Supporting information

FLAG Tagging by CuAAC and Nanogram-scale Purification of the Target Protein for a Bioactive Metabolite Involved in Circadian Rhythmic Leaf Movement in the Leguminosae

Yoshiyuki Manabe, Makoto Mukai, Satoko Ito, Nobuki Kato and Minoru Ueda

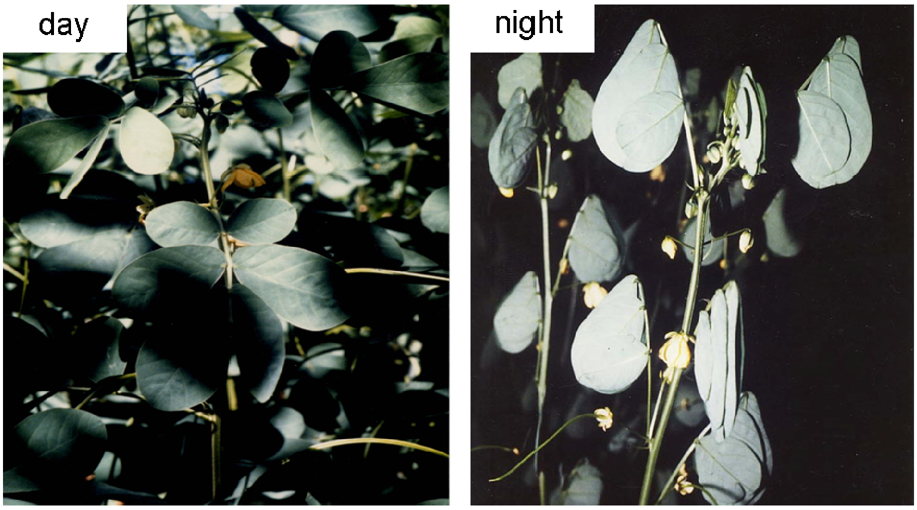


Figure S1. Nyctinastic leaf-movement of *Cassia obutusifolia*

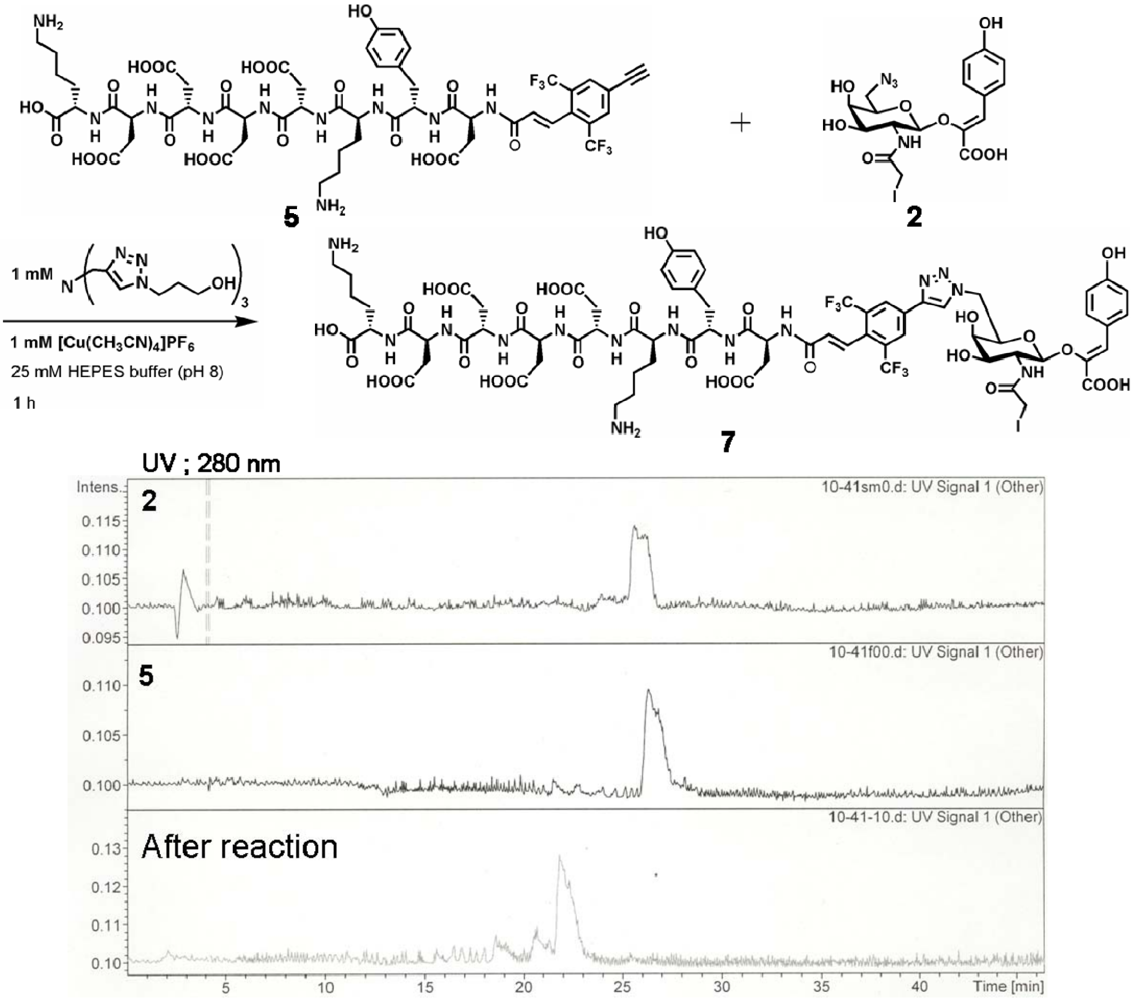
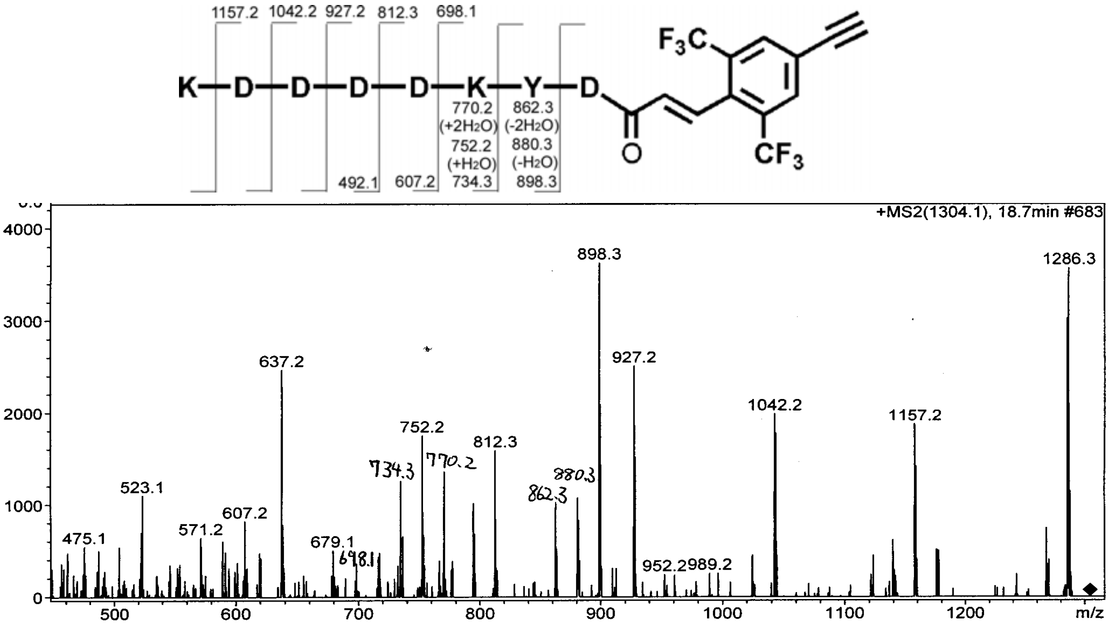


Figure S2. LC-MS analysis of CuAAC between **2** and **5**

Compound **5**

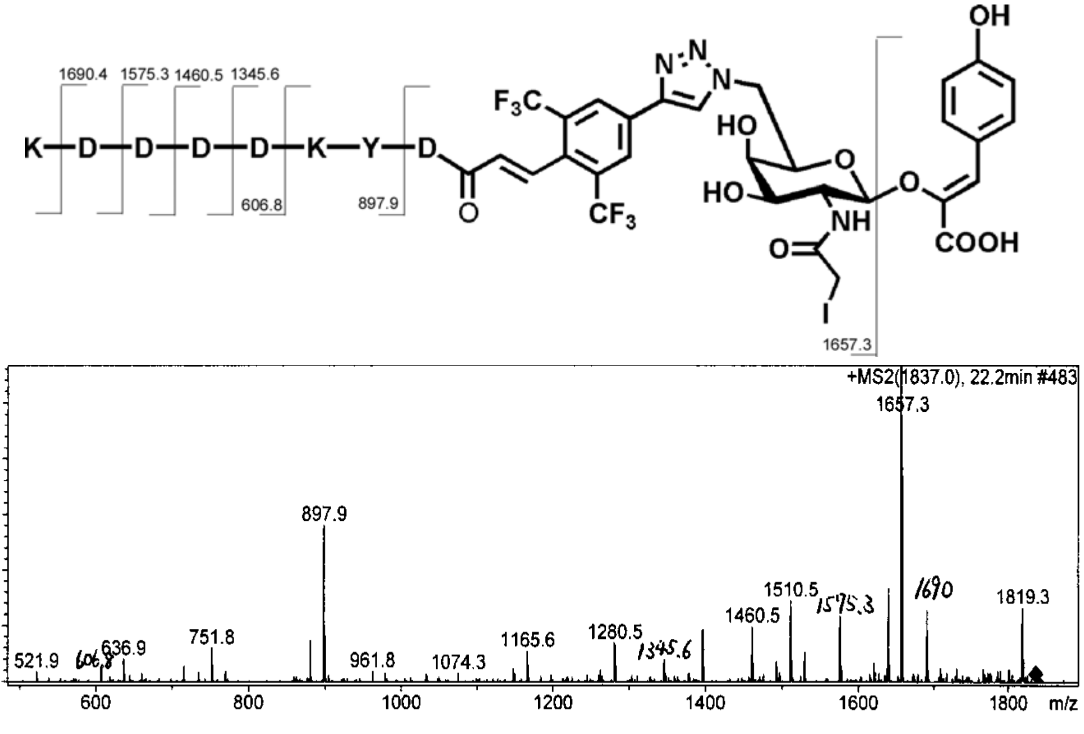
HRMS (ESI, positive) *m/z* $[M+2H]^{2+}$ calcd for $C_{54}H_{66}F_6N_{10}O_{21}$ 652.2149, found 652.2149.

ESI MS/MS analysis (positive mode, precursor ion; m/z =1304.1 $[M + H]^+$)



Compound **7**

ESI MS/MS (positive mode, precursor ion; m/z =1836.4 $[M + H]^+$)



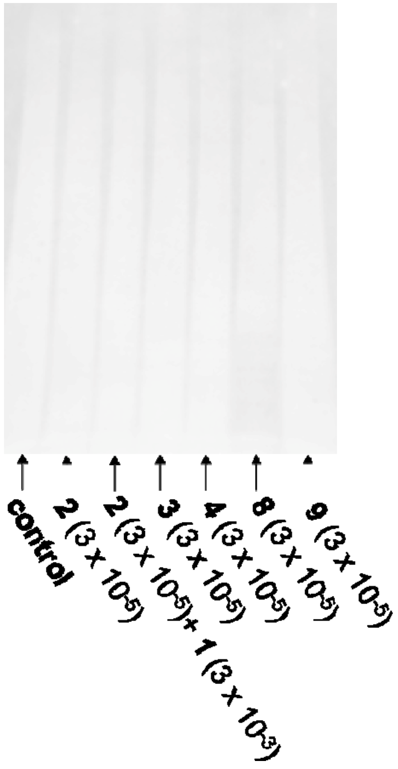
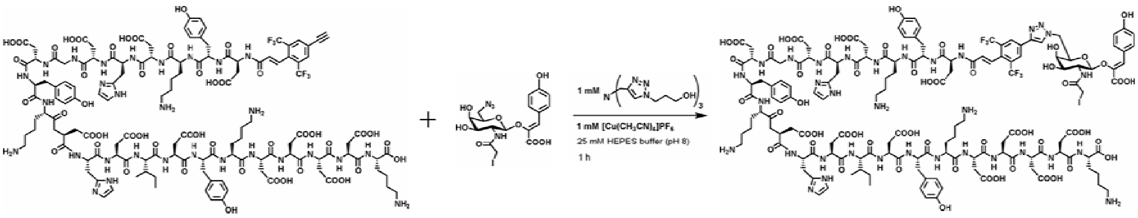


Figure S3. SDS-PAGE analysis of FLAG-tagged proteins in the membrane fraction of *Cassia* motor cell: from the left, blank membrane proteins as control, membrane proteins of motor cells treated with 3 × 10^{-5} M of **2,** 3 × 10^{-5} M of **2** + 3 × 10^{-3} M of **1,** 3 × 10^{-5} M of **3,** 3 × 10^{-5} M of **4,** 3 × 10^{-5} M of **8,** and 3 × 10^{-5} M of **9**, respectively.

Figure S4. Comparison of efficiencies between probes **2** and **7**.



10 **2** **11**

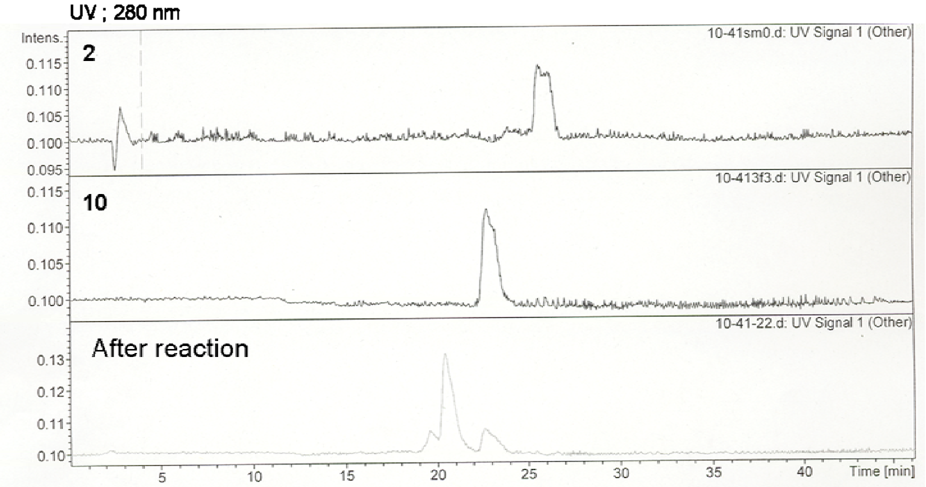
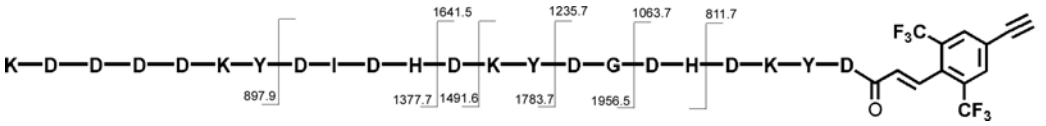


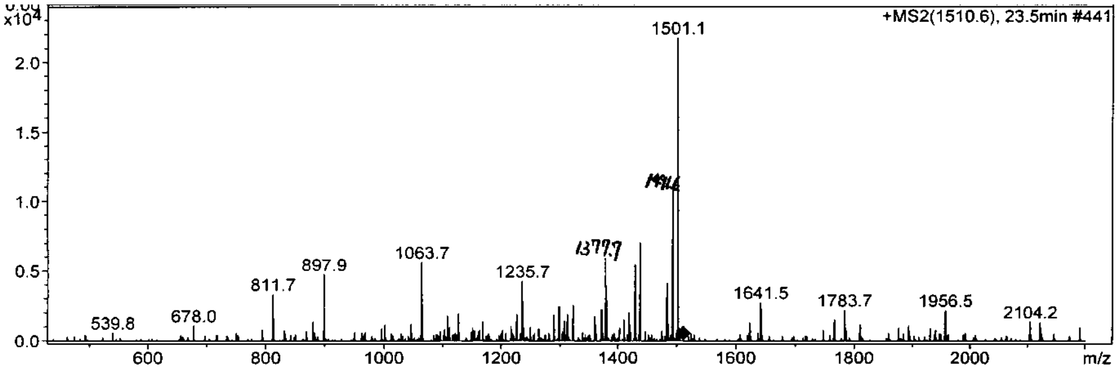
Figure S5. LC-MS analysis of CuAAC between **2** and **10**

Compound **10**

HRMS (ESI, positive) *m/z* $[M+3H]^{3+}$ calcd for $C_{128}H_{164}F_6N_{30}O_{49}$ 756.0378, found 756.0377.

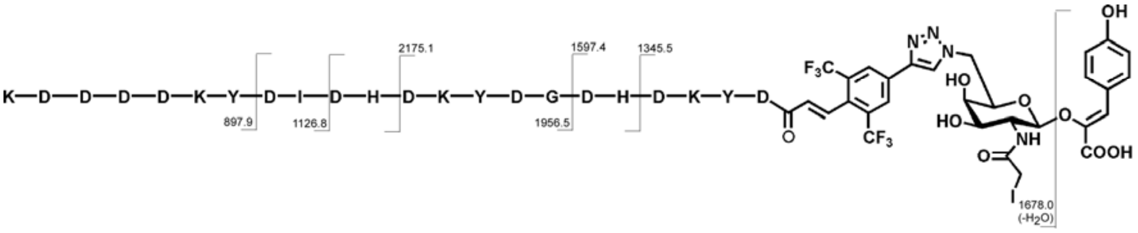
ESI MS/MS (positive mode, precursor ion; m/z = 1510.2 $[M + 2H]^{2+}$)

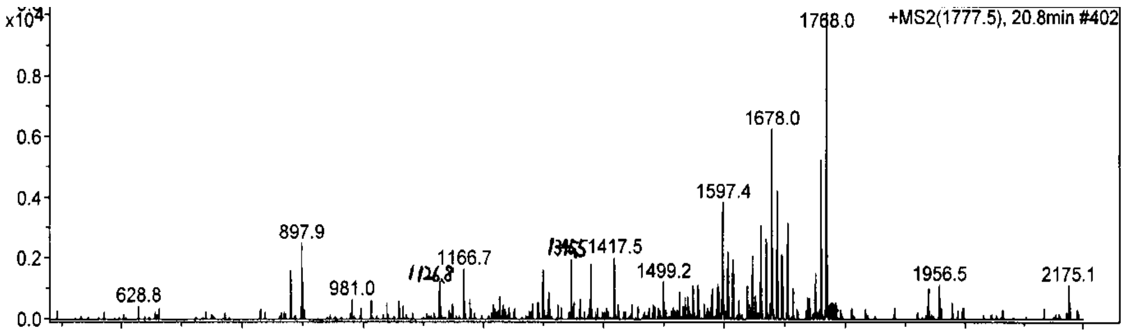


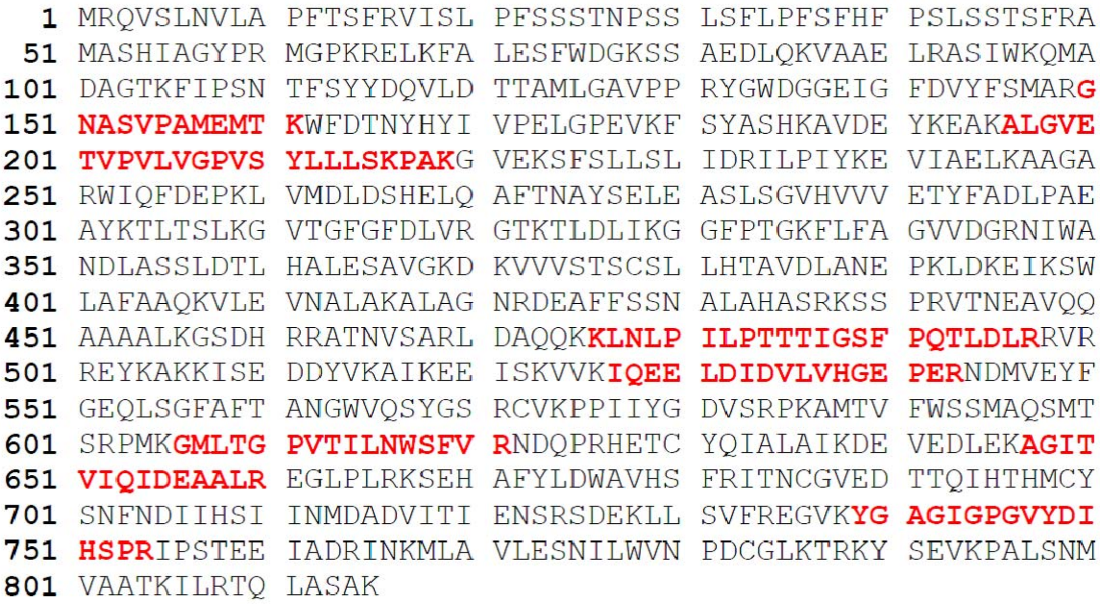


Compound **7**

ESI MS/MS (positive mode, precursor ion; m/z = 1777.0, $[M + 2H]^{2+}$)







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101 DAGTKFIPSN TFSYYDQVLD TTAMLGAVPP RYGWDGGEIG FDVYFSMAR**G**
151 **NASVPAMEMT K**WFDTNYHYI VPELGPEVKF SYASHKAVDE YKEAK**ALGVE**
201 **TVPVLVGPVS YLLLSKPAK**G VEKSFSLLSL IDRILPIYKE VIAELKAAGA
251 RWIQFDEPKL VMDLDSHELQ AFTNAYSELE ASLSGVHVVV ETYFADLPAE
301 AYKTLTSLKG VTGFGFDLVR GTKTLDLIKG GFPTGKFLFA GVVDGRNIWA
351 NDLASSLDTL HALESAVGKD KVVVSTSCSL LHTAVDLANE PKLDKEIKSW
401 LAFAAQKVLE VNALAKALAG NRDEAFFSSN ALAHASRKSS PRVTNEAVQQ
451 AAAALKGSDH RRATNVSARL DAQQK**KLNLP ILPTTTIGSF PQTLDLR**RVR
501 REYKAKKISE DDYVKAIKEE ISKVVK**IQEE LDIDVLVHGE PER**NDMVEYF
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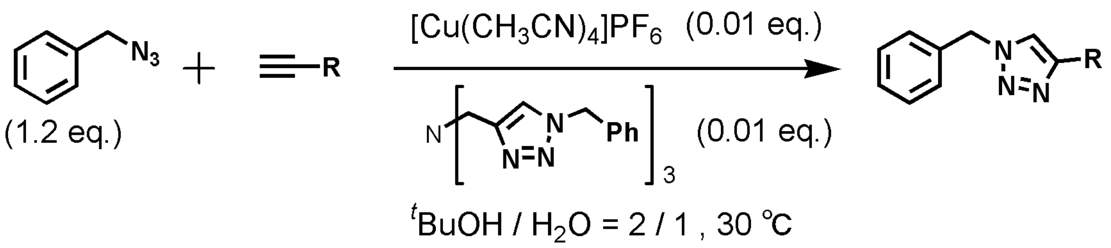
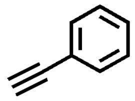
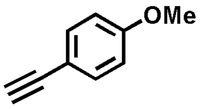
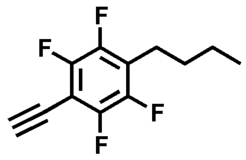
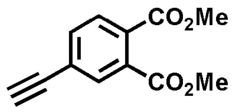
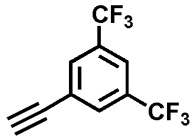
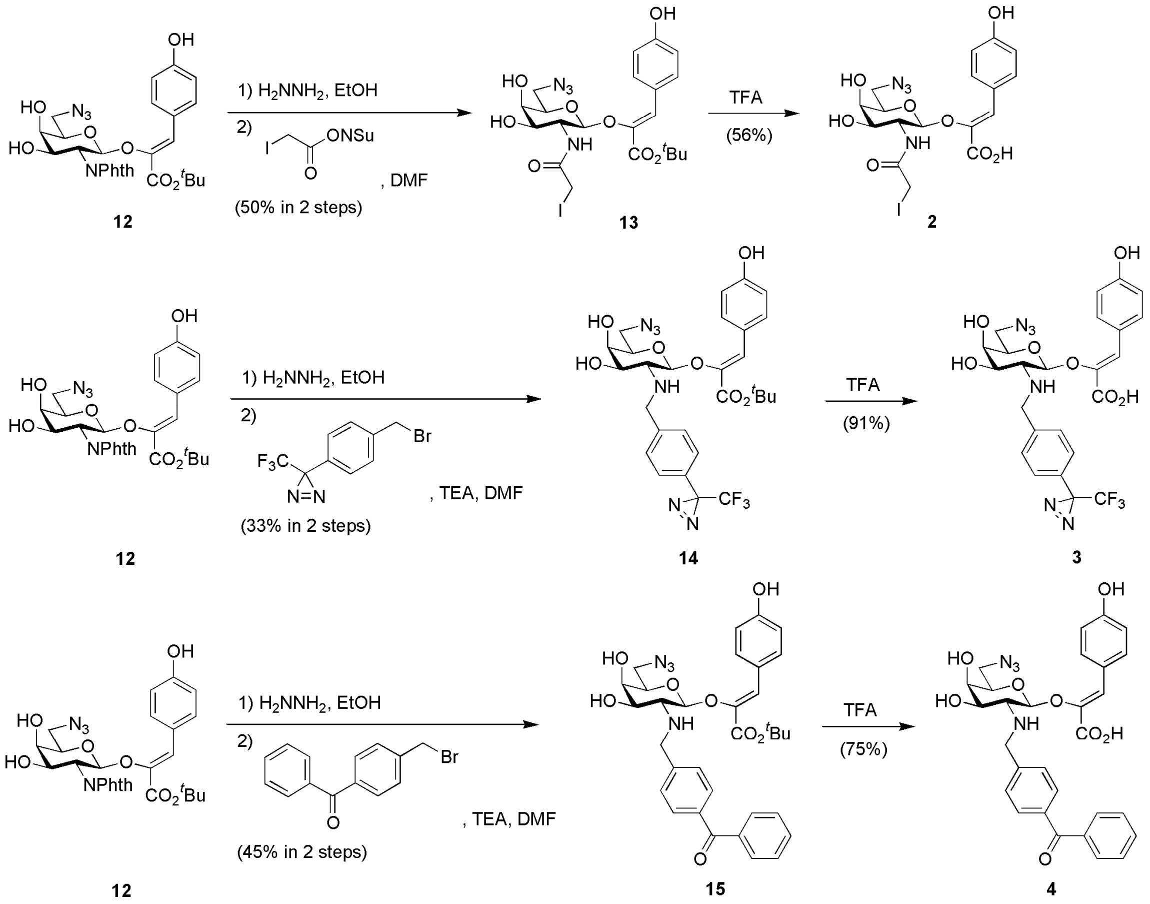
601 SRPMK**GMLTG PVTILNWSFV R**NDQPRHETC YQIALAIKDE VEDLEK**AGIT**
651 **VIQIDEAALR** EGLPLRKSEH AFYLDWAVHS FRITNCGVED TTQIHTHMCY
701 SNFNDIIHSI INMDADVITI ENSRSDEKLL SVFREGVK**YG AGIGPGVYDI**
751 **HSPR**IPSTEE IADRINKMLA VLESNILWVN PDCGLKTRKY SEVKPALSNM
801 VAATKILRTQ LASAK

Figure S6. A sequence of MetE from *Ricinus communis*: Matched peptides of CTPL in LC-MS/MS analysis were shown in red.

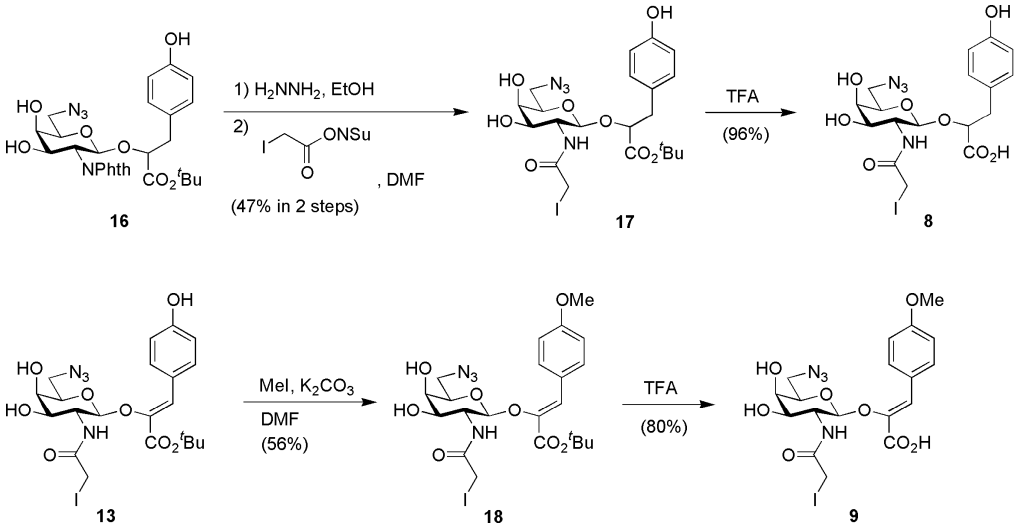
Table S1. Comparison of some alkynes in CuAAC reactivity with benzyl azide



Entry	Alkyne	Time	Yield
1		23.5 h	97%
2		49.5 h	54%
3		25.5 h	54%
4		7 h	93%
5		4.8 h	96%

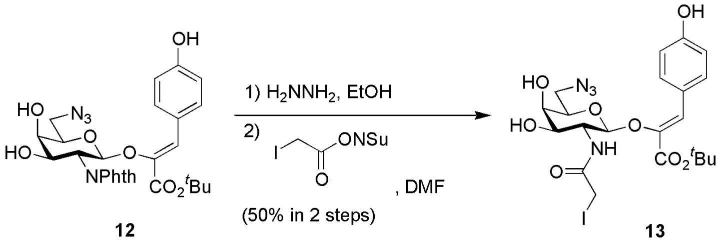


Scheme S1. Syntheses of molecular probes (**2**, **3**, and **4**)



Scheme S2. Syntheses of biologically inactive analogs of molecular probe (**8** and **9**)

Experimental Section



Compound **13**

Compound **12**[22]) (56.5 mg, 0.103 mmol) was dissolved in ethanol (2 mL), and hydrazine monohydrate (40 μL) was added to this solution. After overnight stirring, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc, washed with H_2O, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give crude amine (49.5 mg). Iodoacetic acid N-hydroxysuccinimide ester (5.1 mg, 17.9 μmol) was added to DMF solution of the crude amine (6.3 mg, crude) (0.2 mL), and the mixture was stirred for 6 h at room temperature. The reaction was quenched with AcOH. After dried, the residue was purified by pTLC ($CHCl_3$/MeOH = 10/1) to give **13** (4.0 mg, 50% in two steps).

^{1}H NMR (500 MHz, CD_3OD) δ 7.68 (d, *J* = 8.5 Hz, 2H), 6.82 (s, 1H), 6.76 (d, *J* = 8.5 Hz, 2H), 5.32 (d, *J* = 8.5 Hz, 1H), 4.21 (dd, *J* = 11.0, 8.5 Hz, 1H), 3.77 (s, 2H), 3.74 (dd, *J* = 3.5, 1.0 Hz, 1H), 3.67 (dd, *J* = 11.0, 3.5 Hz, 1H), 3.59 (ddd, *J* = 8.5, 4.0, 1.0 Hz, 1H), 3.50 (dd, *J* = 12.5, 8.5 Hz, 1H), 3.23 (dd, *J* = 12.5, 4.0 Hz, 1H), 1.55 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.0, 165.0, 159.5, 140.4, 133.7, 126.2, 125.8, 116.2, 100.5, 82.8, 76.2, 73.0, 70.0, 54.9, 52.4, 28.5, -1.58; IR (film) 3295, 2978, 2927, 2102, 1698, 1606, 1584, 1556, 1512, 1369, 1316, 1276, 1254, 1158, 1115, 1072, 837, 755 cm^{-1}; $[\alpha]_D^{27}$ -52.7 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{21}H_{27}IN_4O_8$, 613.0771, found 613.0800.

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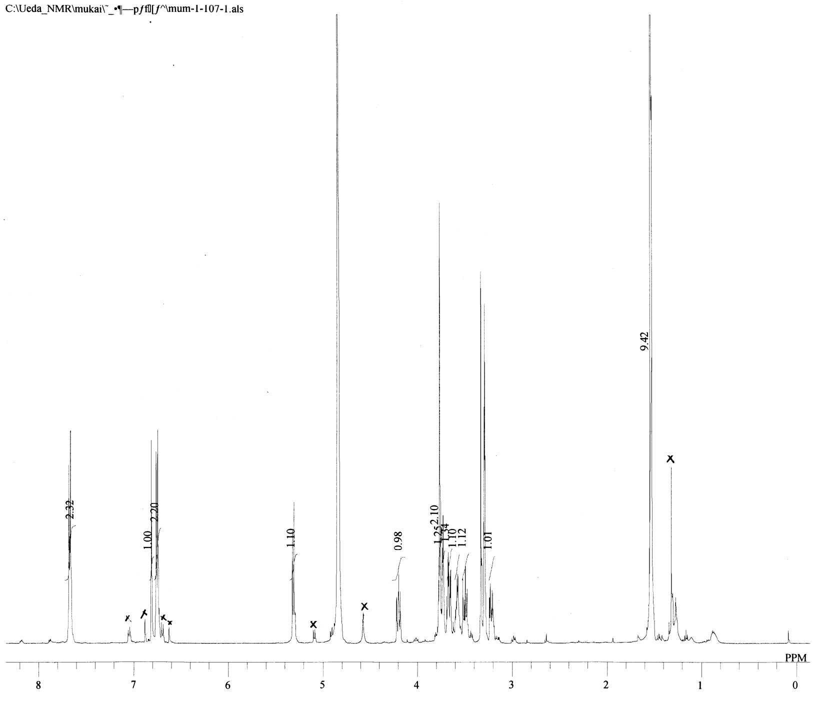

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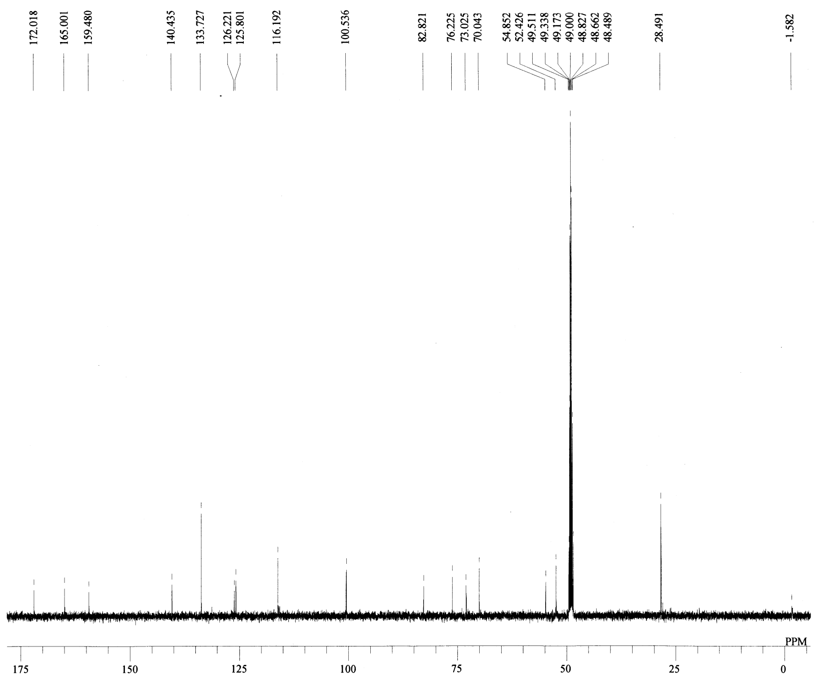

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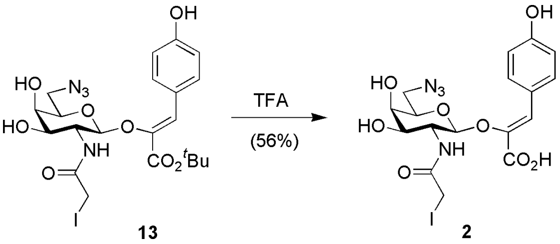

Compound **2**

TFA (1 mL) was added to compound **13** (12.5 mg, 21.2 mmol) in 10-mL round-bottom flask. After staring for 5 minutes, the reaction mixture was dried *in vacuo* using vacuum line. The residue was purified by HPLC [COSMOSIL 5C18-AR (*ϕ* 4.6 × 250 mm), 25% CH_3CNaq. containing 0.1% TFA, 5 mL / min.] to give **2** (6.3 mg, 56%).

^{1}H NMR (500 MHz, CD_3OD) δ 7.71 (d, *J* = 8.5 Hz, 2H), 7.00 (s, 1H), 6.76 (d, *J* = 8.5 Hz, 2H), 5.26 (d, *J* = 8.5 Hz, 1H), 4.21 (dd, *J* = 10.5, 8.5 Hz, 1H), 3.77 (s, 2H), 3.74 (dd, *J* = 3.0, 0.5 Hz, 1H), 3.66 (dd, *J* = 10.5, 3.0 Hz, 1H), 3.60 (ddd, *J* = 8.0, 4.5, 0.5 Hz, 1H), 3.49 (dd, *J* = 13.0, 8.0 Hz, 1H), 3.23 (dd, *J* = 13.0, 4.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.2, 167.6, 159.7, 139.8, 133.9, 127.2, 126.2, 116.1, 101.2, 76.1, 73.3, 70.0, 55.0, 52.3, -1.71; $[\alpha]_D^{23}$ -24.6 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{17}H_{19}IN_4O_8$, 557.0145, found 557.0157.

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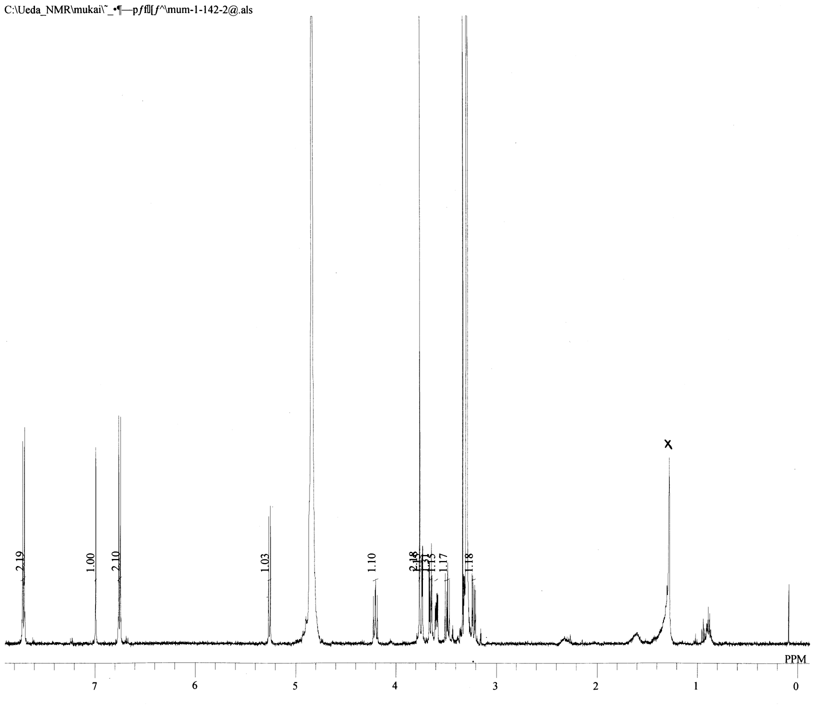

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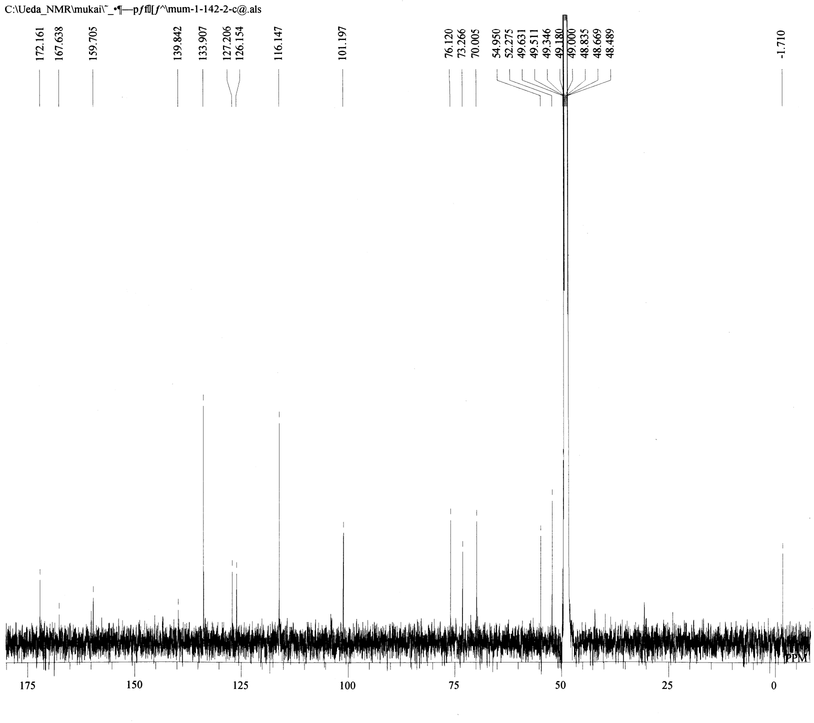

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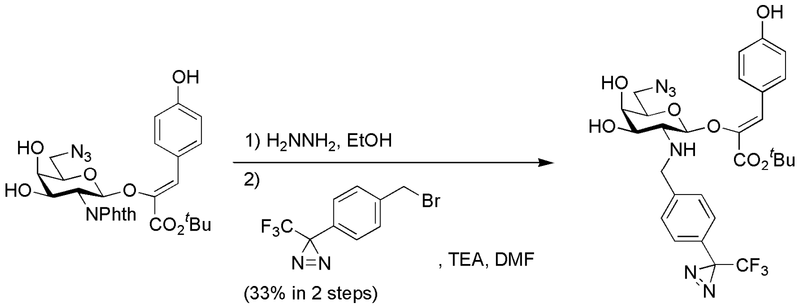

12 **14**

Compound **14**

Compound **12** (28.0 mg, 50.7 mmol) was dissolved in ethanol (1 mL), and hydrazine monohydrate (20 μL) was added to this solution. After overnight stirring, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc, washed with H_2O, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give crude amine (26.1 mg).

3-[4-(Bromomethyl)phenyl]-3-(trifluoromethyl)diazirine (28.2 mg, 0.101 mmol) and TEA (21.2 ml, 0.152 mmol) were added to the solution of the crude amine (26.1 mg, crude) in DMF (0.5 mL), and the mixture was stirred for 1 h at 0 ºC. Then, the mixture was slowly allowed to warm to room temperature. After overnight stirring, the reaction was quenched with AcOH. After dried, the residue was purified by pTLC ($CHCl_3$/MeOH = 10/1) to give **14** (12.5 mg, 33% in two steps).

^{1}H NMR (500 MHz, CD_3OD) δ 7.67 (d, *J* = 8.5 Hz, 2H), 7.48 (d, *J* = 8.5 Hz, 2H), 7.16 (d, *J* = 8.5 Hz, 2H), 6.91 (s, 1H), 6.71 (d, *J* = 8.5 Hz, 2H), 5.11 (d, *J* = 8.5 Hz, 1H), 4.15 (d, *J* = 13.5 Hz, 1H), 4.09 (d, *J* = 13.5 Hz, 1H), 3.68 (d, *J* = 3.5 Hz, 1H), 3.56 (m, 2H), 3.44 (dd, *J* = 13.0, 8.5 Hz, 1H), 3.16 (dd, *J* = 13.0, 4.0 Hz, 1H), 3.01 (d, *J* = 8.5 Hz, 1H), 1.54 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 165.2, 159.7, 143.9, 140.5, 133.7, 130.4, 128.5, 127.5, 127.0, 126.1, 123.5 (q, *J* = 261.2 Hz), 116.1, 103.8, 82.9, 76.1, 74.2, 69.9, 60.5, 53.4, 52.4, 29.5 (q, *J* = 40.2 Hz), 28.5; IR (film) 3312, 2980, 2932, 2103, 1697, 1606, 1585, 1513, 1455, 1394, 1370, 1346, 1316, 1277, 1254, 1233, 1158, 1114, 1067, 759 cm^{-1}; $[\alpha]_D^{27}$ -11.1 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+H]^+$ calcd for $C_{28}H_{31}F_3N_6O_7$, 621.2285, found 621.2259.

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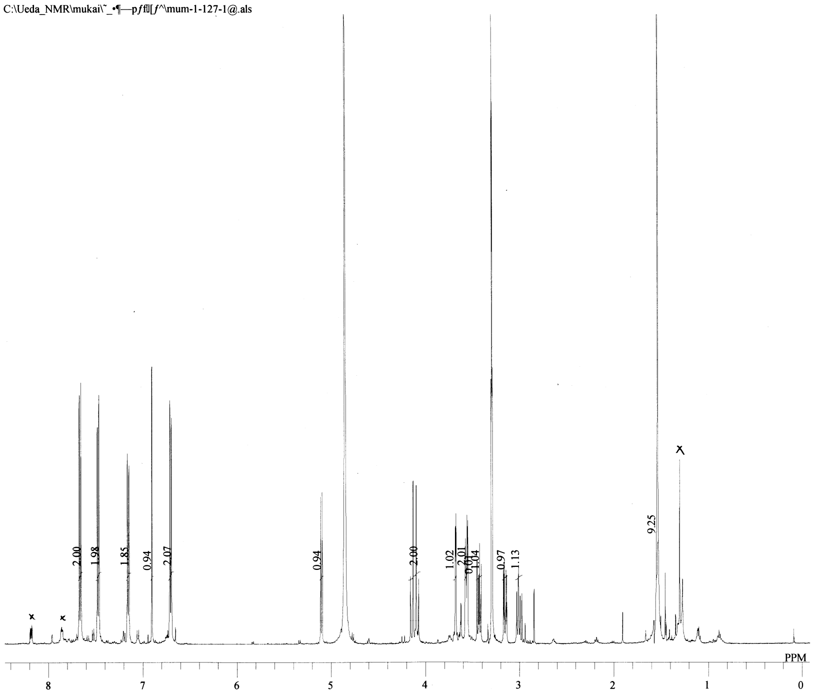

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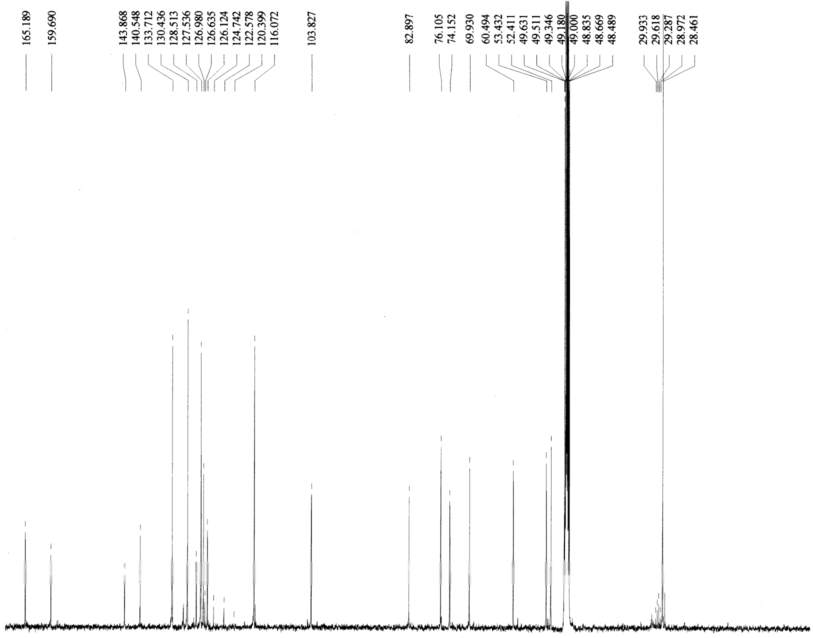

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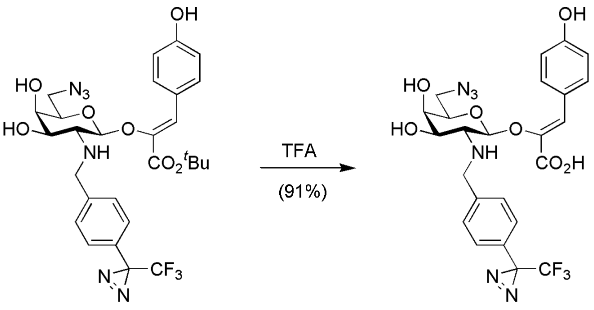


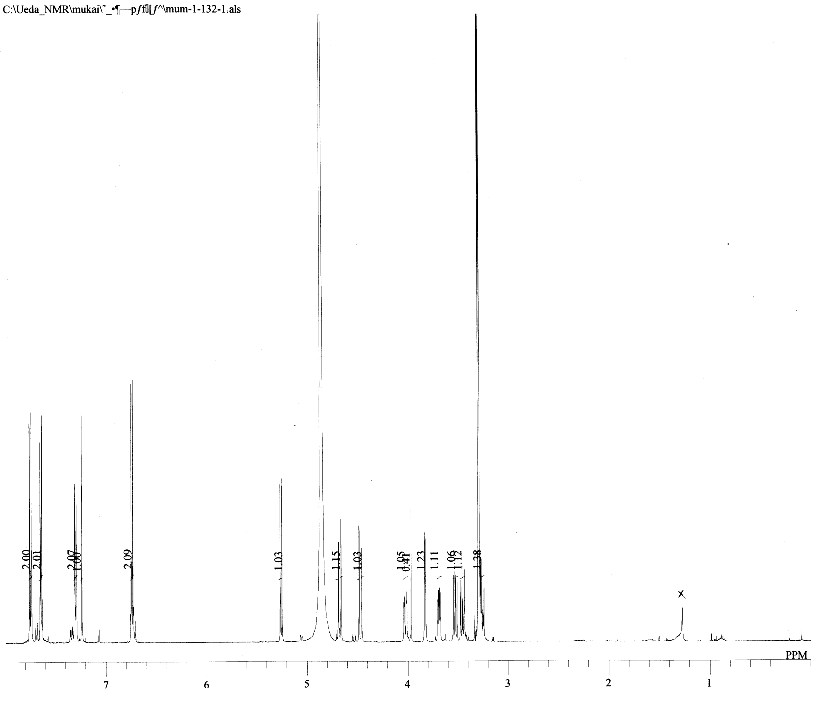
14 **3**

Compound **3**

TFA (1 mL) was added to compound **14** (8.2 mg, 13.2 mmol) in a 10-mL round-bottom flask. After staring for 1 hour, this flask was dried *in vacuo* using a vacuum line. The residue was purified by HPLC [COSMOSIL 5C18-AR (*ϕ* 4.6 × 250 mm), 40% CH_3CNaq. containing 0.1% TFA, 5 mL / min.] to give **3** (6.8 mg, 91%).

^{1}H NMR (500 MHz, CD_3OD) δ 7.75 (d, *J* = 8.5 Hz, 2H), 7.65 (d, *J* = 8.5 Hz, 2H), 7.31 (d, *J* = 8.5 Hz, 2H), 7.24 (s, 1H), 6.74 (d, *J* = 8.5 Hz, 2H), 5.26 (d, *J* = 8.5 Hz, 1H), 4.68 (d, *J* = 13.0 Hz, 1H), 4.48 (d, *J* = 13.0 Hz, 1H), 4.03 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.83 (dd, *J* = 3.0, 0.5 Hz, 1H), 3.69 (ddd, *J* = 8.0, 5.0, 0.5 Hz, 1H), 3.52 (dd, *J* = 11.0, 8.5 Hz, 1H), 3.46 (dd, *J* = 13.0, 8.0 Hz, 1H), 3.26 (dd, *J* = 13.0, 5.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 169.2, 160.7, 134.8, 134.3, 132.8, 132.1, 131.3, 129.8, 128.2, 125.1, 123.4 (q, *J* = 273.1 Hz), 116.1, 101.0, 76.5, 76.2, 70.6, 69.6, 61.4, 52.0, 29.3 (q, *J* = 40.3 Hz); IR (film) 3311, 2926, 2855, 2358, 2106, 1672, 1606, 1513, 1439, 1374, 1261, 1232, 1175, 1156, 1079, 940, 838, 801, 763, 722, 505, 458, 444, 418 cm^{-1}; $[\alpha]_D^{27}$ +20.1 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+H]^+$ calcd for $C_{24}H_{23}F_3N_6O_4$, 320.1862, found 320.1875.

C:\Ueda_NMR\mukai\˜_•¶—pƒf[ƒ^\mum-1-132-1.als



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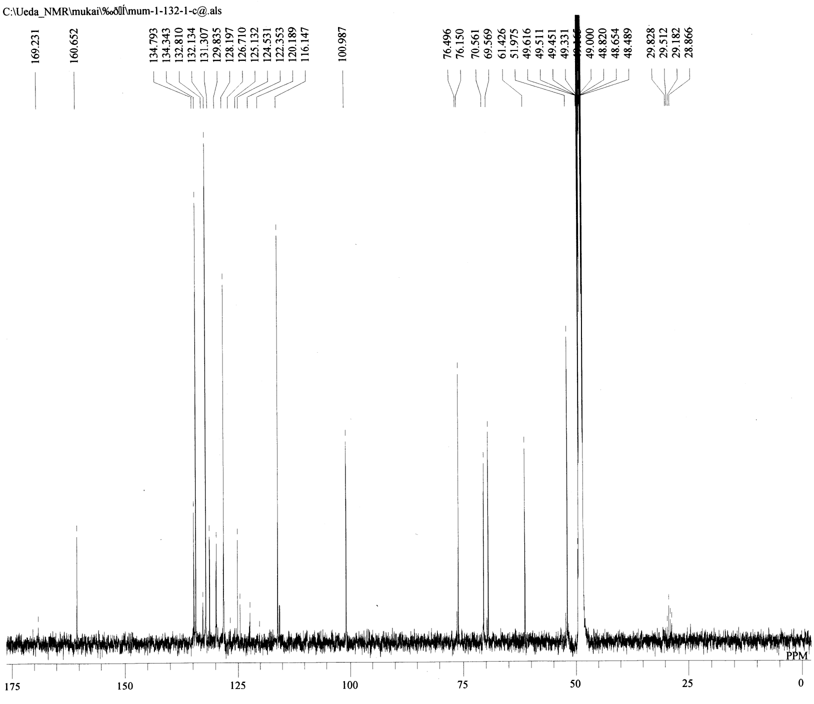

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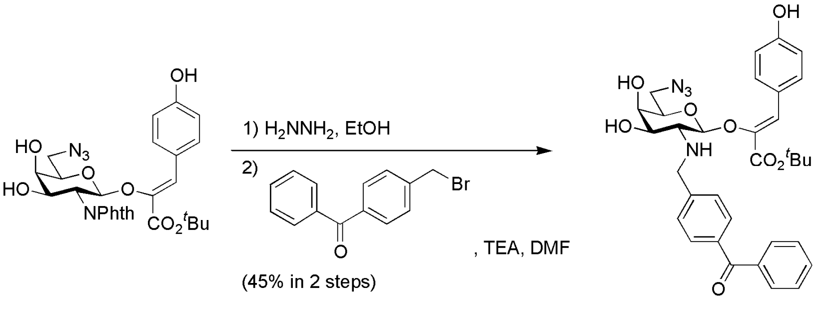


12 **15**

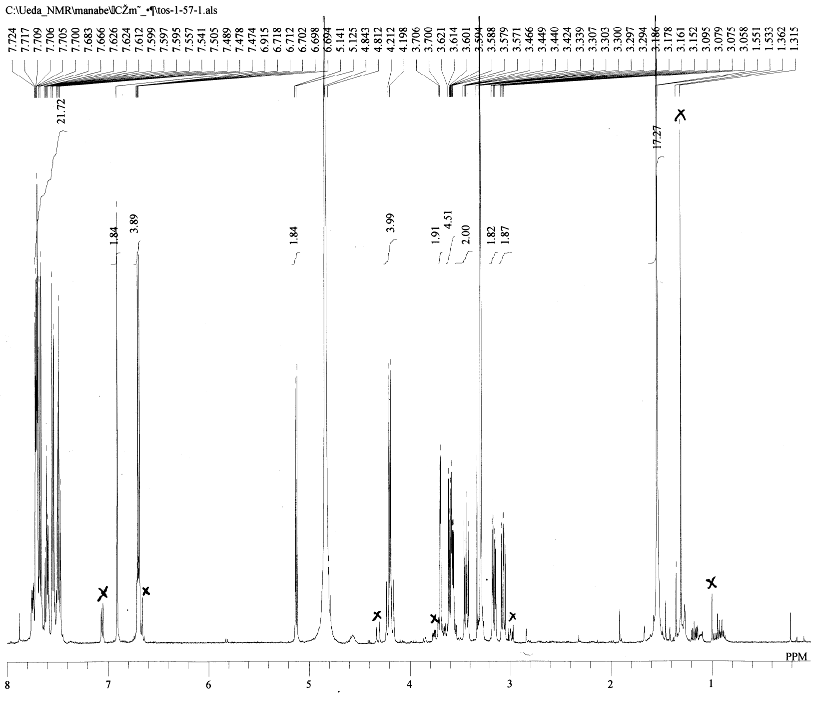
Compound **15**

Compound **12** (32.8 mg, 59.4 mmol) was dissolved in ethanol (2 mL), and hydrazine monohydrate (40 μL) was added to this solution. After overnight stirring, the reaction mixture was evaporated to dryness. The residue was purified by RP-pTLC (water/acetonitrile = 1/1, containing 1% TFA) to give crude amine (33.0 mg, crude).

4-bromomethylbenzophenone (32.5 mg, 0.119 mmol) and TEA (41.3 ml, 0.297 mmol) were added to the DMF solution of the crude amine (33.0 mg, crude) (1 mL), and the mixture was stirred for 2.5 h at room temperature. After overnight stirring, the reaction was quenched with AcOH. After dried, the residue was purified by pTLC ($CHCl_3$/MeOH = 10/1) to give **15** (26.5 mg, 45% in two steps).

^{1}H NMR (500 MHz, CD_3OD) δ 7.75−7.67 (m, 6H), 7.61 (t, *J* = 6.5 Hz, 1H), 7.55 (d, *J* = 8.5 Hz, 2H), 7.49 (t, *J* = 7.8 Hz, 2H), 6.92 (s, 1H), 6.70 (d, *J* = 8.5 Hz, 2H), 5.13 (d, *J* = 8.0 Hz, 1H), 4.22 (d, *J* = 13.5 Hz, 1H), 4.19 (d, *J* = 13.5 Hz, 1H), 3.70 (d, *J* = 3.0 Hz, 1H), 3.61 (dd, *J* = 10.0, 3.5 Hz, 1H), 3.58 (m, 1H), 3.44 (dd, *J* = 13.0, 8.3, 1H), 3.17 (dd, *J* = 13.0, 4.3, 1H), 3.07 (dd, *J* = 10.0, 8.3, 1H), 1.55 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 198.5, 165.2, 159.7, 147.0, 140.6, 139.0, 137.4, 133.7, 131.3, 130.9, 129.8, 129.5, 126.9, 126.1, 116.1, 103.8, 82.9, 76.1, 74.3, 70.0, 60.5, 53.7, 52.4, 28.5; IR (film) 3381, 2978, 2931, 2102, 1698, 1651, 1606, 1512, 1369, 1317, 1279, 1159 cm^{-1}; $[\alpha]^{22}_D$ −43.8 (*c* 0.10, CH_3OH); HRMS (ESI, positive) *m/z* $[M+H]^+$ calcd for $C_{33}H_{37}N_4O_8$ 617.2606, found 617.2602.

C:\Ueda_NMR\manabe\ICŽm˜_•¶\tos-1-57-1.als



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RGAIN            18
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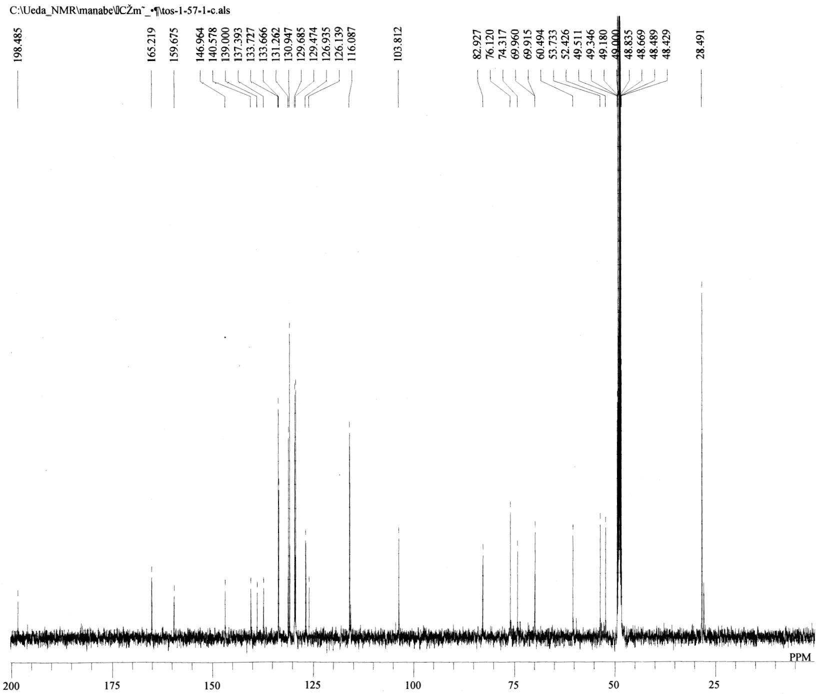

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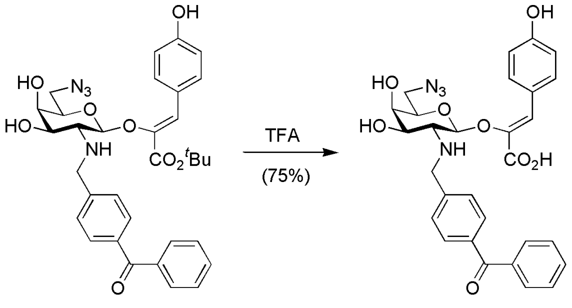
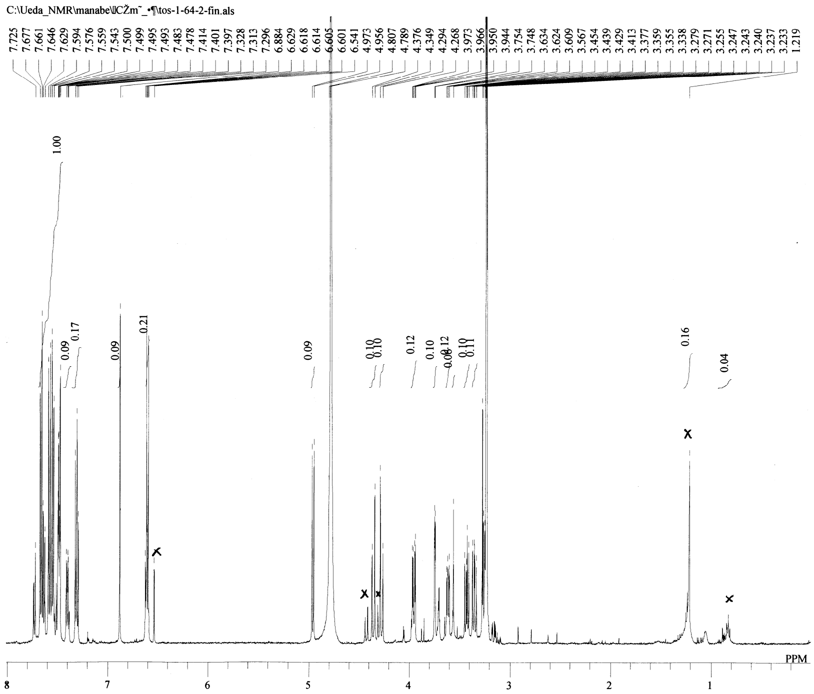


15 **4**

Compound **4**

TFA (1 mL) was added to compound **14** (8.2 mg, 13.2 mmol) in a 10-mL round-bottom flask. After staring for 1 hour, the raction mixture was dried *in vacuo* using a vacuum line. The residue was purified by purified by RP-pTLC (water/acetonitrile = 1/1, containing 1% TFA) to give **4** (5.0 mg, 75%).

^{1}H NMR (500 MHz, CD_3OD) δ 7.74−7.30 (m, 11H), 6.89 (s, 1H), 6.62 (d, *J* = 8.5 Hz, 2H), 4.96 (d, *J* = 8.5 Hz, 1H), 4.36 (d, *J* = 13.0 Hz, 1H), 4.28 (d, *J* = 13.0 Hz, 1H), 3.96 (dd, *J* = 11.0, 3.0 Hz, 1H), 3.75 (d, *J* = 3.0 Hz, 1H), 3.63 (m, 1H), 3.42 (dd, *J* = 12.5, 7.8, 1H), 3.35 (dd, *J* = 11.0, 8.5, 1H), 3.26 (dd, *J* = 12.5, 4.0, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 197.7, 169.3, 160.7, 139.8, 138.4, 137.1, 134.4, 134.0, 131.5, 131.4, 130.9, 130.1, 129.6, 126.5, 125.1, 116.2, 101.0, 76.2, 70.7, 69.6, 61.3, 52.3, 52.0; IR (film) 3304, 2924, 2102, 1658, 1607, 1512, 1366, 1279, 1064, 703 cm^{-1}; $[\alpha]^{22}_{D}$ −38.3 (*c* 0.10, CH_3OH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{29}H_{28}N_4O_8Na$ 583.1799, found 583.1801



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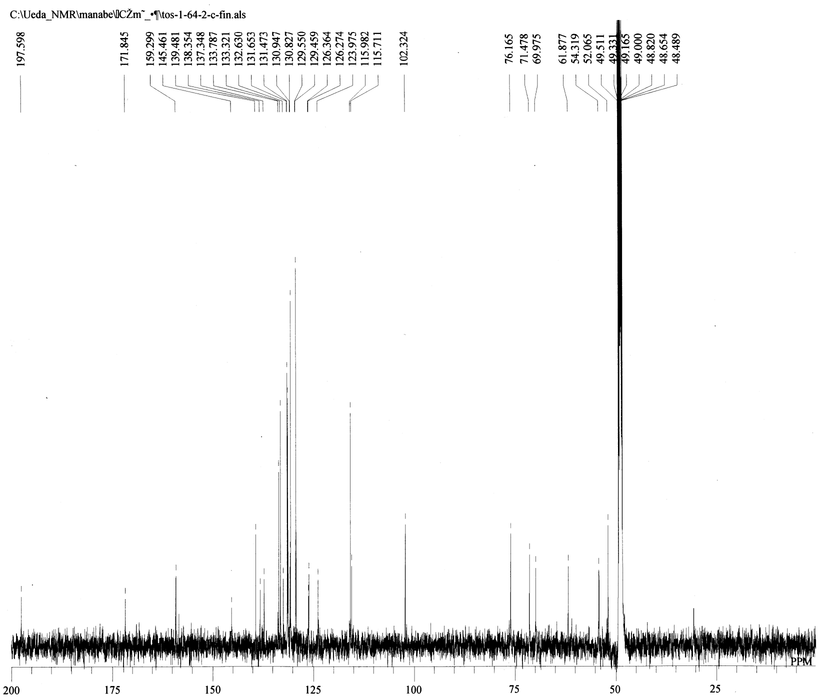

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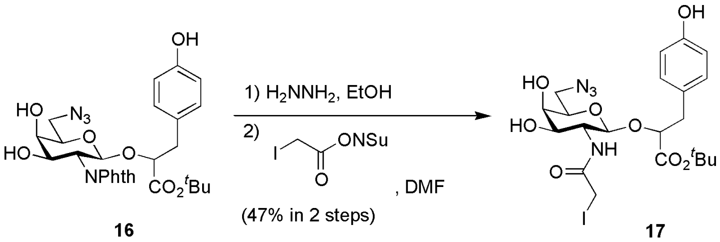

16 **17**

Compound **17**

Compound **16**^{22}) (22.1 mg, 39.8 mmol) was dissolved in ethanol (2 mL), and hydrazine monohydrate (40 μL) was added to this solution. After overnight stirring, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc, washed with H_2O, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give crude amine (22.3 mg). Iodoacetic acid N-hydroxysuccinimide ester (22.6 mg, 79.8 μmol) was added to a DMF solution of crude amine (22.3 mg, crude) (1 mL), and the mixture was stirred for 45 minutes at room temperature. The reaction was quenched with AcOH. After dried, the residue was purified by pTLC ($CHCl_3$/MeOH = 10/1) to give **17** (11.1 mg, 47% in two steps).

^{1}H NMR (500 MHz, CD_3OD) ™ 7.04 (d, *J* = 8.5 Hz, 2H), 6.65 (d, *J* = 8.5 Hz, 2H), 4.53 (d, *J* = 8.5 Hz, 1H), 4.51 (t, *J* = 6.0 Hz, 1H), 3.92 (dd, *J* = 10.5, 8.5 Hz, 1H), 3.75 (s, 2H), 3.72 (d, *J* = 3.0 Hz, 1H), 3.66 (m, 1H), 3.22 (q, *J* = 8.5 Hz, 1H), 2.97 (dd, *J* = 14.0, 6.0 Hz, 1H), 2.90 (dd, *J* = 14.0, 6.0 Hz, 1H), 1.33 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.3, 171.8, 157.1, 132.0, 128.4, 115.8, 101.0, 82.9, 77.7, 76.2, 72.7, 70.2, 54.8, 52.5, 39.3, 28.3, -1.21; IR (film) 3319, 2979, 2931, 2100, 1720, 1656, 1517, 1369, 1250, 1154, 1114, 1073, 757cm^{-1}; $[\alpha]_D^{27}$ -24.4 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* [M+Na]$^+$ calcd for $C_{21}H_{29}IN_4O_8$, 615.0928, found 615.0935.

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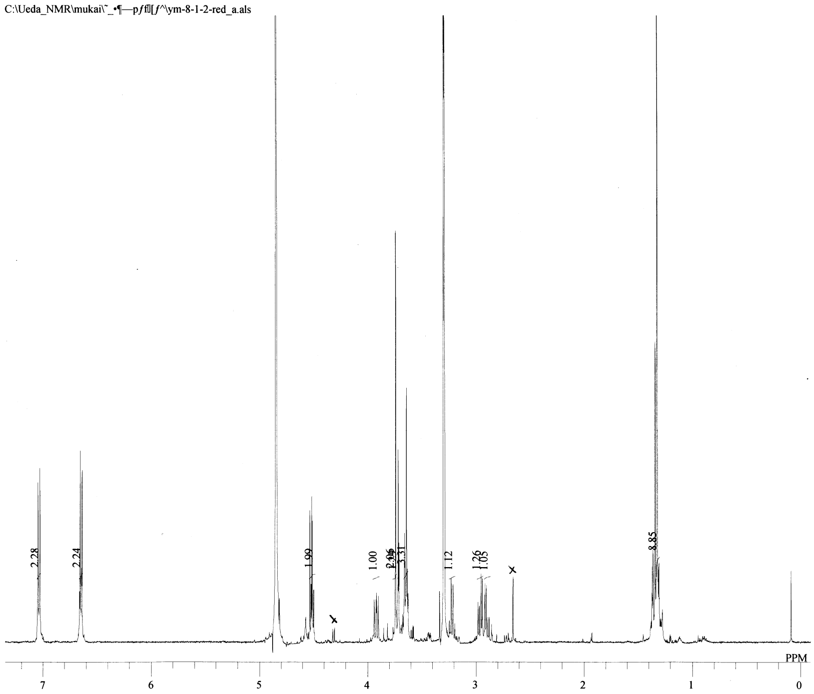

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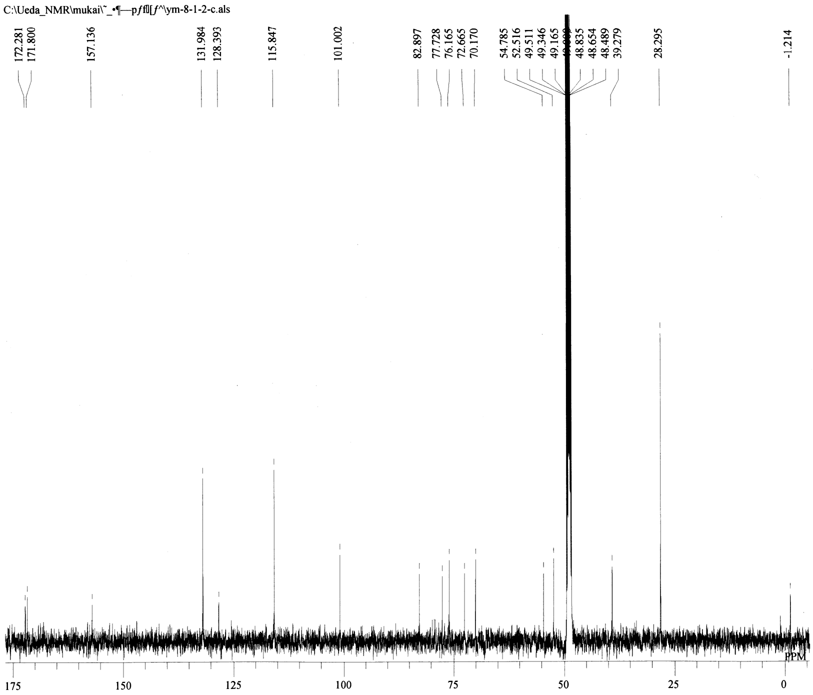

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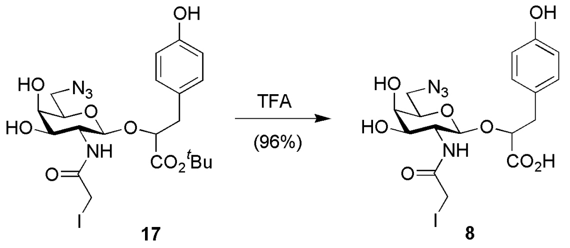

Compound **8**

TFA (1 mL) was added to compound **17** (5.3 mg, 8.95 mmol) in a 10-mL round-bottom flask. After staring for 5 minutes, the reaction mixture was dried *in vacuo* using a vacuum line. The residue was purified by HPLC [COSMOSIL 5C18-AR (ϕ 4.6 × 250 mm), 25% CH_3CNaq. containing 0.1% TFA, 5 mL / min.] to give **8** (4.6 mg, 96%).

^1H NMR (500 MHz, CD_3OD) ™ 7.05 (d, *J* = 8.5 Hz, 2H), 6.64 (d, *J* = 8.5 Hz, 2H), 4.58 (t, *J* = 6.0 Hz, 1H), 4.54 (d, *J* = 8.0 Hz, 1H), 3.91 (dd, *J* = 11.0, 8.0 Hz, 1H), 3.71 (d, *J* = 3.0 Hz, 1H), 3.70 (s, 2H), 3.64 (m, 3H), 3.23 (q, *J* = 8.5 Hz, 1H), 2.99 (dd, *J* = 14.5, 6.0 Hz, 1H), 2.96 (dd, *J* = 14.5, 6.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 181.2, 174.9, 157.1, 131.9, 128.6, 115.9, 101.2, 77.8, 76.1, 72.9, 70.2, 54.9, 52.5, 39.1, -1.41; IR (film) 3290, 2924, 2358, 2342, 2105, 1733, 1653, 1558, 1541, 1517, 1457, 1122, 1069, 470, 456, 426 cm^{-1}; $[\alpha]_D^{27}$ -12.7 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{17}H_{21}IN_4O_8$, 559.0302, found 559.0317.

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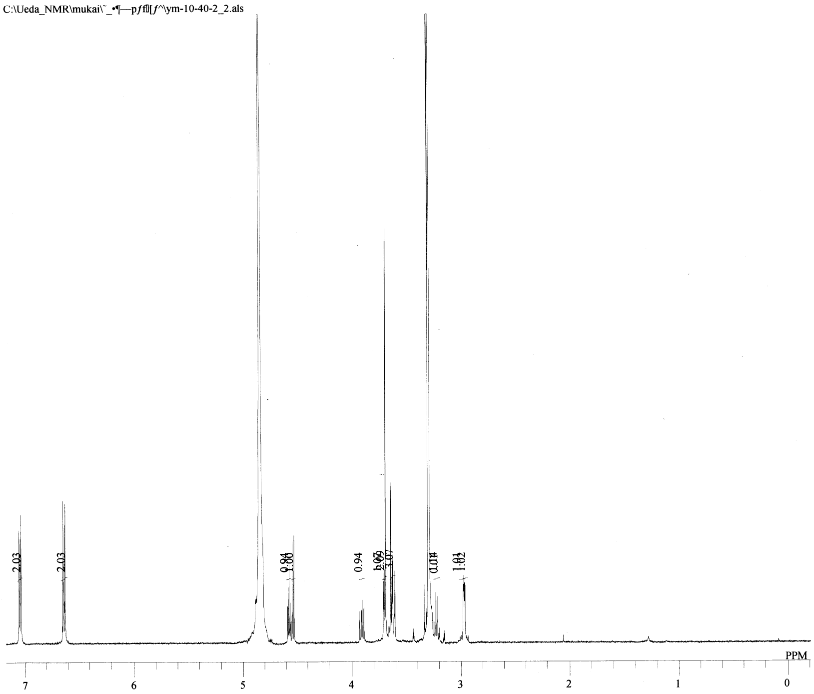

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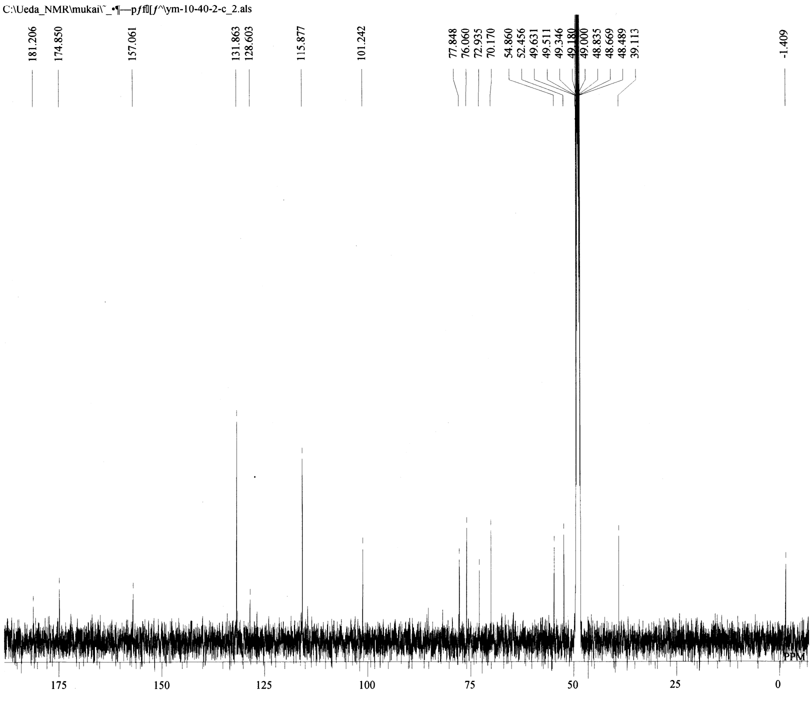

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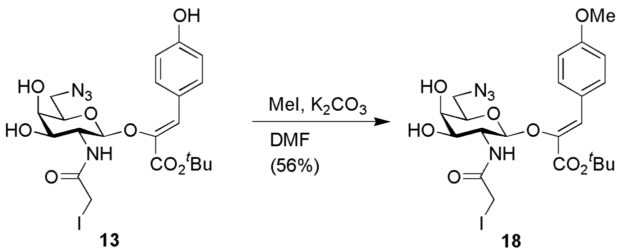
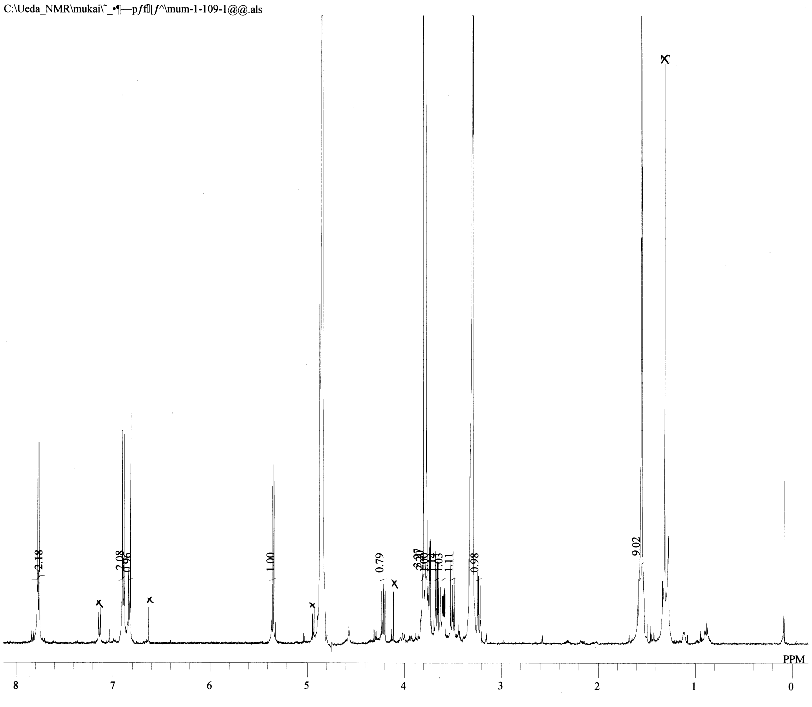


13 **18**

Compound **18**

K_2CO_3 (0.8 mg, 5.93 mmol) and MeI (3.7 ml, 59.3 mmol) were added to the solution of **13** (3.5 mg, 5.93 mmol) in DMF (0.6 mL). After the mixture was stirred for 4 h at room temperature, the reaction was quenched by the addition of water. After partition with EtOAc, the organic layer was dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated. The residue was purified by PLC ($CHCl_3$/MeOH = 10/1) to give **18** (2.0 mg, 56%).

^{1}H NMR (500 MHz, CD_3OD) ™ 7.68 (d, *J* = 9.0 Hz, 2H), 6.81 (d, *J* = 9.0 Hz, 2H), 6.74 (s, 1H), 5.26 (d, *J* = 8.5 Hz, 1H), 4.13 (dd, *J* = 11.0, 8.5 Hz, 1H), 3.72 (s, 3H), 3.69 (s, 2H), 3.65 (dd, *J* = 3.5, 1.0 Hz, 1H), 3.58 (dd, *J* = 11.0, 3.5 Hz, 1H), 3.51 (ddd, *J* = 8.5, 4.0, 1.0 Hz, 1H), 3.42 (dd, *J* = 13.0, 8.5 Hz, 1H), 3.14 (dd, *J* = 13.0, 4.0 Hz, 1H), 1.47 (s, 9H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.0, 164.8, 161.6, 141.0, 133.5, 127.4, 125.2, 114.8, 100.5, 82.9, 76.3, 73.0, 70.1, 55.7, 54.8, 52.5, 28.5, -1.67; IR (film) 3309, 3084, 2931, 2102, 1704, 1604, 1551, 1510, 1369, 1320, 1303, 1254, 1159, 1119, 1074, 1034, 757 cm^{-1}; $[\alpha]_D^{27}$ -60.7 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{22}H_{29}IN_4O_8$, 627.0928, found 627.0951.



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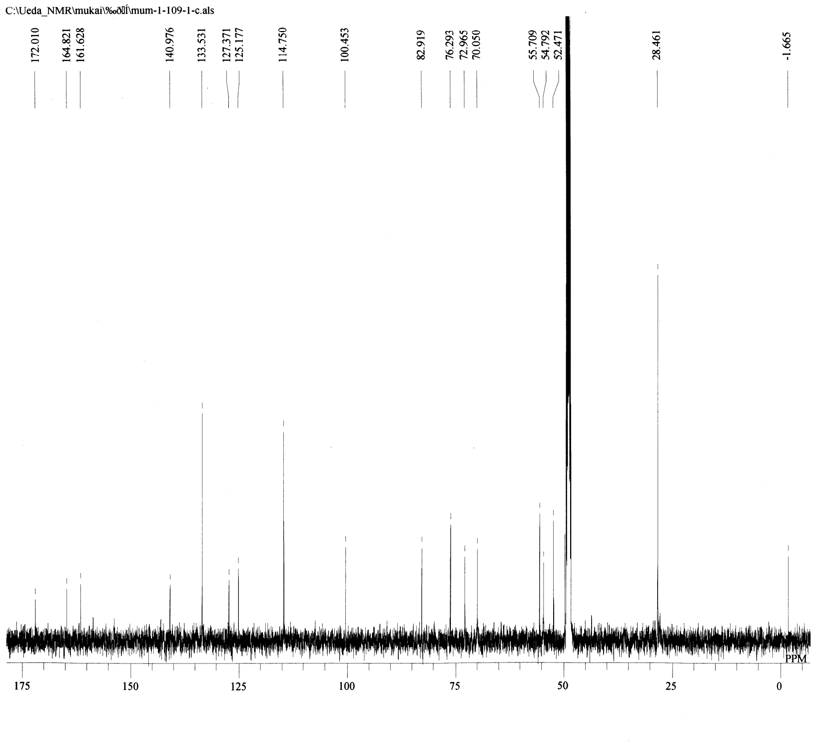

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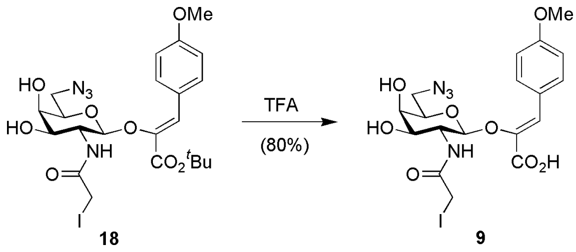
BF	2.00 Hz
RGAIN	22

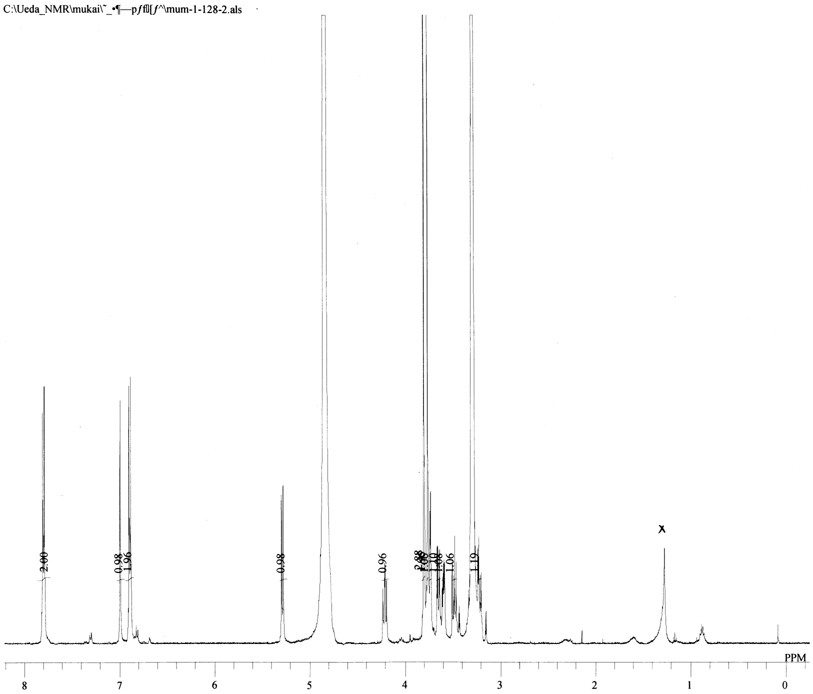


Compound **9**

TFA (1 mL) was added to compound **18** (2.5 mg, 4.1 mmol) in a 10-mL round-bottom. After staring for 5 minutes, the reaction mixture was dried up *in vacuo* using a vacuum line. The residue was purified by HPLC [COSMOSIL 5C18-AR (ϕ 4.6 × 250 mm), 35% CH_3CNaq. containing 0.1% TFA, 5 mL / min.] to give **9** (1.8 mg, 80%).

^{1}H NMR (500 MHz, CD_3OD) ™ 7.81 (d, *J* = 9.0 Hz, 2H), 7.00 (s, 1H), 6.90 (*J* = 9.0 Hz, 2H), 5.30 (d, *J* = 8.5 Hz, 1H), 4.22 (dd, *J* = 10.5, 8.5 Hz, 1H), 3.81 (s, 3H), 3.77 (s, 2H), 3.74 (dd, *J* = 3.5, 1.0 Hz, 1H), 3.66 (dd, *J* = 10.5, 3.5 Hz, 1H), 3.61 (ddd, *J* = 8.0, 4.5, 1.0 Hz, 1H), 3.49 (dd, *J* = 13.0, 8.0 Hz, 1H), 3.23 (dd, *J* = 13.0, 4.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 172.1, 167.4, 161.8, 140.5, 133.7, 127.3, 126.6, 114.7, 101.1, 76.2, 73.3, 70.0, 55.7, 55.0, 52.3, -1.70; IR (film) 3309, 2925, 2854, 2104, 1700, 1604, 1558, 1511, 1458, 1424, 1303, 1254, 1175, 1121, 1069, 1030, 883, 831 cm^{-1}; $[\alpha]_D^{27}$ -41.4 (*c* 0.10, MeOH); HRMS (ESI, positive) *m/z* $[M+Na]^+$ calcd for $C_{18}H_{21}IN_4O_8$, 571.0302, found 571.0327.

C:\Ueda_NMR\mukai\˜_•¶—pƒfl[ƒ^\mum-1-128-2.als



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PD	1.6587 sec
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SLVNT	CD3OD
EXREF	3.30 ppm
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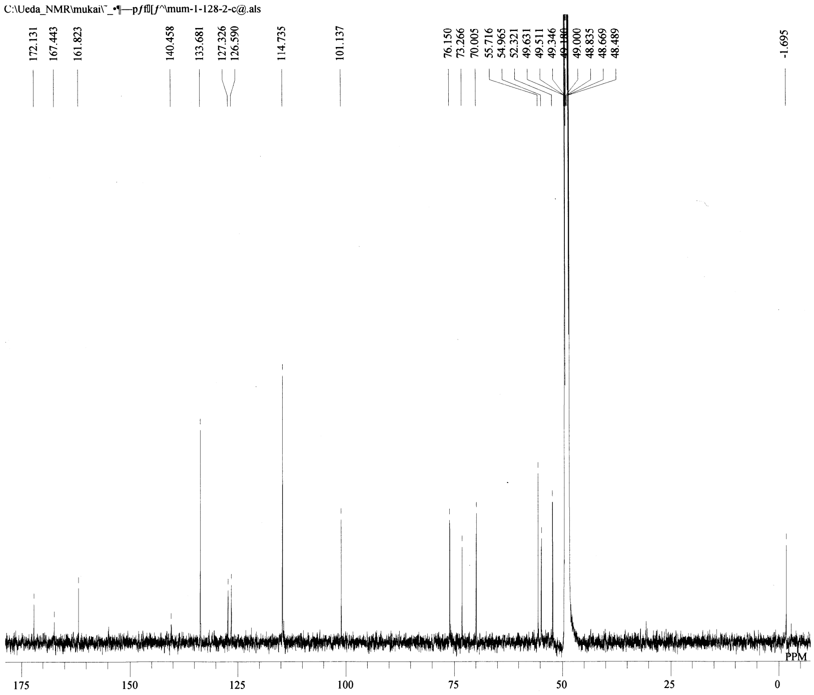
C:\Ueda_NMR\mukai\˜_•¶—pƒfl[ƒ^\mum-1-128-2-c@.als



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OBSET	0.00 KHz
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POINT	16384
FREQU	30959.75 Hz
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ACQTM	0.5292 sec
PD	2.5000 sec
PW1	5.00 usec
IRNUC	1H
CTEMP	29.9 c
SLVNT	CD3OD
EXREF	49.00 ppm
BF	2.00 Hz
RGAIN	22

FLAG-tagging of CTPL using protoplasts of *Cassia occidentalis* motor cell

Protoplasts were prepared from pulvini of *Cassia occidentalis* as described in Supporting Information of ref.5. Each probe (**2**, **3**, **4**, **8**, **9** at 6×10^{-10} mol) was added to a suspension of protoplasts (about 5×10^{4} protoplasts in 20 μL of wash solution [25 mM HEPES-KOH (pH 7), Complete protease inhibitor cocktail (Roche, 1 tablet/500 mL), 0.6 M sorbitol]). Crosslinking with CTPL was conducted at 4 °C as follows: crosslinking using iodoacetoamide-type probes (**2**, **8**, and **9**) was achieved by incubation for 5 min, whereas photocrosslinking using photoaffinity-type probes (**3** and **4**) was achieved by UV irradiation (365 nm, 1820 $\mu W/cm^2$) at a distance of 5 cm from UV lamp for 30 min with ice-cooling at 4 °C. After crosslinking, the protoplasts were sedimented twice by centrifugation (100 x *g*, 7 min, 4 °C) with wash solution and the supernatant was decanted. The sediment was resuspended in 25 mM HEPES buffer (pH 8) (6 μL); FLAG unit **5** (1×10^{-9} mol) in 2 μL of 25 mM HEPES buffer (pH 8) was added to this suspension. The CuAAC reaction was started by the addition of 2 μL of 25 mM HEPES buffer (pH 8)-5% DMSO solution containing ligand **6** (1×10^{-8} mol) and $[Cu(CH_3CN)_4]PF_6$ (1×10^{-8} mol, Aldrich). After incubation for 1 h at 30 °C, 40 μL of extraction buffer [25 mM Tris-MES buffer (pH 7.2) containing 0.25 M sucrose, 3 mM EDTA-2K, 2.5 mM DTT, and Complete protease inhibitor cocktail (1 tablet/mL)] was added to this solution and then the protoplasts were crushed by ultrasonification. Centrifugation of the lysate twice (1st: 25,000 × *g*, 15 min, 4 °C; 2nd: 100,000 × *g*, 1 h, 4 °C) gave a crude cytosolic homogenate as the supernatant (this fraction was designated crude CTPL) with a crude membrane fraction as the pellet. The crude cytosolic and membrane fractions were each suspended in 10 μL of extraction buffer. Electrophoresis buffer (0.3 M Tris-HCl buffer (pH 6.8) containing 10% SDS, 30% glycerol, and 9.3% DTT) was added to each fraction and each solution was heated at 95 °C for 5 min. The reaction mixtures were analyzed by SDS-PAGE (Ready Gel J 7.5% polyacrylamide gels, Bio-Rad Laboratories, Inc.) with a molecular weight marker. After western blotting using Hybond-P PVDF membrane (GE Healthcare UK), the membrane was washed twice with PBS-T buffer and then treated with an anti-FLAG IgG antibody (1:10,000; Delta Biolabs,), anti IgG-HRP conjugate (1:20,000; SCB), and anti-IgG-HRP conjugate (1:20,000; SCB,). Then, chemiluminescence detection was carried out using an ECL Advance western blotting detection kit (GE Healthcare UK) with an LAS-4000 Bioimager (Fujifilm Corp.).

Immunoprecipitation of CTPL

The crude CTPL was mixed with 4 volumes of acetone and allowed to stand for 1 h at -80 °C. After centrifugation (10,000 × *g*, 15 min, 4 ˚C), the pellet was dissolved in 100 μL of lysis buffer [50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100]. Anti-FLAG M2 (mouse IgG1)-Agarose Affinity Gel (10 μL; Sigma Co.) was added to this solution, and then the mixture was incubated for 15 h at 4 ˚C. After centrifugation (5,000 × *g*, 4 ˚C, 30 sec), the supernatant was lyophilized, whereas the precipitate was suspended in wash buffer [50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl] and washed by centrifuging five times (5,000 × *g*, 4 ˚C, 30 sec). The precipitate was suspended in 50 μL of wash buffer, and FLAG-tagged CTPL was eluted by incubation with 2 μL 3×FLAG (5 μg/5 μL, Sigma) at 4 °C for 1 h. After centrifugation (5,000 × *g*, 4 ˚C, 30 sec), the supernatant was lyophilized to give purified CTPL. The purified CTPL was analyzed using an ECL Advance western blotting detection kit (GE Healthcare UK) or silver-staining kit (Wako Co., Ltd.). The amount of purified CTPL and the ratio of supernatant to precipitate in Figure 5B were obtained using an LAS-4000 Bioimager (Fujifilm Corp.). The amount of purified CTPL was estimated from a comparison of the CTPL band with a standard protein in silver staining.

Microsequencing analysis of CTPL

Purified CTPL (5 ng) was analyzed by SDS-PAGE (1 ng × 5 lanes), and stained using Silver Stain MS Kit (Wako Pure Chemical Industries, Ltd.). Protein bands were excised from the gel, destained, and in gel digested with trypsin at 35 ˚C for 20 hours. The peptide fragments were analyzed using Positive mode nanoflow-LC ESI MS (Q-Tof2, Waters Micromass, UK) equipped with L-column ODS (*f* 0.1 × 50 mm). The linear gradient conditions were set as follows: 95% A : 5% B to 45% A : 55% B during 0-35 min (Solvent A: 2% $CH_3CNaq.$ containing 0.1% HCOOH, Solvent B: 90% $CH_3CNaq.$ containing 0.1% HCOOH).