Mutagenicity of Steviol and Its Oxidative Derivatives in *Salmonella typhimurium* TM677

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Stevioside is natural non-caloric sweetner isolated from *Stevia rebaudiana* BERTONI, which has been used as a non-caloric sugar substitute in Japan. Pezzuto *et al.* demonstrated that steviol shows a dose-dependent positive response in forward mutation assay using *Salmonella typhimurium* TM677 in the presence of metabolic activation system (Aroclor induced rat liver S9 fraction). Our studies were carried out to identify the genuine mutagenic active substance from among the eight steviol derivatives. Steviol indicate almost similar levels of mutagenicity under the presence of S9 mixture, as reported by Pezzuto *et al.* 15-Oxo-steviol was found to be mutagenic at the one tenth the level of steviol itself under the presence of S9 mixture. Interestingly, specific mutagenicity of the lactone derivative under the presence of S9 mixture was ten times lower than that of the lactone derivative without the addition of S9 mixture.

Key words steviol; steviol derivative; mutagenicity; *Salmonella typhimurium* TM677

Stevioside[1] is natural non-caloric sweetener from *Stevia rebaudiana* BERTONI, which has been used for a long time in Paraguay and Brazil. Purified stevioside is an odorless powder approximately 250 to 300-fold sweeter than sucrose, and is composed of steviol, a diterpenic hydroxy carboxylic acid and three glucose molecules. At present, stevioside is widely used as a non-caloric sugar substitute, and in various kinds of drinks and food products in many countries. In Japan alone, approximately 200 t of *S. rebaudiana* extract are consumed each year. Stevioside has been subjected to various assessments for safety, and to date, no serious toxic effects have been reported.[2,3] Stevioside and the crude extract of *S. rebaudiana* have been determined as non-mutagenic in many bacterial test systems, such as some test strains of *Salmonella typhimurium*, *Escherichia coli*, and *Bacillus subtillis*, either in the presence or absence of metabolic activation, which is mostly derived from the rat liver S9 fraction.[4] However, it is known that stevioside is converted to its aglycone, steviol, by intestinal bacteria when orally administered to rats.[5] Pezzuto *et al.*[6] demonstrated that steviol causes a dose-dependent positive response in the forward mutation assay using *S. typthimurium* TM677 in the presence of a metabolic activation system (Aroclor-induced rat liver S9 fraction).

Recently Suttajit *et al.*[7] showed that stevioside, at a high dosage (50 mg/plate), possesses a weak mutagenic activity S9 fraction. Since this observed mutagenic effect could be caused by the impurities present in stevioside. Matsui *et al.*[8] examined seven mutagenicity tests using bacteria (reverse mutation assay, forward mutation assay, umn test and rec assay), cultured mammalian cells (chromosomal aberration test and gene mutation assay), and mice (micronucleus test). Consequently, the aglycone, steviol, was shown to produce dose-related positive responses with the same mutagenicity, *i.e.* forward mutation assay using *S. typhimurium* TM677, and chromosomal aberration test using Chinese hamster lung fibroblast cell (CHL) line, and gene mutation assay using CHL. Herein, we wish to identify the genuine mutagenic active substance that is mutagenic toward *S. typhimurium* in the absence of S9 in steviol derivatives. The present paper describes the mutagenicity (forward mutation assay) studies, which were carried out with steviol and several of its oxidative derivatives.

We report that steviol (**2**), steviol-16α,17-epoxide (**3**), 15-hydroxy-steviol (**4**), 15-oxo-steviol (**5**), steviol methyl ester (**6**), 16-oxo-steviol methyl ester (**7**), 3,16-*seco*-13-oxo-steviol methyl ester (**8**), 3,16-*seco*-13-hydroxy-steviol methyl ester (**9**), and steviol methyl ester 8,13-lactone (**10**) are mutagenic toward *S. typhimurium* strain TM677, in the presence of a 9000×***g*** supernatant fraction (S9) obtained from the liver of Aroclor 1254-pretreated rats. While only steviol methyl ester 8,13-lactone (**10**) showed mutagenicity toward the same strain in the presence and in the absence of S9, it is interesting to note that the mutagenicity of lactone **10** in the S9-free system is ten times higher than that of lactone **10** in the presence of S9, in other words, the mutagenicity of lactone **10** is suppressed by addition of S9.

Results and Discussion

Steviol and eight chemically synthesized derivatives of steviol were applied to forward mutation assay using *S. typhimurium* TM677. The results were shown in Table 1 as their specific mutation ratio (mutation frequency/μg sample/plate). Steviol indicated almost similar levels of mutagenicity under the presence of S9 mixture, as reported by Pezzuto *et al.*[6] or Matsui *et al.*[8] Of the eight synthesized derivatives, four compounds [steviol-16,17-epoxide (**3**), steviol methylester (**6**), 13,16-*seco*-13-oxo-steviol methylester (**8**), and steviol methylester 8,13-lactone (**10**)] were found to be mutagenic at nearly the same level as steviol (**2**) itself only under the presence of S9 mixture, and the other three compounds has no activity since over the detection limit. Regardless of the presence or absence of the rat liver ribosomal fraction, 15-hydroxy steviol (**4**), 16-oxo-steviol methylester (**7**) and 3,16-*seco*-13-hydroxy-steviol methylester (**9**) were found to be non-mutagenic or below detection limit in the test system. 15-Oxo-steviol (**5**) was found to be mutagenic at the one tenth the level of steviol itself under the presence of S9 mixture. Interestingly, the specific mutagenicity of the lactone derivative (**10**) under the presence of S9 mixture was ten

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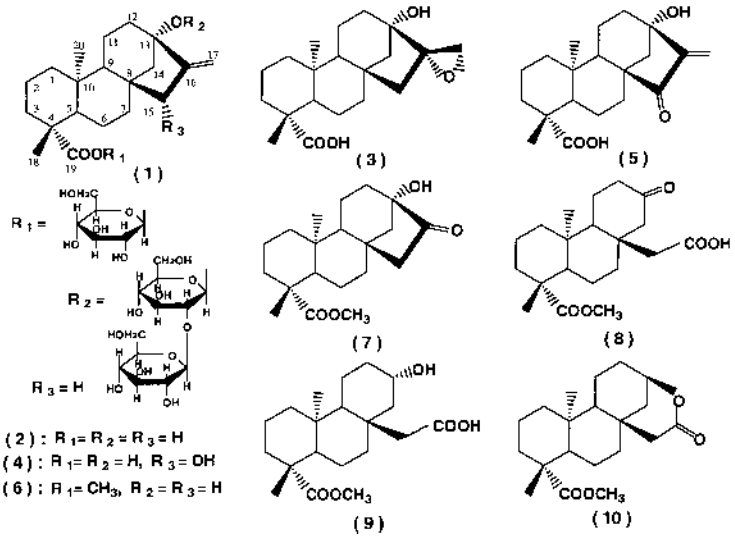

Fig. 1. Structures of Steviol and Its Derivatives

times lower than that observed without the addition of rat liver ribosomal fraction to the test system. This fact suggests the mutagenicity given by the lactone structure can be decreased by one-tenth under the presence of S9 mixture.

It is instructive to consider the one of detoxification of the lactone with the enzyme in liver. In comparison to the *in vitro* tests using the TM677 strain, the activity of steviol (**2**) and steviol methylester (**6**) were nearly identical, indicating that the methyl group attached to C19 does not have an influence on the mutagenicity. However, the steviol methylester (**6**) may be hydrolyzed by enzymaticaly hydrolysis with S9 mixture. The specific activity of steviol (**2**), as measured by forward mutation assay, was determined to be 3.58×10^{-7}/μg/plate, which suggests that the probability of mutation induced by this compound is 3.58 out of ten million microorganisms. By comparing to the mutagenicity of BaP (3,4-benzopyrene), the activity of steviol was calculated to be 0.03% or 1/3000 of authentic mutagenic compounds that are widely distributed in smoke, diesel exhausted gas, and an over heated meat. Specific mutagenicity of the lactone form derived from steviol was 4.2×10^{-6}/μg sample/plate and calculated as 0.004% or 1/24500 of AF2 (furylfuramide), which was one of the official positive compounds for the Ames test in the absence of S9 mixture.

In this report, we have roughly estimated the influence of the mutagenicity of steviol, used as low calorie sweetener in our daily life, in comparison to that of AF2, which was previously used as an authorized food preservative. In the case of a cup of coffee, with the assumption of the common size of commercial individually packed sugar in Japan as ranging from three to five grams, the average amount of sugar can be assumed as four grams per cup. To provide similar sweetness, only 16 mg of stevia is necessary as the sugar substitute for the same cup of coffee. According to the specific mutagenic values of our experimental data, as shown in Table 1, only 0.6 mg stevia is necessary to indicate the similar strength in mutagenicity under the absence of S9 mixture as that given by 16 mg of stevia. Using values for AF2, whose levels were permitted up to 2 mg for preserving 100 g of fish sausage products from 1965 to 1974 in Japan, the mutagenic activity given by 2 mg of AF2 is calculated to be equivalent to 48 g of stevia, which translates to 3000 cups of coffee. From this simulation, the genetic toxicity of stevia can be regarded as negligible and safe, as long as we limit its use in ordinary amount for our daily life. Although further studies, such as the effect of heating on the activity of stevia, or the metabolic fate of its lactone derivatives in our body are necessary, the present paper suggests that there are no serious genotoxic problem with the daily usage of this low-calorie sweetener.

Table 1. Mutagenicity[a] of Steviol and Its Derivatives

Compound	+S9	−S9
3,4-Benzopyrene (BaP)	1.12×10^{-3}	NT
Furylfuramide (AF2)	NT	1.03×10^{-1}
Steviol (**2**)	3.58×10^{-7}	—
Steviol-16,17-epoxide (**3**)	3.80×10^{-7}	—
15-Hydroxy steviol (**4**)	—	—
15-Oxo-steviol (**5**)	2.96×10^{-6}	—
Steviol methylester (**6**)	7.05×10^{-7}	—
16-Oxo-steviol methylester (**7**)	—	—
13,16-*Seco*-13-oxo-steviol methylester (**8**)	5.46×10^{-7}	—
13,16-*Seco*-13α-hydroxy-steviol methylester (**9**)	—	—
Steviol methylester 8,13-lactone (**10**)	4.29×10^{-7}	4.20×10^{-6}

—: Below detection limit. NT: Not tested. *a*) The mutation ratio was calculated by the following formula: mutation ratio (mutation frequency/μg/sample/plate)=(number of colonies of mutants−number of colonies for negative control)/number of colonies for viable cells.

Experimental

Melting points (mp) are uncorrected. IR spectra were measured using a Shimadzu IR-430 instrument. ^{1}H- and ^{13}C-NMR were measured using a Unity-300 (Varian Co.) spectrometer in $CDCl_3$ or pyridine-d_5 with tetramethylsilane (TMS) as the internal standard. The ^{1}H- and ^{13}C-NMR signals of steviol and its derivatives were assigned using H–H correlation spectroscopy (COSY) or C–H COSY and by comparison with spectra of known derivatives. ^{13}C-NMR chemical shifts of steviol and its derivatives were shown in Table 2. Mass spectra were determined using a Shimadzu QP-5000 mass spectrometer.

Preparation of Steviol (2) from Stevioside A soil bacterial strain (unidentified), which was selected by repeated cultivation on a synthetic medium[9] containing stevioside as a sole carbon source, was cultivated on the same synthetic medium (11 medium containing 30 g of stevioside) at

Table 2. ^{13}C-NMR Chemical Shifts (δ) of Steviol and Its Derivatives

C No.	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	41.0	41.0	40.7	40.7	41.7	40.6	41.5	41.4	38.0
2	19.8	19.8	19.9	19.7	19.5	19.4	19.6	19.7	16.6
3	38.6	38.7	38.8	38.6	38.3	37.2	37.9	33.5	29.2
4	43.9	43.9	44.1	44.0	43.9	43.9	43.8	43.8	43.7
5	57.0	56.9	57.4	56.4	56.9	56.6	56.4	57.0	56.5
6	22.6	22.6	22.2	21.3	22.4	21.2	20.4	21.0	20.3
7	41.8	41.9	41.3	40.3	41.7	41.2	39.0	41.6	41.0
8	41.9	41.5	46.6	55.4	41.7	39.5	41.3	39.8	39.5
9	54.3	54.1	53.4	51.2	54.1	53.0	49.6	50.6	53.1
10	39.8	39.8	40.3	40.5	39.5	39.4	39.5	39.0	34.0
11	20.8	19.9	20.7	21.0	20.8	20.0	20.9	20.5	19.5
12	40.8	36.6	44.4	45.4	40.7	38.1	47.2	38.1	33.0
13	79.8	75.1	79.0	76.5	79.8	80.3	211.7	65.8	75.8
14	48.2	48.2	36.4	33.8	48.1	44.4	48.5	42.2	42.9
15	47.5	47.7	82.0	209.1	47.4	53.3	38.6	47.5	48.4
16	157.7	65.2	163.4	154.2	157.6	220.1	173.9	175.0	171.6
17	102.9	46.4	107.2	114.1	103.7	—	—	—	—
18	29.3	29.3	29.4	29.3	28.7	28.6	28.5	28.7	28.6
19	180.1	180.1	180.2	180.0	177.7	177.6	177.7	178.0	177.7
20	15.9	16.1	16.3	15.9	15.2	15.9	16.4	18.3	16.0
$-OCH_3$	—	—	—	—	51.5	51.2	51.2	51.1	51.2

35 °C for 2 weeks. The resulting white crystalline precipitate was isolated from the culture medium, and recrystallized from methanol to yield pure steviol (**2**) (5.5 g): mp 206—207 °C; *Anal.* Calcd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.54; 9.41. The IR and NMR spectral data of steviol were identical to those published in literature.[9,10]

Preparation of Steviol-16α,17-epoxide (3) To a solution of steviol (1.0 g) in dichloromethane (40 ml), *m*-chloroperbenzoic acid (660 mg) was added, and the solution was stirred at room temperature for 1 h. The mixture was treated with 10%-$Na_2S_2O_3$ solution. The organic layers was washed with 5%-$NaHCO_3$ solution, then with water, dried over an hydrous Na_2SO_4, and evaporated to give an oily product. The product was recrystallized from ethyl acetate to afford epoxide **3** (854 mg): mp 207—210 °C; IR (Nujol) cm^{-1}: 3255 (OH), 1715 (C=O), 1245 (epoxide); ^{1}H-NMR (pyridine-d_5) δ: 1.20 (3H, s, C20-H_3), 1.36 (3H, s, C18-H_3), 1.81 (2H, s, C15-H_2), 2.94 (2H, s, C17-H_2).

Preparation of 15α-Hydroxy-steviol (4) To a solution of steviol (500 mg) in dioxane (30 ml) and water (10 ml), SeO_2 (20 mg) and aqueous H_2O_2 (36%, 2 ml) were added, and the solution was stirred at 60 °C for 2 h. The mixture was evaporated and extracted with ethyl acetate (3×30 ml). The combined ethyl acetate layer were dried over anhydrous Na_2SO_4 and evaporated to give an oily product. The product was recrystallized from $CHCl_3$: MeOH (99 : 1) to give alcohol **4** (395 mg): mp 271—275 °C; IR (Nujol) cm^{-1}: 3468, 3382 (OH), 1698 (C=O); ^{1}H-NMR (pyridine-d_5) δ: 1.27 (3H, s, C20-H_3), 1.37 (3H, s, C18-H_3), 4.29 (1H, s, C15-H), 5.67, 5.74 (each 1H, d, J=1.8 Hz, C17-H_2). MS *m/z*: 334 (M^+), 316, 301, 270, 255, 237, 221, 209, 189.

Preparation of 15-Oxo-steviol (5) To a solution of 15-hydroxy-steviol (**4**) (500 mg) in pyridine (20 ml), pyridinium chlorochromate (PCC) (490 mg) was added, and the solution was stirred at room temperature for 4 h. The mixture was poured into ice-water (30 ml) and extracted with diethyl ether (3×30 ml). The combined diethyl ether layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated to give an oily product. The product was recrystallized from diethyl ether to give 15-ketone **5** (144 mg): mp 216—218 °C; IR (Nujol) cm^{-1}: 3518 (OH), 1720, 1697 (C=O); ^{1}H-NMR (pyridine-d_5) δ: 1.22 (3H, s, C20-H_3), 1.34 (3H, s, C18-H_3), 5.47, 6.27 (each 1H, d, J=1.8 Hz, C17-H_2); MS *m/z*: 332 (M^+), 314, 304, 286, 268, 253.

Preparation of Steviol Methyl Ester (6) To a diazomethane–diethyl ether solution (12 ml), steviol (1.00 g) was added. The mixture was shaken and allowed to stand overnight at room temperature. The mixture was concentrated and the residue was recrystallized from hexane–diethyl ether to give ester **6** (930 mg): mp 113—115 °C; IR (Nujol) cm^{-1}: 3483, 3426 (OH), 1732, 1709 (C=O); ^{1}H-NMR (pyridine-d_5) δ: 0.87 (3H, s, C20-H_3), 1.17 (3H, s, C18-H_3), 3.62 (3H, s, –COOC$\underline{H}_3$); *Anal.* Calcd for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70, Found: C, 75.77; H, 9.62.

Preparation of 17-Nor-17-oxo-steviol Methyl Ester (7) and 13,16-*Seco*-13-oxo-steviol Methyl Ester (8) A solution of steviol methyl ester (1.00 g) in dichloromethane (10 ml) was cooled to 0 °C and subjected to a passing stream of oxygen that contain ozone for 10 h. The mixture was treated with Zn–AcOH, then the solvent was removed *in vacuo*, and the residue was purified by chromatography (silica gel, Wakogel C-300) by gradient elution of hexane to ethyl acetate. The fraction eluted with hexane : ethylacetate (1 : 1) afforded 16-ketone **7** (317 mg): mp 207—208 °C; IR (Nujol) cm^{-1}: 3447 (OH), 1744, 1721 (C=O), 1237, 1208; ^{1}H-NMR (pyridine-d_5) δ: 0.89 (3H, s, C20-H_3), 1.18 (3H, s, C18-H_3), 1.74, 2.09 (each 1H, d, J=2.1 Hz, C15-H_2), 3.66 (3H, s, –COOC$\underline{H}_3$). The fraction eluted with hexane : ethylacetate (1 : 2) afforded keto carboxylic acid **8** (218 mg): mp 170—172 °C; IR (Nujol) cm^{-1}: 1721, 1694 (C=O), 1237, 1154, 972; ^{1}H-NMR (pyridine-d_5) δ: 0.95 (3H, s, C20-H_3), 1.15 (3H, s, C18-H_3), 1.65 (1H, m, C14-H_2), 2.44, 2.93 (each 1H, m, C15-H_2), 2.52 (1H, m, C12-H_2), 3.66 (3H, s, –COOC$\underline{H}_3$).

Preparation of 13,16-*Seco*-13-hydroxy-steviol Methyl Ester (9) and Steviol Methyl Ester 8,13-Lactone (10) To a solution of 13,16-*seco*-13-oxo-steviol methyl ester (**8**) (1.0 g) in 1-butanol (50 ml), $NaBH_4$ (250 mg) was added, and the solution was stirred at room temperature for 1 h. The mixture was neutralized with 5%-HCl, the solvent was removed *in vacuo*, and the residue was extracted with ethyl acetate (3×20 ml). The combined ethyl acetate layers were dried over anhydrous Na_2SO_4, and evaporated to give a mixture of esters **9** and **10** as an oily product. The mixture was purified by chromatography on silica gel by a gradient elution of chloroform to chloroform : methanol (7 : 3) to yield 13,16-*seco* ester **9** (540 mg) and lactone **10** (270 mg). Compound **9**: mp 150—154 °C; IR (Nujol) cm^{-1}: 3460 (OH), 1727 (C=O), 1234, 1150 (–C–O); ^{1}H-NMR (pyridine-d_5) δ: 0.97 (3H, s, C20-H_3), 1.15 (3H, s, C18-H_3), 2.44, 2.93 (each 1H, d, J=13.0 Hz, C15-H_2), 3.56 (3H, s, –COOC$\underline{H}_3$), 4.54 (1H, m, C13-H). Compound **10**: mp 149—151 °C; IR (Nujol) cm^{-1}: 1238, 1184 (–C–O), 1720 (C=O), 1730 (C=O, lactone). ^{1}H-NMR (pyridine-d_5) δ: 0.75 (3H, s, C20-H_3), 1.17 (3H, s, C18-H_3), 1.96, 2.38 (each 1H, d, J=12.9 Hz, C15-H_2), 3.66 (3H, s, –COOC$\underline{H}_3$), 4.69 (1H, m, C13-H).

Forward Mutation Assay To assess the mutagenicity of steviol and its chemically synthesized derivatives, we employed forward mutation assay using *Salmonella typhimurium* TM677 and 8-azaguanine (8-AG, Tokyo Kasei Kogyo) as the detection reagent, as proposed by Skopek *et al.*,[11,12] modified by Takagi *et al.*,[13] and reduced in size by the authors.[14] In our experiment, each test samples (100 μl; sterilized by membrane filtration, Millipore, pore size, 0.45 mm) and an equal volume of cultured bacteria suspension, which was diluted to OD_{600}=0.12 with ME culture medium[12] after three hours pre-incubation at 37 °C, were combined into a micro-titer plate equipped with a plastic cover (96 holes, γ-radiation sterilized, Corning), and shaken at 37 °C for 2 h. The incubated mixture (25 μl) was transferred to a test tube equipped with an aluminum cap, which was sterilized at 180 °C for 4 h. Due to the low solubility of the test samples in 5% dimethylsulfoxide (DMSO), the top dose of each sample was varied between 100 and 200 μg/100 μl of the solvent. Test samples were successively diluted five-fold by addition of the same amount of 5% DMSO. For the analyses of mutagenic compounds that required enzymatic activation, S9 mixture (a mixture of cofactors and S9 obtained from homogenates of livers of rats to induce the cytochrome P-450 mono-oxygenase system) was added to the cultured bacteria suspension. Soft agar (2.5 ml) containing 8-AG (1 mg) was added to the test tube and spread over a minimum glucose agar plate. Each measurement was made in triplicate at each sample dose. Benzo[*a*]pyrene (BaP) and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF2) were used as the positive controls for +S9 and −S9 analyses, respectively. Sterilized 5% DMSO was used as the negative control for both +S9 and −S9 analyses. We calculated the specific mutagenicity (expressed as mutagenicity of 1 μg test samples per plate) at the slope where the liner dose-response curve was observed, using the least square method, at the dose range with no direct toxicity of samples. Mutagenicity was determined to be positive in the case where the number of mutants was more than double that of the negative control and to be negative (below detection limit) where the number was less than double. The mutation ratio was calculated by the following formula: mutation ratio (/μg sample/plate)=(number of colonies of mutants−number of colonies for negative control)/numbers of colonies for viable cells.

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