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# Identification of functional bitter taste receptors and their antagonist in chickens

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ABSTRACT

Elucidation of the taste sense of chickens is important not only for the development of chicken feedstuffs for the chicken industry but also to help clarify the evolution of the taste sense among animals. There are three putative chicken bitter taste receptors, chicken T2R1 (cT2R1), cT2R2 and cT2R7, which were identified using genome information and cell-based assays. Previously, we have shown that cT2R1 is a functional bitter taste receptor through both cell-based assays and behavioral tests. In this study, therefore, we focused on the sensitivities of the other two bitter receptors, cT2R2 and cT2R7, by using their agonists in behavioral tests. We tested three agonists of cT2R2 and three agonists of cT2R7. In a 10-min drinking study, the intakes of cT2R2 agonist solutions were not different from that of water. On the other hand, the intakes of cT2R7 agonist solutions were significantly lower compared to water. In addition, we constructed cT2R1-and cT2R7-expressing cells in order to search for an antagonist for these functional bitter taste receptors. By using $Ca^{2+}$ imaging methods, we found that 6-methoxyflavanone (6-meth) can inhibit the activities of both cT2R1 and cT2R7. Moreover, 6-meth also inhibited the reduction of the intake of bitter solutions containing cT2R1 or cT2R7 agonists in behavioral tests. Taken together, these results suggested that cT2R7 is a functional bitter taste receptor like cT2R1, but that cT2R2 is not, and that 6-meth is an antagonist for these two functional chicken bitter taste receptors. This is the first identification of an antagonist of chicken bitter receptors.



## 1. Introduction

Elucidation of the taste sense of chickens is important not only to improve and develop new chicken feedstuffs to meet the future demand for chicken products but also to clarify the evolution of the taste sense among animals. Recent studies of chicken taste systems have revealed that chickens have many taste buds in the oral cavity by using a new chicken taste bud marker, vimentin, and whole-mount tissues from the oral epithelial sheet [1,2], and that chickens can detect some taste qualities such as fat, bitter, and umami *via* these taste receptors [3–5]. In this study, we focused on the ability of chickens to detect bitter taste. Bitterness is one of the five basic tastes, and bitter taste perception in vertebrates is important to enable them to avoid harmful and toxic substances. We previously showed that chickens avoided the intake of bitter solutions in behavioral tests, and an isolated cluster of taste buds was activated by a bitter compound, quinine chloride, as shown using the $Ca^{2+}$ imaging method [6,7]. There are three putative bitter taste receptors in chickens, T2R1 (cT2R1), T2R2 (cT2R2) and T2R7 (cT2R7), which were earlier identified using genome information [8]. Behrens et al. reported that many bitter compounds activated cT2R1, cT2R2 and cT2R7 in a cell-based assay [9]. Hirose et al. showed that cT2R1 activities in the cell-based assay were compatible with behavioral sensitivity to bitterness in chickens [3], and their study suggested that cT2R1 is a functional bitter receptor *in vivo*. However, there is no evidence as to whether cT2R2 and cT2R7 are functional receptors at the *in vivo* level. Thus, we first performed behavioral tests using several of the cT2R2 and cT2R7 agonists identified in the previous report [9].

We also considered that it would be useful to identify an antagonist of functional chicken bitter taste receptors for use in chicken farming. Most chicken feedstuff ingredients are derived from plants, and feed ingredients that originate from plants are generally bitter. If antagonists of chicken bitter taste receptors

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could be identified, they would be useful for reducing the bitter taste of unused resources, which are too bitter for chickens to consume, in order to make new feedstuffs for use in the animal industry. Moreover, an antagonist of the chicken bitter taste receptors would be desirable because, in general, antagonists are a powerful tool for revealing the receptor function in animals. Thus, a secondary goal of this study was to identify an antagonist of chicken functional bitter taste receptors.

Roland et al. showed that 6-methoxyflavanone (6-meth) inhibits the activity of human TAS2R39, a human bitter taste receptor [10]. 6-meth is a flavonoid that occurs in many plants and is widely present in the human diet. Because the cT2R1 gene is close to the human TAS2R39 and TAS2R40 genes [8,11], and agonists of cT2Rs partly overlap among the three cT2Rs [9], we thought that 6-meth might also inhibit the activity of the chicken's bitter taste receptors. Thus, in this research, we investigated the characteristics of 6-meth as a putative antagonist to the functional bitter taste receptors in chickens. We confirmed that cT2R1 and cT2R7 are the functional bitter taste receptors in chicken oral tissues, and 6-meth successfully inhibited the agonist-mediated activities of these functional bitter taste receptors in chickens both *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. Chemicals

Dextromethorphan hydrochloride (Dex), caffeine and colchicine were purchased from Sigma-Aldrich (St. Louis, MO, USA), and chloramphenicol (Chlor), coumarin and andrographolide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Parthenolide and 6-meth were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo). For $Ca^{2+}$ imaging, these compounds were dissolved in ultra-pure water to make stock solutions, stored at −20 °C and diluted with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose at pH 7.4, adjusted with NaOH just before each experiment. For behavioral experiments, taste solutions were prepared using normal tap water before each experiment.

### 2.2. Animals

Rhode Island Red (RIR) chicks of one to 2 weeks in age were used for this research. The study was carried out according to the Guide for Animal Experiments issued by Kyushu University, the Law Concerning the Human Care and Control of Animals (Law No. 105; October 1, 1973), the Act on the Welfare and Management of Animals (Law No. 105, 1973, the Ministry of Environment) and the Japanese Government Notification on the Feeding and Safekeeping of Animals (Notification No. 6; March 27, 1980).

### 2.3. Behavioral test

One-bowl drinking tests were conducted based on our previous report [3] with slight modifications. Briefly, the behavioral test took place over 12 consecutive days, where the first six days were considered to be a training period for the chicks and days 7–12 were considered the experimental period. The chicks were kept together for the first two days to allow them to overcome psychological stress and then separated into individual pens. Commercial layer feed was fed to the chicks *ad libitum* throughout the whole experiment (Powerlayer 17Y; JA Kitakyushu Kumiai Shiryo, Fukuoka, Japan). On the first day, the chicks were supplied normal tap water for 24 h and then the drinking time was restricted to only 10 min in each 24-h period beginning at 17:00 to train them in drinking for a short period of time. Over the experimental period (days 7–12), the chicks were supplied either water or test solutions (bitter, 6-meth, or bitter with 6-meth) for 10 min as in the training period. The water and test solution were given on a randomized basis over the experimental period. To compensate for the evaporation loss from the bowl in the 10 min of exposure, control tap water was set in a brooder box, and the amount of evaporation was subtracted from the volume of water or test solution intake.

### 2.4. Construction of plasmids

Total RNA was isolated from the palates of chicks, which are rich in taste buds [12], and first-strand cDNA was synthesized by reverse transcription. Then, deduced open reading frames (ORFs) of *cT2R7* were amplified and sequenced. The polymerase chain reaction (PCR) primers were designed based on the NCBI nucleotide databases of the *cT2R7* (NM_001080719.1). The PCR products of the ORFs were sub-cloned into the pDisplay (Life Technologies Japan, Tokyo) mammalian expression vector by using an In-Fusion HD Cloning Kit (Takara Bio, Otsu, Japan). $G\alpha_{16/gust44}$/pcDNA3.1 (+) was kindly donated by Dr. Takashi Ueda (Nagoya City University) and *cT2R1/pDisplay* was taken from previous research [3]. The entire sequence of *cT2R7* derived from RIR chicks was compared with the genomic NCBI databases of *cT2R7* (Supplementary Fig. 1).

### 2.5. Cell culture

Human embryonic kidney (HEK)-derived 293T (HEK 293T) cells were maintained in Dulbecco's Eagle's medium (DMEM high glucose; Wako) containing 10% fetal bovine serum (FBS; GE Healthcare, Buckinghamshire, UK) and penicillin-streptomycin solution (×100) (Wako) at 37 °C and 5% $CO_2$.

### 2.6. Measurement of cytosolic $Ca^{2+}$ concentrations

For the $Ca^{2+}$ imaging experiments, HEK293T cells were transfected with either empty vector pDisplay for mock cells or co-transfection of $G\alpha_{16/gust44}$/pcDNA3.1 (+) with cT2R1/pDisplay or cT2R7/pDisplay by using Screen*Fect*™A (Wako) on coverslips coated with poly-D-lysine (0.1 mg/mL; Wako). After transfection, the cells were incubated for 48 h at 37 °C and 5% $CO_2$. Then, the cells were loaded with 1.25 μM Fluo 4-AM solution for 30 min at 37 °C and 5% $CO_2$ in the dark. Fluo 4-AM solution was prepared according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan).

The coverslips were washed with the standard bath solution, and Fluo-4 fluorescence was measured in the standard bath solution using a confocal laser-scanning microscope (Nikon A1R; Nikon Co., Tokyo). The coverslips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical stimulation was applied by running a bath solution containing various chemical reagents. Cell viability was confirmed by responses to 5 μM ATP.

### 2.7. Statistical analysis

The data are expressed as means ± SE. Statistical analyses were done using the paired *t*-test. The analyses, calculations of $IC_{50}$ values, and illustrations of fitting curves using the Hill equation were performed using the IGOR Pro software package (Version 6.34J; WaveMetrics, Portland, OR), and differences with *p*-values < 0.05 were considered to be significant.

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