular probes.[4]

Recently, Miyairi and Okuno, authors of the present paper, found *endo*-PG 1 to be the substance responsible for silver leaf disease on apples from the pathogenic *Stereum purpureum*.[5] This glycosidase causes degradation of pectin in the leaf cell wall, inducing the disease. Interestingly, *endo*-PG 1 makes single crystallines that provide an X-ray crystallographic structure of extremely high quality (less than 0.96 Å resolution, PDB ID: 1K5C).[6] However, soaking experiments have not provided the complex of *endo*-PG 1 with the oligogalacturonate as a reactant, because enzymatic hydrolysis occurred in the crystalline to afford only the complex with the hydrolysates. Thus, we planned to investigate in detail the reaction mechanism of this enzyme employing stable oligogalacturanate mimics. We report here the design and synthesis of sulfur-substituted analogue **3**, designed as a stable mimic of natural substrate **2** against *endo*-PG 1.

We expected that replacing one acetal oxygen cleaved by the enzyme would result in tolerance to the enzyme with minimum structural alteration.[7] On the other hand, Miyairi studied the kinetic properties of *endo*-PG 1 revealing that the minimum size as a substrate is trimer **2**.[6] As shown in Figure 1, the enzyme hydrolyzes **2** site-specifically at the glycoside bond between C4O and C1′. Tetramer **1** was hydrolyzed more easily by this enzyme, but, site specificity decreased, also cleaving the bond between C4′O and C1″ at a ratio of 10%. This suggests that the enzyme can slide on the sugar chain. In contrast, in these experiments, the terminal pyranose unit of **2** binds selectively with the subsite +1 in *endo*-PG 1. Accordingly, replacement of the glycosidic oxygen (atom X in Fig. 1) with a sulfur atom would realize resistance to *endo*-PG 1 with minimum structural alteration, and this analogue would create a homogeneous complex with the target enzyme. We expected that replacing only

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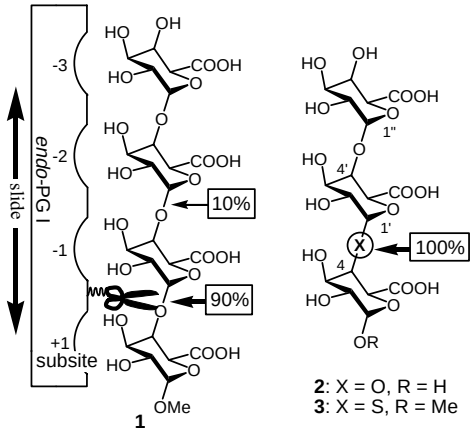


Figure 1.

one oxygen would not cause serious structural alteration in complexation.

Prior to the synthesis, we evaluated analogue **3** by molecular dynamic simulations for the matrix of *endo*-PG 1 and **3** including 8633 waters.[8] These simulations suggested that analogue **3** would bind to the enzyme in almost the same hydrogen-bonding manner (Fig. 2) as the presumed native complex. This native complex was constructed based on two X-ray structures, a complex with two mono-galacturonic acids that bind at the subsites +1 and −1 individually (PDB ID: 1KCD) obtained by using excess mono-galacturonic acid, and a complex with a pentamer at subsite −1, −2, −3, −4 prepared by soaking at higher pH solution in which the enzyme was inactivated.[9] Neither complex involved the glycoside bonds between −1 and +1 (the reaction subsite). Although the substituting glycosidyl oxygen to sulfur did cause a minor conformational alteration, it did not greatly disturb the network of hydrogen bondings between the enzyme and the substance as shown in Figure 2. Accordingly, analogue **3** would be an ideal molecular probe for mechanistic investigation.

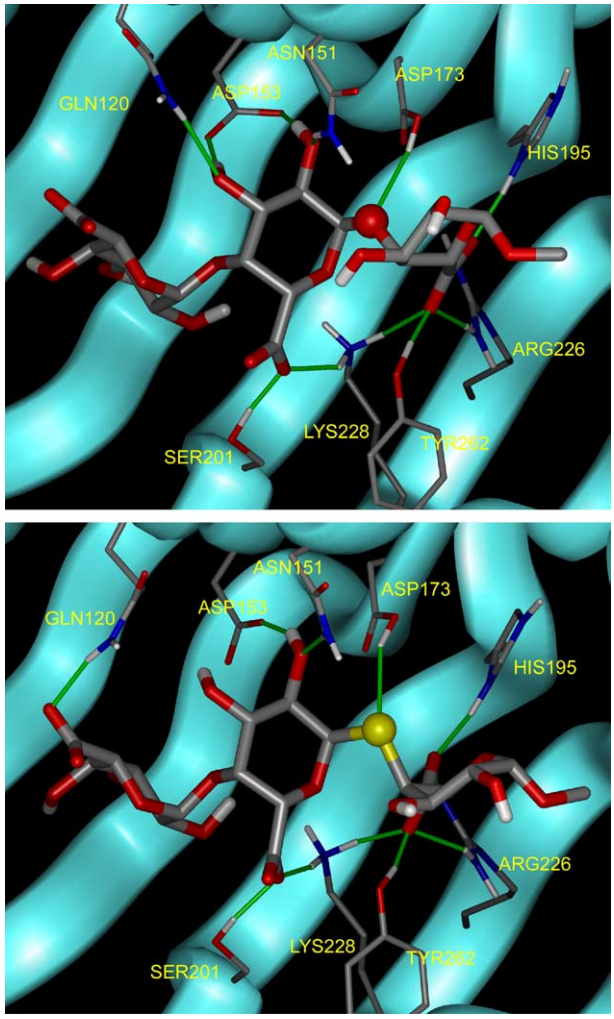
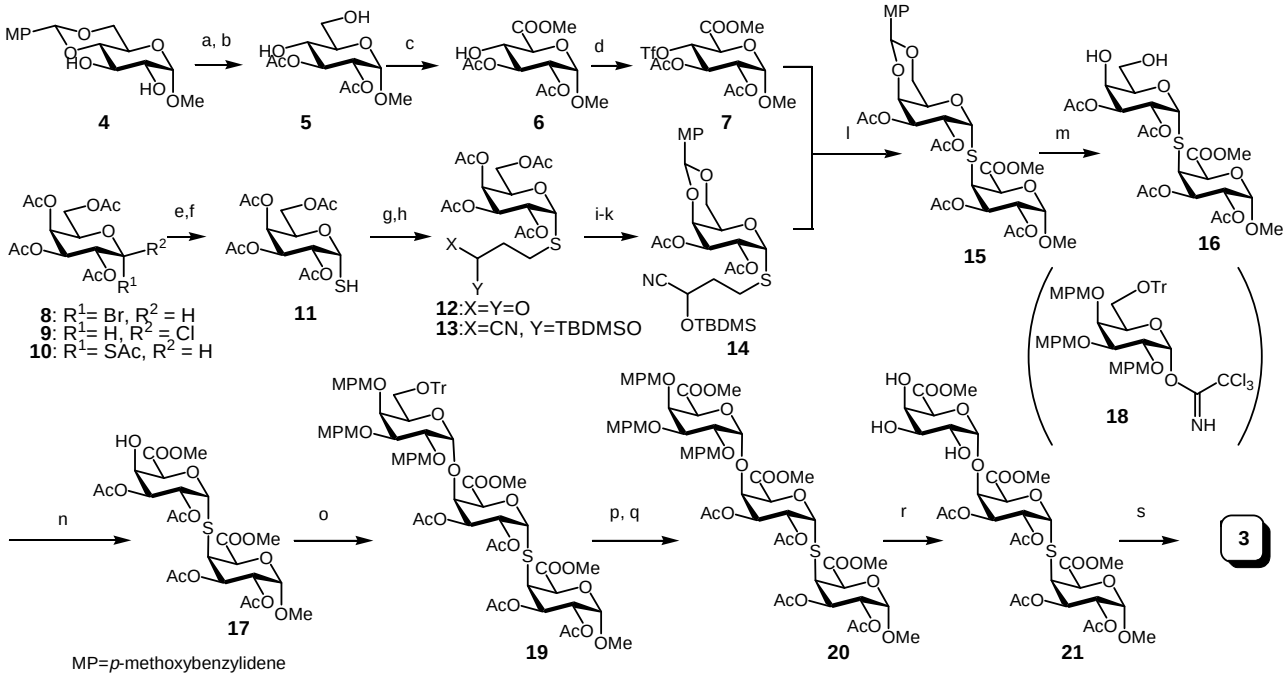


Figure 2. The matrix of *endo*-PG 1 with natural substrate **2** (upper) and sulfur analogue **3** (lower) expected by the calculation using extended COSMOS90. The initial geometries were constructed based on the X-ray structure of *endo*-PG 1 with galacturonic acids at subsites (+1, −1) and (−1, −2, −3, −4) obtained by the soaking experiments.

These computer modeling experiments motivated us to conduct the synthesis. Our preliminary experiments suggested that the glycosylation of thiols using glycosyl trichloroacetimidates provides the desired glycoside only in low yields despite some successful reports.[10] Thus, we focused on introducing thioglycoside linkage by coupling 1-thioderivative **14** with glucopyranose derivative **7**, carrying a trifluoro-methanesulfonate leaving group at the C4 position.

Triflate **7** was first prepared as shown in Scheme 1. After acetylation of methyl 4,6-*O*-(4-methoxy)benzylidene glucopyranoside (**4**),[11] the benzylidene moiety was removed by acetic acid to provide diol **5** in 90% yield in two steps. Selective oxidation of the primary alcohol of **5** was achieved by employing TEMPO and $PhI(OAc)_2$ under aqueous conditions.[12] The following treatment with diazomethane gave methyl ester **6** in 91% in two steps. The C4-OH was then converted into the sulfonate with Tf_2O/pyridine, giving **7** in 89% yield.

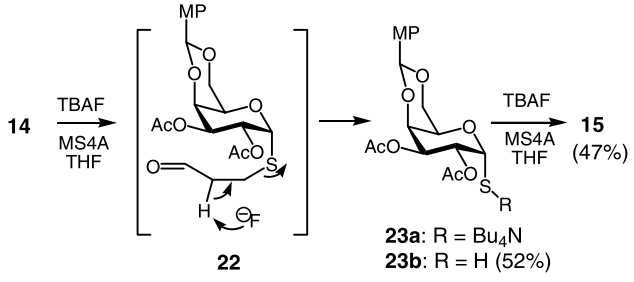
The thiol part was then synthesized. The α-thioacetyl group was introduced stereoselectively at the anomeric position via the double inversion of α-galactosyl bromide **8**. Treatment of **8** with Bu_4NCl in DMPU at room temperature provided β-chloride **9**,[13] which was further treated with potassium thioacetate at 50 °C using a one-pot procedure to give α-thioacetate **10** stereoselectively in 73% overall yield. Prior to manipulating 2,3,4,6-*O*-functions, the 1-*S*-acetyl group was replaced with a newly developed protective group, 3-cyano-3-*tert*-butyldimethylsilylpropyl (abbreviated to CSP group) thioether. The 1-*S*-acetyl group of **10** was removed by basic methanolysis at −25 °C to give thiol **11** in 89% yield. Treatment of **11** with acrolein (1.2 equiv) in DMF resulted in the Michael addition of thiol group. Following aqueous work-up, the aldehyde function in the resulting Michael adduct **12** was further converted into O-silylated cyanohydrin by TBDMSCN in the presence of KCN in CH_3CN to provide **13** in 73% yield in two steps. The ^1H NMR spectrum indicated that **13** is a 1:1 mixture of diastereomers regarding



Scheme 1. Reagents and conditions; (a) Ac_2O, Py (91%); (b) AcOH, H_2O, 65 °C (99%); (c) TEMPO, $PhI(OAc)_2$, acetone, H_2O, then CH_2N_2, Et_2O (91%); (d) Tf_2O, Py, CH_2Cl_2 (83%); (e) Bu_4NCl, DMPU r.t., then KSAc 50 °C (73%); (f) NaOMe, MeOH, −25 °C (89%); (g) acrolein, DMF; (h) TBDMSCN, KCN CH_3CN (73%, two steps); (i) NaOMe, MeOH; (j) *p*-CH_3O-$PhCH(OCH_3)_2$, CSA, DMF (69%, two steps); (k) Ac_2O, Py (93%); (l) TBAF, MS4A, THF (94%); (m) AcOH, H_2O, 65 °C (86%); (n) TEMPO, $PhI(OAc)_2$, CH_2Cl_2, H_2O, then CH_2N_2, Et_2O (53%); (o) **18**, TESOTf, CH_2Cl_2, −78 °C (73%); (p) AcOH, H_2O, 60 °C (80%); (q) TEMPO, $PhI(OAc)_2$, CH_2Cl_2, H_2O, then CH_2N_2, Et_2O (97%); (r) DDQ, CH_2Cl_2, H_2O (79%); (s) NaOH, THF, H_2O (99%).

the cyanohydrin moiety. This protective function was found to be stable during the following treatments. After all acetyl groups in **13** were removed by basic hydrolysis, the C4- and C6-OH were protected in the form of *p*-methoxybenzylidene acetal under the usual conditions. The remaining C2 and C3 alcohols were acetylated again to provide **14** in good overall yield.

Treatment of **14** with TBAF in THF in the presence of molecular sieves 4A and the following addition of triflate **7** after 5 min using a one-pot procedure resulted in a coupling reaction to provide thioglycoside **15** in 94% yield. The fluoride ion under aprotic conditions induced the *retro*-Michael reaction of regenerated aldehyde **22**, giving **23a** in a form of highly reactive tetrabutylammonium mercaptide. This method was more efficient than the following stepwise transformations. Aqueous work-up prior to the addition of **7** gave thiol **23b** in 52% yield (Scheme 2). The basic treatment



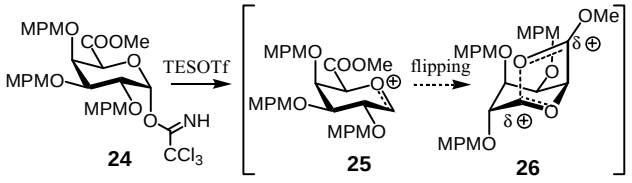
Scheme 2.

of pure **23b** with TBAF/MS4A gave **15** in only a 47% yield. Notably, the coupling reaction with pure **23b** proceeded more smoothly to give **15** in 81% yield by employing sodium hydride as the base.

Glycoside **15** was then converted into glycosyl acceptor **17** by acidic cleavage of the 4-methoxybenzylidene acetal (→**16**, 86% yield), oxidation of the primary alcohol with TEMPO/$PhI(OAc)_2$ followed by treatment with CH_2N_2 (→**17**, 53% yield).

We failed in the glycosylation of **17** when galacturonate **24** (Scheme 3)[12,14] was employed as the donor although the glucuronate derivative (the C4 equatorial isomer) gave the adduct under the same conditions. Carbocation **25** produced from **24** was likely deactivated by the C6-carbonyl group after flipping of the pyranose ring. Thus, we decided to construct the carboxyl group at the non-reducing end after glycosylation. As expected, glycosylation of acceptor **17** with trityl ether protected imidate **18** took place to produce trimer **19** stereoselectively in 73% yield by employing TESOTf as the activator.[15] Stereochemistry of **19** of the newly furnished glycoside bond was confirmed by observing a small coupling constant between C1″H and C2″H (J = 1.5 Hz) in ^{1}H NMR.

The trityl ether in **19** was selectively cleaved by aqueous acetic acid at 60 °C in 80% yield. Oxidation of the resulting alcohol with TEMPO/$PhI(OAc)_2$ was also effective and was followed by treatment with diazomethane to provide methyl ester **20** in 97% yield in three steps.



Scheme 3.

Finally, all protective groups of **20** were removed. The MPM ethers were cleaved by DDQ oxidation without affecting the sulfide function[4] to give triol **21** in 88% yield. The following basic treatment hydrolyzed all ester groups. Ion exchange column chromatography (Dowex 50W, H^+ form) after the reaction provided a pure sample of **3** as a white powder.[16] Preliminary enzymatic studies revealed that **3** inhibited hydrolysis of oligogalacturonic acid by *endo*-PG 1 and was stable under these conditions. The experiments employing surface plasmon resonance[17] revealed the K_D value of **3** to be 0.2 μmol/L.[18]

As described, we designed and synthesized a sulfur analogue of trigalacturonic acid **3** as a stable mimic of the natural substrate. Computer modeling calculations suggested that **3** would be a stable analogue that can bind with *endo*-PG 1 in the same manner as the natural substrate. In fact, **3** was not only stable in the presence of *endo*-PG 1 but also made a stable complex with the target enzyme. In these studies, we also demonstrated that the CSP group is a useful protective group that can be readily activated by TBAF under aprotic conditions. These findings resulted in the successful preparation of **3** in sufficient quantity for the enzymatic studies (>100 mg). Detailed structural and thermodynamic studies on the complex using X-ray crystallographic analysis, calorimetric experiments, and longer-time MD simulations are under investigation in our laboratories.

Acknowledgments

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16. $[\alpha]_D^{25} = +133°$ (*c* 0.50, H_2O), ^1H NMR (400 MHz, 6.7 mg/1.0 mL of D_2O) δ 3.28 (3H, s, OCH_3), 3.44 (1H, dd, $J = 3.9$, 10.4 Hz, C2*H*), 3.57 (1H, dd, $J = 1.8$, 4.6 Hz, C4*H*), 3.61 (1H, dd, $J = 3.9$, 10.5 Hz, C2′*H*), 3.78 (1H, dd, $J = 5.4$, 11.4 Hz, C3″*H*), 3.80 (1H, dd, $J = 4.6$, 10.4 Hz, C3′*H*), 3.98 (1H, dd, $J = 5.4$, 10.2 Hz, C2″*H*), 4.02 (1H, dd, $J = 4.4$, 10.2 Hz, C3*H*), 4.20 (1H, dd, $J = 1.4$, 3.0 Hz, C4′*H*), 4.30 (1H, d, $J = 3.0$ Hz, C4″*H*), 4.75 (2H, d, $J = 3.9$ Hz, C1*H*, C5*H*), 4.929 (1H, s, C5′*H*), 4.933 (1H, d, $J = 3.9$ Hz, C1′*H*), 5.05 (1H, s, C5″*H*), 5.39 (1H, d, $J = 5.4$ Hz, C1″*H*), ^{13}C NMR (100 MHz, D_2O) δ 50.34, 55.51, 67.32, 67.68, 67.71, 68.60, 68.74, 69.26, 69.84, 69.86, 70.11, 70.84, 77.73, 87.29, 99.43, 99.94, 171.67, 172.18, 172.68 ppm. ESIMS (positive) $m/z = 599$ $(M+Na)^+$, (negative), $m/z = 575$ $(M-H)^-$.
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