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Synthesis of *N*-functionalized oleamide derivatives

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Abstract—Oleamide is an interesting compound, which shows various pharmacological activities including the inhibitory effect of gap junction formation. Recently, the studies of the gap junction have been some of the hot topics in biology and its inhibitors have become more useful tools [Cravatt, B. F.; Garcia, O. P.; Siuzdak, G.; Gilula, N. B.; Henriksen, S. J.; Boger, D. L.; Lerner, R. A. *Science* **1995**, *268*, 1506–1509; Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580–590; Guan, X; Cravatt, B. F.; Ehring, G. R.; Hall, J. E.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *J. Cell Biol.* **1997**, *139*, 1785–1792; Boger, D. L.; Patterson, J. E.; Guan, X.; Cravatt, B. F.; Lerner, R. A.; Gilula, N. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4810–4815; Ito, A.; Morita, N.; Miura, D.; Koma, Y.; Kataoka, T. R.; Yamasaki, H.; Kitamura, Y.; Kita, Y.; Nojima, H. *Carcinogenesis* **2004**, *25*, 2015–2022]. However, many reports suggest that the functionalizations of oleamide to retain its biological activity were difficult [Boger, D. L.; Patterson, J. E.; Guan, X.; Cravatt, B. F.; Lerner, R. A.; Gilula, N. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4810–4815; Ito, A.; Morita, N.; Miura, D.; Koma, Y.; Kataoka, T. R.; Yamasaki, H.; Kitamura, Y.; Kita, Y.; Nojima, H. *Carcinogenesis* **2004**, *25*, 2015–2022]. The synthesis of the functionalized oleamide derivatives, whose biological activity is not blocked, has become attractive in both the biological and chemical fields.
Herein, by linking the fluorophore to the oleamide by alkyl chains, we synthesized the fluorescently tagged oleamide whose biological feature is similar to that of oleamide. Moreover, we also synthesized other bioactive derivatives tagged by other groups such as the sugars and biotin via alkyl chain linkers.

1. Introduction

Oleamide, an endogenous fatty acid with a primary amide, is known to accumulate in the cerebrospinal fluid under sleep deprivation conditions.[1] Oleamide induces physiological sleep in mice by ip or iv injection. Oleamide also inhibits the gap junction-mediated intercellular communications (GJICs) without any effect on the calcium wave transmission.[2,3] We also found that it inhibits both connexin (Cx)43/Cx26-mediated GJICs, a non-selective connexin inhibitor.[4] Cx belongs to the cell membrane protein family that constitutes a gap junction and nearly 22 kinds of its subtypes have already been identified. Connexon, a hexamer of the Cx molecule, is a pipe-shaped structure and penetrates the cell membrane and enables the passage of small molecules, such as ions and proteins with low molecular weight through cells.[5]

Recently, the studies of the gap junction have been some of the hot topics in biology. For example, many Cx-subtypes that constitute the gap junction were discovered and many functions of them were found in the last decade.[1–4] Then, its inhibitors have become more useful tools. The synthesis of the functionalized oleamide derivatives, whose biological activity is not blocked, has become attractive in both the biological and chemical fields. However, many reports suggest that the derivatizations of oleamide to retain the inhibitory effect of GJICs were difficult.[3,4]

Now, we report that the fluorescently tagged oleamides, which inhibit the Cx43-mediated GJICs at a level similar to oleamide, were synthesized. And various functionalized oleamide derivatives were also synthesized.

2. Results and discussion

2.1. Design and synthesis of fluorescently tagged oleamide

Oleamide has an unsaturated alkyl chain and primary amide. For derivatization of the oleamide into functionalized

Keywords: Gap junction; Oleamide derivatives; Functional groups; Alkyl linker.
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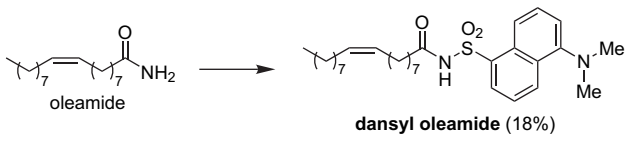
analogues, two strategies are available: (1) the derivatization of its alkyl chain and (2) the introduction of the functional groups on its amide unit. Boger and co-workers reported that the oleamide was the most potent inhibitor of GJICs and the changes in its alkyl chain reduced its activity.[3] We also found that the alkyl chain of the oleamide was critical for its potent biological activity.[4] The oleamide structure is similar to half of phospholipids consisting of the plasma membrane. Therefore, the derivatizations of its alkyl chain may lead to the reduction of its similarity to the phospholipids and loss of its biological activity. As described above, the functionalizations of oleamide for retaining its biological activity were difficult.[3,4] Thus, we tried to modify its amide unit by labeling with functional groups.

First, the fluorescent 5-(dimethylamino)naphth-1-yl sulfonyl (dansyl) group as the functional group was introduced into the oleamide. We previously reported that the amide part of oleamide derivatives substituted by the electron withdrawing groups such as acyl and sulfonyl, the aromatic and the hydrophilic groups, did not inhibit the GJICs.[4] Indeed, the oleamide derivative labeled on its amide part by the dansyl group,[6] namely, the dansyl oleamide, was synthesized. However, it did not inhibit the Cx43-mediated GJICs (Scheme 1, Table 1).



Scheme 1. Reaction conditions: NaH, DnsCl, DMF, room temperature. Dns (dansyl)=5-(dimethylamino)naphth-1-yl sulfonyl.

Therefore, a new dansyl oleamide design is necessary. Based on these results, we decided to attach the dansyl group to the oleamide via the alkyl chain linkers that show the electron donating, less bulky, and hydrophobic nature. Since various fluorescent groups could be tagged by a simple procedure, we selected diamines as the linker. Two synthetic methodologies are available: (1) oleamide part is first constructed and tagged by the dansyl group and (2) the dansyl group is first linked to the diamine and then the oleamide part is introduced (Scheme 2).

For the first method, the mono-substituted amines including the oleamide part were obtained. However, their yields were low because the formations of its dimeric compounds mainly occurred. Moreover, they were insoluble solids, which were difficult to react with the dansyl group. Thus, we chose the latter method. Dansyl chloride (1 equiv) was reacted with the diamines (3 equiv) to afford the mono-dansylated amines **1**. They were easily detected by UV and easy to handle. The C*n*-linked dansyl oleamides were obtained in high yield by the reaction of **1** with oleic chloride prepared from oleic acid and oxalyl chloride (Scheme 3).

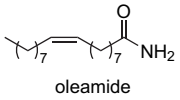
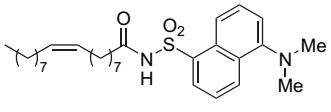
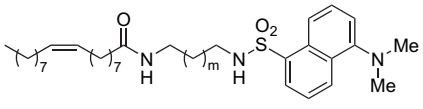
2.2. Cell study of fluorescently tagged oleamide

All the C*n*-linked dansyl oleamides inhibited the Cx43-mediated GJICs at a level similar to the oleamide (Table 1). Among them, the biological activity of the C4/C10-linked dansyl oleamides was more similar to the oleamide (92%, 87%) than that of the C2-linked dansyl oleamide (67%).

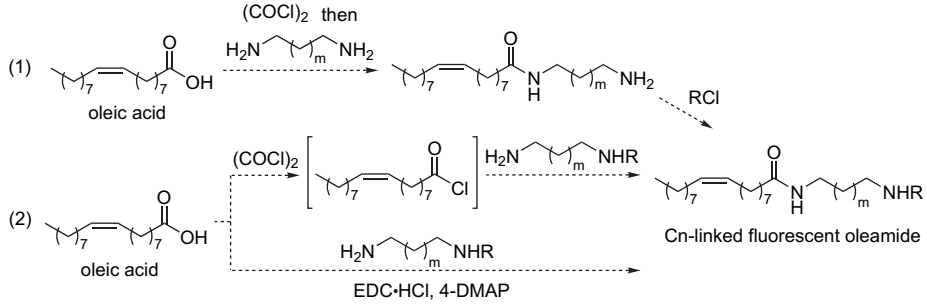
2.3. Synthesis and biological evaluation of functionalized oleamide via the alkyl chain linker

Based on these results, various functionalized oleamide derivatives via the alkyl chain linkers were synthesized (Scheme 4). The oleamide derivatives tagged by the 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) group,[8] that are

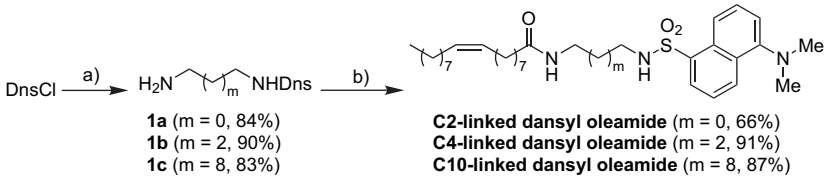
Table 1. Dye transfer assay of C*n*-linked dansyl oleamides using HeLa-Cx43[7]

Agent	Cx43-mediated GJIC score[a]	Inhibition rate[b] (%)
Control	5.18±1.10	—
 oleamide	2.82±0.81*	100
 dansyl oleamide	4.77±0.87	20
 Cn-linked dansyl oleamide		
C2-linked dansyl oleamide (*m*=0)	3.61±0.68*	67
C4-linked dansyl oleamide (*m*=2)	3.17±0.62*	85
C10-linked dansyl oleamide (*m*=8)	3.00±0.68*	92

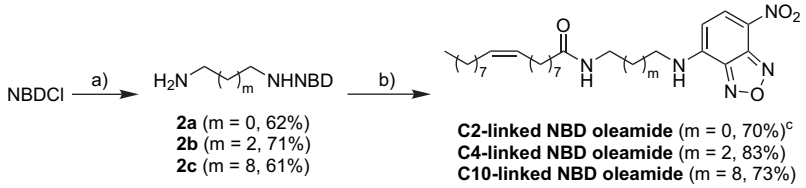
*$p<0.05$; $n>20$.
[a] Donor and recipient cells were co-cultured in the presence of the agents at a concentration of 40 μM. The standard deviations (SD) are shown as ±.
[b] The percent inhibition of the transfer of the calcein dye from the donor cells to recipient cells was determined for comparison with the oleamide (100%).



Scheme 2. Synthetic methodologies of C*n*-linked functional oleamide. R=functional groups, EDC·HCl=1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 4-DMAP=4-dimethylamino-pyridine.



Scheme 3. Reaction conditions: (a) diamines, CH_2Cl_2, room temperature; (b) oleic chloride, CH_2Cl_2, room temperature. Dns (dansyl)=5-(dimethylamino)-naphth-1-yl sulfonyl.

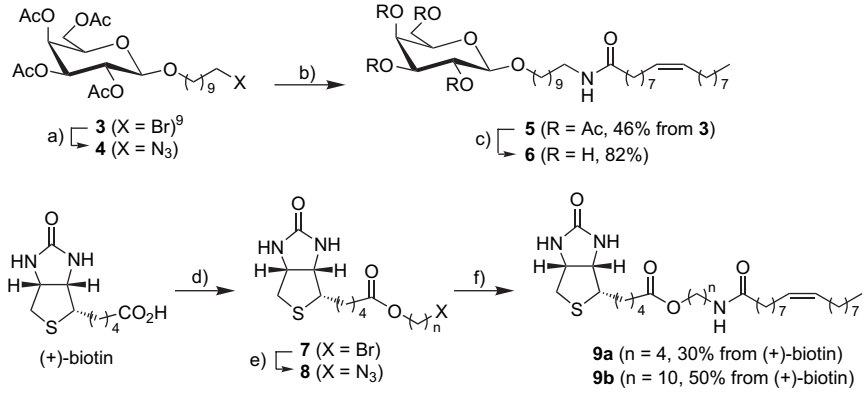


Scheme 4. Reaction conditions: (a) diamines, $NaHCO_3$, THF/EtOH, room temperature; (b) oleic acid, EDC·HCl, 4-DMAP, DMF, room temperature. NBD=7-nitro-2,1,3-benzoxadiazol-4-yl, EDC·HCl=1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 4-DMAP=4-(dimethylamino)pyridine; (c) when **2a** was reacted with oleic acid in the presence of EDC·HCl and 4-DMAP, only the diacylated compound was formed. Thus, oleic chloride was used in this case.

known to show the different λ_{exc} (315 nm for dansyl moiety and 460–480 nm for NBD moiety), were synthesized by a method similar to the C*n*-linked dansyl oleamides. Although the exactly same method failed to give the C*n*-linked NBD oleamides, these were obtained in high yields by the condensation reaction of **2** with oleic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl).

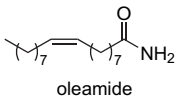
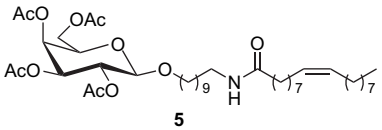
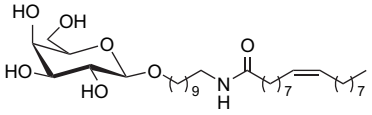
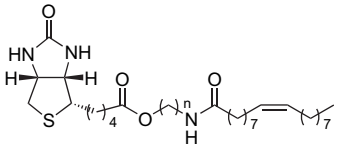
Moreover, the oleamide derivatives labeled by (+)-biotin or sugar (galactose) via the alkyl chain linkers were also synthesized by the simple procedure (Scheme 5).

The inhibitory effects of the Cx43-mediated GJICs by these functionalized oleamides were examined (Table 2). Since the NBD group has the same excitation spectrum of the dyes used in the dye transfer assay,[4] the functionalized



Scheme 5. Reaction conditions: (a) NaN_3, DMF, 60 °C; (b) PPh_3, CH_2Cl_2, then oleic chloride, CH_2Cl_2, room temperature; (c) NaOEt, EtOH, room temperature; (d) 1-bromo-4-butanol or 1-bromo-10-decanol, EDC·HCl, 4-DMAP, DMF, room temperature; (e) NaN_3, DMF, 60 °C; (f) (i) Lindlar catalyst, H_2 (1 atm), MeOH, room temperature; (ii) oleic chloride, CH_2Cl_2, room temperature.

Table 2. Dye transfer assay of the functionalized oleamide derivatives using the alkyl linker[7]

Agent	Cx43-mediated GJIC score[a]	Inhibition rate[b] (%)
Control	4.34±0.49	—
 oleamide	2.00±0.57*	100
 **5**	2.76±0.81*	68
 **6**	2.69±0.85*	71
 **9**		
9a (n=4)	2.72±0.78*	69
9b (n=10)	2.63±0.73*	73

*$p<0.05$; $n>30$.

[a] Donor and recipient cells were co-cultured in the presence of the agents at a concentration of 40 μM. The standard deviations (SD) are shown as ±.

[b] The percent inhibition of the transfer of the calcein dye from the donor cells to recipient cells was determined for comparison with the oleamide (100%).

oleamides excluding the C*n*-linked NBD oleamides were used in this study. As expected, all the oleamide derivatives functionalized by the various groups via the alkyl chain linkers inhibited the Cx43-mediated GJICs at a level similar to oleamide. These results suggested that the functionalizations of the oleamide could be accomplished by using the alkyl chain linkers without its biological activity being influenced by these groups.

3. Conclusion

We synthesized the C*n*-linked dansyl oleamides whose biological activities are retained. Especially, the C4/C10-linked dansyl oleamides show the inhibitory effect of GJICs at a level similar to the oleamide. Moreover, we found that other functional groups such as NBD, sugars, and (+)-biotin could be introduced into the oleamide via alkyl chain linkers without affecting its biological activity. By the strategy, various functions would be fused into oleamide. This method presented here is very useful in various fields.

4. Experimental

4.1. General

The NMR spectra were measured using 300 MHz or 270 MHz spectrometer with tetramethylsilane as the internal standard at 20–25 °C. IR spectra were recorded by a diffuse reflectance measurement of samples dispersed in KBr powder. E. Merck silica gel 60 for column chromatography and E. Merck pre-coated TLC plates, silica gel F_{254}, for preparative thin-layer chromatography were used.

4.2. Synthetic chemistry

4.2.1. Synthesis of dansyl oleamide. Under a nitrogen atmosphere, a solution of NaH (60% dispersion in mineral oil, 21 mg, 0.53 mmol) in THF (2.5 mL) was cooled to 0 °C, and oleamide (100 mg, 0.36 mmol) in THF (1.0 mL) was added. The reaction mixture was stirred at room temperature for 30 min and dansyl chloride (94 mg, 0.36 mmol) was added to the reaction mixture at 0 °C. The reaction mixture was stirred at room temperature for 3 h and poured into H_2O. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 4/1) gave dansyl oleamide (32 mg, 18%) as slightly green oil.

IR (KBr): 3234, 1693, 1454 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.87 (3H, t, J=6.2 Hz), 1.11–1.62 (22H, m), 1.91–2.05 (4H, m), 2.21 (2H, t, J=7.5 Hz), 2.90 (6H, s), 5.29–5.34 (2H, m), 7.19 (1H, d, J=7.2 Hz), 7.59 (2H, m), 8.21 (1H, d, J=9.0 Hz), 8.50 (2H, m), 8.62 ppm (1H, d, J=8.4 Hz). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 171.1, 152.2, 133.2, 132.3, 131.9, 130.0, 129.7, 129.7, 129.5, 128.8, 123.3, 118.0, 115.2, 45.4 (2C), 36.2, 31.9, 29.7, 29.6, 29.5, 29.3 (2C), 29.0, 28.9, 28.6, 27.2, 27.1, 24.2, 22.6, 14.1 ppm. LRMS (FAB) m/z 537 (MNa^+): HRMS (FAB) calcd for $C_{30}H_{46}O_3N_2SNa$: 537.3127. Found: 537.3118 (MNa^+).

4.2.2. Synthesis of C*n*-linked dansyl oleamide.

4.2.2.1. C2-linked dansyl oleamide. Under a nitrogen atmosphere, the solution of 1,2-ethylenediamine (90 mg, 1.50 mmol) in CH_2Cl_2 (3 mL) was cooled to 0 °C, and dansyl chloride (135 mg, 0.50 mmol) in CH_2Cl_2 (2.0 mL) was added. The reaction mixture was stirred at room temperature for 6 h and poured into 10% NaOHaq. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 2/1) gave **1a** (123 mg, 84%).

^1H NMR ($CDCl_3$, 270 MHz) δ: 2.72 (2H, t, J=6.5 Hz), 2.89–3.01 (2H, m), 2.89 (6H, s), 7.20 (1H, d, J=7.3 Hz), 7.55 (2H, m), 8.28 (2H, m), 8.54 ppm (1H, d, J=8.6 Hz).

Under a nitrogen atmosphere, the solution of oleic acid (50 mg, 0.18 mmol) in CH_2Cl_2 (3.4 mL) was cooled to 0 °C, and oxalyl chloride (64 μL, 0.72 mmol) was added. The reaction mixture was stirred at room temperature for 4 h and concentrated in vacuo to give the residue.

Under a nitrogen atmosphere, the residue (oleic chloride) in CH_2Cl_2 (2.0 mL) was slowly added to the solution of **1a** (44 mg, 0.15 mmol) in CH_2Cl_2 (1.0 mL). The reaction mixture was stirred at room temperature for 3 h and poured into H_2O. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and

concentrated in vacuo. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 1/1) gave *C2-linked dansyl oleamide* (54 mg, 66%) as slightly green oil.

IR (KBr): 3394, 1653, 1219 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 0.80 (3H, t, J=7.3 Hz), 1.19–1.49 (22H, m), 1.89–1.98 (6H, m), 2.83 (6H, s), 2.94–2.98 (2H, m), 3.19–3.25 (2H, m), 5.26 (3H, br s), 5.63 (1H, br s), 7.19 (1H, d, J=7.3 Hz), 7.48–7.57 (2H, m), 8.15–8.18 (2H, m), 8.50 ppm (1H, d, J=8.6 Hz). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 174.3, 151.9, 134.4, 130.6, 130.0, 129.9, 129.7, 129.6, 129.5, 128.5, 123.2, 118.7, 115.3, 45.4 (2C), 43.2, 39.1, 36.4, 31.9, 29.7, 29.7, 29.5, 29.3 (2C), 29.2 (2C), 29.1, 27.2, 27.2, 25.5, 22.6, 14.1 ppm. LRMS (FAB) m/z 558 (MH^+): HRMS (FAB) calcd for $C_{32}H_{52}O_3N_3S$: 558.3729. Found: 558.3735 (MH^+).

4.2.2.2. C4-linked dansyl oleamide. Similarly to the preparation of **1a**, 1,4-butanediamine (132 mg, 1.50 mmol) was treated with dansyl chloride (135 mg, 0.50 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and worked up as usual. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 2/1) gave **1b** (145 mg, 90%).

^1H NMR ($CDCl_3$, 270 MHz) δ: 1.40–1.52 (2H, m), 2.65 (2H, t, J=5.4 Hz), 2.82–2.91 (2H, m), 2.82 (6H, s), 7.17 (1H, d, J=7.3 Hz), 7.48–7.56 (2H, m), 8.22 (1H, d, J=8.6 Hz), 8.33 (2H, d, J=8.9 Hz), 8.52 ppm (1H, d, J=8.6 Hz).

Similarly to the preparation of *C2-linked dansyl oleamide*, **1b** (48 mg, 0.15 mmol) was treated with oleic chloride (ca. 0.18 mmol) prepared from oleic acid (50 mg, 0.18 mmol) and oxalyl chloride (64 μL, 0.72 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 4 h and worked up as usual. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 1/1) gave *C4-linked dansyl oleamide* (80 mg, 91%) as slightly green oil.

IR (KBr): 3300, 1651, 1315 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7.0 Hz), 1.26–1.59 (26H, m), 1.99–2.18 (6H, m), 2.90 (6H, s), 2.89–2.91 (2H, m), 3.12–3.15 (2H, m), 4.88 (1H, br s), 5.34 (2H, br s), 7.21 (1H, d, J=7.6 Hz), 7.50–7.60 (2H, m), 8.22–8.30 (2H, m), 8.55 ppm (1H, d, J=8.4 Hz). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 173.4, 151.9, 134.7, 130.3, 129.9, 129.8, 129.7, 129.5, 129.4, 128.3, 123.1, 118.7, 115.1, 45.3 (2C), 42.8, 38.6, 36.6, 31.8, 29.7, 29.7, 29.4, 29.2 (2C), 29.2 (2C), 29.1, 27.1, 27.1, 26.7, 26.5, 25.7, 22.6, 14.1 ppm. LRMS (FAB) m/z 586 (MH^+): HRMS (FAB) calcd for $C_{34}H_{56}O_3N_3S$: 586.4042. Found: 586.4033 (MH^+).

4.2.2.3. C10-linked dansyl oleamide. Similarly to the preparation of **1a**, 1,10-decanediamine (228 mg, 1.50 mmol) was treated with dansyl chloride (135 mg, 0.50 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and worked up as usual. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 2/1) gave **1c** (168 mg, 83%).

^1H NMR ($CDCl_3$, 270 MHz) δ: 1.00–1.52 (16H, m), 2.67 (2H, t, J=6.8 Hz), 2.85–2.90 (2H, m), 2.85 (6H, s), 7.20 (1H, d, J=7.3 Hz), 7.53 (2H, m), 8.26 (2H, m), 8.54 ppm (1H, d, J=8.9 Hz).

Similarly to the preparation of *C2-linked dansyl oleamide*, **1c** (30 mg, 0.074 mmol) was treated with oleic chloride (ca. 0.09 mmol) prepared from oleic acid (25 mg, 0.09 mmol) and oxalyl chloride (32 μL, 0.36 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 4 h and worked up as usual. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 1/1) gave *C10-linked dansyl oleamide* (42 mg, 87%) as slightly green solid.

Mp 40–43 °C. IR (KBr): 3290, 1651, 1454 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 0.88 (3H, t, J=6.2 Hz), 1.11–1.58 (38H, m), 1.99–2.18 (6H, m), 2.84 (6H, s), 2.84–2.90 (2H, m), 3.19–3.26 (2H, m), 4.57 (1H, br s), 5.34 (2H, br s), 7.22 (1H, d, J=8.4 Hz), 7.51–7.60 (2H, m), 8.24–8.30 (2H, m), 8.54 ppm (1H, d, J=8.4 Hz). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 173.2, 151.7, 134.8, 130.3, 130.0, 129.9, 129.8, 129.7, 129.6, 128.3, 123.3, 118.0, 115.2, 45.4 (2C), 43.3, 39.5, 36.9, 33.9, 31.9 (2C), 29.7, 29.7, 29.6, 29.5, 29.3 (2C), 29.1, 29.1, 28.8, 27.2 (2C), 27.2, 26.8, 26.3, 25.8, 24.7, 22.7 (2C), 14.1 ppm. LRMS (FAB) m/z 670 (MH^+): HRMS (FAB) calcd for $C_{40}H_{68}O_3N_3S$: 670.4981. Found: 670.5003 (MH^+).

4.2.3. Synthesis of C*n*-linked NBD oleamide.

4.2.3.1. C2-linked NBD oleamide. Under a nitrogen atmosphere, 1,2-ethylenediamine (12 mg, 0.20 mmol) and $NaHCO_3$ (25 mg, 0.30 mmol) in THF/EtOH (v/v=1/1, 1.0 mL) were cooled to 0 °C, and NBD chloride (20 mg, 0.10 mmol) in THF/EtOH (v/v=1/1, 1.0 mL) was slowly added over 15 min. The reaction mixture was stirred at room temperature for 12 h and poured into H_2O. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 2/1) gave **2a** (14 mg, 62%).

IR (KBr): 3250 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 3.17 (2H, t, J=5.4 Hz), 3.50 (2H, t, J=5.4 Hz), 6.18 (1H, d, J=7.8 Hz), 8.50 ppm (1H, d, J=8.1 Hz).

Similarly to the preparation of *C2-linked dansyl oleamide*, **2a** (12 mg, 0.05 mmol) was treated with oleic chloride (ca. 0.11 mmol) prepared from oleic acid (30 mg, 0.11 mmol) and oxalyl chloride (33 μL, 0.37 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and worked up as usual. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 1/2) gave *C2-linked NBD oleamide* (18 mg, 70%) as yellow solid.

Mp 118–120 °C. IR (KBr): 3288, 1622, 1306 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 0.81 (3H, t, J=7.0 Hz), 1.19–1.56 (22H, m), 1.92–1.98 (4H, m), 2.20 (2H, t, J=7.0 Hz), 3.55–3.64 (4H, m), 5.23–5.26 (2H, m), 5.91 (1H, br s), 6.08 (1H, d, J=8.6 Hz), 7.79 (1H, br s), 8.41 ppm (1H, d, J=8.4 Hz). ^{13}C NMR (67.8 MHz) δ: 176.1, 144.4, 144.2, 136.6, 130.0, 129.6, 98.4, 45.9, 38.5, 36.4, 31.9, 29.7, 29.6, 29.5, 29.3 (2C), 29.2 (2C), 29.1, 27.2, 27.1, 25.6, 22.7, 14.1 ppm. LRMS (FAB) m/z 488 (MH^+): HRMS (FAB) calcd for $C_{26}H_{42}O_4N_5$: 488.3237. Found: 488.3249 (MH^+).

4.2.3.2. C4-linked NBD oleamide. Similarly to the preparation of **2a**, 1,4-butanediamine (18 mg, 0.20 mmol) was treated with NBD chloride (20 mg, 0.10 mmol) and $NaHCO_3$ (25 mg, 0.30 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and worked up as usual. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 2/1) gave **2b** (18 mg, 71%).

IR (KBr): 3300 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ: 1.72 (2H, qui, J=6.3 Hz), 1.96 (2H, t, J=6.3 Hz), 2.87 (2H, t, J=6.0 Hz), 3.44 (2H, t, J=6.0 Hz), 6.07 (1H, d, J=9.0 Hz), 8.48 (1H, d, J=8.7 Hz).

Under a nitrogen atmosphere, the solution of **2b** (25 mg, 0.10 mmol) and oleic acid (28 mg, 0.10 mmol), 4-DMAP (13 mg, 0.11 mmol) in DMF (1 mL) was cooled to 0 °C, and EDC·HCl (21 mg, 21 mmol) was added. The reaction mixture was stirred at room temperature for 12 h and poured into H_2O. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 1/2) gave *C4-linked NBD oleamide* (43 mg, 83%) as yellow solid.

Mp 75–77 °C. IR (KBr): 3306, 1634, 1258 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 0.87 (3H, t, J=6.8 Hz), 1.25–1.87 (26H, m), 1.98–2.01 (4H, m), 2.20 (2H, t, J=7.2 Hz), 3.20–3.35 (2H, m), 3.45–3.62 (2H, m), 5.30–5.34 (3H, m), 5.60 (1H, br s), 6.2 (1H, d, J=8.4 Hz), 8.49 ppm (1H, d, J=8.7 Hz). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 174.0, 144.2, 143.9, 136.6, 130.0, 129.6, 98.8, 43.8, 38.6, 36.8, 31.8, 29.7, 29.6, 29.5, 29.3 (4C), 29.1, 27.7, 27.2, 27.2, 25.8, 25.1, 22.6, 14.1 ppm. LRMS (FAB) *m/z* 516 (MH^+): HRMS (FAB) calcd for $C_{28}H_{46}O_4N_5$: 516.3549. Found, 516.3561 (MH^+).

4.2.3.3. C10-linked NBD oleamide. Similarly to the preparation of **2a**, 1,10-decanediamine (34 mg, 0.20 mmol) was treated with NBD chloride (20 mg, 0.10 mmol) and $NaHCO_3$ (25 mg, 0.30 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and worked up as usual. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 2/1) gave **2c** (20 mg, 61%).

IR (KBr): 3300 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 1.25–1.84 (16H, m), 2.69 (2H, t, J=6.6 Hz), 3.48 (2H, t, J=7.3 Hz), 6.17 (1H, d, J=8.9 Hz), 8.50 ppm (1H, d, J=8.6 Hz).

Similarly to the preparation of *C4-linked NBD oleamide*, **2c** (21 mg, 0.06 mmol) was treated with oleic acid (17 mg, 0.06 mmol), 4-DMAP (8 mg, 0.07 mmol), and EDC·HCl (13 mg, 0.07 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12 h and worked up as usual. Purification of the residue by column chromatography (*n*-hexane/AcOEt, 1/1) gave *C10-linked NBD oleamide* (27 mg, 73%) as yellow solid.

Mp 71–73 °C. IR (KBr): 3315, 1634, 1258 cm^{-1}. ^1H NMR ($CDCl_3$, 270 MHz) δ: 0.81 (3H, t, J=6.8 Hz), 1.19–1.76 (38H, m), 1.92–1.98 (4H, m), 2.09 (2H, t, J=7.8 Hz), 3.16–3.18 (2H, m), 3.41–3.43 (2H, m), 5.23–5.27 (3H, m), 6.11 (1H, d, J=8.9 Hz), 6.27 (1H, br s), 8.43 ppm (1H, d, J=8.9 Hz). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 173.2, 144.3, 144.1, 136.6, 130.0, 129.7, 98.4, 44.0, 39.4, 39.4, 36.9, 31.9, 29.7, 29.7, 29.6, 29.5, 29.3 (2C), 29.2 (2C), 29.2, 29.1, 29.0, 28.4, 27.2, 27.1, 26.8, 26.8, 26.7, 25.8, 22.6, 14.1 ppm. LRMS (FAB) *m/z* 600 (MH^+): HRMS (FAB) calcd for $C_{34}H_{58}O_4N_5$: 600.4489. Found: 600.4515 (MH^+).

4.2.4. Synthesis of functionalized oleamide.

4.2.4.1. Oleamide with 2,3,4,6-tetra-*O*-acetyl-β-D-galactose via alkyl chain linker (5). Under a nitrogen atmosphere, NaN_3 (180 mg, 2.76 mmol) was added to the solution of **3** (370 mg, 0.63 mmol)[9] in DMF (6.3 mL). The reaction mixture was stirred at 60 °C for 3 h and poured into H_2O. The aqueous layer was extracted with AcOEt and washed with H_2O, dried over Na_2SO_4, and concentrated in vacuo to give the unpurified **4**.

Under a nitrogen atmosphere, PPh_3 (150 mg, 0.63 mmol) was added to the solution of unpurified **4** in CH_2Cl_2 (2.2 mL) and stirred at room temperature for 1.5 h. The reaction mixture was cooled to 0 °C, and the solution of oleic chloride (ca. 0.63 mmol) in CH_2Cl_2 (0.3 mL) prepared from oleic acid (178 mg, 0.63 mmol) and oxalyl chloride (0.186 mL, 2.12 mmol) was slowly added. The resulting mixture was stirred at room temperature for 30 min and poured into H_2O. The aqueous layer was extracted with AcOEt, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by column chromatography (hexane/AcOEt, 3/2) gave **5** (200 mg, 46% from **3**) as colorless oil.

IR (KBr): 1754, 1644 cm^{-1}. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.78–0.90 (3H, t, J=7.0 Hz), 1.20–1.52 (38H, m), 1.92 (3H, s), 1.92–1.98 (4H, m), 1.98 (6H, s), 2.06–2.11 (2H, m), 2.08 (3H, s), 3.12–3.20 (2H, m), 3.38–3.41 (1H, m), 3.78–3.86 (2H, m), 4.02–4.13 (2H, m), 4.37–4.40 (1H, d, J=7.3 Hz), 4.92–4.97 (1H, dd, J=13.8, 3.2 Hz), 5.10–5.17 (1H, dd, J=10.5, 8.1 Hz), 5.27–5.32 (1H, m), 5.31–5.32 (2H, d, J=3.2 Hz), 5.40 ppm (1H, br s). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ: 172.9, 170.2, 170.0, 170.0, 169.2, 129.8, 129.6, 101.3, 70.9, 70.5, 70.3, 68.9, 67.0, 61.2, 39.5, 36.9, 31.9, 29.8, 29.7, 29.7, 29.6, 29.5, 29.5, 29.4, 29.3, 29.3, 29.3, 29.3, 29.3, 29.3, 29.2, 27.2, 27.2, 26.9, 25.9, 25.8, 22.7, 20.8, 20.7, 20.7, 20.7, 14.2 ppm. LRMS (FAB) *m/z* 769 (MH^+): HRMS (FAB) calcd for $C_{42}H_{74}O_{11}N$: 768.5260. Found: 768.5261 (MH^+).

4.2.4.2. Oleamide with β-D-galactose via alkyl chain linker (6). Under a nitrogen atmosphere, the solution of **5** (50 mg, 0.06 mmol) in EtOH (0.6 mL) was cooled to 0 °C, and NaOEt (22 mg, 0.27 mmol) was added. The reaction mixture was stirred at room temperature for 2 h, and large amount (ca. 30 mg) of ammonium chloride was dropped. The resulting mixture was filtrated to remove ammonium chloride and concentrated in vacuo. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 10/1) gave **6** (32 mg, 82%) as white solid.

Mp 127–127 °C. IR (KBr): 3269, 1622 cm^{-1}. ^1H NMR (270 MHz, CD_3OD) δ: 0.78–0.80 (3H, t, J=6.2 Hz), 1.22–1.60 (38H, m), 1.76–1.98 (4H, m), 2.04–2.12 (2H, t, J=7.2 Hz), 3.03–3.08 (2H, t, J=6.8 Hz), 3.38–3.44 (4H, m), 3.62–3.65 (2H, m), 3.73 (1H, br s), 3.73–3.81 (1H, m),

4.10–4.12 (1H, d, J=6.5 Hz), 5.10–5.58 ppm (2H, m). ^{13}C NMR (67.8 MHz, $CDCl_3$) δ: 175.9, 130.7, 130.6, 104.9, 76.5, 75.0, 72.5, 70.8, 70.2, 66.9, 62.4, 40.4, 37.2, 33.1, 30.9, 30.9, 30.8, 30.7, 30.7, 30.7, 30.7, 30.5, 30.5, 30.4, 30.4, 30.3, 30.3, 28.2, 28.1, 27.2, 27.2, 23.8, 15.5, 14.6 ppm. LRMS (EI$^+$) m/z 438: HRMS (EI$^+$) calcd for $C_{34}H_{65}O_7N$: 599.4761. Found: 599.4744.

4.2.4.3. C4-linked biotinated oleamide (9a). Under a nitrogen atmosphere, (+)-biotin (100 mg, 0.41 mmol), 4-DMAP (75 mg, 0.62 mmol), and EDC·HCl (118 mg, 0.62 mmol) were added to the solution of 1-bromo-4-butanol (118 mg, 0.62 mmol) in DMF (4.1 mL) at room temperature. The reaction mixture was stirred at 60 °C for 2 h and poured into H_2O. The aqueous layer was extracted with AcOEt. The organic layer was washed with H_2O and 1 N NaOHaq and 1 N HClaq and brine, dried over Na_2SO_4, and concentrated in vacuo to give the residue including **7a**.

Under a nitrogen atmosphere, NaN_3 (80 mg, 1.23 mmol) was added to the solution of the residue (unpurified **7a**) in DMF (4.1 mL), at room temperature. The reaction mixture was stirred at 80 °C for 3 h and poured into H_2O. The aqueous layer was extracted with AcOEt. The organic layer was washed with H_2O and brine, dried over Na_2SO_4, and concentrated in vacuo. The unpurified product **8a** was used without further purification.

To the solution of unpurified **8a** in MeOH (2.2 mL), Lindlar catalyst (palladium on barium sulfate, 15 mg) was added under H_2 (1 atm). The reaction mixture was stirred at room temperature for 12 h. Lindlar catalyst was filtrated by Celite and concentrated in vacuo.

Under a nitrogen atmosphere, oleic chloride (ca. 0.44 mmol) in CH_2Cl_2 (1.0 mL) prepared from oleic acid (124 mg, 0.44 mmol) and oxalyl chloride (168 mg, 1.32 mmol) was slowly added to the solution of the residue in CH_2Cl_2 (1.2 mL). The reaction mixture was stirred at room temperature for 3 h and poured into H_2O. The aqueous layer was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the residue by column chromatography (CH_2Cl_2/MeOH, 10/1) gave **9a** (70 mg, 30% from (+)-biotin) as white solid.

Mp 159–161 °C. IR (KBr): 3206, 1703, 1634 cm^{-1}. ^{1}H NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=7.1 Hz), 1.25–1.72 (32H, m), 2.03 (4H, m), 2.18 (2H, t, J=7.3 Hz), 2.34 (2H, t, J=7.3 Hz), 2.75 (1H, d, J=12.9 Hz), 2.94 (1H, dd, J=12.9, 5.0 Hz), 3.18 (1H, m), 3.28 (2H, t, J=7.1 Hz), 4.08 (2H, t, J=5.4 Hz), 4.35 (1H, m), 4.54 (1H, m), 5.33 ppm (2H, m). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 173.5, 173.0, 163.5, 129.7, 129.5, 63.9, 61.8, 60.0, 55.4, 40.5, 39.0, 36.7, 33.8, 31.8, 29.7, 29.7, 29.6, 29.4, 29.3, 29.1 (3C), 28.2, 28.1, 27.2, 27.1, 26.3, 26.1, 25.8, 24.7, 22.6, 14.1 ppm. LRMS (FAB) m/z 580 (MH$^+$): HRMS (FAB) calcd for $C_{32}H_{57}O_4N_3S$: 580.4178. Found: 580.4151 (MH$^+$).

4.2.4.4. C10-linked biotinated oleamide (9b). Similarly to the preparation of **9a**, from 10-bromo-1-decanol (294 mg, 1.24 mmol), (+)-biotin (200 mg, 0.82 mmol), 4-DMAP (150 mg, 1.24 mmol), EDC·HCl (236 mg, 1.24 mmol), NaN_3 (160 mg, 2.46 mmol), Lindlar catalyst (palladium on barium sulfate, 64 mg), H_2 (1 atm), and oleic chloride (ca. 1.32 mmol), **9b** (272 mg, 50% from (+)-biotin) was obtained as amorphous solid by column chromatography (CH_2Cl_2/MeOH, 10/1).

IR (KBr): 3302, 1711, 1634 cm^{-1}. ^{1}H NMR ($CDCl_3$, 270 MHz) δ: 0.88 (3H, t, J=6.9 Hz), 1.25–1.73 (44H, m), 2.01 (4H, m), 2.16 (2H, t, J=7.4 Hz), 2.33 (2H, t, J=7.4 Hz), 2.75 (1H, d, J=12.7 Hz), 2.93 (1H, dd, J=12.7, 4.9 Hz), 3.19 (3H, m), 4.06 (2H, t, J=6.6 Hz), 4.32 (1H, m), 4.53 (1H, m), 5.34 ppm (2H, m). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ: 173.5, 172.8, 163.6, 129.7, 129.5, 64.4, 61.9, 60.0, 55.4, 40.5, 39.4, 36.8, 33.9, 31.8, 31.7, 29.7, 29.6, 29.5, 29.4, 29.2 (2C), 29.1 (7C), 28.9, 28.6, 28.3, 28.2, 27.2, 26.8, 25.8, 24.8, 22.6, 14.1 ppm. LRMS (FAB) m/z 665 (MH$^+$): HRMS (FAB) calcd for $C_{38}H_{70}O_4N_3S$: 664.5087. Found: 664.5099 (MH$^+$).

4.3. Cell biology (dye transfer assay)

Dye transfer assay was performed as the previous method.[4] The donor HeLa-Cx43 cells were labeled with a gap-junction impermeable dye (DiI) plus a gap-junction permeable dye (calcein-AM)[10] and then placed sparsely on top of unlabeled recipient HeLa-Cx43 cells. The number of DiI-negative recipient cells that received calcein-AM was then assessed under a microscope. Donor cells were scored as 0 for no dye-coupling, and as 1 for dye-coupling from a donor cell to an adjacent recipient cell. When the dye-coupling is of intermediate level, we gave the scores of either 0.25 or 0.5, respectively. Thus, the cells were scored as 2 when the adjacent cell transferred calcein-AM to a subsequent adjacent cell in a direction away from the donor cell, as score 3 when the transfer was to a third cell away from the donor cell, and so on. Experiments were repeated three times, with similar results. Scoring was performed for at least 20 donor cells per agent group on the condition that the donors were single cells that were at least 20 cells away from other donor cells. The mean and standard deviation (SD) of the score counts were calculated for each agent group and expressed as GJIC scores. The oleamide and its derivatives tested in this report are dissolved in ethanol and added to the medium for culture of HeLa-Cx43 cells to make a final concentration of 40 μM.

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