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for

Photolabile Protection for One-Pot Sequential Native Chemical Ligation

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General: Exact mass (HRMS) spectra were recorded on a JEOL JMS-01SG-2 or JMS-HX/HX 110A mass spectrometer. Ion-spray mass (ISMS) spectra were obtained with an API*III*E triple quadrupole mass spectrometer. ^{1}H NMR spectra were recorded using a JEOL AL-400 spectrometer at 400 MHz frequency in $CDCl_3$ or CD_3OD. Chemical shifts are calibrated to the solvent signals. For HPLC separations, a Cosmosil 5C18-ARII analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1 mL/min) or a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 12 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution.

2-Bromo-1-(4-methoxyphenyl)ethanone (5a): To a stirred solution of 4-methoxyacetophenone (**4a**) (15.0 g, 100 mmol) in dioxane (150 mL), was added dropwise a solution of bromine (5.6 mL, 110 mmol) in diethyl ether (120 mL) at room temperature, and the mixture was stirred at 40 °C for 2 h, and then cooled to room temperature. The mixture was washed with water and the organic layer was dried over $MgSO_4$. Concentration under reduced pressure followed by recrystallization with EtOAc-diisopropyl ether gave 19.9 g (87.1 mmol, 87% yield from **4a**) of **5a** as white crystals. m.p. 64-66 °C; ^{1}H NMR (400 MHz, $CDCl_3$) d = 3.89 (s, 3H), 4.41 (s, 2H), 6.98 (d, J =

9.0 Hz, 2H), 7.99 (d, *J* = 9.0 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_9H_{10}BrO_2$ (MH^+) 230.0790, found: 230.0785.

2-Bromo-1-(4-hydroxyphenyl)ethanone (5b): To a stirred solution of $CuBr_2$ (23.0 g, 103 mmol) in EtOAc (100 mL), was added dropwise a solution of 4-hydroxyacetophenone (**4b**) (13.0 g, 94.0 mmol) in $CHCl_3$ (100 mL) at room temperature, and the mixture was refluxed for 2 h, then cooled to room temperature. The mixture was washed with water and the organic layer was dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc-*n*-hexane (3:1) gave 11.8 g (54.6 mmol, 58% yield from **4b**) of **5b** as white crystals. m.p. 124-128 °C; ^{1}H NMR (400 MHz, CD_3OD) *d* = 4.59 (s, 2H), 6.92 (d, *J* = 8.9 Hz, 2H), 7.97 (d, *J* = 8.9 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_8H_8BrO_2$ (MH^+) 216.0519, found: 216.0556.

2-Bromo-1-(4-dimethylaminophenyl)ethanone (5c): Title compound was prepared by the method of Diwu et. al.[1] to give 9.92 g (41.0 mmol, 81% from **4c**) of **5c**. m.p. 89-92 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *d* = 3.09 (s, 6H), 4.36 (s, 2H), 6.66 (d, *J* = 10.0 Hz, 2H), 7.89 (d, *J* = 10.0 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{10}H_{13}BrNO$ (MH^+) 243.1203, found: 243.1212.

***Z*-Glycine 2-(4-methoxyphenyl)-2-oxoethyl ester (7a)**: To a stirred suspention of *Z*-Gly-OH (4.07 g, 19.4 mmol) in benzene (60 mL) were added dropwise DBU (2.47 mL, 16.5 mmol) and **5a** (3.43 g, 15.0 mmol) at 0 °C, and the mixture was stirred at room temperature for 3 h. The reaction mixture was concentrated under reduced pressure. The residue was extracted with EtOAc, and the extract was washed successively with saturated aqueous $NaHCO_3$, saturated aqueous citric acid and brine and dried over $MgSO_4$. Concentration under reduced pressure followed by recrystallization with EtOAc-Et_2O gave 4.71 g (13.2 mmol, 88% from **5a**) of **7a** as white crystals. m.p. 87-89 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *d* = 3.88 (s, 3H), 4.19 (d, *J* = 5.5 Hz, 2H), 5.34 (br, 1H), 5.36 (s, 1H), 6.95 (d, J = 8.8 Hz, 2H), 7.30-7.40 (m, 5H), 7.87 (d, *J* = 9.0 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{19}H_{20}NO_6$ (MH^+) 358.3652, found: 358.3650.

***Z*-Glycine 2-(4-hydroxyphenyl)-2-oxoethyl ester (7b)**: By use of a procedure identical with that described for the preparation of **7a** from **6a**, **6b** was converted into 1.90 g (5.53 mmol, 72% from **6b**) of **7b** as white crystals. m.p. 111-114 °C; ^{1}H NMR (400 MHz, CD_3OD) *d* = 4.06 (s, *J* = 5.6 Hz, 2H), 5.10 (s, 2H), 5.41 (s, 2H), 6.86 (d, *J* = 8.8

Hz, 2H), 7.25-7.36 (m, 5H), 7.85 (d, *J* = 8.8 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{18}H_{18}NO_6$ $[M+H]^+$ 344.3386, found: 344.3380.

***Z*-Glycine 2-(4-dimethylaminophenyl)-2-oxoethyl ester (7c)**: By use of a procedure identical with that described for the preparation of **7a** from **6a**, **6c** was converted into 2.23 g (6.03 mmol, 81% from **6c**) of **7c** as white crystals. m.p 111-113 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *d* = 3.07 (s, 6H), 4.20 (d, 2H), 5.32 (br, 1H), 5.35 (s, 2H), 6.65 (d, *J* = 9.0 Hz, 2H), 7.29-7.37 (m, 5H), 7.79 (d, *J* = 8.8 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{20}H_{23}N_2O_5$ $[M+H]^+$ 371.4070, found: 371.4081.

***Z*-Glycine 2-phenyl-2-oxoethyl ester (7d)**: By use of a procedure identical with that described for the preparation of **7a** from **6a**, **6d** was converted into 2.94 g (9.00 mmol, 93% from **6d**) of **7d** as white crystals. m.p. 89-91 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *d* = 4.21 (d, *J* = 5.6 Hz, 2H), 5.14 (s, 2H), 5.31 (br, 1H), 5.42 (s, 1H), 7.31-7.40 (m, 5H), 7.45-7.51 (m, 3H), 7.62 (t, 1H), 7.89 (d, *J* = 7.4 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{18}H_{18}NO_5$ $[M+H]^+$ 328.3392, found: 328.3399.

Carbonic acid 2-(4-hydroxyphenyl)-2-oxoethyl ester 4-nitro-phenyl ester (6b): A solution of **5b** (3.40 g, 15.6 mmol) and sodium formate (10.6 g, 156 mmol) in 80% ethanol (90 mL) was refluxed for 7 h. The mixture was concentrated under reduced pressure and diluted with water to yield the precipitate of 1-(4-hydroxyphenyl)-2-hydroxyethanone. Resulting precipitate was isolated by filtration and dried in vacuo. m.p. 135-145 °C; ^{1}H NMR (400 MHz, CD_3OD) *d* = 4.81 (s, 3H), 6.86 (d, *J* = 8.8 Hz, 2H), 7.85 (d, *J* = 9.2 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_8H_8O_3$ $[M+H]^+$ 153.1553, found: 153.1589.

To a solution of 1-(4-hydroxyphenyl)-2-hydroxyethanone (1.90 g, 12.5 mmol) in pyridine (25 mL) were added *p*-nitrophenyl chlorocarbonate (3.26 g, 16.2 mmol) at 0 °C, and the reaction mixture was stirred at room temperature for 10 h. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane (2:1) gave 2.52 g (7.96 mmol, 51% from **5b**) of **6b** as yellow crystals. m.p. 126-130 °C; ^{1}H NMR (400 MHz, $CDCl_3$) *d* = 3.08 (s, 6H), 5.95 (s, 2H), 6.67 (d, *J* = 9.2 Hz, 2H), 7.46 (d, *J* = 8.8 Hz, 2H), 7.81 (d, *J* = 8.8 Hz, 2H), 8.28 (d, *J* = 9.2 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{15}H_{12}NO_7$ $[M+H]^+$ 318.2583, found: 318.2589.

Carbonic acid 2-(4-dimethylaminophenyl)-2-oxoethyl ester 4-nitrophenyl ester (6c): A solution of **5c** (5.57 g, 23.0 mmol) and sodium formate (12.2 g, 188 mmol) in

80% ethanol (100 mL) was refluxed for 6 h. The mixture was concentrated under reduced pressure and diluted with water to yield the precipitate of 1-(4-dimethylamino-phenyl)-2-hydroxyethanone. Resulting precipitate was isolated by filtration and dried in vacuo. m.p. 131-133 °C; ^{1}H NMR (400 MHz, $CDCl_3$) d = 3.08 (s, 6H), 3.70 (t, J = 4.4 Hz, 1H), 4.77 (d, J = 4.4 Hz, 2H), 6.66 (d, J = 9.2 Hz, 2H), 7.81 (d, J = 9.2 Hz, 2H); HRMS (FAB), m/z calcd for $C_{10}H_{14}O_2$ $[M+H]^+$ 180.2237, found: 180.2229.

To a solution of 1-(4-dimethylaminophenyl)-2-hydroxyethanone (3.63 g, 20.3 mmol) in CH_2Cl_2 (100 mL), were added *p*-nitrophenyl chlorocarbonate (4.91 g, 24.3 mmol) and pyridine (1.96 mL, 24.3 mmol) at 0 °C, and the reaction mixture was stirred at room temperature for 5 h. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–n-hexane (2:1) gave 6.09 g (17.7 mmol, 77% from **5c**) of **6b** as yellow crystals. m.p. 127-129 °C; ^{1}H NMR (400 MHz, $CDCl_3$) d = 5.55 (s, 2H), 6.89 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 9.2 Hz, 2H), 7.89 (d, J = 8.8 Hz, 2H), 8.33 (d, J = 9.2 Hz, 2H); HRMS (FAB), m/z calcd for $C_{17}H_{17}N_2O_6$ $[M+H]^+$ 345.3267, found: 345.3263.

2(*S*)-2-[2-(4-Hydroxyphenyl)-2-oxo-ethoxycarbonylamino]-3-phenylpropionic acid methyl ester (8b): To a solution of **6b** (1.90 g, 6.0 mmol) in DMF (30 mL), were added L-phenylalanine methylester hydrochloride (1.55 g, 7.2 mmol) and Et_3N (1.0 mL, 7.2 mmol) at 0 °C, and the reaction mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure. The residue was extracted with EtOAc, and the extract was washed successively with saturated aqueous citric acid and brine and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc-n-hexane (2:3) and subsequent recrystallization with EtOAc-Et_2O gave 1.52 g (4.26 mmol, 71% from **6b**) of **8b** as white crystals. m.p. 115-119 °C; ^{1}H NMR (400 MHz, $CDCl_3$) d = 3.11-3.17 (m, 2H), 3.72 (s, 3H), 4.67 (br, 1H), 5.22 (m, 2H), 5.66 (br, 1H), 6.81 (d, J = 8.8 Hz, 2H), 7.16-7.32 (m, 5H), 7.71 (d, J = 8.8 Hz, 2H); HRMS (FAB), m/z calcd for $C_{19}H_{20}NO_6$ $[M+H]^+$ 358.3652, found: 358.3657.

2(*S*)-2-[2-(4-Dimethylaminophenyl)-2-oxo-ethoxycarbonylamino]-3-phenylpropionic acid methyl ester (8c): To a solution of **6c** (1.0 g, 2.9 mmol) in pyridine (40 mL), were added L-phenylalanine methylester hydrochloride (0.82 g, 3.77 mmol) and Et_3N (524 µl, 3.77 mmol) at 0 °C, and the reaction mixture was stirred at room temperature for 15 h. The reaction mixture was concentrated under reduced pressure. The residue

was extracted with EtOAc, and the extract was washed successively with saturated aqueous citric acid and brine and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc-*n*-hexane (2:1) and subsequent recrystallization with EtOAc-Et_2O gave 1.03 g (2.69 mmol, 93% from **6c**) of **8c** as white crystals. m.p. 117-120 °C; ^{1}H NMR (400 MHz, $CDCl_3$) d = 3.06 (s, 6H), 3.15 (d, J = 5.6 Hz, 2H), 3.71 (s, 3H), 4.68 (br, 1H), 5.25 (m, 2H), 5.47 (d, J = 8.0 Hz, 1H), 6.64 (d, J = 9.2 Hz, 2H), 7.16-7.33 (m, 5H), 7.81 (d, J = 8.8 Hz, 2H); HRMS (FAB), *m/z* calcd for $C_{21}H_{25}N_2O_5$ $[M+H]^+$ 385.4336, found: 385.4342.

General procedure for photolysis (7a-7d, 8b and 8c): A solution of compound (0.1 mmol) in EtOH (100 mL) was irradiated under argon at 25 °C using a Riko UVL-100HA-100P photochemical reactor fitted with 100 W high-pressure Hg lump ($h\nu$ > 300 nm). After photolysis, the solution was concentrated under reduced pressure and dissolved in 1.0 mL of EtOH. This sample solution was analyzed by RP-HPLC. The yield of Z-Gly-OH (for **7a**-**7d**) or H-Phe-OMe (for **8b** and **8c**) was calculated using a working curve from the standard EtOH solution of Z-Gly-OH or H-Phe-OMe, respectively, which was analyzed using the same HPLC conditions. Data are expressed as the mean value of two independent experiments.

Preparation of Mapoc protected 12-mer peptide thioester 9: To a 30 mL round-bottomed flask were added 4-sulfamylbutyryl AM-PEGA resin (360 mg, 0.1 mmol), DMF (3 mL), DIPEA (157 μL, 0.90 mmol), and Fmoc-Gly-OH (119 mg, 0.4 mmol). The reaction mixture was stirred for 20 min followed by cooling to -20 °C. After 20 min, PyBOP (156 mg, 0.3 mmol) was added to the reaction mixture as a solid. The reaction mixture was stirred at -20 °C for 8 h. The resin was filtered, washed with DMF and DCM. The loading yield was estimated by Fmoc-quantification[2] (0.22 mmol/g (78%)). All subsequent amino acids were coupled by stepwise Fmoc-based solid-phase synthesis and the N^{α}-Mapoc group was introduced at the end of cycle of the solid phase protocols by **6c** (134 mg. 0.39 mmol) in the presence of DIPEA (67.9 μL, 0.39 mmol) in DMF for 12 h. The protected resin was treated with iodoacetonitrile (180 μL, 2.5 mmol) and DIPEA (200 μL, 1.1 mmol) in NMP (4 mL) for 24 h. The resin was washed with NMP and then treated with ethyl-3-mercaptopropionate (633 μL, 5.0 mmol) and DIPEA (870 μL, 5.0 mmol) in DMF (3 mL) for 24 h. The resin was separated from the solution by filtration and washed three times with 3 mL portions of DMF. Combined filtrates were collected and concentrated to dryness. The side-chain protected crude

peptide were treated with triisopropylsilane/*m*-cresol/thioanisole/H_2O/TFA (5:5:5:5:80 (*v*/*v*), 7 mL) at room temperature for 2 h. Cooled Et_2O was added to the reaction mixture and resulting precipitate was collected by centrifugation. The precipitate was washed three times with Et_2O and purified by RP-HPLC to gave 23 mg of peptide as a freeze-dried powder.

Preparation of peptide thioesters 14 and 15: DMF solution (2 mL) of Gly trithioester (104 mg, 0.4 mmol) was added to 3,5-dimethoxy-4-formyl-phenoxymethyl resin (240 mg, 0.2 mmol) followed by successive treatment of $NaBH_4CN$ (25 mg, 0.4 mmol each: *t*/min = 5 and 120). After 2 h, the resin was washed with DMF, DIPEA-CH_2Cl_2 (1:19), CH_2Cl_2 and DMF. Protected peptide resins for **14** and **15** were manually constructed in 0.1 mmol scale on the above *o*-BAL resin by Fmoc-methodology with HATU/HOAt activation method (Fmoc-AA-OH (5 equiv), HATU (4.9 equiv), HOAt (10 equiv), DIPEA (5 equiv)). N^{α}-Mapoc group for peptide **14** was introduced at the end of cycle of the solid phase protocols by **6c** (172 mg. 0.5 mmol) in the presence of DIPEA (89 μl, 0.5 mmol) in DMF for 12 h. Each protected peptide resin was treated with triisopropylsilane/*m*-cresol/thioanisole/H_2O/TFA (5:5:5:5:80 (*v*/*v*), 7 mL) at room temperature for 2 h. Cooled Et_2O was added to the reaction mixture and resulting precipitate was collected by centrifugation. The precipitate was washed with Et_2O (3x) and purified by RP-HPLC to gave purified peptides **14** (45 mg) and **15** (36 mg).

Photo-triggered intramolecular native chemical ligation**:** N^{α}-Mapoc peptide thioester **9** (200 μg, 0.10 μmol) and sodium mercaptoethanesulfonate (3.0 mg, 18 μmol, (1% *w*/*v*)) was dissolved in 400 μL of 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine hydrochloride. The mixture was photo-irradiated (*hn* > 300 nm) at RT for 20 min and the aliquot was analyzed with RP-HPLC. HPLC condition: liner gradient of solvent B in solvent A, 20 to 80% over 50 min. 9: t_R = 23.5 min, ISMS (reconstructed) 1718.0 (1717.9 calcd for $C_{75}H_{112}N_{16}O_{26}S_2$); 10: t_R = 18.8 min, ISMS (reconstructed) 1584.0 (1583.7 calcd for $C_{70}H_{102}N_{16}O_{24}S$); 12: t_R = 11.2 min ISMS (reconstructed) 1379.0 (1378.5 calcd for $C_{59}H_{91}N_{15}O_{21}S$).

Synthesis of hBNP-32 (17) utilizing the one-pot sequential native chemical ligation: The first native chemical ligation (peptide **13** (4.99 mg, 2.50 μmol) and peptide **14** (2.28 mg, 2.50 μmol)) was performed in 2.5 mL of 0.1 M phosphate buffer (pH 7.6) containing 6 M guanidine hydrochloride and 0.3% thiophenol (*v*/*v*) at 37 °C for 1 h. The N^{α}-Mapoc group was removed by photo-irradiation (*hn* > 300 nm) at 25 °C for 30

min. The second native chemical ligation was achieved in the reaction mixture by the addition of thiophenol (1%, *v*/*v*) and peptide thioester **15** (2.34 mg, 2.50 μmol) at 37 °C for 1 h. The reaction mixture was diluted threefold with 0.1 M phosphate buffer (pH 7.6) followed by addition of DMSO (10% *v*/*v*) and stirred for 3 h. At the completion of the reaction, the solution was diluted threefold by the 0.1% TFA aq. (solvent A) and loaded directly onto a preparative RP-HPLC column. The desired peptide was then eluted with a liner gradient of solvent B in solvent A, 25 to 35% over 40 min (hBNP: 4.82 mg). Analytical HPLC condition: liner gradient of solvent B in solvent A, 5 to 45% over 40 min. **13**: t_R = 13.3 min, ISMS (reconstructed) 935.0 (934.1 calcd for $C_{38}H_{67}N_{11}O_{12}S_2$); **14**: t_R = 13.3 min, ISMS (reconstructed) 1951.0 (1950.3 calcd for $C_{82}H_{132}N_{24}O_{25}S_3$); Cyclic thioester of **14**: t_R = 27.8 min, ISMS (reconstructed) 1889.0 (1888.1 calcd for $C_{80}H_{126}N_{24}O_{25}S_2$); Mapoc-(10-32)-OH: t_R = 30.9 min, ISMS (reconstructed) 2800.0 (2799.3 calcd for $C_{118}H_{196}N_{40}O_{33}S_3$); H-(10-32)-OH (S-S form): t_R = 22.1 min, ISMS (reconstructed) 2593.0 (2592.04 calcd for $C_{107}H_{183}N_{39}O_{30}S_3$); H-(10-32)-OH (SH form): t_R = 24.5 min, ISMS (reconstructed) 2595.0 (2594.1 calcd for $C_{107}H_{185}N_{39}O_{30}S_3$); **15**: t_R = 18.9 min, ISMS (reconstructed) 935.0 (934.1 calcd for $C_{38}H_{67}N_{11}O_{12}S_2$); 2Cys-SH hBNP-32 (**16**): t_R = 27.0 min, ISMS (reconstructed) 3467.0 (3466.1 calcd for $C_{143}H_{246}N_{50}O_{42}S_4$); hBNP-32 (**17**): t_R = 24.9 min, ISMS (reconstructed) 3465.0 (3464.04 calcd for $C_{143}H_{244}N_{50}O_{42}S_4$).

References

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