Unnatural Tripeptides as Potent Positive Allosteric Modulators of T1R2/T1R3

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Supporting Information

ABSTRACT: T1R2/T1R3 belongs to G protein coupled receptors, which recognizes diverse natural and synthetic sweeteners. A novel class of positive allosteric modulators (PAMs) of T1R2/T1R3 was identified through high-throughput screening campaign. Comparing the structure of the potent compound with previously known PAM, we classified the structure of known PAM into three parts, defined as “head”, “linker”, and “tail”. We then investigated the linker–tail structure. It was suggested by molecular docking models of T1R2/T1R3 that an amine that we introduced in the tail was the key for interaction with the receptor binding pocket. We thus synthesized various molecules and found unnatural tripeptide-PAMs, which potently enhance the sweetness of sucrose in sensory evaluation tests.

KEYWORDS: Positive allosteric modulators, Class C GPCR, T1R2/T1R3 receptor, Unnatural peptide, Sweetness enhancer

T1R2/T1R3 belongs to the class C G protein coupled receptors (GPCRs), which was found to recognize diverse natural and synthetic sweeteners and to be involved in sweet taste modulation.^{1–3} The extracellular venus fly trap domain in the T1R2/T1R3 receptor recognizes various sweeteners and contains an orthosteric ligand binding site.^{1,2,4} In 2000, Kunishima et al. determined three different crystal structures of the class C GPCR mGluR1 extracellular ligand-binding region.^{5,6} The structural dynamics of glutamate binding, in which the protein flexibly changed domain arrangements to form an “open” or “closed” conformation, were elucidated. These dynamics also apply to other class C GPCRs such as metabotropic glutamate receptors,^{7,8} umami taste receptors,^{1,9} the Ca²⁺-sensing receptor,¹⁰ the γ -aminobutyric acid type B receptor,^{11,12} pheromone receptors,¹³ as well as the T1R2/T1R3 sweet receptor.

In the field of food chemistry, sweeteners have long inspired chemists and biologists.^{14,15} High-intensity sweeteners, alternatives to sucrose, have lower calorie and might contribute beneficially to human health.^{16,17} Type II diabetes and obesity crises are observed in many countries. It is presumed that high-intensity sweeteners may assist in weight loss and contribute to reduce these diseases. However, some high-intensity sweeteners are perceived as a nonpreferred sweet taste depending on

their time intensity of sweet taste^{14,15} or aftertaste. In 2010, a novel class of positive allosteric modulators (PAMs) was discovered by using cell-based assay of the T1R2/T1R3 receptor.^{18–20} These compounds enhance the sweet taste of sucrose in vivo, but they are not sweet on their own. Since these compounds are very potent and tasteless, they are able to be used as a taste to modify or enhance the taste profile of a variety of foods and beverages. These substances were reviewed by the Expert Panel of the Flavor and Extract Manufacturers Association of the United States (FEMA) and determined to be generally recognized as safe (GRAS) for their intended use as flavor ingredients, and therefore they are available for use in human food in the United States as FEMA GRAS flavor ingredients.^{21–23}

FEMA4774 (1, CAS No. 1359963-68-0) is the novel T1R2/T1R3 positive allosteric modulator, and this compound shows obvious enhancement of the sweetness of sucrose in sensory evaluation tests.²⁴ Here, we report novel positive allosteric modulators of T1R2/T1R3, which have an unnatural tripeptide structure. Initially, the biaryl scaffold 3 was identified

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through high-throughput screening campaign. As we compared the hit compound **3** with **1** and **2**, we classified the structure of **1** into three parts (“head”, “linker”, and “tail”) to identify the role of each part of the structure in enhancer activity. By linker–tail optimization, we obtained compounds **15**, **17**, and **19**. These compounds were evaluated in vivo sensory study, and they enhanced the sweetness of sucrose.

The potency and efficacy of **1** and **2** were determined by calcium influx assay using PEAK^{rapid} cells stably expressing modified human sweet taste receptor. Basically, calcium influx assay was performed with sucrose,^{25–27} since all tested compounds did not have any agonist activity against sweet receptor. Under this condition with sucrose, the half-maximal effective concentration (EC₅₀) value of sucrose with **1** (30 μM) was shifted from 31.7 mM to 0.6 mM, and with **2** (50 μM) it was 15.9 mM.

We explored positive allosteric modulator with a novel core biaryl structure (**3**, **4**, and **5**) for sweetness enhancer through a high-throughput screening campaign with calcium assay using our chemical library. By comparing the structures of **1**, **2**, and **3**, we divided the structure of **1** into three parts, namely, Head–Linker–Tail (Figure 1c). Head–linker is the essential part in

Table 1. SAR Study of Biaryl Derivatives^a

Compound	R
		EC ₅₀ (mM)	Efficacy (%)
3		21.5±1.2	80±2.9
4		16.8±1.1	83±2.3
5		21.0±1.2	85±3.5

^aThe EC₅₀ value of sucrose was determined with each compound at 50 μM. The efficacy is expressed as the percentage of the maximum activation obtained with **2** (50 μM). Data are presented as the mean ± SEM of three separate experiments.

Figure 1. (a) Structure of compounds **1** and **2**. (b) Representative concentration–response curves of sucrose with compound **1** (30 μM) or **2** (50 μM) in calcium influx assays. Data are presented as the mean ± standard error of measure (SEM) of three separate experiments. (c) Structure of **1** divided and classified in three parts.

this molecule for enhancer activity. The tail part determines the level of activity. Using this information, we planned to first optimize the head–linker structure and later connect tail structures to try to elevate the enhancer activity. Compounds were initially evaluated by cell-based assay. These head–linker structures had a weak enhancer activity at 50 μM, compared with **2** (Table 1). The efficacy of each compound was calculated as the percentage of the maximum activation obtained with **2** (50 μM). Then, we searched for the best tail structure, focusing on biaryl derivative **4** as the head–linker.

On the basis of the structure of **1** (Figure 1a), we initially connected the tail to position 5 in the furan of biaryl derivative **4**. Moreover, we hypothesized that an amide bond with a hydrophobic substituent is important for the tail structure to elevate enhancer activity. Unfortunately, however, compounds **6–11** did not show increased enhancer activity compared with **4** (Table 2).

To address these issues, we used molecular docking models of compounds that might have strong enhancing activity against T1R2/T1R3. The binding site of most sweet substances such as sucrose and aspartame is reported to be the extracellular domain of T1R2. Therefore, a model structure of the extracellular domain of T1R2 was constructed by homology modeling based on the X-ray crystal structure of mGluR1 (PDB code: 1EWK).⁵ In our docking model, nitrogens in the pyridine and the carboxylic acid in the head structure of **1** interact with residues D307 and K65 in the T1R2 receptor, respectively (Figure 2). The same interactions were observed in our docking model for compound **8**. Furthermore, the α-position of the carbonyl moiety in the tail structure was close to the backbone amide of T1R2 receptor residue R378 (~3.1 Å apart). We speculated that we could obtain new interactions that would elevate enhancer activity by incorporating an amine at the α-position of the carbonyl moiety in the tail structure.

As we expected, comparing compounds **8** and **12**, the enhancer activity was elevated when we introduced an amine at the α-position of the carbonyl group in the tail structure. We also evaluated some other amino acids in the tail instead of valine (which was the tail amino acid in compound **12**). When we connected hydrophobic amino acids to the tail structure, the enhancer activity was higher than that of **12**. Compound **15** had the best in vitro activity in this chemical series, with an EC₅₀ value of 5.8 mM. We thus concluded that a cyclohexyl glycine in the tail is significant.

From our SAR study of the biaryl chemical series, we deduced that a 4-aminonicotinate head structure and L-cyclohexyl glycine tail structure make important contributions to enhancer activity. Thus, next, we attempted to change the linker structure to obtain compounds with greater enhancer activity than **15**. Because the yield of C–C coupling of the pyridine moiety and furan segment by the Suzuki–Miyaura

Table 2. SAR Study of Tail Structures^a

Compound	Tail	EC ₅₀ (mM)	Efficacy (%)
6		27.1±1.2	33±1.9
7		22.1±1.2	37±1.9
8		18.3±1.2	42±1.6
9		24.5±1.2	41±2.2
10		21.6±1.2	48±2.8
11		26.5±1.2	43±2.2
12		14.6±1.2	52±1.9
13		10.0±1.2	55±2.2
14		11.1±1.2	61±1.9
15		5.8±1.3	72±2.8

^aThe EC₅₀ value of sucrose was determined with each compound at 30 μ M. The efficacy of each compound is expressed as the percentage of the maximum activation obtained with **1** (30 μ M). Data are presented as the mean $\pm$ SEM of three separate experiments.

reaction was low, we chose to insert an amide bond between the head and linker.

To design, synthesize, and evaluate unnatural tripeptide derivatives, we first synthesized and incorporated 4-amino butanoic acid as the linker (**16**; Table 3). There was an obvious enhancer activity (EC₅₀ 16.2 mM). Then, we extended the linker moiety by incorporating 5-amino hexanoic acid (**17**) and 6-amino pentanoic acid (**18**). As we expected, **17** and **18** both had enhancer activity, but **17** had much greater enhancer activity, with an EC₅₀ value of 7.8 mM, similar to the enhancer activity of compound **15**. Compounds **15** and **17** both have a 4-aminonicotinate head and L-cyclohexyl glycine tail but different linker structures. From a superposition of **15** and **17**, we concluded that hydrophobicity of the alkyl part in the 5-amino hexanoic acid linker is important for enhancer activity (because **15** has a lower enhancer activity than **17**; see Figure 3 and also see Supporting Information Figure S2 for a superposition of **15** and **17** in three-dimensional (3D) docking model). Thus, if the linker is shorter than 5-amino hexanoic acid, the lack of hydrophobicity decreases enhancer activity, and if the linker is longer than 5-amino hexanoic acid, molecular overlapping with compound **15** might be worse. This result encouraged us to try a 3,3'-dimethyl- β -alanine linker (**19**), because this structure has the appropriate length and also similar hydrophobicity to the 5-amino hexanoic acid

Figure 2. Molecular docking study of compound **1** and compound **8** in a model structure of the extracellular domain of T1R2 obtained by homology modeling based on the X-ray crystal structure of mGluR1 (PDB code: 1EWK)⁵ using Prime (Schrödinger, LLC) for 3D modeling and Maestro (Schrödinger, LLC) for 2D. (a) Molecular docking study of compound **1**. (b) Molecular docking study of compound **8**.

linker of **17**. Gratifyingly, the 3,3'-dimethyl- β -alanine linker (**19**) resulted in the best in vitro activity in this chemical series, with an EC₅₀ of 2.2 mM.

On the basis of these results, we aimed to further enhance the activity and investigated the effect of introducing various alkyl chains in the linker. Interestingly, a 3-monomethyl- β -alanine linker (**20**), 3-isopropyl- β -alanine linker (**21**), and 3-isobutyl- β -alanine linker (**22**) decreased enhancer activity compared with **19**. We also synthesized and evaluated a 4-amino butanoic acid derivative linker with an alkyl substituent in the γ -position. When we introduced a 2-butane group (**24**) or benzyl group (**25**), the enhancer activity increased, but compound **19** still had the lowest EC₅₀ in our in vitro evaluation (Figure 4).

We evaluated the sweetness-enhancing activity of obtained active compounds in human sensory tests. We selected compounds **15**, **17**, and **19** for evaluation, because they had quite low EC₅₀ values. These three compounds were

Table 3. SAR Study of Linker Structures^a

Compound	Linker	EC ₅₀ (mM)	Efficacy (%)
16		16.2 ± 1.2	37 ± 1.6
17		7.8 ± 1.4	45 ± 2.8
18		20.2 ± 1.3	36 ± 2.2
19		2.2 ± 1.6	67 ± 3.7
20		10.2 ± 1.2	44 ± 1.9
21		18.4 ± 1.3	35 ± 2.2
22		22.7 ± 1.3	38 ± 2.8
23		10.5 ± 1.3	45 ± 1.9
24		8.9 ± 1.3	51 ± 1.9
25		8.5 ± 1.4	55 ± 3.7

^aThe EC₅₀ value of sucrose was determined with each compound at 30 μ M. The efficacy of each compound is expressed as the percentage of the maximum activation obtained with 30 μ M compound 1. Data are presented as the mean $\pm$ SEM of three separate experiments.

synthesized as 2HCl salts, and the sweetness enhancing activity was evaluated. The compounds were tested at 10 ppm in combination with 5% sucrose (Table 4). Compound 15 enhanced the sweetness of the sucrose to 1.77 times, of 17 to 1.96 times, and of 19 to 2.05 times the original level. In our

Figure 4. In vitro assay results for compounds 15, 17, and 19. Representative concentration–response curves of sucrose with tested compounds 15, 17, and 19 (30 μ M) in calcium influx assays. Data are presented as the mean $\pm$ SEM of at least three separate experiments.

Table 4. Sensory Evaluation to Investigate Sweetness Enhancer Activity^a

compound	1	15	17	19
enhancer activity	1.97	1.77	1.96	2.05

^aSee Supporting Information page 10 for details.

sensory evaluation test, 1 enhanced the sweetness to 1.97 times the original level. Therefore, we conclude that compound 19 was a stronger positive allosteric modulator than 1.

Distinct activity results were obtained in vitro assays and sensory evaluation tests. In our cell-based (in vitro) assay, the EC₅₀ value of sucrose with 1 was 0.6 mM, and with compound 19 it was 2.2 mM. But in the sensory evaluation, compound 19 had a stronger enhancer activity than 1. We think this distinction was because (i) the evaluated sucrose concentration was different in the in vitro assay (<80 mM) and sensory evaluation (5%, 146 mM), and (ii) we only performed calcium influx assay using PEAK^{rapid} cells heterologously expressing sweet taste receptor and chimeric G protein to detect intracellular Ca²⁺ influx in vitro. Therefore, the signaling molecules in vitro, including α -gustducin,²⁵ are different from those in taste cells, and multiple signaling pathways may be involved in sweet taste transduction in vivo.²⁶

In conclusion, we succeeded to design and synthesize compounds 15, 17, and 19, which are potent positive allosteric modulators of T1R2/T1R3. Especially, compound 19, an unnatural tripeptide showed stronger sweet taste-enhancing

Figure 3. Superposition of the speculated binding mode of compounds 15 and 17. Also see Supporting Information Figure S2.

activity than **1** in sensory evaluation tests. The main findings by SAR in this study are (i) the head–linker moiety of the modulator was essential, and biaryl derivatives show allosteric modulator activity, even if it was weak, (ii) the nitrogen that we introduced in the α -position of the carbonyl moiety in the tail structure interacted with the T1R2 receptor, increasing the allosteric modulator activity, (iii) hydrophobic interaction in the tail structure was also important, and cyclohexane interacted with a hydrophobic pocket in the T1R2 receptor (by comparing compounds **12–15**, it was clear that more hydrophobic substituents elevated the enhancer activity), (iv) insertion of an amide bond between the head and linker structures made it possible to obtain unique unnatural tripeptides (the interaction between compound **19** with T1R2 receptor is in Supporting Information Figure S3), and (v) the distance from the head to the tail was important for enhancer activity. Our new unnatural tripeptide sweetness enhancers would be useful in the field of food chemistry, and also for human health by helping to decrease calorie intake.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00051.

General methods, synthetic chemistry, procedure, physical properties, HEK293 cell-based assay, sensory evaluation, molecular docking model, abbreviations (PDF)

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Author Contributions

K.Y., M.N., and K.M. designed, synthesized, and analyzed the compounds. H.T. and U.T. constructed the T1R2/T1R3 in silico model and performed docking study. K.H., R.M., and W.S. performed sensory evaluation. S.M. and S.K. performed the in vitro assay. K.Y. wrote the manuscript. U.T., K.H., and S.K. edited the manuscript.

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Notes

The authors declare no competing financial interest.

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