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Brief Report

Synthesis of *N*-alkylated octopamine derivatives and their interaction with octopamine receptor BmOAR1

Kenji Oshima,[1] Ryunosuke Yamamoto,[1] Haruna Yamasaki,[1] Maki Katayama,[1] Keita Noda,[2] Tomohiro Oishi[3] and Hiroto Ohta[4,*]

[1] *Department of Biological and Chemical Systems Engineering, National Institute of Technology, Kumamoto College, 2627 Hirayamashin-machi, Yatsushiro, Kumamoto 866–8501, Japan*
[2] *Graduate School of Science and Technology, Kumamoto University, 2–39–1 Kurokami, Kumamoto 860–8555, Japan*
[3] *Technical Division, Kumamoto University, 2–39–1 Kurokami, Kumamoto 860–8555, Japan*
[4] *Department of Applied Microbial Engineering, Faculty of Life Sciences, Sojo University, 4–22–1 Ikeda, Nishi-ku, Kumamoto 860–0082, Japan*

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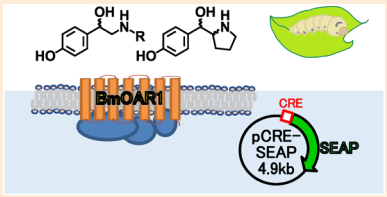
Supplementary material

A series of *N*-alkylated octopamine derivatives was synthesized, and the structure–activity relationships of these derivatives with the silkworm *Bombyx mori* octopamine receptor BmOAR1 were evaluated using a secreted placental alkaline phosphatase reporter assay system. The *N*-alkyl moiety on the ligand affected the intensity of the agonist activity in the order: CH_3>(H)>C_2H_5. Although linear alkyl chains of C3 or higher did not exhibit any activity, the fixed C3 alkyl group forming a pyrrolidine ring showed significant activity. These results suggest that BmOAR1 has a relatively small space around the amine-binding site, and the alkyl part constituting the cyclic amine could exert the same effect as the small alkyl group.



Keywords: octopamine receptor, octopamine analogue, SEAP reporter assay.

Introduction

Octopamine (OA), present in various invertebrates, has been well studied and established as a neurotransmitter, neuromodulator, and neurohormone.[1–4] The binding of OA to specific OA receptors (OARs), typical G protein-coupled receptors, elicits its physiological responses. Insect OARs have been characterized by studies in neuronal and non-neuronal tissues to monitor OA-induced changes at intercellular Ca^{2+} and cAMP levels.[5–8] The structure and function of OARs are closely related to mammalian adrenergic receptors from a molecular evolutionary perspective. Based on their similarities of OARs to adrenergic receptors, OARs are classified as α-adrenergic-like and β-adrenergic-like.[9,10] α-Adrenergic-like OARs induce cAMP elevation and Ca^{2+} mobilization, while β-adrenergic-like OARs cause only cAMP elevation.[10]

As OARs are only present in invertebrates, they are considered attractive targets for insecticides showing selective toxicity. The insecticidal activity of demethylchlorodimeform (DMCDM) is attributed to its interaction with OAR,[11] and DMCDM-related compounds (such as clonidine, the Amitraz metabolite, and NC-7) have been used to characterize OARs.[7,12–14]

In contrast, prior to the discovery of the pharmaceutical isoproterenol, several adrenaline derivatives with alkyl groups instead of CH_3 on the amino moiety were investigated, and the isopropyl form was found to be an effective bronchodilator.[15]

However, in insect OARs, it remains unclear to what extent the introduction of alkyl groups contributes to agonist activity, and what types of structural space are allowed at the amine-binding site of the receptor. In this study, we describe the synthesis of *N*-alkylated OA derivatives (Fig. 1) and their agonistic activity toward insect OAR (BmOAR1). BmOAR1 was isolated from the silkworm *Bombyx mori* and characterized as an α-adrenergic-like OAR.[16,17] Additionally, the importance of the aspartic acid residue Asp103 as the binding site for the amine

* To whom correspondence should be addressed.
E-mail: hiohta@bio.sojo-u.ac.jp
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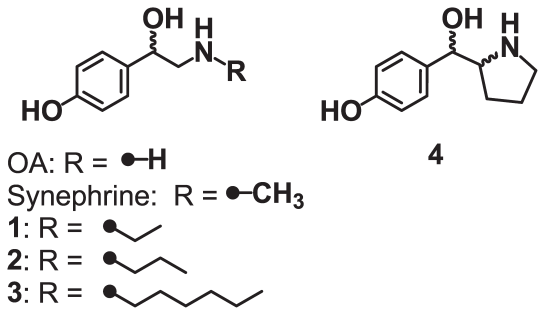

Fig. 1. Octopamine (OA) and its *N*-alkylated derivatives

moiety of OA was noted.[18] The series of compounds in Fig. 1 shows a simple variation in the OA structure. Among the synthesized compounds, one (**4**) was designed to form a cyclic amine by fixing the end of the alkyl group. This design provides a new perspective on the size effect by fixing alkyl groups and the stereoisomeric effect of introducing a new chiral center.

Materials and methods

1. Chemicals

(±)-Octopamine hydrochloride and synephrine were purchased from Sigma-Aldrich. Silica gel for flush chromatography was obtained from Kanto Kagaku (silica gel 60 [spherical] 40–50 μm). The activated alumina used for flush chromatography was procured from MP Biochemicals (MP Alumina N 32-63, active).

2. Apparatus

NMR spectra were recorded on a JEOL JNM-ECZ400R spectrometer. GC/MS spectra were obtained using an Agilent Technologies 7890A/5975C. HRMS spectra were measured using an Agilent Technologies Accurate-Mass Q-TOF LC/MS 6520 system in ESI positive mode with a fragmentor voltage of 100 V. Diastereomeric purity was analyzed using a Hitachi L2000 HPLC system equipped with a DAICEL Crownpak CR(+) column. MM2 forcefield calculations were performed using the function included in CambridgeSoft ChemBio3D Ultra 13.0.

3. Synthesis of ligands

The synthesis and analytical data for compounds **1**, **2** and **3** are provided in Supplementary Material. Compound **4** was synthesized as shown in Fig. 2 and separated into two components by chromatography: Under nitrogen atmosphere, 37 mL of *sec*-butyllithium solution (0.59 mol/L in cyclohexane, 22 mmol) was added dropwise at −75°C to a mixture of 3.8 mL of *N*-Boc-pyrrolidine (22 mmol) and 4.8 mL of *N,N,N′,N′*-tetramethylethylenediamine (32 mmol) in 150 mL of dry ether. After stirring for 4 hr, 4.93 g of *tert*-butyl 4-formylphenyl carbonate (22 mmol) in 30 mL dry ether was added dropwise at −75°C and stirred at −75°C for 16 hr; the reaction was quenched with 50 g/L-citric acid aqueous solution. The organic layer was separated, washed with saturated $NaHCO_3$ solution, and dried over Na_2SO_4. Following solvent removal, 9.11 g of yellow oil was obtained. The crude yellow oil was treated with 50 mL of 2 mol/L-HCl at room temperature for 24 hr and then rendered slightly alkaline (pH approximately 9) with Na_2CO_3. After degassing under reduced pressure, the reaction mixture was lyophilized, extracted with $CHCl_3$–CH_3OH (8:2), and evaporated to yield a dark brown oil. The dark brown oil was subjected to flash chromatography on silica gel and eluted with $CHCl_3$–CH_3OH–NH_3 aq. (70:30:2) to yield the diastereomeric mixture of **4** as R_f 0.2 fraction (1.80 g, 9.3 mmol, 42% yield over three steps). The diastereomers were further separated by flash chromatography on activated alumina and eluted with $CHCl_3$–CH_3OH–NH_3 aq. (90:10:1). From the R_f 0.3 fraction, 72 mg of a pale-yellow powder was obtained, which was named KM017-A (0.37 mmol, 1.7% yield overall). The R_f 0.2 fraction from a similar experiment yielded 11 mg of a pale-yellow powder, which was named HY065-F5 (0.06 mmol, 0.3% yield overall). HPLC purity (monitored at 275 nm) was 98.7% for KM017-A and 98.0% for HY065-F5. HRMS m/z $[M+H]^+$: Calcd. for $C_{11}H_{16}NO_2$: 194.1181, Found in KM017-A: 194.1173, Found in HY065-F5: 194.1175. ^{1}H NMR δ_H for KM017-A (DMSO-d_6): 7.11 (2H, d, J=8.5 Hz, Ar–H), 6.68 (2H, dd, J=8.5 and 2.0 Hz, Ar–H), 4.29 (1H, d, J=6.0 Hz, O–CH), 3.03 (1H, dt, J=6.0 and 6.5 Hz, N–CH), 2.82 and 2.64 (2H, m, N–CH_2), 1.56 (4H, m, CH_2CH_2). ^{13}C NMR δ_X for KM017-A (DMSO-d_6): 156.2, 134.9, 127.5, 114.7, 74.5, 64.6, 46.5, 26.9, 25.4. ^{1}H NMR δ_H for HY065-F5 (DMSO-d_6): 7.10 (2H, d, J=8.5 Hz, Ar–H), 6.68 (2H, dd, J=8.5 and 2.0, Ar–H), 4.10 (1H, d, J=7.5 Hz, O–CH), 3.09 (1H, dt, J=7.5 and 7.5 Hz, N–CH), 2.81 (2H, m, N–CH_2), 1.60 (2H, m, CH_2), 1.32 (2H, m, CH_2). ^{13}C NMR δ_X for HY065-F5 (DMSO-d_6): 156.2, 134.2, 127.8, 114.5, 75.3, 64.6, 45.6, 27.6, 25.0. The ^{1}H, ^{13}C, COSY, and HMQC spectra of KM017-A and HY065-F5 in DMSO-d_6 are presented in Supplementary Figs. S4–S11 and the HPLC chromatograms are shown in Supplementary Fig. S12. The ^{1}H, COSY, and NOESY spectra in acetic ac-

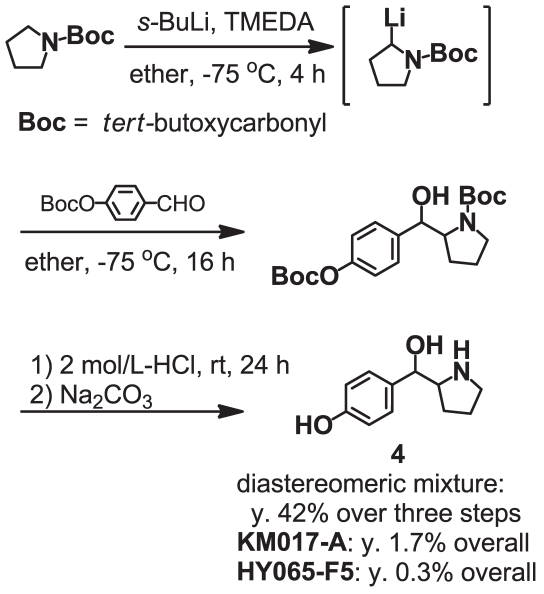

Fig. 2. Synthesis of compound **4**

id-d_4/D_2O (v/v=1:1) are shown in Supplementary Figs. 13–18. ^{1}H NMR δ_H for KM017-A (acetic acid-d_4/D_2O (v/v=1:1)): 7.28 (2H, d, J=8.5 Hz, ArH), 6.89 (2H, dd, J=8.5 and 2.0 Hz, ArH), 5.05 (1H, d, J=5.0 Hz, O–CH), 3.90 (1H, m, N–CH), 3.37 (2H, dd, J=6.0 and 5.0 Hz, N–CH_2), 2.09–1.89 (4H, m, CH_2CH_2). δ_H for HY065-F5 (acetic acid-d_4/D_2O (v/v=1:1)): 7.28 (2H, dd, J=8.5 and 2.0 Hz, ArH), 6.89 (2H, dd, J=8.5 and 2.0 Hz, ArH), 4.70 (1H, d, J=9.0, O–CH), 3.88 (1H, dt, J=9.0 and 7.5 Hz, N–CH), 3.43 (2H, m, N–CH_2), 2.10–1.96 (2H, m, N–CH–CH_2), 1.80 and 1.67 (2H, m, N–CH_2–CH_2).

4. Dose–response analysis of BmOAR1 ligands using secreted placental alkaline phosphatase (SEAP) reporter assay

The dose dependence of BmOAR1 stimulation by each ligand was evaluated as described previously.[17] This chemiluminescent reporter gene assay system was performed using human embryonic kidney (HEK)-293 cells stably expressing BmOAR1 (accession number AB255163) (hereafter called HEK-BmOAR1 cells). Briefly, HEK-BmOAR1 cells were grown at 37°C and 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine rerum (FBS; Invitrogen). The pCRE-SEAP vector (Clontech, Mountain View, CA) (1 μg) was transiently transfected into HEK-BmOAR1 cells (6×10^5 cells/35 mm diameter dish) using transfection reagent GeneJuice® (Novagen, Gibbstown, NJ) following the manufacturer's protocol. After 24 hr of transfection, the cells were reseeded at 1×10^4 cells in each well of a 96-well culture plate and cultured for 1 day (37°C, 5% CO_2). Subsequently, each ligand was added to the cells at a concentration of 10^{-9} to 10^{-4} M and incubated for 1 day (37°C, 5% CO_2). SEAP activity in the culture medium was measured using a chemiluminescent reporter gene assay (Phospha-Light™ system; Applied Biosystems, Foster City, CA, USA). OA (10^{-5} M)-induced SEAP activity is shown as 100%. EC_{50} values were estimated by the Probit method.

Results and discussion

*1. Synthesis of compound **4** and separation of diastereomers*

The crude product of **4** was considered a mixture of the four stereoisomers because the two chiral carbons yielded *erythro*- and *threo*-diastereomers, each containing an enantiomer. Although, compound **4** had poor solubility in low-polarity solvents and typical chromatographic solvents due to its zwitterionic nature, a small amount could be separated chromatographically into two components, KM017-A and HY065-F5, using $CHCl_3$–CH_3OH–NH_3 aq. The HRMS data, with mass errors of −4.1 and −3.1 ppm, respectively, showed sufficiently high agreement with the calculated values. Based on the NMR spectra, the two components were identified as *erythro*/*threo* isomers as follows.

The spin coupling constant (J) value of vicinal C–H in the ^{1}H NMR spectra strongly depends on the dihedral angle, as established by the Karplus correlation. The J value is 8–9 Hz when the dihedral angle is close to 0° or 180°, with a minimum value when the angle is close to 90°. This correlation is useful in determining the relative configuration of the 1,2-bifunctional carbon skeleton. In the case of acyclic vicinal diols, if the conformation resulting from an intramolecular hydrogen bond is predominant, the J values for O–*CH*–*CH*–O tend to be relatively small (below 5.0 Hz) or relatively large (above 6.0 Hz). This phenomenon is clearly demonstrated when acetic acid-d_4/D_2O is used as well as $CDCl_3$ as the solvent,[19] and this solvent system offers the possibility to predict the *erythro*/*threo* configuration. The J values observed for O–*CH*–*CH*–N region of KM017-A and HY065-F5 in acetic acid-d_4/D_2O (v/v=1:1) are 5.0 Hz and 9.0 Hz, respectively. The Newman projection in Supplementary Fig. S19 provides the clues and an overview of the results of the conformational analysis: the MM2 forcefield analysis of the staggered conformation of compound **4** in the *erythro* configuration suggests a preferred N^+(H)—O hydrogen bond distance of 0.259 to 0.266 nm and a CH–CH dihedral angle of 44° to 53°. The estimated angle is reasonable for the J value of 5.0 Hz observed for KM017-A. On the other hand, for the *threo* configuration, the N^+(H)—O hydrogen bond distance of 0.258 to 0.264 nm and the CH–CH dihedral angle of 172° to 180° are shown to be preferred. This corresponds to the J value of 9.0 Hz observed for HY065-F5.

When considering the stereochemistry of compound **4**, previous studies on ephedrine by NMR spectroscopy are very instructive. This is because the two chiral centers in compound **4** are composed of the same type of functional groups as those in ephedrine. In a study on the conformational analysis of (−)-ephedrine and (+)-pseudoephedrine in $CDCl_3$ by NMR spectroscopy, the J values of the CH–CH region were reported to be 4.0 Hz for (−)-ephedrine, the *erythro* isomer, and 8.2 Hz for (+)-pseudoephedrine, the *threo* isomer.[20] Our results agree with these values.

Another feature is the difference of 0.35 ppm in the chemical shifts of the O–CH protons observed in KM017-A (5.05 ppm) and HY065-F5 (4.70 ppm). This would be explained by magnetic anisotropy, which is also present in the σ-bond skeletons. In the *threo* configuration of compound **4**, the O–CH proton of the proposed conformer is partially eclipsed by the C–C bond of the pyrrolidine ring, which probably has a diamagnetic influence that shifts the O–CH resonance upfield. The observed difference in chemical shift is comparable to the 0.49 ppm difference observed between ephedrine (5.08 ppm) and pseudoephedrine (4.59 ppm) in methanol-d_4,[21] suggesting a similar involvement of the C–CH_3 bond located close to the O–CH of pseudoephedrine.[20]

In addition, the NOE interactions observed in KM017-A and HY065-F5 confirm the above assignment of the diastereomers, as shown in Supplementary Figs. S16 and S18. Consequently, it was concluded that KM017-A is the *erythro* isomer and HY065-F5 is the *threo* isomer.

2. Dose-response relationship of BmOAR1 ligands

As shown in Fig. 3, synephrine (an *N*-methyl derivative of OA) exhibited maximum activity at 10^{-6} M, which was the highest agonist activity among the tested derivatives. The EC_{50} value

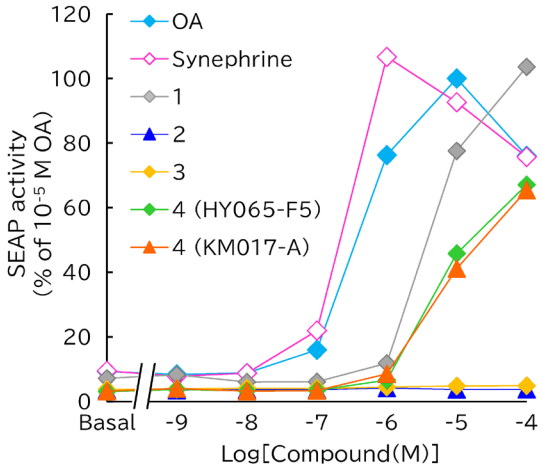

Fig. 3. Dose-response relationship of octopamine (OA) and its derivatives in the CRE-SEAP assay using HEK-BmOAR1 cells. Data represent the mean of duplicate measurements. "Basal" indicates basal levels (SEAP activity without agonists).

of synephrine was 0.22 μM (95% confidence interval; 0.18–0.27 μM), which was approximately two-fold smaller than that of OA (0.47 μM, 95% confidence interval; 0.37–0.61 μM). *N*-Ethyloctopamine (**1**) retained significant activity at 10^{-5} to 10^{-4} M with the EC_{50} value of 5.17 μM (95% confidence interval; 4.12–6.55 μM), whereas compounds substituted with propyl (**2**) or hexyl (**3**) showed no activity at any concentrations tested. This suggests the existence of a small space around the amine-binding site of BmOAR1 capable of accommodating a small alkyl moiety (C2 or lower). Notably, compound **4**, with a fixed C3 alkyl group forming a pyrrolidine ring, showed significant activity, whereas the propyl derivative (**2**) showed no activity. The alkyl part of the cyclic amine is expected to exert the same effect as the small alkyl group. Additionally, this structural design provided a new chiral center, although no diastereomeric differences in activity were detected between HY065-F5 and KM017-A. The agonist activities of HY065-F5 and KM017-A were not very high, with the EC_{50} values estimated at concentrations up to 100 μM, 6.06 μM (95% confidence interval; 4.62–8.32 μM) and 6.76 μM (95% confidence interval; 5.07–9.56 μM), respectively. It is likely that the alkyl portion of the ring did not fully enter the cavity of BmOAR1 that accommodates the *N*-alkyl group, thereby failing to distinguish their agonist activities.

In summary, BmOAR1 is considered to have a small space around the amine-binding site that fits the methyl group while also allowing the ethyl group and a part of the pyrrolidine ring. Our assay results do not completely rule out the possibility that compounds with more extended chain compounds behave as antagonists, resulting in an apparent loss of agonist activity. However, given our interest in the interaction between the small nitrogen-containing rings of the ligand and the amine-binding site of the receptor, we are further pursuing the synthesis and assay of ligands with small cyclic moieties, such as the azetidine ring, to obtain detailed structural information on the ligand binding-sites in combination with molecular modeling, docking simulations, and, in the future, biophysical structural analysis of OA receptors.

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Declarations of interest

None

Electronic supplementary materials

The online version of this article contains supplementary material, which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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