An Implication of Molecular Mechanism for Eliciting Sweetness of Anionic β -Glycyrrhetic Acid Derivatives

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Abstract: Anionic derivatives of β -glycyrrhetic acid (GA) containing a carboxylate- and a sulfate-terminated substituent at the C-3 position were prepared and assessed for their relative sweetness. A molecular mechanism for sweet taste expression of these derivatives was discussed assuming CPK molecular models of the N-terminal α -helix fragment of the T1R3 protein, which was a likely candidate for the binding site of GA derivatives and helped well explain their sweetness.

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Key words: β -glycyrrhetic acid derivative, sweet-taste mechanism, sweet-taste receptor with α -helix, CPK model

1 Introduction

Glycyrrhizin (GK2; **Fig. 1** a) has been employed not only as a sweetener but also as a Chinese medicine against inflammatory illnesses from the ancient times (1). As for the anti-inflammation mechanism of GK2 families, there have recently been numerous studies in the realms of enzyme chemistry (2-5) and medicine (6). Thus, search has begun for developments of a variety of useful GK2 variants (7).

However, there have been few reports which pay attention to the sweet-taste mechanism for GK2, except our recent one (8). GK2 families would have the potential for being medicinally useful as substitutes for sugars. There is a great diversity of sweet-tasting substances, natural or nonnatural, inorganic or organic, such as lead salts, sugars, amino acids, peptides, proteins, nitroanilines, terpenes, sulfamates (9). In sweet molecules with a relatively small molecular weight, the role of bifunctional AH and B entities complementary to the protein receptive-sites (B-HA) has been recognized as a common, fundamental structural feature of sweet substances, in which the AH and B sites are separated by 2.5-4.0 Å according to Shallenberger's hypo-

thetical theory. This theory has been successfully adopted to understand the sweetness of amino acids, aspartame, and dihydrocarcones (10). Furthermore, in some series of sweet substances, the sweetness is presumed to be enhanced by a third hydrophobic component (X) located at 3.5 and 5.5 Å far from the AH and B sites, respectively (9).

However, there are remaining unraveled issues with regard to the assignment of the AH and B moieties. Particularly, the AH and B sites in sweet molecules containing relatively large peptide-, protein- and terpene-scaffolds have not been identified yet and might be separated at a long distance; for example, amphiphilic GK2 widely used as a natural sweetener consists of hydrophobic β -glycyrrhetinate aglycon part of 13 Å long and hydrophilic diglucuronate part (**Fig. 1**).

Recently, we have extended Shallenberger's approach to elucidation of the molecular origin of sweetness of GK2, a typical large molecule, concluding that the presence of both the undissociated carboxy functionality (acting as an AH component) at the C-20 position and the dissociated anionic carboxylate functionality (acting as a B component) in the side chain of position C-3 was essential to exhibit sweet taste, where the AH and B sites are separated by as much as at least 13 Å (8).

In the present work we have prepared a variety of GA-based anionic carboxylate derivatives through its

Fig. 1 Chemical Structures of (a) GA and GK2 and (b) GA Derivatives Prepared in This Study.

hydroxyl group at the C-3 position (**Fig. 1 b**), and found out another structural requirements necessary for enhancing sweetness.

2 Experimental

2.1 Materials

Tetrahydrofuran (THF) was freshly distilled from sodium benzophenone ketyl. All other solvents were distilled after drying before use. Reagents were used as received. β -glycyrrhetic acid (GA) were purchased from Maruzen Pharmaceuticals Co., Ltd. and used without further treatment. Malonyl and Fumaryl chlorides, succinic, glutaric, diglycolic and maleic anhydrides, and bromoacetyl bromide were purchased from Wako Pure Chemical Industries, Ltd., Tokyo Kasei Kogyo Co., Ltd., and Kishida Chemical Co., Ltd., respectively, and used without further purification.

3-O-Malonyl-GA (1) and 3-O-Fumaryl-GA (6). GA (1.0 g, 2.1 mmol) was reacted with 1.6 g (4.2 mmol) of malonyl chloride in the presence of powdered KOH (1.3 g, 21 mmol) in THF (30 mL). The solution was evaporated *in vacuo* and then acidified with aqueous HCl to afford precipitates. Its reprecipitation from water and purification by column-chromatography using silica gel (CHCl_3 / MeOH=6:1) afforded 3-O-malonyl-GA (**1**): yield 25.9%; mp 237-239°C; IR(cm^{-1}) 1730(COOCH), 1701(COOH), 1658(C=C-C=O); $^1\text{H-NMR}$ (CDCl_3) δ 0.84, 0.87, 0.90, 1.13, 1.16, 1.22, 1.36 (each 3H, each s, C-CH₃), 3.37 (1H, s, CO-CH₂-COOH), 4.58 (1H, m, CH-OH), 5.71 (1H, s, C12-H); MS Calcd for $\text{C}_{33}\text{H}_{47}\text{O}_7$ ($[\text{M-H}]^-$) 555.33, Found 555.3. 3-O-Fumaryl-GA (**6**) was prepared according to the procedure as described above. This compound was recrystallized from CHCl_3 / MeOH: yield 53.1%; mp 274-278°C; IR(cm^{-1}) 1732(COOCH), 1714(COOH), 1620(C=C-C=O); $^1\text{H-NMR}$ (DMSO-d_6) δ 0.76, 0.85, 0.88, 0.98, 1.05, 1.08, 1.37 (each 3H, each s, C-CH₃), 4.55 (1H, m, CH-OH), 5.41 (1H, s, C12-H), 6.68 (2H, s, CO-CH=CH-COOH); MS Calcd for $\text{C}_{34}\text{H}_{47}\text{O}_7$ ($[\text{M-H}]^-$) 567.33, Found 567.52.

3-O-Succinyl-GA (2). GA (5.0 g, 11 mmol) was allowed to react with 2.2 g (22 mmol) succinic anhydride in pyridine at 90°C for 5 days (8). The solution was evaporated *in vacuo* and then acidified with aqueous HCl to afford precipitates. The precipitates were recrystallized from MeOH / H₂O to give 4.4 g of **2** in 70% isolated yield: dec. >300°C; IR(cm^{-1}) 1740(COOCH), 1705(COOH), 1651(C=C-C=O); $^1\text{H-NMR}$ (CDCl_3) δ 0.84, 0.88, 0.88, 1.13, 1.16, 1.22, 1.39 (each 3H, each s, C-CH₃), 2.15-2.19 (4H, m, CO(CH₂)₂COOH), 4.56 (1H, m, CH-OH), 5.69 (1H, s, C12-H).

3-O-Glutaryl-GA (3), 3-O-Diglycolyl-GA (4), and 3-O-Maleyl-GA (5). GA (1.0 g, 2.1 mmol) was reacted with 1.5 equiv of glutaric, diglycolic, and maleic anhydrides in the presence of 0.1 equiv of p-toluenesulfonic acid monohydrate in refluxing toluene (70 mL) for 24 hours. Usual treatment of the reaction mixture and then purification by column-chromatography using silica gel (CHCl_3 / MeOH=10:1) gave the title compounds. 3-O-Glutaryl-GA (**3**): yield 9.83%; mp 291-296°C; IR(cm^{-1}) 1730(COOCH), 1708(COOH), 1652(C=C-C=O); $^1\text{H-NMR}$ (DMSO-d_6) δ 0.65, 0.76, 0.82, 1.04, 1.06, 1.10, 1.35 (each 3H, each s, C-CH₃), 2.07 (2H, m, CH₂-CH₂-CH₂), 2.32 (2H, m, CO-CH₂-CH₂-CH₂-COOH), 2.49 (2H, m, CO-CH₂-CH₂-CH₂-COOH), 4.44 (1H, m, CH-

OH), 5.41 (1H, s, C12-H); MS Calcd for $C_{35}H_{51}O_7$ ($[M-H]^-$) 583.37, Found 583.6. 3-O-Diglycolyl-GA (**4**): yield 62.8%; mp 249-252°C; IR(cm^{-1}) 1724(COOCH), 1708(COOH), 1651(C=C-C=O); 1H -NMR ($CDCl_3$) δ 0.84, 0.88, 0.90, 1.13, 1.16, 1.19, 1.36 (each 3H, each s, C-CH₃), 3.37 (4H, m, CH₂-O-CH₂), 4.63 (1H, m, CH-OH), 5.66 (1H, s, C12-H); MS Calcd for $C_{34}H_{49}O_8$ ($[M-H]^-$) 585.3, Found 585.3. 3-O-Maleyl-GA (**5**): this compound was recrystallized from MeOH/H₂O in 54% yield; mp 274-278°C; IR(cm^{-1}) 1733(COOCH), 1716(COOH), 1660(C=C-C=O); 1H -NMR (DMSO- d_6) δ 0.76, 0.84, 0.89, 0.98, 1.04, 1.07, 1.36 (each 3H, each s, C-CH₃), 4.52 (1H, m, CH-OH), 5.41 (1H, s, C12-H), 6.33 (2H, m, CO-CH=CH-COOH); MS Calcd for $C_{34}H_{47}O_7$ ($[M-H]^-$) 567.33, Found 567.

GA-3-O-(COCH₂CONHCH₂COOK) (7). One g (1.8 mmol) of the GA benzyl ester (GABn), which was protected beforehand with benzyl group at 20-COOH position, and 380 mg (2.6 mmol) of malonyl chloride were dissolved in THF. To this solution was added KOH (150 mg) in a small quantity of water dropwise at 0°C. The mixture was treated as usual and the crude product was purified by column-chromatography on silica gel (CHCl₃/MeOH=10:1) to give the malonyl monoester of the GABn, which was further condensed with glycine benzyl ester using DCC, HOBT, and DMAP in CH₂Cl₂ at 0°C. Treatment as usual and purification by preparative TLC afforded 100 mg of the precursor, which was subjected to debenzilation in MeOH/THF with Pd(CH₃COO)₂ at 40°C, followed by neutralization with 2 equiv of KOH to give 22 mg of the desired product GA-3-O-(COCH₂CONHCH₂COOK) (**7**): yield 1.8 %; dec. >250°C; 1H -NMR (D₂O) δ 0.75, 0.92 (each 3H, each s, CH₃), 1.00 (6H, s, 2CH₃), 1.18, 1.20, 1.28 (each 3H, each s, CH₃), 5.56 (1H, s, C12-H); MS Calcd for $C_{35}H_{49}K_2NO_8$ (M^+) 689.3, Found 689.9.

3-O-bromoacetyl-GA. To a stirred solution of 1.0 g (2.1 mmol) of GA in 30 mL of THF was added 290 μ L (3.3 mmol) of bromoacetyl bromide at 0°C. A 260 μ L (3.2 mmol) pyridine solution in 2 mL of THF was added dropwise to the mixture. The mixture was allowed to warm to room temperature and stirred overnight. Then the mixture was centrifuged. The supernatant was concentrated under vacuum to small volume. The resulting residue was diluted with chloroform and acidified with aqueous HCl. The chloroform was removed under reduced pressure and the resulting residue was passed through a silica gel column (CHCl₃ /

MeOH=15:1) to give 970 mg of 3-O-bromoacetyl-GA: mp 163-164°C; 1H -NMR($CDCl_3$) δ 0.91 (3H, s, CH₃), 1.01 (6H, s, 2CH₃), 1.13, 1.17, 1.21, 1.36 (each 3H, each s, CH₃), 3.79-3.88 (2H, s, COCH₂Br), 4.56-4.61 (1H, m, CH-OH), 5.72 (1H, s, C12-H); MS Calcd for $C_{32}H_{47}BrO_5$ (M^+) 591.6, Found 591.4.

GA-3-O-[COCH₂SCH₂CH(NH₂)COOK] (9a and 9b). 3-O-bromoacetyl-GA (50 mg, 0.085 mmol) was dissolved in 10 mL ethanol. To the solution was added a solution of 50 mg of potassium hydroxide and 30 mg (0.24 mmol) of L-cysteine in 1 mL of water. The mixture was concentrated and the crude product was purified by HPLC to give GA-3-O-[COCH₂SCH₂CH(NH₂)COOK] (**9a**): dec >220°C; 1H -NMR (CD₃OD) δ 0.82 (3H, s, CH₃), 0.93 (6H, s, 2CH₃), 1.08, 1.14, 1.17, 1.36 (each 3H, each s, CH₃), 4.56-4.61 (1H, m, CH-OH), 5.70 (1H, s, C12-H); MS Calcd for $C_{35}H_{52}K_2NO_7S$ ($[M+H]^+$) 708.3, Found 708.2. Compound **9b** was prepared according to the same procedure as described above: MS Calcd for $C_{35}H_{52}K_2NO_7S$ ($[M+H]^+$) 708.3, Found 708.4.

GA-3-O-(COCH₂SSO₃K) (13). To a solution of 70 mg (0.12 mmol) of 3-O-bromoacetyl-GA and 4 mg (0.02 mmol) of tetrabutylammonium bromide in 3 mL of DMF was added 30 mg (0.12 mmol) of powdered Na₂S₂O₃·5H₂O and 100 μ L of H₂O. Then, the mixture was stirred for 3 hours. The solvent was evaporated *in vacuo* and the resulting residue was extracted with hot water being sonicated and was passed through an ion-exchange resin column (Dowex 50w-X-8). A fraction that exhibited sweetness was freeze-dried to give white powders, which was purified again by preparative TLC to give 60 mg (yield 64%) of GA-3-O-COCH₂SSO₃K (**13**): 1H -NMR($CDCl_3$) δ 4.59 (1H, m, CH-OH), 5.70 (1H, s, C12-H); MS Calcd for $C_{32}H_{48}O_5S$ (M^+) 700.03, Found 700.19.

Benzyl benzyloxyacetate. A suspension of sodium hydride (60% in oil, 1.18 g, 30 mmol) in THF (20 mL) was added to benzyl alcohol (3g, 30 mmol) at 0°C. To this solution was added dropwise benzyl bromoacetate (6.9 g, 30 mmol). After being stirred for 24 hours, the reaction mixture was poured into water and extracted twice with ether. The combined organic extracts were dried over anhydrous Na₂SO₄ and concentrated. Purification on a silica gel column (hexane / ethyl acetate=4:1) yielded the title compound as an oil: yield 62.5%; IR(cm^{-1}) 1735(COOBn), 1190(C-O-C); 1H -NMR ($CDCl_3$) δ 4.14 (2H, s, O-CH₂-COO), 4.63 (2H,

s, O-CH₂-ph), 5.20 (2H, s, COO-CH₂-ph), 7.35 (10H, m, C₆H₅).

Benzyloxyacetic acid. To a solution of benzyl benzyloxyacetate (4.7 g, 18 mmol) in MeOH was added a solution of 1.1 g (20 mmol) of KOH in MeOH (20 mL). After 2 hours, the solvent was removed *in vacuo*. The residue was washed with benzene, and acidified with aqueous HCl and then taken up in CHCl₃. The CHCl₃ layer was dried over Na₂SO₄. Removal of the solvent under reduced pressure gave 285 mg of benzyloxyacetic acid in 85% yield: IR(cm⁻¹) 1720(COOH), 1116(C-O-C); ¹H-NMR (CDCl₃) δ 4.13 (2H, s, O-CH₂-COOH), 4.65 (2H, s, ph-CH₂-O), 7.37 (5H, m, C₆H₅).

(3-O-Benzyloxymethylcarbonyl)-GABn. Benzyloxyacetic acid (0.90 g, 5.4 mmol) thus obtained was condensed with GABn (3.0 g, 5.4 mmol) in the presence of WSCD (1.08 g, 5.7 mmol), DMAP (30 mg, 0.24 mmol), and HOBt (720 mg, 5.4 mmol) in 90 mL of THF at 0°C. After overnight stirring, the reaction mixture was treated as usual, and then the crude product was purified by preparative TLC using acetone/hexane=3:10, affording 900 mg of 3-O-(benzyloxymethylcarbonyl)-GABn in 23.7% yield: mp 135-140°C; IR(cm⁻¹) 1751(COOCH), 1728(COOCH), 1652(C=C-C=O); ¹H-NMR (CDCl₃) δ 0.73, 0.87, 0.88, 1.10, 1.15, 1.16, 1.35 (each 3H, each s, C-CH₃), 4.10 (2H, s, OCO-CH₂-OH), 4.64 (2H, s, OCO-CH₂-OBn), 4.65 (1H, m, CH-OH), 5.14 (2H, m, COO-CH₂-Bn), 5.54 (1H, s, C12-H), 7.36 (10H, m, C₆H₅).

3-O-(Hydroxymethylcarbonyl)-GA. 3-O-(Benzyloxymethylcarbonyl)-GABn (900 mg, 1.7 mmol) was successively debenzylated in a 100ml of 1:1 MeOH/THF mixture with 100 mg Pd(OAc)₂ as the catalyst under hydrogen for 1 hour at 40°C, followed by solvent removal and then purification by preparative TLC (CHCl₃/MeOH=20:1) to give 560 mg of 3-O-(hydroxymethylcarbonyl)-GA in 78% yield: IR(cm⁻¹) 1747(COOCH), 1712(COOH), 1652(C=C-C=O); ¹H-NMR (CDCl₃) δ 0.84, 0.87, 0.89, 1.13, 1.16, 1.23, 1.37(each 3H, each s, C-CH₃), 4.15 (2H, s, OCO-CH₂-OH), 4.66 (1H, m, CH-OH), 5.72 (1H, s, C12-H).

GA-3-O-(COCH₂OSO₃K) (12). SO₃·pyridine complex (75 mg, 0.6 mmol) was added to a stirred THF solution (20 mL) containing 50 mg (0.1 mmol) of 3-O-(hydroxymethylcarbonyl)-GA at ambient temperature and then the mixture was heated under reflux. After overnight stirring, the solvent was removed under reduced pressure. The residue was dissolved in MeOH

and the solution was made alkaline to pH 10 with 2M KOH solution in MeOH. The precipitated K₂SO₄ was filtered off. The filtrate was subjected to HPLC purification to give 20 mg of GA-3-O-(COCH₂OSO₃K) (12): yield 33.8%; IR(cm⁻¹) 1747(COOCH), 1701(COOH), 1654(C=C-C=O); ¹H-NMR (CDCl₃) δ 0.61, 0.67, 0.92, 0.96, 0.99, 1.17, 1.33 (each 3H, each s, C-CH₃), 3.95 (2H, s, CO-CH₂-OSO₃K), 4.44 (1H, m, CH-OH), 5.47 (1H, s, C12-H); MS Calcd for C₃₂H₄₇O₉S ([M-H]⁻) 607.3, Found 607.3.

GA-3-O-[COCH₂OPO₂(OH)K] (14) and GA-3-[OPO₂(OH)K] (16). The solution of 3-O-(hydroxymethylcarbonyl)-GA (1.3 g, 2.5 mmol) in 20 mL THF was added to a stirred solution containing 1.8 g (12.5 mmol) of phosphoryl chloride in 10 mL THF at -20°C. To the solution was added dropwise pyridine (2 g, 25 mmol). After 2 hours stirring, the mixture was filtered, and the filtrate was acidified with a small quantity of aqueous HCl. The solution was made alkaline to pH 10 with 2M KOH solution in MeOH. The precipitated GA-3-O-[COCH₂OPO₂(OH)K] (14) was filtered off and dried under vacuum; yield 1.5%; IR(cm⁻¹) 1732(COOCH), 1697(COOH), 1656(C=C-C=O); ¹H-NMR (CDCl₃) δ 0.70, 0.72, 0.83, 0.90, 0.99, 1.04, 1.32 (each 3H, each s, C-CH₃), 3.96 (2H, s, OCO-CH₂-PO₃H₂), 4.50 (1H, m, CH-OH), 5.60 (1H, s, C12-H).

GA-3-O-[PO₂(OH)K] (16) was prepared according to the procedure as described above with a minor modification; yield 5.1%; IR(cm⁻¹) 1703(COOH), 1660(C=C-C=O); MS Calcd for C₃₀H₄₇O₇ ([M-H]⁻) 549.3, Found 549.3.

2.2 Measurements

Instruments. IR spectra were recorded on a Shimadzu FT-IR 8200 spectrometer. ¹H-NMR spectra were measured using a Nihondensi Detum α-400 spectrometer operating at 400 MHz. ¹H-NMR spectra were obtained in CDCl₃ or DMSO-d₆ and chemical shifts were referenced to tetramethylsilane. FAB-MS spectra were recorded on a Nihon Bunko JMS-7000 spectrometer.

Sensory evaluation for Relative Sweetness. Evaluation of sweetness relative to sucrose was accomplished for sample solutions of 25°C with a human sensory panel. Typically, a solution of known concentration of about 4 mM of a compound was diluted to desired concentrations with distilled water. The concentration at which the taste was closest to that of 2 or 4% (W/V)

sucrose solution was determined by tasting the sample solutions of the different concentrations.

3 Results and Discussion

3.1 Construction of an α -Helical Protein Receptor Using CPK Models

There have recently been increasing interests in isolation and structural characterization of sweet-taste receptor proteins. Fisher proposed a sweet-taste chemoreceptor based on an α -helix protein (11). Suami have proposed the complementary interactions of AH, B, and X groups on sweet molecules such as D- α -amino acids with the L-Asp unit located at the N-terminus of a right-handed α -helical receptor protein, suggesting that the sequence of AH, B, and X groups occurs in a clockwise orientation (12). Recently, based on the nucleotide

sequence of the genomic DNA and cDNA derived from a human taste cDNA library, TAS1R3 was predicted to encode a protein (T1R3) of 843 amino acids with 7 transmembrane helices and an extracellular domain of 558 amino acids (13). The sequence of the N-terminal fragment of T1R3 was as follows:

M1 P2 A3 L4 A5 I6 M7 G8 L9 S10
L11 A12 A13 F14 L15 E16 L17 G18 M19 G20
A21 S22 L23 C24 L25...

The understanding of factors that are responsible for sweetness expression is of great importance. To properly confront these issues, it is necessary to seek the assistance of molecular modeling. Thus, CPK modeling studies were performed for reasonable understandings of the degree and mechanism for sweet tasting of GA

Fig. 2 Schematic Drawing (a) and CPK Space-filling Representation (b) of the Receptor. The T1R3 backbone●; the hydrophobic amino acid side chains○; the hydrogen-bonding and electrostatically interacting groups●.

derivatives. We present here a hypothesis that suggests that GK2 should bind to an α -helical fragment to form a complex on the biomembrane of the taste cell. The detailed specifications of the peptide structure in question are presented below.

It is presumed, therefore, that the present peptide-model consists of the initial 15-mer fragment residing on the T1R3 protein mentioned above (14), and all the amino acid residues have the same geometry; the relevant peptide bonds are all in the trans conformation, which serves to direct the peptide conformation to an α -helix ($3.613\text{\AA}$ / turn), as illustrated in **Fig. 2**.

Ala-3 (A3), Ile-6 (I6), Met-7 (M7), Leu-11 (L11), Phe-14 (F14) side chains projecting from the α -helical core would stabilize the α -helical conformation. The so constructed CPK model of the helical peptide complex with GA exhibits several characteristic features: (1) The terminal M1 residue possesses a positive charge NH_3^+ ,

which could play a crucial role in providing significant contribution to complex stability via favorable contact geometries; (2) The five-membered proline ring of Pro-2 (P2) restricts the rotational freedom around P2 residue, and interacts with the highly hydrophobic isobutyl group L4 to place the helix tail in a favorable position for complexation; (3) The right-handed helix begins from A3; (4) The hydroxyl of Ser-10 (S10) is capable of donating a well-placed hydrogen bond to the enone oxygen of GA; (5) The main chain amide carbonyl oxygen of S10 has the potential to form an amide-based hydrogen-bond to the C-20 carboxylic acid of GA; (6) The hydrophobic GA scaffold is able to lie side-by-side along the α -helix core with the long axis of the GA molecule tilting slightly where the hydrophobic GA scaffold would be stabilized by van der Waals contacts with the hydrophobic I6, M7, L11, and F14 side chains projecting from the host core, as depicted in **Fig.**

Fig. 3 Schematic Drawing (a) and CPK Space-filling Representation (b) of the Receptor-MGGR Adduct. MGGR●; T1R3 backbone●; the hydrophobic amino acid side chains●; the interaction sites●.

3. Thus, CPK molecular models of the GA backbone docked to the helix suggest good surface complementarity between the two components.

3.2 Synthesis of Sweet Substances and their Sweetness assessment

The results of sweet potency are summarized in **Tables 1** and **2** along with some reported thus far. In general, reduced conformational freedoms of two interacting molecules will favor the complexation between them owing to an entropic advantage. It is quite natural to speculate, therefore, that sweetness also should increase with decreasing conformational freedoms of the relevant molecules. Actually, as inspection of **Table 1** reveals, the observed sweetness decreased exponentially with the increasing number of inserted methylene groups (compounds **1**, **2**, and **3**). This fact shows that a receptive NH_3^+ site which electrostatically interacts with the terminal anionic COO^- substituent on GA must be found at a distance of 6 Å or less away from the C-3 carbon atom of GA; thus, the NH_3^+ moiety on **M1** should be a likely candidate for the proton-donation site. As judged from comparison of compound **12** with **14** and **15** with **16** (**Table 2**), the observed sweetness is independent of the basicity of the anion centers, although the pKa values of, for example, sulfate anions

differ greatly from those of phosphate anions, indicating that the electrostatic Coulombic interaction rather than any other attractive intermolecular interactions such as hydrogen bonding plays a major role in the binding.

Meanwhile, to provide a more conclusive test for the geometrical or conformational effect of substituents linked to position C-3, the *cis*- and *trans*-geometrical isomers, i. e., compounds **5** and **6**, have been compared for sweetness; the former is six times sweeter than the latter. This observation can be readily accounted for by careful inspection of CPK models, which implies that the carboxylate group of the *cis*-isomer is in narrow confines in any time, but that of the *trans*-isomer is not always, in spite of both carboxylate groups rotating freely around the C-O σ -bond (**Fig. 4 a** and **4 b**); namely, a time-averaged ion-pair contact in the *trans* isomer is much poorer than that in the *cis* isomer.

As compared to compounds **1** and **3** bearing merely a polymethylene as a spacer, compounds **7** and **8** bearing an amide group-inserted spacer exhibited a fourfold increase in sweetness regardless of the latter spacer being longer than the former spacer, probably because suitable positioning of an N-H moiety in the C-3 substituent is capable of producing an additional AH/B hydrogen-bonding pair with the backbone amide oxy-

Table 1 Relative Sweetness of GA Carboxylates to 2-4% Sucrose.

Compound	R	Sweetness ^{a)}
1	-OCOCH ₂ CO ₂ K	90
2	-OCOCH ₂ CH ₂ CO ₂ K	30 ¹⁾
3	-OCOCH ₂ CH ₂ CH ₂ CO ₂ K	20
4	-OCOCH ₂ OCH ₂ CO ₂ K	15
5	$\begin{array}{c} \text{H} \quad \text{H} \\ \diagdown \quad \diagup \\ -\text{OCOC}=\text{CCO}_2\text{K} \end{array}$ (<i>cis</i>)	200
6	$\begin{array}{c} \text{H} \\ \diagdown \\ -\text{OCOC}=\text{CCO}_2\text{K} \end{array}$ (<i>trans</i>)	10
7	-OCOCH ₂ CONHCH ₂ CO ₂ K	130
8	-OCOCH ₂ CH ₂ CONHCH ₂ CO ₂ K	80
9a	-OCOCH ₂ SCH ₂ CHCO ₂ K (L-cysteine)	190 ^{b)}
9b	$\begin{array}{c} \text{NH}_3^+ \\ \\ -\text{OCOCH}_2\text{SCH}_2\text{CHCO}_2\text{K} \end{array}$ (D-cysteine)	260 ^{b)}
10	$\begin{array}{c} \text{OCH-CO}_2\text{K} \\ \\ -\text{O}-\text{CH}-\text{CH}-\text{OH} \\ \quad \\ \text{HO}-\text{C}-\text{CH}-\text{OH} \end{array}$ (MGGR)	900 ¹⁵⁾
11	-OCOCHCH ₂ OH (D-Serine)	600 ^{c)16)}
	$\begin{array}{c} \text{NH}_2 \\ \\ -\text{OCOCHCH}_2\text{OH} \end{array}$ (L-Serine)	300 ^{c)16)}

a) Tasted as its pH 7 solution. b) As its pH 9 solution. c) As its NH_3^+ - EtOH-H₂O solution.

Table 2 Relative Sweetness of GA Sulfates and Phosphates to 2-4% Sucrose.

Compound	R	Sweetness ^{a)}
12	-OCOCH ₂ OSO ₃ ⁻	180
13	-OCOCH ₂ SSO ₃ ⁻	30
14	-OCOCH ₂ OP(OH)O ₂ ⁻	80
15	-OSO ₃ ⁻	150 ³⁾
16	-OP(OH)O ₂ ⁻	50

a) Tasted as its pH 7 solution.

gen of A3 on the taste receptor. Furthermore, as comparison of compound **9** (D- or L- enantiomer) with compound **3** or **8** indicates, incorporation of an amino group in the neighboring position to the terminal COO⁻ group results in more sweetness-enhancement. Obviously, these enhanced sweetness appears to be attributed to the existence of the N-H functionality that is able to interact with the carbonyl oxygen of A3 main chain to provide an additional hydrogen-bonding pair. Incidentally, the D-enantiomer (**9a**) exhibited a 1.2 times sweeter taste than the corresponding L-enantiomer (**9b**); according to careful inspection of the constructed CPK molecular models of the complexes, the amino acid α -methine proton of the L-enantiomer renders the formation of the amide-carbonyl hydrogen-bonding pair sterically more unfavorable (**Fig. 5 a** and **5 b**), when the carboxylate tails come first into close contact with the NH₃⁺ moiety on the M1 residue. Finally, we must mention the origin of the sweetness of GK2 or MGGR, a hydrolysis product of GK2 (compound **10**). These compounds possess at least one glucuronate group; its 5'-CO₂⁻ moiety acts as a proton acceptor (B) toward the NH₃⁺ moiety of M1 and the 2'-OH as a proton donor toward the backbone amide oxygen of A3. In these complexes, restriction of conformational motion of the 5'-COO⁻ and 2'-OH group by the pyranose ring should contribute to complex stability and thereby resulted in substantial sweetness enhancement. The plausible situation for its complexation is depicted in **Fig. 2**. The same argument might hold for compound **11** with a C-3 substituent of comparatively short length, which are co-functionalized with an NH₂ (instead of COO⁻) as a proton acceptor and a OH as a proton donor.

4 Conclusion

Anionic β -glycyrrhetic acid derivatives having a terminated carboxylate or sulfate group at the 3-O posi-

tion elicited sweetness. The sweetness mechanism was well accounted for by assuming molecular models of the N-terminus of the T1R3 α -helix protein that is recognized as a sweetness receptor on the mouse taste bud cell. Thus, the α -helix modeling proposed in this study was found to be a valuable methodology to explain the observed sweetness of the GA carboxylate derivatives. This approach would not only be useful for understanding and optimizing the sweetness of a GA family, but

Fig. 4 Schematic Drawings of the Complexes of (a) the *cis*- and (b) *trans*-GA Derivatives with the Helical Receptor. For simplicity only upper part of the complexes is depicted.

Fig. 5 Schematic Drawings of the Complexes of (a) the D- and (b) L-cysteine GA Derivatives with the Helical Receptor. For simplicity only upper part of the complexes is depicted.

also be applicable to designing and discovering a new class of sweeteners.

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