Electronic Supplementary Information

Creation of an artificial metalloprotein with a Hoveyda-Grubbs catalyst moiety through the intrinsic inhibition mechanism of α-chymotrypsin

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CONTENTS

1. Experimental

Materials

All chemicals were obtained from conventional commercial sources and used as received unless otherwise noted. α-Chymotrypsin from bovine pancreas (Type II, lyophilized) was purchased from Sigma-Aldrich (C4129) and used as received. Inhibitors with Hoveyda-Grubbs catalyst moiety (**1**$_{\text{-L}}$ and **1**$_{\text{-D}}$) were synthesized from the inhibitor/linker part **2** and the Hoveyda-Gubbs catalyst part **3** as described below (Scheme S1). *N*-Succinyl L-phenylalanine *p*-nitroanilide (SucPhePNA) was prepared by the acid deprotection of *N*-benzyloxycarbonyl L-phenylalanine *p*-nitroanilide and the condensation with succinic anhydride in the presence of diisopropylamine in CH_2Cl_2.[1] *N,N'*-Diallylammonium hydrochloride (DAA, **4**) was prepared by bubbling HCl gas into a solution of *N,N'*-diallylamine in ether. *N,N'*-Diallyl-[3-(1-D-glucopyranosyl)oxy]propanamide (GlcDAA, **5**) was synthesized as described below (Scheme S2). *N*-Tosyldiallylamine (TDA) was prepared according to the reported method.[2]

General procedures

Air-sensitive and/or moisture-sensitive compounds were manipulated using conventional Schlenk technique under a nitrogen atmosphere or treated in a glove-box filled with nitrogen (O_2 < 1ppm).

Instruments

^{1}H-NMR spectra were collected using a JEOL JNM-ECA 600 (600 MHz) or a JEOL JNM-ECP 400 (400 MHz) NMR spectrometer. The ^{1}H NMR chemical shifts are reported in ppm relative to tetramethylsilane (TMS) or the residual solvent resonances. ESI-MS analyses were carried out using a JEOL JMS-T100LC mass spectrometer. MALDI-TOF-MS analyses were conducted using a Bruker Autoflex II mass spectrometer with sinapinic acid as the matrix. UV-vis spectra were measured using a Shimadzu UV-2550 spectrophotometer. CD spectra were measured using a JASCO J-725 circular dichroism spectrophotometer. FPLC analyses were conducted using a BioRad Biologic Duoflow liquid chromatography system operated in a chromatochamber (4 ºC). HPLC analyses were conducted using a Shimadzu HPLC system equipped with an LC-liquid chromatograph pump, an SPD-M20A diode array detector, a 20-μL sample loop and a Shimadzu Shim-Pack ODS(H) column (I.D. 4.6 mm × 250 mm). The MMFF calculation for the structural optimization of **1**$_{\text{-L}}$ was carried out by using Spartan '08 (Wavefunction Inc.). B3LYP functional with

LANL2DZ and 6-31G* basis set was employed for the calculations.

Modification of α-chymotrypsin with Hoveyda-Grubbs catalyst inhibitor 1

As a typical procedure, α-chymotrypsin (33 mg) was dissolved in 40 mL of 1 mM HEPES buffer (pH = 7.0) containing KCl (100 mM). The protein solution was incubated at 25 ºC. A solution of the Hoveyda-Grubbs inhibitor (**1**$_{\text{-L}}$ or **1**$_{\text{-D}}$, 2.8 mg) in MeOH (0.8 mL) was slowly added to the protein solution with gently shaking (final concentrations: [α-chymotrypsin] = 33 μM, [inhibitor] = 66 μM, MeOH content = 2% (v/v)) The solution was incubated at 25 ºC for 2 h. If needed, the hydrolase activity was checked. After centrifuging to remove insoluble materials, the protein solution was concentrated by ultrafiltration (cutoff M_w = 10,000) and subjected to FPLC purification (CM Sepharose FF 5 mL, GE Healthcare) with elution of 100 mM KCl*aq* (1 CV) followed by a linear gradient to 1 M KCl*aq* over 4 CV. The fractions eluted at 12–20 mL were collected and concentrated. The concentrated protein solution was frozen and kept at –80 ºC before use (ε_{280} = 57,000 $M^{-1}cm^{-1}$).

Evaluation of hydrolase activity

The concentration of a protein solution of unmodified and modified protein was adjusted to 6 μM in 10 mM HEPES buffer (pH = 7.0) containing 100 mM KCl*aq*. After addition of SucPhePNA in MeCN to the protein solution ([SucPhePNA] = 0.6 mM and [MeCN] = 2% (v/v) in final), the absorbance change at 377 nm (the release of *p*-nitroaniline) was followed at 25 ºC.

CD measurements

A solution of the modified or unmodified α-chymotrypsin in 100 mM KCl*aq* was put into a quartz cuvette (L = 0.1 mm) and scanned in a range of appropriate wavelengths at 25 ºC. Based on the CD signals, the UV-vis spectra obtained in a separate experiment were analyzed by Gaussian-type functions using ORIGIN Pro 8.0J software (OriginLab Corporation), and the best-fit wave shapes were calculated.

Evaluation of metathesis activity of modified α-chymotrypsin toward GlcDAA and DAA

A solution of the modified α-chymotrypsin in D_2O containing KCl (100 mM) was degassed and kept under a nitrogen atmosphere. To a solution of a substrate in the same solvent was added the protein solution, and the mixture was stood at 37 ˚C with N_2 stream. The final concentrations of the reactants are [substrate] = 8.0 mM and

[α-chymotrypsin] = 50 μM (0.63 mol% for the reaction with GlcDAA) or 0.25 mM (2.5 mol% for the reaction with DAA). In the reaction of GlcDAA, the appearance of the peaks at 2.65 ppm and 4.30 ppm in the ^{1}H NMR spectrum is indicative of the product formation of the ring closing metathesis (Fig. S7). No appearance of new peaks was observed in the reaction of DAA. The turnover numbers were calculated based on the peak intensity at 5.85 ppm (an overlapped signal of the starting compounds and the ring-closed products).

Evaluation of metathesis activity of 1 (without protein) toward DAA and GlcDAA

A substrate solution in D_2O containing KCl (100 mM) was degassed and kept under a N_2 atmosphere. Hoveyda-Grubbs catalyst inhibitor **1** was dissolved in MeOH (for the reaction with DAA) or DMSO (for the reaction with GlcDAA). The catalyst solution was added to the substrate solution and the mixture was stood at 37 °C with N_2 stream. The final concentrations of the reactants are [substrate] = 8.0 mM and (0.63 mol% for the reaction with GlcDAA) or 0.2 mM (2.5 mol% for the reaction with DAA). The content of MeOH or DMSO was set to 10% (v/v). The product detection in the reaction of GlcDAA was conducted in the same manner as descried above. For the reaction of DAA, the product amount was calculated from the peak intensity at 3.92 ppm.

Evaluation of metathesis activity of modified α-chymotrypsin toward TDA

A solution of the modified α-chymotrypsin in D_2O containing KCl (100 mM) was degassed and kept under a nitrogen atmosphere. A mixed solution of TDA and 1-phenylethanol (internal standard for HPLC analysis) in DMSO was added to the protein solution, and the mixture was stood at 37 °C with N_2 stream. The final concentrations of the reactants are [TDA] = 1.0 mM, [α-chymotrypsin] = 25 μM (2.5 mol%) and [1-phenylethanol] = 0.64 mM. The content of DMSO should be 10% (v/v) because of serious denaturation of the protein in the presence of higher contents of DMSO. The reaction under the high concentrations of TDA was impossible due to low solubility of TDA. The reaction mixture was sampled and subjected to an HPLC analysis. The separation conditions were as follows: Elution flow rate 1.0 mL/min; Solvent 90% H_2O/10% MeCN (0–5 min), linear gradient to 100% MeCN (5–12 min), 100% MeCN (15–20 min); Detection 210 nm. The retention times of the components are 14.07 min for 1-phenylethanol, 15.56 min for *N*-tosyl 2,5-dihydropyrrole (RCM product) and 16.56 min for *N*-tosyldiallylamine. A typical chromatogram is shown in Fig. S8.

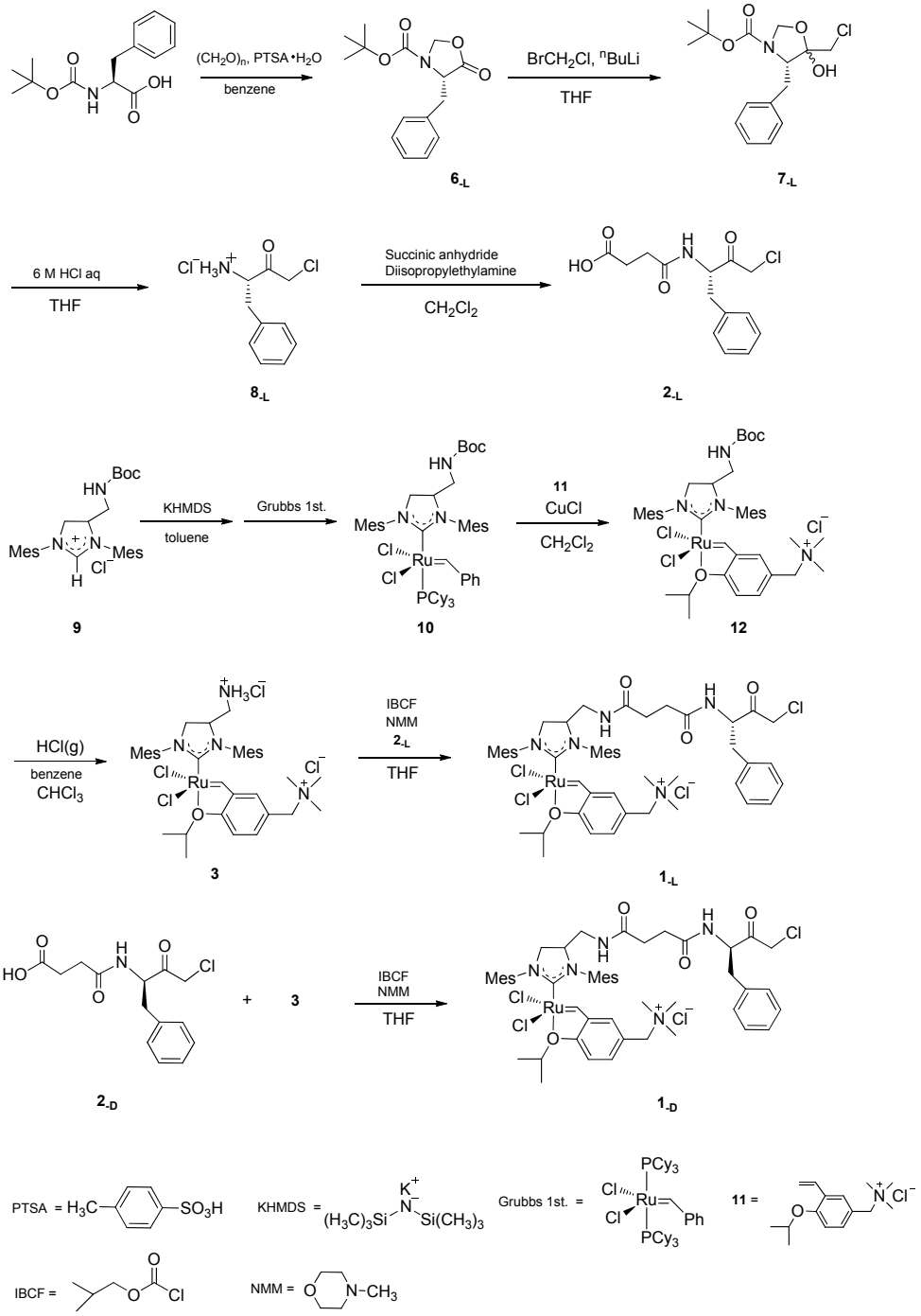
Evaluation of metathesis activity of 1 toward TDA

A solution of **1** in D_2O containing KCl (100 mM) and DMSO (10 %(v/v)) was degassed and kept under a nitrogen atmosphere. A solution containing TDA and 1-phenylethanol in DMSO was added to the catalyst solution, and the mixture was stood at 37 °C with N_2 stream. The final concentrations of the reactants are [TDA] = 1.0 mM, [**1**] = 25 μM (2.5 mol%) and [1-phenylethanol] = 0.64 mM. The product detection was conducted in the same manner as descried above.

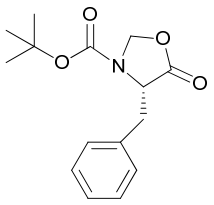
2. Synthesis of inhibitors with Hoveyda-Grubbs catalyst moiety 1_{-L} and 1_{-D}

The synthetic route is depicted in Scheme S1. Compounds **9** and **11** were prepared by the reported method.[3] The D-form inhibitor ($\mathbf{1_{-D}}$) was synthesized from the D-inhibitor part $\mathbf{2_{-D}}$ with **3** by the same manner as the synthesis of L-form inhibitor $\mathbf{1_{-L}}$, which is described below.

Scheme S1

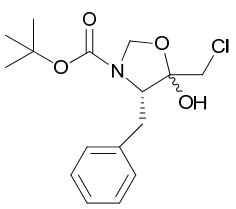

(CH2O)n, PTSA•H2O
benzene
BrCH2Cl, nBuLi
THF
6-L
7-L
6 M HCl aq
THF
Succinic anhydride
Diisopropylethylamine
CH2Cl2
8-L
2-L
KHMDS
toluene
Grubbs 1st.
11
CuCl
CH2Cl2
9
10
12
HCl(g)
benzene
CHCl3
IBCF
NMM
2-L
THF
3
1-L
2-D
1-D
PTSA = H3C SO3H
KHMDS = (H3C)3Si N K Si(CH3)3
Grubbs 1st. =
11 =
IBCF =
NMM = O N-CH3

Compound 6-L.[4] In a 300-mL three-necked flask equipped with a Dean-Stark trap and a condenser, *N*-Boc-L-phenylalanine (5.30 g, 20 mmol), paraformaldehyde (1.40 g) and *p*-toluenesulfonic acid monohydrate (PTSA·H_2O, 0.200 g, 1.1 mmol) were suspended in benzene (200 mL) and warmed up to 90 °C. After 1.5 h, the reaction mixture became a pale yellow solution. After cooling, the solution was diluted with AcOEt (50 mL), washed with 5% citric and brine, and dried over Na_2SO_4. The solvent was evaporated to obtain **6-L** as pale yellow solids with 68% yield (3.70 g). The product was used for the next reaction without further purification.



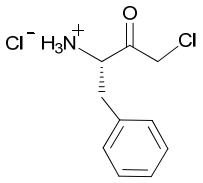
6-L

Compound 7-L.[5] In a 200-mL three-necked flask equipped with a dropping funnel, compound **6-L** (4.20 g, 15 mmol) and bromochloromethane (2.52 g, 20 mmol) were dissolved in dry THF (42 mL) and cooled to –78 °C. To the solution was dropwise added *n*-butyllithium in hexane (1.6 M, 12.2 mL, 20 mmol). After stirring for 1 h, 5% $KHSO_4$*aq* was added and stirred for 30 min. The reaction mixture was diluted with AcOEt (30 mL), washed with water twice and dried over Na_2SO_4. The solvent was evaporated and the obtained pale yellow oil was subjected to silica gel column chromatography (hexane/AcOEt = 5/2, R_f = 0.48 on silica TLC). Compound **7-L** was obtained as pale yellow oil with 69% yield (3.40 g) ^{1}H NMR: δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.48 (9H, s, Boc), 2.92 (1H, m, -CHC$H_{2\alpha}$Ph), 3.02 (1H, m, -CHC$H_{2\beta}$Ph), 3.80 (1H, m, -C$H_{2\alpha}$Cl), 3.92 (1H, m, -C$H_{2\beta}$Cl), 4.30 (1H, m, -NC*H*(CH_2Ph)C-), 4.87 (2H, m, -NCH_2O-), 5.20 (1H, br, -OH), 7.10–7.40 (phenyl protons).



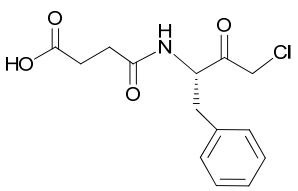
7-L

Compound 8-L.[5] In a 50-mL flask, compound **6-L** (1.20 g, 3.7 mmol) was dissolved in THF (15 mL) and 6 M HCl*aq* (5 mL) was added. The solution was stirred at 50 °C for 50 min. After the solution was cooled to room temperature, the solvent was evaporated. The formed solid was dissolved in MeOH, re-precipitated by addition of ether and collected by suction. Compound **8-L** was obtained as white solids with 80 % yield (0.690 g) ^{1}H NMR: δ_H (400 MHz; DMSO-d_6; residual proton in solvent) 3.04 (1H, dd, *J* = 14.4, 6.6 Hz, -CHC$H_{2\alpha}C_6H_5$), 3.16 (1H, dd, *J* = 14.4, 6.6 Hz, -CHC$H_{2\beta}C_6H_5$), 4.47-4.51 (1H, m, -C*H*$CH_2C_6H_5$), 4.51(1H, dd, *J* =17.4 Hz, -C$H_{2\alpha}$Cl), 4.68 (1H, dd, *J* =17.4 Hz, -C$H_{2\beta}$Cl), 7.36-7.28 (m, 5H, -C_6H_5), 8.49 (3H, br, -NH_3).



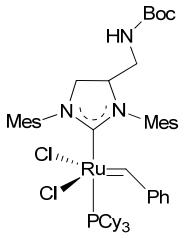
8-L

Inhibitor moiety $\mathbf{2}_{\text{-L}}$. In a 50-mL two-necked flask equipped with a $CaCl_2$ tube, compound $\mathbf{8}_{\text{-L}}$ (0.300 g, 1.3 mmol) and cracked succinic anhydride (0.149 g, 1.5 mmol) was suspended in CH_2Cl_2 (7.5 mL). The mixture was cooled to –20 °C with an ice-salt bath. Diisoprolylethylamine (DIPEA, 0.331 g, 2.6 mmol) in 1 mL of CH_2Cl_2 was dropwise added, and the solution was stirred in the ice bath for 1 h and at room temperature for 1 h. The solvent was evaporated, and the resulted residue was triturated with AcOEt (25 mL) and 0.1 M HCl*aq* (5 mL). The organic phase was separated, washed with brine and dried over Na_2SO_4. After the solvent was evaporated, addition of hexane gave compound $\mathbf{2}_{\text{-L}}$ as white solids with 74% yield (0.283 g). ^{1}H NMR δ_H (400 MHz; DMSO-d_6; residual protons in solvent) 2.23-2.45 (4H, m, -HOOCCH_2CH_2CONH), 2.80 (1H, dd, J = 13.8, 5.4 Hz, -CHC$H_{2\beta}$C$_6H_5$), 3.08 (1H, dd, J = 13.8, 5.4 Hz, -CHC$H_{2\alpha}$C$_6H_5$), 4.45-4.51 (1H, m, -CHCH$_2$C$_6H_5$), 4.48 (1H, d, J =17.4 Hz, -C$H_{2\alpha}$Cl), 4.60 (1H, d, J =17.4 Hz, -C$H_{2\beta}$Cl), 7.19-7.29 (5H, m, -C$_6H_5$), 8.47 (1H, s, -NHCO). ^{13}C NMR δ_c (100 MHz, DMSO *d*-6; carbon in solvent) 30.96 (-HOOCCH$_2$CH_2CONH), 31.57 (-HOOCCH$_2$CH$_2$CONH), 39.17 (-CONHCH(CH_2C$_6$H$_5$)CO), 46.32 (-COCH_2Cl), 54.24 (-CONHCH(CH$_2$C$_6$H$_5$)CO), 109.1 (*ortho*-Ph), 110.5 (*para*-Ph), 111.2 (*meta*-Ph), 117.9 (ipso-Ph), 145.3 (-CONH-), 147.0 (HOOC-), 168.3 (-COCH$_2$Cl).



$\mathbf{2}_{\text{-L}}$

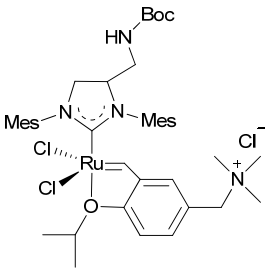
Grubbs 2nd generation catalyst 10.[3] In a glovebox, NHC ligand **5** (138 mg, 0.292 mmol) was suspended in 2 mL of dry toluene in a 30-mL round-bottom flask. Potassium hexamethyldisilazane (KHMDS, 57.5 mg, 0.292 mmol) in 1 mL of toluene was added, and the mixture was stirred at room temperature for 1 h. After the solution became yellow, Grubbs 1st generation catalyst (200 mg, 0.243 mmol) was added to the solution. The reaction mixture was transferred into a Schlenk tube equipped with a condenser. The tube was out of the glovebox and the solution was stirred at 50 ºC for 2 h and at room temperature for 3 h under a nitrogen atmosphere. The reaction mixture was dried up and the resulted purple residue was subjected to silica gel column chromatography (elution: CH_2Cl_2/MeOH = 13/1) with bubbling N_2. The second fraction (with magenta in color) was collected and the solvent was evaporated to afford **10** as purple solid (205 mg) with 86% yield. Appearance of a broad peak at 19.08–19.24 ppm (from the benzylidene proton) in the ^{1}H NMR spectrum (400 MHz; $CDCl_3$; Me_4Si) confirms the ligand exchange of the PCy_3 ligand with the NHC ligand. Further assignment of the peaks was impossible because of the free



10

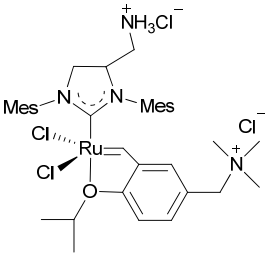
rotation of the NHC ligand moiety.[3]

Hoveyda-Grubbs catalyst 12. In a Schlenk tube equipped with a condenser, Grubbs 2nd generation catalyst **10** (286 mg, 0.292 mmol), Hoveyda-type ligand **11** (78.8 mg, 0.292 mmol) and CuCl (28.9 mg, 0.292 mmol) were dissolved in CH_2Cl_2 under a nitrogen atmosphere. The reaction mixture was stirred at 45 ºC for 1 h and turned to be green in color. After the reaction mixture was dried up, the resulted residue was subjected to alumina column chromatography (neutral; activity I; CH_2Cl_2/MeOH = 13/1). The leading band (R_f = 0.9 on TLC with elution of CH_2Cl_2/MeOH = 13/1) was collected. The solvent was evaporated to afford compound **12** as dark green solid with 206 mg (82% yield). ^{1}H NMR: δ_H (400 MHz; $CDCl_3$; Me_4Si) 1.16 (6H, t, J = 6.4 Hz, -CH(CH_3)$_2$), 1.54 (9H, s, Boc), 2.20–2.47 (18H, br, CH_3 × 6 in mestyl groups), 3.04 (9H, s, -N(CH_3)$_3$), 3.35 (2H, br, -CH_2NHBoc), 4.00 (1H, m, -MesNC$H_{2\alpha}$CH(CH$_2$NHBoc)NMes), 4.26 (1H, m, -MesNC$H_{2\beta}$CH(CH$_2$NHBoc)NMes), 4.55 (2H, br, –CH_2N(CH$_3$)$_3$), 4.80 (1H, br, -MesNCH$_2$CH(CH$_2$NHBoc)NMes), 4.90 (1H, m, -CH(CH$_3$)$_2$), 6.65–7.60 (7H, m, phenyl protons), 8.05 (1H, br, -NHBoc), 16.4 (1H, s, Ru=CHAr).



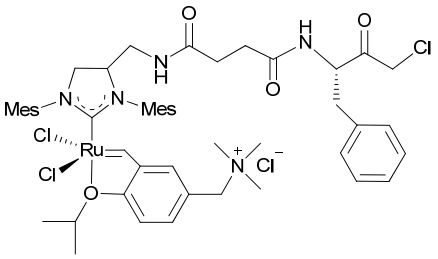
12

Deprotected Hoveyda-Grubbs catalyst 3. In a 50-mL flask, Hoveyda-Grubbs catalyst **12** (198 mg, 0.230 mmol) was dissolved in benzene/$CHCl_3$ (= 1/1, 10 mL). HCl gas (generated by the reaction of NaCl with concH_2SO_4) was bubbled to the solution at room temperature for 1 h. During the bubbling, viscous green material was deposited at the bottom the flask. The solvent was evaporated, and the residue was dissolved in MeOH. Addition of ether gave precipitation of compound **3** as dark green solid (167 mg, 88 % yield). ^{1}H NMR: δ_H (400 MHz; D_2O, residual protons in solvent) 1.16 (6H, m, -OCH(CH_3)$_2$), 2.20–2.47 (18H, m, CH_3 × 6 in mestyl groups), 3.03 (9H, s, -N(CH_3)$_3$), 3.35–3.36 (2H, m, -CH_2NH$_3^+$Cl$^-$), 4.18 (1H, m, -MesNC$H_{2\alpha}$CH(CH$_2$NHBoc)NMes), 4.47 (1H, m, -MesNC$H_{2\beta}$CH(CH$_2$NHBoc)NMes), 4.51 (2H, m, –CH_2N(CH$_3$)$_3$), 4.84–5.09 (2H, m, -MesNCH$_2$CH(CH$_2$NHBoc)NMes and -CH(CH$_3$)$_2$), 7.07 (1H, s, phenyl proton), 7.16–7.28 (5H, m, phenyl protons), 7.90 (1H, m, phenyl proton), 16.85 (1H, s, Ru=CHAr) ESI-HR-MS (positive mode)**:** Calcd. for $C_{36}H_{51}Cl_2N_4ORu^+$ ([M–H–Cl$_2$]$^+$), 727.2562; Obsd: 727.2478.



3

Inhibitor with Hoveyda-Grubbs catalyst moiety $\mathbf{1}_{\text{-L}}$. In a 50-mL three-necked flask with a three-way cock and a dropping funnel, compound **2** (27 mg, 0.091 mmol) was dissolved in dry THF under a nitrogen atmosphere and the solution was cooled to –20 ºC. *N*-Methylmorpholine (NMM, 12 μL, 0.10 mmol) was added thorough a microsyringe and isobutyl chloroformate (IBCF, 14 μL, 0.10 mmol) was sequentially added. While the solution was stirred at –20 ºC, compound **3** (68 mg, 0.090 mmol) in dry DMF was charged into the dropping funnel with avoiding air contact. After 15 min, the solution of **3** was slowly added to the solution of **2** from the dropping funnel. During the addition of **3**, NMM (12 μL, 0.10 mmol) was slowly added thorough a microsyringe. After the complete of the addition, the reaction solution was stirred at –20 ºC for 15 min and at 0 ºC for 30 min. Ether was added before the reaction mixture was transferred into test tubes. The reaction mixture was centrifuged and the supernatant was removed and ether was added to be centrifuged. Green precipitate was dissolved in MeOH and subjected to gel column chromatography (Sephadex LH-20 (GE Healthcare), L = 1 m, elution : MeOH). Green band was collected and the solvent was evaporated to afford $\mathbf{1}_{\text{-L}}$ as green solid (35 mg, 37 % yield). ^{1}H NMR: δ_{H} (400 MHz; CD_3OD, residual protons in solvent) 1.19-1.24 (6H, m, -OCH(CH_3)$_2$), 2.25–2.60 (22H, br, CH_3 × 6 in mestyl groups and –HNCOCH_2CH_2CONH-), 3.10 (9H, s, -N(CH_3)$_3$), 4.57–4.65 (2H, m, -CH_2N(CH_3)$_3$), 4.84–5.09 (2H, m, -MesNCH$_2$CH(CH_2NHBoc)NMes and -CH(CH_3)$_2$), 7.04–7.79 (11H, m, phenyl protons), 8.00 (1H, m, phenyl proton), 16.66 (1H, s, Ru=CHAr). Other peaks were not assignable due to overlapping with solvent and water peaks and very complicated peak patterns. ESI-HR-MS (positive mode)**:** Calcd. for $C_{50}H_{65}Cl_3N_5O_4Ru^+$ ([M–Cl$^-$]$^+$), 1006.3140; Obsd: 1006.3156.

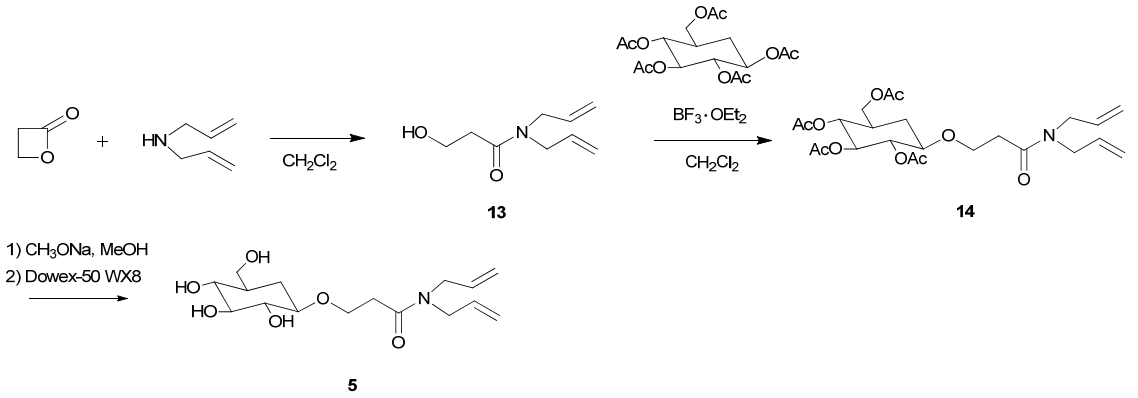


$\mathbf{1}_{\text{-L}}$

3. Synthesis of GlcDAA (5)

The synthetic route is depicted in Scheme S2.

Scheme S2



***N,N*-Diallyl-3-hydroxypropanamide (13).** In a 100-mL two-necked flask, β-propiolactone (3.40 g, 34.7 mmol) was dissolved in 20 mL of dry CH_2Cl_2 under a nitrogen atmosphere and cooled to 0 ºC. After *N,N'*-diallylamine (2.50 g, 34.7 mmol) was slowly added, the solution was stirred at 0 ºC for 1 h and at room temperature for 6 h. The reaction solution was diluted with water and the organic phase was separated. The organic phase was washed with sat. $NaHCO_3$*aq* and brine. After dried over Na_2SO_4, the solvent was evaporated. The resulted residue was purified by silica gel column chromatography eluted by AcOEt and compound **13** was obtained as colorless oil (1.57 g, 27% yield). ^{1}H NMR: δ_H (400 MHz; CD_3OD, residual protons in solvent) 2.56 (2H, t, J = 5.2 Hz, -COCH_2CH$_2$OH), 3.50 (1H, br, -COCH$_2$CH$_2$OH), 3.87 (4H, m, -NCH_2CH=CH$_2$ x 2), 4.01 (2H, m, -COCH$_2$CH_2OH), 5.17 (4H, m, -NCH$_2$CH=CH_2 x 2), 5.73 (2H, m, -NCH$_2$CH=CH$_2$ x 2).

***N,N'*-Diallyl-[3-(1-D-pentaacetylglucopyranosyl)oxy]propanamide (14).** In a 100-mL two-necked flask, β-D-pentaacetylglucopyranoside (1.23 g, 3.15 mmol) was dissolved in 7 mL of dry CH_2Cl_2 under a nitrogen atmosphere and cooled to 0 ºC. After BF_3•OEt_2 (0.671 g, 4.73 mmol) and compound 13 (0.800 g, 4.73 mmol) were sequentially added, the reaction mixture was stirred at room temperature overnight. The solution was cooled before quenched with sat$NaHCO_3$*aq*. The solution was transferred into a separatory funnel and the organic phase was separated. The water phase was extracted with CH_2Cl_2 twice. The organic phases were combined and washed with brine. After dried over Na_2SO_4, the solvent was evaporated. The

resulted residue was purified by silica gel column chromatography (elution: hexane/AcOEt = 1/4) and compound **14** was obtained as colorless oil (0.879 g, 56% yield). ^{1}H NMR: δ_H (400 MHz; $CDCl_3$, Me_4Si) 1.99–2.09 (12H, m, -CH_3), 2.55 (1H, m, -$CH_2CH_\alpha CON$-), 2.68 (1H, m, -$CH_2CH_\beta CON$-), 3.70 (1H, d, J = 7.8 Hz, Glc-5H), 3.92 (4H, m, -$NCH_2CH{=}CH_2$ x 2), 3.97 (1H, m, - $CH_\alpha CH_2CON$-), 4.10 (1H, m, -$CH_\beta CH_2CON$-), 4.12 (1H, d, J = 7.8 Hz, Glc-6H$_\alpha$), 4.27 (1H, d, J = 7.8 Hz, Glc-6H$_\beta$), 4.56 (1H, d, J = 7.8 Hz, Glc-1H), 4.96 (1H, dd, J = 7.8 Hz, 6.6 Hz, Glc-2H), 5.04 (1H, dd, J = 7.8 Hz, 6.6 Hz, Glc-4H), 5.09 (4H, m, -$NCH_2CH{=}CH_2$ x 2), 5.14 (1H, J = 7.8 Hz, 6.6 Hz, Glc-3H) , 5.74 (2H, m, -$NCH_2CH{=}CH_2$ x 2).

***N,N'*-Diallyl-[3-(1-D-glucopyranosyl)oxy]propanamide (GlcDAA, 5).** In a 50-mL flask, compound **14** (0.300 g, 0.600 mmol) was dissolved in 5 mL of MeOH. After sodium methoxide (0.136 g, 2.52 mmol) was added, the solution was stirred at room temperature for 20 min. The solution was treated with Dowex-50 WX8 (5.0 g) and filtered. The solvent was evaporated to afford **15** as colorless oil (0.194 g, 97% yield). ^{1}H NMR: δ_H (400 MHz; D_2O, residual protons) 2.78 (2H, t, J = 6.4 Hz, -OCH_2CH_2CON-), 3.23 (1H, dd, J = 8.8 Hz, 8.8 Hz, Glc-2H), 3.36 (1H, d, J = 8.4 Hz, 8.8 Hz, Glc-4H), 3.42 (2H, m, Glc-3H and -5H), 3.68 (1H, dd, J = 6.0 Hz, 8.4 Hz, Glc-6H$_{2\alpha}$), 3.87 (1H, d, J = 6.0 Hz,8.4 Hz, Glc-6H$_{2\beta}$), 3.90 (1H, m, -$OCH_{2\alpha}CH_2CON$-), 3.97 (2H, m, -$NCH_{2\alpha}CH{=}CH_2$), 4.04 (2H, m, -$NCH_{2\beta}CH{=}CH_2$), 4.14 (1H, m, -$OCH_{2\beta}CH_2CON$-), 4.44 (1H, J = 8.8 Hz, Glc-1H), 5.10–5.30 (4H, -$NCH_2CH{=}CH_2$ x 2), 5.70–5.91 (2H, m, -$NCH_2CH{=}CH_2$ x 2).

References

1. Y. Kawai, T. Matsuo and A. Ohno, *J. Chem. Soc. PerkinTrans 2*, 2000, **4**, 887–891.
2. S. Varray, R. Lazaro, J. Martinez and F. Lamaty, *Organometallics*, 2003, **22**, 2426–2435.
3. J. P. Jordan and R. H. Grubbs, *Angew. Chem. Int. Ed.*, 2007, **46**, 5152–5155.
4. M. R. Paleo, M. I. Calaza, P. Graña and F. J. Sardina, *Org. Lett.*, 2004, **6**, 1061–1063.
5. T. Onishi, N. Hirose, T. Nakano, M. Nakazawa and K. Izawa, *Tetrahedron. Lett.*, 2001, **42**, 5883–5885.

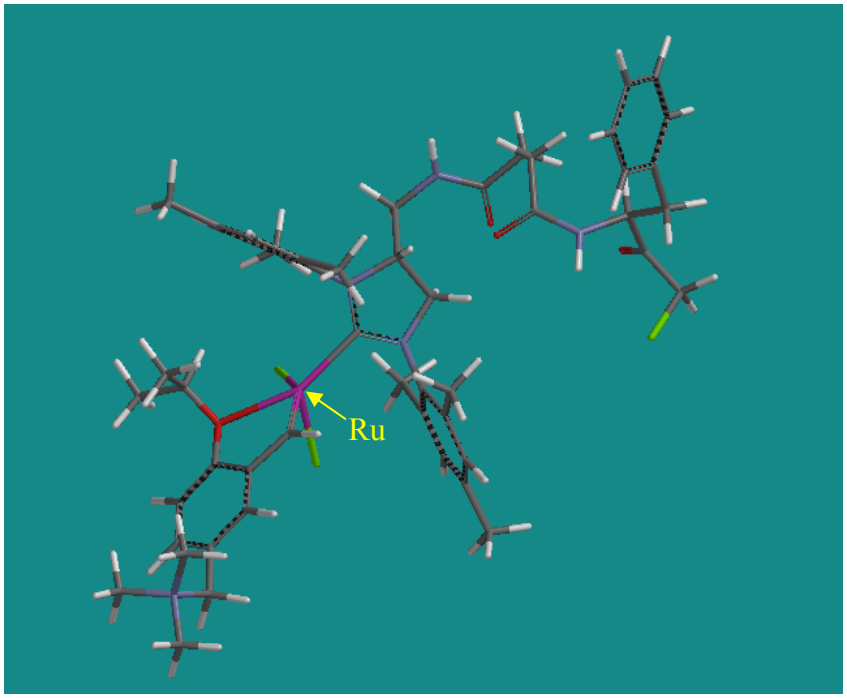


Fig. S1. Structure of $\mathbf{1}_{-L}$ optimized by MMFF calculation.

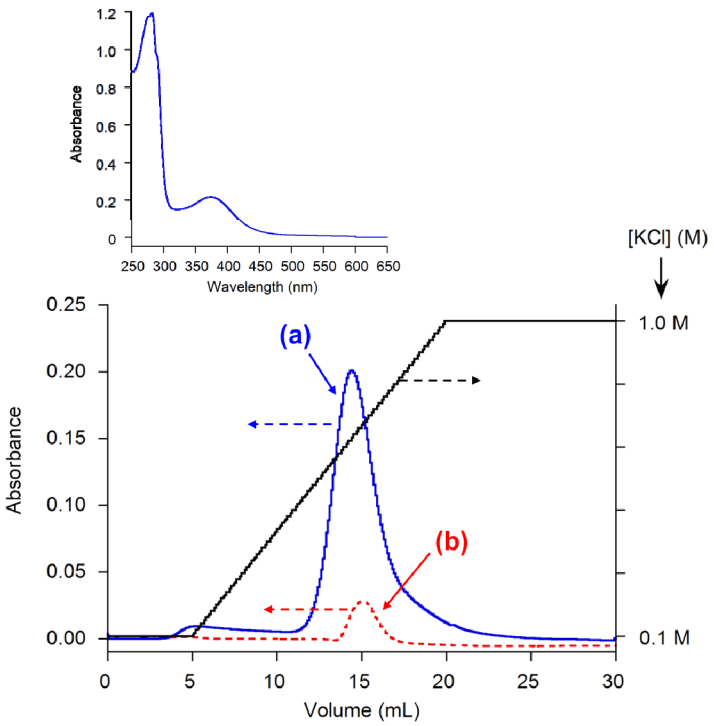


Fig. S2. FPLC chromatograms for the modified α-chymotrypsin with $\mathbf{1}_{\text{-L}}$ (chromatogram (a), blue line), the unmodified α-chymotrypsin (chromatogram (b), red line) and UV-vis spectrum of the modified α-chymotrypsin, which was obtained at elution volume of 12–18 mL (inset); CM FF column (5 mL, GE Healthcare), 0.5 mL/min, detection: 280 nm, 10 mM HEPES (pH = 7.0) → KCl Gradient.

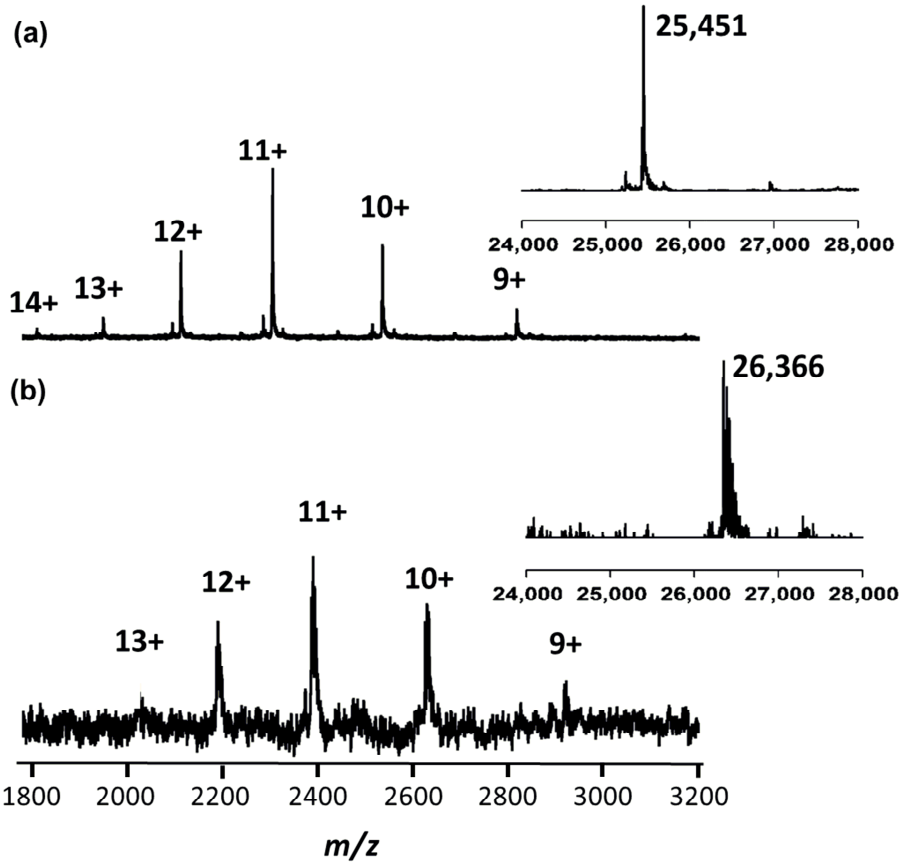


Fig. S3. Electrospray ionization mass (ES-MS) spectra of α-chymotrypsins and their deconvoluted spectra (insets); (a) Unmodified α-chymotrypsin; (b) Modified α-chymotrypsin with $\mathbf{1}_{\text{-L}}$.

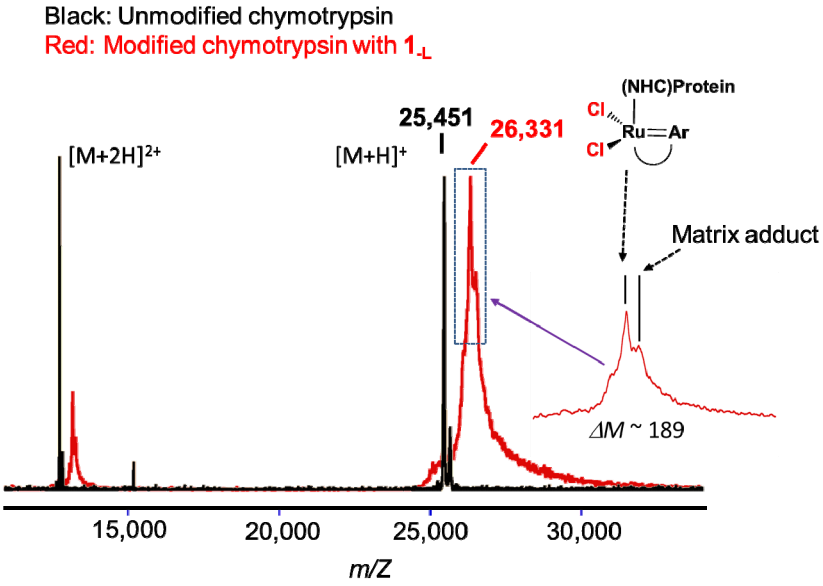


Fig. S4. MALDI-TOF-MS spectra of the unmodified α-chymotrypsin (black spectrum) and the modified α-chymotrypsin (red line); Linear mode; Matrix: sinapinic acid.

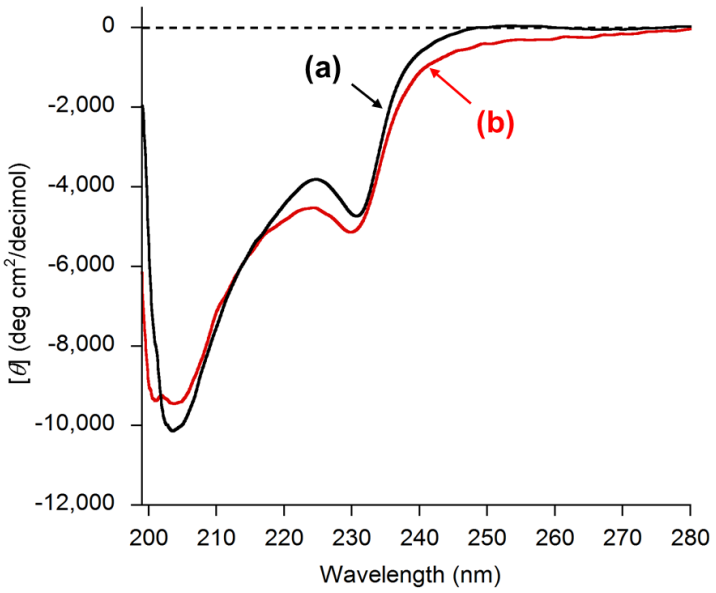


Fig. S5. CD spectra of the unmodified α-chymotrypsin (spectrum (a), black line) and the modified α-chymotrypsin (spectrum (b), red line) in high energy region; 100 mM KCl*aq* at 25 ºC.

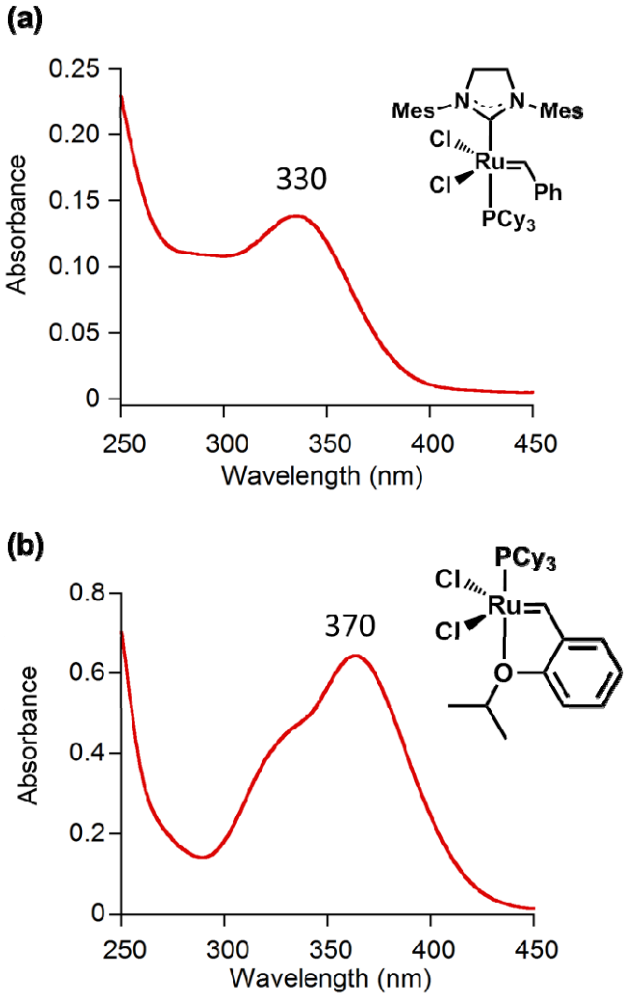


Fig. S6. UV-vis spectra of Grubbs 2nd generation catalyst (spectrum (a)) and Hoveyda-Grubbs catalyst (spectrum (b)) in CH_2Cl_2.

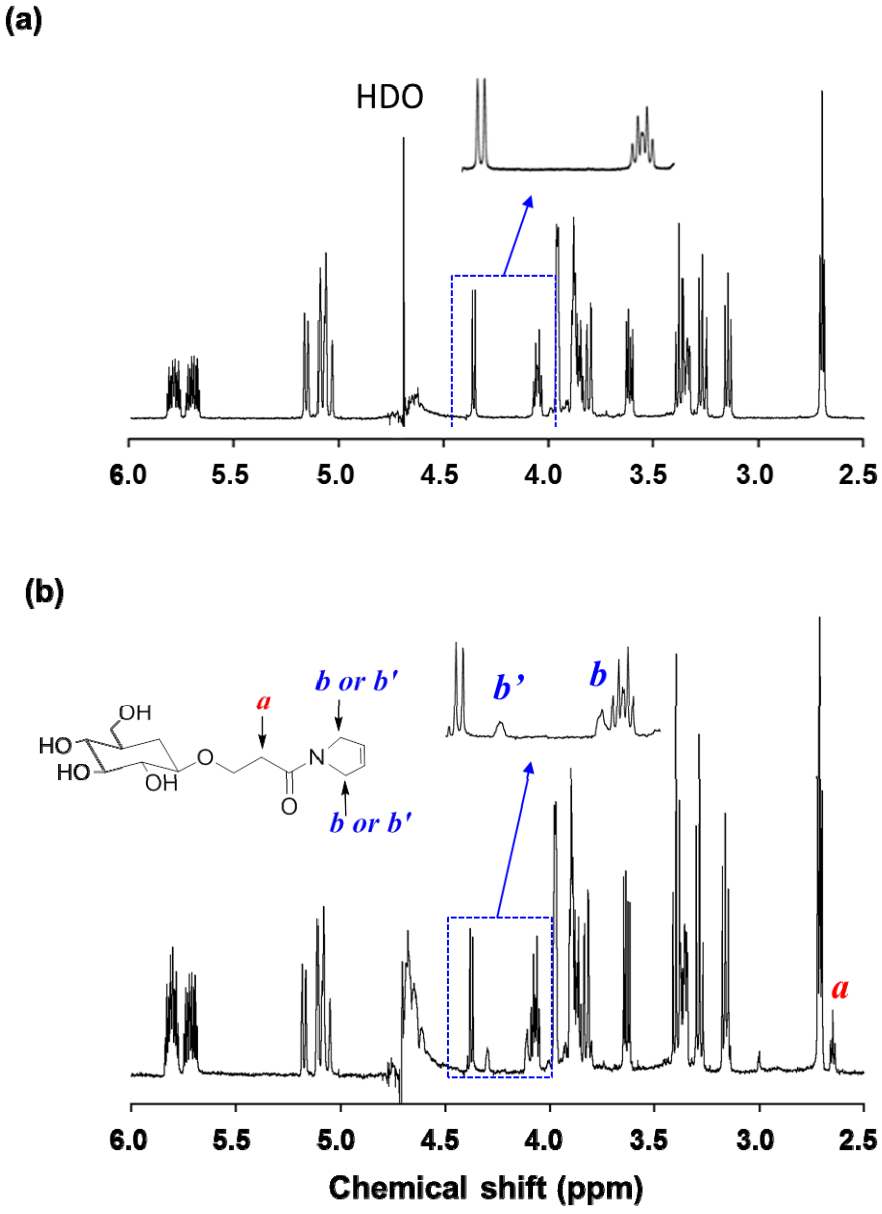


Fig. S7. ^{1}H NMR spectra (in D_2O/H_2O = 1/1) of GlcDAA (Spectrum (a)) and of the mixture of GlcDAA and the α-chymotrypsin modified with $\mathbf{1}_{\mathbf{L}}$ (Spectrum (b); [GlcDAA] = 9.3 mM, [α-chymotrypsin] = 50 μM). Spectrum (b) was taken 2 h after the reaction started. Both spectra were collected with presaturation of the peak originated from HDO (4.65 ppm). The peak assignment indicated in Spectrum (b) was confirmed by the ring closing product of GlcDAA obtained by the reaction of GlcDAA in the presence of Hoveyda-Grubbs catalyst **3** in 100 mM KCl*aq* at 50 °C.

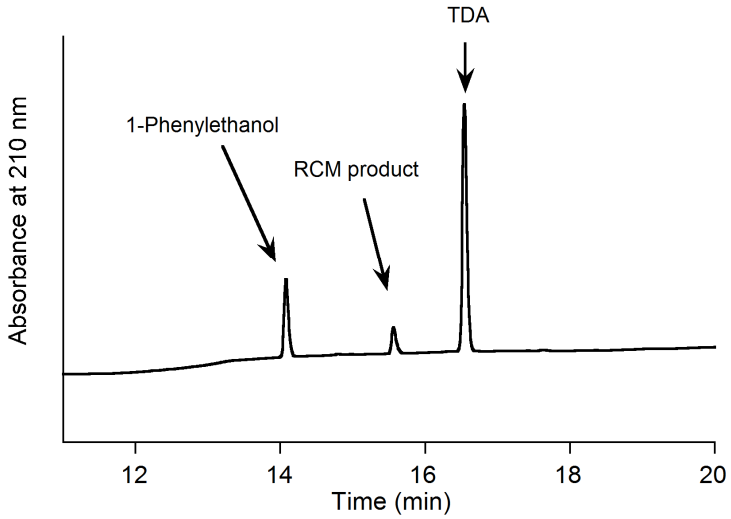


Fig. S8. HPLC chromatogram taken at 2 h in RCM of TDA catalyzed of α-chymotrypsin modified with **1**. [TDA] = 1.0 mM, [α-chymotrypsin] = 25 μM (2.5 mol%) and [1-phenylethanol] = 0.64 mM (internal standard); Column: Shimadzu Shim-Pack ODS(H) (I.D. 4.6 mm × 250 mm) column; Separation condition: Elution flow rate 1.0 mL/min; Solvent 90% H_2O/10% MeCN (0–5 min), linear gradient to 100% MeCN (5–12 min) and 100% MeCN (12–20 min); Detection 210 nm.