Supplementary Information

Simple Purification of Small-Molecule-Labelled Peptides via Palladium Enolate Formation from β-Ketoamide Tags

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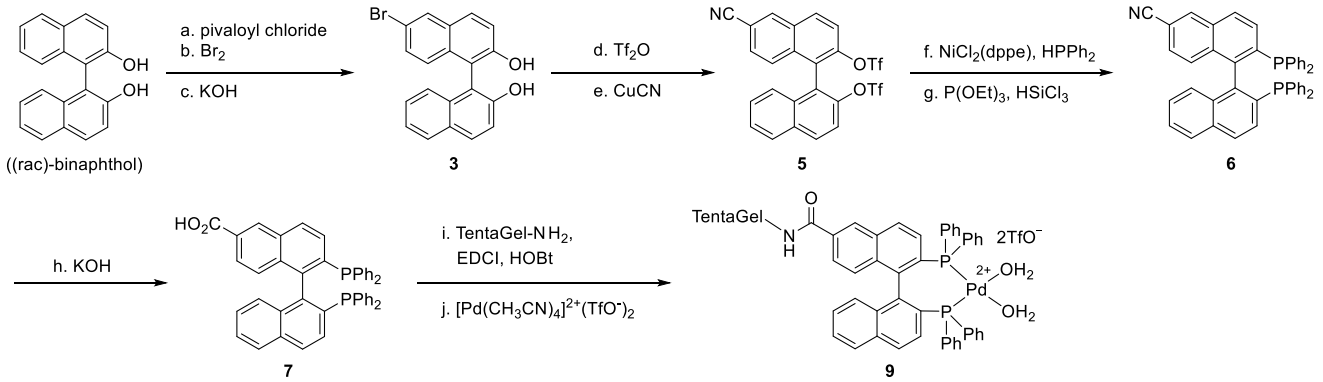
General Information

Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECS 400 spectrometer. The proton chemical shift values are reported in parts per million downfield from tetramethylsilane and referenced to the proton resonance of $CHCl_3$. Coupling constants (*J*) are reported in Hz. The carbon chemical shift values are reported in parts per million and referenced to the carbon resonance of $CDCl_3$. ^{31}P NMR data is reported as chemical shift relative to triphenylphosphine as an external standard. Multiplicities are reported using the following abbreviations: s, singlet; d, doublet; t, triplet; q; quartet; m, multiplet; br, broad. Mass spectra were recorded on a Bruker microTOF-QII-RSL. MALDI-TOF/MS was taken on a Bruker Daltonics autoflex speed with matrix dimer and external peptide calibration standards (including angiotensin I, angiotensin II, substance P, bonbesin, and ACTH clip 18-39). Optical rotations were measured on a JASCO P-2200 polarimeter at rt using the sodium D line. The purification efficiencies were quantified by HPLC (UltiMate 3000, Thermo Fisher Scientific). Flash column chromatography was done with standard silica gel (Silica gel 60N, spherical, neutral, 100 ~ 210 μm, Kanto Chemical Co. Ltd.). Gel permeation chromatography (GPC) was performed with JAI LC-918. Dehydrated solvents (MeOH, EtOH, THF, and toluene) were purchased from Kanto Chemical Co. Ltd. and used as received, and other reagents were also used without any purification. TentaGel beads were mixed in an RT-30mini (Taitec Corp.).

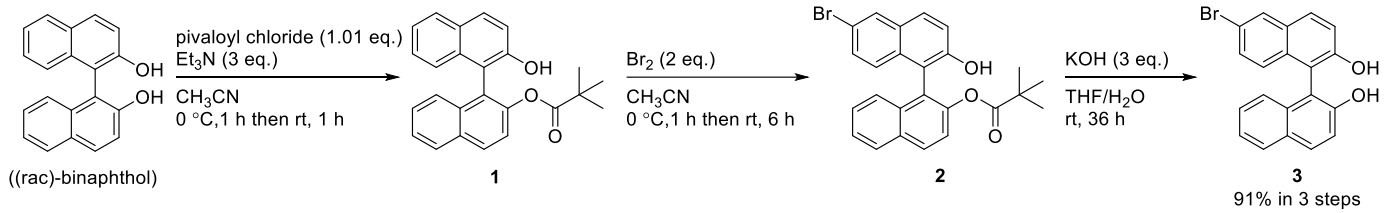
Preparation of TentaGel-Supported Pd Aqua Complex (Scheme S1)

Scheme S1



Reagents and conditions: (a) pivaloyl chloride, Et_3N, CH_3CN, 0 °C, 1 h then rt, 4 h; (b) Br_2, CH_3CN, 0 °C, 1 h then rt, 6 h; (c) KOH, THF/H_2O, rt, 36 h, 91% in 3 steps; (d) Tf_2O, pyridine, CH_2Cl_2, rt, 20 h; (e) CuCN, NMP, 180 °C, 4 h, 73% in 2 steps; (f) $NiCl_2$(dppe) (15 mol%), $HPPh_2$, DMF, 100 °C, 24 h; (g) $P(OEt)_3$, $HSiCl_3$, 100 °C, 3 h, 90% in 2 steps; (h) KOH, dioxane/MeOH/H_2O, reflux, 46 h, 46%; (i) TentaGel-NH_2, EDCI, HOBt, DMF, rt, 13 h; (j) $[Pd(CH_3CN)_4]^{2+}(TfO^-)_2$, wet acetone, rt, 7 h, 96% in 2 steps.

(*rac*)-6-bromo-2,2'-dihydroxy-1,1'-binaphthyl (3)[1]



To a solution of (*rac*)-2,2'-dihydroxy-1,1'-binaphthyl (4.0 g, 14 mmol) and triethylamine (5.8 mL, 42 mmol) in CH_3CN (22.0 mL) was added dropwise a solution of pivaloyl chloride (1.76 mL, 14.1 mmol) in CH_3CN (20.0 mL) over a period of 1 h at 0 °C. The reaction mixture was stirred for 1 h at rt, and then diluted with Et_2O. The organic solution was washed with aqueous 1 *N* HCl, saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated *in vacuo* to give (*rac*)-2-hydroxy-2'-pivaloyloxy-1,1'-binaphthyl (**1**).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.08 (1H, d, *J* = 9.2 Hz), 7.98 (1H, d, *J* = 8.4 Hz), 7.88 (1H, d, *J* = 8.4 Hz), 7.83 (1H, d, *J* = 7.6 Hz), 7.51 (1H, dd, *J* = 7.4, 2.0 Hz), 7.39-7.23 (6H, m), 7.06 (1H, d, *J* = 8.8 Hz), 5.18 (1H, s), 0.77 (9H, s).

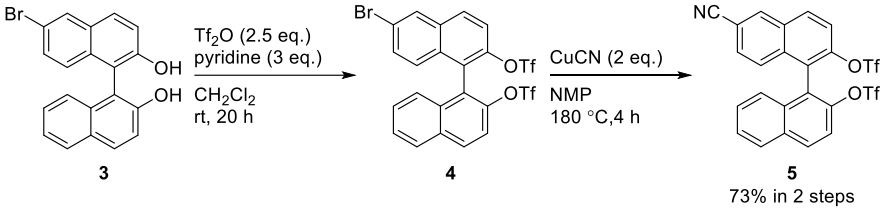
To a solution of **1** in CH_3CN (67.0 mL), bromine (1.36 mL, 26.6 mmol) was slowly added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 6 h at rt, and subsequently quenched with saturated aqueous Na_2SO_3. The mixture was extracted with Et_2O, and the organic solution was washed with saturated aqueous $NaHCO_3$, 1 *N* HCl, and brine, dried over Na_2SO_4, and concentrated *in vacuo* to give (*rac*)-6-bromo-2-hydroxy-2'-pivaloyloxy-1,1'-binaphthyl (**2**).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.08 (1H, d, *J* = 9.2 Hz), 7.98 (2H, m), 7.79 (1H, d, *J* = 9.2 Hz), 7.52 (1H, t, *J* = 7.2 Hz), 7.39-7.25 (5H, m), 6.93 (1H, d, *J* = 8.8 Hz), 5.26 (1H, s), 0.77 (9H, s).

To a solution of **2** in THF/H_2O (3/2, 70 mL) was added potassium hydroxide (2.36 g, 42 mmol). The reaction mixture was stirred for 36 h at rt and extracted with AcOEt. The organic solution was washed with aqueous 1 *N* HCl, saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography (SiO_2, eluent; $CHCl_3$) to give (*rac*)-6-bromo-2,2'-dihydroxy-1,1'-binaphthyl (**3**) (4.66 g, 91% in 3 steps) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.05 (1H, d, *J* = 8.0 Hz), 7.98 (1H, d, *J* = 8.4 Hz), 7.90 (1H, d, *J* = 8.0 Hz), 7.89 (1H, d, *J* = 8.8 Hz), 7.42-7.30 (5H, m), 7.10 (1H, d, *J* = 8.0 Hz), 7.02 (1H, d, *J* = 9.2 Hz), 5.12 (1H, s), 5.02 (1H, s); HRMS (ESI) Calcd. for $C_{20}H_{13}BrO_2$ $[M+Na]^+$ 386.9997; Found 386.9985.

(*rac*)-6-cyano-2,2'-bis(trifluoromethanesulfonyloxy)-1,1'-binaphthyl (5)[2]



3 **4** **5**

73% in 2 steps

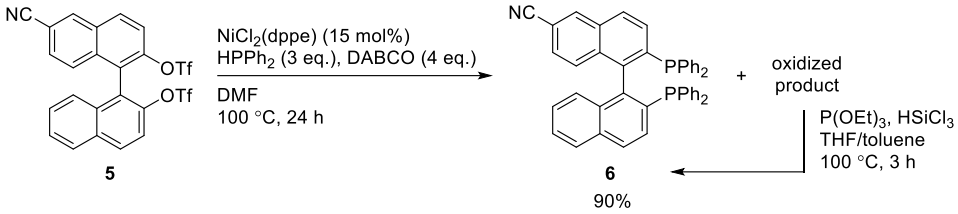
To a solution of **3** (4.58 g, 12.6 mmol) and pyridine (3.0 mL, 37.2 mmol) in CH_2Cl_2 (25.0 mL) was added trifluoromethanesulfonic anhydride (5.1 mL, 31.4 mmol) at 0 °C. The reaction mixture was stirred for 20 h at rt, and then the solvent was removed under reduced pressure. The residue was taken up in AcOEt, and washed with aqueous 1 *N* HCl, saturated aqueous $NaHCO_3$, and brine. The organic solution was dried over Na_2SO_4 and concentrated *in vacuo* to give (*rac*)-6-bromo-2,2'- bis(trifluoromethanesulfonyloxy)-1,1'-binaphthyl (**4**).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.18 (1H, d, *J* = 2.0 Hz), 8.15 (1H, d, *J* = 9.2 Hz), 8.05 (1H, d, *J* = 9.2 Hz), 8.02 (1H, d, *J* = 8.0 Hz), 7.64 (1H, d, *J* = 8.8 Hz), 7.61 (1H, d, *J* = 8.8 Hz), 7.62-7.58 (1H, m), 7.47 (1H, dd, *J* = 8.8 Hz, 2.0 Hz), 7.43 (1H, t, *J* = 7.7 Hz), 7.20 (1H, d, *J* = 8.0 Hz), 7.12 (1H, d, *J* = 9.2 Hz).

A solution of **4** and cuprous cyanide (2.26 g, 25.2 mmol) in 1-methyl-2- pyrrolidinone (28.5 mL) was stirred for 4 h at 180 °C. After cooling to 100 °C, the reaction mixture was slowly poured into 15% aqueous NH_3 (100 mL), and extracted with benzene. The organic solution was washed with 5% aqueous NH_3 and brine, and then dried over Na_2SO_4. The solvent was removed *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; Et_2O/Hex = 1/4) to give (*rac*)-6-cyano-2,2'-bis(trifluoromethanesulfonyloxy)-1,1'-binaphthyl (**5**) (5.25 g, 73%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.41 (1H, s), 8.22 (1H, d, *J* = 9.2 Hz), 8.19 (1H, d, *J* = 9.2 Hz), 8.04 (1H, d, *J* = 8.0 Hz), 7.76 (1H, d, *J* = 9.2 Hz), 7.62 (1H, t, *J* = 8.0 Hz), 7.62 (1H, d, *J* = 9.2 Hz), 7.54 (1H, d, *J* = 8.8 Hz), 7.44 (1H, t, *J* = 8.0 Hz), 7.37 (1H, d, *J* = 8.8 Hz), 7.15 (1H, d, *J* = 8.8 Hz); HRMS (ESI) Calcd. for $C_{23}H_{11}F_6NO_6S_2$ $[M+Na]^+$ 597.9830; Found 597.9816.

(*rac*)-6-cyano-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (6)[2,3]

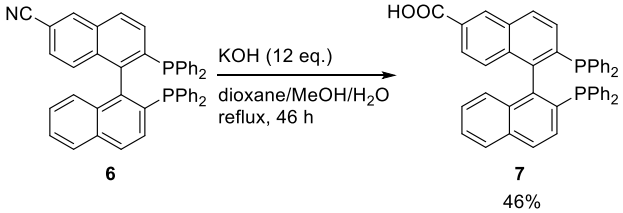


To a solution of $NiCl_2$(dppe) (649 mg, 1.23 mmol) in DMF (15.0 mL) was added diphenylphosphine (1.05 mL, 6.12 mmol) at rt, and the reaction mixture was stirred for 30 min at 100 °C. Then DABCO (3.68 g, 32.8 mmol) and a solution of **5** (4.70 g, 8.20 mmol) in DMF (25.0 mL) were added. The reaction mixture was kept at 100 °C, and three additional portions of diphenylphosphine (1.05 mL x 3, 18.4 mmol) were added at 1, 3, and 7 h later. The reaction mixture was stirred at 100 °C for 24 h, and then cooled to rt, and acidified with 1 *N* HCl. The organic layer was extracted with AcOEt. The organic solution was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by recrystallization from MeOH to give (*rac*)-6-cyano-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (**6**) (3.45 g, 65%) and an oxidized product (1.50 g).

The oxidized product (1.50 g) and triethylphosphite (3.80 mL, 21.9 mmol) were mixed in degassed THF/toluene (1/1, 60.0 mL) under a nitrogen atmosphere. Trichlorosilane (8.65 mL, 87.5 mmol) was added to the solution at rt. The reaction mixture was stirred at 100 °C for 3 h, cooled to rt, diluted with Et_2O, and then quenched with aqueous 1*N* NaOH at 0 °C. The resulting mixture was stirred for 30 min, then extracted with AcOEt. The organic solution was washed with saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was washed with CH_2Cl_2 and purified by recrystallization from MeOH to give **6** (1.31 g, 25% from **5** in 2 steps) as a white solid[3].

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.18 (1H, s), 7.91 (2H, dd, *J* = 8.0, 2.4 Hz), 7.83 (1H, d, *J* = 8.4 Hz), 7.57 (1H, dd, *J* = 8.8, 2.4 Hz), 7.42 (1H, dd, *J* = 8.8, 2.8 Hz), 7.34 (1H, t, *J* = 7.4 Hz), 7.25-7.15 (10H, m), 7.11-7.07 (6H, m), 7.03-6.95 (4H, m), 6.90-6.86 (2H, m), 6.74 (1H, d, *J* = 8.8 Hz), 6.65 (1H, d, *J* = 8.0 Hz); ^{31}P-NMR (160 MHz, $CDCl_3$): δ –14.0 (br-s); HRMS (ESI) Calcd. for $C_{45}H_{31}NP_2[M+Na]^+$ 670.1829; Found 670.1856.

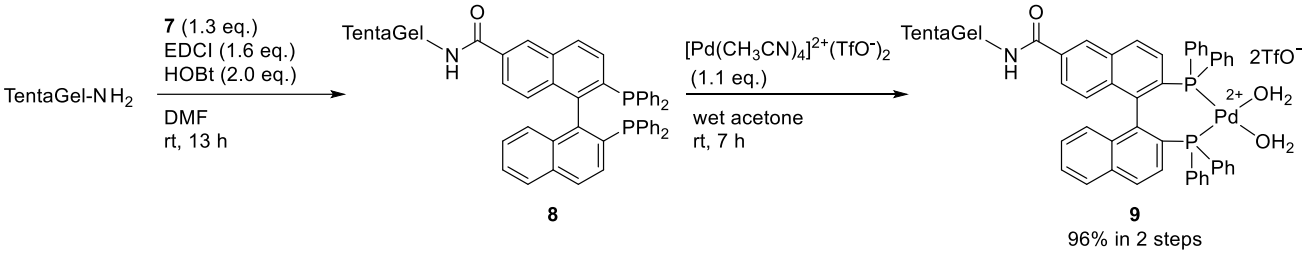
(*rac*)-6-hydroxycarbonyl-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (7)[2]



To a solution of **6** (460 mg, 0.71 mmol) in dioxane /MeOH/H_2O (7.0 mL/7.0 mL/2.6 mL) was added KOH (478 mg, 8.5 mmol) at rt. The reaction mixture was refluxed for 46 h, then acidified with 1 *N* HCl at 0 °C and extracted with AcOEt. The organic solution was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/1) to give (*rac*)-6-hydroxycarbonyl-2,2'-bis(diphenylphosphino) -1,1'-binaphthyl (**7**) (218 mg, 46%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.67 (1H, s), 8.02 (1H, d, *J* = 8.4 Hz), 7.91 (1H, d, *J* = 8.0 Hz), 7.84 (1H, d, *J* = 8.4 Hz), 7.54 (1H, dd, *J* = 8.6, 2.2 Hz), 7.46 (2H, td, *J* = 7.9, 2.2 Hz), 7.34 (1H, t, *J* = 7.5 Hz), 7.23-7.01 (20H, m), 6.89 (1H, t, *J* = 7.6 Hz), 6.82 (1H, d, *J* = 8.8 Hz), 6.72 (1H, d, *J* = 8.0 Hz); ^{31}P-NMR (160 MHz, $CHCl_3$): δ –14.2 (br-s); HRMS (ESI) Calcd. for $C_{45}H_{32}O_2P_2$[M+Na]$^+$ 667.1956; Found 667.1934.

TentaGel-supported Pd aqua complex (9)[2,4]



TentaGel®S-NH_2 beads (HiPep Laboratories, 0.26 g, 0.070 mmol) were washed with CH_3CN (5.0 mL x 5) and $CHCl_3$ (5.0 mL x 5) prior to use. To a suspension of **7** (61 mg, 0.091 mmol) and TentaGel S-NH_2 beads in DMF (3.0 mL) was added EDCI·HCl (22 mg, 0.11 mmol) and HOBt (19 mg, 0.14 mmol). The progress of the reaction was monitored by means of the Kaiser test (ninhydrin test). The mixture was stirred for 13 h at rt, and then filtered. The beads were washed with DMF (5.0 mL x 5) and $CHCl_3$ (5.0 mL x 5), and dried *in vacuo* to give TentaGel-supported (*rac*)-binap (**8**).

^{31}P-NMR (160 MHz, THF): δ –14.7 (s).

To a suspension of **8** in wet acetone (2.0 mL, containing 0.5 v/v% H_2O) was added $[Pd(CH_3CN)_4]^{2+}(TfO^-)_2$ (44 mg, 0.077 mmol) at rt. The mixture was stirred for 7 h at rt, and then filtered. The beads were washed with $CHCl_3$ (5.0 mL x 5), and dried *in vacuo* to give TentaGel-supported Pd aqua complex (**9**) (331 mg, 96%) as reddish black beads[4].

^{31}P-NMR (160 MHz, $CDCl_3$): δ 34.1 (s).

NMR Measurement of Pd Enolate Formation in Aqueous Solution

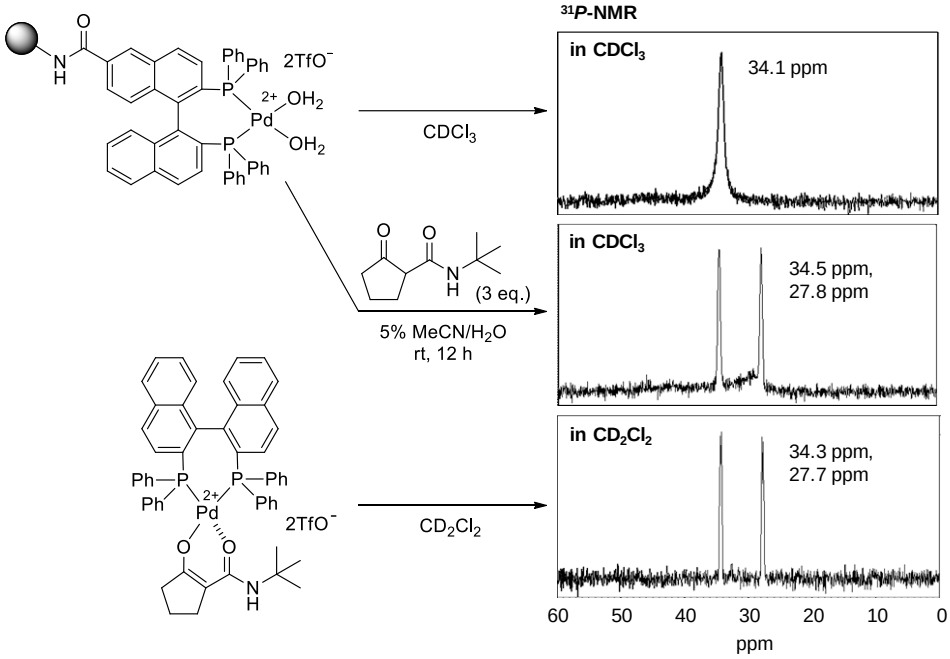
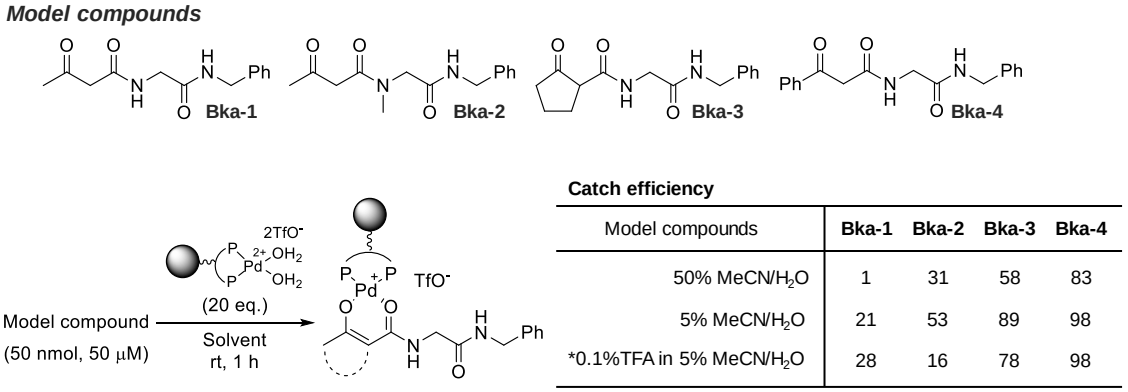


Fig. S1

To a suspension of TentaGel-supported Pd aqua complex (65 mg, 13 μmol) in 5% MeCN (2.0 mL) was added cyclic β-ketoamide (7.2 mg, 39 μmol) at rt. The reaction mixture was rotated for 12 h at rt, and then the beads were collected by filtration and washed with 50% MeCN (x 3) and $CHCl_3$ (x 3). The washed beads were dried *in vacuo*, and the ^{31}P-NMR spectrum was measured in $CDCl_3$. The bottom chart shows the ^{31}P-NMR spectrum of the isolated Pd enolate complex derived from the same substrate[5]. The characteristic peaks precisely match the observed peaks indicated in the above chart.

Comparison of Tag Structures Based on Catch Efficiencies in Aqueous Solution

To compare the affinities of various compounds having β-ketoamide structure, model compounds **Bka-1-4** were synthesized and their reaction with TentaGel-supported palladium complex was examined.



Catch efficiency

Model compounds	Bka-1	Bka-2	Bka-3	Bka-4
50% MeCN/H_2O	1	31	58	83
5% MeCN/H_2O	21	53	89	98
*0.1%TFA in 5% MeCN/H_2O	28	16	78	98

*100 eq. of beads were used.

Fig. S2

To a solution of β-ketoamide-tagged model compounds (50 nmol) in 50% CH_3CN/H_2O, 5% CH_3CN/H_2O, or 0.1% TFA in 5% CH_3CN/H_2O (1 mL) was added TentaGel-supported Pd aqua complex (5.0 mg, 1 μmol). The mixture was rotated for 1 h at rt, and then filtered. The beads were washed with the same solvent (3 times). The supernatant and washing solutions were analyzed by HPLC.

*HPLC conditions and calibration curves of model compounds

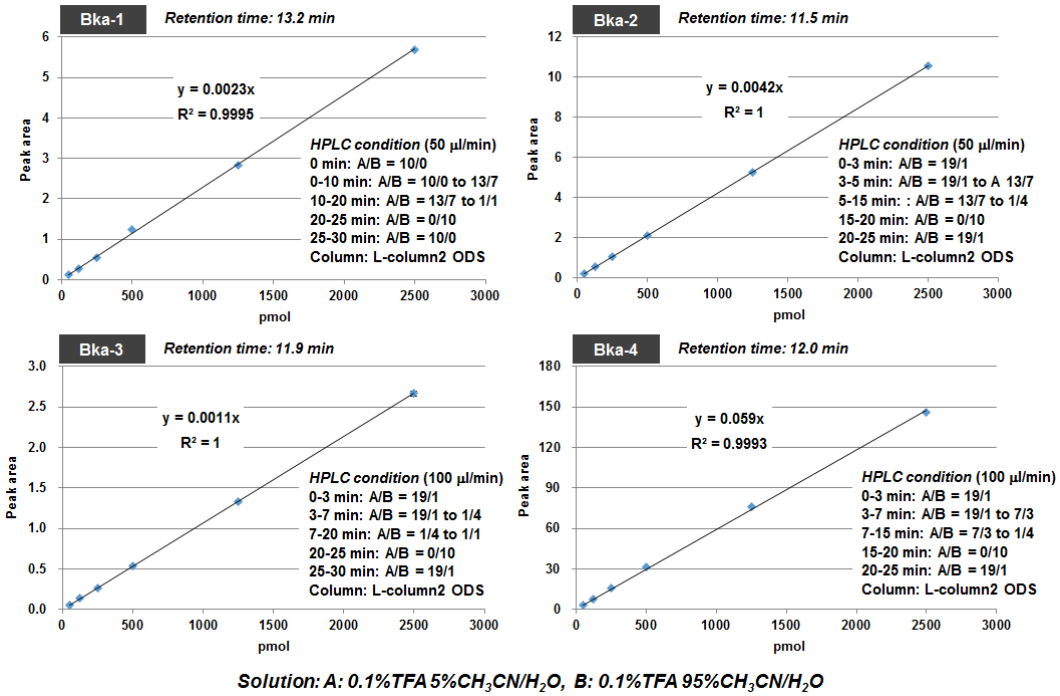
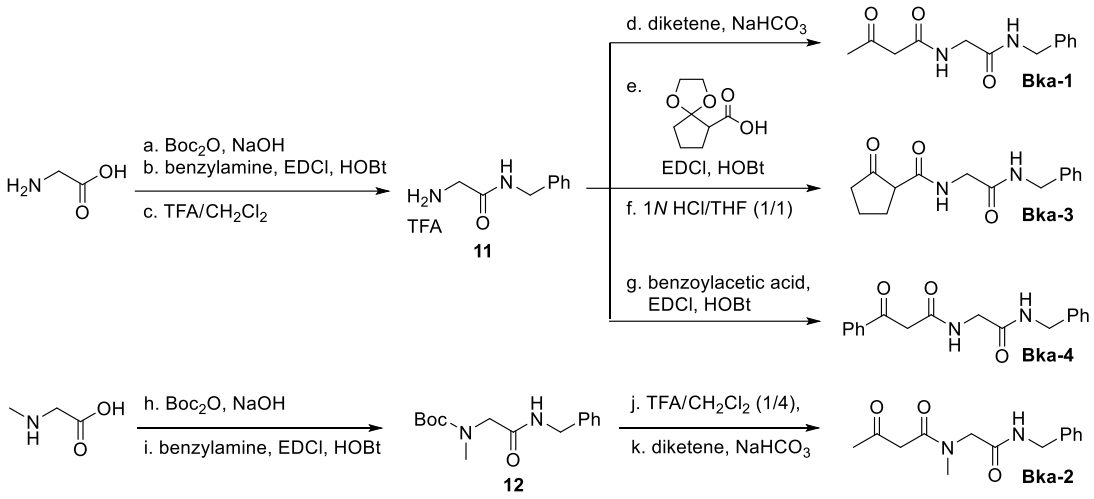


Fig. S3

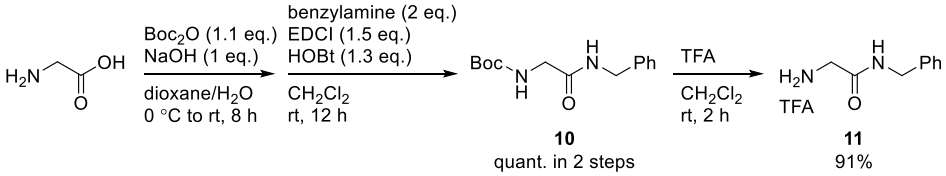
Synthesis of β-Ketoamide-Tagged Model Compounds

Scheme S2



Reagents and conditions: (a) Boc_2O, NaOH, dioxane/H_2O (2/1), 0 °C to rt, 8 h; (b) benzylamine, EDCI, HOBt, CH_2Cl_2, rt, 12 h; (c) TFA/ CH_2Cl_2 (1/4), rt, 2 h, 93% in 3 steps; (d) diketene, $NaHCO_3$, toluene, 0 °C to rt, 1 h; (e) protected β-ketocarboxylic acid, EDCI, HOBt, CH_2Cl_2, rt, 13 h, 18%; (f) 1*N* HCl/THF (1/1), rt, 24 h, 41% in 2 steps; (g) benzoylacetic acid, EDCI, HOBt, Et_3N, CH_2Cl_2, rt, 12 h, 54%; (h) Boc_2O, NaOH, dioxane/H_2O (2/1), 0 °C to rt, 14 h; (i) benzylamine, EDCI, HOBt, CH_2Cl_2, rt, 15 h, 94% in 2 steps; (j) TFA/CH_2Cl_2 (1/4), rt, 1 h; (k) diketene, $NaHCO_3$, toluene, 0 °C to rt, 1 h, 56% in 2 steps.

2-amino-*N*-benzylacetamide TFA salt (11)[6]



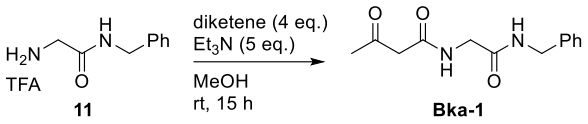
To a solution of glycine (1.49 g, 19.8 mmol) in dioxane/H_2O (2/1, 45.0 mL) was added di-*tert*-butyl dicarbonate (4.95 mL, 21.8 mmol) and NaOH (792 mg, 19.8 mmol) at 0 °C. The reaction mixture was stirred for 8 h from 0 °C to rt, and then the solvent was removed *in vacuo*. The resulting residue was dissolved in H_2O, and the aqueous layer was extracted with AcOEt. The aqueous phase was acidified with 1 *N* HCl and extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4, and concentrated *in vacuo* to afford *N*-(*tert*-butoxycarbonyl)glycine (3.6 g, quant.).

To a solution of *N*-(*tert*-butoxycarbonyl)glycine (3.6 g, 19.8 mmol) in CH_2Cl_2 (100 mL) was added EDCI·HCl (5.69 g, 29.7 mmol), HOBt (3.94 g, 25.7 mmol), and benzylamine (4.33 mL, 39.6 mmol) at 0 °C. The reaction mixture was stirred for 13 h at rt and quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with CH_2Cl_2, and the organic solution was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/1 to 7/3) to afford *tert*-butyl (2-(benzylamino)-2-oxoethyl)carbamate (**10**) (5.55 g, quant in 2 steps) as a white solid.

To a solution of **10** (4.55 g, 17.2 mmol) in CH_2Cl_2 (64.0 mL) was added TFA (14.0 mL) at 0 °C. The reaction mixture was stirred for 2 h at rt. The solvent was removed *in vacuo*, and residual TFA was removed azeotropically with $CHCl_3$. The residue was purified by recrystallization from Et_2O/CH_2Cl_2 to afford 2-amino-*N*-benzylacetamide TFA salt (**11**) (4.09 g, 91%) as a white solid.

^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.90 (1H, t, *J* = 5.2 Hz), 8.14 (3H, br-s), 7.36-7.26 (5H, m), 4.35 (2H, d, *J* = 5.2 Hz), 3.63 (2H, s); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 166.0, 138.7, 128.4 (2C), 127.4 (2C), 127.1, 42.3, 40.2; HRMS (ESI) calcd for $C_9H_{12}N_2O$ $[M+H]^+$ 165.1022; found 165.1026.

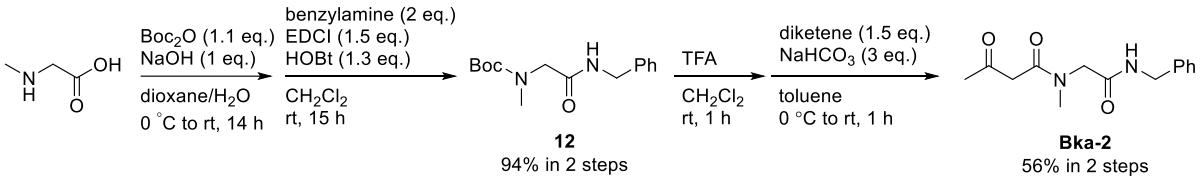
N-(2-(benzylamino)-2-oxoethyl)-3-oxobutanamide (Bka-1)[7]



To a solution of **11** (1.00 g, 3.83 mmol) in MeOH (75.0 mL) was added Et_3N (2.67 mL, 19.1 mmol) and diketene (1.18 mL, 15.3 mmol) at 0 °C. The reaction mixture was stirred for 15 h at rt, and the solvent was removed *in vacuo*. The residue was purified by recrystallization from Et_2O/CH_2Cl_2 to afford **Bka-1** (250 mg, 26%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.39 (2H, br-s), 7.33-7.23 (5H, m), 4.30 (2H, d, *J* = 6.0 Hz), 3.77 (2H, d, *J* = 5.6 Hz), 3.39 (2H, s), 2.16 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 203.5, 168.7, 166.6, 139.3, 128.3 (2C), 127.2 (2C), 126.8, 51.1, 42.2, 42.0, 30.0; HRMS (ESI) calcd for $C_{13}H_{16}N_2O_3$ $[M+Na]^+$ 271.1053; found 271.1053.

N-(2-(benzylamino)-2-oxoethyl)-*N*-methyl-3-oxobutanamide (Bka-2)



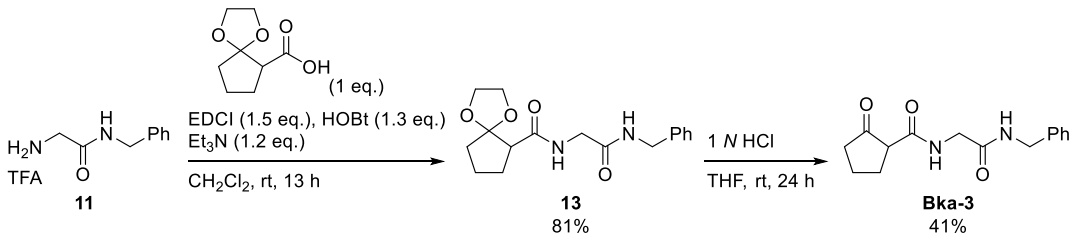
To a solution of *N*-methylglycine (2.00 g, 22.4 mmol) in dioxane/H_2O (2/1, 50.0 mL) was added di-*tert*-butyl dicarbonate (5.39 g, 24.7 mmol) and NaOH (896 mg, 3 portions, 22.4 mmol) at 0 °C. The reaction mixture was stirred for 14 h from 0 °C to rt, and then the solvent was removed *in vacuo*. The residue was dissolved in H_2O, and the aqueous layer was extracted with AcOEt. The aqueous phase was acidified with 1 *N* HCl and extracted with CH_2Cl_2. The organic solution was dried over Na_2SO_4 and concentrated *in vacuo* to afford *N*-*tert*-butoxycarbonyl-*N*-methylglycine. To a solution of *N*-*tert*-butoxycarbonyl-*N*-methylglycine (22.4 mmol) in CH_2Cl_2 (112 mL) was added EDCI·HCl (6.44 g, 33.6 mmol), HOBt (4.46 g, 29.1 mmol), and benzylamine (4.90 mL, 44.8 mmol) at 0 °C. The reaction mixture was stirred for 15 h at rt, and quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with CH_2Cl_2, and the organic solution was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 3/7 to 1/1) to afford *tert*-butyl (2-(benzylamino)-2-oxoethyl)(methyl)carbamate (**12**) (5.89 g, 94% in 2 steps) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.34-7.24 (5H, m), 4.46 (2H, d, *J* = 6.0 Hz), 3.89 (2H, s), 2.93 (3H, s), 1.40 (9H, s); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 169.4, 156.6, 138.1, 128.8 (2C), 127.7 (3C), 81.0, 53.4, 43.4, 36.0, 28.3 (3C); HRMS (ESI) calcd for $C_{15}H_{22}N_2O_3$ $[M+Na]^+$ 301.1523; found 301.1520.

To a solution of **12** (1.00 g, 3.60 mmol) in CH_2Cl_2 (14.4 mL) was added TFA (3.60 mL) at 0 °C. The reaction mixture was stirred for 1 h at rt. The solvent was removed *in vacuo*, and residual TFA was removed azeotropically with $CHCl_3$ to afford *N*-benzyl-2-(methylamino)acetamide TFA salt. To a solution of *N*-benzyl-2-(methylamino)acetamide TFA salt (3.60 mmol) in toluene (3.60 mL) was added $NaHCO_3$ (605 mg, 7.20 mmol) and diketene (832 μL, 10.8 mmol) at 0 °C. The reaction mixture was stirred for 48 h at rt and quenched with saturated aqueous NH_4Cl. The organic solution was washed with saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/1) to afford **Bka-2** (532 mg, 56% in 2 steps) as a pale-yellow oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.30-7.21 (5H, m), 4.44 (2H, d, *J* = 4.4 Hz), 4.05 (2H, br-s), 3.60 (2H, s), 2.98 (3H, s), 2.20 (3H, s); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 203.4, 168.0, 167.5, 138.3, 128.7 (2C), 127.7 (2C), 127.3, 52.0, 49.9, 43.4, 37.4, 30.4; HRMS (ESI) calcd for $C_{14}H_{18}N_2O_3$ $[M+Na]^+$ 285.1210; found 285.1209.

_N_-(2-(benzylamino)-2-oxoethyl)-2-oxocyclopentane-1-carboxamide (Bka-3)



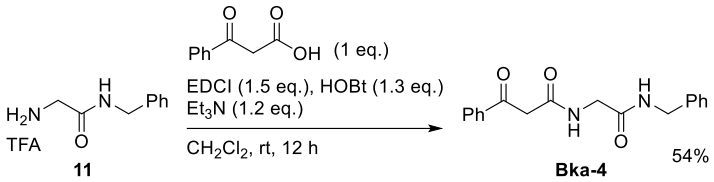
To a solution of **11** (567 mg, 2.17 mmol) in CH_2Cl_2 (20.0 mL) was added EDCI·HCl (491 mg, 2.56 mmol), HOBt (400 mg, 2.96 mmol), 1,4-dioxaspiro[4.4]nonane-6-carboxylic acid (340 μL, 1.97 mmol), and triethylamine (330 μL, 2.40 mmol). The reaction mixture was stirred for 13 h at rt and quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with CH_2Cl_2, and the organic solution was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 7/3) to afford *N*-(2-(benzylamino)-2-oxoethyl)-1,4-dioxaspiro[4.4]nonane-6-carboxamide (**13**) (507 mg, 81%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.32-7.23 (5H, m), 7.03 (1H, br-s), 6.55 (1H, br-s), 4.50 (1H, dd, *J* = 6.4, 14.6 Hz), 4.35 (1H, dd, *J* = 5.6, 14.6 Hz), 4.20 (1H, dd, *J* = 6.4, 17.2 Hz), 3.91-3.75 (3H, m), 3.65-3.54 (2H, m), 2.80 (1H, t, *J* = 8.4 Hz), 2.15-2.05 (1H, m), 1.93-1.85 (1H, m), 1.80-1.60 (4H, m); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.7, 169.2, 138.1, 128.8 (2C), 128.0 (2C), 127.6, 117.8, 64.4, 51.9, 43.6, 43.6, 35.4, 25.5, 21.1.

To a solution of *N*-(2-(benzylamino)-2-oxoethyl)-1,4-dioxaspiro[4.4]nonane-6-carboxamide (507 mg, 1.60 mmol) in THF (20.0 mL) was added 1 *N* HCl (20.0 mL). The mixture was stirred for 24 h at rt. The aqueous layer was extracted with AcOEt, and the organic solution was washed with H_2O and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The product was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/1 to 1/0) to afford **Bka-3** (179 mg, 41%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.31-7.21 (6H, m), 7.04 (1H, br-s), 4.43 (1H, dd, *J* = 6.0, 14.9 Hz), 4.37 (1H, dd, *J* = 6.0, 14.9 Hz), 4.04 (1H, dd, *J* = 6.4, 16.4 Hz), 3.86 (1H, dd, *J* = 4.8, 16.4 Hz), 2.99 (1H, t, *J* = 9.4 Hz), 2.37-2.14 (4H, m), 2.07-1.99 (1H, m), 1.84-1.72 (1H, m); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 216.1, 168.9, 168.0, 138.1, 128.7 (2C), 127.7 (2C), 127.5, 54.9, 43.5, 43.5, 38.6, 25.8, 20.6; HRMS (ESI) Calcd for $C_{15}H_{18}N_2O_3$ $[M+Na]^+$ 297.1210; found 297.1235.

***N*-(2-(benzylamino)-2-oxoethyl)-3-oxo-3-phenylpropanamide (Bka-4)**



To a solution of **1** (650 mg, 2.49 mmol) in CH_2Cl_2 (10.0 mL) was added EDCI·HCl (715 mg, 3.74 mmol), HOBt (496 mg, 3.24 mmol), benzoylacetic acid (347 mg, 2.49 mmol), and triethylamine (434 µL, 2.49 mmol). The reaction mixture was stirred for 12 h at rt and quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with CH_2Cl_2, and the organic solution was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 7/3 to 1/0) to afford **Bka-4** (418 mg, 54%) as a white solid.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.52-8.40 (2H, m), 7.97 (2H, d, *J* = 8.2 Hz), 7.63-7.30 (1H, m), 7.53 (1H, dd, *J* = 7.6, 8.2 Hz), 7.50-7.46 (1H, m), 7.34-7.21 (5H, m), 4.32 (2H, d, *J* = 5.6 Hz), 4.01 (2H, s), 3.80 (2H, d, *J* = 5.6 Hz); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 195.1, 168.6, 166.8, 139.2, 136.2, 133.5, 128.7 (2C), 128.4 (2C), 128.3 (2C), 127.2 (2C), 126.8, 46.7, 42.3, 42.0; HRMS (ESI) calcd for $C_{18}H_{18}N_2O_3$ $[M+Na]^+$ 333.1210; found 333.1210.

Synthesis of Bza-peptide

The peptides were prepared by solid-phase synthesis (433A Peptide Synthesizer, Applied Biosystems) using Fmoc-protected amino acids. Bza-tagged glycine was used to introduce the Bza tag. Synthesized peptides were purified by HPLC (L-2000, Hitachi) to > 90% purity. HPLC conditions: Inertsil ODS-3 (250 x 4.6 mm I.D.) column, 25-55% MeCN (containing 0.1% TFA) (30 min) mobile phase, 1.0 mL/min flow rate and UV (215 nm) light source.

HPLC Analysis

HPLC chromatogram of synthetic peptide Bza-GLYEIAR

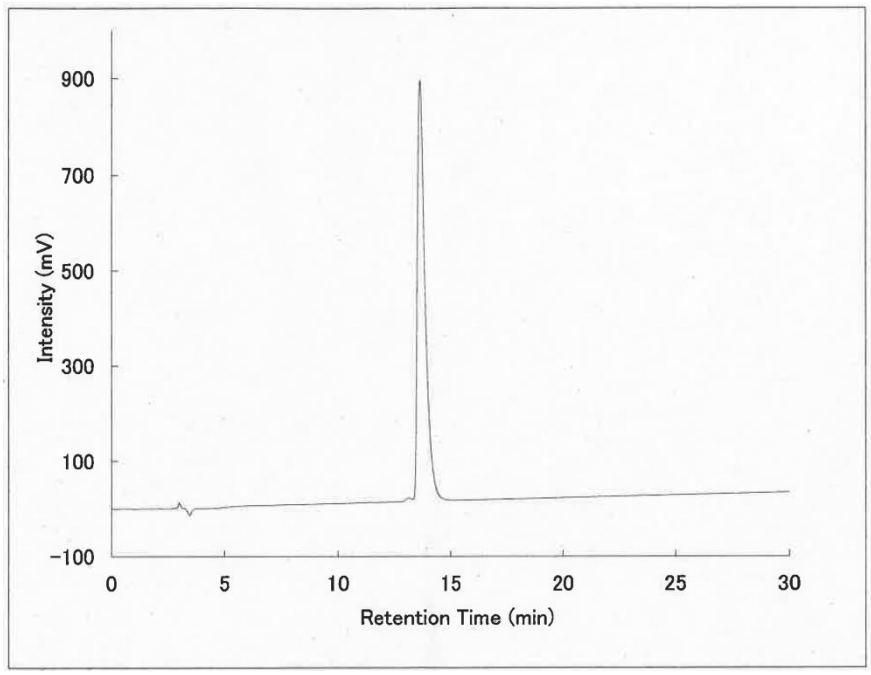


Fig. S4

HPLC conditions

Column: Inertsil ODS-3 (250 x 4.6 mm I.D.)

Column temp.: 25 °C

Mobile phase: 25-55% MeCN (containing 0.1% TFA), 30 min

Flow rate: 1.0 mL/min

Detection: UV at 215 nm

MALDI TOF-MS analysis

Calcd. For $C_{46}H_{67}N_{10}O_{13}$ $[M+H]^+$ 967.5

Found: $[M+H]^+$ 967.2

Investigation of Appropriate Solutions for the Purification of Bza-Peptide

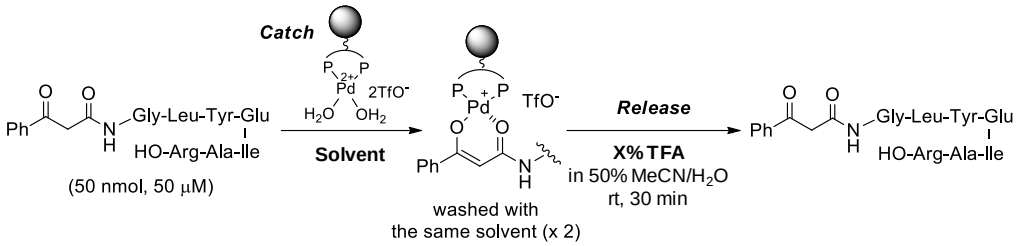


Fig. S5

Solvent

Phosphate buffer: 25 mM phosphate-Na, pH 7.0

Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4

Catch

To a solution of Bza-peptide (50 nmol) in 1 mL of solvent (phosphate buffer or PBS buffer or 0.1%TFA in 5% CH_3CN/H_2O) was added TentaGel-supported Pd aqua complex (5.0 mg, 1 μmol). The mixture was rotated for 1 or 2 h at rt, and then filtered. The recovered beads were washed with the same solvent (1 mL, twice). The supernatant and combined washing solution were analyzed by HPLC.

Release

The recovered beads were exposed to 0.5 mL of acidic solution (0.1% TFA in 50% CH_3CN/H_2O or 0.3% TFA in 50% CH_3CN/H_2O or 0.5% TFA in 50% CH_3CN) for 30 min at rt. The mixture was filtered, and the recovered beads were washed with 0.5 ml of the same solvent. The obtained solutions were analyzed by HPLC. 0.1% TFA in 50% CH_3CN/H_2O and 0.5% TFA in 50% CH_3CN/H_2O were similarly investigated.

HPLC conditions and calibration curve of Bza-peptide

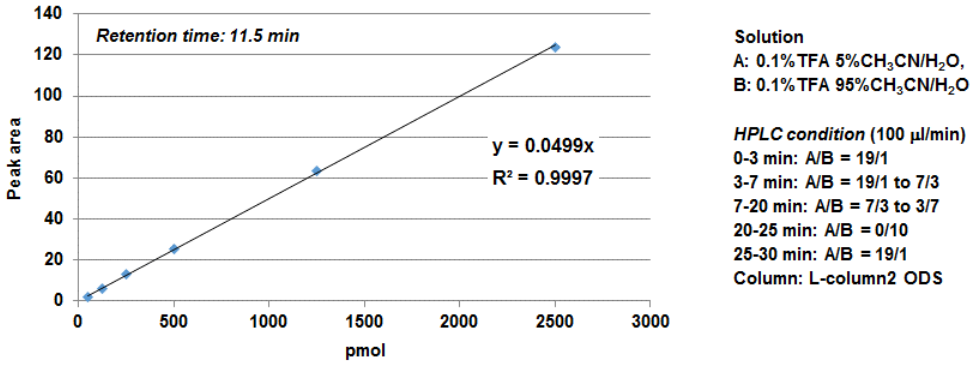


Figure S6

Typical HPLC charts obtained from the purification experiment using Bza-peptide

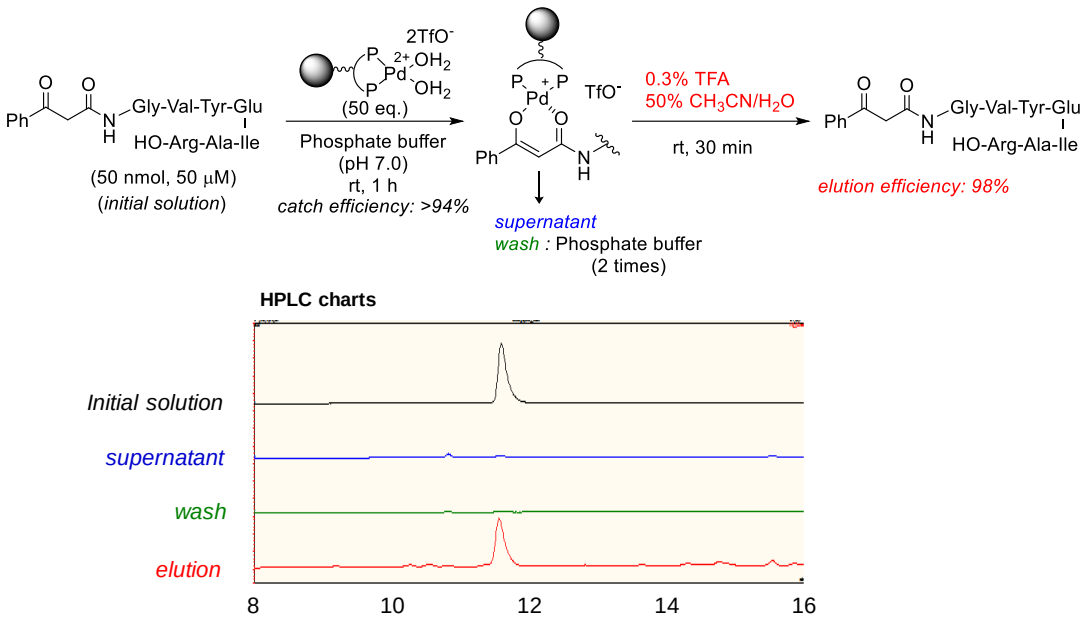


Fig. S7

To a solution of Bza-peptide (50 nmol) in 1 mL of phosphate buffer (pH 7.0) was added TentaGel-supported Pd aqua complex (12.5 mg, 2.5 μmol). The mixture was rotated for 1 h at rt, and then filtered. The recovered beads were washed with the same solvent (1 mL, twice). The supernatant and washing solutions were analyzed by HPLC: the catch efficiency was > 94%. The recovered beads were exposed to 0.5 mL of 0. 3% TFA in 50% CH_3CN/H_2O for 30 min at rt. The mixture was filtered, and the recovered beads were washed with 0.5 ml of the same solvent. The obtained solutions were analyzed by HPLC: the elution efficiency was 98%. HPLC analysis was performed in the same way as for **Figure S5**.

Determination of dissociation constant (Kd) between Pd complex and ketoamide tag.

NMR experiment of Pd aqua complex and Bka-4 in acetone.

We examined the dissociation constant of Pd complex and simple ketoamide compound **Bka-4** in organic solvent. To a solution of Pd aqua complex (3.2 mg, 3.0 μmol) and trimethoxybenzene (internal standard, 6.5 μmol) in acetone-d_6 (400 μL), different amounts of **Bka-4** (1.5, 2.4, 4.5, 6.0, or 12.0 μmol, 0.5-4 eq.) were added in the solvent (200 μL). After more than 1 h at rt, the ^{1}H NMR spectra were measured. The amount of Pd enolate complex was determined based on the integration of signal 5.73 ppm, which was quantified using the internal standard (**Fig. S8**).

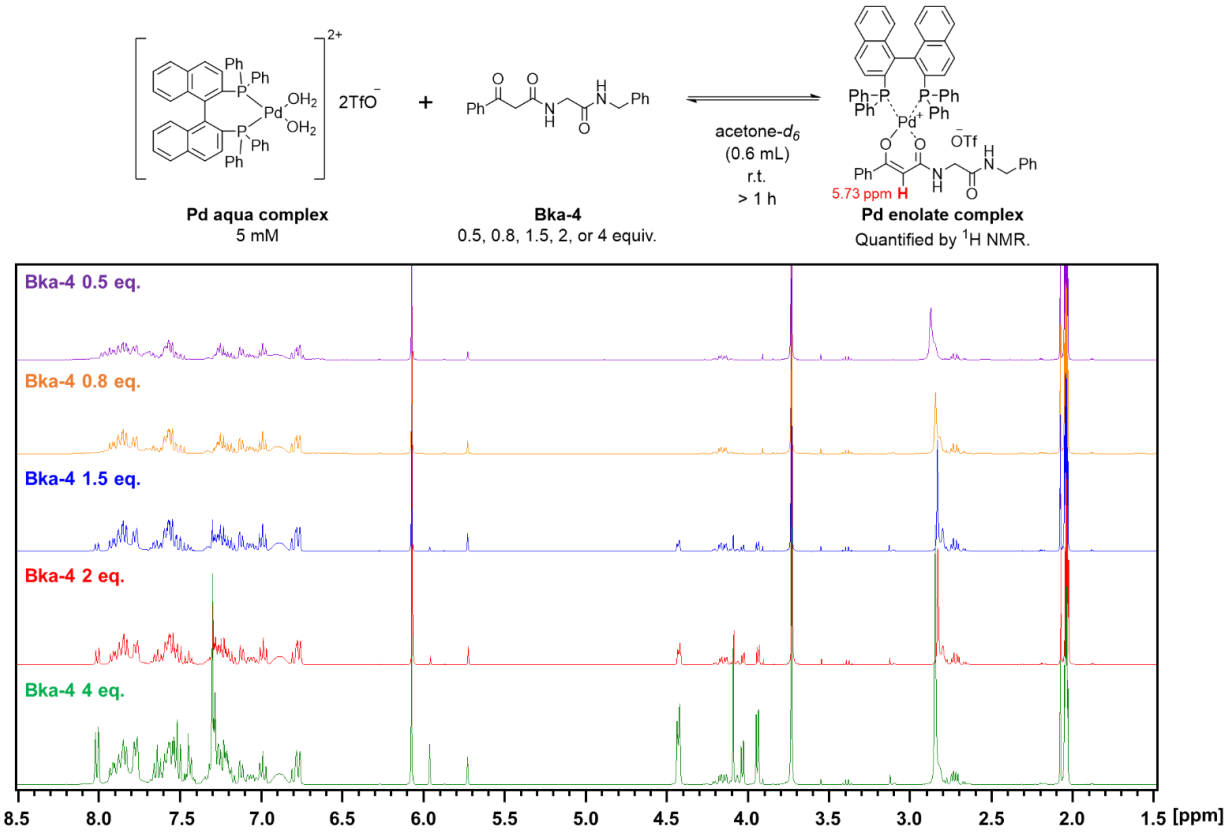


Fig. S8

Using the quantified [Pd enolate complex], we made the Scatchard plot with [bound **Bka-4**] and [bound/free **Bka-4**]. The Kd value was calculated as 1.12 x 10^{-3} from the slope of the approximate line (**Fig. S9**).

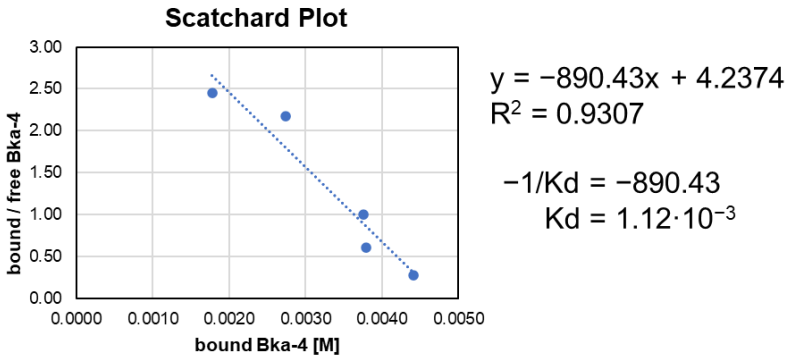


Fig. S9

Analysis of the Kd value between Tentagel-supported Pd aqua complex and Bza-peptide in aqueous solution. To estimate the Kd value in aqueous solution, the water-solubilities of Pd aqua complex and **Bka-4** were insufficient. Instead, we analyzed the dissociation constant between Pd complex immobilized on TentaGel and ketoamide-tagged peptide. To a suspension of different amounts of TentaGel-supported Pd aqua complex in phosphate buffer (1 mL), Bza-tagged peptide (68.0 nmol) at rt was added. The mixture was rotated for 1 h at rt, subsequently filtered, and the recovered beads were washed with 1 mL of phosphate buffer (x 2). The washed beads were exposed to 0.3% TFA 50% CH_3CN/H_2O (250 µL) for 30 min at rt. The mixture was filtered, and the recovered beads were washed with 250 µL of the same solvent. The obtained solutions (initial solution, supernatant, washing solution, and eluted solution) were analyzed by HPLC (**Fig. S10**). HPLC analysis was performed in the same way as for **Fig. S6**.

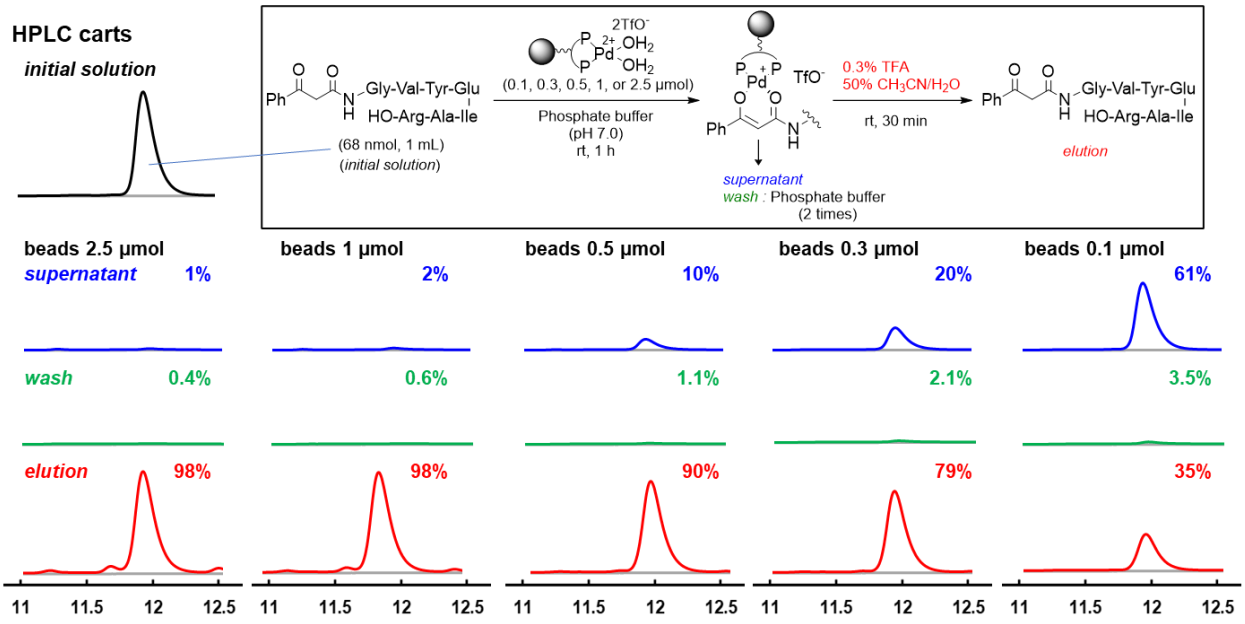


Fig. S10

Using the quantified [Pd enolate complex], we made the Scatchard plot with [bound Pd complex] and [bound/free Pd complex]. The Kd value was calculated as 9.98 x 10^{-5} from the slope of the approximate line (**Fig. S11**).

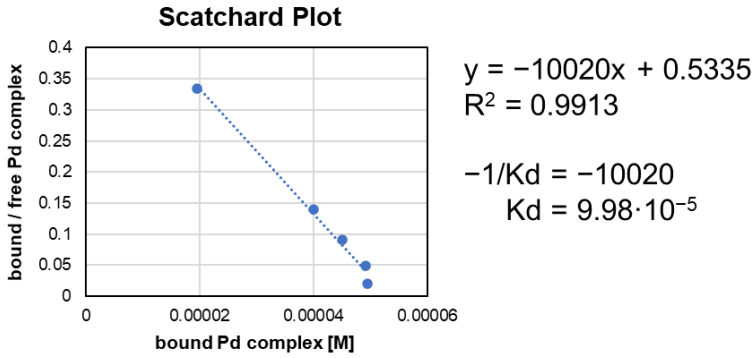


Fig. S11

Estimation of essential amount of Pd complex for the efficient purification.

To estimate the essential conditions for the purification, the following equation were derived from the dissociation constant (Kd)

$$\mathrm{Kd} = \frac{[\mathrm{Pd}] \cdot [\text{ketoamide}]}{[\text{Pd-ketoamide}]} = \frac{([\mathrm{Pd}]_0 - [\text{Pd-ketoamide}]) \cdot ([\text{ketoamide}]_0 - [\text{Pd-ketoamide}])}{[\text{Pd-ketoamide}]}$$

[Pd]: concentration of palladium complex

[ketoamide]: concentration of ketoamide

[Pd-ketoamide]: concentration of palladium-ketoamide complex

$[\mathrm{Pd}]_0$: initial concentration of palladium complex

$[\text{ketoamide}]_0$: initial concentration of ketoamide

Considering the experimental conditions, we can introduce the following approximation, which means excess amount of Pd complex compared with labeled peptides.

$$[\mathrm{Pd}]_0 \gg [\text{ketoamide}]_0 \geq [\text{Pd-ketoamide}]$$

$$[\mathrm{Pd}]_0 - [\text{Pd-ketoamide}] \cong [\mathrm{Pd}]_0$$

Using the above approximation, the following equation can estimate the catch efficiency.

$$\mathrm{Kd} = \frac{[\mathrm{Pd}]_0 \cdot ([\text{ketoamide}]_0 - [\text{Pd-ketoamide}])}{[\text{Pd-ketoamide}]}$$

$$\frac{\mathrm{Kd}}{[\mathrm{Pd}]_0} = \frac{[\text{ketoamide}]_0 - [\text{Pd-ketoamide}]}{[\text{Pd-ketoamide}]} = \frac{[\text{ketoamide}]_0}{[\text{Pd-ketoamide}]} - 1$$

$$\frac{\mathrm{Kd} + [\mathrm{Pd}]_0}{[\mathrm{Pd}]_0} = \frac{[\text{ketoamide}]_0}{[\text{Pd-ketoamide}]}$$

$$\text{Catch Efficiency} = \frac{[\text{Pd-ketoamide}]}{[\text{ketoamide}]_0} = \frac{[\mathrm{Pd}]_0}{\mathrm{Kd} + [\mathrm{Pd}]_0}$$

Based on the above equation, if higher concentration of palladium complex than Kd value is used, the catch efficiency is constant and approaches quantitative. To confirm this, we examined the catch efficiency with excess concentration of Pd (2.5×10^{-3} M) and different concentration of ketoamide-tagged peptide (**Fig. S12**).

To a suspension of TentaGel-supported Pd aqua complex (12.5 mg, 2.5 μmol) in phosphate buffer (1 mL), different equivalents of Bza-tagged peptide (3.5, 7.1, 14.9, 30.8, 37.5, or 76.5 nmol) at rt were added. The mixture was rotated for 1 h at rt, subsequently filtered, and the recovered beads were washed with 1 mL of phosphate buffer (x 2). The washed beads were exposed to 0.3% TFA 50% CH_3CN/H_2O (500 μL) for 30 min at rt. The mixture was filtered, and the recovered beads were washed with 500 μL of the same solvent. The obtained solutions (initial solution, supernatant, washing solution, and eluted solution) were analyzed by HPLC (**Fig. S12**). HPLC analysis was performed in the same way as for **Fig. S6**.

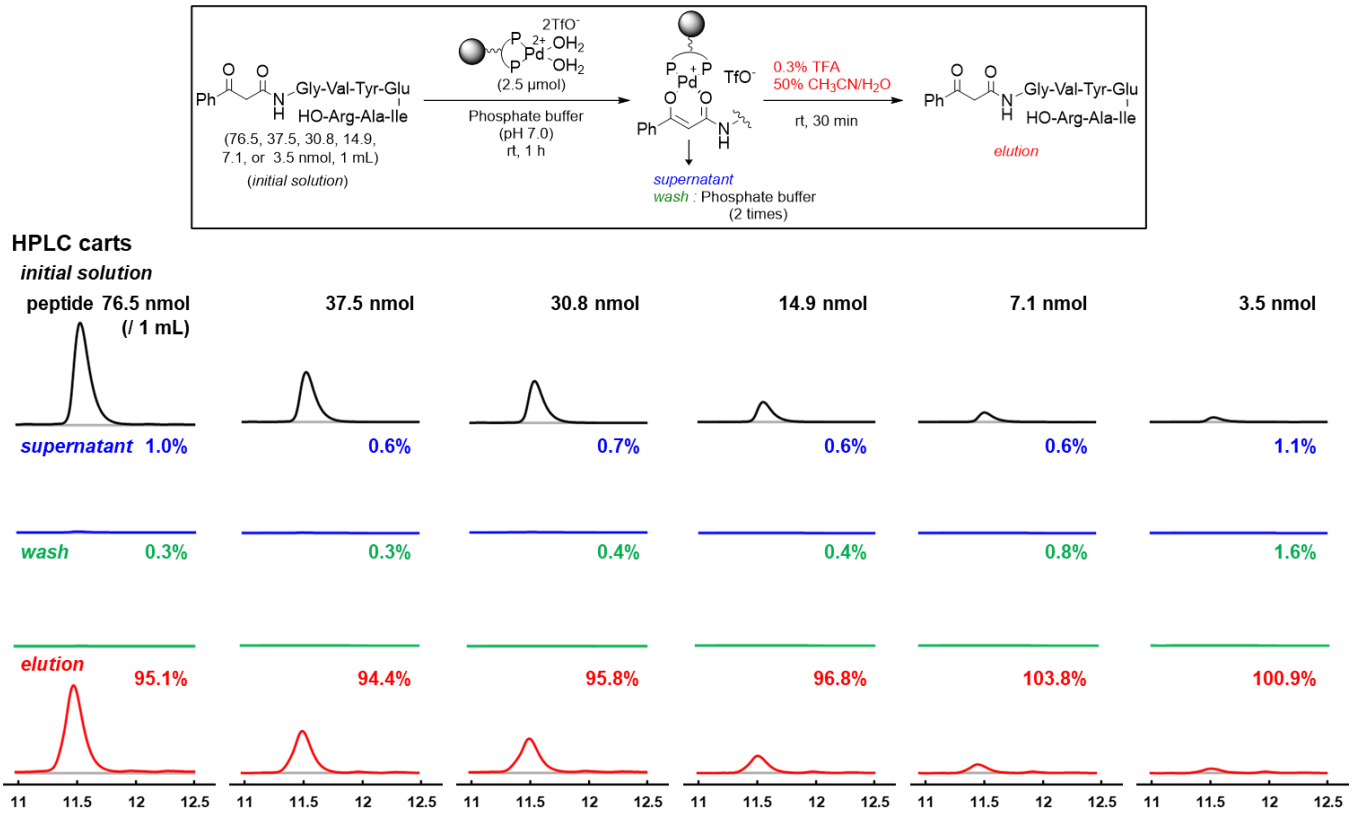


Fig. S12

Consequently, the catch efficiency was consistently quantitative, supporting the above hypothesis. Therefore, we estimated the minimum essential amount of Pd complex as 10^{-3} M.

Purification of Bza-Peptide from Tryptic Digest of BSA

Preparation of BSA tryptic digest

Bovine serum albumin (10 mg) was dissolved in 1 mL of denaturing buffer (7 M guanidine hydrochloride, 1 M Tris-HCl (pH 8.5), 65 mM dithiothreitol (DTT)) and incubated for 10 min at 95 °C and 1 h at 37 °C. After addition of 162.5 mM iodoacetamide (IAM), the sample solution was incubated for 1 h at rt. The protein solution was desalted by a Zeba Desalt Spin column 7K MWCO (BIO-RAD), and then digested with trypsin (sequencing grade modified trypsin, Promega) at 37 °C overnight. The solution was lyphilized and resolved in water before use. The amount of peptide was determined by amino acid analysis.

Procedure for the purification of Bza-peptide from tryptic digest of BSA

To a solution of Bza-peptide (500 pmol) and BSA tryptic digest (540 pmol BSA) in 0.1%TFA in 5% CH_3CN/H_2O (1.00 mL) was added TentaGel-supported Pd aqua complex (10.0 mg, 2 μmol). The mixture was rotated for 1 h at rt, then filtered, and the recovered beads were washed with 2.00 mL of 0.1% TFA 5% CH_3CN/H_2O (x 10). The washed beads were exposed to 0.3% TFA 50% CH_3CN/H_2O (500 μL) for 30 min at rt. The obtained solutions (initial solution, supernatant, washing solution, and eluted solution) were analyzed by MALDI-TOF-MS. Among various BSA peptides, $BSA_{347\text{-}359}$ (blue, **Fig. S13**) showed specific binding to TentaGel-supported Pd aqua complex (**Figure 5c**).

matured BSA

```
  1 MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIA
 51 FSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCK
101 VASLRETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEF
151 KADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGAC
201 LLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAE
251 FVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKE
301 CCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFL
351 GSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKL
401 KHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVS
451 RSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCC
501 TESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQT
551 ALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVV
601 STQTALA
```

Fig. S13 Amino acid sequence of BSA (UniprotKB – P02769)

$BSA_{161\text{-}167}$ and $BSA_{347\text{-}359}$ are shown in red and blue, respectively. Bza-peptide was designed from $BSA_{161\text{-}167}$.

Assay System for Inhibitory Activities against Cathepsin B

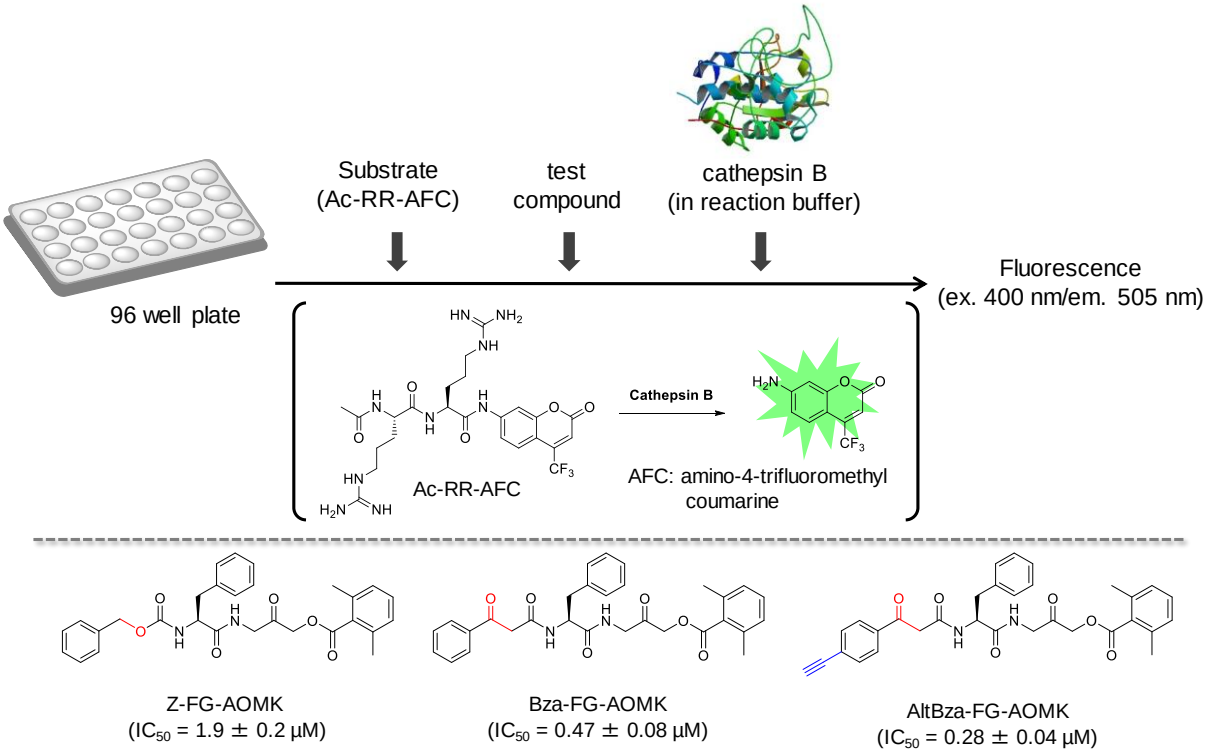


Fig. S14

The cathepsin-inhibitory activities of compounds were measured by using a cathepsin B activity fluorometric assay kit (K140-100, BioVision). Briefly, test compound and substrate were mixed in a 96-well plate (50 μL/well), and human liver cathepsin B (Calbiochem, catalog No. 219362) was added (0.050 μg, 50 μL/well). The plate was incubated at 37 °C for 10 min, and the activity of cathepsin B was determined based on the increase of fluorescence (Ex 400 nm/Em 505 nm) measured with a 96-well plate reader (Spectra Max M2e, Molecular Devices). The dose-response curves and IC_{50} values of compounds were calculated by Origin 9.0 software, and data are presented as mean ± S.D. (n = 3).

Purification and Identification of Bza-FG-AOMK-labelled peptide

Preparation of tryptic digest of labelled cathepsin B

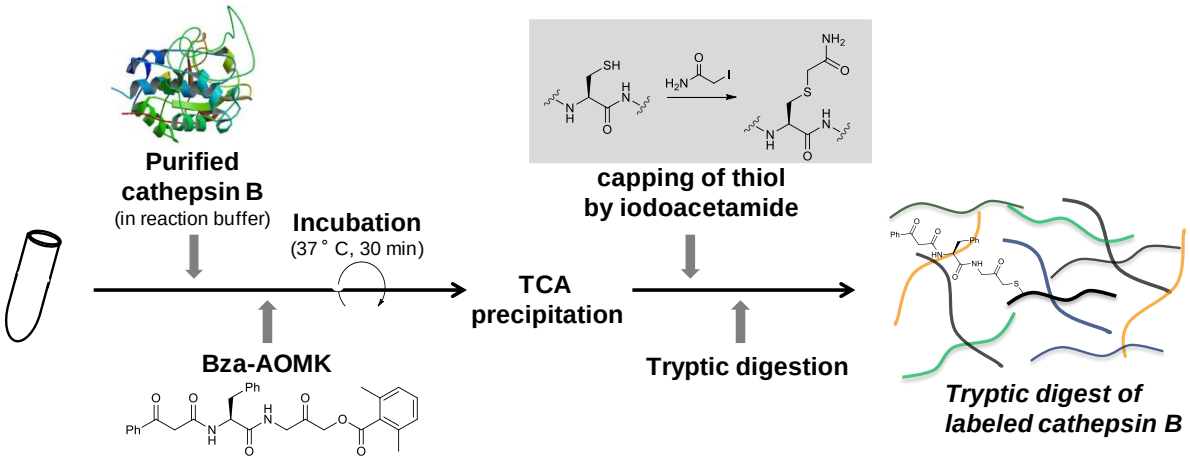


Fig. S15

Human liver cathepsin B (6.5 μg, Calbiochem, catalog No. 219362) was dissolved in 1.0 mL of buffer (50 mM sodium acetate (pH 5.6), 5 mM $MgCl_2$, 2 mM DTT). Then 5 μL of 6 mM Bza-FG-AOMK in DMSO was added (final concentration 30 μM), and the mixture was incubated for 30 min at 37 °C. After incubation, cathepsin B was precipitated by adding trichloroacetic acid (TCA). The precipitate was washed with acetone and dissolved in denaturing buffer (trifluoroethanol 15 μL, 100 mM ammonium bicarbonate 15 μL, and 200 mM DTT 1.5 μL). The solution was incubated for 1 h at 60 °C. After addition of 6 μL of 200 mM iodoacetamide (IAM), incubation was continued for 1 h at rt, and then 1 μL of 200 mM DTT was added to quench excess IAM. The solution was diluted with 240 μL of 25 mM ammonium bicarbonate buffer containing *n*-decyl-β-D-glucopyranoside (DG, final concentration: 0.05 w/v %). After addition of trypsin (200 ng), the sample solution was incubated overnight at 37 °C and used as the tryptic digest of cathepsin B (240 pmol/280 μL, approximately).

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  1 MWQLWASLCCLLVLANARSRPSFHPLSDELVNYVNKRNTTWQAGHNFYNV
                                          matured cathepsin B
 51 DMSYLKRLCGTFLGGPKPPQRVMFTEDLKLPASFDAREQWPQCPTIKEIR
                          light chain      heavy chain
101 DQGSCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTCCGSMCGDGC

151 NGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGSRPPCTG

201 EGDTPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVE

251 GAFSVYSDFLLYKSGVYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSW
                          matured cathepsin B
301 NTDWGDNGFFKILRGQDHCGIESEVVAGIPRTDQYWEKI
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Fig. S16 Amino acid sequence of human cathepsin B (UniprotKB – P07858)

Catalytic cysteine residue (Cys108) and $CatB_{246\text{-}263}$ are shown in red and blue, respectively.

Purification of the peptide labelled by Bza-FG-AOMK

To a solution of tryptic digest of BzaFG-AOMK-labelled cathepsin B (250 pmol) in 0.1% TFA in 5% CH_3CN/H_2O (1.0 mL) was added TentaGel-supported Pd aqua complex (500 nmol, 2000 eq.). The mixture was rotated for 1 h at rt, then filtered. The recovered beads were washed with 2.0 mL of 0.1% TFA in 5% CH_3CN/H_2O (5 times), and then the beads were exposed to 0.3% TFA in 50% CH_3CN/H_2O (500 µL) for 30 min at rt. After filtration, the beads were washed with 0.3% TFA in 50% CH_3CN/H_2O (500 µL). The obtained solutions, initial solution, supernatant, washing solution, and eluted solution, were analyzed by MALDI-TOF-MS (**Fig. 6c** and **S17** left).

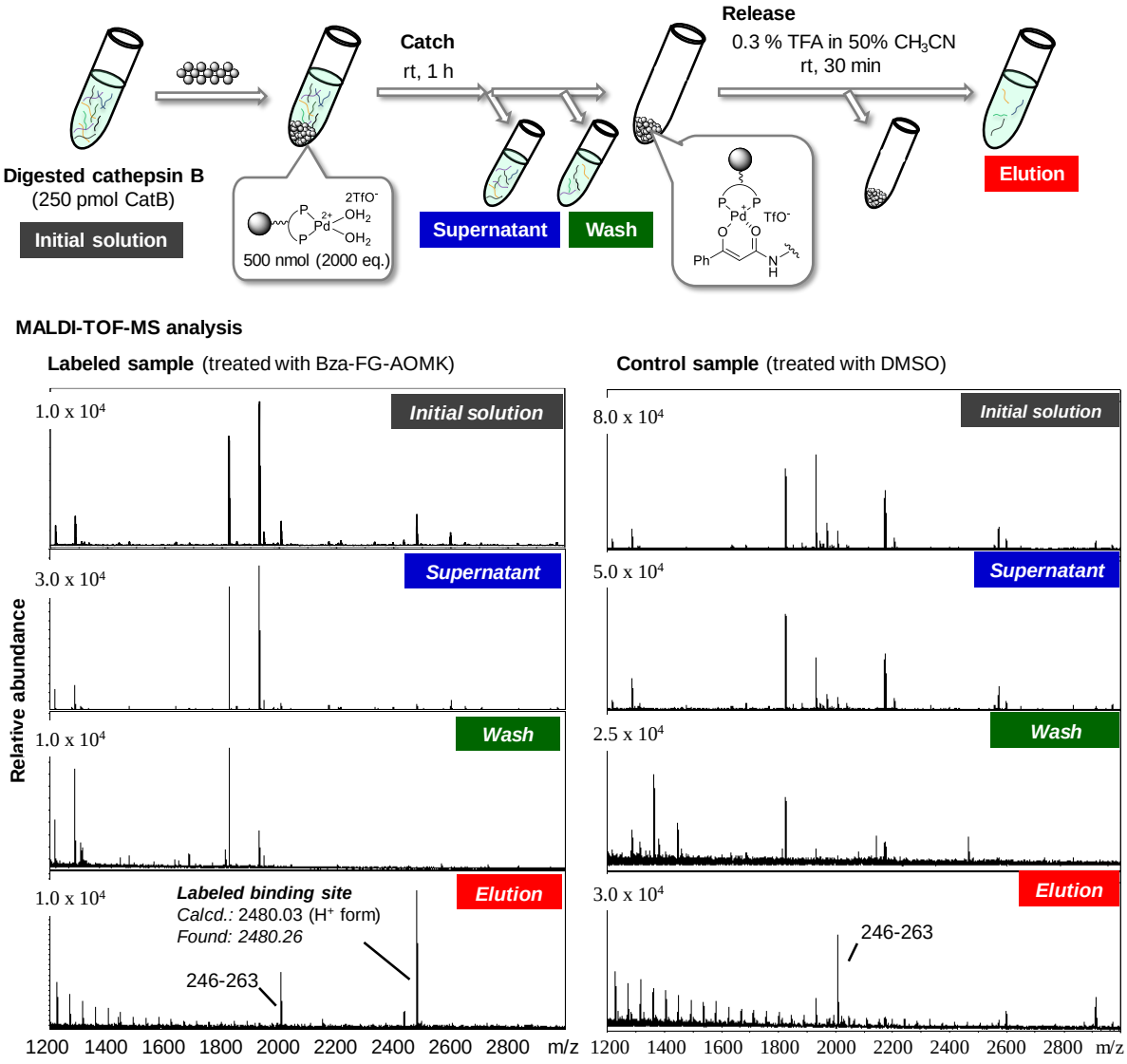


Fig. S17

Purification was also performed using the tryptic digest of non-labelled cathepsin B as an initial solution (**Fig. S17** right). In this case, the target peptide was not observed in the eluate. However, a strong signal of $CatB_{246\text{-}263}$ was detected in the same way as in the Bza-labelled sample (**Fig. S17** left). Based on this observation, $CatB_{246\text{-}263}$ (blue in **Fig. S16**) appears to have some affinity with TentaGel-supported Pd aqua complex. One possible explanation is hydrophobic interaction with the polystyrene regions of TentaGel. Another is coordination of some specific sequence

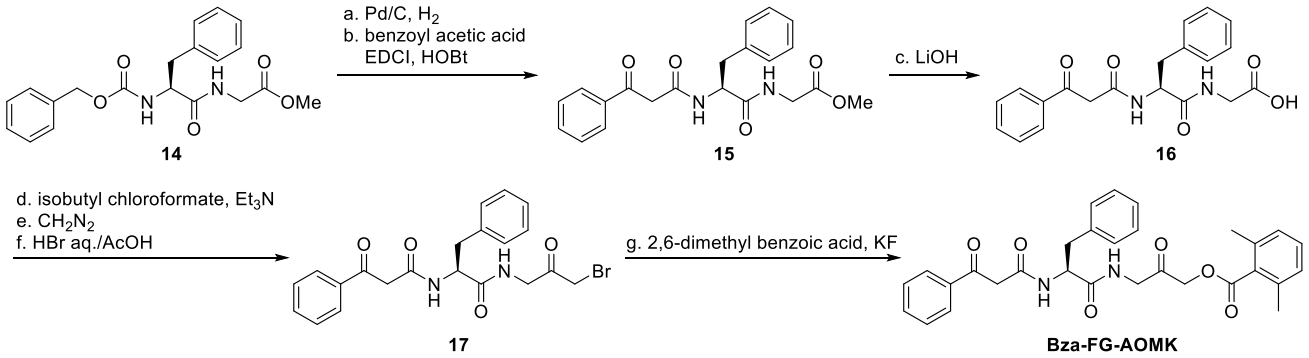
of amino-acid residues of $CatB_{246-263}$ to the Pd complex. In addition, the region from 1200 to 1800 m/z contains many peaks related to polyethylene glycol (PEG), which is thought to be derived from the PEG linker of TentaGel.

LC-ESI-MS/MS analysis for the identification of the binding site

The eluted solution was also analyzed by LC-ESI-MS/MS to identify the binding site. Mass spectra were acquired using a LTQ Orbitrap XL source (Thermo Fisher Scientific) equipped with a nano electrospray ionization (nanoESI) source (Nikkyo Technos Co, Ltd.). Full mass scan was acquired in the FT mode (resolution 60,000) and MS/MS scan (CID) was acquired in the iontrap (IT) mode or FT mode. On a nanoflow HPLC system (nanoLC) (UltiMate 3000 nano LC system, Thermo Fisher Scientific), Acclaim PepMap100 C18 nanoViper (75 μm i. d. x 150 mm, 3 μm, 100 Å, Thermo Fisher Scientific) and μ-precolumn cartridge (Acclaim PepMap100 C18, 300 μm i. d. x 5 mm, 100 Å, 5 μm, Thermo Fisher Scientific) were used as the analytical column and trap column, respectively. For the analytical column, mobile-phase A consisted of 0.1% FA, 4% MeCN in distilled water; mobile-phase B consisted of 100% MeCN containing 0.1% FA. For the trap column, mobile-phase C consisted of 0.1% TFA in distilled water. The gradient method was used with mobile-phase A and mobile-phase B at a flow rate of 250 nL/min. A representative gradient was as follows; 0% B (0 - 10 min), 0 – 40% B (10 – 40 min), 40 – 70% B (40 – 45 min), 70 – 100% B (45 – 46 min), total 60 min run. The MS and MS/MS data were searched against protein database using Proteome Discoverer (Thermo Fisher Scientific) with MASCOT (Matrix Science). Theoretical peptide mass value was calculated by a Xcalibur Qual Browser (Thermo Fisher Scientific).

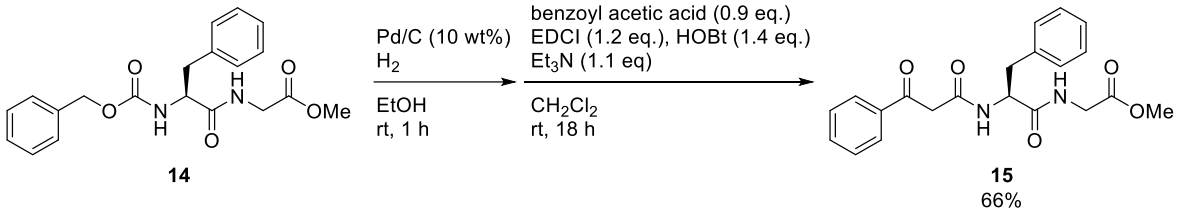
Synthesis of Bza-FG-AOMK

Scheme S3



Reagents and conditions: (a) Pd/C (10 wt%), H_2, EtOH, rt, 1 h; (b) benzoyl acetic acid, EDCI, HOBt, Et_3N, CH_2Cl_2, rt, 10 h, 66% in 2 steps; (c) LiOH, THF/MeOH/H_2O, rt, 2 h; (d) isobutyl chloroformate, Et_3N, THF, -40 °C, 30 min then 0 °C, 30 min; (e) diazald, KOH, Et_2O/MeOH/H_2O, 0 °C, 1 h; (f) 48% HBr aq./AcOH (1/1), 0 °C, 10 min; (g) 2,6-dimethylbenzoic acid, KF, DMF, rt, 14 h, 50% in 5 steps.

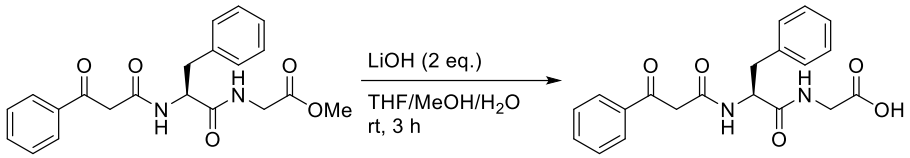
Bza-FG-OMe (15)



To a solution of Cbz-FG-OMe (**14**)[8] (2.5 g, 6.7 mmol) in EtOH (68.0 mL) was added Pd/C (250 mg, 10 wt%). The suspension was stirred for 1 h under a hydrogen atmosphere, and then filtered through a Celite pad. The filtrate was concentrated under reduced pressure and the residue was dissolved in CH_2Cl_2 (34.0 mL). To this solution was added benzoylacetic acid (1.0 g, 6.1 mmol), EDCI·HCl (1.4 g, 7.3 mmol), and HOBt (1.1 g, 8.2 mmol) at rt. The reaction mixture was stirred for 18 h, then the solvent was removed *in vacuo*. The crude mixture was diluted with AcOEt, and washed with saturated aqueous NH_4Cl, saturated aqueous $NaHCO_3$, and brine. The organic solution was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by recrystallization from CH_2Cl_2/hexane to give Bza-FG-OMe (**15**) (1.54 g, 66%) as a white solid.

$[a]_D^{24}$: -42.9 (c 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, CD_3OD): δ 7.96 (2H, d, *J* = 8.1 Hz), 7.74-7.72 (1H, m), 7.62 (1H, t, *J* = 7.7 Hz), 7.48 (2H, dd, *J* = 7.7, 8.1 Hz), 7.43-7.38 (1H, m), 7.29-7.18 (5H, m), 4.73 (1H, dd, *J* = 5.4, 9.4 Hz), 3.99 (2H, s), 3.72 (3H, s), 3.31 (2H, s), 3.26 (1H, dd, *J* = 5.4, 14.0 Hz), 2.92 (1H, dd, *J* = 9.4, 14.0 Hz); ^{13}C-NMR (100 MHz, CD_3OD): δ 196.6, 173.9, 171.5, 169.6, 138.5, 137.5, 134.9, 130.2 (2C), 129.9 (2C), 129.6 (2C), 129.5 (2C), 127.8, 56.0, 52.6, 41.9, 38.6, 32.7; HRMS (ESI) calcd for $C_{21}H_{22}N_2O_5$ $[M+Na]^+$ 405.1385; found 405.1421.

Bza-FG-OH (16)

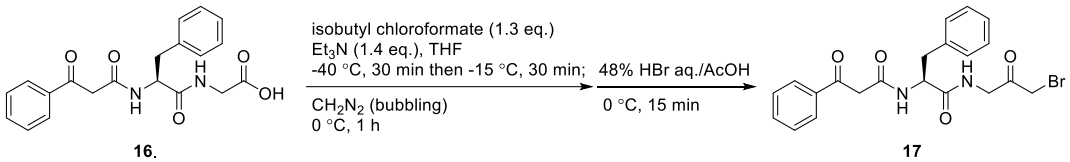


15 16

To a solution of **15** (1.5 g, 3.92 mmol) in THF/MeOH/H_2O (2/1/1, 80 mL) was added LiOH•H_2O (329 mg, 7.8 mmol). The reaction mixture was stirred for 3 h at rt, and then quenched with 1*N* HCl. The aqueous phase was extracted with AcOEt. The combined organic solution was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by recrystallization from CH_2Cl_2/hexane to afford Bza-FG-OH (**16**) (1.47 g including inseparable impurities) as a white solid.

$[a]_D^{21}$: -31.2 (c 0.54, MeOH); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.54-8.42 (2H, m), 7.83 (2H, d, *J* = 8.0 Hz), 7.62 (1H, t, *J* = 7.8 Hz), 7.46 (2H, dd, *J* = 7.8, 8.0 Hz), 7.29-7.18 (5H, m), 4.60-4.55 (1H, m), 3.94-3.79 (4H, m), 3.07 (1H, dd, *J* = 3.9, 13.6 Hz), 2.77 (1H, dd, *J* = 9.2, 13.6 Hz); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 194.5, 171.3, 171.1, 166.2, 137.8, 136.2, 133.4, 129.2 (2C), 128.7 (2C), 128.3 (2C), 128.1 (2C), 126.3, 53.8, 46.7, 40.7, 37.8; HRMS (ESI) calcd for $C_{20}H_{20}N_2O_5$ $[M-H]^-$ 367.1288; found 367.1308.

Bza-FG-Br (17)

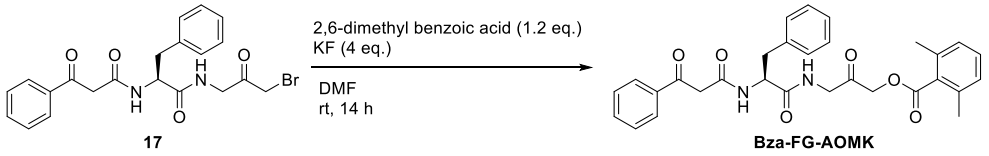


16. 17

To a solution of **16** (400 mg, 1.08 mmol) in THF (10 mL) was added triethylamine (211 µL, 1.51 mmol) and isobutyl chloroformate (184 µL, 1.41 mmol) at -40 °C. The reaction mixture was stirred for 30 min at -40 °C, and for 30 min at -15 °C. In another flask, a solution of Diazald (1.07 g, 5.0 mmol) in Et_2O (10 mL) was added slowly over 1 h to a solution of KOH (841 mg, 15.0 mmol) in MeOH/H_2O/Et_2O (2/1/1, 24.0 mL) at rt. The generated CH_2N_2 was added to the reaction solution by bubbling, and the reaction mixture was stirred for 1 h at 0 °C. Then, 48% aqueous HBr/AcOH (1/1, 4.0 mL) was added, and stirring was continued for 15 min at 0 °C. The reaction mixture was quenched with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with AcOEt, and the organic solution was washed with H_2O and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/1) to afford Bza-FG-Br (**17**) (396 mg including inseparable impurities) as a white solid.

$[a]_D^{21}$: -22.1 (c 0.83, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.90 (2H, d, *J* = 7.6 Hz), 7.71-7.69 (1H, m), 7.59 (1H, t, *J* = 7.4 Hz), 7.46 (2H, dd, *J* = 7.4, 7.6 Hz), 7.42-7.35 (1H, m), 7.28-7.17 (5H, m), 4.86-4.81 (1H, m), 4.31 (1H, dd, *J* = 5.4, 18.9 Hz), 4.21 (1H, dd, *J* = 5.2, 18.9 Hz), 3.90 (2H, d, *J* = 8.0 Hz), 3.89 (2H, s), 3.18 (1H, dd, *J* = 6.4, 14.1 Hz), 3.08 (1H, dd, *J* = 7.8, 14.1 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 197.7, 195.5, 171.4, 166.7, 136.5, 136.0, 134.3, 129.4 (2C), 129.0 (2C), 128.8 (2C), 128.7 (2C), 127.2, 54.7, 47.2, 45.6, 37.8, 31.8; HRMS (ESI) calcd for $C_{21}H_{21}N_2O_4$ $[M+Na]^+$ 469.0558; found 469.0511.

Bza-FG-AOMK



To a solution of **17** (330 mg, 0.74 mmol) in DMF (7.4 mL) was added 2,6-dimethyl benzoic acid (134 mg, 0.89 mmol) and KF (172 mg, 2.96 mmol) at rt. The reaction mixture was stirred for 14 h at rt and quenched with H_2O. The aqueous layer was extracted with AcOEt and the organic solution was washed with H_2O, saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 7/3) to afford **Bza-FG-AOMK** (235 mg, 50% in 5 steps from compound **15**) as a white solid.

$[a]_D^{22}$: -22.2 (c 0.63, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.92 (2H, d, *J* = 7.6 Hz), 7.72-7.71 (1H, m), 7.60 (1H, t, *J* = 7.5 Hz), 7.46 (2H, dd, *J* = 7.5, 7.6 Hz), 7.38-7.36 (1H, m), 7.28-7.17 (5H, m), 7.09-7.03 (3H, m), 4.89 (2H, d, *J* = 2.4 Hz), 4.85-4.80 (1H, m), 4.26 (1H, dd, *J* = 5.0, 19.1 Hz), 4.15 (1H, dd, *J* = 5.2, 19.1 Hz), 3.93 (2H, d, *J* = 8.8 Hz), 3.18 (1H, dd, *J* = 6.6, 14.2 Hz), 3.12 (1H, dd, *J* = 7.4, 14.2 Hz), 2.36 (6H, s); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 198.9, 195.6, 171.2, 169.1, 166.5, 136.4, 136.0, 135.8 (2C), 134.3, 132.4, 130.0, 129.4 (2C), 129.0 (2C), 128.9 (2C), 128.7 (2C), 127.9 (2C), 127.2, 66.9, 54.8, 46.9, 45.5, 37.8, 20.1 (2C); HRMS (ESI) calcd for $C_{30}H_{30}N_2O_6$ $[M+Na]^+$ 537.1996; found 537.1995.

Comparison with Purification Method Using Biotin-Avidin System and Click Reaction

To compare our method with the widely used alkyne-tag-click reaction method, alkyne-tagged Bza-FG-AOMK (AltBza-FG-AOMK) was prepared and used in our method or the general method using click reaction and biotin-avidin system.

Purification of the peptide modified by AltBza-FG-AOMK by using Pd beads

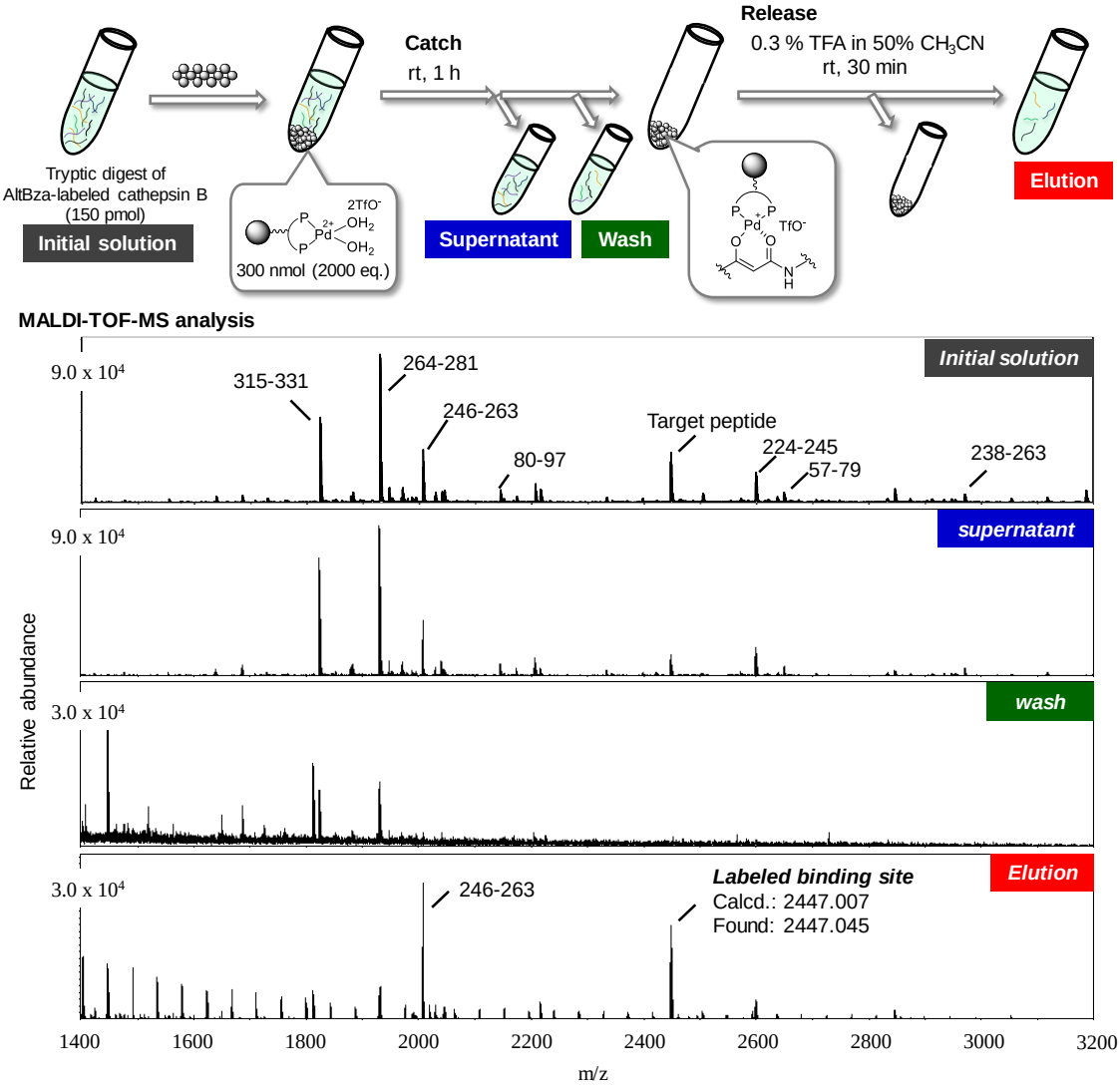


Fig. S18

The tryptic digest of AltBza-FG-AOMK-labelled cathepsin B (150 pmol) was prepared by using the same method as **Fig. S15**. Even in the case of this sample, we succeeded in purification of the AltBza-FG-AOMK-labelled peptide by our method.

*In the case of AltBza-FG-AOMK, one of the cysteine residues in target peptide was not capped efficiently by iodoacetamide.

Purification of the peptide modified by AltBza-FG-AOMK by using biotin-avidin system

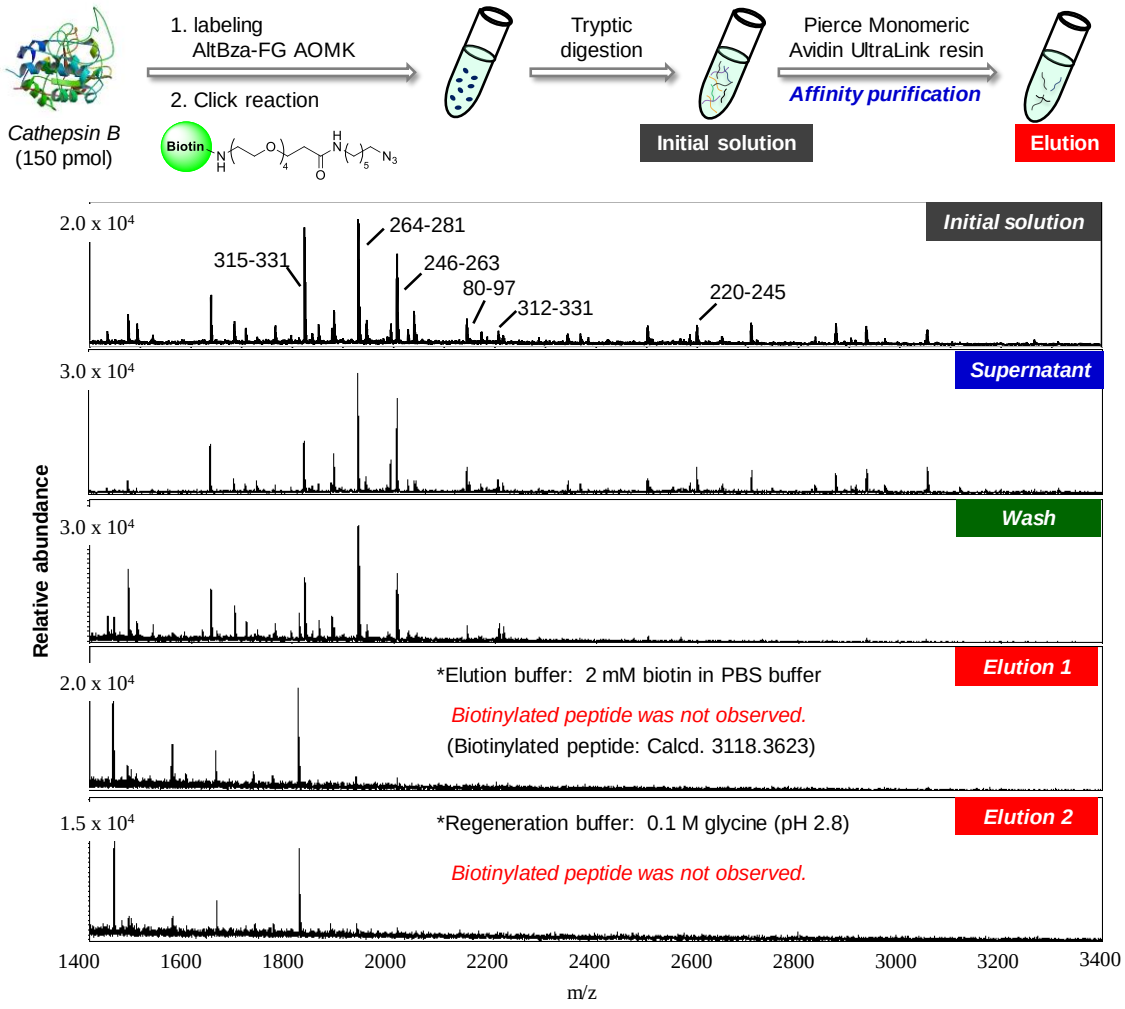


Fig. S19

Human liver cathepsin B (4 μg, 150 pmol) was incubated with AltBza-FG-AOMK (final concentration 30 μM) for 30 min at 37 °C, then cathepsin B was precipitated with TCA and washed with acetone. The precipitate was dissolved in 100 μL of SDS buffer (1% SDS, 50 mM Tris-HCl, pH 8.0). Introduction of a biotin tag was performed by click reaction using Click-it Protein Reaction Buffer Kit (Thermo Fisher Scientific). After click reaction, reagents were removed by MeOH/$CHCl_3$ precipitation. The precipitate was digested with trypsin after capping of cysteine residues using the same method as for **Fig. S15**. We tried to purify the AltBza-FG-AOMK-labelled peptide by using the biotin-avidin system according to the supplier's protocol. Pierce Monomeric Avidin UltraLink resin (100 μL, 50% aqueous slurry, ≥ 900 pmol binding capacity) was added to a tube, and then washed 5 times with PBS buffer (0.5 mL). The tryptic digest of cathepsin B was mixed with the avidin beads in PBS buffer (0.5 mL), and the mixture was incubated for 1 h at 4 °C. The supernatant was recovered after centrifugation, and the beads were washed 5 times with PBS buffer (0.5 mL). The washed beads were incubated in 2 mM biotin in PBS buffer (0.1 mL) for 1 h at 4 °C, and the supernatant was recovered as eluate 1. Additionally, the beads were incubated in regeneration buffer (0.1 M glycine buffer, pH 2.8) for 1 h at rt, and then the supernatant was recovered as eluate 2. The obtained solutions were analyzed by MALDI-TOF-MS (**Fig. S19**) and LC-ESI-MS.

Non-labelled target peptide ($CatB_{101-120}$) was not observed in the initial solution. This indicates that the labelling with AltBza-FG-AOMK proceeded successfully. However, the biotinylated target peptide was not detected even in the initial solution. Possible reasons for this are that the biotinylated peptide may have low ionization efficiency or may have precipitated due to the low solubility of the biotin tag.

Western blotting analysis of cathepsin B labelled by AltBza-FG-AOMK with click reaction.

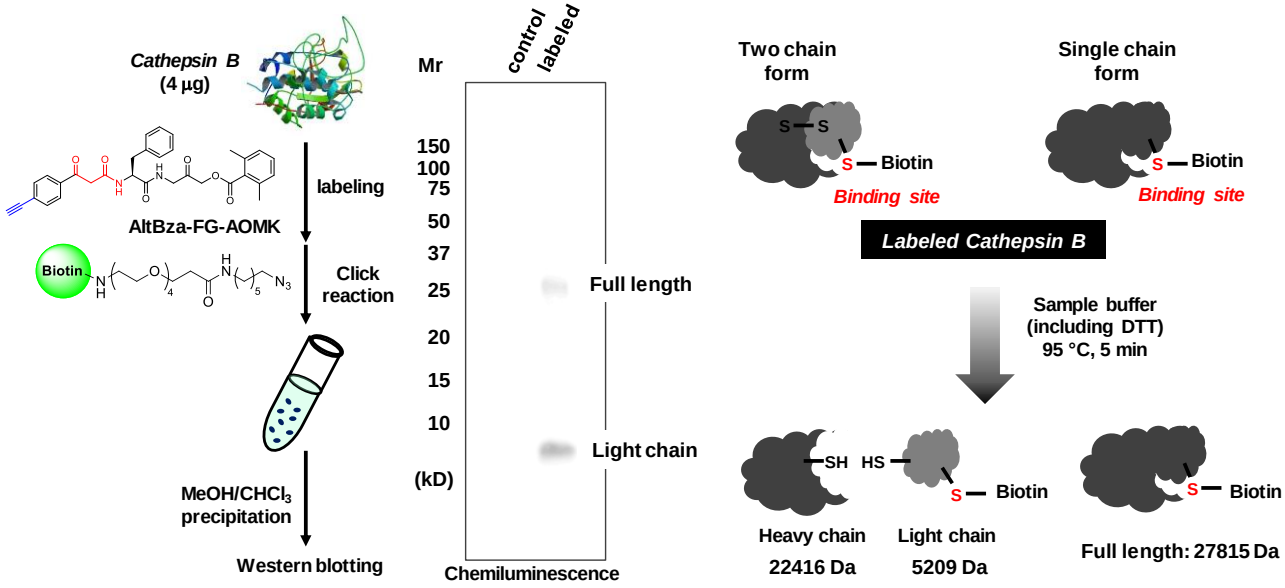
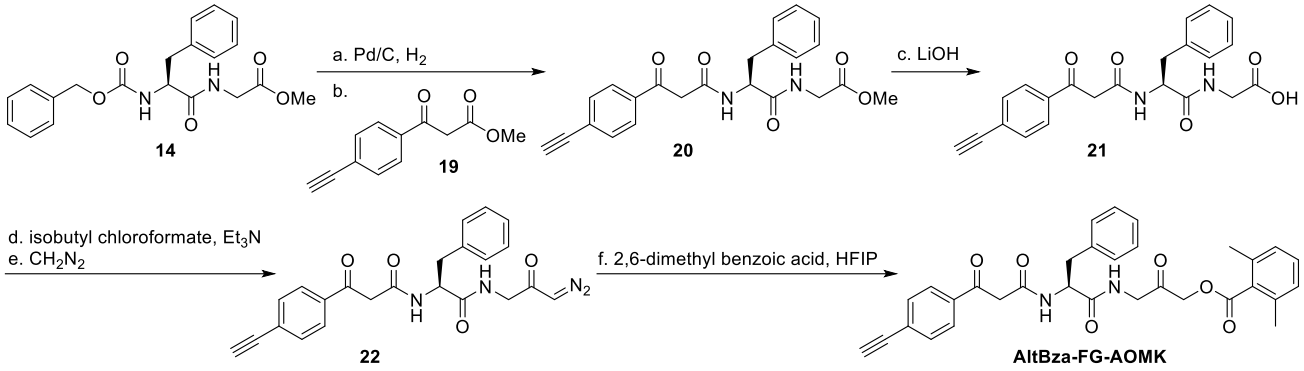


Fig. S20

To confirm the introduction of the biotin tag by click reaction, Western blotting analysis was performed (**Fig. S20**). In the same manner as for figure S14, human liver cathepsin B (4 μg, 150 pmol) was treated with or without AltBza-FG-AOMK, and the biotin tag was introduced by click reaction. The biotinylated cathepsin B was dissolved in SDS sample buffer, and reductive alkylation was performed with a sample buffer kit (Apro Science; acrylamide as an alkylation reagent). The obtained samples were separated by SDS-PAGE and analyzed by Western blotting using streptavidin-HRP (GE Healthcare). The chemiluminescence image was obtained by using LAS4000 (GE-Helthcare). As shown in the right part of **Fig. S20**, cathepsin B has two forms: a single chain form and a two-chain form. The two-chain form was mainly included in our sample, and the binding site is in the light chain. Indeed, the light chain was clearly observed by chemiluminescence, indicating that the introduction of the biotin tag proceeded successfully with AltBza-FG-AOMK and click reaction.

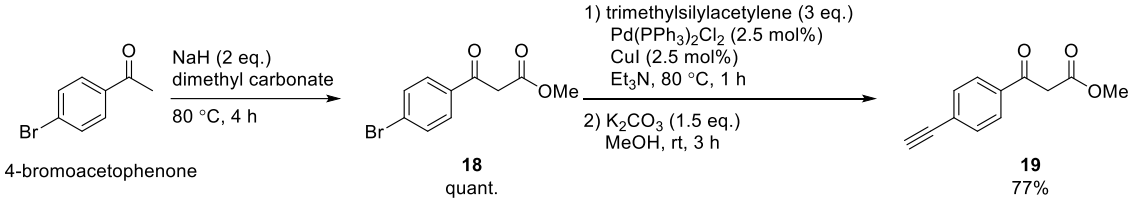
Synthesis of AltBza-FG AOMK

Scheme S4



Reagents and conditions: (a) Pd/C (10 wt%), H_2, THF/EtOH, rt, 1.5 h; (b) methyl 4-(ethynyl)benzoylacetate, toluene, reflux, 6 h, 54% in 2 steps; (c) LiOH, THF/MeOH/H_2O, rt, 2 h, 94%; (d) isobutyl chloroformate, Et_3N, THF, -60 °C, 30 min then -40 °C, 30 min; (e) diazald, KOH, Et_2O/MeOH/H_2O, 0 °C, 2 h, 43% in 2 steps; (f) 2,6-dimethyl benzoic acid, HFIP, rt, 3 h, 14%.

methyl 3-(4-ethynylphenyl)-3-oxopropanoate (19)[9,10]



To a solution of 4-bromoacetophenone (10.0 g, 50.0 mmol) in dimethyl carbonate (50.0 mL) was added NaH (4.40 g, 55% dispersion in mineral oil, 100 mmol) at rt and the mixture was stirred for 4 h at 80 °C. The reaction mixture was quenched with 1 *N* HCl, and the whole was extracted with AcOEt two times. The combined organic solution was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/9) to give methyl 4-bromobenzoylacetate (**18**) (13.5 g, quant.) as a pale yellow oil[9].

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.80 (2H, d, *J* = 8.6 Hz), 7.63 (2H, d, *J* = 8.6 Hz), 3.97 (2H, s), 3.75 (3H, s); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 191.5, 167.7, 134.7, 132.3, 130.1, 127.7, 52.7, 45.7.

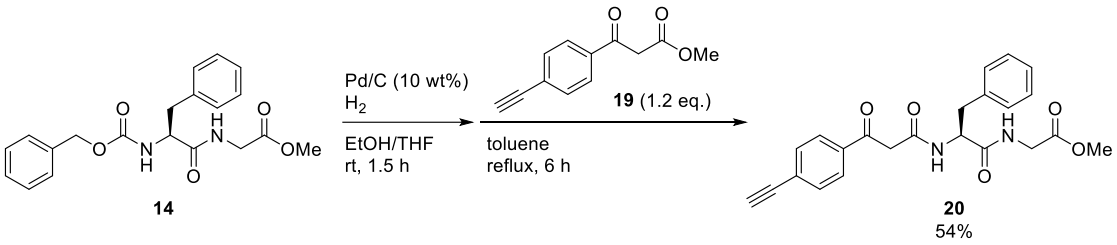
To a solution of **18** (12.2 g, 47.6 mmol) and trimethylsilylacetylene (19.8 mL, 143 mmol) in Et_3N (450 mL) was added bis(triphenylphosphine)palladium dichloride (842 mg, 1.20 mmol) and copper iodide (229 mg, 1.20 mmol) at rt. The reaction mixture was stirred for 1 h at 80 °C, then the solution was filtered through a Celite pad. The filtrate was concentrated under reduced pressure, and the residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 1/19) to give the TMS-protected product[10]. To a solution of the TMS-protected compound in MeOH (250 mL) was added K_2CO_3 (9.90 g, 71.4 mmol) at rt. The reaction mixture was stirred for 3 h at rt, then saturated aqueous NH_4Cl was added, and the mixture was extracted with CH_2Cl_2 two times. The combined organic solution was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash

column chromatography (SiO_2, eluent; AcOEt/Hex = 1/9) to give methyl 3-(4-ethynylphenyl)-3-oxopropanoate (**19**) (7.36 g, 77%) as a pale-yellow solid.

Enol form (major): ^{1}H-NMR (400 MHz, $CDCl_3$): δ 12.5 (1H, s), 7.73 (2H, d, *J* = 8.4 Hz), 7.53 (2H, d, *J* = 8.4 Hz), 5.68 (1H, s), 3.81 (3H, s), 3.20 (1H, s); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 173.5, 170.5, 133.6, 132.4 (2C), 126.1 (2C), 125.1, 87.9, 83.1, 79.6, 51.7; HRMS (ESI) Calcd. for $C_{12}H_{10}O_3Na$: 225.0522. Found: 255.0521.

*Keto form: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.90 (2H, d, *J* = 8.8 Hz), 7.59 (2H, d, *J* = 8.8 Hz), 3.94 (2H, s), 3.75 (3H, s), 3.28 (1H, s); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 191.7, 167.8, 135.7, 132.6 (2C), 128.5 (2C), 127.8, 82.7, 81.1, 52.7, 45.8.

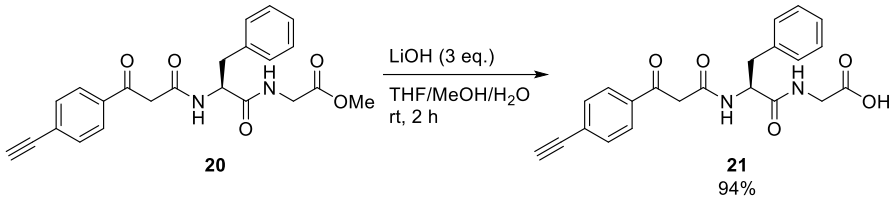
AltBza-FG-OMe (20)[11]



To a mixture of **14**[8] (1.53 g, 4.10 mmol) in EtOH/THF (4/1, 40.0 mL) was added Pd/C (153 mg, 10 wt%). The suspension was stirred for 1.5 h under a hydrogen atmosphere, and then the mixture was filtered through a Celite pad. The filtrate was concentrated under reduced pressure. The residue was dissolved in toluene (40.0 mL), and then **19** (1.00 g, 4.90 mmol) was added to the solution at rt. The reaction mixture was refluxed for 6 h, then diluted with AcOEt. The organic solution was washed with saturated aqueous NH_4Cl, saturated aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, eluent; MeOH/$CHCl_3$ = 1/49), and recrystallization from CH_2Cl_2/hexane to give AltBza-FG-OMe (**20**) (901 mg, 54%) as a white solid.

white solid; $[a]_D^{24}$: -47.2 (c 1.0, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.87 (2H, d, *J* = 8.2 Hz), 7.57 (2H, d, *J* = 8.2 Hz), 7.31-7.18 (6H, m), 6.76 (1H, dd , *J* = 5.4, 4.9 Hz), 4.79 (1H, dd, *J* = 7.6, 6.4 Hz), 4.07 (1H, dd, *J* = 18, 5.4 Hz), 3.92 (1H, dd, *J* = 18, 4.9 Hz), 3.90 (2H, d, *J* = 4.8 Hz), 3.72 (3H, s), 3.29 (1H, s), 3.15 (1H, dd, *J* = 13.8, 6.4 Hz), 3.15 (1H, dd, *J* = 13.8, 7.6 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$, D_2O 1 drop): δ 194.6, 171.2, 170.2, 166.3, 136.6, 135.6, 132.6 (2C), 129.4 (2C), 128.7 (2C), 128.5 (2C), 127.1, 125.8, 82.6, 81.3, 54.6, 52.5, 45.9, 41.3, 37.8; HRMS (ESI) Calcd. for $C_{23}H_{22}N_2O_5$ $[M+Na]^+$ 429.1421; Found 429.1412.

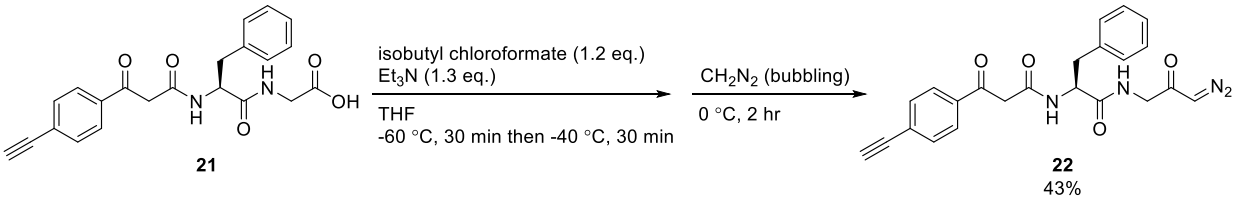
AltBza-FG-OH (21)



To a solution of AltBza-FG-OMe (800 mg, 1.97 mmol) in THF/MeOH/H_2O (2/1/1, 21 mL) was added lithium hydroxide monohydrate (250 mg, 5.96 mmol) at rt. The reaction mixture was stirred at rt for 2 h, and then diluted with AcOEt and H_2O. The mixture was separated, and the aqueous layer was acidified with 1 *N* HCl. The acidified aqueous layer was extracted with AcOEt twice. The combined organic solution was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was crystallized from CH_2Cl_2/hexane to give AltBza-FG-OH (**21**) (723 mg, 94%) as a white solid.

white solid; $[a]_D^{25}$: -44.1 (c 1.00, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$, DMSO-d_6 1 drop): δ 7.82 (2H, d, *J* = 8.4 Hz), 7.51 (2H, d, *J* = 8.4 Hz), 7.28-7.13 (7H, m), 4.73 (1H, dd, *J* = 7.8, 6.0 Hz), 4.05-3.80 (4H, m), 3.26 (1H, s), 3.17 (1H, dd, *J* = 14.1, 6.0 Hz), 2.98 (1H, dd, *J* = 14.1, 7.8 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$/DMSO-d_6 (4/1)): δ 193.7, 171.1, 170.7, 165.9, 136.7, 135.2, 131.6 (2C), 128.7 (2C), 127.8 (2C), 127.6 (2C), 125.8, 124.8, 81.9, 80.9, 53.8, 46.3, 40.7, 37.0; HRMS (ESI) Calcd. for $C_{22}H_{19}N_2O_5$ $[M-H]^-$ 391.1299; Found 391.1323.

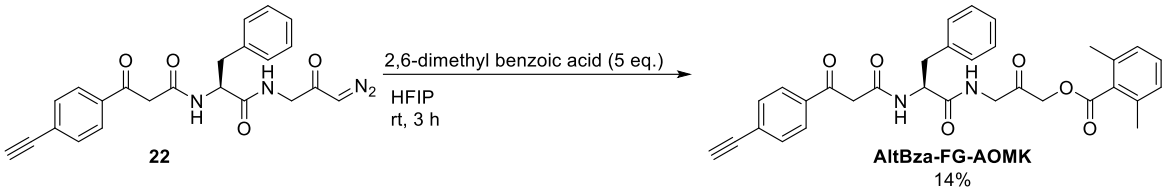
AltBza-FG-N_2 (22)[10]



To a solution of AltBza-FG-OH (680 mg, 1.73 mmol) and triethylamine (313 μL, 2.24 mmol) in THF (17 mL) was added isobutyl chloroformate (270 μL, 2.08 mmol) at -60 °C. The reaction mixture was stirred at -60 °C for 30 min, and then at -40 °C for 30 min. In another flask, a solution of Diazald (1.85 g, 8.65 mmol) in Et_2O (17.0 mL) was added slowly over 1 h to a solution of KOH (1.46 g, 26.0 mmol) in Et_2O/MeOH/H_2O (1/2/1, 40.0 mL) at rt. The generated CH_2N_2 was added to the reaction solution by bubbling, and stirring was continued for 2 h at 0 °C. The reaction mixture was quenched with AcOH, and diluted with AcOEt. The organic solution was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt), and recrystallization from CH_2Cl_2/hexane to give AltBza-FG-N_2 (**22**) (308 mg, 43%) as a white solid.

white solid; $[a]_D^{25}$: -50.9 (c 0.50, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.87 (2H, d, *J* = 8.6 Hz), 7.57 (2H, d, *J* = 8.6 Hz), 7.33-7.20 (6H, m), 7.11 (1H, br-s), 5.34 (1H, s), 4.76 (1H, dd, *J* = 7.8, 6.4 Hz), 4.00-3.86 (4H, m), 3.30 (1H, s), 3.20 (1H, dd, *J* = 13.3, 6.4 Hz), 3.11 (1H, dd, *J* = 13.3, 7.8 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$, D_2O 1 drop): δ 194.8, 190.4, 171.0, 166.4, 144.6, 136.4, 135.5, 132.7(2C), 129.4 (2C), 128.9 (2C), 128.6 (2C), 127.3, 125.8, 82.6, 81.5, 54.9, 53.9, 46.9, 37.6; HRMS (ESI) Calcd. for $C_{23}H_{20}N_4O_4[M+Na]^+$ 439.1377; Found 439.1376.

AltBza-FG-AOMK[12]



To a solution of **22** (100 mg, 0.240 mmol) in HFIP (500 μL) was added 2,6-dimethylbenzoic acid (180 mg, 1.20 mmol) at rt. The reaction mixture was stirred for 3 h at rt, and then diluted with AcOEt. The organic solution was washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, eluent; AcOEt/Hex = 7/3), PTLC (eluent; AcOEt/Hex = 1/1), and GPC ($CHCl_3$) to give AltBza-FG-AOMK (18 mg, 14%) as a white solid.

white solid; $[a]_D^{25}$: -26.0 (c 0.50, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.88 (2H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 8.4 Hz), 7.30-7.20 (7H, m), 7.05 (2H, d, *J* = 8.0 Hz), 6.89 (1H, br-s), 4.90 (2H, s), 4.79 (1H, dd, *J* = 7.4, 6.6 Hz), 4.28 (1H, dd, *J* = 19, 5.4 Hz), 4.16 (1H, dd, *J* = 19, 4.6 Hz), 3.95 (1H, d, *J* = 17 Hz), 3.89 (1H, dd, *J* = 17 Hz), 3.29 (1H, s), 3.19 (1H, dd, *J* = 14.2, 6.6 Hz), 3.13 (1H, dd, *J* = 14.2, 7.4 Hz), 2.37 (6H, s); ^{13}C-NMR (75 MHz, $CDCl_3$, D_2O 1 drop): δ 199.0, 194.8, 171.1, 169.2, 166.2, 136.4, 135.8 (2C), 135.6, 132.6 (2C), 132.3, 130.0, 129.4 (2C), 128.8 (2C), 128.6 (2C), 127.9 (2C), 127.2, 125.8, 82.6, 81.3, 66.9, 54.6, 46.8, 37.7, 20.1 (2C); HRMS (ESI) Calcd. for $C_{32}H_{30}N_2O_6$ $[M+Na]^+$ 561.1996; Found 561.2000.

Purification and Identification of Bza-VAD(OMe)-FMK-labelled peptide

To examine the generality of our method, we applied the same approach for another enzyme inhibitor. The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has inflicted severe damage globally. To overcome this pandemic, various therapeutic approaches were examined.[13] Among them, SARS-CoV-2 main protease (M^{pro}) was expected to be an antiviral target to suppress the virus replication.[14] Z-VAD(OMe)-FMK was identified as a potent inhibitor for SARS-CoV-2 M^{pro} by chemical screening from a library of 4198 chemical entities.[15] A fluoromethylketone (FMK) group of Z-VAD(OMe)-FMK was identified for creating a covalent bond with catalytic cysteine of the protease.[15] We synthesized Bza-VAD(OMe)-FMK from Z-VAD(OMe)-FMK and applied it for the purification of the labelled peptide.

Assay System for Inhibitory Activities against SARS-CoV-2 Main Protease (M^{pro})

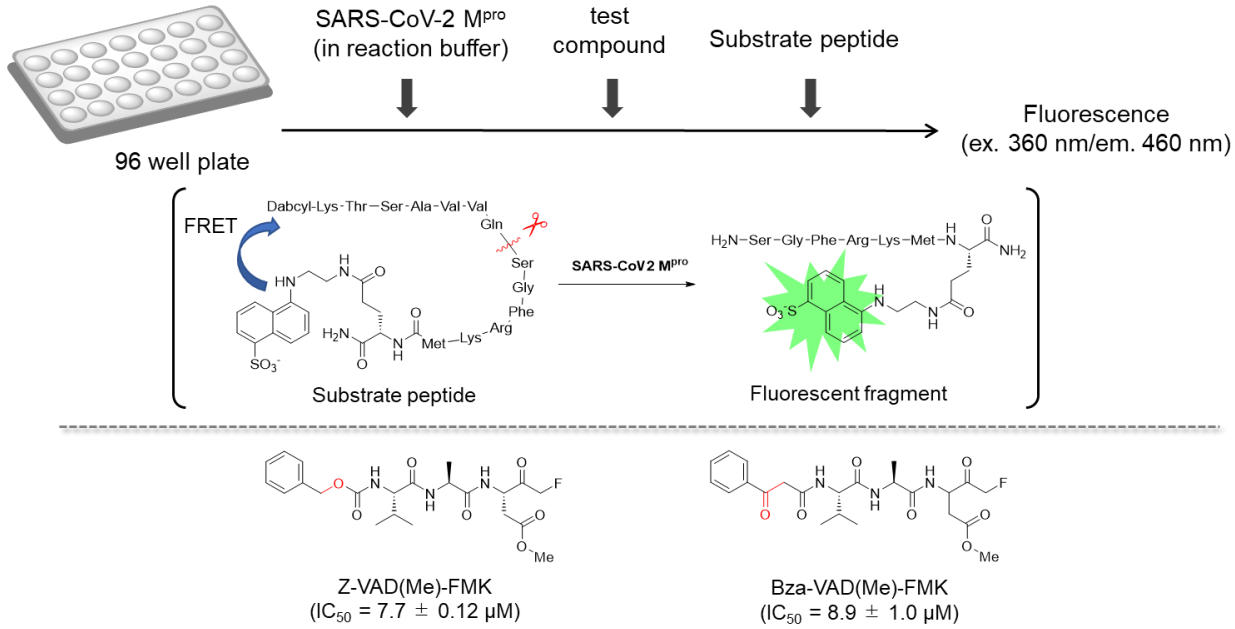


Fig. S21

The protease-inhibitory activities of compounds were measured by using a fluorogenic substrate peptide (PEPTIDE INSTITUTE, INC., code: 3249-v). Briefly, test compound and SARS-CoV-2 M^{pro} (Sigma-Aldrich, catalog No. SAE0172-200UG) were mixed in a 96-well plate (0.70 μg, 50 μL/well, final 0.20 μM), and the fluorogenic substrate was added (50 μL/well, final 20 μM). The plate was incubated at 30 °C for 15 min, and the activity of SARS-CoV-2 M^{pro} was determined based on the increase of fluorescence (Ex 360 nm/Em 460 nm) measured with a 96-well plate reader (Spectra Max M2e, Molecular Devices). The dose-response curves and IC_{50} values of compounds were calculated by Origin 2023 software, and data are presented as mean ± S.D. (n = 3). Bza-VAD(OMe)-FMK showed a comparable protease-inhibitory activity to Z-VAD(OMe)-FMK.

Preparation of enzymatic digest of labelled SARS-CoV-2 M^{pro}

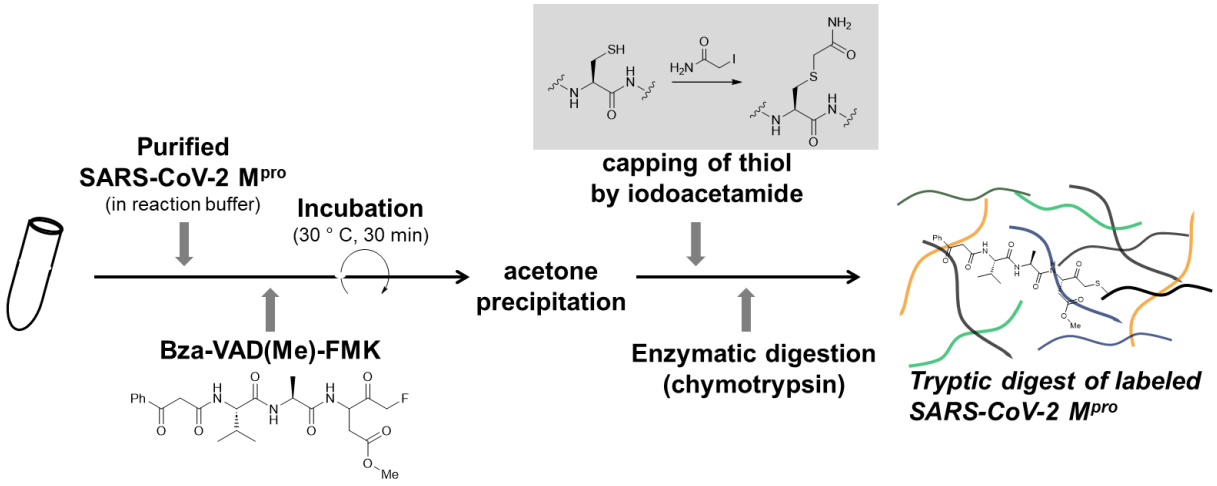


Fig. S22

Recombinant SARS-CoV-2 M^{pro} (11.2 µg, Sigma-Aldrich, catalog No. SAE0172-200UG) was dissolved in 1.6 mL of reaction buffer (20 mM Tris·HCl (pH 7.2), 100 mM NaCl, 1 mM EDTA and 1 mM DTT). Then 16 µL of 3 mM Bza-VAD(Me)-FMK in DMSO was added (final concentration 30 µM), and the mixture was incubated for 30 min at rt. After incubation, SARS-CoV-2 M^{pro} was precipitated by adding cold acetone. The precipitate was washed with acetone and dissolved in denaturing buffer (7 M urea, 1 M Tris·HCl (pH 8.5) and 5 mM TCEP). The solution was incubated for 30 min at rt. After addition of 3 µL of 200 mM iodoacetamide (IAM), incubation was continued for 30 min at rt. Then, the solution was diluted with 480 µL of water containing *n*-decyl-β-D-glucopyranoside (DG, final concentration: 0.05 w/v %). After addition of chymotrypsin (300 ng), the sample solution was incubated for 4 h at rt and used as the enzymatic digest of SARS-CoV-2 M^{pro} (320 pmol/540 µL, approximately).

```
  1 SGFRKMAFPSGKVEGCMVQVTCGTTTLNGLWLDDVVYCPRHVICTSEDML
 51 NPNYEDLLIRKSNHNFLVQAGNVQLRVIGHSMQNCVLKLKVDTANPKTPK
101 YKFVRIQPGQTFSVLACYNGSPSGVYQCAMRPNFTIKGSFLNGSCGSVGF
151 NIDYDCVSFCYMHHMELPTGVHAGTDLEGNFYGPFVDRQTAQAAGTDTTI
201 TVNVLAWLYAAVINGDRWFLNRFTTTLNDFNLVAMKYNYEPLTQDHVDIL
251 GPLSAQTGIAVLDMCASLKELLQNGMNGRTILGSALLEDEFTPFDVVRQC
301 SGVTFQ
```

Fig. S23 Amino acid sequence of SARS-CoV-2 M^{pro} (NCBI Accession Number: YP_009725301.1)
Catalytic cysteine residue (Cys145) is shown in red.

Purification of the peptide labelled by Bza-VAD(Me)-FMK

To a solution of enzymatic digest of Bza-VAD(Me)-FMK-labelled SARS-CoV-2 M^{pro} (160 pmol) in 0.3% TFA in 5% CH_3CN/H_2O (1.0 mL) was added TentaGel-supported Pd aqua complex (200 nmol, 1250 eq.). The mixture was rotated for 1 h at rt, then filtered. The beads were washed with 2.0 mL of 0.3% TFA in 5% CH_3CN/H_2O (5 times), and then the beads were exposed to 0.3% TFA in 50% CH_3CN/H_2O (500 µL) for 30 min at rt. After filtration, the beads were washed with 0.3% TFA in 50% CH_3CN/H_2O (500 µL). The obtained solutions, initial solution, supernatant, washing solution, and eluted solution, were washed with $CHCl_3$/MeOH. The organic solvents contained in the water layer were removed under reduced pressure, and the residue was concentrated by C18 spin column (GL Science, MonoSpin® C18) and analyzed by MALDI-TOF-MS.

Although non-specific peptides were also detected, three Bza-labelled peptides were successfully identified by the comparison with a control sample (Fig S24). One was a reported peptide,[15] and the others were unreported peptides derived from the difference of chymotrypsin cleavage pattern and the hydrolysis of methyl ester. This result indicates the advantage of our method for the identification of unexpected modification of labelled peptides.

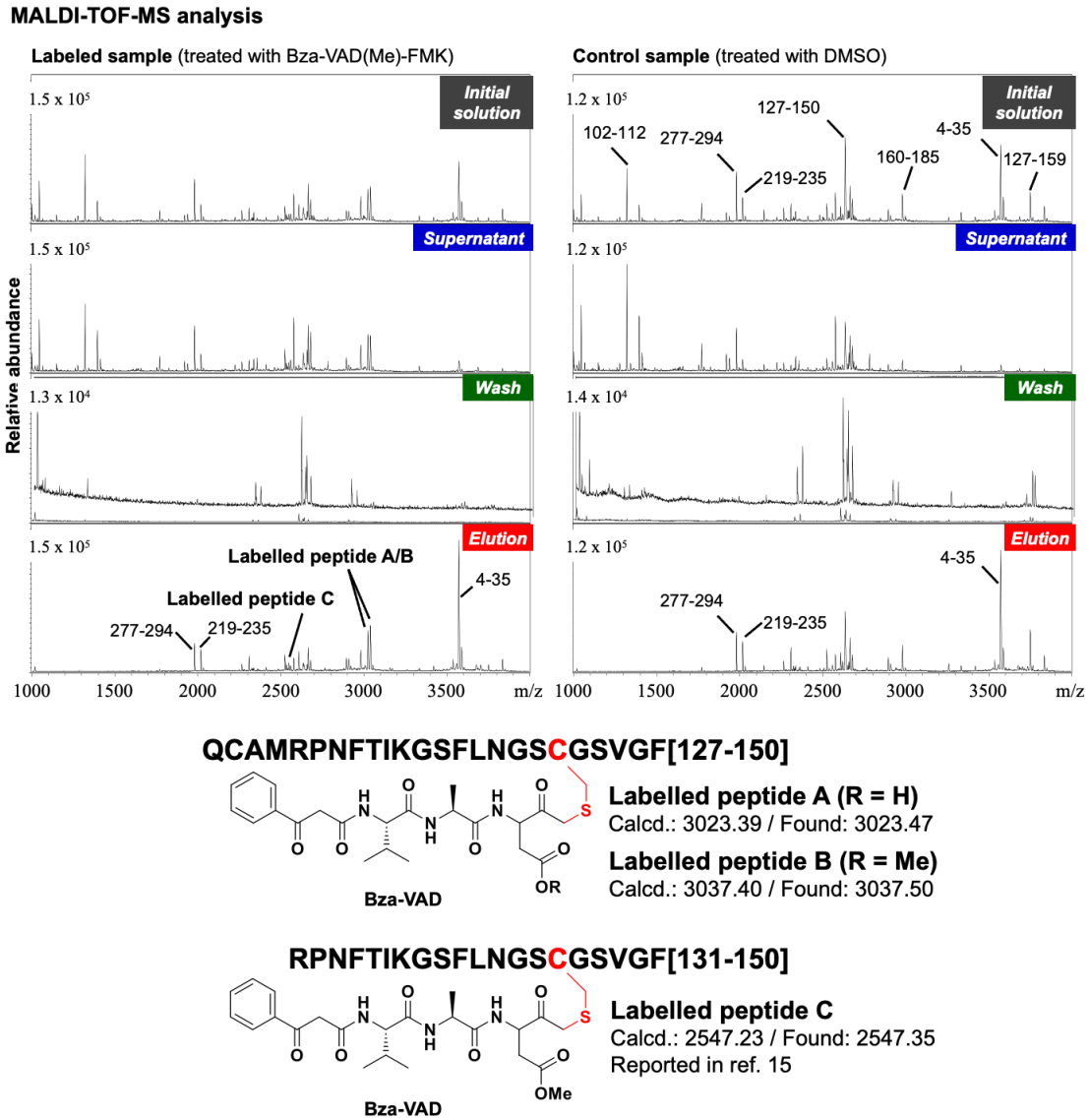
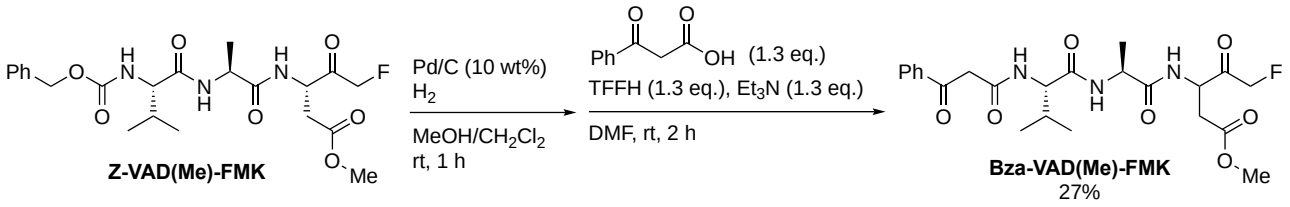


Fig. S24

Synthesis of Bza-VAD(Me)-FMK

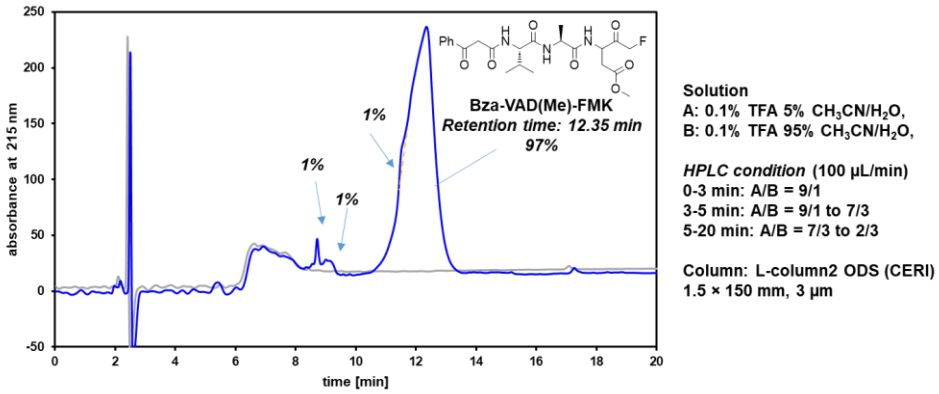


To a solution of Z-VAD(Me)-FMK (20 mg, 0.043 mmol, PEPTIDE INSTITUTE, INC., code: 3188-v) in MeOH (4.5 mL) and CH_2Cl_2 (0.5 mL) was added Pd/C (2.0 mg, 10 wt%). The suspension was stirred for 1 h under a hydrogen atmosphere, and then filtered through a Celite pad. The filtrate was concentrated under reduced pressure and the residue was dissolved in DMF (3.0 mL). To this solution was added benzoylacetic acid (9.1 mg, 0.056 mmol), Et_3N (7.7 µL, 0.056 mmol), and TFFH (14.7 mg, 0.056 mmol) at rt. The reaction mixture was stirred for 2 h, then the solvent was removed *in vacuo*. The crude mixture was diluted with CH_2Cl_2 and washed with water and brine. The organic solution was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography (SiO_2, eluent; AcOEt/Hex = 7/3 to 9/1) twice to give Bza-VAD(Me)-FMK (5.5 mg, 27%) as a white solid. We confirmed the purity of compound by HPLC analysis, and it showed a single peak. However, careful NMR analysis indicated that the compound was obtained as inseparable 1:1 diastereomeric mixture due to epimerization of aspartic acid. We applied this sample for the labelling experiments, because it showed a comparable protease-inhibitory activity to Z-VAD(OMe)-FMK (Fig. S21).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.97 (2H, d, *J* = 7.8 Hz), 7.65 (1H, t, *J* = 7.4 Hz), 7.52 (2H, dd, *J* = 7.8, 7.4 Hz), 7.44-7.38 (1H, br), 7.31 (1H, br), 7.16-7.10 (1H, br), 5.31-4.92 (total 2H, m), 4.87-4.79 (1H, m), 4.57-4.50 (1H, m), 4.29-4.24 (1H, m), 4.13 (1H, d, *J* = 17.5 Hz), 4.04 (1H, d, *J* = 17.5 Hz), 3.64 and 3.55 (total 3H, s, ca 1:1), 2.94-2.90 (1H, m), 2.87-2.83 (1H, m), 2.35-2.28 (1H, m), 1.46 and 1.45 (total 3H, d, *J* = 7.2 Hz, ca 1:1), 1.02 (3H, d, *J* = 6.9 Hz), 0.972 and 0.966 (total 3H, d, *J* = 6.9 Hz, ca 1:1) ^{13}C-NMR (100 MHz, $CDCl_3$):δ 202.5 (d, *J* = 16.4 Hz), 196.1, 172.6, 171.1, 170.8, 167.8, 135.6, 134.5, 129.0, 128.5, 84.1 (d, *J* = 183.0 Hz), 59.9, 52.2, 52.0, 49.2, 45.9, 34.7, 29.7, 19.5, 17.8, 17.5; HRMS (ESI) calcd for $C_{23}H_{30}FN_3O_7$ $[M+Na]^+$ 502.1960; found 502.1981.

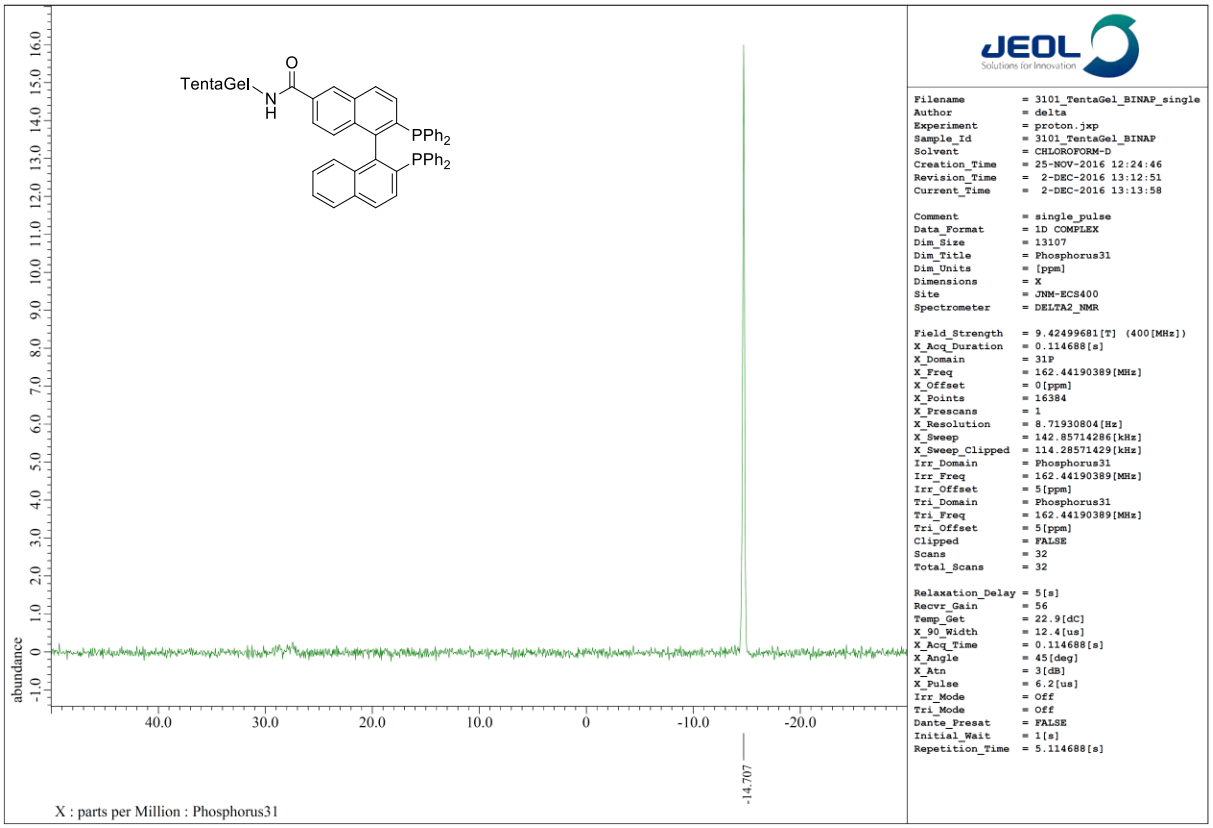
HPLC chromatogram of Bza-VAD(Me)-FMK

Column: SERI L-column ODS (1.5 x 0150 mm I.D., 3 µm)
Column temp.: 35 °C
Mobile phase: 9.5-57% MeCN (containing 0.1% TFA), 20 min
Flow rate: 100 µL/min
Detection: UV at 215 nm

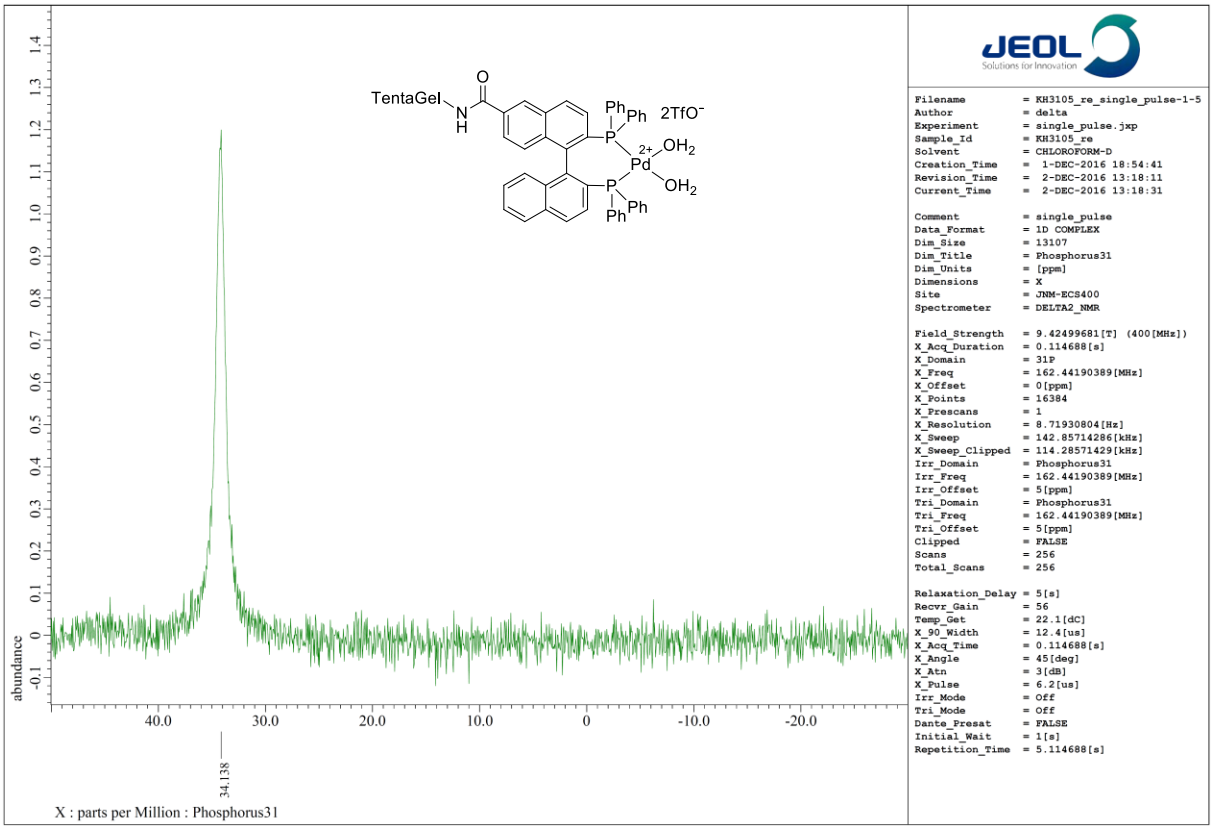


NMR Spectra

TentaGel-supported BINAP (^{31}P-NMR)

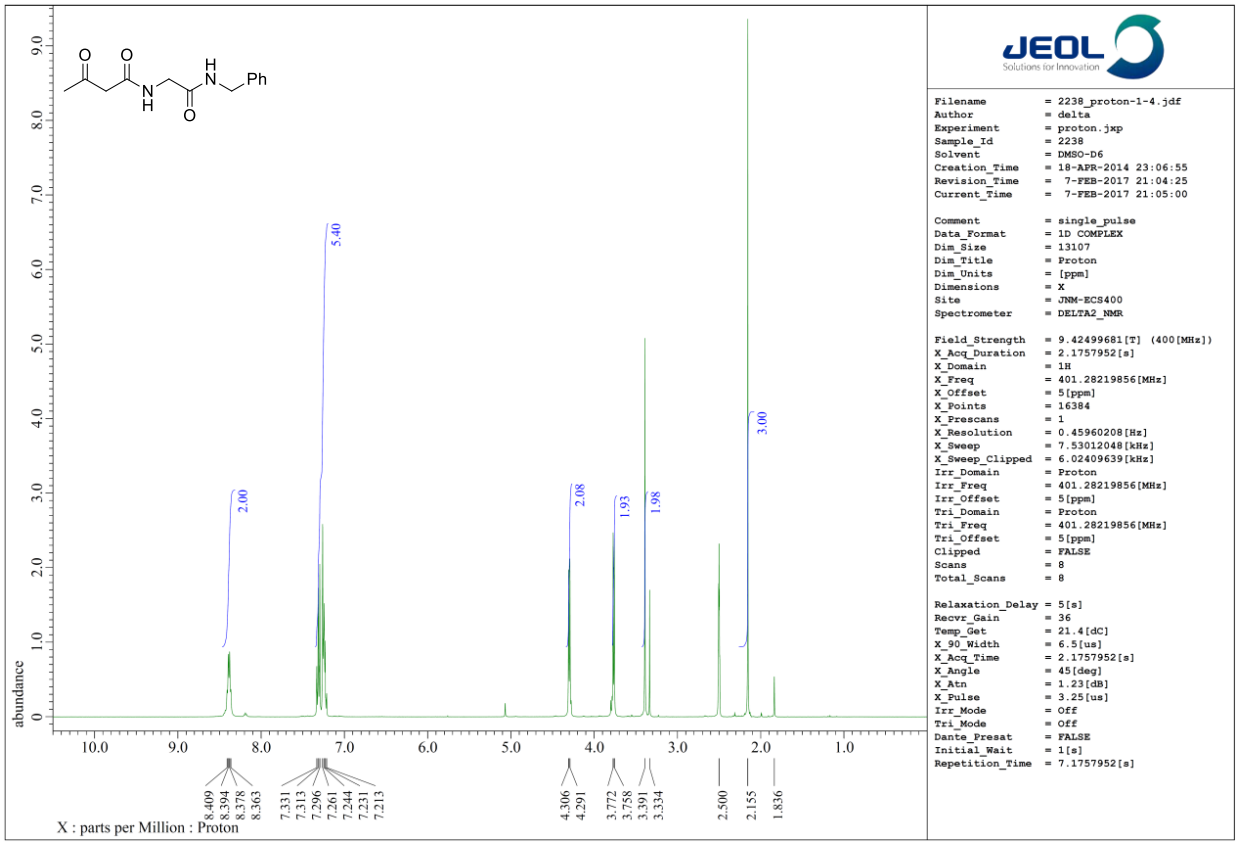


TentaGel-supported Pd aqua complex (^{31}P-NMR)

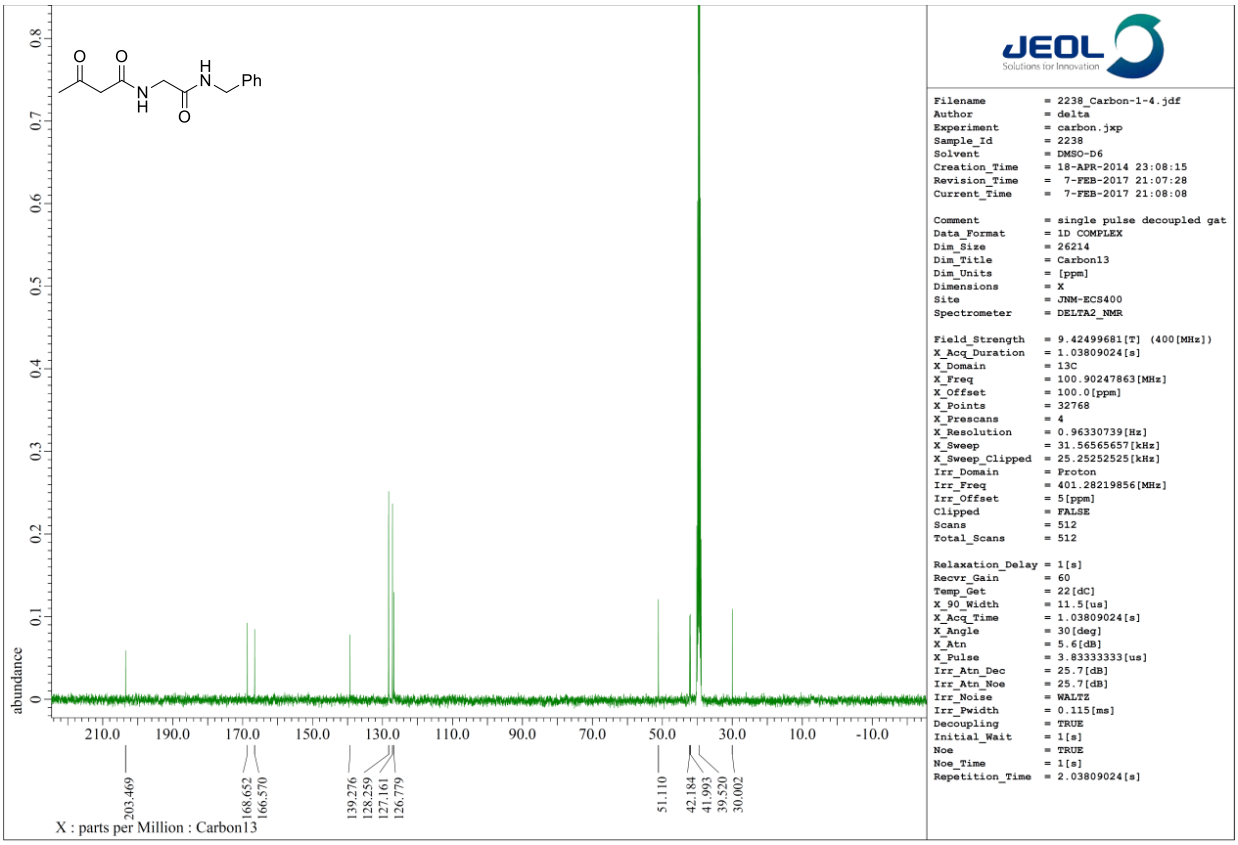


Bka-1

^{1}H-NMR

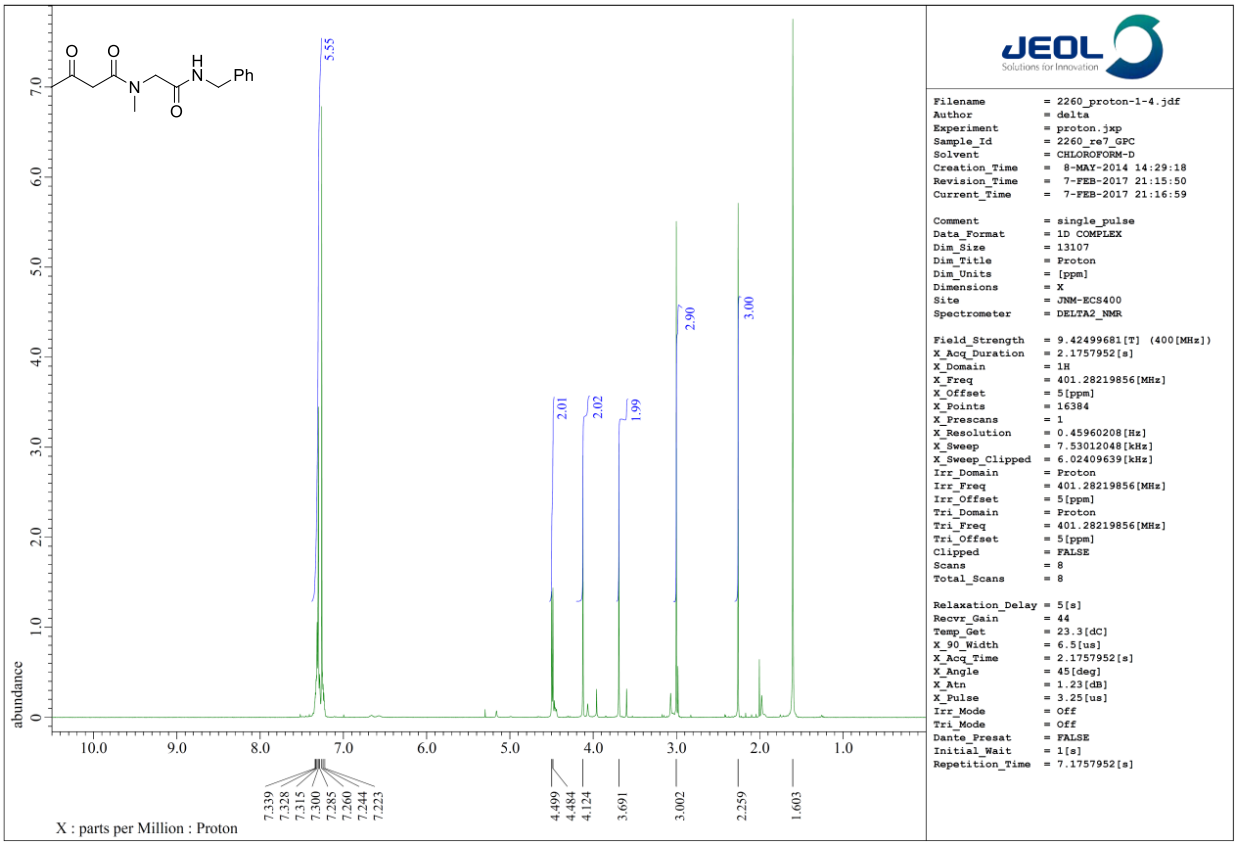


^{13}C-NMR

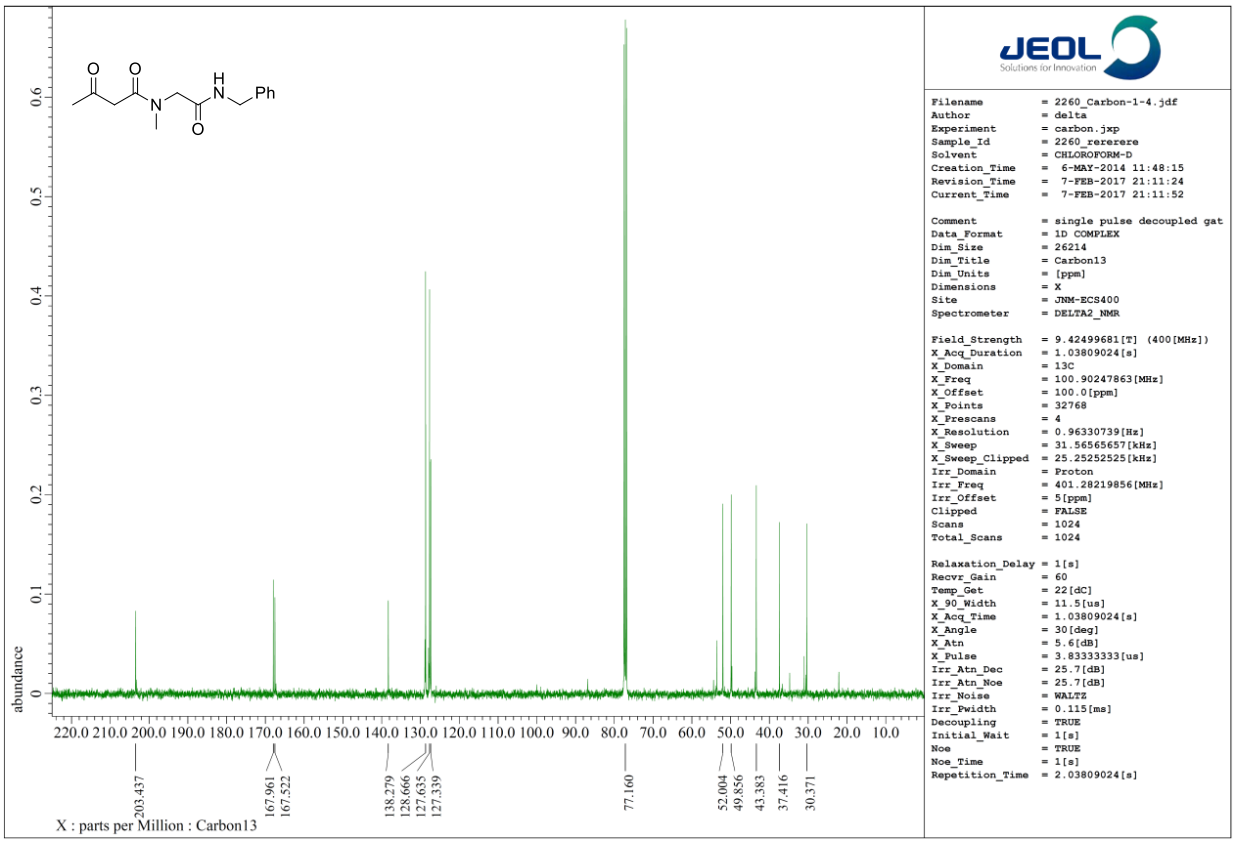


Bka-2

^{1}H-NMR

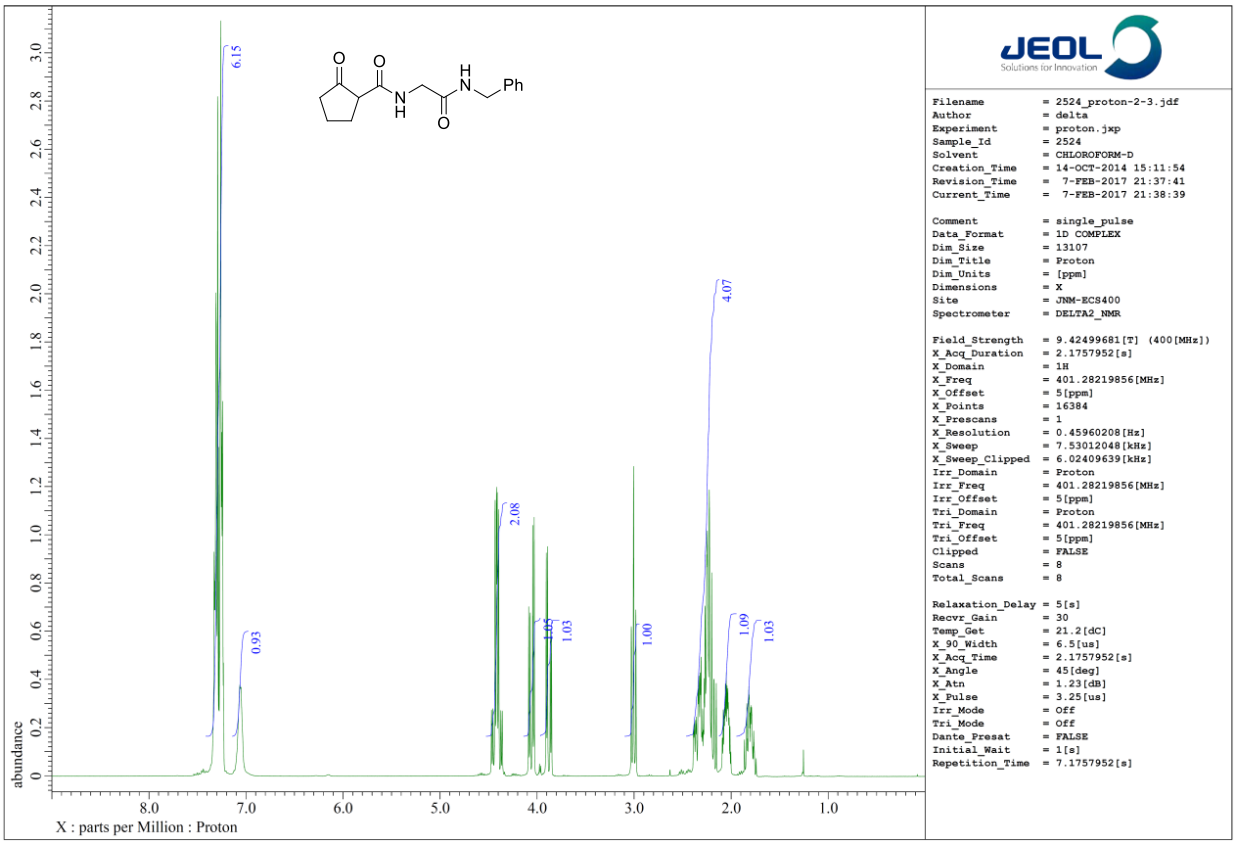


^{13}C-NMR

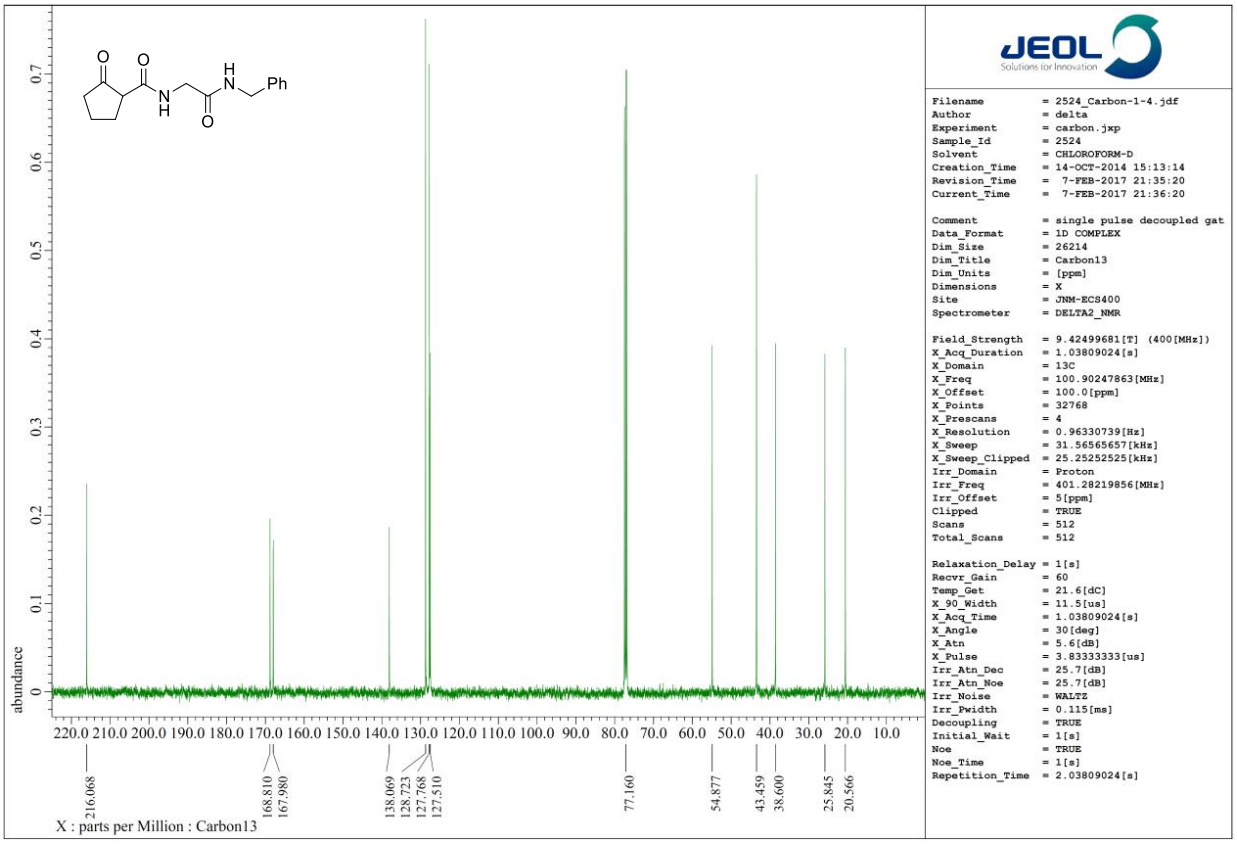


Bka-3

^{1}H-NMR

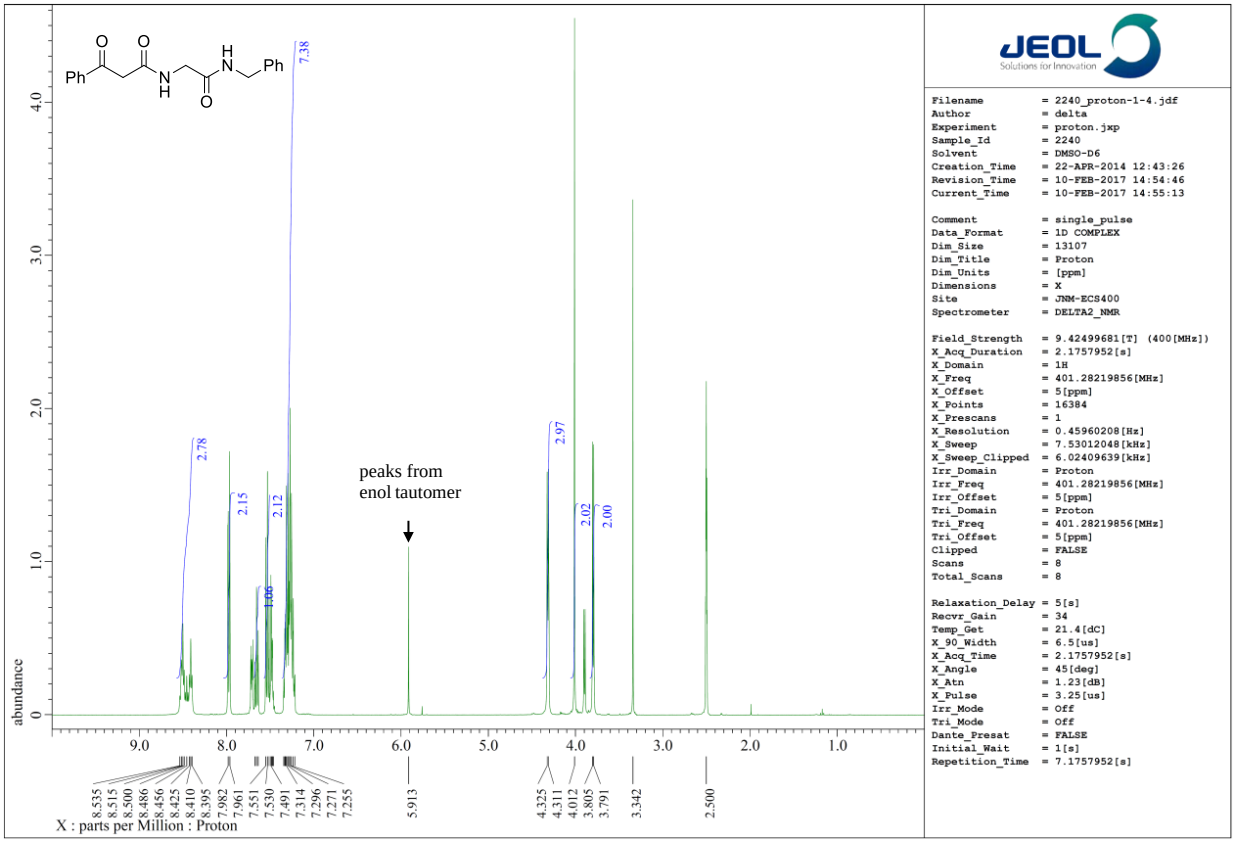


^{13}C-NMR

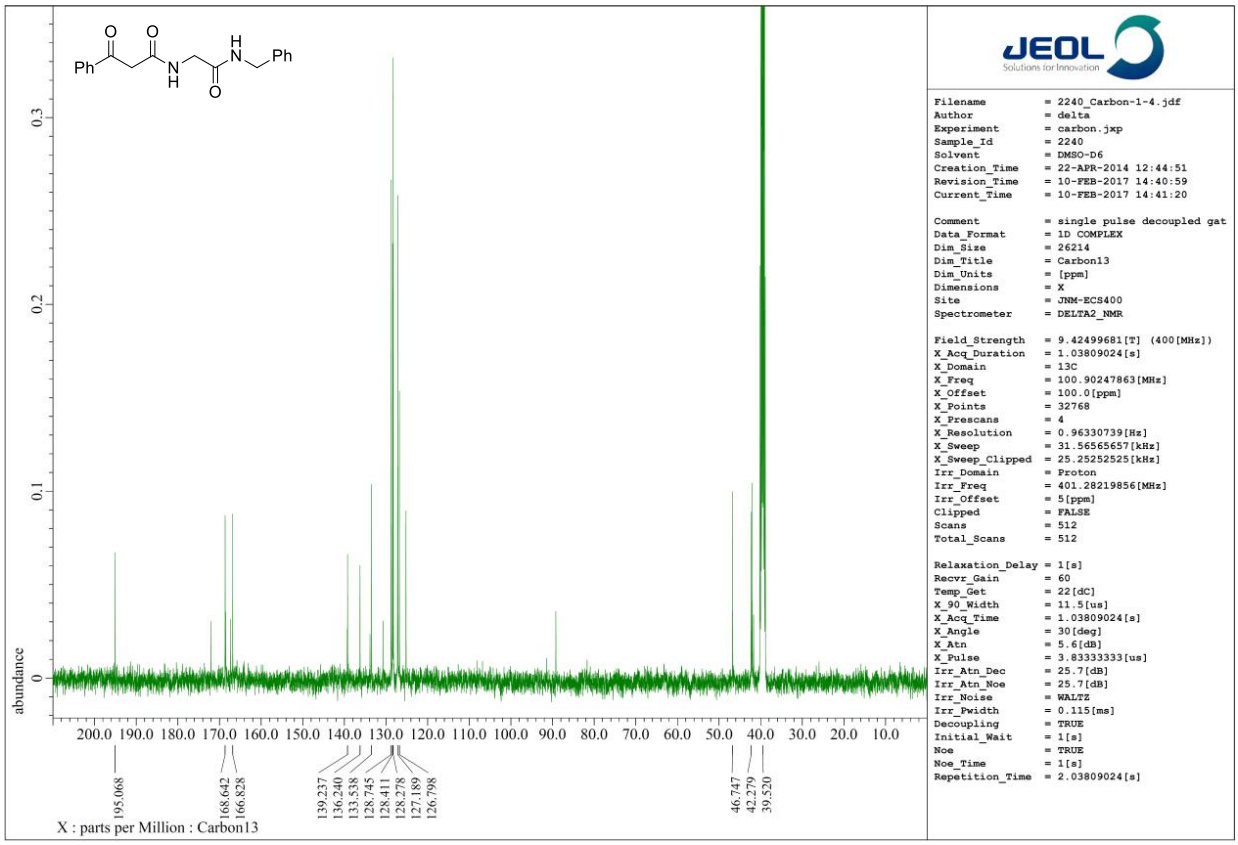


Bka-4

^{1}H-NMR

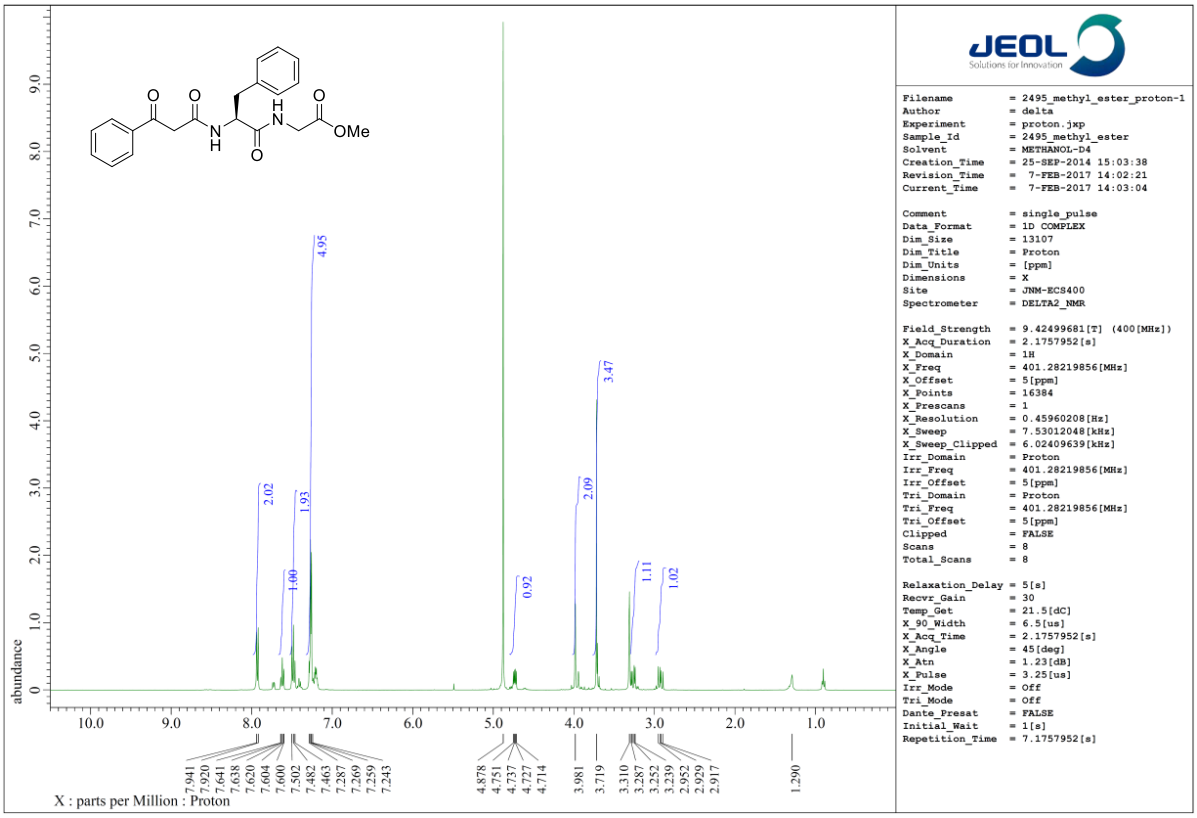


^{13}C-NMR

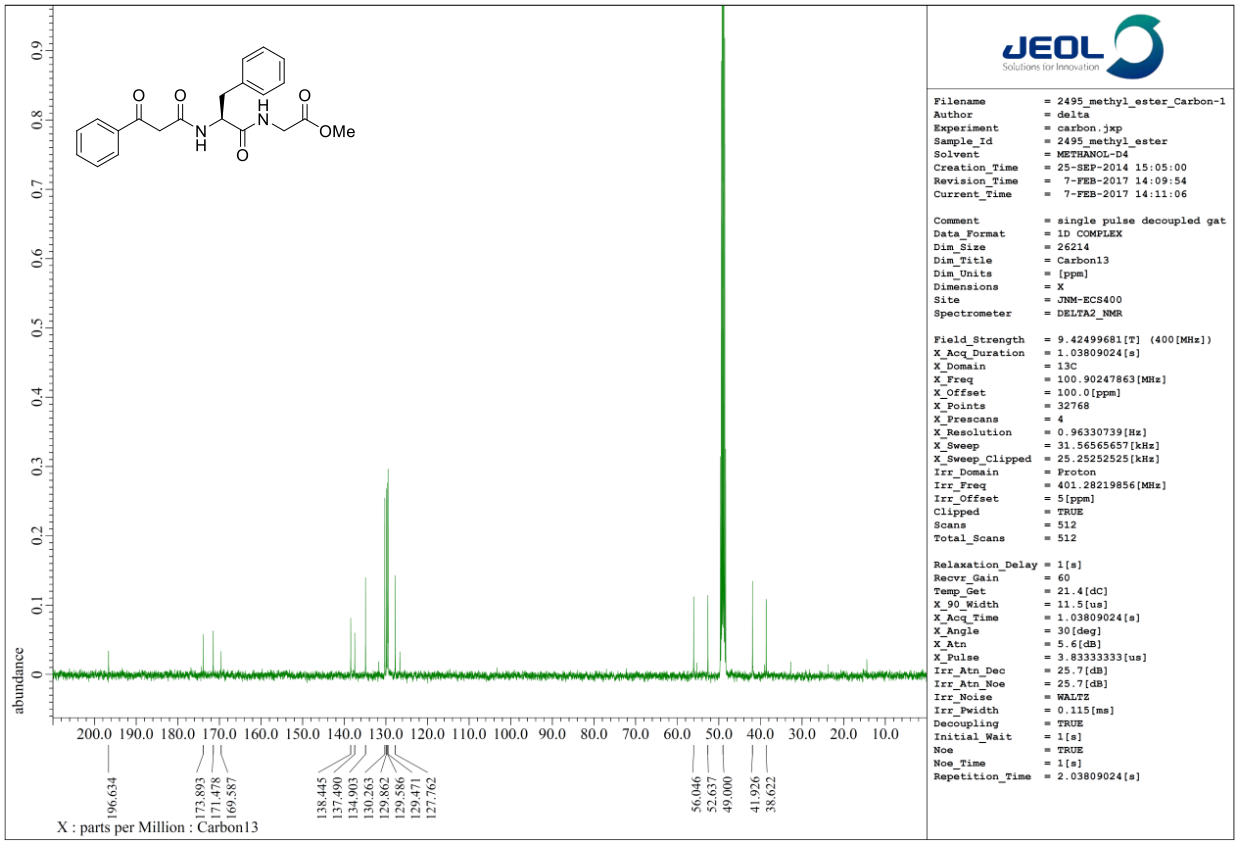


Bza-FG-OMe (15)

^{1}H-NMR

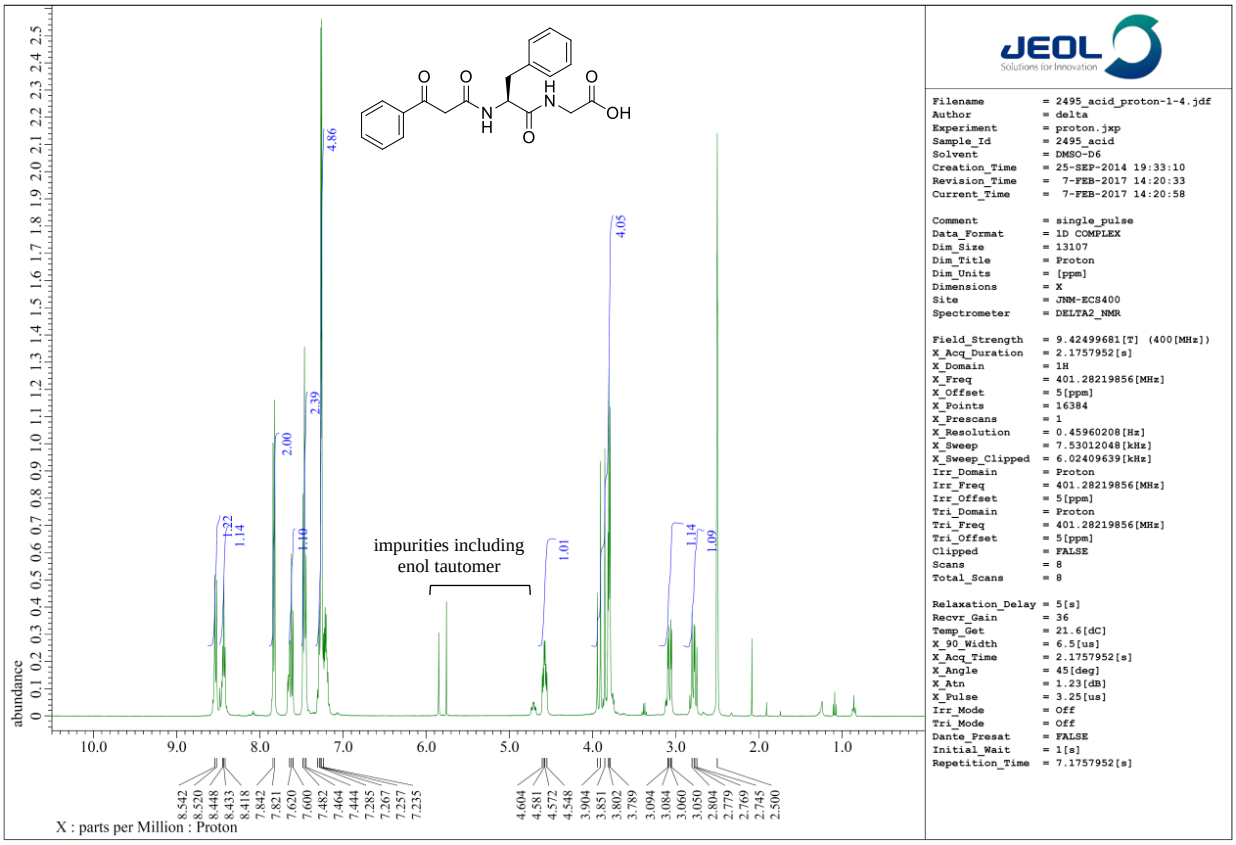


^{13}C-NMR

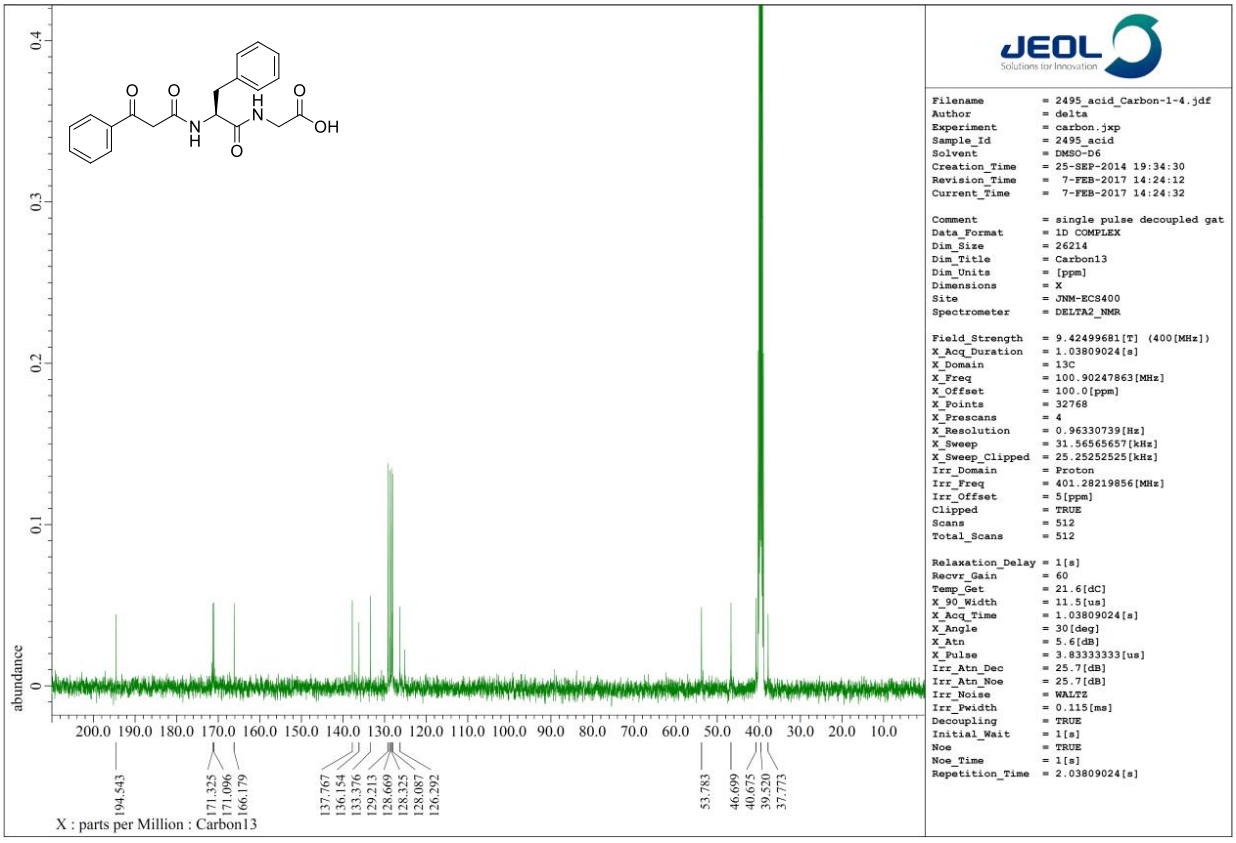


Bza-FG-OH (16)

^{1}H-NMR

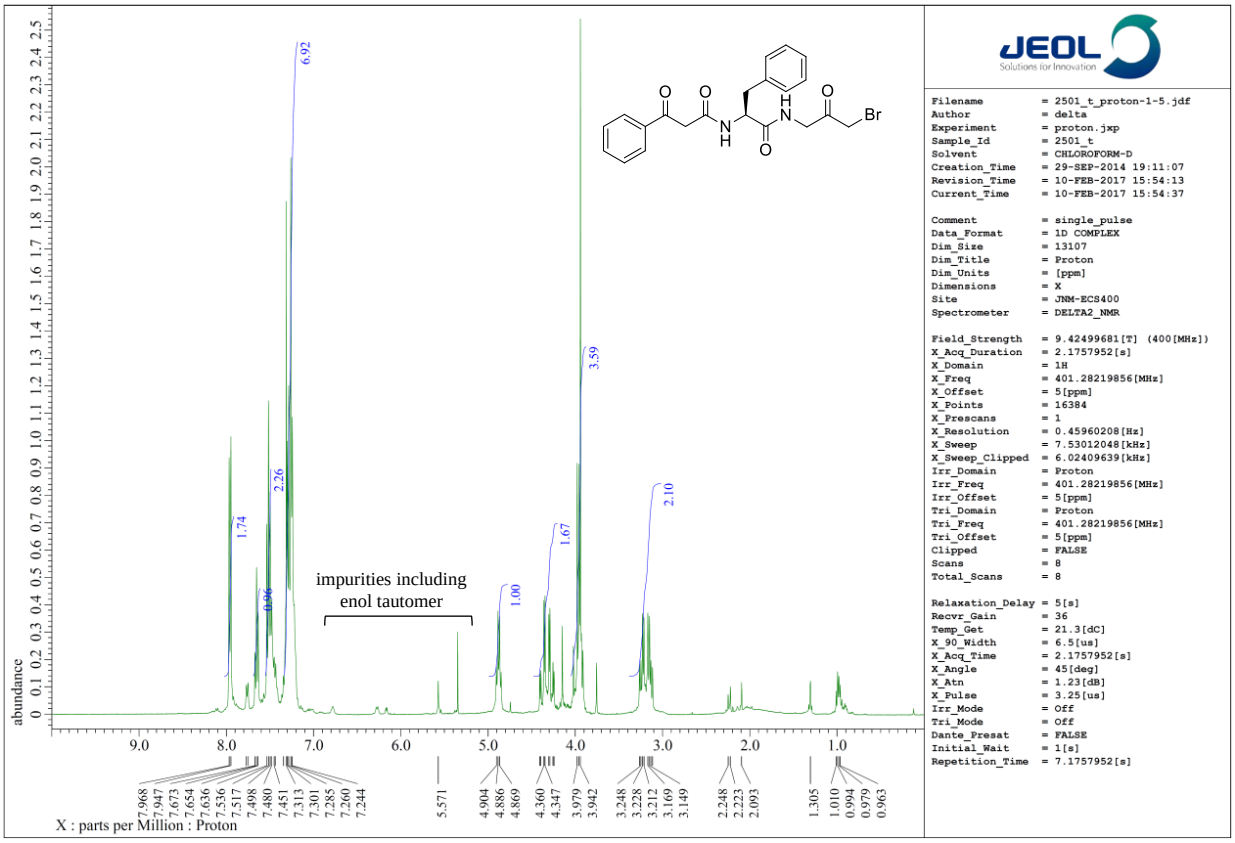


^{13}C-NMR

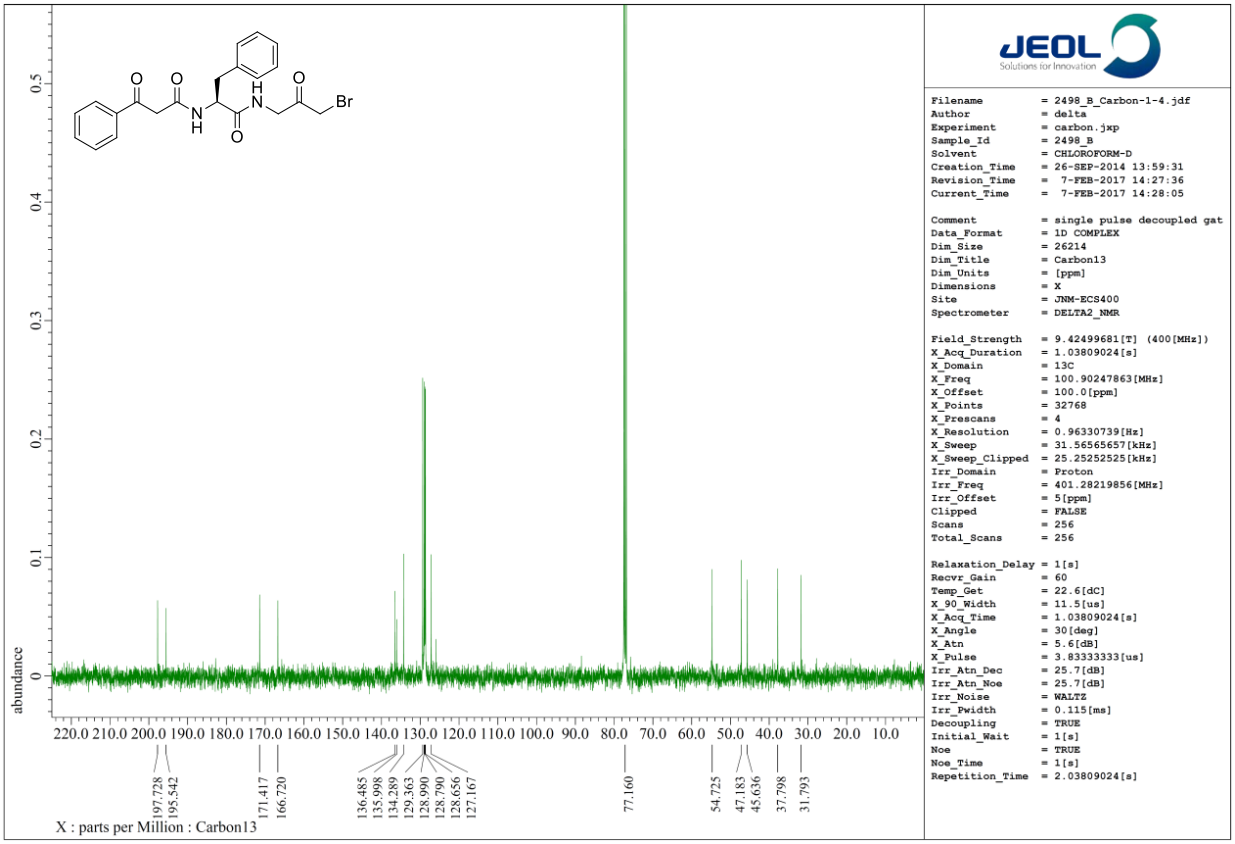


Bza-FG-Br (17)

^{1}H-NMR

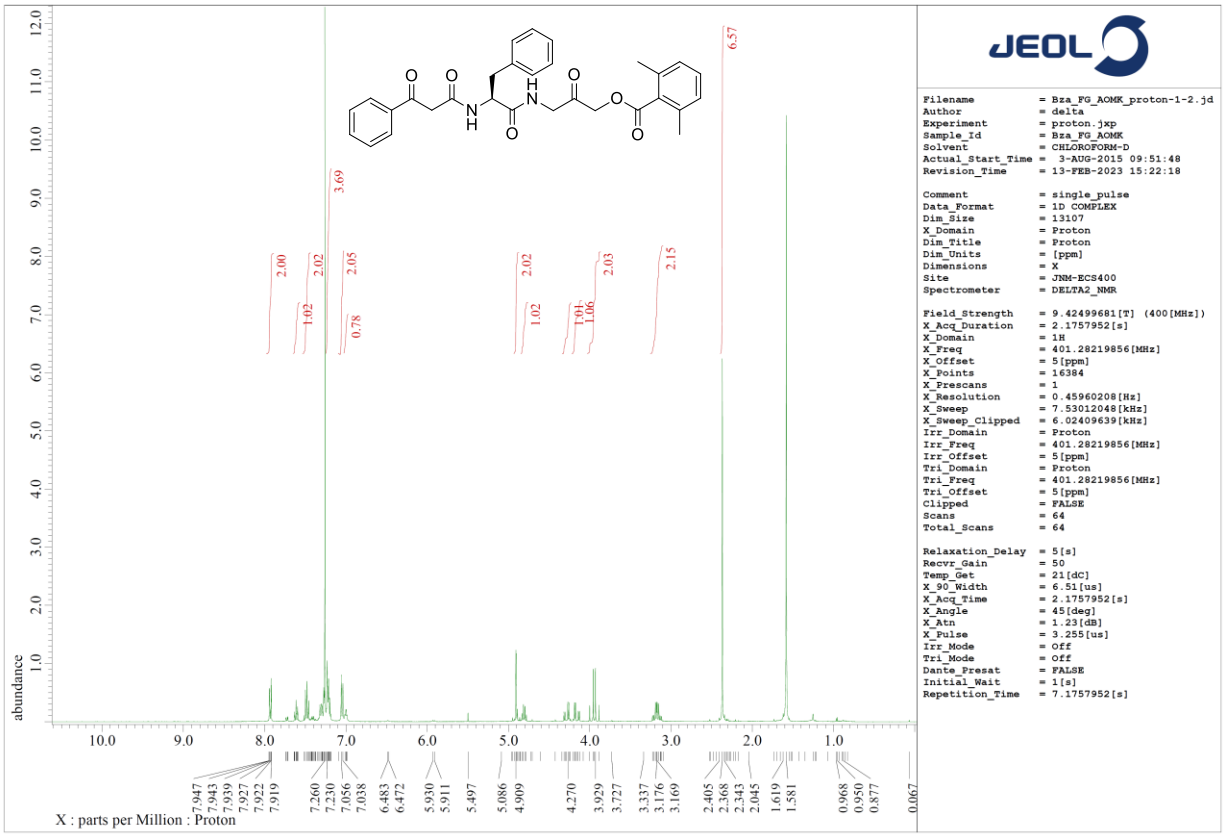


^{13}C-NMR

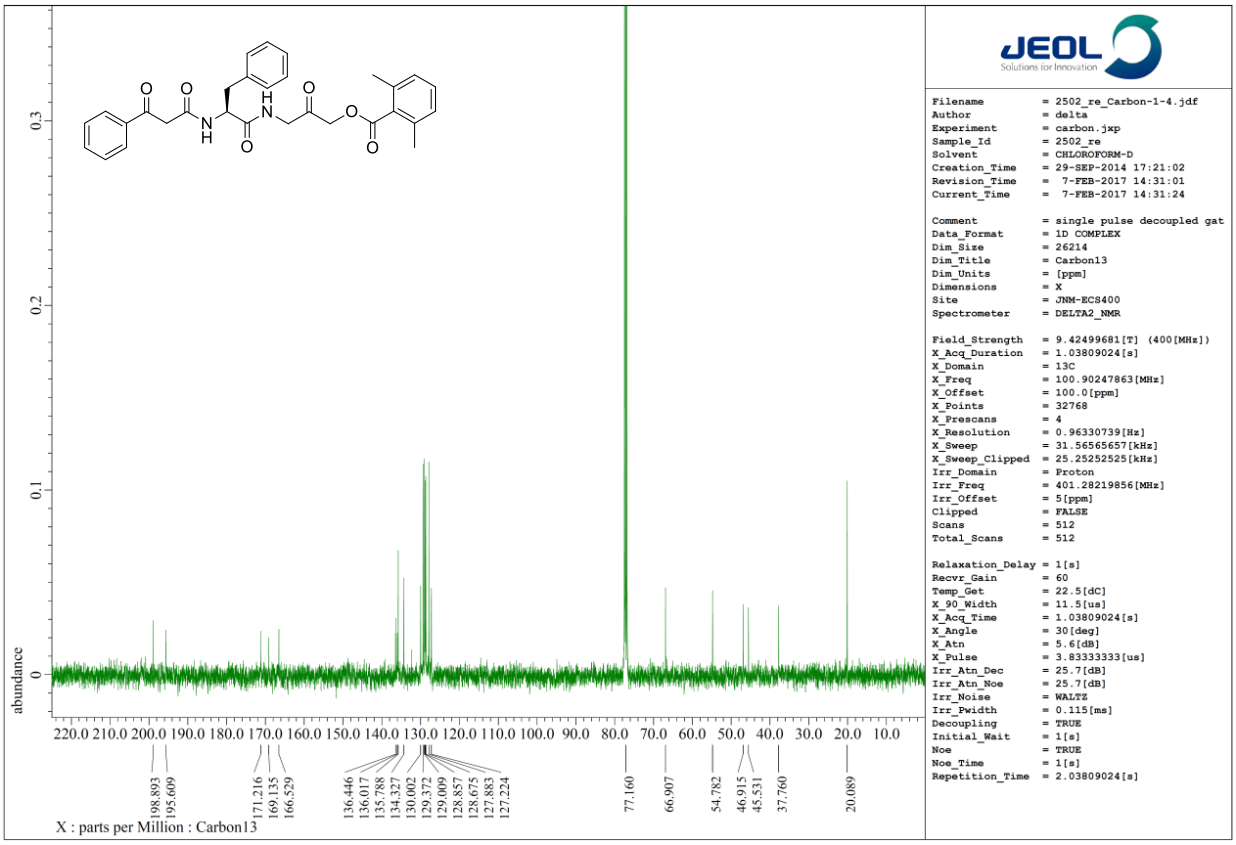


Bza-FG-AOMK

^{1}H-NMR

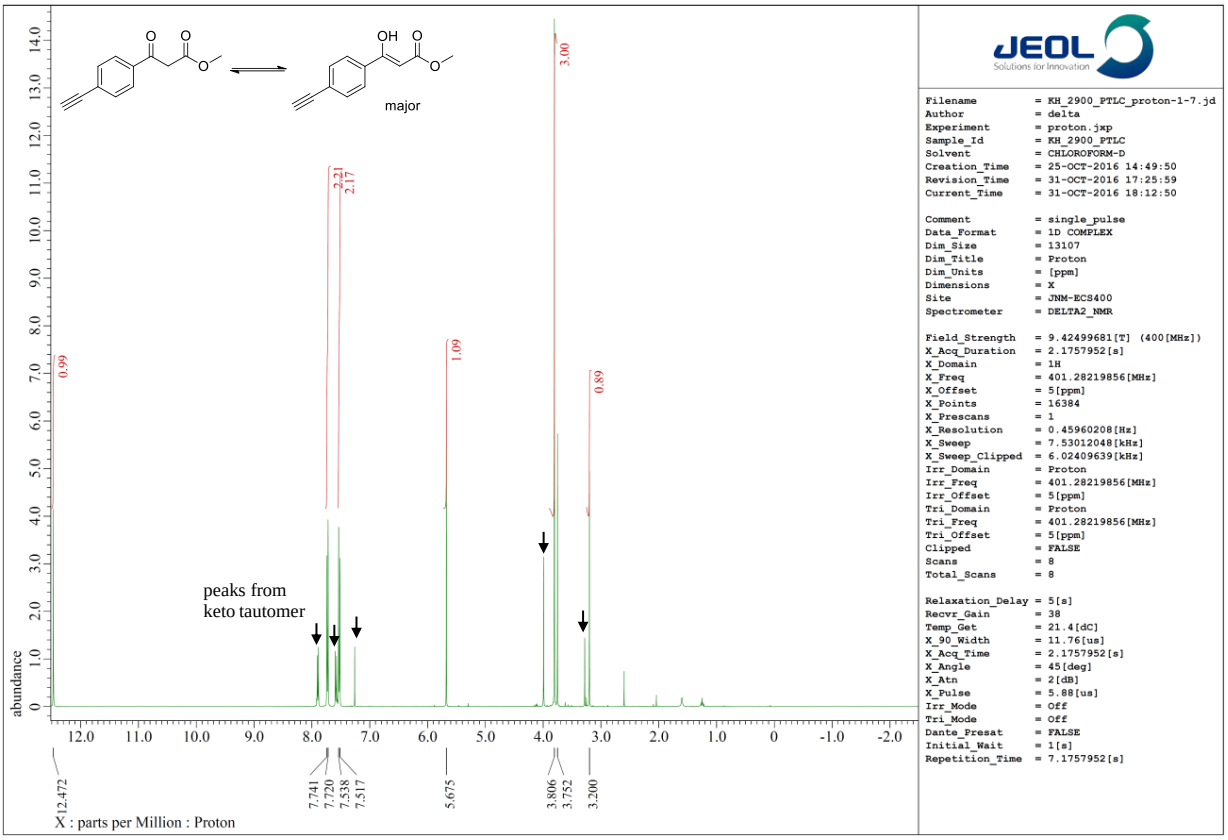


^{13}C-NMR

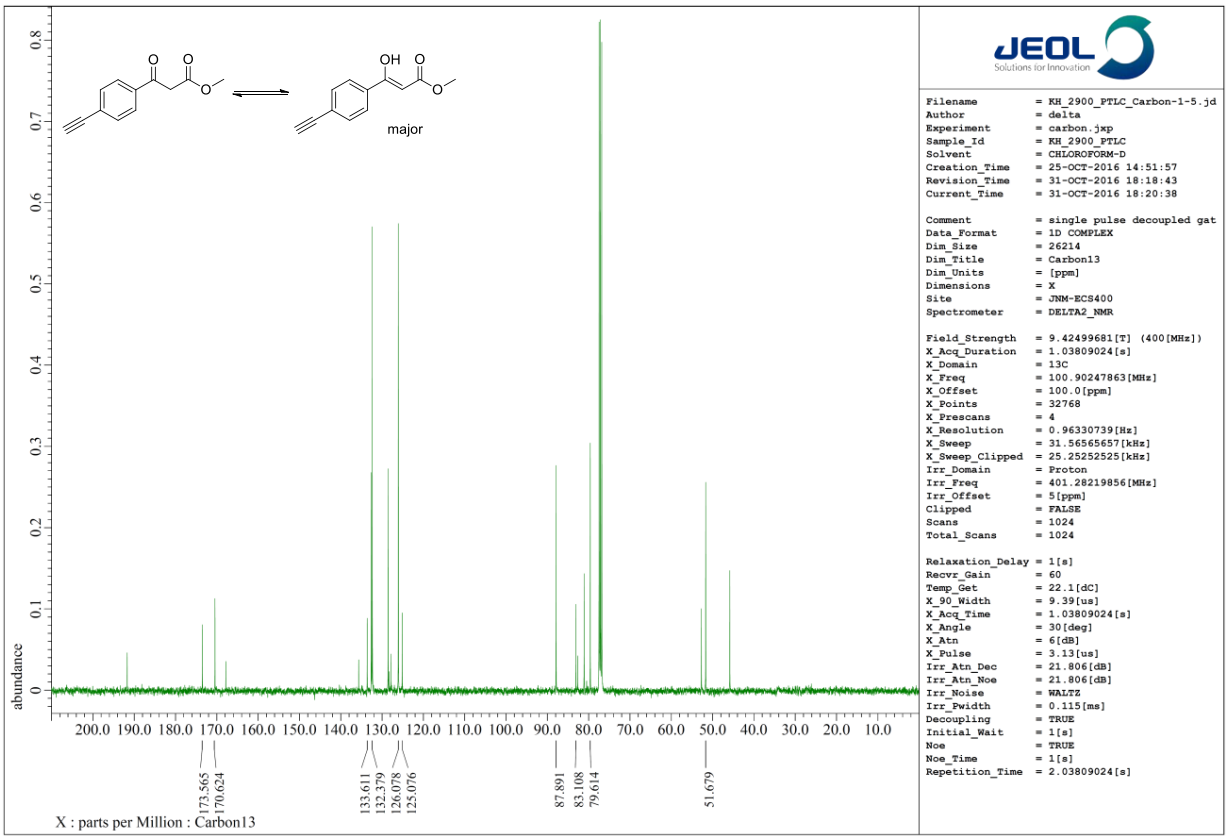


methyl 3-(4-ethynylphenyl)-3-oxopropanoate (19)

^{1}H-NMR

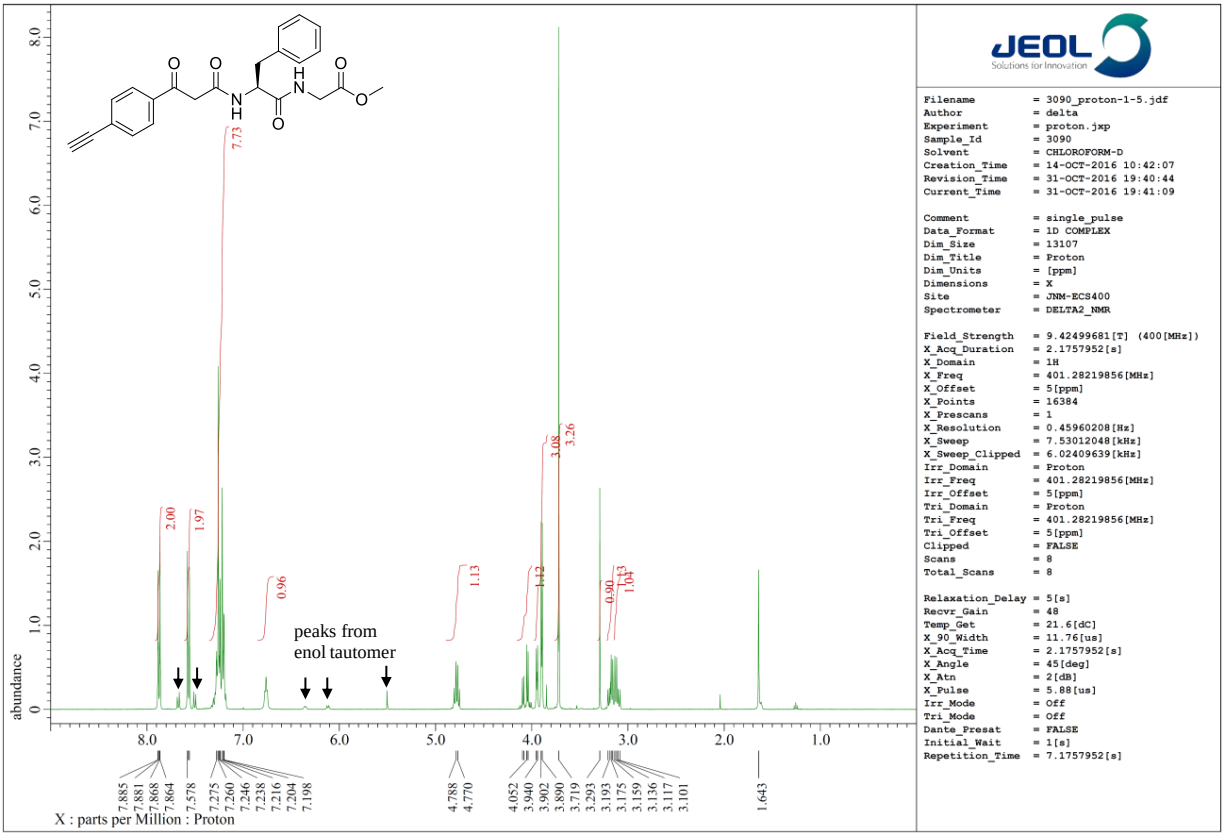


^{13}C-NMR

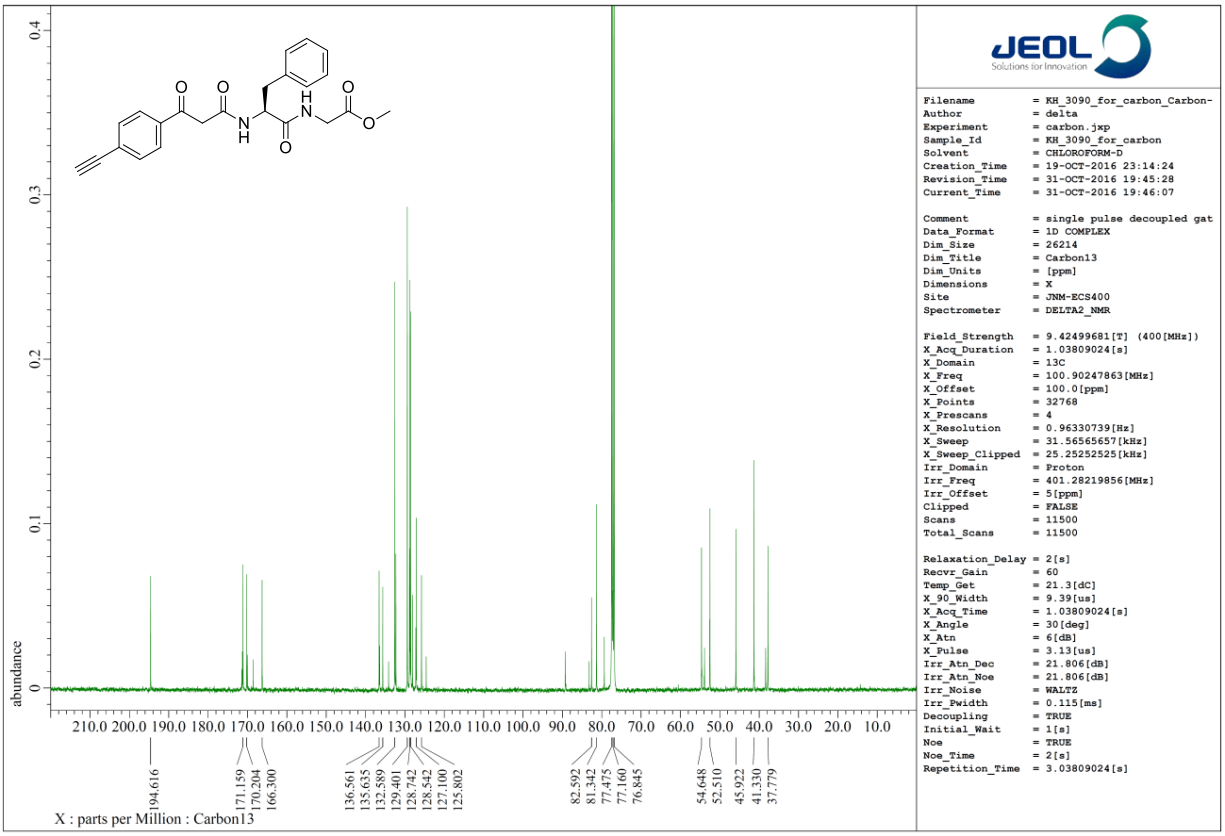


AltBza-FG-OMe (20)

^{1}H-NMR

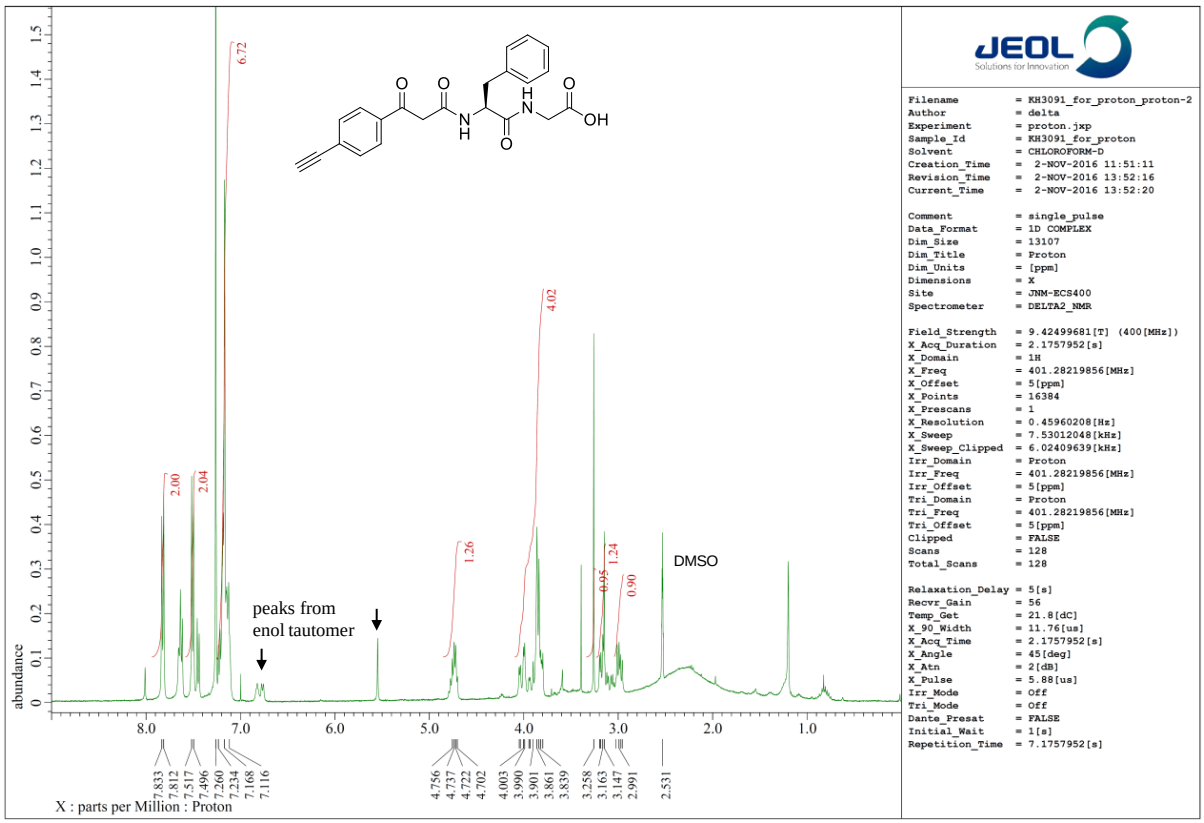


^{13}C-NMR

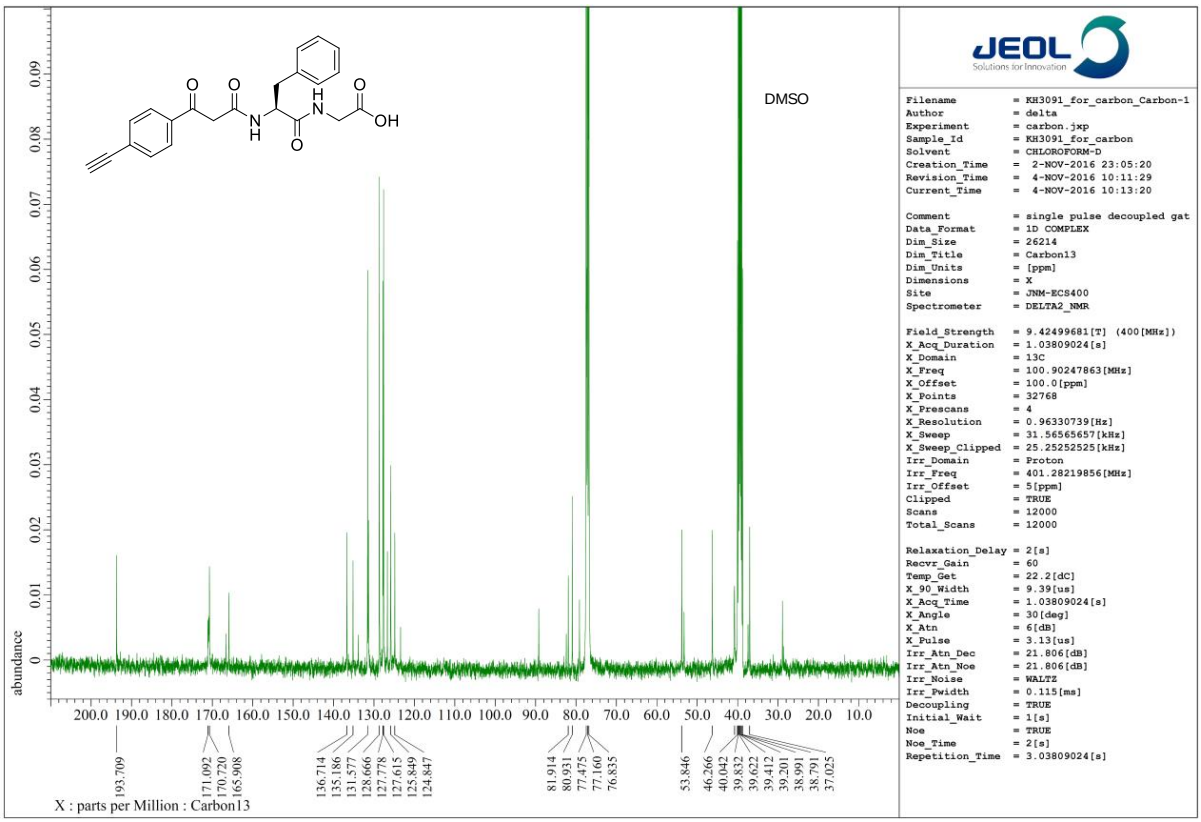


AltBza-FG-OH (21)

^{1}H-NMR

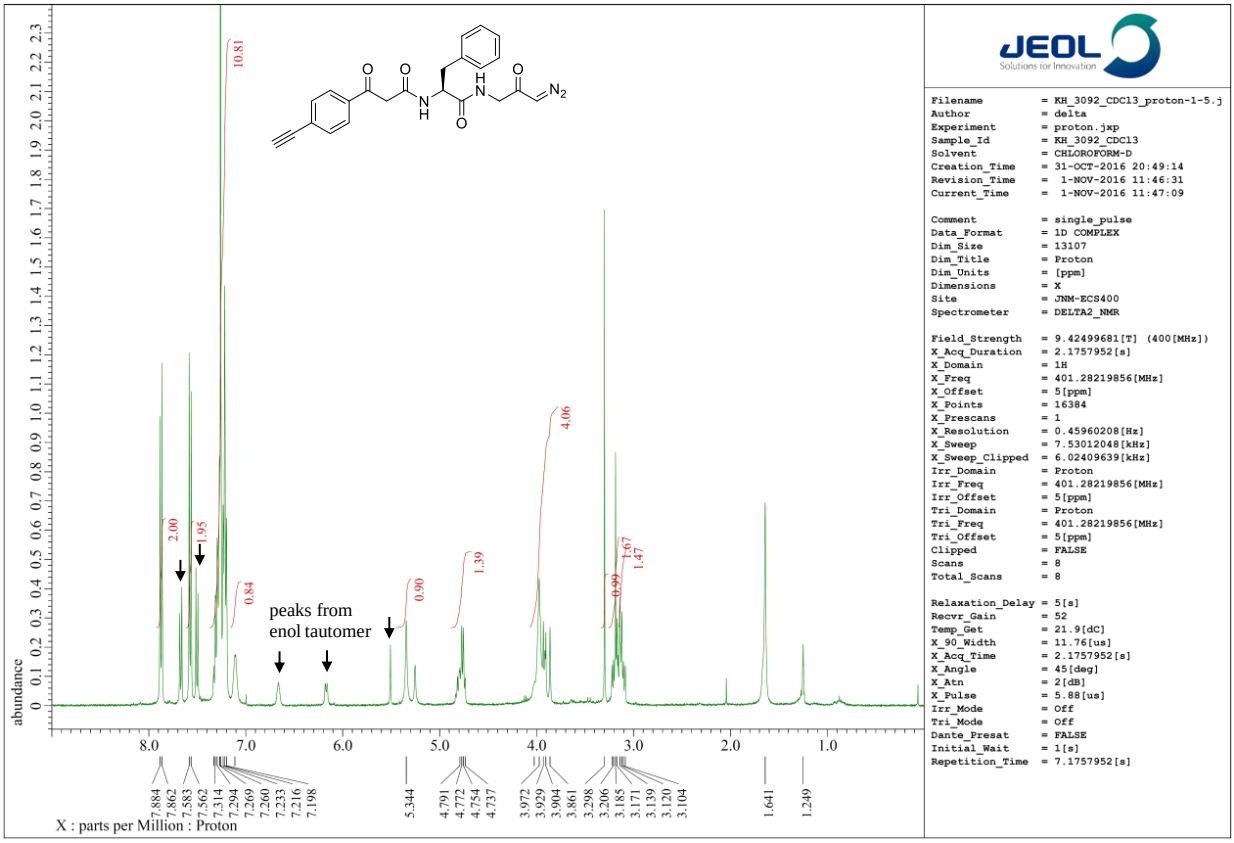


^{13}C-NMR

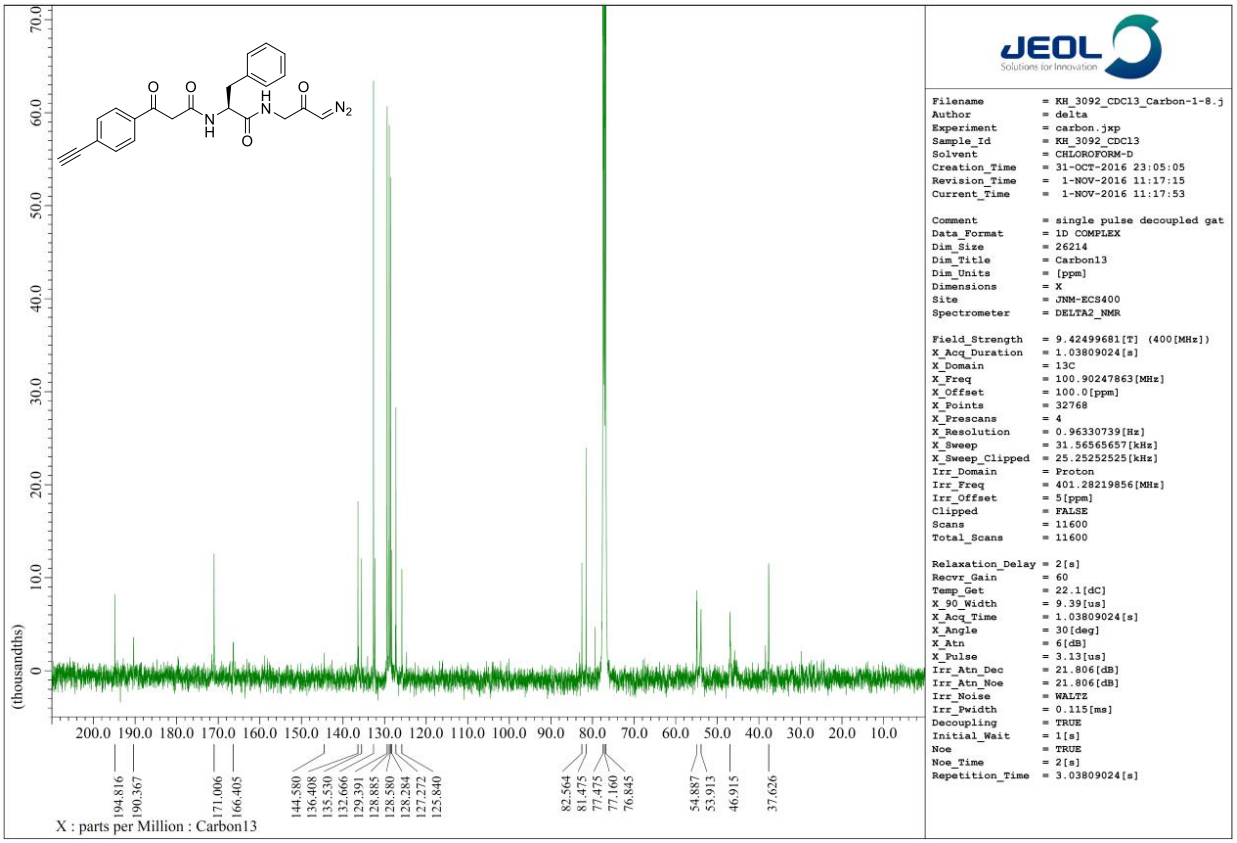


AltBza-FG-N_2 (22)

^{1}H-NMR

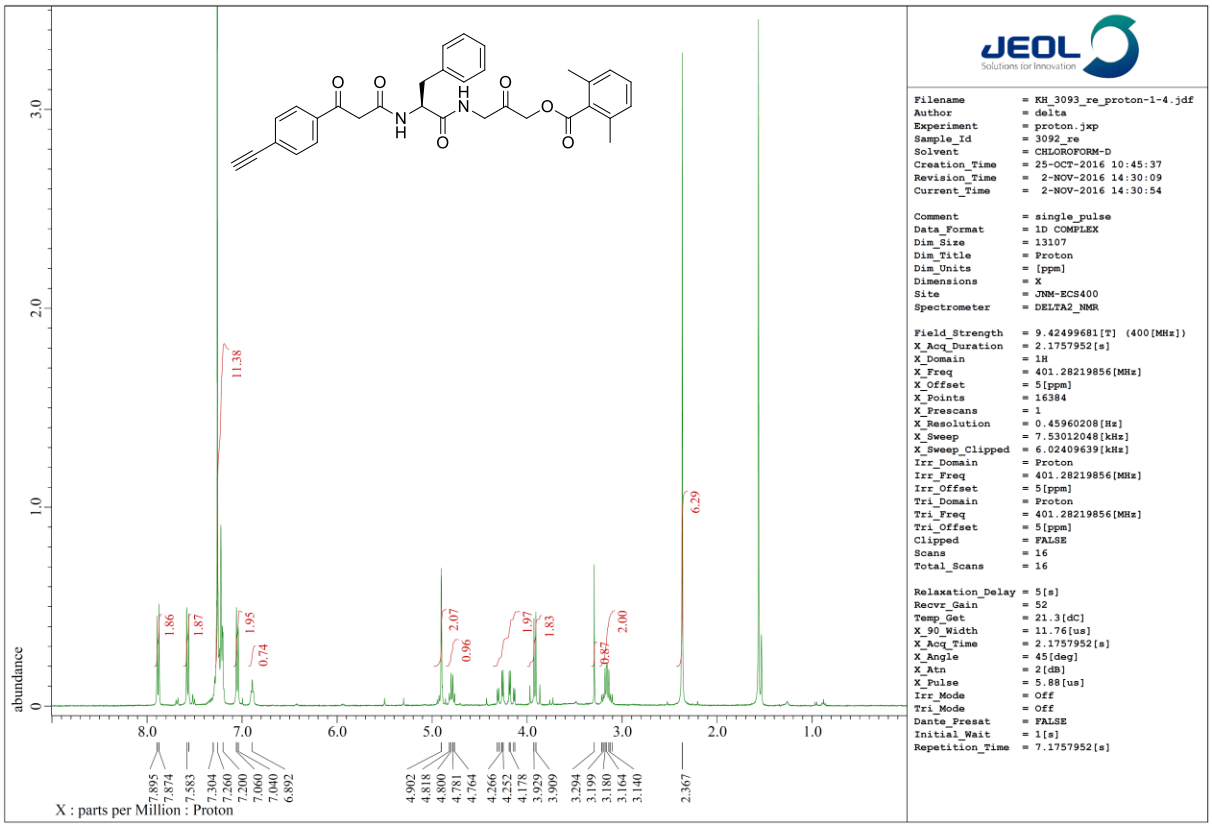


^{13}C-NMR

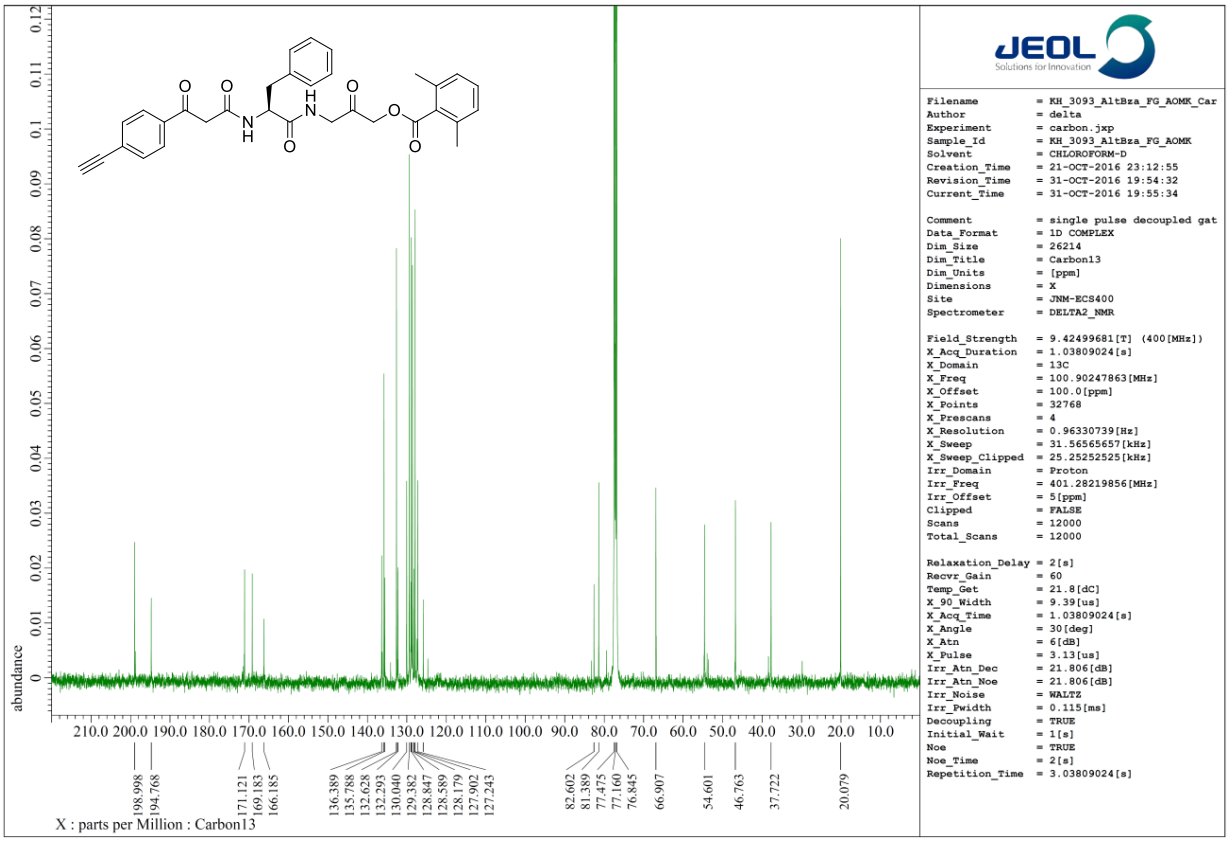


AltBza-FG-AOMK

^{1}H-NMR

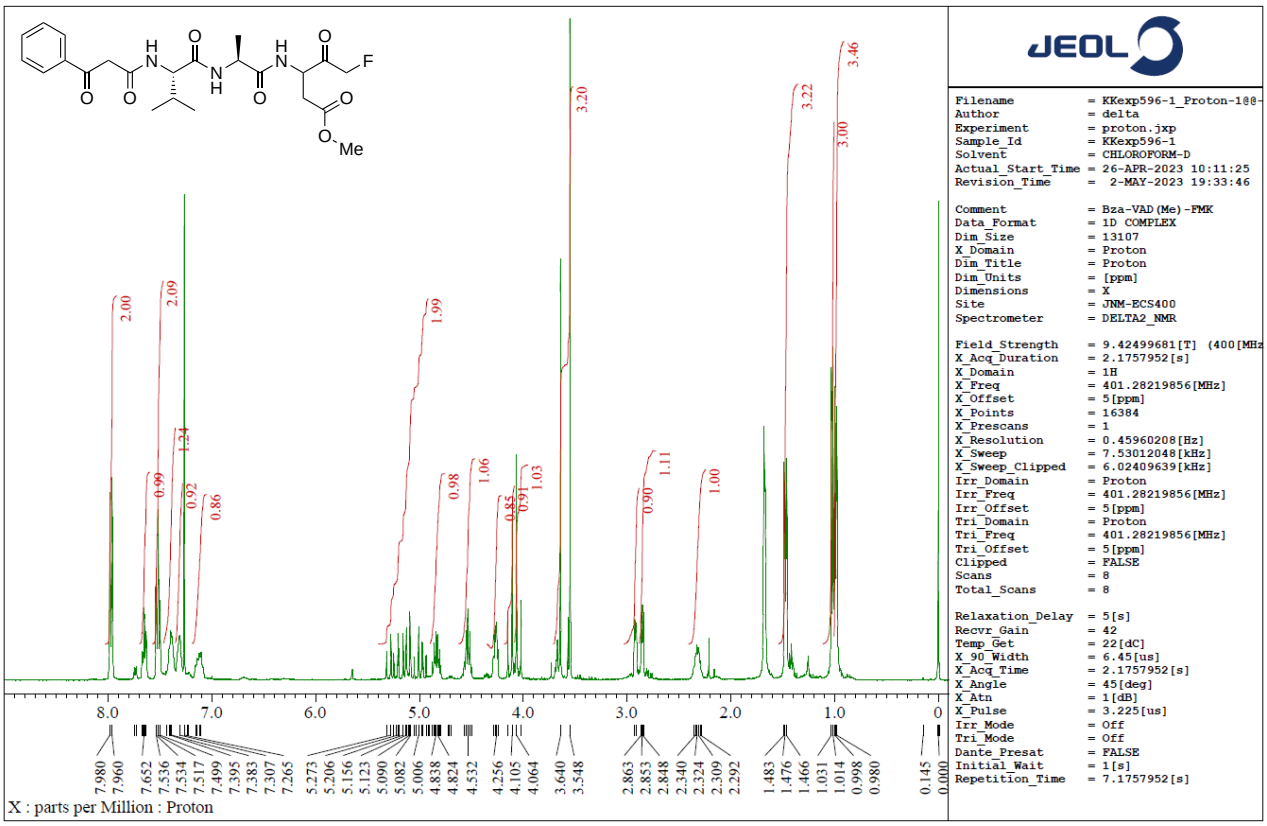


^{13}C-NMR

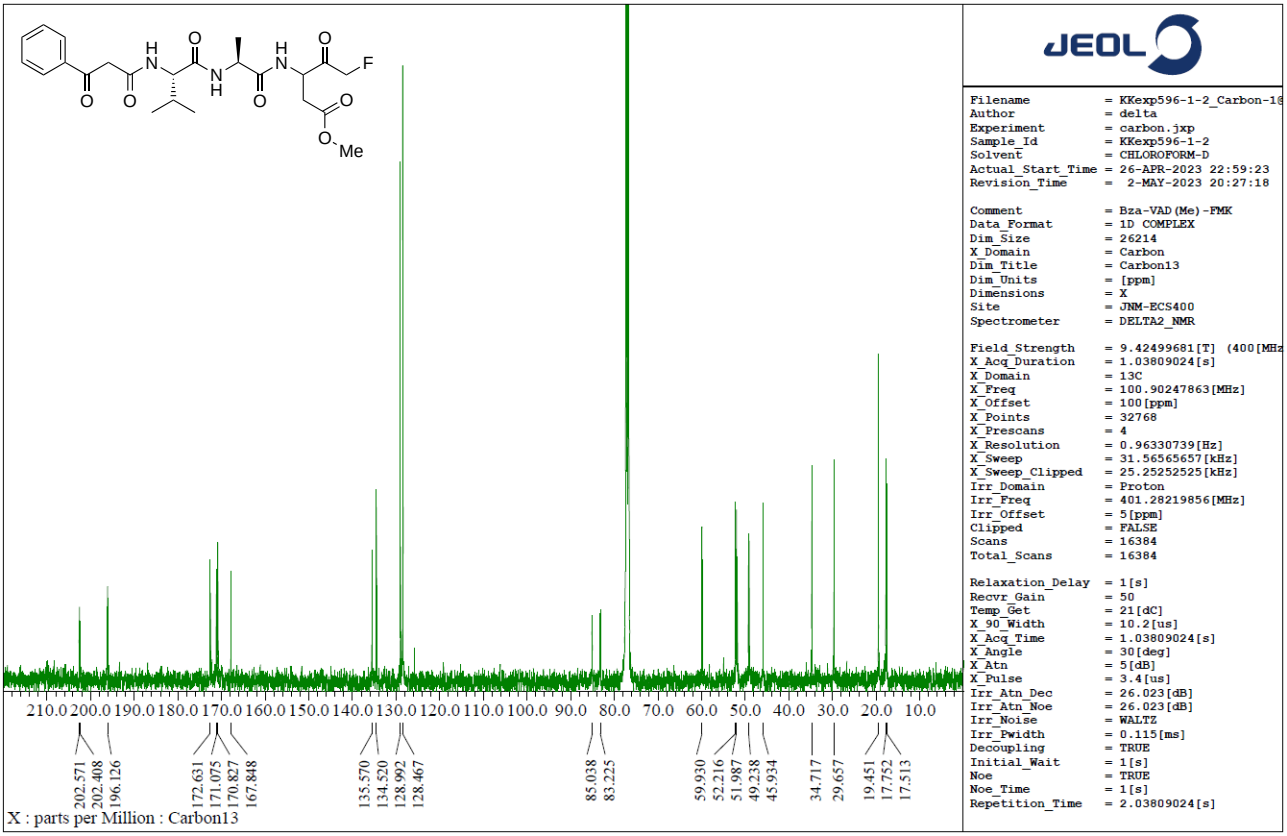


Bza-VAD(Me)-FMK

^{1}H-NMR



^{13}C-NMR



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