

Supplementary Information

Turn-ON Fluorescent Affinity Labeling Using a Small Bifunctional *O*-Nitrobenzoxadiazole Unit

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Supplementary Results

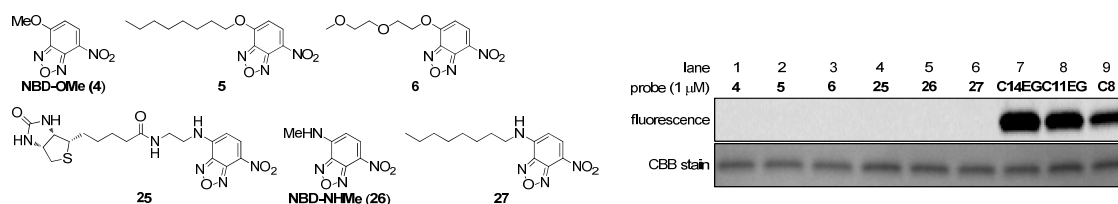


Figure S1. In-gel fluorescence analysis of avidin treated with *O*-NBD or *N*-NBD compounds (fluorescence image at 530 nm after excitation at 488 nm). Labeling conditions: 1 μM avidin, 1 μM compound, 25 mM phosphate buffer, pH 7.0, 0 °C, and 1 h. Comments: No labeling was observed when avidin was treated with *O*-NBD compounds lacking the ligand unit.

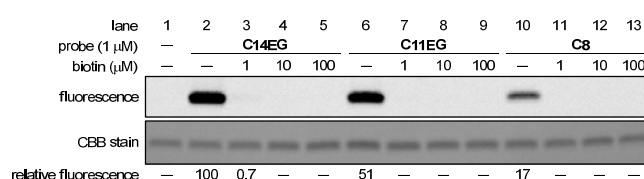


Figure S2. In-gel fluorescence analysis of avidin treated with *O*-NBD probes in the absence or presence of biotin (fluorescence image at 530 nm after excitation at 488 nm). Labeling conditions: 1 μM avidin, 1 μM *O*-NBD probe, 25 mM phosphate buffer, pH 7.0, 0 °C, and 1 h. In lanes 3–5, 7–9, and 11–13, avidin was preincubated with 1–100 μM biotin for 1 h at 0 °C before addition of *O*-NBD probe.

Comments: Pretreatment with 1 equivalent of biotin completely blocked the labeling.

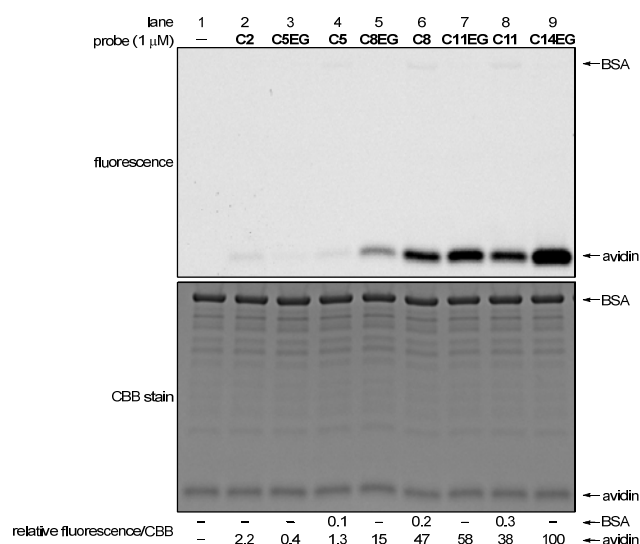
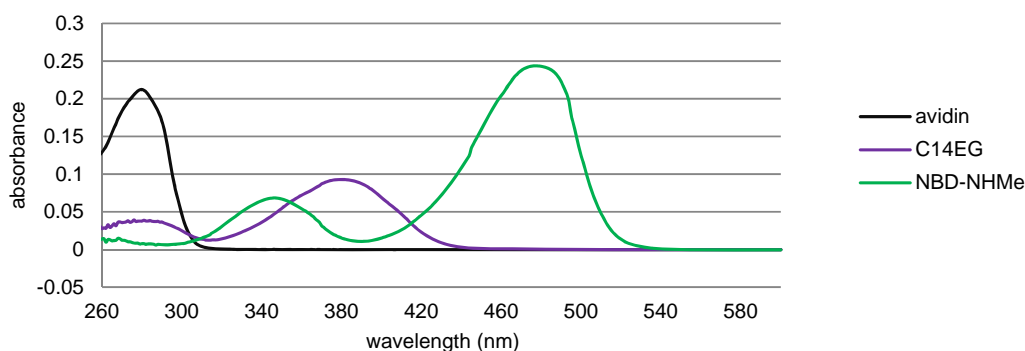


Figure S3. In-gel fluorescence analysis of a mixture of avidin and BSA treated with biotin *O*-NBD probes (fluorescence image at 530 nm after excitation at 488 nm). Labeling conditions: 1 μM avidin, 1 μM BSA, 1 μM biotin *O*-NBD probe, 25 mM phosphate buffer, pH 7.0, 0 °C, for 1 h.

Comments: Selective labeling of avidin was achieved in the presence of a non-target protein, BSA, which contains about sixty lysine residues. The probes having ethylene glycol linkers (C8EG, C11EG and C14EG) exhibited extremely high target-selectivity (>99.9%).

Comments: Increase in the labeling temperature from 0 °C to room temperature resulted in faster labeling, but did not reduce the target-selectivity.



Comments: The absorbance of NBD-NHMe at 478 nm was found to be 0.244.

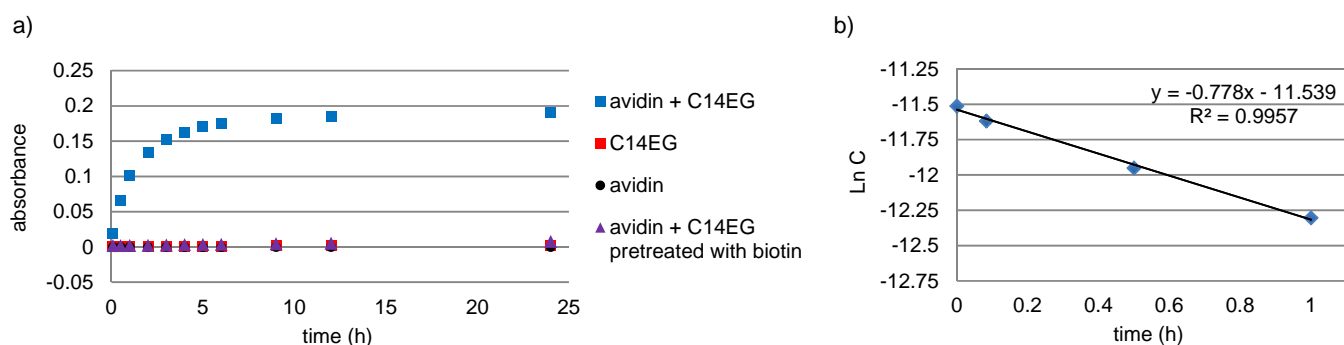


Figure S6. (a) Time course of the absorbance at 478 nm. Conditions for blue squares: 10 μ M avidin, 10 μ M **C14EG**, 25 mM phosphate buffer, pH 7.0, and room temperature. Conditions for red squares: 10 μ M **C14EG**, 25 mM phosphate buffer, pH 7.0, and room temperature. Conditions for black circles: 10 μ M avidin, 25 mM phosphate buffer, pH 7.0, and room temperature. Conditions for purple triangles: 10 μ M avidin (preincubated with 100 μ M biotin for 1 h at room temperature before addition of **C14EG**), 10 μ M **C14EG**, 25 mM phosphate buffer, pH 7.0, and room temperature. Absorbance of a mixture of avidin and **C14EG** at 478 nm: 0.019 (5 min), 0.066 (30 min), 0.101 (1 h), 0.134 (2 h), 0.152 (3 h), 0.163 (4 h), 0.171 (5 h), 0.175 (6 h), 0.182 (9 h), 0.185 (12 h), and 0.190 (24 h). (b) Plots of the reaction time versus Ln C (concentration of **C14EG** (M)): 10 μ M avidin, 10 μ M **C14EG**, 25 mM phosphate buffer, pH 7.0, and room temperature. Concentration of **C14EG** was calculated from UV absorbance at 478 nm, regarding “absorbance = 0.185 (absorbance at 12 h)” as full conversion.

Comments: The labeling yield was calculated based on the absorbance at 478 nm. For example, the labeling yield after 12 h was found to be 76% (0.185/0.244). According to the supplier’s data sheet, avidin used in our experiments has ~ 80% biotin-binding activity (12 units/mg). This would be a key reason to why the maximum labeling yield was 78% (after 24 h). The reaction rate constant of avidin and **C14EG** at room temperature (23 °C) was found to be 0.78/h.

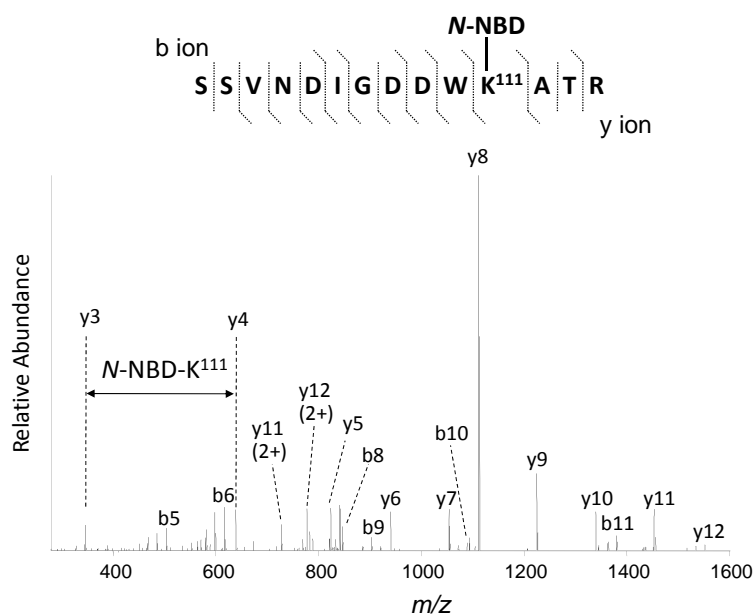


Figure S7. Enlarged MS/MS spectrum from ion at m/z 863.86 (see also Figure 4c). The labeling of avidin was carried out under the following conditions: 1 μ M avidin, 1 μ M **C14EG**, 25 mM phosphate buffer, pH 7.0, 0 °C, for 1 h. Main fragmentation series (y-carboxy and b-amino) are indicated. The details are summarized in Table S1.

Comment: The MS/MS analysis identified the labeled amino acid residue as K111, which is located near the biotin binding-site.

Table S1. Simulated fragment ion pattern of NBD-modified peptide [SSVNDIGDDWK¹¹¹(N-NBD)ATR]. Bold blue text indicates the ions assigned by MASCOT search.

b#	b (+)	b (2+)	sequence	y (+)	y (2+)	y#
1	88.039	44.5233	S			14
2	175.071	88.039	S	1639.710	820.358	13
3	274.140	137.574	V	1552.678	776.842	12
4	388.183	194.595	N	1453.609	727.308	11
5	503.210	252.108	D	1339.566	670.287	10
6	616.294	308.651	I	1224.539	612.773	9
7	673.315	337.161	G	1111.455	556.231	8
8	788.342	394.675	D	1054.434	527.721	7
9	903.369	452.188	D	939.407	470.207	6
10	1089.448	545.228	W	824.380	412.694	5
11	1380.545	690.776	N-NBD-K111	638.301	319.654	4
12	1451.582	726.295	A	347.204	174.106	3
13	1552.630	776.819	T	276.167	138.587	2
14			R	175.119	88.063	1

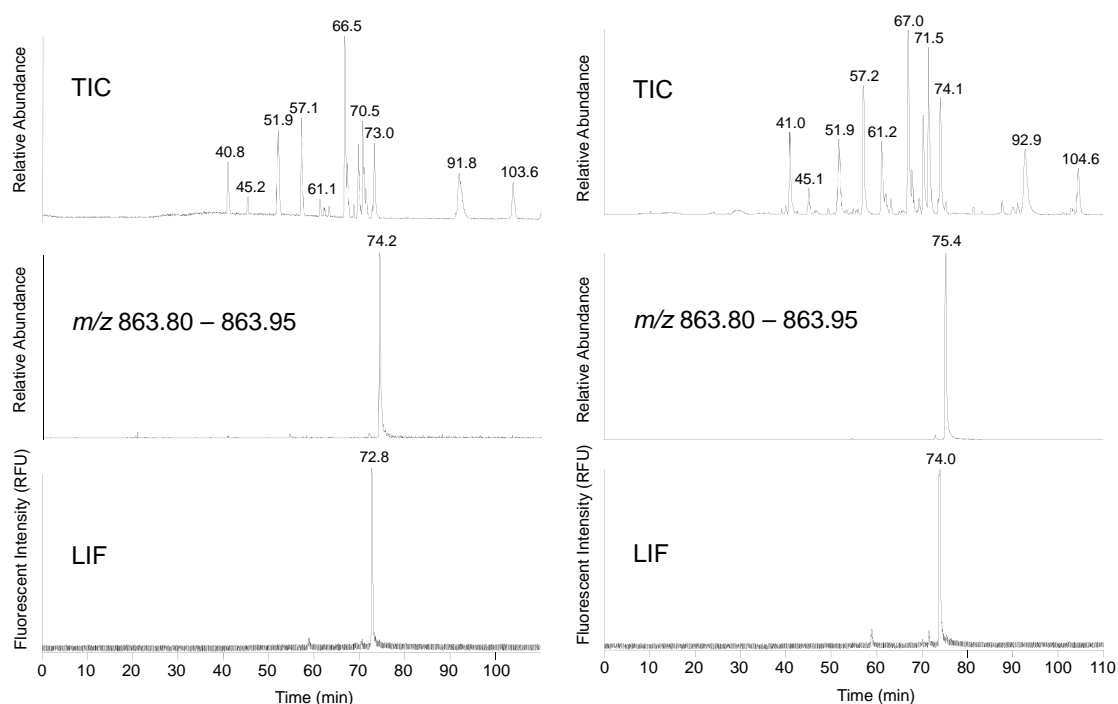


Figure S8. LC-LIF-MS analysis of trypsin digests of avidin labeled with **C8** (left) or **C11EG** (right). Labeling of avidin was carried out under the following conditions: 1 μ M avidin, 1 μ M *O*-NBD probe, 25 mM phosphate buffer, pH 7.0, 0 $^{\circ}$ C, and 1 h. The labeled avidin was subjected to in-gel tryptic digestion and analyzed by LC-LIF-MS. Chromatograms monitored by total ion current (TIC) (top), extracted ion in the range of m/z 863.80–863.95, corresponding to N-NBD-[Ser101-Arg114] (middle), and laser-induced fluorescence (LIF) detector (bottom, λ_{ex} = 473 nm, λ_{em} = 500–600 nm). The delay time from the fluorescence chromatogram to the ion chromatogram was determined to be 1.4 min by using authentic fluorescent peptides.

Comments: Avidin labeled with **C8** (or **C11EG**) exhibited a single peak in the LIF chromatogram. We analyzed this peak (MS/MS analysis), and identified the NBD-modified peptide residue as K111 [SSVNDIGDDWK¹¹¹(N-NBD)ATR].

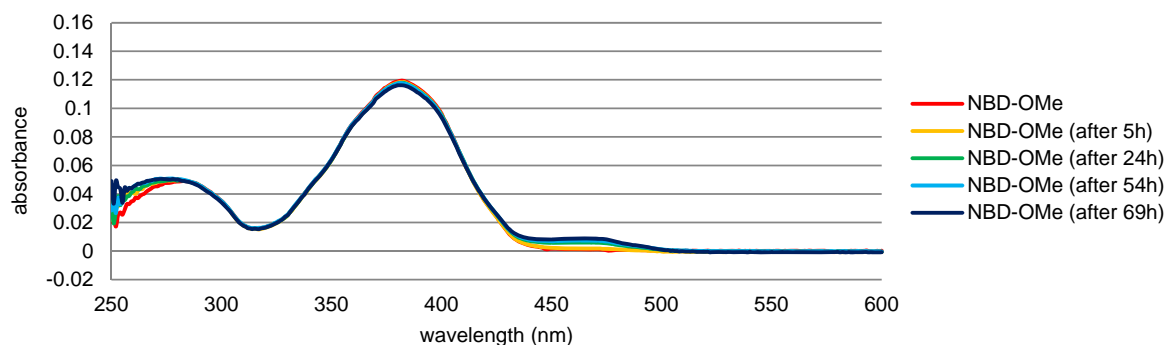


Figure S9. Time-dependent spectral change of NBD-OMe: 10 μ M NBD-OMe in 25 mM phosphate buffer (pH 7.0) after 0 min (red), 5 h (yellow), 24 h (green), 54 h (light blue), and 69 h (navy blue).

Comments: NBD-OMe was slightly hydrolyzed in phosphate buffer after 69 h at room temperature.

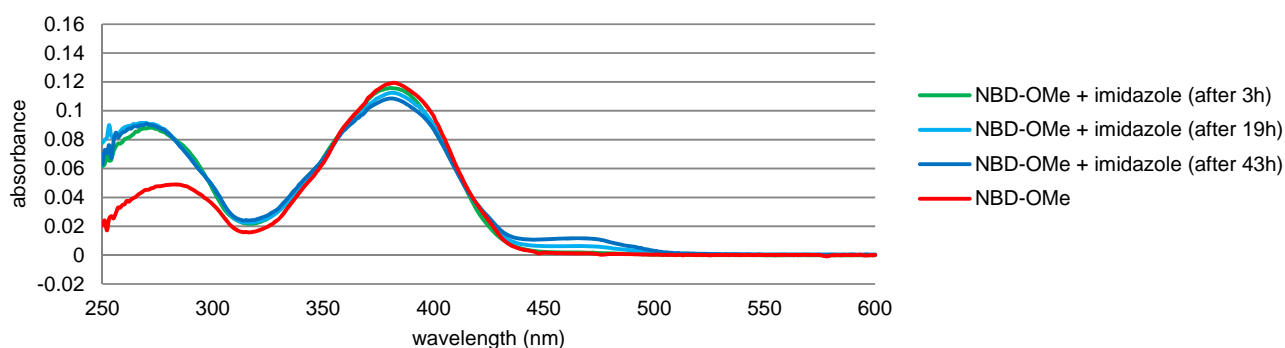


Figure S10. Time-dependent spectral change of NBD-OMe in the presence of imidazole: 10 μ M NBD-OMe and 10 mM imidazole in 25 mM phosphate buffer (pH 7.0) after 3 h (green), 19 h (light blue), and 43 h (blue). The absorption spectrum of NBD-OMe is shown by a red line.

Comments: Almost no reaction took place when imidazole was added to NBD-OMe, although the hydrolysis of NBD-OMe seemed to be slightly accelerated.

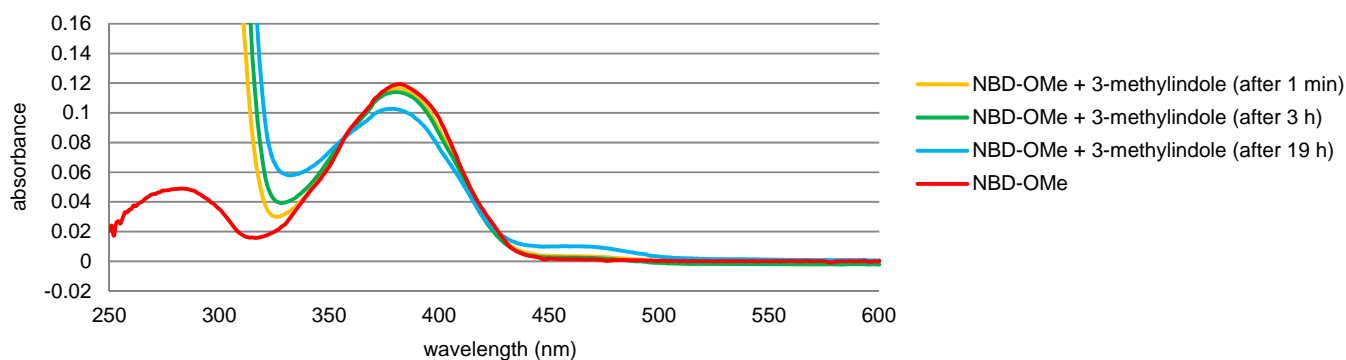


Figure S11. Time-dependent spectral change of NBD-OMe in the presence of 3-methylindole: 10 μ M NBD-OMe and 10 mM 3-methylindole in 25 mM phosphate buffer (pH 7.0) after 1 min (yellow), 3 h (green), and 19 h (light blue). The absorption spectrum of NBD-OMe is shown by a red line.

Comments: Almost no reaction took place when 3-methylindole was added to NBD-OMe, although the hydrolysis of NBD-OMe seemed to be slightly accelerated.

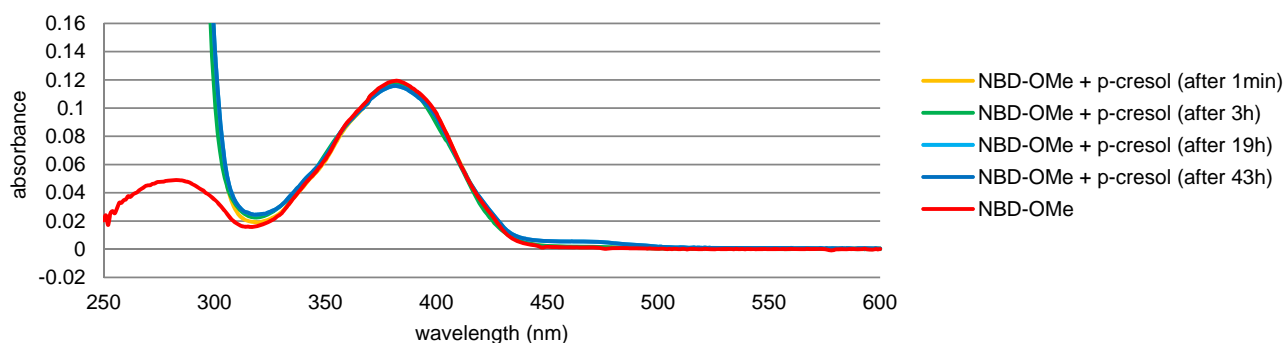


Figure S12. Time-dependent spectral change of NBD-OMe in the presence of *p*-cresol: 10 μ M NBD-OMe and 10 mM *p*-cresol in 25 mM phosphate buffer (pH 7.0) after 1 min (yellow), 3 h (green), 19 h (light blue), and 43 h (blue). The absorption spectrum of NBD-OMe is shown by a red line.

Comments: No reaction took place when *p*-cresol was added to NBD-OMe.

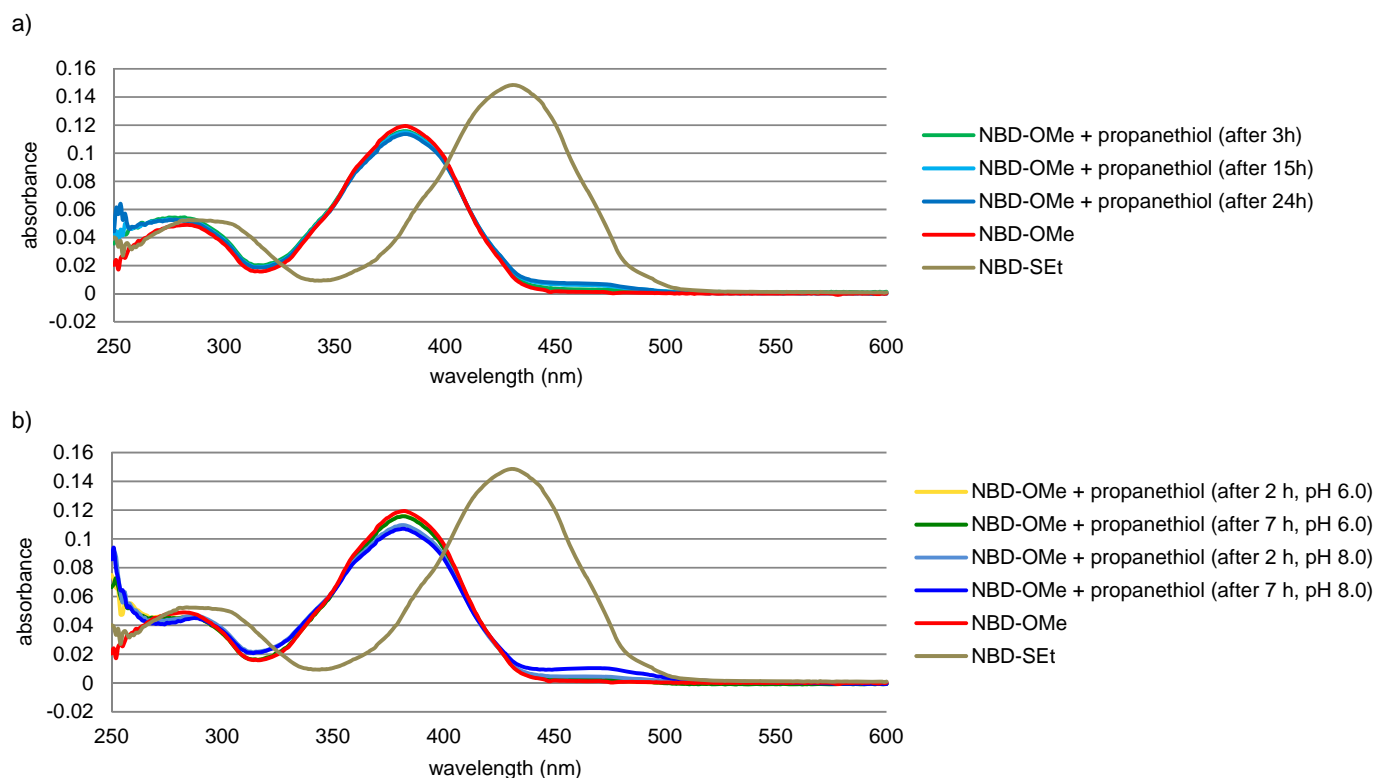


Figure S13. (a) Time-dependent spectral change of NBD-OMe in the presence of 1-propanethiol: 10 μ M NBD-OMe and 10 mM propanethiol in 25 mM phosphate buffer (pH 7.0) after 3 h (green), 15 h (light blue), and 24 h (blue). The absorption spectra of NBD-OMe and NBD-SEt are shown by red and other lines, respectively. (b) Time-dependent spectral change of NBD-OMe in the presence of 1-propanethiol at pH 6.0 or 8.0: 10 μ M NBD-OMe and 10 mM propanethiol in 25 mM phosphate buffer (pH 6.0 or 8.0).

Comments: No reaction took place when 1-propanethiol was added to NBD-OMe, although the hydrolysis of NBD-OMe seemed to be slightly accelerated under basic conditions (pH 8.0).

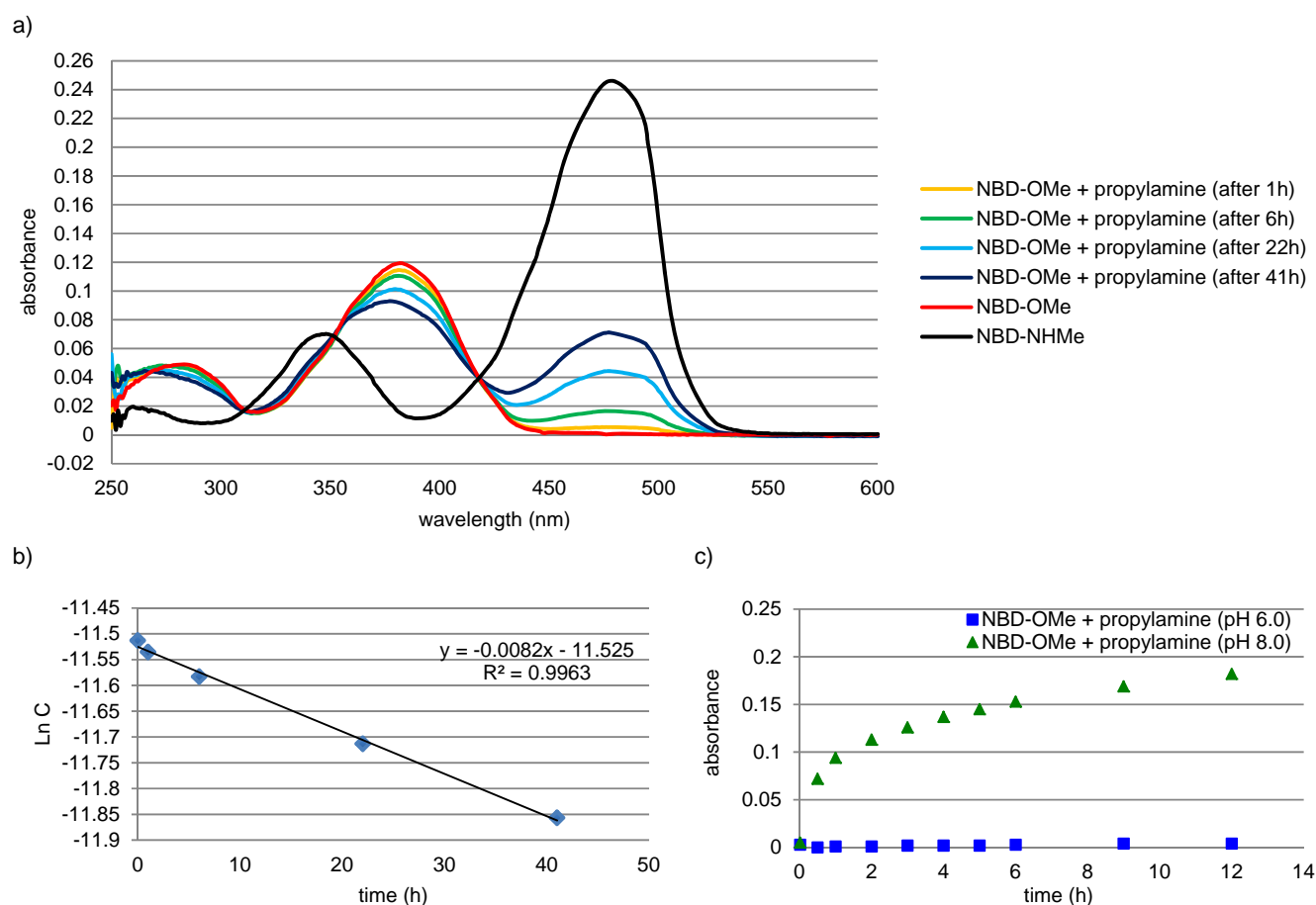


Figure S14. (a) Time-dependent spectral change of NBD-OMe in the presence of 1-propylamine: 10 μ M NBD-OMe and 10 mM propylamine in 25 mM phosphate buffer (pH 7.0) after 1 h (yellow), 6 h (green), 22 h (light blue), and 41 h (blue). The absorption spectra of NBD-OMe and NBD-NHMe are shown by red and black lines. (b) Plots of the reaction time versus $\ln C$ (concentration of NBD-OMe (M)). Conditions: 10 μ M NBD-OMe and 10 mM propylamine in 25 mM phosphate buffer (pH 7.0) at room temperature. The concentration of NBD-OMe was calculated from UV absorbance at 478 nm regarding “absorbance = 0.244” as full conversion (see also Figure S5). (c) Time course of the UV absorbance at 478 nm of the mixture of NBD-OMe and 1-propylamine. Conditions: 10 μ M NBD-OMe, 10 mM 1-propylamine, 25 mM phosphate buffer, pH 6.0 (blue squares) or pH 8.0 (green triangles), and room temperature.

Comments: 1-Propylamine (1000 equiv.) slowly reacted with NBD-OMe to give amino NBD at pH 7.0. The reaction yield after 6 h was calculated to be 6.8% based on the absorbance at 478 nm. The reaction rate constant of NBD-OMe and 1-propylamine at pH 7.0 was found to be 0.0082/h (pseudo-first-order reaction). The reaction was accelerated under basic conditions (pH 8.0).

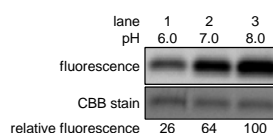


Figure S15. In-gel fluorescence analysis of avidin treated with C14EG at different pH values (fluorescence image at 530 nm after excitation at 488 nm). Labeling conditions: 1 μ M avidin, 1 μ M C14EG, 25 mM phosphate buffer, pH 6.0–8.0, 0 $^{\circ}$ C, and 1 h.

Comments: The labeling of avidin was accelerated under basic conditions, suggesting that deprotonation of K111 ($-\text{NH}_3^+$, amine salts) is involved in the labeling event.

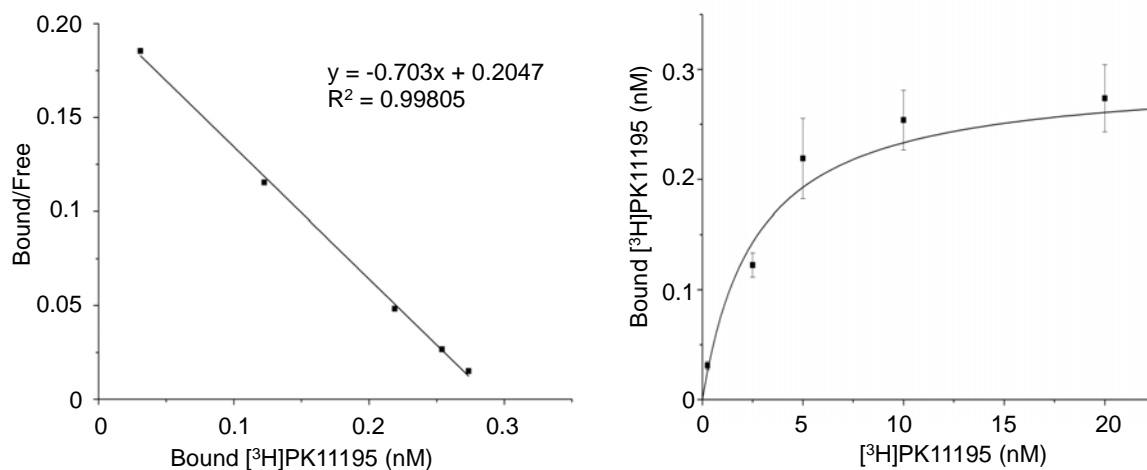


Figure S16. Scatchard plot and saturation curve of specific [³H]PK11195 binding to mouse kidney mitochondria. Assays were carried out utilizing 0.25–20 nM [³H]PK11195. Non-specific binding was measured in the presence of 1 μ M PK11195. Data are averages of the three experiments.

Comments: The K_d value of PK11195 was found to be 1.4 nM.

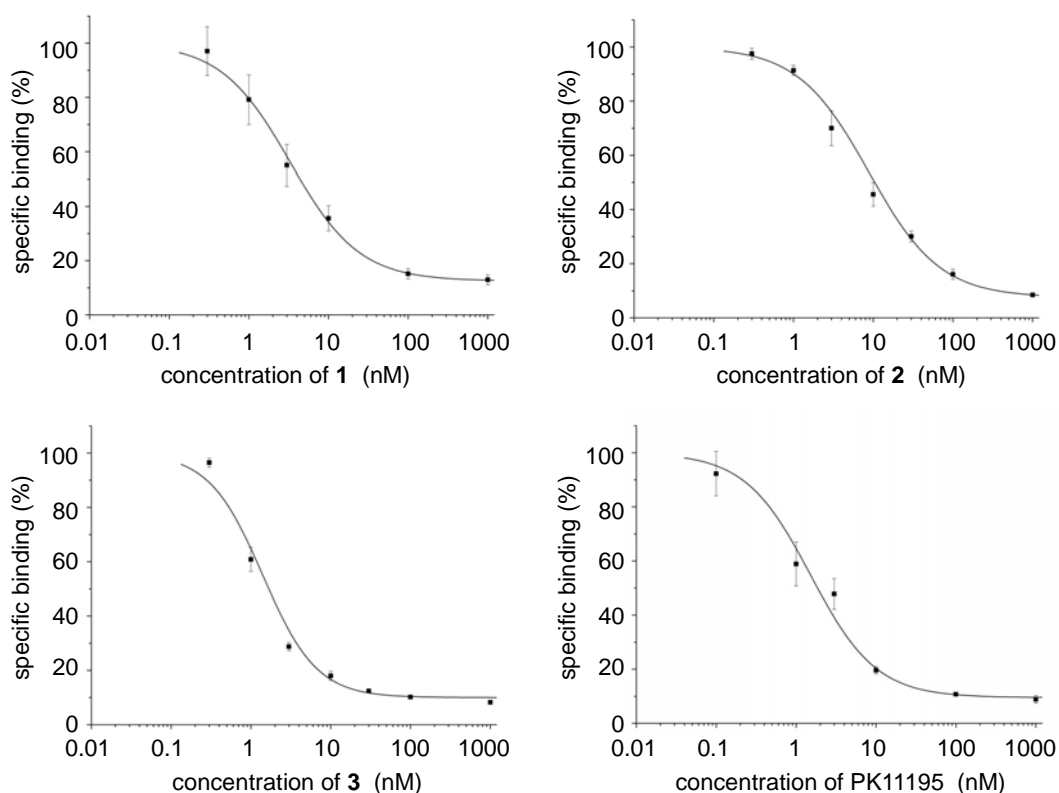


Figure S17. Displacement of [³H]PK11195 binding in mouse kidney mitochondria. Bound radioactivity is shown as a percentage of specific binding in the absence of competitor. Data points represent the means \pm SEM of triplicate determinations. IC_{50} values (nM): 4.4 (1), 9.8 (2), 1.7 (3), 1.9 (PK11195).

Table S2. Binding affinities of the synthetic compounds to TSPO.

compound	K_i (nM)
1	3.2
2	7.2
3	1.3
PK11195	1.4

The K_i values were derived from IC_{50} values in accordance with the equation of Cheng and Prusoff (see below).

$$K_i = IC_{50} / [1 + L / K_d(L)]$$

L: concentration of [3H]PK11195 (0.5 nM)

$K_d(L)$: K_d of PK11195 (1.4 nM)

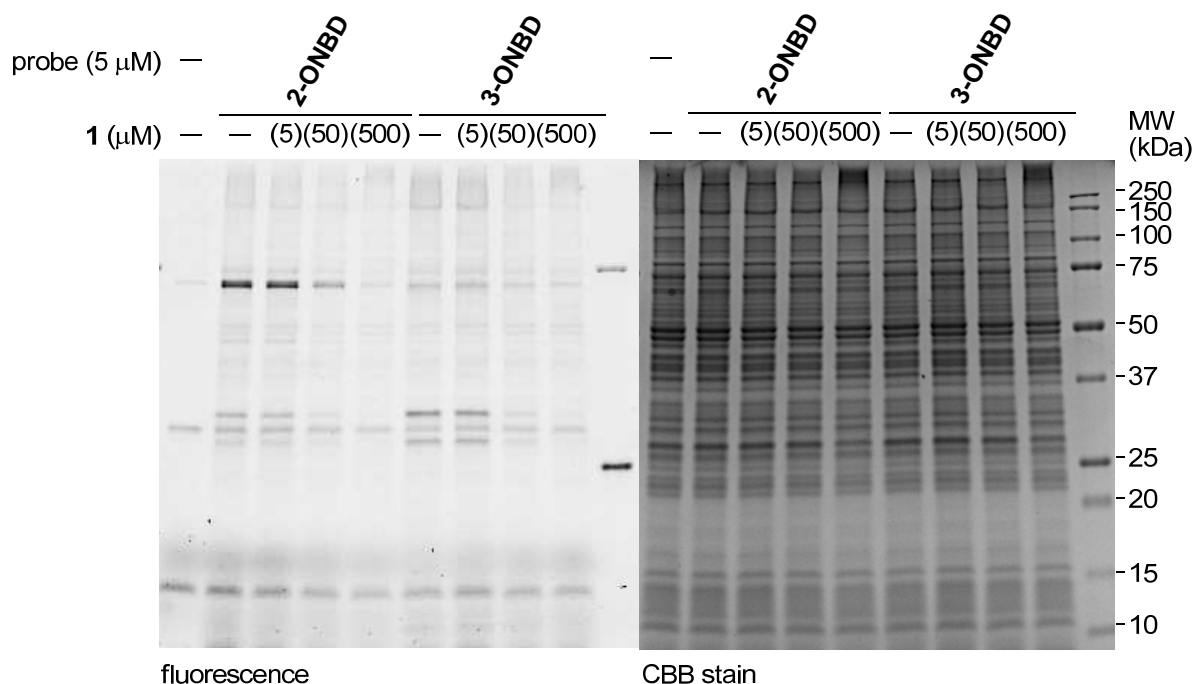


Figure S18. In-gel fluorescence analysis of mouse kidney mitochondria labeled with **2-ONBD** or **3-ONBD** in the absence/presence of **1** (fluorescence image at 530 nm after excitation at 488 nm). Labeling conditions: 0.15 mg/mL mitochondria, 5 μ M *O*-NBD probe, \pm **1** (5, 50 and 500 μ M), 5 mM Hepes-KOH, pH 7.2, 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 30 $^{\circ}$ C, for 1 h. In the competition analysis, the mitochondria were preincubated with **1** for 10 min before addition of *O*-NBD probes.

Comments: Labeling of 70, 32 and 28 kDa bands was inhibited by **1** in a dose-dependent manner, suggesting that these bands were specifically labeled in response to the target-ligand interactions. Although the use of 500 μ M **1** completely inhibited the labeling, we observed precipitation of **1**. Thus, this condition was not used for competition analyses.

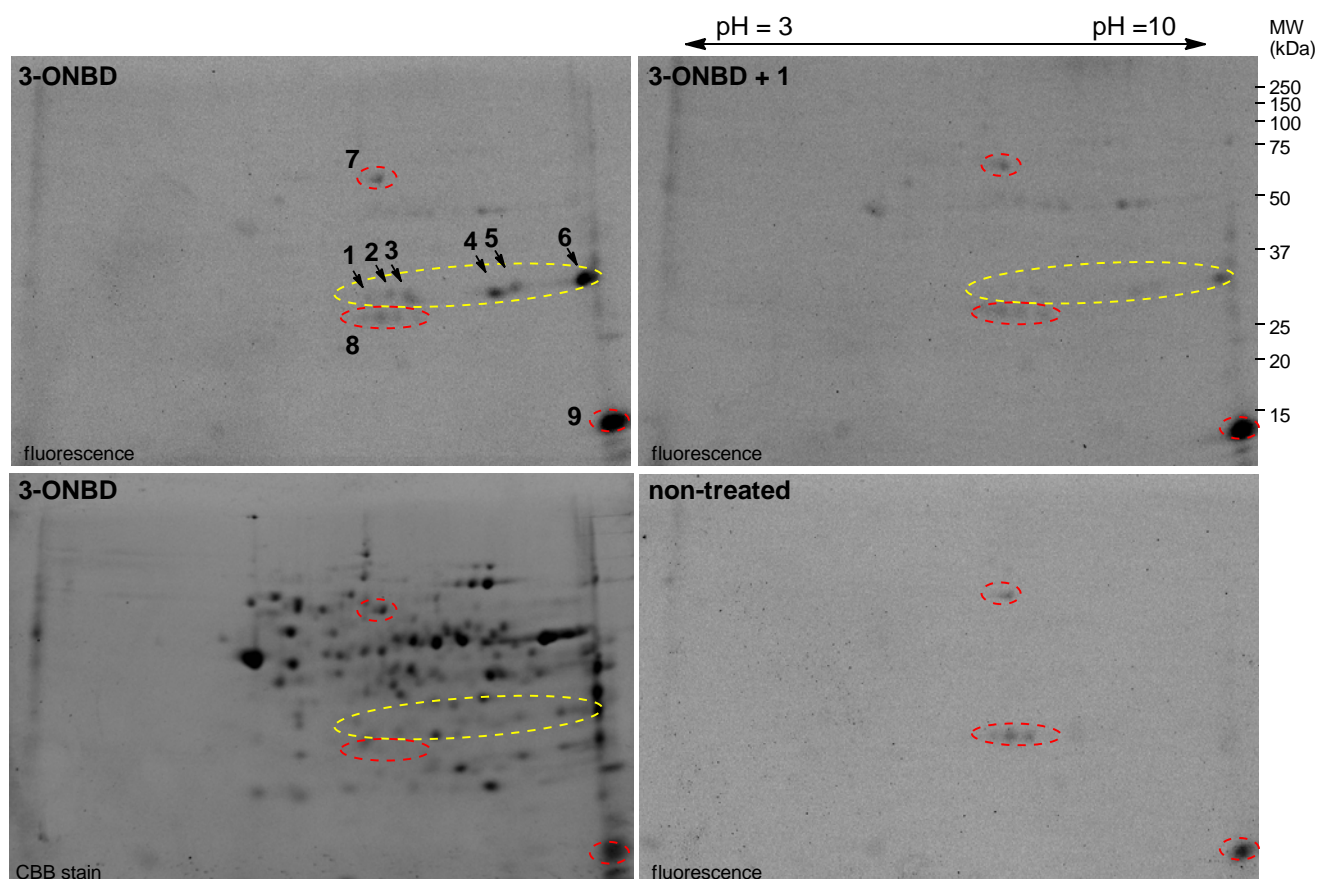


Figure S19. 2-D PAGE analysis of labeled mitochondria. Mitochondria labeled with **3-ONBD** were subjected to 2-D PAGE and visualized with an in-gel fluorescence imager (upper left) and by CBB staining (lower left). Labeling conditions: 5 μM **3-ONBD**, 0.15 mg/mL mouse kidney mitochondria, 5 mM Hepes-KOH, pH 7.2, 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 30 $^{\circ}\text{C}$, and 1 h. The upper right gel shows mitochondria labeled with 5 μM **3-ONBD** in the presence of 50 μM **1**. The lower right gel shows mitochondria not treated with any probe. The fluorescent spots encircled in red indicate autofluorescent proteins of mouse kidney mitochondria.

Comments: The spots circled in yellow almost completely disappeared after pretreatment with 50 μM **1**. Therefore, these fluorescent spots show specifically labeled proteins. Meanwhile, fluorescent spots around 40–55 kDa were not affected by pretreatment of **1** (see upper gels), thus, these spots show non-specifically labeled proteins. However, it is also obvious that these 40–55 kDa fluorescent spots contain abundant proteins of mouse kidney mitochondria as seen from the CBB stained gel (lower left gel). Thus, it is confirmed that **3-ONBD** selectively labels 32 kDa proteins (spots 1–6).

Table S3. Proteins identified from the fluorescent spots 1–9 (Figure S19, upper left gel). Each spot was subjected to in-gel tryptic digestion and analyzed by LC-MS. The identified proteins were ranked by ion score. The proteins in the top 5 or showing over 60% sequence coverage are listed. VDACs are indicated in blue.

spot	identified protein	accession number	M.W.	pI	score	sequence coverage (%)
1	delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor [Mus musculus]	gi 7949037	36095	7.6	2222	69
	citrate lyase beta subunit [Mus musculus]	gi 19073015	37495	8.76	2045	69
	electron transfer flavoprotein subunit alpha, mitochondrial [Mus musculus]	gi 227500281	34988	8.62	1394	66
	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	948	68
	fumarylacetoacetate hydrolase domain-containing protein 2A [Mus musculus]	gi 29366814	34654	8.42	809	62
2	citrate lyase beta subunit [Mus musculus]	gi 19073015	37495	8.76	2252	73
	fumarylacetoacetate hydrolase domain containing 2A, isoform CRA_a [Mus musculus]	gi 148696242	36351	8.73	1834	84
	electron transfer flavoprotein subunit alpha, mitochondrial [Mus musculus]	gi 227500281	34988	8.62	1054	68
	voltage-dependent anion-selective channel protein 2 [Mus musculus]	gi 6755965	31713	7.44	183	45
	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	126	44
3	electron transfer flavoprotein subunit alpha, mitochondrial [Mus musculus]	gi 227500281	34988	8.62	2927	83
	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	2099	83
	prostaglandin E synthase 2 [Mus musculus]	gi 260763900	43296	9.11	1558	63
	voltage-dependent anion-selective channel protein 2 [Mus musculus]	gi 6755965	31713	7.44	1218	63
	citrate lyase beta subunit [Mus musculus]	gi 19073015	37495	8.76	578	52
4	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	3878	82
	Sdha protein, partial [Mus musculus]	gi 15030102	72280	7.11	1228	24
	carbonyl reductase [NADPH] 1 [Mus musculus]	gi 113680352	30622	8.53	1005	64
	pyrroline-5-carboxylate reductase 2 [Mus musculus]	gi 19526878	33638	7.67	1004	55
	Electron transferring flavoprotein, alpha polypeptide [Mus musculus]	gi 13097375	35018	8.62	788	54
	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor [Mus musculus]	gi 111038118	34442	8.76	706	72
	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor [Mus musculus]	gi 111038118	34442	8.76	2489	85
5	thiosulfate sulfurtransferase [Mus musculus]	gi 6678449	33445	7.71	1290	53
	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	983	76
	Sdha protein, partial [Mus musculus]	gi 15030102	72280	7.11	440	22
	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	4365	86
	D-beta-hydroxybutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	gi 170014720	38274	9.14	2424	59
6	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor [Mus musculus]	gi 111038118	34442	8.76	1857	87
	V-type proton ATPase subunit E 1 [Mus musculus]	gi 45504359	26141	8.44	878	53
	ATP synthase subunit gamma, mitochondrial isoform b [Mus musculus]	gi 163838648	30237	8.86	657	46
	Sdha protein, partial [Mus musculus]	gi 15030102	72280	7.11	6855	72
	omega-amidase NIT2 [Mus musculus]	gi 12963555	30483	6.44	804	57
7	nitrilase homolog 1 isoform 2 [Mus musculus]	gi 336088630	31866	6.59	643	62
	enoyl-CoA hydratase domain-containing protein 3, mitochondrial precursor [Mus musculus]	gi 31980955	32382	8.6	615	62
	Electron transferring flavoprotein, alpha polypeptide [Mus musculus]	gi 13097375	35018	8.62	505	28
	hydroxyacylglutathione hydrolase, mitochondrial isoform 1 precursor [Mus musculus]	gi 227499238	34082	8.06	441	30
	cytochrome c, somatic [Mus musculus]	gi 6681095	11598	9.61	1766	74
8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7 [Mus musculus]	gi 12963571	12568	10.17	1262	84
	acyl-coenzyme A thioesterase 13 [Mus musculus]	gi 13385260	15173	8.95	1133	60
	cytochrome b-c1 complex subunit 7 [Mus musculus]	gi 13385726	13553	9.1	1123	63
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 [Mus musculus]	gi 13385492	15273	10.1	793	62

Comments: VDACs are the only proteins that exist in all specifically labeled spots (Figure S19, upper left gel, spots 1–6). In addition to this result, Figure S20 indicated that **3-ONBD** selectively labeled VDACs, partner proteins of TSPO. The autofluorescent spots 7–9 were found to contain flavoproteins or cytochromes.

Table S4. Proteins identified from the 70, 32 and 28 kDa bands (Figure 6a, lane 4). The 70, 32 and 28 kDa bands were subjected to in-gel tryptic digestion and analyzed by LC-MS. The identified proteins were ranked by ion score. The proteins in the top 10 or showing over 60% sequence coverage are listed. VDACs and ANTs are indicated in blue.

band	identified protein	accession number	M.W.	pI	score	sequence coverage (%)
70 kDa	SdhA protein, partial [Mus musculus]	gi 15030102	72280	7.11	10910	75
	very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor [Mus musculus]	gi 23956084	70831	8.91	9922	82
	calcium-binding mitochondrial carrier protein Aralar2 isoform 1 [Mus musculus]	gi 7657583	74420	8.77	6637	77
	calcium-binding mitochondrial carrier protein Aralar1 [Mus musculus]	gi 27369581	74523	8.43	6637	75
	Na(+)/H(+) exchange regulatory cofactor NHE-RF3 [Mus musculus]	gi 225579120	56463	5.34	6415	84
	apoptosis-inducing factor 1, mitochondrial precursor [Mus musculus]	gi 6755004	66724	9.23	5738	66
	carnitine O-palmitoyltransferase 2, mitochondrial precursor [Mus musculus]	gi 162138915	73934	8.59	5561	68
	transketolase [Mus musculus]	gi 6678359	67588	7.23	4469	78
	ATP-binding cassette sub-family D member 3 [Mus musculus]	gi 60218877	75426	9.32	3263	66
	mitochondrial inner membrane protein isoform 1 [Mus musculus]	gi 70608131	83848	6.18	3240	54
32 kDa	voltage-dependent anion-selective channel protein 1 [Mus musculus]	gi 6755963	30737	8.62	9746	76
	hydroxyacyl-coenzyme A dehydrogenase, mitochondrial precursor [Mus musculus]	gi 111038118	34442	8.76	7058	86
	D-beta-hydroxybutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	gi 170014720	38274	9.14	4578	60
	2,4-dienoyl-CoA reductase, mitochondrial precursor [Mus musculus]	gi 13385680	36191	9.1	4293	69
	electron transfer flavoprotein subunit alpha, mitochondrial [Mus musculus]	gi 227500281	34988	8.62	4275	73
	prohibitin 2 [Mus musculus]	gi 126723336	33276	9.83	3696	66
	sideroflexin-1 [Mus musculus]	gi 15147224	35626	9.35	3603	62
	mitochondrial ATP synthase, H+ transporting F1 complex beta subunit [Mus musculus]	gi 89574015	48047	4.9	2513	51
	voltage-dependent anion-selective channel protein 2 [Mus musculus]	gi 6755965	31713	7.44	2698	59
	hydroxymethylglutaryl-CoA lyase, mitochondrial precursor [Mus musculus]	gi 171543858	34217	8.7	2495	64
	Ndufa9 protein, partial [Mus musculus]	gi 13543186	39454	9.55	2311	66
	fumarylacetoacetate hydrolase domain-containing protein 2A [Mus musculus]	gi 29366814	34654	8.42	2299	74
	citrate lyase beta subunit [Mus musculus]	gi 19073015	37495	8.76	2054	69
	delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor [Mus musculus]	gi 7949037	36095	7.6	1972	61
	guanine nucleotide-binding protein G(i)/G(s)/G(t) subunit beta-2 [Mus musculus]	gi 13937391	37307	5.6	1914	73
28 kDa	ADP/ATP translocase 2 [Mus musculus]	gi 22094075	32910	9.74	10960	88
	ADP/ATP translocase 1 [Mus musculus]	gi 148747424	32883	9.73	8022	83
	phosphate carrier protein, mitochondrial precursor [Mus musculus]	gi 19526818	39606	9.36	5305	72
	prohibitin [Mus musculus]	gi 6679299	29802	5.57	3980	84
	mitochondrial 2-oxoglutarate/malate carrier protein [Mus musculus]	gi 21312994	34133	9.95	3522	76
	enoyl-Coenzyme A delta isomerase 3 [Mus musculus]	gi 21312210	35208	9.22	2417	64
	Cyc1 protein, partial [Mus musculus]	gi 52350626	34383	9.02	2194	70
	Auh protein, partial [Mus musculus]	gi 20072952	32621	9.57	1886	58
	enoyl-CoA hydratase domain-containing protein 2, mitochondrial isoform 1 precursor [Mus musculus]	gi 225543400	31832	9.03	1859	54
	enoyl-CoA hydratase domain-containing protein 3, mitochondrial precursor [Mus musculus]	gi 31980955	32382	8.6	1824	58
	H2-K region expressed gene 6, isoform CRA_e [Mus musculus]	gi 148678295	24988	8.75	1579	66
	peroxisomal 2,4-dienoyl-CoA reductase [Mus musculus]	gi 6753622	31280	9.04	1429	60

Comments: VDACs and ANTs were found from the 32 kDa and 28 kDa bands, respectively. However, after careful 2-D PAGE separation, 28 kDa fluorescent spots were not observed (Figure 6b, Figure S19 and Table S3). For this reason, it remains unclear whether **3-ONBD** has labeled ANT. The 70 kDa band contained several mitochondrial membrane proteins. Although we could not find connections between these proteins and TSPO, the result might suggest that the 70 kDa band contains PIGA-interacting protein(s).

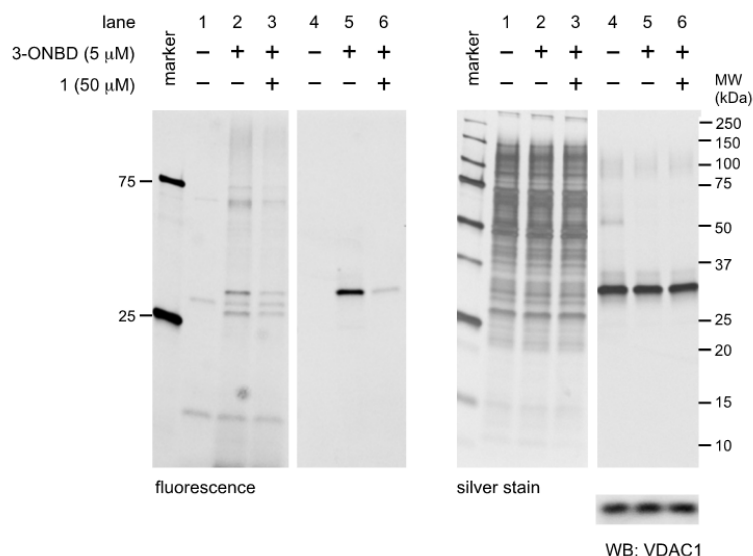


Figure S20. In-gel fluorescence analysis of VDACs purified from the labeled mitochondria (fluorescence image at 530 nm after excitation at 488 nm). The labeled mitochondria (lanes 1–3) and purified VDACs (lanes 4–6) are shown. Labeling condition: 5 μ M **3-ONBD**, 0.15 mg/mL mouse kidney mitochondria, 5 mM Hepes-KOH (pH 7.2), 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 30 $^{\circ}$ C, and 1 h. In the competition analysis, the mitochondria were preincubated with 50 μ M **1** for 10 min. VDACs were then purified from the labeled mitochondria according to the reported VDAC-purification method.^{S1,S2} Initially, the labeled mitochondria were disrupted by freeze-thaw and centrifuged at 15000 g for 10 min. The pellet was resuspended in VDAC purification buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 3% Triton X-100, 0.1 mM PMSF, 0.5 μ g/mL leupeptin). After inverting for 30 min at 4 $^{\circ}$ C and centrifugation at 32000 g for 1 h, the obtained supernatant was loaded to a hydroxyapatite/cerite (10:1 w/w) column. The endogenous VDAC in the unbound fractions was analyzed by SDS-PAGE. Western blotting analysis was performed using mouse monoclonal antibody to VDAC1 (abcam).

Comments: Mouse kidney mitochondria were labeled by **3-ONBD** under the general conditions, and the labeled mitochondria were purified according to the VDAC-isolation protocol and analyzed by SDS-PAGE. The result revealed that **3-ONBD** selectively labeled VDACs in response to PIGA-target interactions.

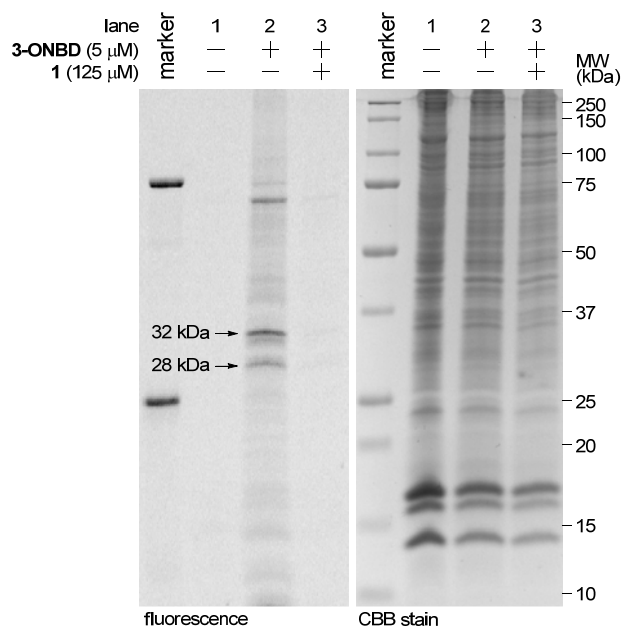


Figure S21. In-gel fluorescence analysis of HEK293T cells labeled with **3-ONBD** (fluorescence image at 530 nm after excitation at 488 nm). Labeling condition: 5 μ M **3-ONBD**, 37 $^{\circ}$ C, for 1.5 h. In the competition analysis, the cells were preincubated with 125 μ M **1** for 0.5 h at 37 $^{\circ}$ C. The labeled cells were lysed with solubilization buffer (20 mM Hepes–Na (pH 7.0), 1 mM EDTA, 0.5% Triton X-100, and complete mini (Roche, 1 tablet/10 mL)) and analyzed by SDS-PAGE.

Comments: The 32 and 28 kDa proteins were specifically labeled by **3-ONBD**, and the labeling was subject to competition by **1**. The 70 kDa protein was also labeled. In labeling experiments using HEK293T mitochondria, we identified heat shock proteins (HSPs) from the 70 kDa band. Recently, cytosolic chaperones have been suggested to be involved in the transportation of TSPO from cytosol to mitochondria.^{S3} Our *O*-NBD probe **3-ONBD** might also label this putative TSPO-HSP complex, although this remains to be confirmed.

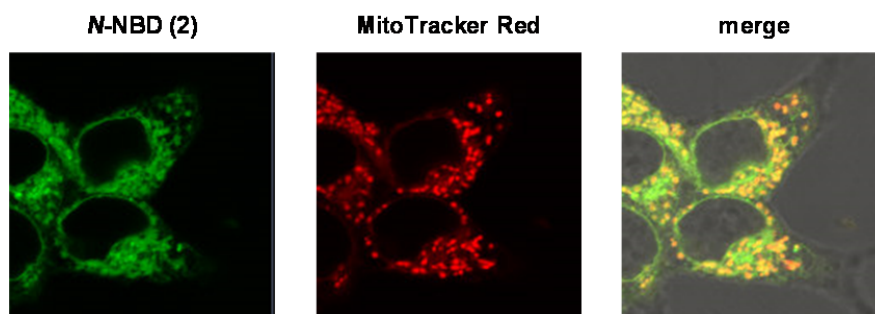


Figure S22. Mitochondrial localization of **2**. HEK293T cells were preincubated with 12.5 nM MitoTracker Red CMXRos for 30 min before addition of 2.5 μ M **2**. After incubation at 37 $^{\circ}$ C for 1 h, fluorescent images were obtained. Fluorescence images of *N*-NBD (left, Ex. 488 nm, Em. 493-548 nm) and MitoTracker Red CMXRos (middle, Ex. 561 nm, Em. 578-678 nm) are shown. The merged image is also presented with the bright field image (right).

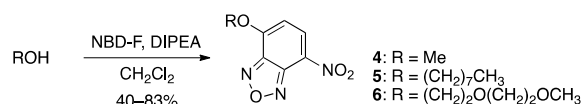
Comments: The known fluorescent TSPO ligand **2** was mainly localized in mitochondria.^{S4} However, as compound **2** itself is fluorescent, in addition to the fluorescence signals from TSPO-binding probe at mitochondria, signals of the free probe at other hydrophobic parts of the cells were also observed. In contrast, **3-ONBD** clearly labeled mitochondria possibly because only the PIGA-interacting proteins were labeled with turn-ON fluorescence (Figure 7).

Supplementary Methods

Synthetic Procedure

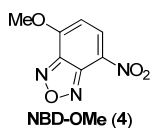
General Information

All chemicals and reagents were purchased from Sigma-Aldrich Co., LLC, Kanto Chemical Co., Inc., Tokyo Chemical Industry Co., Ltd., Acros Organics or Wako Pure Chemical Industries, Ltd., and used without further purification. Reactions were performed under an atmosphere of nitrogen, unless noted otherwise, and monitored by thin-layer chromatography (TLC, Merck silica gel 60 F₂₅₄) plates. Bands were visualized using UV light or appropriate reagents followed by heating. Flash chromatography was carried out with silica gel (Silica gel 60N, 40–50 µm particle size) purchased from Kanto Chemical Co., Inc. NMR spectra were recorded on a JEOL JNM-AL400 or ECP400 spectrometer, operating at 400 MHz for ¹H-NMR and at 100 MHz for ¹³C-NMR. Proton and carbon chemical shifts were referenced to residual solvent peaks.^{S7} High-resolution mass spectra were obtained using a Bruker micrOTOF-Q II-RSL mass spectrometer. Melting points were determined by using a Yanagimoto hot-stage melting point apparatus.



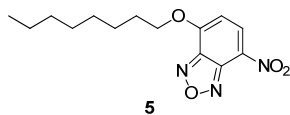
Scheme S1. Synthesis of alkoxy NBD compounds **4**, **5** and **6**.

Synthesis of NBD-OMe (4) (Compound **4** was prepared by a different method from the reported procedure.^{S6a})



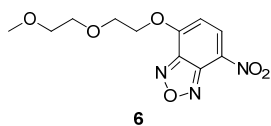
MeOH (2.2 µL, 55 µmol) and *N,N*-diisopropylethylamine (9.5 µL, 55 µmol) were added to a solution of NBD-F (10 mg, 55 µmol) in anhydrous CH₂Cl₂ (1.5 mL), and the mixture was stirred for 24 h at room temperature. After removal of the solvent, the residue was purified by preparative TLC (CHCl₃) to afford the product NBD-OMe (8.5 mg, 80%) as a yellow solid. NBD-OMe: ¹H NMR (400 MHz, CDCl₃) δ 4.24 (s, 3H), 6.71 (d, *J* = 8.4 Hz, 1H), 8.56 (d, *J* = 8.4 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 57.9, 104.0, 130.1, 134.1, 144.1, 145.3, 155.5; HRMS (ESI) Calcd. for C₇H₅N₃O₄Na: 218.0172, Found 218.0181.

Synthesis of 5

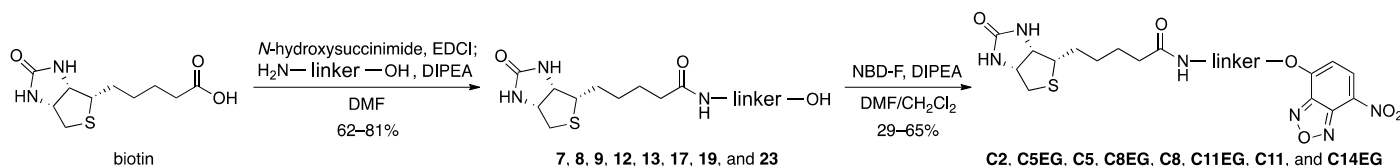


1-Octanol (15 µL, 0.27 mmol) and *N,N*-diisopropylethylamine (16 µL, 0.27 mmol) were added to a solution of NBD-F (18 mg, 0.27 mmol) in anhydrous CH₂Cl₂ (1.5 mL), and the mixture was stirred for 17 h at room temperature. After removal of the solvent, the residue was purified by preparative TLC (CHCl₃) to afford the product **5** (24 mg, 83%) as a yellow solid. **5**: M.p. 47–49 °C; ¹H NMR (400 MHz, CDCl₃) δ 0.88 (s, 3H), 1.22–1.43 (m, 8H), 1.54 (tt, *J* = 7.4, 7.4 Hz, 2H), 1.99 (tt, *J* = 7.1, 7.1 Hz, 2H), 4.39 (t, *J* = 6.8 Hz, 2H), 6.67 (d, *J* = 8.4 Hz, 1H), 8.54 (d, *J* = 8.8 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 14.2, 22.7, 25.9, 28.7, 29.2, 29.3, 31.9, 71.6, 104.4, 129.6, 134.3, 144.1, 145.4, 155.2; HRMS (ESI) Calcd. for C₁₄H₁₉N₃O₄Na: 316.1268, Found 316.1279.

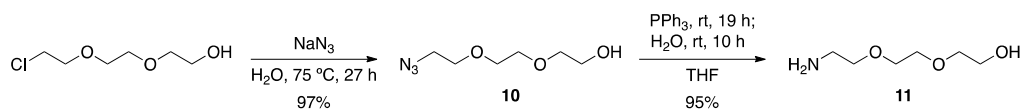
Synthesis of **6**



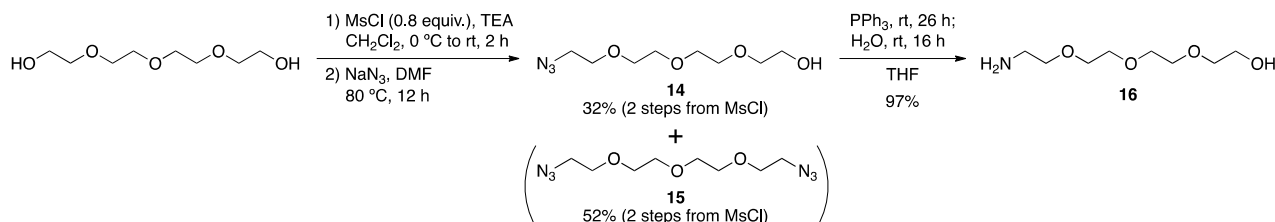
2-(2-Methoxyethoxy)ethanol (15 μ L, 0.13 mmol) and *N,N*-diisopropylethylamine (87 μ L, 0.51 mmol) were added to a solution of NBD-F (47 mg, 0.26 mmol) in anhydrous CH_2Cl_2 (1.5 mL), and the mixture was stirred for 2 d at room temperature. After removal of the solvent, the residue was purified by silica gel column chromatography (AcOEt/hexane, 1:1) to afford the product **6** (15 mg, 40%) as a yellow paste. **6**: ^1H NMR (400 MHz, CDCl_3) δ 3.37 (s, 3H), 3.56 (t, J = 4.6 Hz, 2H), 3.74 (t, J = 4.6 Hz, 2H), 4.03 (t, J = 4.6 Hz, 2H), 4.58 (t, J = 4.6 Hz, 2H), 6.76 (d, J = 8.8 Hz, 1H), 8.53 (d, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 59.2, 69.1, 70.8, 71.2, 72.0, 105.2, 130.0, 134.1, 144.1, 145.4, 154.9; HRMS (ESI) Calcd. for $\text{C}_{11}\text{H}_{13}\text{N}_3\text{O}_6\text{Na}$: 306.0697, Found 306.0700.



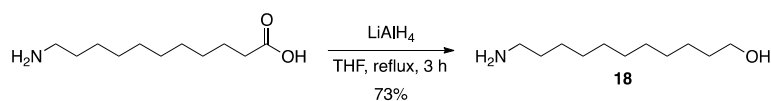
Scheme S2. Synthesis of biotin *O*-NBD probes.



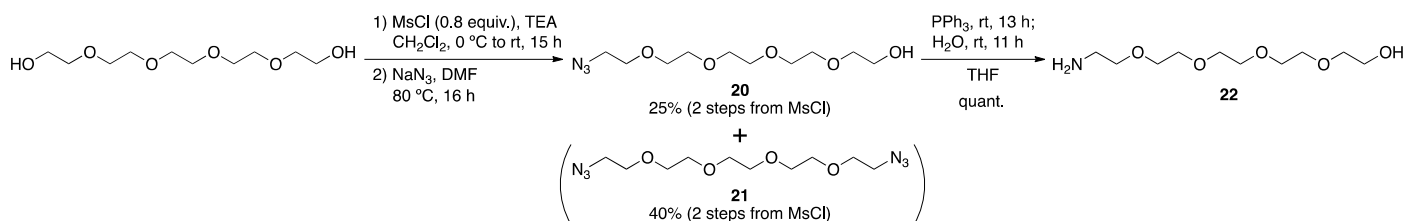
Scheme S3. Synthesis of the linker unit of probe **C8EG**.



Scheme S4. Synthesis of the linker unit of probe **C11EG**.

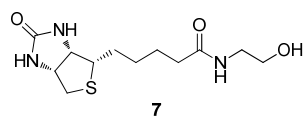


Scheme S5. Synthesis of the linker unit of probe **C11**.



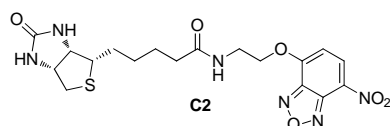
Scheme S6. Synthesis of the linker unit of probe **C14EG**.

Synthesis of 7 (Compound 7 was prepared by a small modification of a known procedure.^{S8})



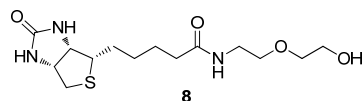
N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 11 h at room temperature. *N,N*-Diisopropylethylamine (210 μ L, 1.2 mmol) and 2-ethanolamine (27 μ L, 0.45 mmol) were then added, and stirring was continued for 3 h. The solvent was removed under reduced pressure, and the residue was taken up in CH_2Cl_2 . Filtration afforded a white solid, which was washed with CH_2Cl_2 to give the product **7** (79 mg, 67%) as a white solid. **7**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 10:1) δ 1.34 (tt, J = 7.3, 7.3 Hz, 2H), 1.47–1.68 (m, 4H), 2.13 (t, J = 7.4 Hz, 2H), 2.63 (d, J = 12.8 Hz, 1H), 2.82 (dd, J = 4.8, 12.8 Hz, 1H), 2.99–3.10 (m, 1H), 3.20–3.29 (m, 2H), 3.54 (t, J = 4.8 Hz, 2H), 4.22 (dd, J = 4.6, 7.4 Hz, 1H), 4.41 (dd, J = 5.2, 7.2 Hz, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 10:1) δ 25.4, 27.9, 28.2, 35.6, 40.3, 41.9, 55.6, 60.2, 61.0, 61.8, 164.3, 174.8; HRMS (ESI) Calcd. for $\text{C}_{12}\text{H}_{21}\text{N}_3\text{O}_3\text{SNa}$: 310.1196, Found 310.1195.

Synthesis of C2



N,N-Diisopropylethylamine (73 μ L, 0.41 mmol) and NBD-F (38 mg, 0.21 mmol) were added to a solution of **7** (20 mg, 70 μ mol) in anhydrous DMF (1.0 mL), and the mixture was stirred for 33 h at room temperature. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$, 10:1 to 8:1), followed by preparative TLC ($\text{CHCl}_3/\text{MeOH}$, 10:1), to afford the product **C2** (17 mg, 53%) as a light brown solid. The starting **7** was recovered in 35% yield. **C2**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 10:1) δ 1.22 (tt, J = 7.7, 7.7 Hz, 2H), 1.33–1.56 (m, 4H), 2.06 (t, J = 7.6 Hz, 2H), 2.50 (d, J = 13.2 Hz, 1H), 2.68 (dd, J = 5.0, 13.0 Hz, 1H), 2.89–2.96 (m, 1H), 3.57 (t, J = 5.2 Hz, 2H), 4.09 (dd, J = 4.6, 8.2 Hz, 1H), 4.29 (t, J = 5.4 Hz, 2H), 4.26–4.33 (m, 1H), 6.69 (d, J = 8.8 Hz, 1H), 8.42 (d, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 10:1) δ 25.2, 27.9, 28.2, 35.3, 38.0, 40.0, 55.4, 60.1, 61.9, 69.4, 105.1, 129.6, 134.6, 143.9, 145.2, 154.2, 174.8 (The ^{13}C signal of the urea, around 164 ppm, was not observed.); HRMS (ESI) Calcd. for $\text{C}_{18}\text{H}_{22}\text{N}_6\text{O}_6\text{SNa}$: 473.1214, Found 473.1212.

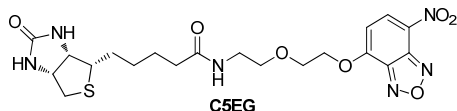
Synthesis of 8 (Compound 8 was prepared by a different method from the reported procedure.^{S9})



N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 3 h at room temperature. *N,N*-Diisopropylethylamine (210 μ L, 1.2 mmol) and 2-(2-aminoethoxy)ethanol (45 μ L, 0.45 mmol) were then added, and stirring was continued for 14 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$, 10:1 to 5:1) to afford the product **8** (110 mg, 81%) as a white solid. **8**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.41 (tt, J = 7.5, 7.5 Hz, 2H), 1.55–1.74 (m, 4H), 2.20 (t, J = 7.4 Hz, 2H), 2.70 (d, J = 6.4 Hz, 1H), 2.88 (dd, J = 4.6, 12.6 Hz, 1H), 3.08–3.15 (m, 1H), 3.40 (t, J = 4.8 Hz, 2H), 3.54 (t, J = 5.0 Hz, 2H), 3.54 (t, J =

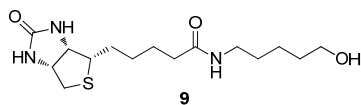
5.0 Hz, 2H), 3.69 (t, $J = 4.2$ Hz, 2H), 4.29 (dd, $J = 4.4, 7.6$ Hz, 1H), 4.48 (dd, $J = 4.8, 7.6$ Hz, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 25.7, 28.0, 28.3, 35.7, 39.2, 40.6, 55.9, 60.3, 61.4, 62.0, 69.9, 72.3, 164.5, 174.2; HRMS (ESI) Calcd. for $\text{C}_{14}\text{H}_{25}\text{N}_3\text{O}_4\text{SNa}$: 354.1458, Found 354.1447.

Synthesis of **C5EG**



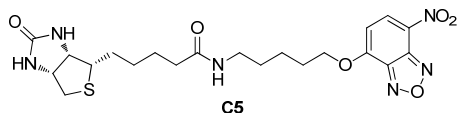
N,N-Diisopropylethylamine (63 μL , 0.36 mmol) and NBD-F (33 mg, 0.18 mmol) were added to a solution of **8** (20 mg, 60 μmol) in anhydrous $\text{CH}_2\text{Cl}_2/\text{DMF}$ (1.4 mL, 5:2), and the mixture was stirred for 4 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$, 10:1 to 6:1) to afford the product **C5EG** (19 mg, 65%) as a light brown solid. The starting **8** was recovered in 21% yield. **C5EG**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 99:1) δ 1.39 (tt, $J = 7.6, 7.6$ Hz, 2H), 1.50–1.73 (m, 4H), 2.21 (dt, $J = 3.6, 7.2$ Hz, 2H), 2.74 (d, $J = 6.6$ Hz, 1H), 2.89 (dd, $J = 4.4, 12.8$ Hz, 1H), 3.13 (dt, $J = 4.0, 7.0$ Hz, 1H), 3.38–3.50 (m, 2H), 3.65 (t, $J = 5.0$ Hz, 2H), 3.95–3.99 (m, 2H), 4.30–4.36 (m, 1H), 4.50–4.56 (m, 3H), 6.77 (d, $J = 8.0$ Hz, 1H), 8.55 (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 99:1) δ 25.4, 28.0, 28.1, 35.6, 39.1, 40.4, 55.5, 60.7, 62.3, 68.4, 70.1, 70.5, 105.3, 130.0, 134.4, 144.1, 145.4, 154.6, 166.8, 174.0; HRMS (ESI) Calcd. for $\text{C}_{20}\text{H}_{26}\text{N}_6\text{O}_7\text{SNa}$: 517.1476, Found 517.1464.

Synthesis of 9 (Compound 9 was prepared by a different method from the reported procedure.^{S10})



N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 3 h at room temperature. *N,N*-Diisopropylethylamine (210 μL , 1.2 mmol) and 5-amino-1-pentanol (49 μL , 0.45 mmol) were then added, and stirring was continued for 14 h. The solvent was removed under reduced pressure, and the residue was taken up in CH_2Cl_2 . Filtration afforded a white solid, which was washed with CH_2Cl_2 to give the product **9** (105 mg, 78%) as a white solid. **9**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.15–1.59 (m, 12H), 2.01 (t, $J = 7.2$ Hz, 2H), 2.56 (d, $J = 13.2$ Hz, 1H), 2.75 (dd, $J = 5.0, 12.6$ Hz, 1H), 2.91–3.10 (m, 3H), 3.40 (t, $J = 6.6$ Hz, 2H), 4.14 (dd, $J = 4.4, 8.0$ Hz, 1H), 4.34 (dd, $J = 6.0, 6.0$ Hz, 1H); HRMS (ESI) Calcd. for $\text{C}_{15}\text{H}_{27}\text{N}_3\text{O}_3\text{SNa}$: 352.1665, Found 352.1658.

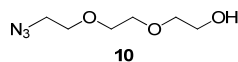
Synthesis of **C5**



N,N-Diisopropylethylamine (63 μL , 0.36 mmol) and NBD-F (33 mg, 0.18 mmol) were added to a solution of **9** (20 mg, 61 μmol) in anhydrous $\text{CH}_2\text{Cl}_2/\text{DMF}$ (1.8 mL, 5:4), and the mixture was stirred for 4 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$, 10:1) to afford the product **C5** (13 mg, 43%) as a light yellow solid. The starting **9** was recovered in 37% yield. **C5**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.40 (tt, $J = 7.4, 7.4$ Hz, 2H), 1.52–1.73 (m, 8H), 1.99 (tt, $J = 7.2, 7.2$ Hz, 2H), 2.17 (dt, $J = 3.6, 7.3$ Hz, 2H), 2.68 (d, $J = 12.8$ Hz, 1H), 2.88 (dd, $J = 5.0, 12.6$ Hz, 1H), 3.09–3.16 (m, 1H), 3.25 (t, $J = 6.2$ Hz, 2H), 4.28 (dd, $J = 5.0,$

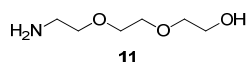
7.8 Hz, 1H), 4.37 (t, J = 6.4 Hz, 2H), 4.47 (dd, J = 5.2, 8.0 Hz, 1H), 6.69 (d, J = 8.8 Hz, 2H), 8.54 (d, J = 8.4 Hz, 2H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 99:1) δ 23.1, 25.4, 27.9, 28.0, 28.1, 29.0, 35.7, 38.9, 40.4, 55.4, 59.9, 61.7, 71.0, 104.5, 124.0, 134.3, 143.9, 145.2, 154.8, 164.9, 173.5; HRMS (ESI) Calcd. for $\text{C}_{21}\text{H}_{28}\text{N}_6\text{O}_6\text{SNa}$: 515.1683, Found 515.1672.

Synthesis of 10 (Compound **10** was prepared by a small modification of a known procedure.^{S11})



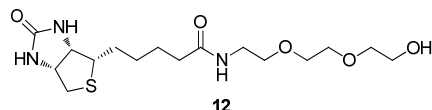
Sodium azide (590 mg, 9.1 mmol) was added to a solution of 2-[2-(2-chloroethoxy)ethoxy]ethanol (770 mg, 4.5 mmol) in water (2.2 mL), and the mixture was stirred for 27 h at 75 °C. After removal of the solvent under reduced pressure, Et_2O was added, and the resulting suspension was filtered. The filtrate was concentrated under reduced pressure to afford the product **10** (770 mg, 97%) as a colorless oil. **10**: ^1H NMR (400 MHz, CDCl_3) δ 2.24 (br-s, 1H), 3.40 (t, J = 5.0 Hz, 2H), 3.61 (t, J = 4.6 Hz, 2H), 3.65–3.71 (m, 6H), 3.74 (t, J = 4.6 Hz, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ 50.8, 61.9, 70.2, 70.5, 70.8, 72.6.

Synthesis of 11 (Compound **11** was prepared by a small modification of a known procedure.^{S12})



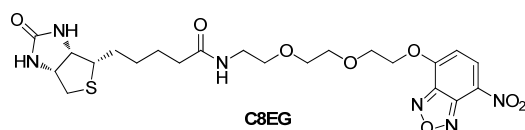
Triphenylphosphine (300 mg, 1.3 mmol) was added to a solution of **10** (200 mg, 1.1 mmol) in THF (5 mL), and the mixture was stirred for 19 h at room temperature. Water (62 μL , 3.4 mmol) was added to the mixture, and stirring was continued for 10 h. The solvent was removed under reduced pressure, and water was added to the residue. The aqueous solution was then washed with toluene, and concentrated under reduced pressure to afford the product **11** (160 mg, 95%) as a colorless oil. **11**: ^1H NMR (400 MHz, CD_3OD) δ 2.81 (t, J = 5.2 Hz, 2H), 3.54 (t, J = 5.4 Hz, 2H), 3.58 (t, J = 4.6 Hz, 2H), 3.61–3.71 (m, 6H); ^{13}C NMR (100 MHz, CD_3OD) δ 42.0, 62.2, 71.3, 71.4, 73.2, 73.7.

Synthesis of 12 (Compound **12** was prepared by a different method from the reported procedure.^{S13})



N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 27 h at room temperature. *N,N*-Diisopropylethylamine (430 μL , 2.5 mmol) and **11** (67 mg, 0.45 mmol) were added, and stirring was continued for 13 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography ($\text{MeOH}/\text{CH}_2\text{Cl}_2$, 1:10 to 1:5) to afford the product **12** (100 mg, 67%) as a white solid. **12**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.31–1.40 (m, 2H), 1.51–1.72 (m, 4H), 2.16 (t, J = 7.6 Hz, 2H), 2.68 (d, J = 13.2 Hz, 1H), 2.83 (dd, J = 4.8, 13.2 Hz, 1H), 3.02–3.11 (m, 1H), 3.32–3.38 (m, 2H), 3.47–3.59 (m, 8H), 3.64–3.69 (m, 2H), 4.25 (dd, J = 4.4, 7.6 Hz, 1H), 4.45 (dd, J = 5.0, 7.8 Hz, 1H), 6.5 (br-s, 1H), 7.22 (t, J = 5.2 Hz, 1H); HRMS (ESI) Calcd. for $\text{C}_{16}\text{H}_{29}\text{N}_3\text{O}_5\text{SNa}$: 398.1720, Found 398.1710.

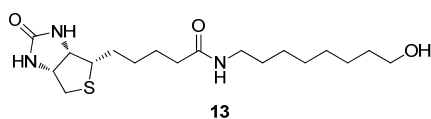
Synthesis of C8EG



N,N-Diisopropylethylamine (70 μL , 0.40 mmol) and NBD-F (37 mg, 0.20 mmol) were added to a solution of **12** (25 mg, 67

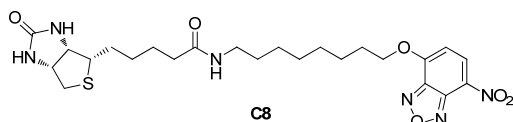
μmol) in anhydrous DMF (1.0 mL), and the mixture was stirred for 2 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by preparative TLC ($\text{CHCl}_3/\text{MeOH}$, 5:1) to afford the product **C8EG** (16 mg, 44%) as a light yellow solid. The starting **12** was recovered in 46% yield. **C8EG**: ^1H NMR (400 MHz, CDCl_3) δ 1.32–1.43 (m, 2H), 1.52–1.75 (m, 4H), 2.21 (t, $J = 7.4$ Hz, 2H), 2.73 (d, $J = 13.2$ Hz, 1H), 2.89 (dd, $J = 4.8, 12.8$ Hz, 1H), 3.10 (dt, $J = 4.4, 7.2$ Hz, 1H), 3.37–3.50 (m, 2H), 3.58 (t, $J = 4.8$ Hz, 2H), 3.64–3.69 (m, 2H), 3.73–3.79 (m, 2H), 4.03 (t, $J = 4.6$ Hz, 2H), 4.27 (dd, $J = 4.6, 8.2$ Hz, 1H), 4.51 (dd, $J = 5.0, 7.8$ Hz, 1H), 4.59 (t, $J = 4.6$ Hz, 2H), 6.76 (br-s, 1H), 6.81 (d, $J = 8.8$ Hz, 1H), 8.57 (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.7, 28.1, 28.2, 35.9, 39.3, 40.6, 55.6, 60.5, 62.0, 68.9, 70.0, 70.2, 70.7, 80.0, 105.3, 130.0, 134.4, 144.2, 145.4, 154.7, 164.1, 173.6; HRMS (ESI) Calcd. for $\text{C}_{22}\text{H}_{30}\text{N}_6\text{O}_8\text{SNa}$: 561.1738, Found 561.1732.

Synthesis of **13**



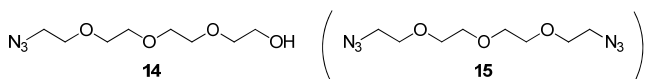
N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 2 h at room temperature. *N,N*-Diisopropylethylamine (210 μL , 1.2 mmol) and 8-amino-1-octanol (65 mg, 0.45 mmol) were then added, and stirring was continued for 1 h. The resulting suspension was filtered to afford a white solid, which was washed with cold water and Et_2O to give the product **13** (120 mg, 81%) as a white solid. **13**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.20 (s, 10H), 1.28–1.47 (m, 6H), 1.47–1.67 (m, 4H), 2.08 (br-s, 2H), 2.63 (d, $J = 12.4$ Hz, 1H), 2.70–2.91 (m, 1H), 3.06 (br-s, 3H), 3.39–3.53 (m, 2H), 4.20 (br-s, 1H), 4.39 (br-s, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 25.5, 25.5, 26.7, 28.0, 28.3, 29.1, 29.2, 29.2, 32.4, 35.8, 39.3, 40.3, 55.6, 60.1, 61.9, 62.2, 164.2, 173.9; HRMS (ESI) Calcd. for $\text{C}_{18}\text{H}_{33}\text{N}_3\text{O}_3\text{SNa}$: 394.2135, Found 394.2131.

Synthesis of **C8**



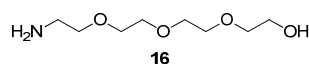
N,N-Diisopropylethylamine (47 μL , 0.27 mmol) and NBD-F (40 mg, 0.22 mmol) were added to a solution of **13** (20 mg, 54 μmol) in anhydrous DMF (2.0 mL), and the mixture was stirred for 27 h at room temperature. The mixture was then diluted with AcOEt , washed with water and brine, and dried over Na_2SO_4 . The solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$, 10:1), followed by preparative TLC ($\text{CHCl}_3/\text{MeOH}$, 20:1), to afford the product **C8** (8.5 mg, 30%) as a light brown solid. The starting **13** was recovered in 66% yield. **C8**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.22–1.41 (m, 10H), 1.41–1.54 (m, 6H), 1.54–1.72 (m, 4H), 1.89–1.98 (m, 2H), 2.16 (br-s, 2H), 2.71 (d, $J = 12.8$ Hz, 1H), 2.75–2.95 (m, 1H), 3.08–3.18 (m, 1H), 3.16 (t, $J = 7.0$ Hz, 2H), 4.29 (br-s, 1H), 4.34 (t, $J = 6.6$ Hz, 2H), 4.49 (br-s, 1H), 6.67 (d, $J = 8.4$ Hz, 1H), 8.52 (d, $J = 8.0$ Hz, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 25.6, 25.8, 26.8, 28.0, 28.2, 28.5, 29.1, 29.1, 29.4, 35.8, 39.5, 40.4, 55.5, 60.4, 62.1, 71.4, 104.6, 129.5, 134.5, 144.1, 145.4, 155.1, 164.0, 173.8; HRMS (ESI) Calcd. for $\text{C}_{24}\text{H}_{34}\text{N}_6\text{O}_6\text{SNa}$: 557.2153, Found 557.2147.

Synthesis of **14** and **15** (Compound **14** was prepared by a small modification of a known procedure.^{S14})



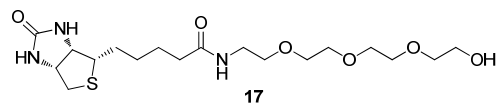
Triethylamine (0.86 mL, 6.2 mmol) was added to a solution of tetraethylene glycol (0.89 mL, 5.2 mmol) in anhydrous CH_2Cl_2 (10 mL). After cooling to 0 °C, methanesulfonyl chloride (0.32 mL, 4.1 mmol) was added dropwise to the solution, and the mixture was stirred for 1 h at 0 °C and for 1 h at room temperature. The reaction mixture was then washed with 2 *N* HCl and brine, and dried over Na_2SO_4 . The solvent was removed under reduced pressure. The residue was then dissolved in DMF (2.0 mL), and sodium azide (301 mg, 4.6 mmol) was added. The mixture was stirred at 80 °C for 12 h, and the solvent was removed under reduced pressure. Et_2O was added to the residue, and the resulting suspension was filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (AcOEt/hexane/MeOH, 1:2:0 to 1:0:0.05) to afford the product **14** (300 mg, 32% from MsCl) and product **15** (260 mg, 52% from MsCl) as colorless oils. **14**: ^1H NMR (400 MHz, CDCl_3) δ 2.63 (br-s, 1H), 3.39 (t, J = 5.0 Hz, 2H), 3.60 (t, J = 4.6 Hz, 2H), 3.57–3.77 (m, 10H), 3.72 (t, J = 4.6 Hz, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ 50.8, 61.8, 70.2, 70.4, 70.7, 70.8, 70.8, 72.6. **15**: ^1H NMR (400 MHz, CDCl_3) δ 3.38 (t, J = 5.0 Hz, 4H), 3.65–3.69 (m, 12H); ^{13}C NMR (100 MHz, CDCl_3) δ 50.8, 70.2, 70.8.

Synthesis of 16 (Compound **16** was prepared by a small modification of a known procedure.^{S14})



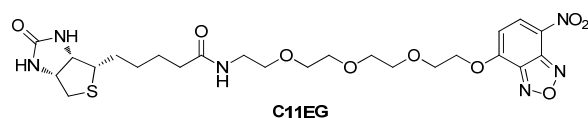
Triphenylphosphine (263 mg, 1.0 mmol) was added to a solution of **14** (200 mg, 0.91 mmol) in THF (5 mL), and the mixture was stirred for 26 h at room temperature. Water (49 μL , 2.7 mmol) was added to the mixture, and stirring was continued for 16 h. The solvent was removed under reduced pressure, and water was added to the residue. The aqueous solution was then washed with toluene, and concentrated under reduced pressure to afford the product **16** (170 mg, 97%) as a colorless oil. **16**: ^1H NMR (400 MHz, CDCl_3) δ 2.77–2.90 (m, 4H), 3.52 (t, J = 5.0 Hz, 2H), 3.57 (t, J = 4.2 Hz, 2H), 3.60–3.68 (m, 6H), 3.69 (t, J = 4.4 Hz, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ 41.5, 61.5, 70.1, 70.3, 70.6, 70.7, 73.0, 73.1.

Synthesis of 17



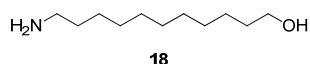
N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 26 h at room temperature. *N,N*-Diisopropylethylamine (210 μL , 1.2 mmol) and **16** (87 mg, 0.45 mmol) were then added, and stirring was continued for 16 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (MeOH/ CHCl_3 , 1:10) to afford the product **17** (106 mg, 62%) as a white solid. **17**: ^1H NMR (400 MHz, CDCl_3) δ 1.39 (tt, J = 7.4 Hz, 2H), 1.56–1.76 (m, 4H), 2.19 (t, J = 7.4 Hz, 2H), 2.71 (d, J = 12.8 Hz, 1H), 2.85 (dd, J = 4.6, 13.0 Hz, 1H), 3.10 (dt, J = 4.4, 7.4 Hz, 1H), 3.35–3.43 (m, 2H), 3.51 (t, J = 4.8 Hz, 2H), 3.55–3.71 (m, 12H), 4.27 (dd, J = 4.6, 7.8 Hz, 1H), 4.47 (dd, J = 5.0, 7.4 Hz, 1H), 6.74 (br-s, 1H), 7.35 (br-s, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.8, 28.2, 28.4, 36.0, 39.2, 40.6, 55.8, 60.4, 61.5, 61.9, 70.0, 70.1, 70.2, 70.4, 70.6, 72.7, 164.4, 173.7; HRMS (ESI) Calcd. for $\text{C}_{18}\text{H}_{33}\text{N}_3\text{O}_6\text{SNa}$: 442.1982, Found 442.1993.

Synthesis of C11EG



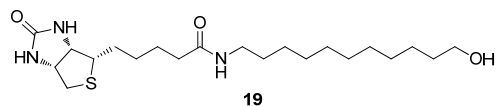
N,N-Diisopropylethylamine (62 μ L, 0.35 mmol) and NBD-F (33 mg, 0.18 mmol) were added to a solution of **17** (25 mg, 60 μ mol) in anhydrous CH_2Cl_2 (2.0 mL), and the mixture was stirred for 2 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (MeOH/ CH_3Cl , 1:10), followed by preparative TLC (MeOH/AcOEt, 3:7), to afford the product **C11EG** (15 mg, 42%) as a light yellow solid. The starting **17** was recovered in 37% yield. **C11EG**: ^1H NMR (400 MHz, CDCl_3) δ 1.34 (tt, J = 7.7, 7.7 Hz, 2H), 1.47–1.71 (m, 4H), 2.17 (t, J = 7.6 Hz, 2H), 2.69 (d, J = 12.4 Hz, 1H), 2.83 (dd, J = 4.8, 12.8 Hz, 1H), 3.05 (dt, J = 4.8, 7.5 Hz, 1H), 3.29–3.41 (m, 2H), 3.50 (t, J = 5.2 Hz, 2H), 3.52–3.62 (m, 6H), 3.68–3.73 (m, 2H), 3.97 (t, J = 4.4 Hz, 2H), 4.25 (dd, J = 4.4, 8.0 Hz, 1H), 4.46 (dd, J = 4.6, 7.4 Hz, 1H), 4.52 (t, J = 4.6 Hz, 2H), 6.76 (d, J = 8.4 Hz, 1H), 6.81 (br-s, 1H), 8.51 (d, J = 8.0 Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.7, 28.1, 28.2, 35.9, 39.4, 40.6, 55.6, 60.6, 62.0, 69.0, 69.9, 70.1, 70.5, 70.6, 70.8, 71.1, 105.4, 129.9, 134.5, 144.1, 145.4, 154.8, 164.2, 173.7; HRMS (ESI) Calcd. for $\text{C}_{24}\text{H}_{34}\text{N}_6\text{O}_9\text{SNa}$: 605.2000, Found 605.1992.

Synthesis of 18 (Compound **18** was prepared according to a known procedure.^{S15})



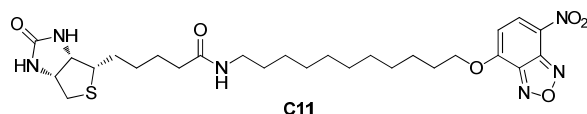
11-Aminoundecanoic acid (500 mg, 2.5 mmol) was added slowly to a suspension of LiAlH_4 (120 mg, 3.0 mmol) in anhydrous THF (5.0 mL) at 0 $^\circ\text{C}$, and the mixture was stirred for 3 h at 65 $^\circ\text{C}$. The solution was cooled to 0 $^\circ\text{C}$, and NaOH aq. (1M, 1 mL) was added to it. Stirring was continued for 30 min at room temperature, and then the mixture was dried over MgSO_4 and filtered through a Celite pad. The filtrate was concentrated under reduced pressure to afford the product **18** (340 mg, 73%) as a white solid. **18**: ^1H NMR (400 MHz, CD_3OD) δ 1.32 (s, 14H), 1.42–1.58 (m, 4H), 2.63 (t, J = 7.4 Hz, 2H), 3.54 (t, J = 6.6 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 27.0, 28.0, 30.6, 30.7, 30.7, 30.8, 33.5, 33.7, 42.5, 63.0.

Synthesis of 19



N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 21 h at room temperature. *N,N*-Diisopropylethylamine (210 μ L, 1.2 mmol) and **18** (84 mg, 0.45 mmol) were then added, and stirring was continued for 2 h. The solvent was removed under reduced pressure, and CH_2Cl_2 was added to the residue. The resulting suspension was filtered to afford a white solid, which was washed with CH_2Cl_2 to give the product **19** (120 mg, 70%) as a white solid. **19**: ^1H NMR (400 MHz, CD_3OD) δ 1.32 (br-s, 14H), 1.39–1.79 (m, 10H), 2.19 (t, J = 7.4 Hz, 2H), 2.71 (d, J = 12.8 Hz, 1H), 2.93 (dd, J = 4.8, 12.8 Hz, 1H), 3.16 (t, J = 7.2 Hz, 2H), 3.16–3.24 (m, 2H), 3.54 (t, J = 6.6 Hz, 2H), 4.30 (dd, J = 4.6, 7.8 Hz, 1H), 4.49 (dd, J = 5.2, 7.6 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 27.0, 28.0, 29.5, 29.8, 30.4, 30.6, 30.7, 30.8, 33.7, 36.8, 40.4, 41.1, 57.0, 61.6, 63.0, 63.4, 166.1, 175.9; HRMS (ESI) Calcd. for $\text{C}_{21}\text{H}_{39}\text{N}_3\text{O}_3\text{SNa}$: 436.2604, Found 436.2590.

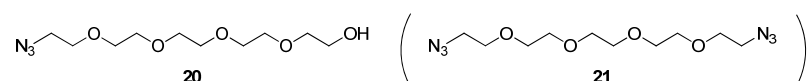
Synthesis of C11



N,N-Diisopropylethylamine (63 μ L, 0.36 mmol) and NBD-F (33 mg, 0.18 mmol) were added to a solution of **19** (25 mg, 60 μ mol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 2 d at room temperature. After removal of the solvent under

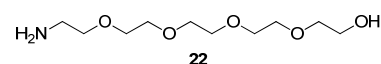
reduced pressure, the residue was purified by silica gel column chromatography (MeOH/CHCl₃, 1:10), followed by preparative TLC (MeOH/CHCl₃, 1:5), to afford the product **C11** (13 mg, 37%) as a light brown solid. The starting **19** was recovered in 55% yield. **C11**: ¹H NMR (400 MHz, CDCl₃) δ 1.29 (br-s, 14H), 1.40–1.59 (m, 6H), 1.59–1.81 (m, 4H), 1.94–2.02 (m, 2H), 2.76 (d, *J* = 12.8 Hz, 1H), 2.93 (dd, *J* = 5.0, 13.0 Hz, 1H), 3.12–3.24 (m, 1H), 3.23 (t, *J* = 7.4 Hz, 2H), 4.33–4.39 (m, 1H), 4.39 (t, *J* = 6.8 Hz, 2H), 4.51–4.59 (m, 1H), 6.03 (br-s, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 8.55 (d, *J* = 8.8 Hz, 1H); HRMS (ESI) Calcd. for C₂₇H₄₀N₆O₆SNa: 599.2622, Found 599.2624.

Synthesis of 20 and 21 (Compound 20 was prepared by a different method from the reported procedure.^{S16})



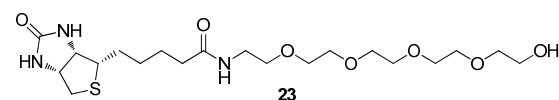
Triethylamine (0.80 mL, 5.7 mmol) was added to a solution of pentaethylene glycol (1.0 mL, 4.7 mmol) in anhydrous CH₂Cl₂ (30 mL). The mixture was cooled to 0 °C, then methanesulfonyl chloride (0.29 mL, 3.8 mmol) was added dropwise to it, and the whole was stirred for 4 h at 0 °C and for 11 h at room temperature. The reaction mixture was washed with 1 *N* HCl and brine, and then dried over Na₂SO₄. The solvent was removed under reduced pressure. The residue was dissolved in DMF (1.5 mL), and sodium azide (280 mg, 4.3 mmol) was added to the resulting solution. The mixture was stirred at 80 °C for 16 h, and the solvent was removed under reduced pressure. Et₂O was added to the residue, and the resulting suspension was filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (CHCl₃/MeOH, 50:1) to afford the product **20** (250 mg, 25% from MsCl) and the product **21** (220 mg, 40% from MsCl) as colorless oils. **20**: ¹H NMR (400 MHz, CDCl₃) δ 2.68 (br-s, 1H), 3.59 (t, *J* = 4.6 Hz, 2H), 3.65 (br-s, 14H), 3.70 (t, *J* = 4.8 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 50.8, 61.8, 70.1, 70.4, 70.7, 70.7, 70.7, 70.7, 70.8, 72.6. **21**: ¹H NMR (400 MHz, CDCl₃) δ 3.37 (dt, *J* = 3.2, 4.8 Hz, 4H), 3.63–3.67 (m, 16H); ¹³C NMR (100 MHz, CDCl₃) δ 50.8, 70.1, 70.7, 70.8, 70.8.

Synthesis of 22



Triphenylphosphine (170 mg, 0.66 mmol) was added to a solution of **20** (160 mg, 0.60 mmol) in THF (1 mL), and the mixture was stirred for 13 h at room temperature. Water (32 μL, 1.8 mmol) was added to it, and the resulting mixture was further stirred 11 h. The solvent was removed under reduced pressure, and water was added to the residue. The aqueous solution was then washed with toluene, and concentrated under reduced pressure to afford the product **22** (140 mg, quant.) as a colorless oil. **22**: ¹H NMR (400 MHz, CD₃OD) δ 2.77 (t, *J* = 5.4 Hz, 2H), 3.51 (t, *J* = 5.2 Hz, 2H), 3.55 (t, *J* = 4.8 Hz, 2H), 3.59–3.69 (m, 14H); ¹³C NMR (100 MHz, CD₃OD) δ 42.1, 62.1, 71.2, 71.4, 71.5, 71.5, 71.5, 71.6, 73.5, 73.7.

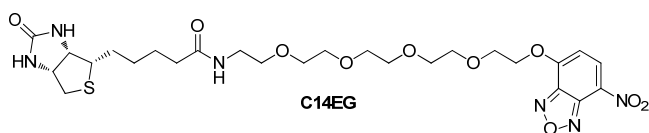
Synthesis of 23



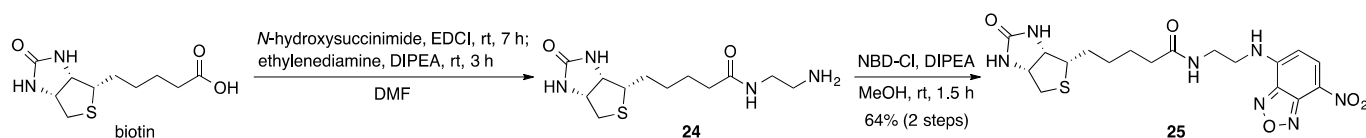
N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 26 h at room temperature. *N,N*-Diisopropylethylamine (210 μL, 1.2 mmol) and **22** (110 mg, 0.45 mmol) were then added, and stirring was continued for 22 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (MeOH/CHCl₃, 1:10 to 1:5), followed by preparative TLC (MeOH/CHCl₃, 1:5), to afford the product

23 (120 mg, 62%) as a white solid. **23**: ^1H NMR (400 MHz, CDCl_3) δ 1.40 (tt, $J = 7.4$ Hz, 2H), 1.56–1.77 (m, 4H), 2.21 (t, $J = 7.2$ Hz, 2H), 2.72 (d, $J = 13.2$ Hz, 1H), 2.87 (dd, $J = 5.0, 13.0$ Hz, 1H), 3.10 (dt, $J = 4.4, 7.2$ Hz, 1H), 3.35–3.45 (m, 2H), 3.53 (t, $J = 4.8$ Hz, 2H), 3.55–3.66 (m, 14H), 3.70 (t, $J = 4.6$ Hz, 2H), 4.29 (dd, $J = 4.8, 8.0$ Hz, 1H), 4.48 (dd, $J = 5.2, 7.6$ Hz, 1H), 5.79 (br-s, 1H), 6.56 (br-s, 1H), 7.17 (br-s, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.7, 28.1, 28.3, 29.8, 35.9, 39.3, 40.6, 55.7, 60.4, 61.6, 62.0, 70.1, 70.1, 70.2, 70.5, 70.5, 70.6, 72.8, 164.3, 173.9; HRMS (ESI) Calcd. for $\text{C}_{20}\text{H}_{37}\text{N}_3\text{O}_7\text{SNa}$: 486.2244, Found 486.2248.

Synthesis of **C14EG**

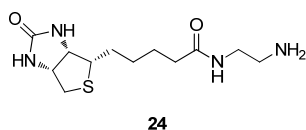


N,N-Diisopropylethylamine (40 μL , 0.23 mmol) and NBD-F (14 mg, 78 μmol) were added to a solution of **23** (18 mg, 39 μmol) in anhydrous CH_2Cl_2 (1.5 mL), and the mixture was stirred for 2 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography ($\text{MeOH}/\text{CHCl}_3$, 1:10 to 1:5), followed by preparative TLC ($\text{MeOH}/\text{CHCl}_3$, 1:5), to afford the product **C14EG** (7.0 mg, 29%) as a light yellow solid. The starting **23** was recovered in 31% yield. **TC14EG**: ^1H NMR (400 MHz, CDCl_3) δ 1.42 (tt, $J = 7.4, 7.4$ Hz, 2H), 1.57–1.78 (m, 4H), 2.22 (t, $J = 7.6$ Hz, 2H), 2.73 (d, $J = 13.2$ Hz, 1H), 2.90 (dd, $J = 4.8, 12.8$ Hz, 1H), 3.13 (dt, $J = 4.4, 7.4$ Hz, 1H), 3.38–3.45 (m, 2H), 3.55 (t, $J = 5.0$ Hz, 2H), 3.60–3.70 (m, 10H), 3.74–3.77 (m, 2H), 4.02 (t, $J = 4.6$ Hz, 2H), 4.31 (dd, $J = 4.6, 7.8$ Hz, 1H), 4.50 (dd, $J = 4.8, 7.6$ Hz, 1H), 4.58 (t, $J = 4.6$ Hz, 2H), 6.38 (br-s, 1H), 6.80 (br-s, 1H), 6.82 (d, $J = 8.4$ Hz, 1H), 8.56 (d, $J = 8.4$ Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.7, 28.2, 28.2, 35.9, 39.3, 40.7, 55.6, 60.4, 61.9, 69.1, 70.0, 70.2, 70.5, 70.6, 70.7, 70.7, 70.8, 71.2, 105.4, 129.9, 134.4, 144.2, 145.4, 154.8, 167.4, 173.5; HRMS (ESI) Calcd. for $\text{C}_{26}\text{H}_{38}\text{N}_6\text{O}_{10}\text{SNa}$: 649.2262, Found 649.2267.



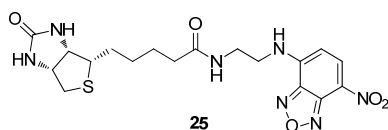
Scheme S7. Synthesis of biotin *N*-NBD probe **25**.

Synthesis of 24 (Compound **24** was prepared by a different method from the reported procedure.^{S17})

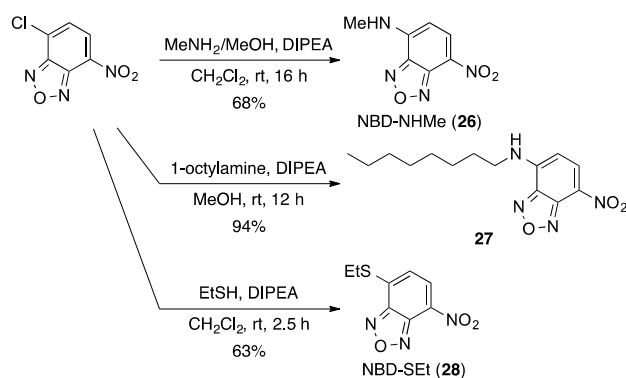


N-Hydroxysuccinimide (52 mg, 0.45 mmol) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (120 mg, 0.61 mmol) were added to a solution of biotin (100 mg, 0.41 mmol) in anhydrous DMF (1.5 mL), and the mixture was stirred for 7 h at room temperature. *N,N*-Diisopropylethylamine (360 μL , 2.0 mmol) and ethylenediamine (140 μL , 2.0 mmol) were added, and stirring was continued for 3 h. The solvent was removed under reduced pressure, and CHCl_3 was added to the residue. After filtration, the white solid was washed with CHCl_3 to afford the product **24** as a white solid. **24**: ^1H NMR (400 MHz, CD_3OD) δ 1.44 (t, $J = 7.6$ Hz, 2H), 1.52–1.82 (m, 4H), 2.24 (t, $J = 7.4$ Hz, 2H), 2.32–2.43 (m, 2H), 2.43–2.51 (m, 2H), 2.70 (d, $J = 13.2$ Hz, 1H), 2.87 (dd, $J = 5.2, 12.8$ Hz, 1H), 3.15–3.40 (m, 1H), 4.30 (dd, $J = 4.4, 8.0$ Hz, 1H), 4.49 (dd, $J = 4.4, 8.0$ Hz, 1H); HRMS (ESI) Calcd. for $\text{C}_{12}\text{H}_{22}\text{N}_4\text{O}_2\text{SNa}$: 309.1356, Found 309.1362.

Synthesis of **25**

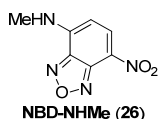


N,N-Diisopropylethylamine (360 μ L, 2.1 mmol) and NBD-Cl (82 mg, 0.41 mmol) were added to a solution of **24** in MeOH (10 mL), and the mixture was stirred for 1.5 h at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (MeOH/ CH_2Cl_2 , 1:10) to afford the product **25** (120 mg, 64% from biotin) as an orange solid. **25**: M.p. 116–119 $^{\circ}\text{C}$; ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 10:1) δ 1.17–1.57 (m, 6H), 2.06 (dt, J = 2.4, 7.2 Hz, 2H), 2.53 (d, J = 13.2 Hz, 1H), 2.72 (dd, J = 4.8, 13.2 Hz, 1H), 2.90–2.96 (m, 1H), 3.34–3.52 (m, 4H), 4.05–4.15 (m, 1H), 4.33 (dd, J = 5.0, 7.8 Hz, 1H), 6.12 (d, J = 8.8 Hz, 1H), 8.33 (d, J = 8.8 Hz, 1H); HRMS (ESI) Calcd. for $\text{C}_{18}\text{H}_{23}\text{N}_7\text{O}_5\text{SNa}$: 472.1374, Found 472.1369.



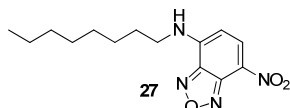
Scheme S8. Synthesis of NBD compounds **26**, **27** and **28**.

Synthesis of NBD-NHMe (26) (Compound **26** was prepared by a different method from the reported procedure.^{S6a})



Methylamine (2 M in MeOH, 0.5 mL, 1.0 mmol) and *N,N*-diisopropylethylamine (175 μ L, 1.0 mmol) were added to a solution of NBD-Cl (100 mg, 0.50 mmol) in anhydrous CH_2Cl_2 (3.0 mL), and the mixture was stirred for 16 h at room temperature. After removal of the solvent, the residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$, 20:1) to afford the product NBD-NHMe (66 mg, 68%) as an orange solid. NBD-NHMe: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 10:1) δ 3.06 (s, 3H), 6.04 (d, J = 8.8 Hz, 1H), 8.40 (d, J = 8.8 Hz, 1H); HRMS (ESI) Calcd. for $\text{C}_7\text{H}_6\text{N}_4\text{O}_3\text{Na}$: 217.0332, Found 217.0338.

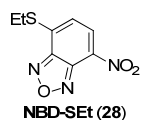
Synthesis of **27**



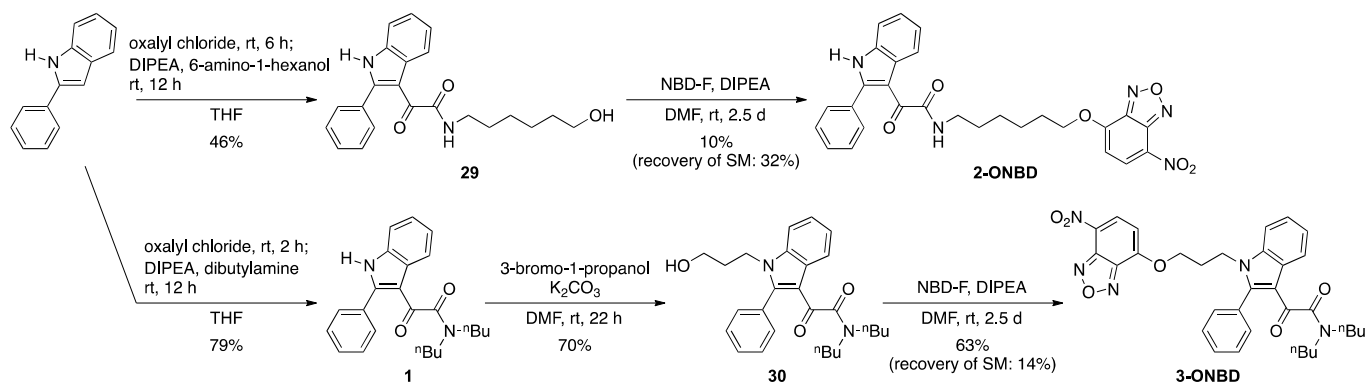
N,N-Diisopropylethylamine (180 μ L, 1.0 mmol) and NBD-Cl (100 mg, 0.50 mmol) were added to a solution of *n*-octylamine (83 μ L, 0.5 mmol) in MeOH (5.0 mL), and the mixture was stirred for 12 h at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (CH_2Cl_2) to afford the product **27** (140 mg, 94%) as a dark red solid. **27**: M.p. 98–100 $^{\circ}\text{C}$; ^1H NMR (300 MHz, CDCl_3) δ 1.88 (t, J = 6.8 Hz, 3H), 1.23–1.53 (m, 10H), 2.81

(tt, $J = 7.4$, 7.4 Hz, 2H), 3.49 (dt, $J = 6.6$, 6.6 Hz, 2H), 6.17 (d, $J = 8.7$ Hz, 1H), 6.27 (br-s, 1H), 8.49 (d, $J = 8.7$ Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.2, 22.7, 27.1, 28.7, 29.2, 29.3, 31.8, 44.2, 98.6, 124.0, 136.7, 144.0, 144.4; HRMS (ESI) Calcd. for $\text{C}_{14}\text{H}_{20}\text{N}_4\text{O}_3\text{Na}$: 315.1428, Found 315.1425.

Synthesis of NBD-SEt (28) (Compound 28 was prepared by a different method from the reported procedure.^{S6a})

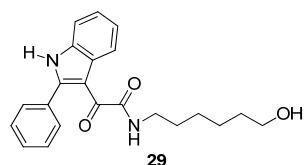


Ethanethiol (37 μL , 0.50 mmol) and *N,N*-diisopropylethylamine (175 μL , 1.0 mmol) were added to a solution of NBD-Cl (100 mg, 0.50 mmol) in anhydrous CH_2Cl_2 (3.0 mL), and the mixture was stirred for 2.5 h at room temperature. After removal of the solvent, the residue was purified by silica gel column chromatography (AcOEt/hexane, 1:3) to afford the product NBD-SEt (71 mg, 63%) as a yellow solid. NBD-SEt: ^1H NMR (400 MHz, CDCl_3) δ 1.52 (t, $J = 7.6$ Hz, 3H), 3.30 (q, $J = 7.3$ Hz, 2H), 7.14 (d, $J = 7.6$ Hz, 1H), 8.40 (d, $J = 7.6$ Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 13.2, 26.2, 120.3, 130.8, 132.7, 142.0, 142.6, 149.3; HRMS (ESI) Calcd. for $\text{C}_8\text{H}_7\text{N}_3\text{O}_3\text{SNa}$: 248.0100, Found 248.0107.



Scheme S9. Synthesis of **2-ONBD** and **3-ONBD**.

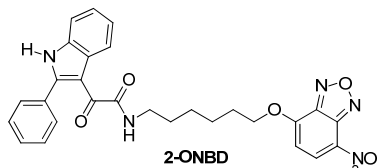
Synthesis of 29



A solution of oxalyl chloride (0.13 mL, 1.6 mmol) in anhydrous THF (1.0 mL) was added dropwise to a stirred solution of 2-phenylindole (0.20 g, 1.0 mmol) in anhydrous THF (3.0 mL) at 0 $^{\circ}\text{C}$, and the mixture was stirred for 6 h at room temperature. 6-Amino-1-propanol (0.36 mg, 3.1 mmol) and *N,N*-diisopropylethylamine (1.1 mL, 6.2 mmol) were added to the mixture, and stirring was continued for 12 h. The solvent was removed under reduced pressure, and the residue was taken up in AcOEt. This solution was washed with water and brine, dried over Na_2SO_4 , and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/ CHCl_3 , 1:20) to afford the product **29** (172 mg, 46%) as a yellow foam. **29**: ^1H NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 1.10–1.26 (m, 4H), 1.28 (tt, $J = 7.2$, 7.2 Hz, 2H), 1.41 (tt, $J = 6.9$, 6.9 Hz, 2H), 2.89 (t, $J = 7.4$ Hz, 2H), 3.47 (t, $J = 6.8$ Hz, 2H), 7.13–7.22 (m, 4H), 7.22–7.31 (m, 2H), 7.31–7.36 (m, 2H), 8.10 (d, $J = 7.2$ Hz, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$, 20:1) δ 25.2, 26.5, 28.7, 32.2, 39.1 and 39.3, 62.2, 109.9 and 109.9, 111.8 and 111.9, 121.4, 122.8, 123.7, 127.9 and 128.0, 128.3, 129.2, 129.4, 131.9 and 132.0, 136.0 and 136.2, 148.3 and 148.4, 165.3 and 165.4,

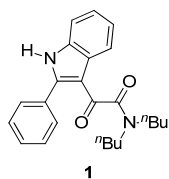
187.2; HRMS (ESI) Calcd. for $C_{22}H_{24}N_2O_3Na$: 387.1679, Found 387.1676.

Synthesis of **2-ONBD**



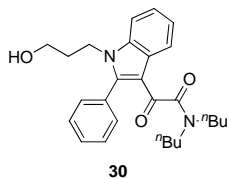
N,N-Diisopropylethylamine (0.11 mL, 0.66 mmol) and NBD-F (60 mg, 0.33 mmol) were added to a solution of **29** (60 mg, 0.11 mmol) in anhydrous DMF (0.7 mL), and the mixture was stirred for 2.5 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography ($CH_3Cl/MeOH$, 30:1), followed by preparative TLC ($CH_3Cl/MeOH$, 30:1), to afford the product **2-ONBD** (6.0 mg, 10%) as a light yellow paste. The starting **29** was recovered in 32% yield. **2-ONBD**: 1H NMR (400 MHz, $CDCl_3$) δ 1.31–1.41 (m, 2H), 1.43–1.54 (m, 2H), 1.87 (tt, J = 6.8, 6.8 Hz, 2H), 3.12 (dt, J = 6.5, 6.5 Hz, 2H), 4.19 (t, J = 6.4 Hz, 2H), 6.49 (d, J = 8.4 Hz, 1H), 6.93 (t, J = 6.0 Hz, 1H), 7.04–7.10 (m, 3H), 7.16 (dd, J = 7.6, 7.6 Hz, 1H), 7.22–7.27 (m, 3H), 7.31 (dd, J = 7.4, 7.4 Hz, 1H), 8.07 (d, J = 7.6 Hz, 1H), 8.44 (d, J = 8.4 Hz, 1H), 9.63 (br-s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.3, 26.2, 28.4, 29.0, 39.1, 71.1, 104.5, 110.1, 111.9, 121.6, 123.0, 123.9, 128.1, 128.6, 129.0, 129.6, 129.6, 132.1, 134.4, 135.8, 144.1, 145.3, 147.7, 154.9, 164.3, 186.8; HRMS (ESI) Calcd. for $C_{28}H_{25}N_3O_6Na$: 550.1697, Found 550.1705.

Synthesis of **1** (Compound **1** was prepared by a small modification of a known procedure.^{S18})



A solution of oxalyl chloride (0.27 mL, 3.1 mmol) in anhydrous THF (2.0 mL) was added dropwise to a stirred solution of 2-phenylindole (0.40 g, 2.1 mmol) in anhydrous THF (6.0 mL) at 0 °C, and the mixture was stirred for 2 h at room temperature. Dibutylamine (1.1 mL, 6.2 mmol) and *N,N*-diisopropylethylamine (2.1 mL, 12 mmol) were added to the mixture, and stirring was continued for 12 h. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography ($AcOEt$ /hexane, 1:3) to afford the product **1** (615 mg, 79%) as a light yellow solid. **1**: 1H NMR (400 MHz, $CDCl_3$) δ 0.78 (t, J = 7.6 Hz, 3H), 0.84 (t, J = 6.4 Hz, 3H), 1.07–1.22 (m, 6H), 1.47 (tt, J = 7.8, 7.8 Hz, 2H), 2.97 (t, J = 7.2 Hz, 2H), 3.07 (t, J = 7.8 Hz, 2H), 6.95 (dd, J = 7.6, 7.6 Hz, 2H), 7.10–7.27 (m, 6H), 8.17 (d, J = 8.0 Hz, 1H), 10.6 (br-s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.7 and 13.9, 20.0 and 20.4, 29.2 and 30.5, 44.8 and 48.4, 110.5, 112.2, 121.8, 122.9, 123.6, 127.4, 128.0, 129.4, 129.6, 130.8, 135.9, 147.7, 168.3, 187.1; HRMS (ESI) Calcd. for $C_{24}H_{28}N_2O_2Na$: 399.2043, Found 399.2043.

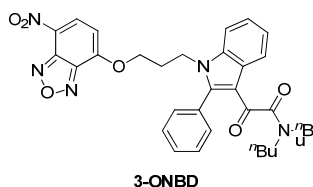
Synthesis of **30**



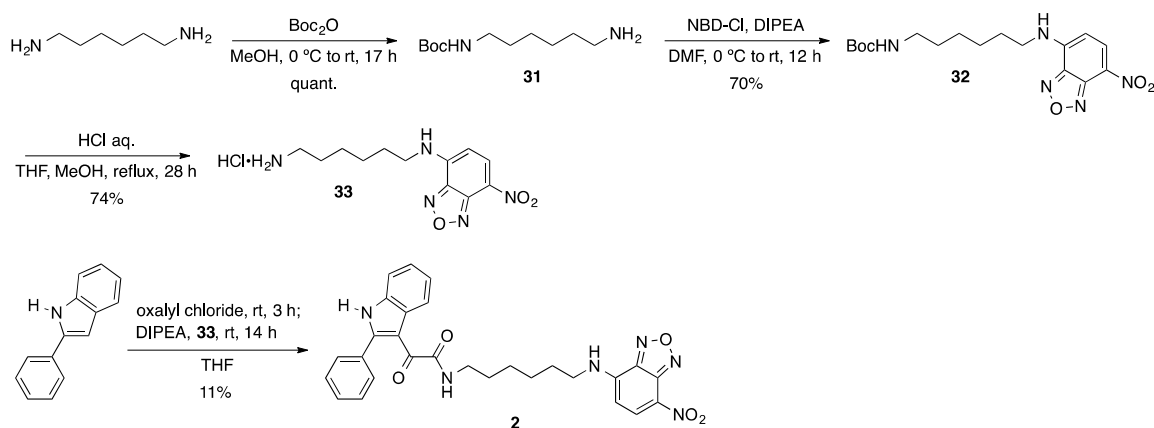
Potassium carbonate (0.11 g, 0.77 mmol) and 3-bromo-1-propanol (28 μ L, 0.32 mmol) were added to a solution of **1** (0.10 g, 0.27 mmol) in anhydrous DMF (1.0 mL), and the mixture was stirred for 22 h at room temperature. The mixture was then diluted with $AcOEt$, washed with water and brine, dried over Na_2SO_4 , and evaporated under reduced pressure. The residue was

purified by silica gel column chromatography (AcOEt/hexane, 1:1) to afford the product **30** (80 mg, 70%) as colorless foam. **30**: ^1H NMR (400 MHz, CDCl_3) δ 0.82 (t, J = 7.8 Hz, 3H), 0.85 (t, J = 7.8 Hz, 3H), 1.02–1.23 (m, 6H), 1.49 (tt, J = 7.6, 7.6 Hz, 2H), 1.69 (tt, J = 6.5, 6.5 Hz, 2H), 2.32 (s, 1H), 2.76 (t, J = 7.8 Hz, 2H), 2.96 (t, J = 7.8 Hz, 2H), 3.39 (t, J = 5.8 Hz, 2H), 3.95 (t, J = 7.4 Hz, 2H), 7.29–7.37 (m, 4H), 7.42–7.52 (m, 4H), 8.39–8.43 (m, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 13.8 and 14.0, 20.0 and 20.4, 29.5 and 30.7, 32.6, 41.1, 44.6 and 48.6, 59.2, 110.4, 112.0, 122.6, 123.4, 124.0, 126.7, 128.2, 129.5, 130.0, 130.8, 136.3, 148.2, 167.7, 187.6; HRMS (ESI) Calcd. for $\text{C}_{27}\text{H}_{34}\text{N}_2\text{O}_3\text{Na}$: 457.2462, Found 457.2453.

Synthesis of **3-ONBD**

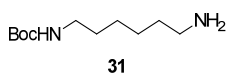


N,N-Diisopropylethylamine (66 μL , 0.39 mmol) and NBD-F (35 mg, 0.19 mmol) were added to a solution of **30** (28 mg, 64 μmol) in anhydrous DMF (1.0 mL), and the mixture was stirred for 2.5 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (AcOEt/hexane, 1:1), followed by preparative TLC (AcOEt/hexane, 1:1), to afford the product **3-ONBD** (24 mg, 63%) as a light yellow paste. The starting **30** was recovered in 14% yield. **3-ONBD**: ^1H NMR (400 MHz, CDCl_3) δ 0.81 (t, J = 7.4 Hz, 3H), 0.84 (t, J = 7.0 Hz, 3H), 1.02–1.23 (m, 6H), 1.46 (tt, J = 7.6, 7.6 Hz, 2H), 2.27 (tt, J = 6.1, 6.1 Hz, 2H), 2.77 (t, J = 7.8 Hz, 2H), 2.93 (t, J = 8.0 Hz, 2H), 4.12 (t, J = 5.8 Hz, 2H), 4.31 (t, J = 6.6 Hz, 2H), 6.42 (d, J = 7.6 Hz, 1H), 7.22–7.34 (m, 5H), 7.34–7.41 (m, 2H), 7.44 (d, J = 8.0 Hz, 1H), 8.40 (d, J = 8.0 Hz, 1H), 8.45 (d, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 13.8 and 14.0, 20.1 and 20.4, 28.7, 29.6 and 30.7, 40.2, 44.6 and 48.5, 67.1, 104.7, 109.9, 112.6, 123.0, 123.7, 124.2, 126.9, 128.3, 129.2, 129.9, 130.1, 130.9, 133.9, 136.2, 144.0, 145.1, 147.7, 154.0, 167.4, 187.7; HRMS (ESI) Calcd. for $\text{C}_{33}\text{H}_{35}\text{N}_5\text{O}_6\text{Na}$: 620.2480, Found 620.2486.



Scheme S10. Synthesis of reported fluorescent TSPO ligand **2**.^{S4}

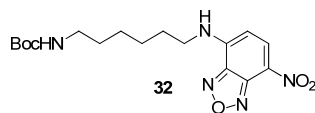
Synthesis of 31 (Compound **31** was prepared by a small modification of a known procedure.^{S19})



Di-*tert*-butyl dicarbonate (0.50 g, 2.3 mmol) in MeOH (10 mL) was added dropwise over 3 h to a solution of hexamethylene diamine (1.1 g, 9.2 mol) in MeOH (20 mL) at 0 °C, and the mixture was stirred for 4 h at the same temperature. The reaction mixture was further stirred for 10 h at room temperature, and then concentrated under reduced pressure. After addition of water,

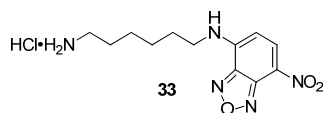
the resulting mixture was extracted with Et₂O. The combined organic phase was dried over Na₂SO₄, and the solvent was removed under reduced pressure to afford the product **31** (500 mg, quant.) as a colorless oil. **31**: ¹H NMR (400 MHz, CD₃OD) δ 1.29–1.36 (m, 4H), 1.42 (s, 9H), 1.42–1.50 (m, 4H), 2.61 (t, *J* = 7.2 Hz, 2H), 3.02 (t, *J* = 7.2 Hz, 2H); ¹³C NMR (100 MHz, CD₃OD) δ 27.7, 27.7, 28.8, 30.9, 33.8, 41.2, 42.5, 79.8, 158.6.

Synthesis of 32 (Compound **32** was prepared by a small modification of a known procedure.^{S4})



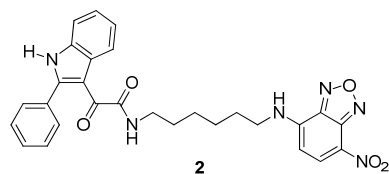
N,N-Diisopropylethylamine (0.71 mL, 4.2 mmol) and NBD-Cl (280 mg, 1.4 mmol) were added to a solution of **31** (300 mg, 1.4 mmol) in anhydrous DMF (3.0 mL) at 0 °C, and the resulting mixture was stirred for 2 d at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (CHCl₃) to afford the product **32** (370 mg, 70%) as a brown paste. **32**: ¹H NMR (400 MHz, CDCl₃) δ 1.33–1.53 (m, 6H), 1.40 (s, 9H), 1.79 (tt, *J* = 7.2, 7.2 Hz, 2H), 3.10 (t, *J* = 6.6 Hz, 2H), 3.44–3.54 (m, 2H), 4.63 (br-s, 1H), 6.15 (d, *J* = 8.8 Hz, 1H), 6.99 (br-s, 1H), 8.44 (dd, *J* = 2.0, 8.8 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 26.1, 26.3, 28.3, 28.5, 30.0, 40.2, 43.8, 79.3, 98.6, 123.3, 136.8, 144.1, 144.3, 156.3.

Synthesis of 33 (Compound **33** was prepared by a small modification of a known procedure.^{S4})

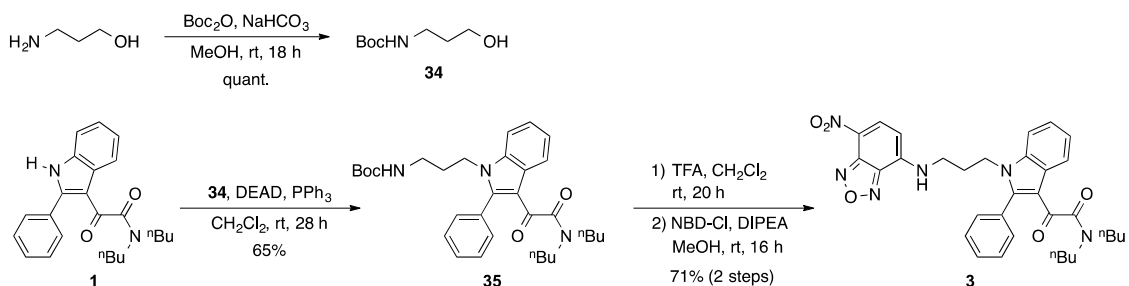


32 (260 mg, 0.69 mmol) was dissolved in THF (2.0 mL) and MeOH (0.5 mL), and 12 M HCl (0.1 mL) was added. The mixture was refluxed for 28 h, and then the solvent was removed under reduced pressure. The residue was washed with CHCl₃ to afford the product **33** (160 mg, 74%) as a red solid. **33**: ¹H NMR (400 MHz, CD₃OD) δ 1.43–1.58 (m, 4H), 1.71 (tt, *J* = 7.3, 7.3 Hz, 2H), 1.81 (tt, *J* = 7.1, 7.1 Hz, 2H), 2.95 (t, *J* = 7.6 Hz, 2H), 3.54 (br-s, 2H), 6.31 (d, *J* = 9.2 Hz, 1H), 8.45 (d, *J* = 9.2 Hz, 1H).

Synthesis of 2 (Compound **2** was prepared by a small modification of a known procedure.^{S4})

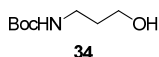


Oxalyl chloride (24 μL, 0.29 mmol) was added to a stirred solution of 2-phenylindole (50 mg, 0.26 mmol) in anhydrous THF (1.5 mL) at 0 °C, and the mixture was stirred for 3 h at room temperature. **33** (90 mg, 0.29 mmol) and *N,N*-diisopropylethylamine (0.27 mL, 1.6 mmol) were then added to the mixture, and stirring was continued for 14 h. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (MeOH/CHCl₃, 1:10), followed by preparative TLC (MeOH/CHCl₃, 1:10), to afford the product **2** (15 mg, 11%) as an orange solid. **2**: ¹H NMR (400 MHz, CDCl₃/CD₃OD, 20:1) δ 1.22–1.32 (m, 2H), 1.32–1.41 (m, 4H), 1.66 (tt, *J* = 7.2 Hz, 2H), 2.96 (t, *J* = 6.6 Hz, 2H), 3.36 (br-s, 2H), 6.03 (d, *J* = 8.8 Hz, 1H), 7.14–7.19 (m, 2H), 7.28–7.35 (m, 4H), 7.42–7.46 (m, 2H), 8.02–8.07 (m, 1H), 8.33 (d, *J* = 8.8 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃/CD₃OD, 20:1) δ 26.1, 26.2, 28.0, 28.7, 38.8, 43.7, 98.5, 109.9, 111.7, 121.4, 122.8, 123.8, 127.1, 127.9, 128.3, 129.2, 129.4, 132.1, 136.1, 137.0, 144.1, 148.4, 165.5, 187.1; HRMS (ESI) Calcd. for C₂₈H₂₆N₆O₅Na: 549.1857, Found 549.1856.



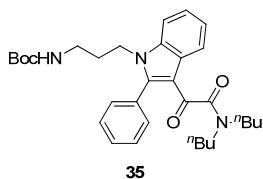
Scheme S11. Synthesis of fluorescent TSPO ligand **3**.

Synthesis of 34 (Compound **34** was prepared by a small modification of a known procedure.^{S20})



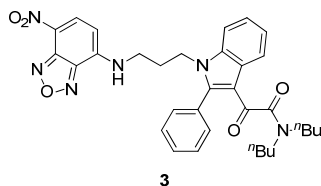
3-Amino-1-propanol (0.18 mL, 2.3 mmol) was added dropwise to a solution of di-*tert*-butyl dicarbonate (0.50 g, 2.3 mmol) and sodium hydrogen carbonate (0.39 g, 4.6 mmol) in MeOH (10 mL), and the mixture was stirred for 18 h at room temperature. After removal of the solvent, the residue was taken up in water and extracted with AcOEt. The combined organic layer was washed with brine and dried over Na₂SO₄. The solvent was removed under vacuum to afford the product **34** (0.40 g, quant.) as a colorless oil. **34**: ¹H NMR (400 MHz, CDCl₃) δ 1.43 (s, 3H), 1.65 (tt, *J* = 5.9 Hz, 2H), 3.27 (t, *J* = 6.4 Hz, 2H), 3.65 (t, *J* = 6.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 28.5, 33.0, 37.1, 59.4, 79.8, 157.3.

Synthesis of 35



Diethyl azodicarboxylate (72 μ L, 0.40 mmol) was added to a solution of triphenylphosphine (110 mg, 0.40 mmol), **1** (100 mg, 0.27 mmol) and **34** (70 mg, 0.40 mmol) in anhydrous CH_2Cl_2 (1.5 mL), and the mixture was stirred for 28 h at room temperature. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (AcOEt/hexane, 1:3 to 1:2), followed by preparative TLC (MeOH/ CHCl_3 , 1:100), to afford the product **35** (92 mg, 65%) as a colorless paste. **35**: ^1H NMR (400 MHz, CDCl_3) δ 0.83 (t, $J = 7.4$ Hz, 3H), 0.85 (t, $J = 7.0$ Hz, 3H), 1.05–1.23 (m, 6H), 1.40 (s, 9H), 1.50 (tt, $J = 7.7, 7.7$ Hz, 2H), 1.81 (tt, $J = 7.1, 7.1$ Hz, 2H), 2.79 (t, $J = 7.8$ Hz, 2H), 2.91–3.01 (m, 4H), 3.93 (t, $J = 7.6$ Hz, 2H), 4.22–4.34 (m, 1H), 7.33–7.37 (m, 3H), 7.37–7.43 (m, 2H), 7.46–7.53 (m, 3H), 8.42–8.47 (m, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 13.9 and 14.0, 20.1 and 20.4, 28.5, 29.6 and 30.7, 30.4, 37.9, 41.7, 44.6 and 48.6, 79.6, 110.1, 112.2, 122.9, 123.4, 124.0, 126.9, 128.3, 129.6, 130.1, 130.9, 136.1, 147.9, 155.9, 167.5, 187.7; HRMS (ESI) Calcd. for $\text{C}_{32}\text{H}_{43}\text{N}_3\text{O}_4\text{Na}$: 556.3146, Found 556.3155.

Synthesis of 3



Trifluoroacetic acid (140 μ L, 1.8 mmol) was added to a solution of **35** (24 mg, 44 μ mol) in CH₂Cl₂ (1.5 mL), and the mixture

was stirred for 20 h at room temperature. Saturated NaHCO_3 was added to it, and the resulting mixture was extracted with CH_2Cl_2 . The combined organic layer was washed with brine, and dried over Na_2SO_4 . The solvent was removed under reduced pressure, and the crude material was dissolved in MeOH (2 mL). *N,N*-Diisopropylethylamine (15 μL , 89 μmol) and NBD-Cl (8.9 mg, 44 μmol) were added to the solution, and stirring was continued for 16 h at room temperature. After removal of the solvent under reduced pressure, the residue was purified by silica gel column chromatography (MeOH/ CHCl_3 , 1:100), followed by preparative TLC (MeOH/ CHCl_3 , 1:100), to afford the product **3** (19 mg, 71%) as an orange solid. **3**: M.p. 88–89 °C; ^1H NMR (400 MHz, CDCl_3) δ 0.83 (t, J = 7.0 Hz, 3H), 0.84 (t, J = 6.8 Hz, 3H), 1.01–1.12 (m, 2H), 1.14 (tt, J = 6.8, 6.8 Hz, 2H), 1.18 (tt, J = 7.2, 7.2 Hz, 2H), 1.48 (tt, J = 7.6, 7.6 Hz, 2H), 2.00 (tt, J = 6.6, 6.6 Hz, 2H), 2.79 (t, J = 7.8 Hz, 2H), 2.95 (t, J = 7.8 Hz, 2H), 3.27–3.36 (m, 2H), 4.02 (t, J = 7.2 Hz, 2H), 5.87 (d, J = 8.4 Hz, 1H), 6.76 (br-s, 1H), 7.25–7.33 (m, 8H), 8.29–8.34 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ 13.8 and 14.0, 20.1 and 20.4, 28.1, 29.5 and 30.7, 40.9, 41.3, 44.7 and 48.6, 98.9, 110.0, 112.2, 122.6, 123.7, 124.3, 126.6, 128.3, 129.1, 130.1, 130.6, 135.8, 136.4, 143.9, 144.1, 147.7, 167.7, 187.6; HRMS (ESI) Calcd. for $\text{C}_{33}\text{H}_{36}\text{N}_6\text{O}_5\text{Na}$: 619.2639, Found 619.2646.

General information for LC-LIF-MS system

Materials for HPLC analysis

Trifluoroacetic acid (TFA), distilled water containing 0.1vol% formic acid (FA), and acetonitrile (MeCN) containing 0.1vol% FA were purchased from Kanto Chemical Co., Inc.

Liquid chromatography and mass spectrometry

Mass spectra were acquired using a LTQ Orbitrap XL equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific, Inc.). For automatic measurement, the system was controlled by Xcalibur software (Thermo Fisher Scientific, Inc.). Full mass scan was acquired in the FT mode (resolution 60,000) and MS/MS scan (CID) was acquired in the ion trap (IT) mode or FT mode. In the nano flow HPLC system (UltiMate 3000 nano LC system, Thermo Fisher Scientific, Inc.), an Acclaim PepMap 100 C18 (0.075 i. d. x 150 mm, Thermo Fisher Scientific, Inc.) and a ZORBAX 300SB-C18 (0.3 i. d. x 5 mm, Agilent Technologies, Inc.) were used as analytical and trap columns, respectively. For the analytical column, mobile-phase A (distilled water containing 0.1vol% FA and 4vol% MeCN) and mobile-phase B (MeCN containing 0.1vol% FA) were utilized. Mobile-phase C (distilled water containing 0.1vol% TFA) and mobile-phase D (MeCN containing 0.1vol% TFA) were used for the trap column. Gradient elution was done with mobile-phase A and mobile-phase B at a flow rate of 250 nL/min. Database search was performed using a peptide sequencing program, Proteome Discoverer (Thermo Fisher Scientific, Inc.) through a database MS/MS ion search, and with MASCOT (www.matrixscience.com). Theoretical mass value of peptides was calculated by Xcalibur software.

Laser-induced fluorescence detector

The laser-induced fluorescence (LIF) detector (LIF 727, GL Sciences, Inc.) was fitted with a 6 nL flow cell and connected to the nano flow HPLC system (UltiMate 3000 nano LC system, Thermo Fisher Scientific, Inc.). Parameters of the LIF 727 were as follows; PMT 700V, response 0.5 sec, output range 1 RFU/FS1V, band pass filter 500–600 nm.

TSPO knock-down

HEK293T cells were plated in 6 well plates at the density of 2.5×10^5 cells per well. Cells were transfected with 40 nM Stealth RNAi (TSPO-HSS141398, TSPO-HSS186329; Invitrogen Life Technologies) using X-tremeGENE siRNA Transfection Reagent (Roche) and Opti-MEM I reduced serum medium (Gibco Life Technologies). Negative Universal control Med (Invitrogen Life Technologies) was used as a negative control. After 24 h incubation, medium was changed to fresh growth medium. After further incubation for 24 h, cells were pretreated with or without **1** (87.5 μ M) for 0.5 h and then treated with **3-ONBD** (0.5 μ M) for 1.5 h. After the treatment of compounds, cells were washed with PBS and lysed with lysis buffer (20 mM Hepes–Na (pH 7.0), 1 mM EDTA, 0.5% Triton X-100, and complete mini (Roche, 1 tablet/10 mL)).

Animal experiment

Kidney mitochondria were prepared from female B6 mice (Nihon SLC, Hamamatsu, Japan). All procedures were conducted according to the RIKEN guidelines for animal research and were approved by the RIKEN Animal Experiments Committee.

Western blotting

To detect VDAC1, TSPO and tubulin, anti-VDAC1/Porin (1:1000, abcam, ab14734), anti-PBR (1:10000, abcam, ab109497) and anti- α -tubulin (1:1000, sigma, T9026) antibodies were used, respectively. Goat anti-mouse IgG (1:2000, Bio-Rad, #170-6516) and goat anti-rabbit IgG (1:2000, Santa Cruz Biotechnology, SC-2004) were used as secondary antibodies. Immunoreactive bands were detected using a chemiluminescence reagent (Immobilon Western chemiluminescent HRP substrate,

Millipore) with a luminescent image analyzer (LAS-4000, Fujifilm).

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