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Development of a fluorescent peptide for the highly sensitive and selective detection of oxytocin

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ABSTRACT

A novel oxytocin-targeting fluorescent peptide (compound **1**), with a cyanopyranyl group as the fluorophore and a peptide as the binding site of oxytocin, was developed. Compound **1** allowed the detection of oxytocin by alteration of its fluorescence quantum yield. When alone in solution and unbound to oxytocin, compound **1** had a weak fluorescence emission, contrary to a much stronger fluorescence emission when associated with oxytocin. The increase in fluorescence intensity was concentration-dependent, from which a good linear relationship was observed by plotting the data as a function of oxytocin concentration. The binding and detection of compound **1** with oxytocin was not affected by the presence of foreign substances, thereby allowing for the highly selective detection of oxytocin. As an application of this fluorescent reagent, compound **1** was immobilized on the surface of magnetic beads *via* covalent bonding under optimal conditions. The fluorescent detection of oxytocin in rat serum was successfully achieved using magnetic beads. Overall, the experimental results of this work clearly show that compound **1** is a good probe for oxytocin detection, and can be widely employed in oxytocin detection protocols in diverse areas of research.

1. Introduction

Oxytocin is a peptide hormone neurotransmitter that plays an essential role in mammalian labor and lactation through its peripheral action. Oxytocin consists of nine amino acids constituting a 20-membered ring. It is produced by neurons in the paraventricular nuclei and supraoptic nucleus of the hypothalamus, and is stored in the posterior pituitary for secretion in blood [1]. Recent studies suggest that oxytocin modulates a number of functions. It is involved in maternal bonding, sexual behavior, and social affiliation [2–4]. In addition, dysregulation of oxytocin levels causes autism and other developmental disorders associated with increased anxiety and deficit in social behavior [5]. Moreover, it has been proposed that the efficacy of oxytocin administration depends on its dosage [6–9]. Animal [10,11] and human studies on diseases other than the autism spectrum disorder [12–14] have shown dose-dependent oxytocin efficacy. Consequently, there is a strong need to develop efficient and rapid methods that can be used to selectively determine and continuously detect changes in oxytocin level in organisms.

Fluorescence spectrometry is a conventional and highly sensitive analytical method. Fluorescent probes that exhibit a spectral response upon binding to ions and neutral organic or inorganic molecules have enabled researchers to investigate the changes in free guest ions or changes in the concentrations of molecules, using a broad range of fluorescence-based techniques such as microscopy, flow cytometry, and spectroscopy [15–19]. Hence, fluorescence-based assays for proteins, inhibitors, and probes can be advantageous for high-throughput analysis, when used for assays that involve an immobilized component or in solution [20].

The parameters considered while designing a fluorescent reagent to detect oxytocin included the production of weak-to-strong fluorescence after binding to or reacting with oxytocin, which provides a reduced background noise and results in the highly sensitive detection of oxytocin; high selectivity and specificity to oxytocin; and reduced interference from foreign substances. In previous studies, several novel fluorescent reagents for detecting biomolecules were developed in our lab, which were sensitive to changes in the external environment. Briefly, the quantum yield of these molecules generally increases as the environment becomes more hydrophobic, which results in dramatic fluorescence spectral changes [21–27]. To take advantage of this phenomenon, novel fluorescent probes were subjected to varying external environmental conditions and the results showed that their quantum

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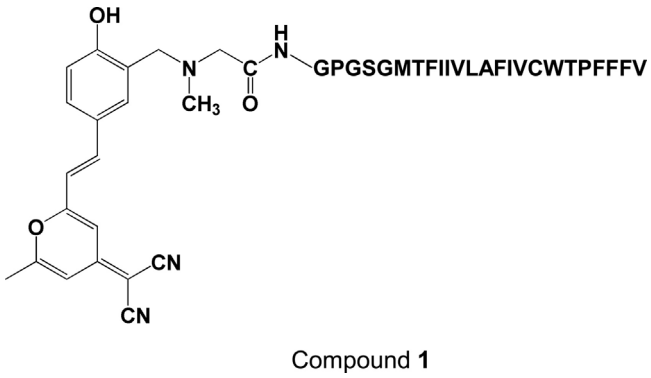


Fig. 1. Chemical structure of the fluorescent reagent (Compound **1**).

yields responded linearly to the concentration of the target compounds.

In this study, a novel technique for oxytocin detection was developed. This technique combined the properties of the above-mentioned fluorescent molecular probes. Using the cyanopyranyl group as the fluorescent emitter and a peptide as the binding site for oxytocin, the amino acid sequence of the probe was designed from the oxytocin receptor [28]. The chemical structure of the molecular probe (compound **1**) is shown in Fig. 1. Compound **1** was successfully synthesized, and the interactions of this molecule with oxytocin in homogenous and heterogeneous solution systems were investigated using magnetic beads. Our experimental results clearly indicated that the compound developed in this study acted as a good fluorescent probe, which could be easily used to detect oxytocin.

2. Materials and methods

2.1. General information

All chemicals used were of analytical grade and were purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Sigma–Aldrich (St. Louis, MO, USA). Magnetic beads were purchased from Tamagawa Seiki Co., Ltd. (Nagano, Japan). Absorption spectra were recorded at 25 °C using the V-670 UV/visible spectrophotometer (JASCO, Tokyo, Japan), and fluorescence spectra were recorded at 25 °C using the JASCO FP-6500 fluorophotometer. ^{1}H NMR spectra were recorded using the Bruker AV-500 spectrometer.

2.2. Synthesis of a fluorescent reagent for the detection of oxytocin

The scheme for the synthesis of a fluorescent reagent is illustrated in Scheme 1, and individual synthetic protocols are detailed below.

2.2.1. Ethyl 2-((5-formyl-2-hydroxybenzyl)(methyl)amino)acetate

Sarcosine ethyl ester hydrochloride (1.5 g, 10.0 mmol) was added to a solution of paraformaldehyde (1.3 g) in 30 mL isopropyl alcohol and 50 mL H_2O, and the solution was stirred for 45 min at 80 °C under N_2 atmosphere. Following the addition of 4-hydroxybenzaldehyde (1.0 g, 8.2 mmol), the reaction mixture was refluxed for 24 h and the solvent was removed *in vacuo*. The resulting residue was dissolved in chloroform, washed with water, and dried over Na_2SO_4. Following the removal of the solvent, the product was purified by column chromatography (Al_2O_3; $CHCl_3$:MeOH = 200:3 v/v). The yield of the product was 78%. ^{1}H NMR ($CDCl_3$, 400 MHz, r.t., TMS, δ/ppm) 1.29 (3H, t), 2.41 (3H, s), 3.36 (2H, s), 3.87 (2H, s), 4.24 (2H, q), 6.96 (1H, d), 7.57 (1H, s), 7.72 (1H, d), 9.82 (1H, s), 10.56 (1H, bs).

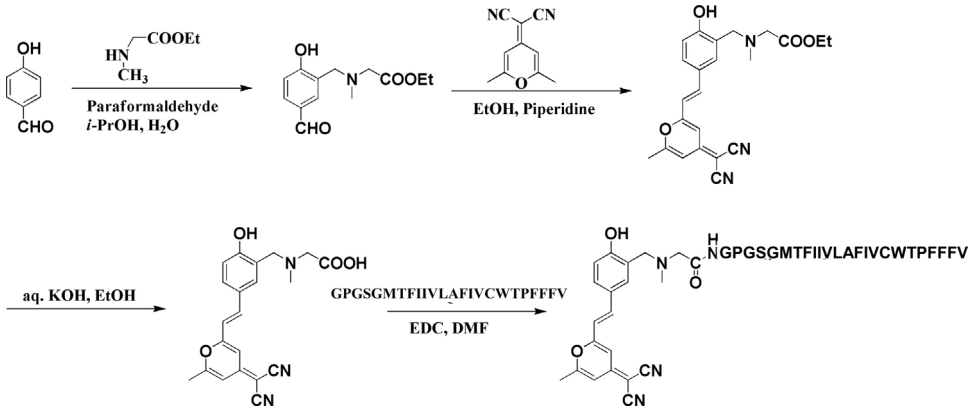
2.2.2. (E)-ethyl 2-((5-(2-(4-(dicyanomethylene)-6-methyl-4H-pyran-2-yl)vinyl)-2-hydroxybenzyl)(methyl)amino)acetate

Here, 4-(dicyanomethylene)-2,6-dimethyl-4*H*-pyran (0.3 g, 1.6 mmol) and piperidine (0.2 g, 1.7 mmol) were added to a solution of ethyl 2-((5-formyl-2-hydroxybenzyl)(methyl)amino)acetate (0.5 g, 1.6 mmol) in 40 mL ethanol, and then refluxed for 12 h under N_2 atmosphere. After removing the solvent *in vacuo*, the residue was dissolved in $CHCl_3$, washed with H_2O, and dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the product was purified by column chromatography (SiO_2; *n*-hexane:ethyl acetate = 3:2 v/v → $CHCl_3$). The yield of the process was 88%.

^{1}H NMR ($CDCl_3$, 400 MHz, r.t., TMS, δ/ppm) 1.28 (3H, t), 2.40 (3H, s), 2.42 (3H, s), 3.36 (2H, s), 3.86 (2H, s), 4.22 (2H, q), 6.50–6.53 (2H, m), 6.65 (1H, s), 6.95 (1H, d), 7.17 (1H, s), 7.40–7.42 (2H, m), 9.95 (1H, bs).

2.2.3. (E)-2-((5-(2-(4-(dicyanomethylene)-6-methyl-4H-pyran-2-yl)vinyl)-2-hydroxybenzyl)(methyl)amino)acetic acid

KOH solution (1 N, 5.0 mL) was added to 25 mL of (*E*)-ethyl2-((5-(2-(4-(dicyanomethylene)-6-methyl-4*H*-pyran-2-yl)vinyl)-2-hydroxybenzyl)(methyl)amino)acetate solution (0.2 g, 0.3 mmol) in ethanol, and stirred for 12 h at 25 °C. After removing the solvent, the residue was dissolved in H_2O, and neutralized with 1 N HCl (pH ~7–8). The product was extracted



Scheme 1. Method for the synthesis of fluorescent peptide (Compound **1**).