Bioorganic & Medicinal Chemistry Letters 20 (2010) 1081–1083

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Photoactive ligands probing the sweet taste receptor. Design and synthesis of highly potent diazirinyl D-phenylalanine derivatives

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ARTICLE INFO

Article history:
Received 24 August 2009
Revised 13 November 2009
Accepted 7 December 2009
Available online 11 December 2009

Keywords:
Sweet taste
D-Amino acid
Photoaffinity label
Diazirine

ABSTRACT

Some D-Amino acids such as D-tryptophan and D-phenylalanine are well known as naturally-occurring sweeteners. Photoreactive D-phenylalanine derivatives containing trifluoromethyldiazirinyl moiety at 3- or 4-position of phenylalanine, were designed as sweeteners for functional analysis with photoaffinity labeling. The trifluoromethyldiazirinyl D-phenylalanine derivatives were prepared effectively with chemo-enzymatic methods using L-amino acid oxidase and were found to have potent activity toward the human sweet taste receptor.

Sweet taste receptor is a member of class C G protein-coupled receptors (GPCRs) and forms heterodimeric structure with T1R2 and T1R3 subunits. Each subunit has a large amino-terminal domain (ATD) linked by a cysteine-rich domain (CRD) at the extracellular site to a seven transmembrane helical domain (TMD).[1] Human heterodimeric sweet taste receptor (hT1R2-hT1R3) responds to a wide variety of chemical substances including naturally-occurring sugars, glycosides, D-amino acids, and artificial chemical compounds such as sucralose, aspartame, and saccharin.[2] Although these sweeteners have various chemical structures, all of the compounds bind to the same sweet taste receptor.[3,4] Since the receptor distinguishes D- and L-amino acids, preferring D-amino acids,[5–7] the structural relationships between D-amino acids and other sweeteners and the structural features of D-amino acid derivatives that favor the activation of the sweet taste receptor have been studied with conformational analysis by crystallography, NMR analysis and molecular modeling of these ligands.[8] However, it is still obscure to understand the structural relations among the sweeteners, since neither the receptor structure nor the structures of the ligands complexed with the receptor is not available so far.

Since T1R2 and T1R3 are orthologs of metabotropic glutamate receptor1 (mGluR1),[9] the crystal structure of which is known, structural models of T1R2 and T1R3 have been constructed with homology modeling and complex structures with some sweetners have been built through ligand-docking in the putative ligand-binding site,[10] although complex models of D-amino acids are not available. Combining the mutational results on T1R2 and T1R3, complex structure models would give a reasonable binding mode of ligands. However, they should be confirmed by appropriate experiments.

Photoaffinity labeling is one of the methods used in the study of the interactions of low molecular bioactive compounds with biomolecules.[11–15] It is suitable for the analysis of biological interactions because it is based on the affinity of the bioactive compound for biomolecules. Selection of photophore for photoaffinity labeling is very critical to obtain better results, but there are no universal photophore selections.[12] Various photophores, such as benzophenone, arylazide, and 3-(trifluoromethyl)phenyldiazirine, are used for the elucidation of the ligand–receptor or substrate–enzyme interactions. In this study, we focused on synthesizing photoreactive D-phenylalanine derivatives, having trifluoromethyldiazirine at 3- or 4- position of phenylalanine benzene ring, based on our modeling work of the ligand–receptor complex, showing that the ligand-binding site has a space for binding the trifluoromethyldiazirine moiety on the phenyl ring of D-phenylalanine. Since preparing the carbene precursor (3-trifluoromethyl)phenyldiazirinyl three-membered ring requires somewhat complicated process, fewer applications have been reported in biomolecular studies than other photophores[13–15] and neither does the synthesis of diazirinyl D-amino acid

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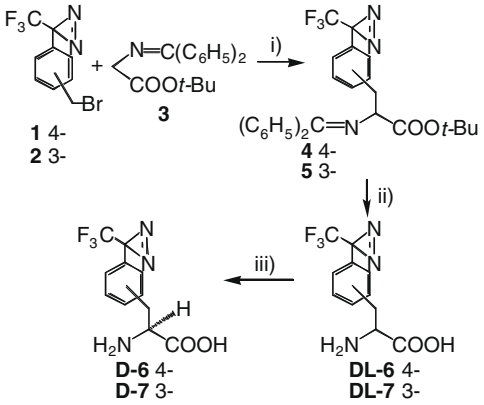
doi:10.1016/j.bmcl.2009.12.029

derivatives. A few cases of asymmetric synthesis of (3-trifluoromethyl)-diazirinyl L-phenylalanine derivatives are reported.[16–20] However, D-selective asymmetric synthesis has not been reported. Although L-amino acid derivatives are prepared by enzymatic resolution of *N*-acyl derivatives using acylase, the residual *N*-acyl D-amino acid compounds were recovered with less effectiveness.

L-Amino acid oxidase is an enzyme for converting L-amino acid to α-keto acid but does not work on the D-amino acid compounds at all.[21] Thus, the enzymatic resolution of D-amino acid from racemic mixture using the oxidase is a useful method and thus we tried to resolve the trifluoromethyldiazirinyl D-phenylalanine derivatives from racemic mixture using L-amino acid oxidase. The D-phenylalanine derivatives were then subjected to calcium imaging assay using hT1R2-hT1R3 expressing cells.

A complex model of D-phenylalanine in the ATD of the hT1R2 has been constructed using the crystal structure of the glutamate bound closed form of the ATD of the metabotropic glutamate receptor type-1(mGluR1, PDB code: 1EWK).[9,22] D-Phenylalanine was docked into the binding site with a guide of the salt bond between the amino group of the ligand and Glu302 of the receptor, which is conserved and crucial in binding in mGluR1 and also suggested to be crucial for binding of some sweeteners such as aspartame by mutational experiments.[3] The phenyl group bound a hydrophobic pocket which also binds a larger indole moiety of D-tryptophan. The docking of the designed diazirinyl D-phenylalanines at the same site suggested that the receptor accepts the diazirinyl moiety at the binding pocket (Fig. 1). Thus, the photoaffinity labeling of the receptor with this photophore would validate the complex structure model.

The 4-diazirinyl benzyl bromide derivatives **1**[24] was reacted with *N*-(diphenylmethylene)glycine *tert*-butyl ester **3** in the presence of CsOH and Bu_4NBr as phase transfer catalyst to afford phenylalanine skeleton **4** at room temperature, followed by deprotection with acidic conditions to afford racemic diazirinyl phenylalanine **DL-6**. The racemate was treated with L-amino acid oxidase



Scheme 1. Synthesis of diazirinyl D-phenylalanine derivatives **D-6** and **D-7**. Reagents and conditions: (i) CsOH, Bu_4NBr, CH_2Cl_2, rt, 12 h, 60–68%; (ii) TFA, rt, 2 h, 91–95%; (iii) L-amino acid oxidase, pH 7.0, 37 °C, 12 h, 40–45%.

in the neutral pH condition at 37 °C[25] to give the D-amino acid derivative (Scheme 1).

The enzymatic product was monitored with chiral HPLC column (CHIROBIOTIC T, with 10% ethanol–water) at 350 nm detection. Although **L-4** was still detected within 2 h, it was completely consumed after 12 h. The keto acid derived from the L-form (Fig. 2B, ~6 min), which is too unstable to isolate in pure form, was removed from the reaction mixture by acidification of the mixture, followed by washing with ethyl acetate. The desired diazirinyl D-amino acid derivative **D-6**[26] was then obtained as a powder after lyophilization in moderate yield (Fig. 2C). The positional isomer, 3-diazirinyl D-phenylalanine derivative **D-7**,[27] was also prepared starting from 3-diazirinyl benzyl bromide derivative **2**[28] with the identical process in a moderate yield (Scheme 1).

The activity of the diazirinyl phenylalanine derivatives **D-6** and **D-7** is shown in Figure 3. The sweetness activity of the D-amino acid derivatives was measured by using HEK293T cells transiently expressing hT1R2-hT1R3 and chimeric G-protein, Gα16-gust44[29],

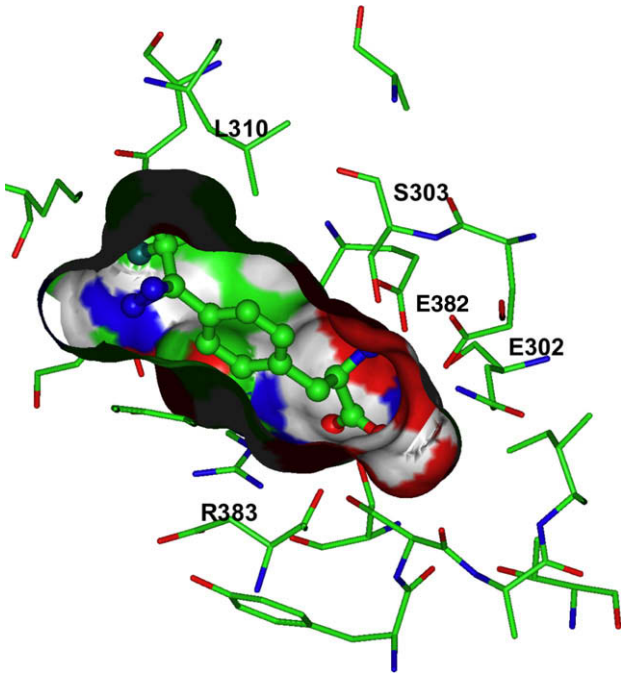


Figure 1. A complex model of the phenylalanine derivative at the ligand-binding site of hT1R2. The residues at the binding site within 5 Å from the ligand (ball-and-stick model) are shown by stick model and Connolly surface.[23] Residue numbers are given by one letter code. The nitrogen, oxygen, fluorine and carbon atoms are colored by blue, red deep green and green, respectively. Hydrogen atoms are omitted for clarity.

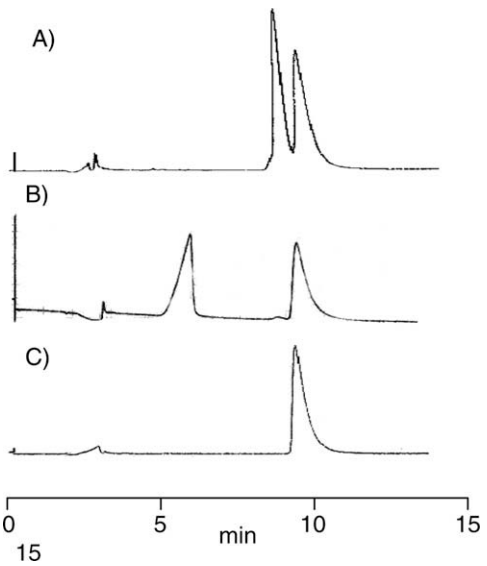


Figure 2. Enzymatic resolution of diazirinyl amino acid derivative (**DL-6**) with L-amino acid oxidase. (A) Before enzymatic resolution, (B) after incubation with L-amino acid oxidase at 37 °C for 12 h and (C) purified **D-6**. Chiral HPLC was performed with Chirobiotic T (4.6 × 250 mm, 10% ethanol, 1 ml/min) at 350 nm detection.

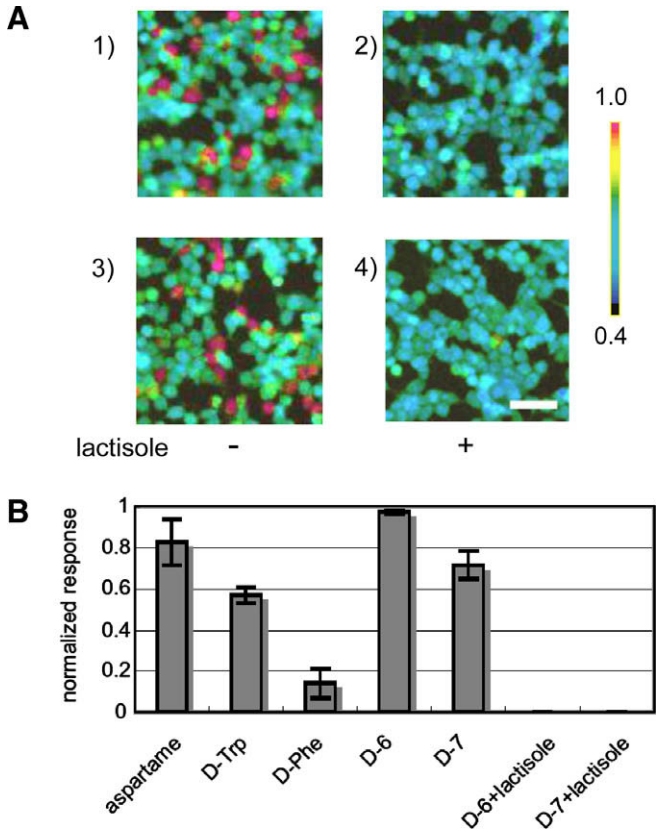


Figure 3. Sweet-tasting effect assay. (A) Representative ratiometric images of calcium ion indicator fura-2 loaded HEK293T cells coexpressing hT1R2-hT1R3 and G16-gust44 with 1.25 mM **D-6** (1) and **D-7** (3). The color scale indicates the F340/F380 ratio, where F340 is fluorescence intensity at 340 nm. Scale bar, 50 μM. Competitive inhibitions were performed by co-incubation with 1.25 mM lactisole (2 for **D-6** and 4 for **D-7**, respectively). Red color images represent responding cells for sweet potential. (B) Sweetness potential for known chemicals and synthetic photoreactive compounds. Cells were defined as responding positively when the F340/F380 increased above 0.15 after addition of a tastant. Number of cell responses, which trigger with 10 mM aspartame, were set as standard. The degree of cell response as the number of positively responding cells at 1.25 mM of each chemical against standard are represented to normalize the response. Each column represents the mean ± SE of three independent experiments.

with Ca^{2+} imaging analysis as described by Nakajima et al.[30] The cells, which responded to the ligands, showed red color in the pseudocolor images. The responses to the diazirinyl phenylalanine derivatives **D-6** and **D-7** were significantly reduced by addition of lactisole, an antagonist against hT1R2-hT1R3[31] (Fig. 3). Both the photoreactive D-amino acids, **D-6** and **D-7**, exhibited higher sweetness activity than D-tryptophan and D-phenylalanine, and a similar activity as aspartame had at the same dose (1.25 mM). Since the trifluoromethyldiazirinyl moiety may occupy the binding site for the phenyl ring of D-tryptophane, the ligand-binding site would have a significantly large space for accepting hydrophobic moieties of ligands.

These results indicate that the preparation of the diazirinyl D-phenylalanine derivatives through the enzymatic resolution with L-amino acid oxidase is effective and that these photoreactive compounds have enough affinity with the sweet taste receptor to elucidate the binding site for the ligands in the sweet taste receptor.

Acknowledgments

This research was partially supported by Ministry of Education, Science, Sports and Culture Grant-in-Aid for Scientific Research on a Priority Area, 18032007, for Scientific Research on Innovative Areas, 20200038 and for Scientific Research (C), 19510210, 21510219 (M.H.) and 16108004 (K.A.), and the Japan Society for the Promotion of Science (JSPS) (to A.K.). We also acknowledge the support by the grant from Research and Development Program for New Bio-industry Initiatives and JST, CREST. M.H. also thanks the Fugaku Foundation and Research for Promoting Technological Seeds for financial support for the study.

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25. Typical procedure for enzymatic resolution; DL-6 was suspended in water (20 ml) and the suspension was adjusted to pH 7 with NH_4OH and formic acid. L-amino acid oxidase (Sigma A-9378) was added to the suspension, The reaction mixture was incubated at 37 °C for 12 h, then made acid with 1 N formic acid and extracted with ethyl acetate. The aqueous layer was concentrated and the residue was subjected to chiral HPLC with Chirobiotic T (4.6 × 250 mm, 10% ethanol, 1 ml/min) at 350 nm, then lyophilized to afford colorless amorphous mass.
26. **D-6**: $[\alpha]_D$ (*c* 0.2, MeOH) +69.0, literature for **L-6** $[\alpha]_D$ (*c* 0.115, MeOH) −70.0[11]). ^{1}H NMR (500 MHz, CD_3OD) δ 7.46 (d, 2H, *J* = 7.9 Hz), 7.27 (d, 2H, *J* = 7.9 Hz), 3.84 (dd, 1H, *J* = 8.2, 4.8 Hz), 3.38 (m, 1H), 3.10 (dd, 2H, *J* = 14.5, 8.2 Hz).
27. **D-7**: $[\alpha]_D$ (*c* 0.2, MeOH) +8.9, ^{1}H-NMR (500 MHz, CD_3OD) δ 7.37 (d, 1H, *J* = 8.2 Hz), 7.18 (d, 2H, *J* = 8.2 Hz), 7.05 (s, 1H), 3.67 (m, 1H), 3.22 (m, 1H), 2.95 (m, 1H).
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