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Synthesis of Oxytocin Derivatives Lipidated via a Carbonate or Carbamate Linkage as a Long-Acting Therapeutic Agent for Social Impairment-Like Behaviors

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Keywords

Lipidation; Long-Acting; Oxytocin; Social Impairment-Like Behavior; Prodrug

Abstract

In the course of our studies of hydrophobic oxytocin (OT) analogues, we newly synthesized lipidated OT (LOT-4a-c and LOT-5a-c), in which a long alkyl chain (C14-C16) is conjugated via a carbonate or carbamate linkage at the Tyr-2 phenolic hydroxy group and a palmitoyl group at the terminal amino group of Cys-1. These LOTs did not activate OT and vasopressin receptors. Among the LOTs, however, LOT-4c, having a C16-chain via a carbonate linkage at the phenolic hydroxyl group of the Tyr-2, showed very long-lasting action for the recovery of impaired social behavior in CD38 knockout mice, a rodent model of autistic phenotypes, whereas the effect of OT itself rapidly diminished. These results indicate that LOT-4c may serve as a potential prodrug in mice.
1. Introduction

Oxytocin (OT) is a cyclic neuropeptide and hormone comprising nine amino acids. OT is synthesized in the neurons of the supraoptic and paraventricular nuclei of the hypothalamus\textsuperscript{1,2} and secreted into the bloodstream from the posterior pituitary lobe.\textsuperscript{3} Binding of OT with OT receptors (OTRs)\textsuperscript{1} in the peripheral organs induces uterine contractions during delivery and lactation in the postpartum period in females.\textsuperscript{4} Thus, OT is used clinically to induce labor as well as to prevent postpartum bleeding.\textsuperscript{5} In the central nervous system (CNS), OT is a neuromodulator with a key role in social memory, parental behavior, and pair-bonding in mammals.\textsuperscript{6} In humans, clinical studies\textsuperscript{7} suggest a role of OT in the modulation of trust\textsuperscript{8}, recognition of facial expressions,\textsuperscript{9} and mother to infant gaze interactions.\textsuperscript{10}

Autism spectrum disorder (ASD) is a set of congenital neurodevelopmental conditions characterized by impairments in social communication, social interactions, and the presence of repetitive behaviors and restricted interests.\textsuperscript{11} Recent clinical trials demonstrated that single intravenous\textsuperscript{12} or intranasal OT administration\textsuperscript{13} in ASD patients led to recovery of emotion recognition and social processing.\textsuperscript{14} Repeated intranasal application of OT, however, failed to have positive effects over the long-term condition in clinical trials.\textsuperscript{15,16} One possible reason for the results of the clinical trials is the pharmacokinetic properties of OT, i.e., short half-life in the blood\textsuperscript{17} and poor penetration of the blood–brain barrier, which is typical of peptides.\textsuperscript{18} Therefore, OT may not be a suitable candidate for the treatment of ASD symptoms, but a valuable lead for the development of new compounds with improved pharmacokinetic profiles.

Lipidation, the conjugation of a peptide with long-chain fatty acids, may extend the half-life of peptides in the blood.\textsuperscript{19} Using this strategy, we previously developed three OT analogues, named lipoxytocins (LOTs, Figure 1), in which a palmitoyl group is conjugated to the terminal amino group of Cys-1 and the phenolic hydroxyl group of Tyr-2 (LOT-1), or to only one of the amino or
phenolic hydroxyl groups (LOT-2, LOT-3). Administration of these analogues and
OT by single intraperitoneal injection (1 ng/g of body weight) led to recovery of social behavior in CD38 knockout (CD38KOCC) mice,\textsuperscript{21,22} a rodent model of autistic phenotypes developed in our laboratory.\textsuperscript{23, 24} It is noteworthy that the recovery effects the LOTs lasted approximately 24 h – remarkably longer than that of OT.\textsuperscript{21,22} Furthermore, elevated OT levels in the cerebrospinal fluid were observed 24 h after a single intraperitoneal injection of LOT-1,\textsuperscript{21} suggesting that LOT-1 was easily transported into the cerebrospinal fluid owing to its lipophilic properties. Because neither of the three LOTs induces OTR-mediated cytosolic Ca\textsuperscript{2+}-mobilization in OTR- expressing HEK 293 cells,\textsuperscript{21} they are likely to be OT prodrugs that release OT in the brain. It is also likely that their long-acting effects result from the slow hydrolysis of their ester and/or amide linkage-conjugating palmitoyl groups.

In the course of our studies on long-acting OT analogues,\textsuperscript{20-24} we newly synthesized additional lipidated OT (LOT-4a-c and LOT-5a-c, Figure 1), which had a carbonate or a carbamate linkage between the Tyr-2 phenolic hydroxy group and a long alkyl chain (C14-C16), unlike the ester linkage in the previous LOTs, but had a palmitoyl group at the terminal amino group of Cys-1 like the previous LOTs. In this paper, we describe the synthesis and results on the pharmacological evaluation of these new lipidated OTs.
Figure 1. Chemical structure of OT and LOTs.

oxytocin (OT): $R^1 = R^2 = H$
LOT-1: $R^1 = R^2 = \text{CO(CH}_2\text{)}_{14}\text{CH}_3$
LOT-2: $R^1 = \text{CO(CH}_2\text{)}_{14}\text{CH}_3, R^2 = H$
LOT-3: $R^1 = H, R^2 = \text{CO(CH}_2\text{)}_{14}\text{CH}_3$

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<table>
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<tbody>
<tr>
<td>LOT-4a</td>
<td>$R^1 = \text{CO(CH}<em>2\text{)}</em>{14}\text{CH}_3, R^2 = \text{COO(CH}<em>2\text{)}</em>{13}\text{CH}_3$</td>
</tr>
<tr>
<td>LOT-4b</td>
<td>$R^1 = \text{CO(CH}<em>2\text{)}</em>{14}\text{CH}_3, R^2 = \text{COO(CH}<em>2\text{)}</em>{13}\text{CH}_3$</td>
</tr>
<tr>
<td>LOT-4c</td>
<td>$R^1 = \text{CO(CH}<em>2\text{)}</em>{14}\text{CH}_3, R^2 = \text{COO(CH}<em>2\text{)}</em>{15}\text{CH}_3$</td>
</tr>
<tr>
<td>LOT-5a</td>
<td>$R^1 = \text{CO(CH}<em>2\text{)}</em>{14}\text{CH}_3, R^2 = \text{CONH(CH}<em>2\text{)}</em>{13}\text{CH}_3$</td>
</tr>
<tr>
<td>LOT-5b</td>
<td>$R^1 = \text{CO(CH}<em>2\text{)}</em>{14}\text{CH}_3, R^2 = \text{CONH(CH}<em>2\text{)}</em>{15}\text{CH}_3$</td>
</tr>
<tr>
<td>LOT-5c</td>
<td>$R^1 = \text{CO(CH}<em>2\text{)}</em>{14}\text{CH}_3, R^2 = \text{CONH(CH}<em>2\text{)}</em>{15}\text{CH}_3$</td>
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2. Results

2.1 Synthesis. All of the newly designed LOTs were synthesized from LOT-2 as shown in Scheme 1. We previously prepared LOT-2 by treating OT with 1.3 equivalents of palmitic anhydride (Pal₂O) in the presence of Et₃N in CH₂Cl₂/DMF. Because the removal of unreacted Pal₂O and its hydrolyzed product palmitic acid is troublesome using this procedure, the purity of LOT-2 was often insufficient. Therefore, we reassessed the procedure and found that we could reproducibly obtain high-purity LOT-2 in high yield when the reaction was carried out with reduced Pal₂O (1.0 equiv) and the product was purified by silica gel column chromatography with a high-polar solvent system (CHCl₃/MeOH = 3:1).

We next investigated the formation of the carbonate or carbamoyl linkage at the phenolic hydroxy group of Tyr-2 in LOT-2 and found that the carbonate linkage was effectively formed by using 4-nitrophenyl carbonates 1a–c. Thus, treatment of LOT-2 with the carbonates 1a–c with a long alkyl-chain in the presence of DMAP and Et₃N in CH₂Cl₂/DMF effectively provided all of desired three LOT-4a–c. On the other hand, formation of the carbamate linkage was unsuccessful by using the corresponded 4-nitrophenylcarbamates. The carbamates LOT-5a–c were effectively prepared, however, when LOT-2 was treated with carbamic chlorides 2a–c in the presence of DMAP and Et₃N in CH₂Cl₂/DMF.
Scheme 1. Synthesis of LOT-4a–c and LOT-5a–c.
**In vitro study.** We examined the ability of the compounds to activate OTRs. In the presence of a compound, we measured \([\text{Ca}^{2+}]_i\), which is dependent on Ca\(^{2+}\)-mobilization from intracellular stores induced by OTR activation in HEK-293 cells expressing human OTRs (hOTRs) or human vasopressin receptors 1a and 1b (hV\(_{1a}\)Rs and hV\(_{1b}\)Rs)\(^{24}\). The overall data, including the half-maximal effective concentration (EC\(_{50}\)) and the maximal effect (E\(_{\text{max}}\)) for OT and the OT analogues are presented in Table 1.

**Table 1. Biological activities of OT and analogues**

<table>
<thead>
<tr>
<th>Agonist potency</th>
<th>Agonist Activity</th>
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<tbody>
<tr>
<td>EC(_{50}), nM (95% confidence interval)</td>
<td>E(_{\text{max}})%</td>
</tr>
<tr>
<td>hOTR</td>
<td>hV(_{1a})R</td>
</tr>
<tr>
<td>OT</td>
<td>0.0046 (0.0013-0.015)</td>
</tr>
<tr>
<td>LOT-4a</td>
<td>2.7 (0.09-73)</td>
</tr>
<tr>
<td>LOT-4b</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>LOT-4c</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>LOT-5a</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>LOT-5b</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>LOT-5c</td>
<td>&gt;100000</td>
</tr>
</tbody>
</table>

EC\(_{50}\) values of agonist activities were determined for hOTR, hV\(_{1a}\)R, and hV\(_{1b}\)R using \([\text{Ca}^{2+}]_i\) measurement with a microspectrofluorometric system. Number of independent experiments: n = 3, each experiment was performed in duplicate, number of measured cells in each cell-dish: 20. \(^*E_{\text{max}}\) values of agonist potencies were calculated from EC\(_{50}\) curves for hOTR, hV\(_{1a}\)R, and hV\(_{1b}\)R.

In the Ca\(^{2+}\)-assay, OT had an EC\(_{50}\) of 0.0046 nM (0.0013-0.015) for hOTRs and 100-fold lower activity for hV\(_{1a}\)Rs and hV\(_{1b}\)Rs (Table 1). These data are similar to our previous results.\(^{24}\) Most of the newly synthesized LOTs, however, exhibited no agonistic effect in this system. Only LOT-4a exhibited very weak partial agonist activity with an EC\(_{50}\) of 2.7 nM (0.09-73) and an E\(_{\text{max}}\) of 15.3%.
2.3. Effects on Parental Nurturing Behavior. CD38 is transmembrane protein, involved in process of OT release in CNS. A single nucleotide polymorphisms of CD38 gene are associated with ASD. According to these findings, we previously developed CD38KOCC mice that display autistic phenotypes features such as deficit in social memory and recognition. One of the features of CD38KOCC mice is a deficit in paternal nurturing behavior. OT administration recovers recovery of this impairment in CD38KOCC mice. Thus, these mice are useful for evaluating the effects of OT-like compounds to recover this autistic phenotype.

Thus, we examined the effects of OT, LOT4-a-c and LOT5-a-c on parental nurturing behavior, i.e., the latency to retrieve pups by CD38KOCC sires at 30 min and at 24 h after treatment with the compounds. Thirty minutes after a single intraperitoneal (ip)- injection of phosphate-buffered saline (PBS), the mean parental scores of CD38KOCC sires to retrieve pups were 0 ± 0 s (n = 10, Table 2). The mean scores of CD38KOCC sires to retrieve pups within 30 min after a single ip-injection of OT (100 ng/100 g body weight) was 5.3 ± 1.14 (p= 0.03), consistent with previous reports. At 30 min after the treatment, among the LOTs, only LOT4a induced a difference in pup retrieval latencies compared with mice treated with PBS (6.8 ± 1.5 vs 0 ± 0, respectively; p = 0.031). A one-way ANOVA revealed significant differences between groups (F8,81 = 3.93, p = 0.0006). At 24 h after administration, however, LOT4c demonstrated clear difference from PBS treated group: 5.2 ± 1 (n =10) vs 0 ± 0 (n = 10), (multiple comparisons with Bonferroni post hoc correction p = 0.011). All of LOTs other than LOT4a did not demonstrated difference compare with PBS treated mice. A one-way ANOVA revealed significant differences between groups (F8,81 = 4.2, p = 0.0003)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parental scores</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>24 h</td>
</tr>
<tr>
<td>PBS</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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Table 2 Pup retrieval behavior by sires.
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<tbody>
<tr>
<td>OT</td>
<td>5.3 ± 1.14</td>
<td>0 ± 0</td>
<td>0.03</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>LOT-4a</td>
<td>6.8 ± 1.5</td>
<td>3.1 ± 1.32</td>
<td>0.002</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>LOT-4b</td>
<td>3.9 ± 1.6</td>
<td>2.9 ± 1.19</td>
<td>0.23</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>LOT-4c</td>
<td>2 ± 1.33</td>
<td>5.2 ± 1</td>
<td>0.99</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>LOT-5a</td>
<td>3.3 ± 1.37</td>
<td>3.6 ± 1.48</td>
<td>0.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>LOT-5b</td>
<td>2 ± 0.85</td>
<td>3.1 ± 1.01</td>
<td>0.99</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>LOT-5c</td>
<td>1.9 ± 1.27</td>
<td>3.4 ± 1.4</td>
<td>0.99</td>
<td>0.27</td>
<td></td>
</tr>
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</table>

The Parental scores by CD38KOCC sires at 30 min and the 24 h after a single intraperitoneal injection of PBS, OT or LOTs (100 ng/100 g body weight); n = 10/group. One-way ANOVA followed by Bonferroni’s post hoc test was performed for 30 min ($F_{8,81} = 3.93, p = 0.0006$), and 24 h ($F_{8,81} = 4.2, p = 0.0003$) P-values indicated Bonferroni post hoc test compared with CD38KOCC treated by PBS.

**Figure 2.** Pup retrieval behavior by sires. The parental scores for pup retrieval CD38KOCC sires. The time-course after a single intraperitoneal injection of PBS, OT, or LOT-4c (100 ng/100 g body weight); n = 10/group. Two-way ANOVA followed by Bonferroni’s test for multiple comparisons was performed for the time-course (time $F_{5,162} = 1.867, p = 0.1$ treatment $F_{2,162} = 5.19, p = 0.0065$, interaction $F_{10, 162} = 1.88, p = 0.05$). * $p < 0.05$ and ** $p < 0.01$ compared to CD38KOCC PBS treated mice with Bonferroni’s correction for multiple comparisons.
We then examined the long-lasting activity of LOT-4c over time based on parental scores. As shown in Figure 2, the activity of OT was lost at 6 h. These data are consistent with previous findings. LOT-4c exhibited a tendency to increase paternal nurturing scores at 12 h and significantly increased paternal nurturing scores at 24 h, but lost its activity at the 48 h time-point. Two-way ANOVA indicated significant effects of treatment conditions ($F_{2, 162} = 5.191$ $P = 0.0065$).
3. Discussion

We evaluated the effects of OT and six lipidated OT analogues, LOT-4a-c and LOT-5a-c, on a typical social behavior, male parental behavior (Table 2). The impairment in paternal retrieval behavior displayed by male CD38KOCC mice induced by the CRISP-Cas9 gene-editing method was identical to that reported in CD38KO mice generated by the classical homologous recombination method.₂⁷ Thirty minutes after administering OT to CD38KOCC sires, OT elicited recovery from the paternal behavior deficit exhibited by PBS-treated mice. Five lipidated analogues, LOT-4a, LOT4b, LOT-5a, LOT-5b, and LOT-5c, exhibited essentially no recovery of function, whereas another analogue, LOT4c, effectively reversed the behavioral deficit. LOT-4c exhibited effects on parental behavior 24 h after injection (Figure 2). The use of novel indicators of other social behaviors evaluated in our previous reports is needed to confirm the benefit of the OT analogue LOT-4c over natural and/or synthetic OT as a long-acting analogue.

In the present study, we tested the analogues in only one social behavioral test, in contrast to our previous papers. Nevertheless, we applied two different scales to measure paternal nurturing behavior. We measured the parental score which reflects paternal behavior for all five pups and the quality of the retrieval behavior. The current judgment on the basis of both retrieval latency and parental score allows for the evaluation of OT, LOT-4, and LOT-5 analogues in the parental behavior test.

The mechanism underlying the activity of LOT-4a to exhibit OT-like in vivo effects for a short-time period (30 min after administration) is unclear. One possible mechanism may be its high permeability through the blood–brain barrier due to lipidation,³⁰ although permeability was not examined in the present study.

A limitation of our study of LOT-4c is the absence of data other than the effects at 30 min and 24 h for recovery of parental behavior in CD38KOCC mice. Experiments with chronic treatment should be performed. On the basis of the beneficial effects of LOT-4a in short-term
activity and LOT-4c in long-term activity experiments, a combination of the two LOTs may be advantageous to improve the behavior. In addition, we do not know where and when the lipidated moieties are removed from LOTs and release OT that can be easily transported to the brain by the receptor for advanced glycation end-products (RAGE), because RAGE is a binding partner and carrier of OT on brain capillary endothelial cell membranes. Therefore, LOTs could serve as prodrugs after cleaving the lipidated moieties, even at the peripheral and central sites, where an intensified OT-induced OT release autoregulation might occur.

4. Conclusion

We identified a potent lipidated OT analogue, LOT-4c having a C16-chain via a carbonate linkage at the phenolic hydroxyl group of Tyr-2, which showed very long-lasting recovery of parenting behavior in CD38KOCC mice, a rodent model of an autistic phenotype. Although the precise mechanism is unknown, LOT-4c is likely to be a prodrug that slowly releases OT in the bloodstream or in the brain. Based on these results together with previous findings, we conclude that some LOTs are beneficial over native OT and potentially useful candidates for the treatment of psychiatric diseases, including ASD.

5. Experimental Section

5.1 Compound Synthesis

5.1.1 General Methods of Chemistry. 1H-NMR spectra were recorded in CDCl₃ at ambient temperature unless otherwise noted, at 400 or 500 MHz, with TMS as an internal standard. 13C NMR spectra were recorded in CDCl₃ at ambient temperature. Silica gel column chromatography was performed with silica gel 60 N (spherical, neutral, 63-210 µm, Kanto Chemical Co., Inc.). Flash column chromatography was performed with silica gel 60 N (spherical, neutral, 40-50 µm, Kanto Chemical Co., Inc.). Purities of final compounds were confirmed as >95% by HPLC:
column, Kanto C18 GP, 250 × 4.6 (mm); column temperature,
25°C; flow rate, 1.0 mL/min; solvent, MeOH/ H2O = 95:5 containing 0.1% TFA; detection, 220 nm.

5.1.2 N-Palmitoyloxytocin (LOT-2). To a solution of oxytocin acetate (12.6 mg, 12.5 µmol) and Et3N (5.2 µL, 37 µmol) in DMF (0.25 mL) was added a solution of Pal2O (6.2 mg, 12.5 µmol) in CH2Cl2 (0.80 mL), and the reaction mixture was stirred for 3 h. After the solvent was evaporated, the residue was purified by silica gel column chromatography (MeOH/ CHCl3 = 1:5) to give the title compound (14.9 mg, 12.0 µmol, 96%) as white powder: 1H NMR (500 MHz, DMSO-d6) δ 9.13 (s, 2H), 8.62 (br, 1H), 8.22-8.08 (m, 4H), 7.92 (m, 2H), 7.62 (br, 1H), 7.35 (s, 1H), 7.28 (s, 1H), 7.09-7.05 (m, 4H), 6.88 (s, 1H), 6.79 (s, 1H), 6.61 (d, 2H, J = 8.0 Hz), 4.95 (br, 1H), 4.69 (m, 1H), 4.55 (m, 1H), 4.38 (m, 1H), 4.30 (m, 1H), 4.16 (m, 1H), 3.95 (br, 1H), 3.88 (br, 1H), 3.64-3.52 (m, 1H), 3.28 (m, 1H), 3.13 (d, J = 12 Hz, 1H), 3.05-2.97 (m, 2H), 2.76-2.66 (m, 2H), 2.62-2.52 (m, 2H), 2.11 (t, J = 7.5 Hz, 2H), 2.03 (t, J = 7.0 Hz, 1H), 2.01-1.71 (m, 6H), 1.62 (m, 1H), 1.57-1.40 (m, 5H), 1.31-1.12 (m, 25H), 0.90-0.80 (m, 15H). The 1H NMR data are in accord with those reported previously.20

5.1.3 Alkyl (4-nitrophenyl) carbonate (1a-c). To a solution of an alcohol (CH3(CH2)nOH, n = 13, 14, 15, 0.50 mmol) in CH2Cl2 (5.0 mL) was added p-nitrophenyl chloroformate (101 mg, 0.50 mmol) and pyridine (81 µL, 1.0 mmol) at 0 °C, and the reaction mixture was stirred at room temperature for 11 h. The mixture was partitioned between hexane and H2O, and the organic layer was dried (Na2SO4) and evaporated. The residue was purified by silica gel column chromatography (EtOAc/ hexane = 1:50) to give the title compound as white powder. 1a (163 mg, 0.429 mmol, 86%): 1H NMR (400 MHz, CDCl3) δ 8.28 (d, J = 9.6 Hz, 2H, aromatic), 7.39 (d, J = 9.6 Hz, 2H, aromatic), 4.29 (t, J = 6.8 Hz, 2H, OCH2), 1.76 (m, 2H, OCH2CH2), 1.42 (m, 2H, OCH2CH2CH2), 1.22-1.38 (m, 20H, CH2×10), 0.88 (t, J = 6.8 Hz, 2H, CH3); 13C NMR (CDCl3, 100 MHz) δ 155.6, 152.5, 145.3, 125.3, 121.8, 69.7, 31.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.4, 29.2, 28.5, 25.6, 22.7, 14.1. 1b (72.6 mg, 0.184 mmol, 37%): 1H NMR (400 MHz, CDCl3) δ 8.28 (d, J = 9.6 Hz, 2H, aromatic), 7.39 (d, J = 9.6 Hz, 2H, aromatic), 4.29 (t, J = 6.8
1H, 2H, OCH₂), 1.76 (m, 2H, OCH₂CH₂), 1.42 (m, 2H, OCH₂CH₂CH₂), 1.22-1.38 (m, 22H, CH₂×11), 0.88 (t, J = 6.8 Hz, 2H, CH₃); ¹³C NMR (CDCl₃, 100 MHz) δ 155.6, 152.5, 145.3, 125.3, 121.8, 69.7, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 28.5, 25.6, 22.7, 14.1. ¹c  

(163 mg, 0.400 mmol, 80%): ¹H NMR (400 MHz, CDCl₃) δ 8.28 (d, J = 9.2 Hz, 2H, aromatic), 7.39 (d, J = 9.2 Hz, 2H, aromatic), 4.29 (t, J = 6.8 Hz, 2H, OCH₂), 1.76 (m, 2H, OCH₂CH₂), 1.42 (m, 2H, OCH₂CH₂CH₂), 1.21-1.38 (m, 22H, CH₂×12), 0.88 (t, J = 6.8 Hz, 2H, CH₃); ¹³C NMR (CDCl₃, 100 MHz) δ 155.6, 152.5, 145.3, 125.3, 121.8, 69.7, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 28.5, 25.6, 22.7, 14.1.

5.1.4 LOT-4a–c. To a solution of LOT-2 (12.5 mg, 10.0 µmol) and Et₃N (4.2 µL, 30.1 µmol) and DMAP (1.2 mg, 10 µmol) in DMF (0.25 mL) was added a solution of alkyl (4-nitrophenyl) carbonate (1a–c, 20 µmol) in CH₂Cl₂ (0.25 mL), and the reaction mixture was stirred at room temperature for 24 h. After evaporation of the solvent, the residue was purified by HPLC (column, Kanto RP-8 GP150-4.6; 0.1% TFA-containing MeOH / H₂O (95: 5); 220 nm; 1.00 mL/min; 25 °C). The eluent was evaporated and lyophilized to give the title compound as white powder. LOT-4a (9.7 mg, 65%): ¹H NMR (500 MHz, DMSO-d₆) δ 8.80 (br, 1H), 8.36-8.17 (m, 3H), 8.11 (d, 1H, J = 9.0 Hz), 7.93 (m, 2H), 7.47 (br, 1H), 7.41-7.26 (m, 4H), 7.10-7.04 (m, 4H), 6.87 (s, 1H), 6.80 (s, 1H), 5.04 (br, 1H), 4.69 (m, 2H), 4.40 (m, 1H), 4.31 (d, J = 7.5 Hz, 1H), 4.15 (m, 3H), 3.97 (br, 1H), 3.84 (br, 1H), 3.65-3.51 (m, 5H), 3.38-3.27 (m, 2H), 3.02-2.82 (m, 3H), 2.65-2.54 (m, 3H), 2.10 (t, J = 7.5 Hz, 2H), 1.96 (m, 4H), 1.93-1.71 (m, 5H), 1.64-1.52 (m, 3H), 1.49-1.38 (m, 4H), 1.31-1.14 (m, 47H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1507.86 [(M+Na)⁺]; HRMS (ESI) calcd for C₇₄H₁₂₄N₁₂O₁₅S₂Na: 1507.8643 [(M+Na)⁺], found: 1507.8615.

LOT-4b (13.1 mg, 87%): ¹H NMR (500 MHz, DMSO-d₆) δ 8.78 (br, 1H), 8.36-8.17 (m, 3H), 8.10 (d, 1H, J = 9.0 Hz), 7.93 (m, 2H), 7.47 (br, 1H), 7.40-7.24 (m, 4H), 7.09-7.04 (m, 4H), 6.87 (s, 1H), 6.80 (s, 1H), 5.03 (br, 1H), 4.69 (m, 2H), 4.39 (m, 1H), 4.31 (d, J = 8.0 Hz, 1H), 4.16 (m, 3H), 3.96 (br, 1H), 3.84 (br, 1H), 3.70-3.56 (m, 5H), 3.36-3.24 (m, 2H), 3.05-2.82 (m, 3H), 2.67-2.54 (m, 3H), 2.11 (t, J = 7.0 Hz, 2H), 1.96 (m, 4H), 1.92-1.71 (m, 5H).
1.52 (m, 3H), 1.52-1.38 (m, 4H), 1.38-1.14 (m, 49H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1521.88 [(M+Na)+]; HRMS (ESI) calcd for C75H126N12O15S2Na: 1521.8799 [(M+Na)+], found: 1522.8769.

LOT-4c (12.3 mg, 81%): 1H NMR (500 MHz, DMSO-d6) δ 8.79 (br, 1H), 8.32-8.19 (m, 3H), 8.12 (d, 1H, J = 8.5 Hz), 7.93 (m, 2H), 7.65 (t, J = 7.5 Hz, 2H), 1.96 (m, 4H), 1.95-1.73 (m, 5H), 1.70-1.53 (m, 3H), 1.51-1.38 (m, 4H), 1.38-1.14 (m, 51H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1535.89 [(M+Na)+]; HRMS (ESI) calcd for C76H128N12O15S2Na: 1535.8956 [(M+Na)+], found: 1535.8941.

5.1.5 Alkylcarbamic chloride (2a–c). To a solution of triphosgene (bis(trichloromethyl) carbonate, 74.2 mg, 0.250 mmol) in CH2Cl2 (1.0 mL) was added an amine (0.500 mmol) and Et3N (112 µL, 0.804 mmol) in CH2Cl2 (3.0 mL) at −20 °C, and the reaction mixture was stirred at room temperature for 3 h. The mixture was filtrated with silica gel and the filtrate was partitioned with saturated aqueous NH4Cl, H2O, and brine. The organic layer was dried (Na2SO4) and evaporated to give the corresponding alkylcarbamic chloride 2a–c as an oil, respectively, which were unstable and therefore immediately subjected to the next reaction without further purification.

5.1.6 LOT-5a–c. To a solution of LOT-2 (12.5 mg, 10.0 µmol) and Et3N (4.2 µL, 30.1 µmol) and DMAP (1.2 mg, 10 µmol) in DMF (0.25 mL) was added a solution of alkylcarbamic chloride (2a–c, 20 µmol) in CH2Cl2 (0.25 mL), and the reaction mixture was stirred at room temperature for 1.5 h. After evaporation of the solvent, the residue was purified by HPLC (column, Kanto RP-8 GP150-4.6; 0.1%TFA-containing MeOH/H2O (95:5); 220 nm; 1.0 mL/min; 25 °C). The eluent was evaporated and lyophilized to give the title compound as white powder. LOT-5a (13.5 mg, 9.09 µmol, 91%): 1H NMR (500 MHz, DMSO-d6) δ 8.79 (br, 1H), 8.31-8.19 (m, 3H), 8.12 (d, 1H, J = 8.5 Hz), 7.93 (m, 2H), 7.65 (t, J = 6.0 Hz, 1H), 7.48 (br, 1H), 7.39-7.33 (m, 2H), 6.87 (s, 1H), 6.80 (s, 1H), 5.04 (br, 1H), 4.69 (m, 2H), 4.40 (m, 1H), 4.31 (d, J = 8.0 Hz, 1H), 4.15 (m, 3H), 3.96 (br, 1H), 3.84 (br, 1H), 3.66-3.53 (m, 5H), 3.36-3.26 (m, 2H), 3.05-2.82 (m, 3H), 2.68-2.56 (m, 3H), 2.10 (t, J = 7.5 Hz, 2H), 1.96 (m, 4H), 1.95-1.73 (m, 5H), 1.70-1.53 (m, 3H), 1.51-1.38 (m, 4H), 1.38-1.14 (m, 51H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1535.89 [(M+Na)+]; HRMS (ESI) calcd for C76H128N12O15S2Na: 1535.8956 [(M+Na)+], found: 1535.8941.
7.36 (s, 1H), 7.28 (m, 4H), 7.12-7.04 (m, 2H), 6.97-6.79 (m, 4H), 5.05 (br, 1H), 4.73-4.62 (m, 2H), 4.39 (m, 1H), 4.31 (d, J = 8.0 Hz, 1H), 4.16 (m, 1H), 3.96 (br, 1H), 3.84 (br, 1H), 3.66-3.53 (m, 5H), 3.33-3.28 (m, 2H), 3.04-2.80 (m, 5H), 2.71-2.55 (m, 3H), 2.11 (t, J = 7.5 Hz, 2H), 2.07-1.72 (m, 9H), 1.69-1.41 (m, 8H), 1.38-1.10 (m, 45H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1506.88 [(M+Na)^+]; HRMS (ESI) calcd for C_{74}H_{125}N_{13}O_{14}S_{2}Na: 1506.8803 [(M+Na)^+], found: 1506.8774.

LOT-5b (8.2 mg, 55%): ^1H NMR (500 MHz, DMSO-d6) δ 8.77 (br, 1H), 8.31-8.19 (m, 3H), 8.12 (d, 1H, J = 8.5 Hz), 7.92 (m, 2H), 7.65 (t, J = 6.0 Hz, 1H), 7.49 (br, 1H), 7.36 (s, 1H), 7.28 (m, 4H), 7.10-7.04 (m, 2H), 6.94-6.78 (m, 4H), 5.04 (br, 1H), 4.75-4.62 (m, 2H), 4.39 (m, 1H), 4.31 (d, J = 8.0 Hz, 1H), 4.16 (m, 1H), 3.97 (br, 1H), 3.84 (br, 1H), 3.65-3.53 (m, 5H), 3.33-3.26 (m, 2H), 3.03-2.79 (m, 5H), 2.68-2.56 (m, 3H), 2.11 (t, J = 7.0 Hz, 2H), 2.05-1.74 (m, 9H), 1.69-1.41 (m, 8H), 1.32-1.10 (m, 47H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1520.89 [(M+Na)^+]; HRMS (ESI) calcd for C_{75}H_{127}N_{13}O_{14}S_{2}Na: 1520.8959 [(M+Na)^+], found: 1520.8940.

LOT-5c (10.1 mg, 6.68 µmol, 53%): ^1H NMR (500 MHz, DMSO-d6) δ 8.78 (br, 1H), 8.29-8.19 (m, 3H), 8.12 (d, 1H, J = 8.5 Hz), 7.92 (m, 2H), 7.65 (t, J = 5.5 Hz, 1H), 7.49 (br, 1H), 7.36 (s, 1H), 7.28 (m, 4H), 7.10-7.06 (m, 2H), 6.94-6.78 (m, 4H), 5.05 (br, 1H), 4.76-4.64 (m, 2H), 4.39 (m, 1H), 4.31 (d, J = 8.0 Hz, 1H), 4.16 (m, 1H), 3.96 (br, 1H), 3.84 (br, 1H), 3.68-3.52 (m, 5H), 3.34-3.28 (m, 2H), 3.02-2.81 (m, 5H), 2.69-2.56 (m, 3H), 2.11 (t, J = 7.5 Hz, 2H), 2.02-1.76 (m, 9H), 1.69-1.40 (m, 8H), 1.30-1.11 (m, 49H), 0.92-0.78 (m, 18H); LRMS (ESI) m/z 1534.91 [(M+Na)^+]; HRMS (ESI) calcd for C_{76}H_{129}N_{13}O_{14}S_{2}Na: 1534.9116 [(M+Na)^+], found: 1534.9098.

5.2 Expression Plasmid Construction, Cell Culture and Transfection.

Plasmid constructs for hOTRs (pcDNAHOXTR)^20, pcDNAHV1AR and pcDNAHV1BR^24 were described previously.

Human embryonic kidney HEK-293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were grown in culture dishes to 80 to 90% confluence
and transfected with pcDNAHOXTR, pcDNAHV1AR or pcDNAHV1BR using GeneJuice Transfection Reagent (EMD Millipore, Temecula, CA, USA) following the manufacturer’s instruction. Cells were selected in DMEM supplemented with 5% FBS and 800 µg/mL G418 (geneticin; Sigma Chemical Co., St Louis, MO, USA). The resulting transformed cells, stably expressing human OXTRs, human V1ARs and V1BRs and those transfected with pcDNA3 (+) (mock-transfected cells) were maintained in DMEM supplemented with 10% FBS and 100 µg/mL G418.

5.3 Intracellular free calcium concentration measurement.

Transfected HEK-293 cells were loaded with fura-2/AM to a final concentration of 1 µmol/mL in complete medium and incubated at 37°C. After 30-min loading, the cells were washed three times with HEPES-buffered saline (HBS) solution (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 20 mmol/L HEPES-NaOH, 2 mmol/L CaCl₂, 20 mmol/L glucose, pH 7.4). The fluorescence of the cells loaded with fura-2/AM was then measured at 37°C, at the determined sites, through a pinhole (10–20 µm in diameter). We used alternating excitation wavelengths of 340 and 380 nm in a Ca²⁺ microspectrofluorometric system (IX-73 Model; Olympus, Tokyo, Japan) and Metafluor software (Molecular Devices, Sunnyvale, CA). The Ca²⁺ emission was detected every 3 sec for 5 min after application of PBS, OT, AVP or analogues. The ratio of fluorescence at 340 nm and 380 nm (F340/F380) was used to determine intracellular free calcium concentrations ([Ca²⁺]ᵢ). All data were normalized to the baseline fluorescence (F0) recorded 10 s before application of compounds and given as percentage from the maximum response obtained by OT for hOTRs, AVP for hV1AR and hV1BRs. OT, AVP and analogues for experiments were diluted in 50% ethanol to a concentration of 10⁻³ mol/L and then diluted in distilled water to obtain the required concentrations. Each experiment was performed 3 times, each triplicated.

5.4 Animals.
Male and female Slc:ICR mice (Institute of Cancer Research of the Charles River Laboratories, Inc., Wilmington, MA, USA) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) through a local distributor (Sankyo Laboratory Service Corporation, Toyama, Japan). The procedure to produce the CD38KOCC was described previously. A male and female of each genotype were paired and housed in a nursing cage in our laboratory under standard conditions (24 °C; 12 h light/dark cycle, lights on at 08:00) with food and water provided ad libitum. All animal experiments were performed in accordance with the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and were approved by the Committee on Animal Experimentation of Kanazawa University (Ethics Approval Code AP-173824).

5.5 Parental Retrieval Test.

The design of the experiments for parental retrieval behavior was described previously. Virgin males and females of identical genotypes were paired at 56–64 days. A single male and a single female were continuously housed together in a standard mouse maternity cage from the mating period to the delivery of pups and then to postnatal days 3–5. All family units consisted of a new sire and dam, and their first litter of each genotype was used. All mice were experimentally naive. Thirty minutes before starting the experiment, the cages with the families were placed in the experimental room for habituation. The sire and dam were placed in a new clean cage with new woodchip bedding for 10 min, while the pups were left in the nest in the original cage. Five pups were randomly selected from the litter and placed individually at a site remote from the nest in the original cage. The sires were returned to the original home cage in the presence of their five biological pups to assess parental behavior. Parental retrieval behavior was measured by observing the parent behavior for 10 min following the reunion. The sire received a single intraperitoneal injection of 0.3 mL of phosphate-buffered saline (PBS) or 0.3
mL of OT (1 mL per 100 g of body weight), compounds at a concentration of 100 ng/mL dissolved in PBS.

For screening experiments parental behavior was examined. Thirty minutes and 24 h after the injection, only once. Paternal retrieval behavior was examined by measuring parental scores for each pup. Each sire or received 2 points for each pup returned completely to the nest, 1 point if the parent had contact with the pup or moved the pup to another place, and 0 if the pup was intact.

For time-course experiment 30 minutes, 6, 12, 24 or 48 h after the injection, The behavioral tests were carried out in a randomly mixed sequence of the experimental groups. For each time-point and each compound 10 mice were used. The experiments were performed between 10:00 and 15:00. For each time-point. One-way ANOVA test was performed for comparison compound effects for 30 min and 24 hours in screening experiment. If ANOVA- test shown significance followed Bonferroni’s post-hoc test was performed. Two-way ANOVA followed by Bonferroni’s post hoc test was performed for time-course.
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33. From the structure–activity relationship viewpoint, it is strange that only LOT-4a had agonistic effect to hOTR (Table 1), because the structure of LOT-4a is very similar to those of other LOT-4b,c and LOT-5a-c. A possibility that only a trace of OT contaminating to LOT-4a sample might affect the agonistic effect in vitro due to the remarkably strong agonistic potency of OT (EC\textsubscript{50} = 4.6 pM) might not be eliminated.