Supporting Information

Unnatural Tripeptides as Potent Positive Allosteric Modulators of T1R2/T1R3

Kei Yamada[1]*, Masakazu Nakazawa[1], Kayo Matsumoto[1], Uno Tagami[1]*, Takatsugu Hirokawa[2], Keisuke Homma[1], Suguru Mori[1], Ryo Matsumoto[1], Wakana Saikawa[1], Seiji Kitajima[1]*

[1]Ajinomoto Co., Inc., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki-shi 210-8681, Japan
[2]Molecular Profiling Research Center of Drug Discovery (molprof), National Institute of Advanced Industrial Science and Technology (AIST), 2-3-26 Aomi, Koto-ku, Tokyo 135-0064, Japan

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General methods

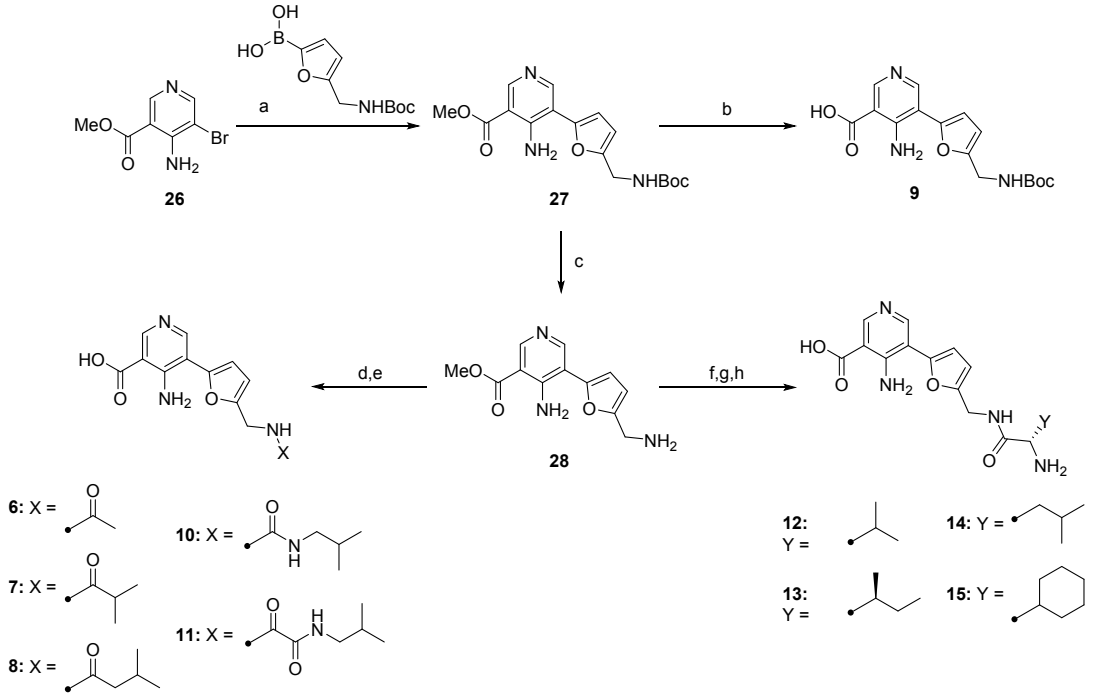
All reagents and solvents were procured from commercial sources and used as received. Thin layer chromatography (TLC) was carried out using Merck GmbH Precoated silica gel 60 F254. Chromatography on silica gel was carried out using prepacked silica gel cartridges (Yamazen Hi-Flash Column Silicagel or Wako Presep Silicagel). Chemical shifts in 1H NMR spectra are reported in δ values (ppm) relative to trimethylsilane. High-performance liquid chromatography (HPLC) analyses were performed in the following conditions: Waters Xbridge C18 column (3.5 µm, 4.6 × 50 mm), 30 °C column temperature, 1.0 mL/min flow rate, photodiode array detection (254 nm), and linear mobile phase gradient of 20%−90% B over 5 min, holding for 3.5 min at 20% B (mobile phase A, 0.05% trifluoroacetic acid in water; mobile phase B, acetonitrile), by which the purities of final compounds were confirmed as >95%. Mass spectra (ESI-MS) were recorded on a Waters 2695 electrospray ionization-mass spectrometer.

Synthetic chemistry, procedure and physical properties

Compounds **3**–**5** were prepared by Suzuki-Miyaura cross-coupling reaction. In the same way, biaryl compounds **6**–**15** were also prepared by Suzuki-Miyaura cross-coupling reaction (**Scheme S1**). After constructing the biaryl component (**27**), the protecting group *tert*-butoxycarbonyl (Boc) was removed to afford an amine derivative (**28**). The intermediate **28** was used for connecting several "tail" structures. Acetylation was used to connect simple alkyl groups, to afford **6**–**8** and **11**. Compound **10** was produced by 1,1'-carbonyldiimidazole-mediated urea formation. From **28**, we condensed several amino acids to afford compounds **12**–**15**.

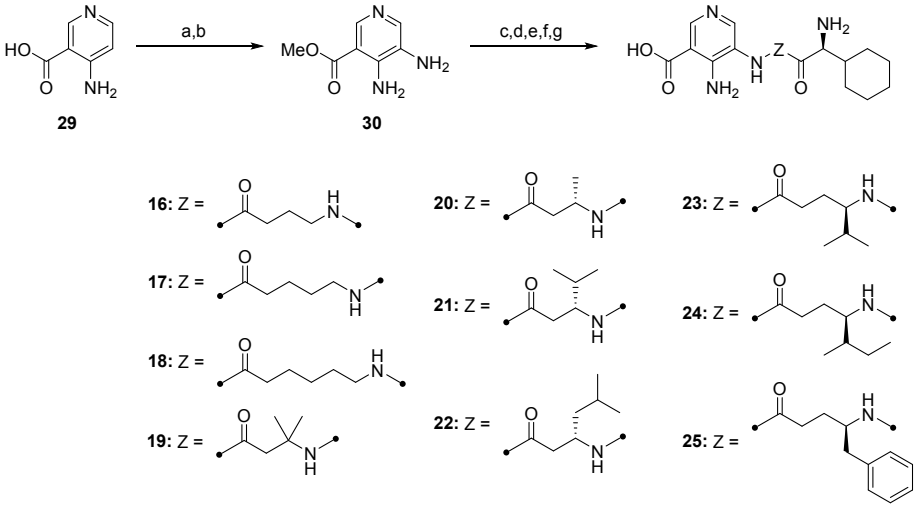
To prepare the unnatural tripeptide derivatives, we first synthesized the "head" structure from 4-aminonicotinate. 3,4-Diamino nicotinate (**30**) was a key intermediate in synthesis of the unnatural tripeptides (**Scheme S2**). From **30**, appropriate amino acids were condensed to the 3-amino group. As we expected, the 3-amine was unreactive, so we used the strong condensation reagent COMU (1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylaminomorpholino)]uroniumhexafluorophosphate). After removal of the Boc group (deprotection), we connected Boc–cyclohexylglycine (Chg)–OH, hydrolyzed the methyl ester group, and deprotected Boc again, to afford **16**–**25**.

Scheme S1. Synthetic route for synthesis of biaryl derivatives[a]



[a]Reagents: (a) $Pd(dppf)(PPh_3)_2$, Na_2CO_3, 1,4-dioxane/H_2O = 4:1; (b) 1 M LiOH, THF/MeOH = 1:1; (c) 4 M HCl in 1,4-dioxane; (d) for **6**,**7**,**8**, **11**, X(C=O)Cl, Et_3N, CH_2Cl_2; for **10**, 1,1'-carbonyldiimidazole, ET_3N, THF then isobutylamine; (e) 1 M LiOH, THF/MeOH = 1:1; (f) Boc–Val–OH (**12**) or Boc–Ile–OH (**13**) or Boc–Leu–OH (**14**) or Boc–Chg–OH (**15**), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), HOBt, Et_3N, CH_2Cl_2; (g) 1 M LiOH, THF/MeOH = 1:1; (h) TFA, CH_2Cl_2.

Scheme S2. Synthetic route for synthesis of unnatural tripeptides[a]



[a]Reagents: (a) KNO_3, H_2SO_4 then MeOH; (b) H_2, Pd/C, MeOH; (c) Boc-(Z)-OH, COMU, *N*-ethyldiisopropylamine, DMF; (d) TFA, CH_2Cl_2; (e) Boc-Chg-OH, WSCI, HOBt, DIPEA, DMF; (f) 1 M LiOH, THF/MeOH = 1:1; (g) TFA, CH_2Cl_2.

4-Amino-5-[5-[(tert-butoxycarbonylamino)methyl]-2-furyl]pyridine-3-carboxylate (27). Methyl 4-amino-5-bromopyridine-3-carboxylate (786 mg, 3.40 mmol), [5-tert-butoxycarbonylamino)methyl]-2- furyl boronic acid (1.15 g, 4.77 mmol), sodium carbonate (901 mg, 8.50 mmol) and [1,1'-bis(diphenylphosphino)ferrocene]dichloropalladium (II) (124 mg, 0.17 mmol) were dissolved in 1,4-dioxane (24 mL)/water (8 mL) at 105 °C for 1.5 h. The reaction solution was concentrated under reduced pressure, ethyl acetate and aqueous sodium hydrogen carbonate solution were added, and insoluble matter was filtered off using celite. The filtrate was extracted with ethyl acetate, the organic layer was dried over anhydrous magnesium sulfate, and the solvent was distilled off under reduced pressure. The resulting residue was purified by basic silica gel chromatography (hexane/ethyl acetate) to give **27** (813 mg, 2.34 mmol). MS (ESI) *m/z*: 348 $[M+H]^+$

4-Amino-5-[5-(aminomethyl)-2-furyl]pyridine-3-carboxylate dihydrochloride (28). **27** (813 mg, 2.34 mmol) was dissolved in 1,4-dioxane, 4 M hydrochloric acid/1,4-dioxane solution (25 mL) was added, and the mixture was stirred at room temperature overnight. The solvent was distilled off under reduced pressure and dried to obtain **28** (750 mg, 2.34 mmol). MS (ESI) *m/z*: 248 $[M+H]^+$

4-Amino-5-[5-[(methylbutanoylamino)methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetic acid salt (8). **28** (46.0 mg, 0.144 mmol) was suspended in dichloromethane (2 mL), triethylamine (0.0539 mL, 0.387 mmol) and isovaleryl chloride (0.0189 mL, 0.155 mmol), and the mixture was stirred overnight at room temperature. The reaction solution was diluted with dichloromethane and washed with an aqueous solution of sodium hydrogen carbonate. The organic layer was dried over anhydrous magnesium sulfate and the solvent was distilled off under reduced pressure to give 4-amino-5-[5-[(3- methylbutanoylamino)methyl]-2-furyl]pyridine-3-carboxylate (44.4 mg, 0.134 mmol, MS (ESI) *m/z*: 332 $[M+H]^+$).

Subsequently, 4-amino-5-[5-[(3-methylbutanoylamino)methyl]-2-furyl]pyridine-3-carboxylate (44.4 mg, 0.134 mmol) was dissolved in THF (1.5 mL)/methanol (1.5 mL), 1 M aqueous lithium hydroxide solution (1.5 mL) was added under ice cooling, and the mixture was stirred at room temperature for 2 h. To the reaction solution was added 2 M trifluoroacetic acid aqueous solution (0.8 mL) under ice cooling. Then the mixture was concentrated under reduced pressure, and the residue obtained was purified by reverse-phase HPLC using octadodecyl chemically bonded silica gel as a packing material, eluted with a mixed solution of water and acetonitrile containing 0.1% trifluoroacetic acid (v/v), and the desired fraction was lyophilized to give **8** (43.8 mg, 0.102 mmol). ^{1}H NMR ($(CD_3)_2SO$) δ = 8.72 (d, *J* = 0.9 Hz, 1H), 8.44 (d, *J* = 0.9 Hz, 1H), 8.30 (t, *J* = 5.6 Hz, 1H), 6.93 (d, *J* = 3.4 Hz, 1H), 4.29 (d, *J* = 5.6 Hz, 2H), 2.02–1.84 (m, 3H), 0.89–0.72 (m, 6H). MS (ESI) *m/z*: 318 $[M+H]^+$

4-amino-5-[5-(Acetamidomethyl)-2-furyl]-pyridine-3-carboxylic acid trifluoroacetate (6): ^{1}H NMR ($(CD_3)_2SO$) δ = 8.72 (d, *J* = 0.9 Hz, 1H), 8.45 (d, *J* = 0.9 Hz, 1H), 8.34 (t, *J* = 5.6 Hz, 1H), 6.93 (d, *J* = 3.4 Hz, 1H), 6.42 (d, *J* = 3.4 Hz, 1H), 4.28 (d, *J* = 5.6 Hz, 2H), 1.80 (s, 3H). MS (ESI) *m/z*: 276 $[M+H]^+$

4-Amino-5-[5-[(2-methylpropanoylamino)methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetate (7): ^{1}H NMR ($(CD_3)_2SO$) δ = 8.72 (s, 1H), 8.44 (s, 1H), 8.26 (t, *J* = 5.6 Hz, 1H), 6.93 (d, *J* = 3.4 Hz, 1H), 6.38 (d, *J* = 3.4 Hz, 1H), 4.28 (d, *J* = 5.6 Hz, 2H), 2.35 (hept, *J* = 6.8 Hz, 1H), 0.96 (d, *J* = 6.8 Hz, 6H). MS (ESI) *m/z*: 304 $[M+H]^+$

4-Amino-5-[5-[(tert-butoxycarbonylamino)methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetate (9): ^{1}H NMR ($(CD_3)_2SO$) δ = 8.73 (s, 1H), 8.45 (s, 1H), 7.40 (t, *J* = 5.7 Hz, 1H), 6.93 (d, *J* = 3.4 Hz, 1H), 6.39 (d, *J* = 3.4 Hz, 1H), 4.15 (d, *J* = 5.8 Hz, 2H), 1.33 (s, 9H). MS (ESI) *m/z*: 334 $[M+H]^+$

4-Amino-5-[5-[[[2-(isobutylamino)-2-oxo-acetyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetate (11): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.24 (t, *J* = 6.0 Hz, 1H), 9.14 (brs, 1H), 8.84–8.66 (m, 2H), 8.46 (d, *J* = 1.0 Hz, 1H), 8.26 (brs, 1H), 6.95 (d, *J* = 3.4 Hz, 1H), 6.45 (d, *J* = 3.4 Hz, 1H), 4.37 (d, *J* = 6.0 Hz, 2H), 2.90 (t, *J* = 6.7 Hz, 2H), 1.74 (hept, *J* = 6.8 Hz, 1H), 0.76 (d, *J* = 6.8 Hz, 6H). MS (ESI) *m/z*: 361 $[M+H]^+$

4-Amino-5-[5-[(isobutylcarbamoylamino)methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetate salt (10). **28** (32.0 mg, 0.100 mmol) and 1,1'-carbonyldiimidazole (19.5 mg , 0.120 mmol) were dissolved in THF (1.5 mL), triethylamine (0.042 mL, 0.300 mmol) was added under ice cooling, and the mixture was stirred at room temperature for 1 h. Isobutylamine (0.015 mL, 0.150 mmol) was added to the reaction solution and the mixture was stirred at room temperature overnight and then concentrated under reduced pressure. The residue was diluted with ethyl acetate and washed with an aqueous solution of sodium bicarbonate. The organic layer was dried over anhydrous magnesium sulfate, and the solvent was distilled off under reduced pressure. The resulting residue was dissolved in a mixture of THF (1.5 mL)/methanol (1.5 mL), 1 M aqueous lithium hydroxide solution (0.5 mL) was added under ice cooling, and the mixture was stirred at room temperature for 4 h. To the reaction solution was added 2 M trifluoroacetic acid aqueous solution (0.8 mL) under ice cooling, and the residue obtained by concentrating under reduced pressure was purified by reverse-phase HPLC in the same manner as in step 4 of the synthesis of **8**, to give **10** (35.2 mg, 0.0789 mmol). ^{1}H NMR ($(CD_3)_2SO$) δ = 9.06 (s, 1H), 8.71 (d, *J* = 0.9 Hz, 1H), 8.45 (d, *J* = 0.9 Hz, 1H), 8.21 (brs, 1H), 6.93 (d, *J* = 3.4 Hz, 1H), 6.35 (d, *J* = 3.4 Hz, 1H), 6.29 (t, *J* = 5.9 Hz, 1H), 6.03 (t, *J* = 5.9 Hz, 1H), 4.22 (d, *J* = 5.5 Hz, 2H), 2.77 (t, *J* = 6.2 Hz, 2H), 1.63–1.45 (m, 1H), 0.76 (d, *J* = 6.7 Hz, 6H). MS (ESI) *m/z*: 333 $[M+H]^+$

4-Amino-5-[5-[[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid ditrifluoroacetate (15). **28** (38.4 mg, 0.120 mmol), N-(tert-butoxycarbonyl)-L-2-cyclohexylglycine (34.0 mg, 0.132 mmol), WSCI (25.3 mg, 0.132 mmol) and HOBt (17.8 mg, 0.132 mmol) were suspended in dichloromethane (2 mL) and triethylamine (0.050 mL, 0.36 mmol), and the mixture was stirred overnight at room temperature. The reaction solution was concentrated under reduced pressure, diluted with ethyl acetate, washed with an aqueous solution of sodium bicarbonate and saturated brine, the organic layer was dried over anhydrous magnesium sulfate, and the solvent was distilled off under reduced pressure. The residue obtained was dissolved in a mixture of THF (1 mL)/methanol (1 mL), 1 M aqueous lithium hydroxide solution (0.600 mL) was added under ice cooling, and the

mixture was stirred at room temperature for 2 h. Then, 2 M trifluoroacetic acid aqueous solution (0.300 mL) was added to the reaction solution under ice cooling, the residue was concentrated under reduced pressure, and the residue obtained was purified by reverse-phase HPLC in the same manner as in step 4 of the synthesis of **8**, to give 4-amino-5-[5-[[[(2S)-2-(tert-butoxycarbonylamino)-2-cyclohexyl-acetyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetate salt (47.6 mg, 0.0812 mmol). MS (ESI) *m/z*: 473 $[M+H]^+$

4-amino-5-[5-[[[(2S)-2-(tert-butoxycarbonylamino)-2-cyclohexyl-acetyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid trifluoroacetate salt (33.6 mg, 0.0573 mmol) was suspended in dichloromethane (2 mL), trifluoroacetic acid (2 mL) was added, and the mixture was stirred at room temperature for 2 h. The solvent was distilled off under reduced pressure, and the residue obtained was dissolved in water and lyophilized, to give **15** (36.0 mg, 0.0600 mmol). ^{1}H NMR ($(CD_3)_2SO$) δ = 8.90 (t, *J* = 5.6 Hz, 1H), 8.70 (s, 1H), 8.42 (s, 1H), 8.25–7.87 (m, 3H), 6.90 (d, *J* = 3.4 Hz, 1H), 6.46 (d, *J* = 3.4 Hz, 1H), 4.50–4.28 (m, 2H), 3.53–3.45 (m, 1H), 1.71–1.41 (m, 6H), 1.13–0.81 (m, 5H). MS (ESI) *m/z*: 373 $[M+H]^+$

4-Amino-5-[5-[[[(2S)-2-amino-3-methyl-butanoyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid ditrifluoroacetate (12): ^{1}H NMR ($(CD_3)_2SO$) δ = 8.95 (t, *J* = 5.6 Hz, 1H), 8.72 (s, 1H), 8.44 (s, 1H), 8.07 (brs, 3H), 6.94 (d, *J* = 3.4 Hz, 1H), 6.47 (d, *J* = 3.4 Hz, 1H), 4.49–4.29 (m, 2H), 3.60–3.41 (m, 1H), 2.06–1.91 (m, 1H), 0.83 (d, *J* = 6.9 Hz, 6H). MS (ESI) *m/z*: 333 $[M+H]^+$

4-Amino-5-[5-[[[(2S,3S)-2-amino-3-methyl-pentanoyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid ditrifluoroacetate (13): ^{1}H NMR ($(CD_3)_2SO$) δ = 8.90 (t, *J* = 5.6 Hz, 1H), 8.71 (s, 1H), 8.42 (s, 1H), 8.06 (s, 3H), 6.92 (d, *J* = 3.4 Hz, 1H), 6.46 (d, *J* = 3.4 Hz, 1H), 4.48–4.29 (m, 2H), 3.59–3.51 (m, 1H), 1.80–1.66 (m, 1H), 1.46–1.31 (m, 1H), 1.10–0.94 (m, 1H), 0.85–0.68 (m, 6H). MS (ESI) *m/z*: 347 $[M+H]^+$

4-Amino-5-[5-[[[(2S)-2-amino-4-methyl-pentanoyl]amino]methyl]-2-furyl]pyridine-3-carboxylic acid ditrifluoroacetate (14): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.09 (t, *J* = 5.6 Hz, 1H), 8.79 (s, 1H), 8.51 (s, 1H), 8.17 (brs, 3H), 6.99 (d, *J* = 3.4 Hz, 1H), 6.54 (d, *J* = 3.4 Hz, 1H), 4.54–4.38 (m, 2H), 3.82–3.73 (m, 1H), 1.69–1.50 (m, 3H), 0.88 (dd, *J* = 8.5, 6.0 Hz, 6H). MS (ESI) *m/z*: 347 $[M+H]^+$

4,5-Diaminopyridine-3-carboxylate (30). 4-Aminopyridine-3-carboxylic acid (**29**) (5.00 g, 36.2 mmol) was dissolved in sulfuric acid (40.0 mL), potassium nitrate (3.66 g, 36.2 mmol) was added under ice cooling, and the mixture was stirred at 75 °C for 5 h. After returning to room temperature, methanol (100 mL) was added, and the mixture was stirred overnight at 60 °C. The reaction solution was slowly added to an aqueous solution of potassium acetate (145 g) and water (271 mL) under ice cooling, and the precipitated crystal was filtered. The crystals were dissolved in ethyl acetate, washed with an aqueous sodium hydrogen carbonate solution, the organic layer was dried over magnesium sulfate, filtered, and the solvent was distilled off to obtain methyl 4-amino-5-nitro-pyridine-3-carboxylate (2.36 g, 12.0 mmol). Methanol (20.0 mL) was added to methyl 4-amino-5-nitro-pyridine-3-carboxylate (2.36 g, 12.0 mmol) and palladium on carbon (100 mg) was added. The inside of the flask was purged with argon, a

balloon was attached, the internal gas was replaced with hydrogen, and the mixture stirred at room temperature for 3 h. The inside of the flask was again purged with argon, the content filtered, and the solvent was distilled off under pressure to obtain **30** (1.87 g, 11.2 mmol).

4-Amino-5-[[3-[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]-3-methyl-butanoyl]amino]pyridine-3-carboxylic acid ditrifluoroacetate (19): 3-(Tert-butoxycarbonylamino)-3-methylbutanoic acid (652 mg, 3.00 mmol) and COMU (1.41 g, 3.30 mmol) were dissolved in DMF (15.0 mL), DIPEA (561 µL, 3.30 mmol) was added, and the mixture was stirred at room temperature for 10 min. Then, methyl 4,5-diaminopyridine-3-carboxylate (500 mg, 3.00 mmol) was added and the mixture was stirred overnight at room temperature. The reaction solution was diluted with ethyl acetate, washed with an aqueous sodium hydrogen carbonate solution and saturated brine, the organic layer was dried over anhydrous magnesium sulfate, and the solvent was distilled off under reduced pressure. The resulting residue was purified by basic silica gel chromatography (hexane/ethyl acetate) to give 4-amino-5-[[3-(tert-butoxycarbonylamino)-3-methyl-butanoyl]amino]pyridine-methyl-3-carboxylate (617 mg, 1.68 mmol).

4-Amino-5-[[3-(tert-butoxycarbonylamino)-3-methyl-butanoyl]amino]pyridine-methyl-3-carboxylate (617 mg, 1.68 mmol) was suspended in dichloromethane (2.00 mL) and trifluoroacetic acid (2.00 mL), and the mixture was stirred at room temperature for 2 h. The solvent was distilled off under reduced pressure, and the residue obtained was dissolved in water and lyophilized to give 4-amino-5-[(3-amino-3-methyl-butanoyl)amino]pyridine methyl trifluoroacetate (590 mg, 1.62 mmol).

4-Amino-5-[(3-amino-3-methyl-butanoyl)amino]pyridine methyl trifluoroacetate (159.0 mg, 0.598 mmol), N-(tert-butoxycarbonyl)-(3-amino-L-2-cyclohexylglycine) (154.0 mg, 0.598 mmol), WSCI (116.0 mg, 0.598 mmol) and HOBt (82.0 mg, 0.598 mmol) were suspended in dichloromethane (2 mL) and the mixture was stirred at room temperature for 2 h. After diluting with ethyl acetate, and washing with aqueous sodium hydrogen carbonate solution and saturated brine, the organic layer was dried over anhydrous magnesium sulfate, and the solvent was distilled off under reduced pressure. The residue obtained was dissolved in methanol (1 mL), 2 M sodium hydroxide aqueous solution (1.00 mL) was added under ice cooling, and the mixture was stirred at room temperature for 2 h. To the reaction solution was added 2 M trifluoroacetic acid aqueous solution (1.00 mL) under ice cooling, the residue was concentrated under reduced pressure, and the residue obtained was purified by reverse-phase HPLC as in step 4 of the synthesis of **8** to give 4-amino-5-[[[3-[(2S)-2-(tert-butoxycarbonylamino)-2-cyclohexyl-acetyl]amino]-3-methyl-butanoyl]amino] pyridine-3-carboxylic acid trifluoroacetic acid salt (209.2 mg, 0.426 mmol).

4-Amino-5-[[[3-[(2S)-2-(tert-butoxycarbonylamino)-2-cyclohexyl-acetyl]amino]-3-methyl-butanoyl]amino] pyridine-3-carboxylic acid trifluoroacetic acid salt (209.2 mg, 0.426 mmol) was dissolved in 1 mL dichloromethane, 1 mL trifluoroacetic acid was added, and the mixture was stirred for 1 h. After distilling off the solvent under reduced pressure and freeze-drying, **19** (165.4 mg, 0.423 mmol) was obtained. ^{1}H NMR ($(CD_3)_2SO$) δ = 9.41 (s, 1H), 8.62 (s, 1H), 8.38 (s, 1H), 8.06 (s, 1H), 7.93 (s, 2H), 2.91–2.73 (m, 2H), 1.57 (dq, *J* = 23.5, 12.5, 11.5 Hz, 6H), 1.36 (d, *J* = 7.8

Hz, 6H), 1.03 (dtd, *J* = 45.5, 23.9, 23.3, 14.4 Hz, 6H). MS (ESI) *m/z*: 392.2 $(M+H)^+$

4-Amino-5-[5-((S)-cyclohexylglycylamino)-pentanoylamino]-nicotinic acid (17): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.57 (s, 1H), 8.72 (s, 1H), 8.53 (s, 1H), 8.42 (t, *J* = 5.6 Hz, 1H), 8.11–8.05 (m, 2H), 3.25 (dd, *J* = 13.3, 6.5 Hz, 5H), 3.08 (dq, *J* = 12.6, 6.4 Hz, 2H), 2.44 (t, *J* = 7.3 Hz, 2H), 1.75–1.56 (m, 8H), 1.49 (p, *J* = 7.0 Hz, 2H), 1.13 (ddd, *J* = 32.9, 15.8, 7.6 Hz, 3H). MS (ESI) *m/z*: 392.1 $(M+H)^+$

4-Amino-5-[7-((S)-cyclohexylglycylamino)-heptanoylamino]-nicotinic acid (18): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 1H), 8.71 (s, 1H), 8.55 (s, 1H), 8.38 (t, *J* = 5.6 Hz, 1H), 8.07 (s, 2H), 3.22 (dt, *J* = 13.1, 6.6 Hz, 2H), 3.03 (dq, *J* = 12.8, 6.5 Hz, 2H), 2.42 (t, *J* = 7.5 Hz, 2H), 1.73 (s, 1H), 1.70 (s, 2H), 1.60 (d, *J* = 6.3 Hz, 3H), 1.44 (d, *J* = 6.9 Hz, 2H), 1.32 (s, 3H), 1.22–1.09 (m, 3H), 1.02 (t, *J* = 14.0 Hz, 2H). MS (ESI) *m/z*: 420.1 $(M+H)^+$

4-Amino-5-[[(3S)-3-[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]butanoyl]amino]pyridine-3-carboxylic acid ditrifluoroacetate (20): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.53 (s, 1H), 8.61 (s, 1H), 8.37 (d, *J* = 8.0 Hz, 1H), 8.34 (s, 1H), 8.04–7.94 (m, 2H), 4.25 (p, *J* = 7.0 Hz, 1H), 2.51 (d, *J* = 7.1 Hz, 2H), 1.49 (q, *J* = 15.8, 14.7 Hz, 7H), 1.12 (d, *J* = 6.6 Hz, 3H), 0.94 (ddt, *J* = 27.4, 16.5, 8.1 Hz, 5H). MS (ESI) *m/z*: 378.2 $(M+H)^+$

4-Amino-5-[[(3R)-3-[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]-4-methyl-pentanoyl]amino]pyridine-3-carboxylic acid ditrifluoroacetate (21): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.64 (s, 1H), 8.74 (s, 1H), 8.48 (s, 1H), 8.11 (s, 3H), 4.38 (d, *J* = 9.6 Hz, 1H), 3.92 (s, 1H), 3.68–3.59 (m, 1H), 3.48 (t, *J* = 8.2 Hz, 1H), 2.98 (dd, *J* = 14.8, 4.5 Hz, 1H), 2.43 (dd, *J* = 14.8, 9.1 Hz, 1H), 2.04–1.87 (m, 3H), 1.84–1.53 (m, 7H), 1.28–0.99 (m, 6H). MS (ESI) *m/z*: 406.2 $(M+H)^+$

4-Amino-5-[[(4R)-4-[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]-5-methyl-hexanoyl]amino]pyridine-3-carboxylic acid ditrifluoroacetate (23): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.55 (s, 1H), 8.71 (s, 1H), 8.49 (s, 1H), 8.13 (d, *J* = 8.9 Hz, 1H), 8.10–8.00 (m, 2H), 3.74–3.55 (m, 1H), 2.39 (t, *J* = 7.8 Hz, 2H), 1.91–1.54 (m, 10H), 1.29–1.00 (m, 5H), 0.90 (dd, *J* = 6.9, 1.5 Hz, 6H). MS (ESI) *m/z*: 420.3 $(M+H)^+$

4-Amino-5-[[(4R)-4-[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]-5-methyl-heptanoyl]amino]pyridine-3-carboxylic acid ditrifluoroacetate (24): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.49 (s, 1H), 8.69 (s, 1H), 8.43 (s, 1H), 8.18 (d, *J* = 8.8 Hz, 1H), 8.04 (d, *J* = 5.2 Hz, 2H), 3.83–3.69 (m, 1H), 3.57 (s, 2H), 2.45–2.29 (m, 2H), 1.94–1.38 (m, 6H), 1.29–0.97 (m, 6H), 0.89 (t, *J* = 7.4 Hz, 6H). MS (ESI) *m/z*: 434.3 $(M+H)^+$

4-Amino-5-[[(4R)-4-[[(2S)-2-amino-2-cyclohexyl-acetyl]amino]-5-phenyl-pentanoyl]amino]pyridine-3-carboxylic acid ditrifluoroacetate (25): ^{1}H NMR ($(CD_3)_2SO$) δ = 9.51 (s, 1H), 8.69 (s, 1H), 8.41 (d, *J* = 6.9 Hz, 2H), 8.07 (s, 2H), 7.37–7.18 (m, 5H), 2.91–2.81 (m, 1H), 2.42 (p, *J* = 8.5, 8.0 Hz, 1H), 1.89–1.56 (m, 11H), 1.13 (tt, *J* = 23.8, 12.1 Hz, 6H). MS (ESI) *m/z*: 468.2 $(M+H)^+$

Calcium influx assay

PEAK^{rapid} cells stably expressing modified human sweet receptor and G protein to improve the assay sensitivity [27] were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum, 1.39 mM glucose and 1% Pen-Strep (Gibco). The cells were seeded into D-lysine coated 96-well plate 7.0×10^4 cells/well, and the cells were cultured over-night. After culture, all the medium from the 96 wells was discarded. Staining solution (200 µl) for measurement of intracellular calcium ions, Calcium Assay Kit Express (Molecular Devices), diluted 80 times with assay buffer (20 mM HEPES, 146 mM NaCl, 1 mM MgSO4, 1.39 mM glucose, 1 mM CaCl2, 2.5 mM probenecid, 0.1% bovine serum albumin) was added to each well, and the plate was left standing at 37 °C for 30 min and then incubated at room temperature for 45 min for staining. After staining, 50 µl of stimulant solution (sucrose and test compound) prepared in the assay buffer was added to each well, and the fluorescence value was measured for 120 s after the stimulation by using fluorometric imaging plate reader FDSSµCELL (Hamamatsu Photonics). By measuring the fluorescence value (Ex. 480 nm, Em. 540 nm) before and after the stimulation, the change of the intracellular free calcium ion concentration via the sweet taste receptor caused by addition of the stimulant was quantitatively investigated. Measurement and analysis of the fluorescence data was performed using the software attached to the FDSSµCELL, and ΔF/F values were calculated according to the equation F/F = (Maximum fluorescence value after stimulation − Minimum fluorescence value after stimulation)/Fluorescence value before stimulation. EC_{50} values and efficacy were calculated using non-linear curve-fitting in Graphpad Prism 7.0 (Graphpad). The data are expressed as the mean ± SEM of three separate experiments.

Sensory evaluation

Human sensory analyses were conducted following the spirit of the Helsinki Declaration and were also approved by the Management Committee of Ajinomoto Co., Inc., and informed consent was obtained from all assessors. To determine the sweet taste enhancing activity for obtained active compounds *in vitro*, human sensory evaluation experiments were conducted. Briefly, test compounds were dissolved in ethanol, then added to 5% sucrose solution at 10 ppm, respectively. Trained panelists were presented eight sucrose solutions (5%, 6%, 7%, 8%, 9%, 10%, 11% and 12%) and 5% sucrose solution including test compound (test sample). They were asked to rate the perceived sweetness intensity of the depicted sucrose solutions and test sample. Test sample was rated and scored on the 8-point scale for sweetness intensity using a percentage of sucrose equivalence scale, where 5 = 5% sucrose and 12 = 12% sucrose. Panelists were instructed to rinse their mouths with deionized water before test and when changing samples. This sensory evaluation was conducted by 3 trained panelists, and the average score was used as the test sample score. Sweetness enhancement activities were calculated as: Sweetness enhancement = average score of panelists/score for 5(%).

Molecular docking model

The binding site of most sweet substances, such as sucrose and aspartame, is reported to be an extracellular domain of T1R2[3]. 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: 1EWK5) using Prime (Schrödinger, LLC). The T1R2 model structure was protonated and refined using Protein Preparation Wizard in Maestro (Schrödinger, LLC). Next, sucrose and the modulators were prepared by Ligprep (Schrödinger, LLC). First, sucrose was docked to the T1R2 model structure using docking program Glide (Schrödinger, LLC) in SP mode, then, the modulators were docked to the sucrose–T1R2 complex structure. Docking pose was selected by the Glide score and visual in-spection (**Figure S1**-**S3**).

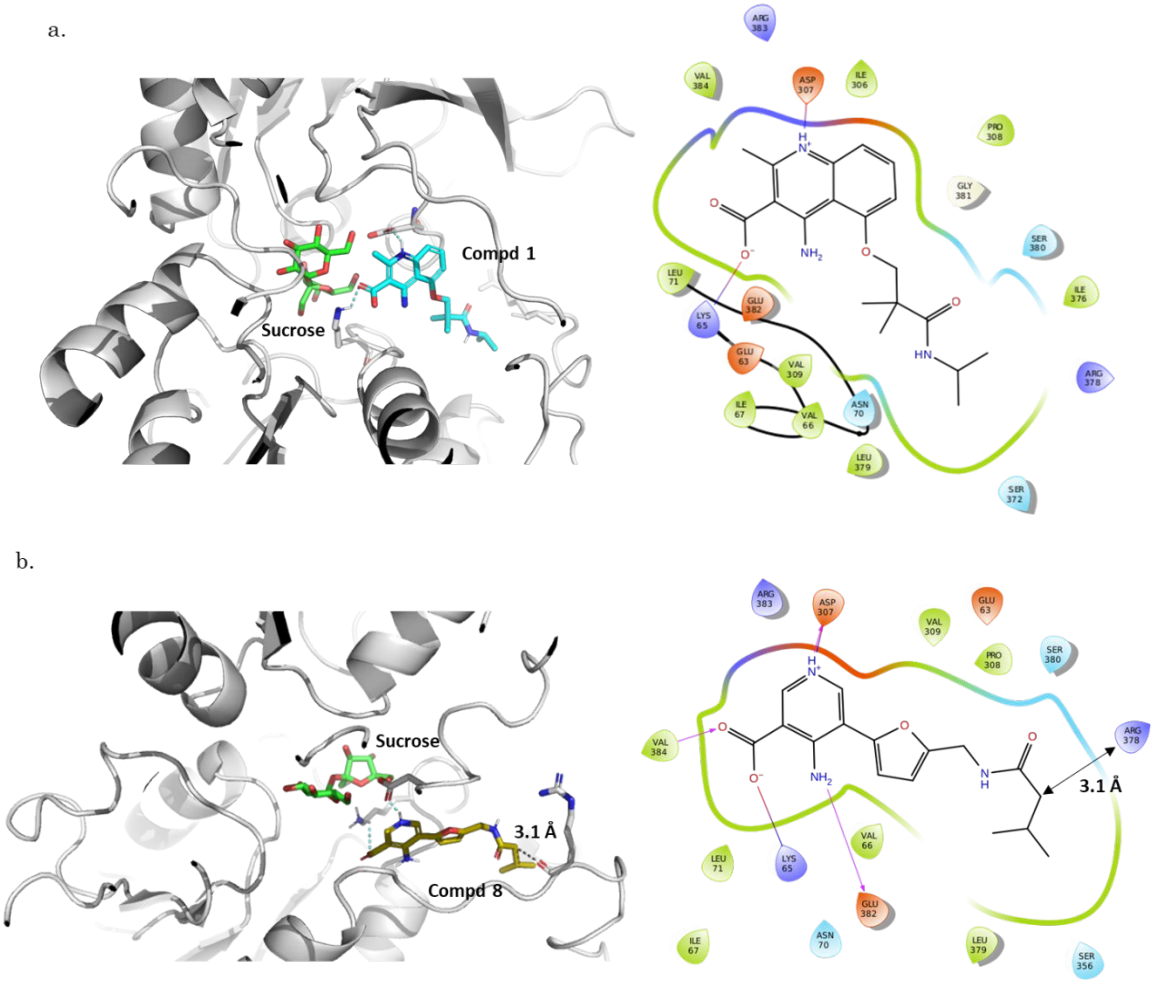


Figure S1. Molecular docking study of FEMA4774 (**1**) and compound **8** in a model structure of the extracellular domain of T1R2 which was constructed by homology modeling based on the X-ray crystal structure of mGluR1 (PDB code: 1EWK[4]) using Prime (Schrödinger, LLC) for 3D modeling and Maestro (Schrödinger, LLC) for 2D. Light blue lines in the 3D docking model show hydrogen bonding between compounds and the T1R2 receptor. a. Molecular docking study of FEMA4774. b. Molecular docking study of compound **8**.

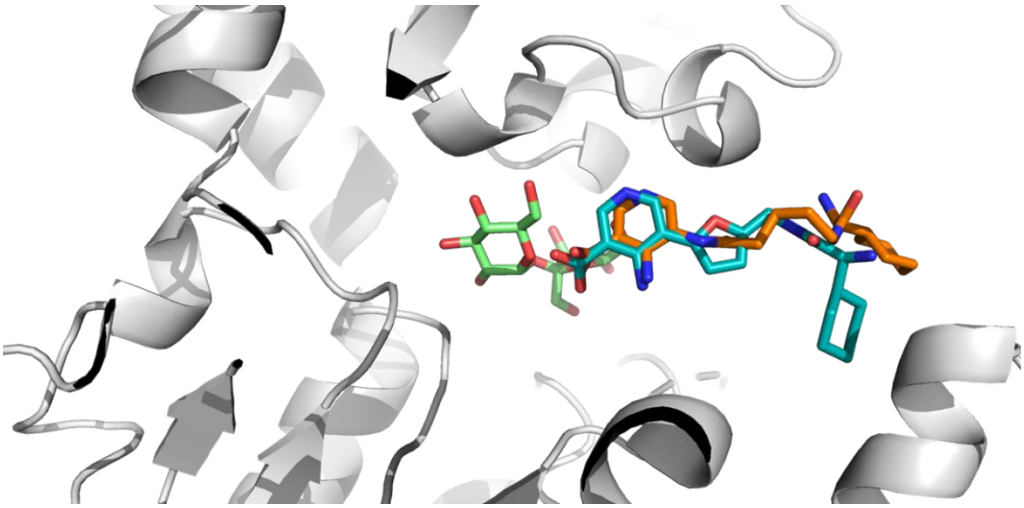


Figure S2. Molecular docking study of **15** and **17** in a model structure of the extracellular domain of T1R2 which was constructed by homology modeling based on the X-ray crystal structure of mGluR1 (PDB code: 1EWK[4]) using Prime (Schrödinger, LLC) for 3D modeling.

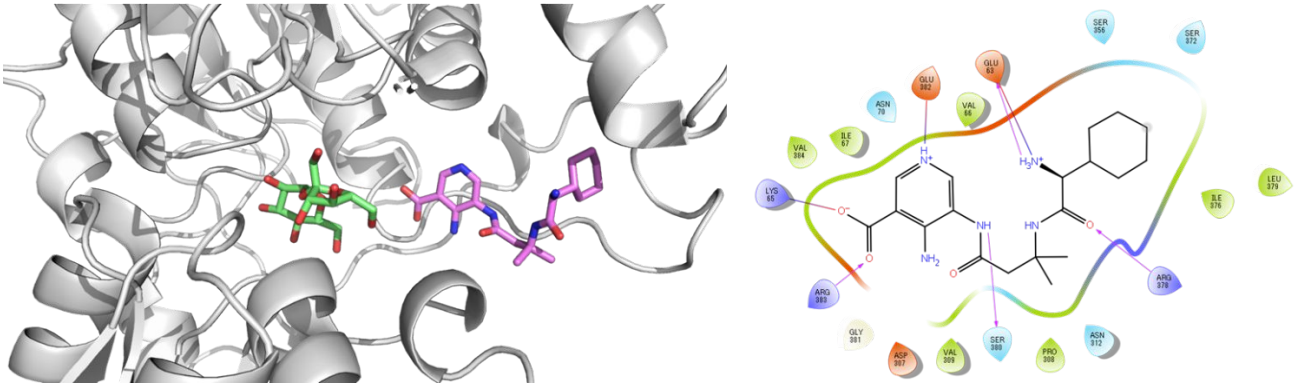


Figure S3. Molecular docking study of **19** in a model structure of the extracellular domain of T1R2 which was constructed by homology modeling based on the X-ray crystal structure of mGluR1 (PDB code: 1EWK[4]) using Prime (Schrödinger, LLC) for 3D modeling and Maestro (Schrödinger, LLC) for 2D.

Abbreviations

Boc, tert-butoxycarbonyl; Chg, Cyclohexylglycine; COMU, (1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylamino-morpholino)]uroniumhexafluorophosphate); DIPEA, N-ethyl-diisopropylamine; dppf, 1,1'-Bis(diphenylphosphino)ferrocene; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOBt, 1-hydroxybenzotriazole.

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