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Production and physicochemical assessment of new stevia amino acid sweeteners from the natural stevioside

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Abstract

New stevia amino acid sweeteners, stevia glycine ethyl ester (ST-GL) and stevia L-alanine methyl ester (ST-GL), were synthesized and characterized by IR, NMR (^{1}H NMR and ^{13}C NMR) and elemental analysis. The purity of the new sweeteners was determined by HPLC and their sensory properties were evaluated relative to sucrose in an aqueous system. Furthermore, the stevia derivatives (ST-GL and ST-AL) were evaluated for their acute toxicity, melting point, solubility and heat stability. The novel sweeteners were stable in acidic, neutral or basic aqueous solutions maintained at 100°C for 2 h. The sweetness intensity rate of the novel sweeteners was higher than sucrose. Stevia amino acid (ST-GL and ST-AL) solutions had a clean sweetness taste without bitterness when compared to stevioside. The novel sweeteners can be utilized as non-caloric sweeteners in the production of low-calorie food.

Keywords: Stevioside, stevia amino acid, glycine, L-alanine, sweeteners, sensory properties, spectroscopic analysis.

1. Introduction

Stevioside is a natural non-caloric sweetener and medical supplementary material, which has received much attention in recent times. Stevioside is an extract of intense sweetness (>80%) obtained from *Stevia rebaudiana* Bertoni, a perennial shrub of the *Asteraceae* (Compositae) family originally grown in South America, particularly in Brazil and Paraguay (Palazzo, Carvalho, Efraim & Bolini, 2011). Structurally, stevioside **1** ($C_{38}H_{60}O_{18}$), 13-[2-O-β-D-glucopyranosyl-α-glucopyranosyl)oxy]kaur-16-en-19-oic-acid β-D-glucopyranosyl ester, which is a glycoside with a glucosyl and a sophorosyl residue attached to the aglycone steviol, which has a cyclopentanonhydrophenanthrene skeleton.

Recently stevia was approved for use as a sweetener by the Joint Food and Agriculture Organization/World Organization Expert Committee on Food Additives (JECFA, 2008; JECFA, 2009). The committee suggested a temporary admissible daily intake (ADI) of steviol of up to 4 mg kg^{-1} body weight (BW), which is equivalent to 10 mg kg^{-1} BW stevioside (JECFA, 2008). Stevioside is a white, crystalline and odourless powder considered about 300 times sweeter than solutions containing 0.4% sucrose (Soejarto, Compadre, Medon, Kamath & Kinghorn, 1983). Stevioside and extracts of *Stevia rebaudiana* leaves are commercially available and used in many countries, including USA, Europe, Japan and several South American countries, as a sweetener for a variety of foods and beverages (Kinghorn & Soejarto, 1985; Panpatil & Polasa, 2008; JECFA,

2008; JECFA, 2009; EFSA (2010); Lemus-Mondaca R., Vega-Gálvez, Zura-Bravo & Ah-Hen, 2012; Abdel-Nabey & Massoud, 2013; Gasmalla, Yang, Musa, Hua & Zhang W., 2014). In different countries, stevioside is used as a sweetener primarily intended for people with diabetes (Puri, Sharma & Tiwari, 2011; Nikolai, Oxana & Alexander, 2001). Over the years, the number of countries in which stevia is available as a sweetener has been increasing. This compound is present in some low-sugar products available in the market, but their acceptability is limited due to undesirable sensory effects, such as the metallic / bitter aftertaste (Alonso & Setser, 1994; Schiffman, Booth, Losee, Pecore, & Warwick, 1995).

Amino acids are commonly used as seasoning to improve sweetness and flavour of foodstuffs (Kirimura, Shimizu, Kimizuka, Ninomiya, & Katsuya, 1969). Of these amino acids, L-alanine is of great interest as it has been shown to reduce non-enzymatic browning, caused by the Maillard reaction, which is a unique characteristic of L-alanine compared to other amino acids. Amino acids may improve the sensory properties of stevioside and reduce the negative aftertaste characteristics. Food technology enables one to modify taste characteristics of any sweetener in a food or beverage by altering the product's flavour chemistry, physical properties or textural ingredients (Wiet and Beyts, 1992).

Therefore, the present study was designed to investigate the production of stevioside coupled with glycine or L-alanine with the aim of generating new sweeteners with improved sensory characteristics.

2. Materials and methods

2.1. Materials

The solvents used were of HPLC reagent grade. All chemicals were purchased from Sigma Aldrich, Germany unless otherwise stated.

Stevioside was obtained from AWA for food additives (Alexandria, Egypt). Sucrose was purchased from El Nasr Pharmaceutical Chemical Co. (Cairo, Egypt).

Purity of the stevioside was determined by reverse-phase high performance liquid chromatography (RP-HPLC) as described by Vanek, Nepovím and Valícek (2001). An agilent HPLC connected to a UV–visible Agilent 1200 PDA detector was used for the analysis. A solution of the obtained stevioside was prepared and filtered through a 0.45 µm filter prior to use in HPLC. The injection volume was 70 µl and the compounds were separated using an Eclipse plus C18 column (3.5 µm 4.6x100 mm). A linear gradient over 20 min (84 to 50 % CH_3CN in H_2O, (pH 5, H_3PO_4)) at a flow rate 2.0 ml/min was used to elute stevioside which was detected at 210 nm. Quantification of stevioside was carried out by the construction of a standard curve using pure stevioside (Sigma-Aldrich, USA).

2.2. Chemical analyses

Melting points were determined using a Mel-Temp apparatus and the values obtained are uncorrected.

The infrared spectra was acquired using a Perkin-Elmer 1600 FTIR spectrophotometer, with He-Ne as the reference, at a resolution of 4 cm^{-1}. The spectra were taken in the region 400-4000 cm^{-1} as KBr pellets.

Nuclear Magnetic Resonance spectra (^1H NMR and ^{13}C NMR spectra) were recorded on a JOEL 500 MHz spectrometer with chemical shifts reported in parts

per million (ppm) and are referenced relative to residual solvent (e.g. MeOH at δ_H 3.35, 4.78 ppm for CD_3-OD). Spin multiplicities are represented by the following signals: s (singlet), br s (broad singlet), d (doublet), br d (broad doublet), dd (doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet), sex (sextet) and m (multiplet).

Elemental analyses were performed on a Perkin-Elmer 2400 elemental analyzer, and the values obtained were within ±0.3% of the theoretical values. Follow-up of the reactions and checking of the purity of the compounds was done by TLC on silica gel-protected aluminum sheets (Type 60 GF254, Merck) and the spots were detected by exposure to a UV-lamp at λ254 nm for a few seconds. The compounds were named using Chem. Draw Ultra version 11, Cambridgesoft Corporation.

2.3. Hydrolysis of stevioside 1 (Hyd. ST)

Stevioside (**1**, 5 g, 6.21 mmol) was added to 50 ml of 100 mmol NaOH (4 g dissolved in 50 ml MeOH at room temperature) and the mixture was heated at 60°C. The mixture was refluxed for 7 hrs under continuous stirring. The mixture was cooled to room temperature and then neutralized with 1 N HCl. To obtain the product, two methods were employed as follows;

Method A: The mixture was concentrated under vacuum to give a white solid, which was recrystallized from methanol-acetone (1:1) to yield pure **2** (2.99 g, 75.0%, mp 198-199ºC).

Method B: The product was extracted with *n*-BuOH. The *n*-BuOH layer was washed with water and concentrated under vacuum to afford a crude solid which

was recrystallized from methanol-acetone (1:1) mixture to yield pure **2** (3.48 g, 87.5%, mp 198-199ºC).

The compound obtained had the following characteristics;

IR (KBr): 3500-3300 (br., O-H), 2917.69 (SP_3 C-H), 1720 (C=O acid), 1642.9 (C=C) cm^{-1}. ^{1}HNMR ($CDCl_3$): δ 0.83-0.86 (m, 1H, CH), 0.94 (s, 3H, CH_3), 0.97-1.09 (m, 2H, CH), 1.16 (s, 3H, CH_3), 1.39-1.43 (m, 2H, CH), 1.48-1.53 (m, 3H, CH), 1.57-1.69 (m, 1H, CH), 1.77-1.93 (m, 6H, CH), 2.01-2.14 (m, 3H, CH), 2.17-2.20 (m, 2H, CH), 3.29-3.62 (m, 16H, 13 CH-O, 3 OH, D_2O exchangeable), 4.58-4.66 (m, 7H, O-CH-O, $=CH_2$, 4 OH), 5.22-5.27 (m, 1H, O-CH-O). ^{13}C NMR: δ 15.23, 15.42, 18.93, 19.89, 20.00, 21.86, 21.93, 28.04, 28.11, 29.43, 37.31, 37.74, 37.89, 39.35, 40.64, 41.28, 41.41, 41.71, 43.37, 44.13, 44.25, 53.67, 53.80, 56.64, 56.73, 61.17, 61.36, 61.89, 62.37, 68.75, 70.07, 70.19, 70.60, 71.09, 74.04, 74.91, 76.44, 76.54, 76.84, 76.90, 76.94, 76.99, 77.16, 77.32, 78.59, 80.71, 86.48, 87.02, 87.74, 95.78, 96.03, 102.37, 102.94, 104.62, 152.32, 180.55. Analytically calculated for $C_{32}H_{50}O_{13}$: C, 59.80; H, 7.84; found: C, 60.08; H, 8.11.

2.4. Synthesis of St-Gly-OEt 3

To a cooled solution of 3.21 g (5 mmol) of **2** in 4 ml CH_2Cl_2, 1.054 g (5.5 mmol) of EDC.HCl (*N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide. hydrochloride) and 0.99 g (5.5 mmol) potassium salt of Oxyma in 8 ml CH_2Cl_2 were added and stirred for 5 min. Then, 5 ml of a solution containing 0.767 g (5.5 mmol) H-Gly-OEt.HCl and 1.2 g (11 mmol) Na_2CO_3 in H_2O was added. The reaction mixture was stirred at 0°C for 1 h and then the reaction mixture was stirred overnight at

148 room temperature. The organic layer was evaporated under vacuum. After
149 dilution with 20 ml *n*-BuOH, the organic layer was washed with 5% citric acid (2
150 × 5 ml) and water (2 × 5 ml), dried over anhydrous Na_2SO_4 and filtered. The
151 solvent was removed using a rotary evaporator and the residue was recrystallized
152 from methanol/ether to give the product in yield (**3**) (0.26 g) 71.4%, mp 190-
153 191ºC (dec). Compound **3** had the following characteristics; IR (KBr): 3450-3350
154 (br., O-H), 2926 (sp_3 C-H), 1733 (C=O acid), 1638 (C=O amide, C=C) cm^{-1}.
155 ^{1}HNMR (MeOD): δ 0.81-0.90 (m, 3H, CH_3), 0.94-0.97 (m, 1H, CH), 0.97- 1.09
156 (m, 2H, 2 CH), 1.13-1.18 (m, 2H, CH), 1.24-1.29 (m, 4H, CH + CH_3), 1.35 (m,
157 4H, CH + CH_3), 1.40-1.43 (m, 1H, CH), 1.51-1.53 (m, 2H, 2 CH), 1.56-1.64 (m,
158 1H, CH), 1.68-2.23 (m, 9H, 9 CH), 3.11-3.21 (m, 4H, 4 CH-O), 3.27-3.31 (m,
159 8H, 5 CH-O, 3 OH, D_2O exchangeable), 3.39-3.47 (m, 3H, 3 CH-O), 3.53-3.90
160 (m, 8H, 2 CH_2, 4 OH), 4.60-4.61 (m, 2H, =CH_2), 5.20-5.21 (m, 2H, 2 O-CH-O),
161 7.96 (s, 1H, NH, exchangeable). ^{13}C NMR (MeOD): δ 12.98, 14.51, 16.22, 17.35,
162 17.70, 18.07, 18.41, 19.60, 19.85, 23.13, 28.35, 30.70, 31.31, 36.82, 39.05, 39.42,
163 39.49, 39.59, 41.24, 42.47, 42.92, 43.86, 51.23, 53.08, 55.19, 60.70, 60.78, 61.55,
164 61.59, 68.37, 69.63, 70.84, 73.47, 75.90, 75.95, 76.18, 78.40, 85.17, 88.19,
165 102.21, 102.26, 158.22, 164.82. Anal. Calcd for $C_{36}H_{57}NO_{14}$: C, 59.41; H, 7.89;
166 N, 1.92; found: C, 59.70, H, 7.61, N, 2.20.
167 The purity of St-Gly-OEt **3** was determined by RP-HPLC using the following
168 conditions; detection at 220 nm and using Agilent 1200 PDA detector. An Eclipse
169 plus C18 column (3.5 µm 4.6x100 mm) was used for the separation of the
170 compounds using a linear gradient of 84 to 55% CH_3CN in H_2O/ (pH 5, H_3PO_4),

over 15 min at a flow rate of 1.0 ml/min. The retention time t_R of St-Gly-OEt was 5.43 min (100%).

2.5. Synthesis of St-Ala-OMe 4

To a solution of 3.21 g (5 mmol) of **2** in 4 ml CH_2Cl_2, 1.054 g (5.5 mmol) of EDC.HCl and 0.99 g (5.5 mmol) potassium salt of Oxyma in 8 ml CH_2Cl_2 were added and stirred for 5 min. Then, 5 ml of a solution containing 0.765 g (5.5 mmol) H-Ala-OMe .HCl and 1.2 g (11 mmol) Na_2CO_3 in H_2O was added after one min preactivation. The reaction mixture was stirred at 0°C for 1 h and left stirring overnight at room temperature. The organic layer was evaporated under vacuum. *n*-BuOH (20 ml) was added and the organic layer was washed with 5 ml of 5% citric acid and 5 ml water, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed with a rotary evaporator and the residue was recrystallized from methanol/ether to give the product yield (**4**) (0.26 g, 71.4%, mp 185-186ºC). Compound **4** had the following characteristics; IR (KBr): 3450-3350 (br., O-H), 2926 (sp_3 C-H), 1730 (C=O acid), 1625 (C=O amide, C=C) cm^{-1}. ^1H NMR (MeOD): δ 0.85-1.02 (m, 6H, 2 CH_3), 1.06-1.17 (m, 5H, 2 CH + CH_3), 1.29-1.54 (m, 8H, 5 CH + CH_3), 1.60-1.71 (m, 1H, CH), 1.78-1.94 (m, 6H, 6 CH), 2.02-2.14 (m, 3H, 3 CH), 2.20-2.33 (m, 1H, CH), 2.75-2.84 (m, 2H, CH_2), 3.23-3.39 (m, 12H, 10 CH-O, 2 OH, D_2O exchangeable), 3.51-3.66 (m, 5H, 1 CH-O, 4 OH, D_2O exchangeable), 3.68-3.72 (m, 2H, CH_2), 3.75-3.88 (m, 3H, CH_2, 1 OH), 4.60-4.61 (m, 2H, =CH_2), 5.21-5.23 (m, 2H, 2 O-CH-O), 7.31 (s, 1H, NH, exchangeable). ^{13}C NMR (MeOD): δ 12.48, 12.90, 13.12, 14.39, 15.41, 15.66, 16.23, 18.69, 18.93, 19.10, 19.87, 19.97, 21.93, 22. 13, 28.02, 28.74, 34.47,

35.53, 36.11, 37.53, 37.76, 37.89, 39.34, 39.67, 40.65, 40.96, 41.28, 41.42, 41.59, 42.08, 43.36, 43.57, 44.11, 51.41, 53.69, 55.12, 56.65, 57.48, 61.18, 61.42, 62.40, 68.81, 70,21, 71.10, 71. 23, 71.31, 74.06, 74.85, 74.92, 76.51, 76.88, 76.98, 77.09, 77.33, 78.62, 86.52, 87.33, 87.71, 95.80, 96.00, 102.39, 102.98, 104.58, 151.55, 152.00, 180.48. Analytically Calculated for $C_{36}H_{57}NO_{14}$: C, 59.41; H, 7.89; N, 1.92; found: C, 59.25, H, 7.72, N, 1.67.

The purity of St-Ala-OMe **4** was by determined by RP-HPLC. The analysis conditions were those described for compound **3**. The retention time t_R for St-Ala-OMe was 3.64 min (100%).

2.6. Physical characteristics of stevia amino acid sweeteners

The melting points of pure sucrose, stevioside (St) and each stevia amino acid sweetener were determined with a Mel-Temp Apparatus. The solubility in water, ethanol, methanol and chloroform was determined according to the methods described by Soejarto, Compadre, Medon, Kamath and Kinghorn (1983). Heat stabilities of stevia amino acid sweeteners in acidic, neutral and alkaline solutions (at pH range of 2.5 to 9) at 60°C and 100°C for 2 h were examined by TLC and confirmed by HPLC as described by Chang and Cook (1983).

2.7 Acute toxicity

The oral acute toxicity of stevia derivatives ST-GL and ST-AL was investigated using male mice (20 g each, Medical Research Institute, Alexandria University) according to previously reported methods (Verma, Tripathi, Saxena, & Shanker, 1994). The animals were divided into groups of six mice each.

The compounds were suspended in 1% acacia gum and administered orally in doses of 50, 150 and 300 mg/kg. The mortality percentage in each group was recorded after 24 h. Additionally, the test compounds were investigated for their parenteral acute toxicity in groups of mice of six animals each. The compounds, their vehicle or propylene glycol (control) were given by intraperitoneal injection

2.8. Sensory studies

Sensory tests were carried out in a standardized room. A total of 40 subjects had participated for sensory studies which included 20 trained taste panel staff members and 20 students from the Food Science and Technology Department, Faculty of Agriculture, Alexandria University. The panelists were from both sexes (aged 22–48 years) who volunteered for the study. The subjects were trained adequately in identifying tastes of various test molecules before the commencement of the tests.

2.8.1. Sweetness intensity evaluation

All the solutions were freshly prepared on the day of testing and were presented to each panellist in coded plastic cups and each served at room temperature (22±2°C) according to Wiet and Beyts (1992). For each sample of stevioside, Gly-ST and Ala-ST solutions were evaluated at four sweetness intensity levels of sucrose. These levels were 2%, 5%, 10% and 20% according to the formulae developed by DuBois, Walters, Schiffman, Warwick, Booth and Pecore (1991); Cardello, Da Silva and Damasio (1999); Silva-Bustos, Narváez-Cuenca and Restrepo-Sánchez (2011); Fujimaru, Park and Lim (2012).

Stevioside concentration in all the samples served was based on sucrose equivalence ratio as per the labeling in the commercial stevioside.

2.8.2. Taste attributes evaluation

Panellists rated the samples for five attributes, including sweetness, bitterness, residual aftertaste: i.e. sweet after taste (sweet. AT), bitter (non-sweet) and off aftertaste. The residual aftertaste was measured approximately 20 seconds after swallowing the sample. The scale used was rated from zero to 10 where zero = the absence of the attribute, while 10 = the attribute is extremely intense (Stone & Sidel, 2004).

3. Result and discussion

3.1. General

The HPLC analysis of the commercial stevioside showed that the sample was 97.8% pure.

3.2. Hydrolysis of stevioside

Stevioside (St) **1** was hydrolyzed to the corresponding acid **2** by the use sodium hydroxide in methanol (Scheme 1). The structure of the obtained product was identified by spectroscopic analysis, IR, ^{1}H NMR, ^{13}C NMR and elemental analysis.

3.3. Synthesis of stevia glycine ethyl ester 3

The obtained acid **2** was allowed to condense with the ethyl ester of glycine using EDC.HCl and the potassium salt of Oxyma as a coupling reagent in a dichloromethane water mixture to afford the desired product stevia glycine ethyl ester **3** (Scheme 1).

Recently OxymaPure was introduced, by our research group (Cherkupally et al, 2013), as an additive for peptide bond formation through a new formulation in which the *N*-hydroxylamine group is replaced by a potassium salt. The complete suppression of its acidity converts K-Oxyma into the most suitable coupling choice when peptides are assembled in highly acid-labile solid-supports. The coupling efficiency and epimerization reduction ability are conserved with regard to the parent OxymaPure. In addition, K-Oxyma displays great solubility in water and a variety of organic solvents and is safer than classical 1-hydroxybenzotriazole additives.

The structure of the obtained product **3** was identified by spectroscopic analysis, IR, ^{1}H NMR, ^{13}C NMR and elemental analysis**.** The purity of the target product was further confirmed by HPLC analysis (Figure 1).

3.4. Synthesis of stevia L-alanine methyl ester 4

The obtained acid **2** was allowed to condense with the methyl ester of L-alanine using EDC.HCl and the potassium salt of Oxyma as a coupling reagent in a dichloromethane water mixture to afford the desired product stevia L-alanine methyl ester **4** (Scheme 1). The structure of the obtained product **4** was identified by spectroscopic analysis, IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. The purity of the target product was further checked by HPLC analysis (Figure 2).

3.5. Physical characteristics of stevia amino acid sweeteners

The melting point of stevia amino acid sweetener crystals was determined on a Mel-Temp apparatus and was found to be 190-191ºC and 185-186ºC for ST-GL and ST-AL, respectively. These melting temperature ranges are lower compared

284 to the melting point of stevioside (200-201°C). The melting point of stevioside in
285 our study was slightly higher than that reported by Nishiyama, Alvarez and Vieira
286 (1992) and Avent, Hanson and DeOliviera (1990), who reported the melting point
287 of stevioside as 198°C, which may be related to the compound purity or
288 instrumental effect in both studies.
289 The solubility results indicated that ST-GL and ST-AL samples were soluble in
290 water at 25ºC and soluble in ethanol. On the other hand, the samples were slightly
291 soluble in methanol and insoluble in acetone, chloroform and ether. Stevioside is
292 similarly soluble in methanol and ethanol and insoluble in acetone, chloroform
293 and ether (Soejarto *et al*., 1983; Moussa, Zeitoun, Zeiton & Massoud, 2003).
294 The aqueous solution of stevia amino acid sweeteners showed good stability over
295 a wide range of pH values and temperatures. The thermal treatment in a pH range
296 of 2.5-9 for 2 h showed good stability and no degradation (decomposition)
297 occurred at 60°C and 100°C. Accordingly, the application of stevia amino acid
298 sweeteners as a sweetening agent might be suitable or recommended in baking
299 and other processes involving thermal treatment within the tested temperature
300 range. Buckenhuskers and Omran (1997) reported that stevioside has excellent
301 heat stability up to 100°C for 1 h at pH range 3-9, but rapid decomposition occurs
302 at pH level greater than 9 under these conditions.
303 *3.6. Acute toxicity*
304 Stevia derivatives ST-GL and ST-AL were further evaluated for their oral acute
305 toxicity in male mice using a previously reported method (Verma, Tripathi,
306 Saxena, & Shanker, 1994). The results indicated that the compounds are nontoxic

and well tolerated by experimental animals up to 300 mg/kg. Moreover, these compounds were tested for their toxicity through the parenteral route (Bekhit & Fahmy, 2003). The results revealed that all test compounds were non-toxic up to 150 mg/kg.

3.7. Sensory properties of stevia sweeteners

The sensory properties of stevia-glycine and stevia-L-alanine sweeteners were evaluated relative to sucrose in a simple aqueous system. Trained panelists were provided with different concentrations of sweeteners for evaluating their intensity and equivalency relative to sucrose.

3.7.1. Sweetness equivalency and potency

The sweetness equivalency values of sucrose in water solutions in comparison to different concentrations of stevia, L-alanine, glycine, Hyd. ST, ST-AL and ST-GL are shown in Table 1. The sensory panelists observed that ST-GL was equi-sweet at 0.02, 0.07, 0.15 and 0.28 g/100ml as sucrose at 2.0, 5.0, 10.0 and 20.0 g/100ml, respectively. ST-AL at 0.016, 0.05, 0.09 and 0.24 g/100ml levels promoted the same level of sweetness as 2.0, 5.0, 10.0 and 20.0 g/100ml of sucrose in aqueous solution, respectively. The sweetness equivalency values for samples are shown in Figure 3. The results also indicate that 0.83 g/100ml aqueous solution of hydrolysed stevioside (Hyd. ST) **2** was equivalent to 10% sucrose, indicating 12 times more potency than sucrose. ST-GL at concentration of 0.15% had equal sweetness to a 10% sucrose solution, indicating 67 times more potency than sucrose. Also the results indicate that the 0.09% aqueous solution of ST-AL was equivalent to 10% sucrose, indicating 111 times more

potency than sucrose. The sweetness potency of all sweeteners decreased as the level of sweetener increased.

3.7.2. Taste/Profile

Sweetness intensity scores, sweetness aftertaste and other taste persistent after swallowing were evaluated for different concentrations of the stevia amino acid sweeteners in aqueous solutions and were compared with the sucrose aqueous solution. The results of the corresponding sweetness intensity for stevioside and stevia amino acid sweetener concentration are given in Table 2. The data indicated that stevia amino acid sweetener (ST-GL and ST-AL) solutions appeared to have a clean sweetness taste, and were not bitter.

The panellists observed that stevioside solution had a high score of bitterness and bitterness aftertaste which was more obvious as the concentration of stevioside increased. According to Moussa *et al.* (2003), the aftertaste of stevia sweetener was a mixed taste of sweetenss and bitterness. Comparing stevioside with stevia amino acid sweetener solutions, it is clear that the stevia amino acid sweetener solutions had a lower score of sweetness, while the score of bitterness disappeared. This is probably due to the influence of the sweet aftertaste of amino acids on the non-sweet aftertaste of stevioside. Schiffman *et al.* (1995) found that sweeteners can be used in binary combination to take the advantage of their synergistic effects.

The degree of sweetness of stevia amino acid solutions increased at high concentrations. By increasing the stevia amino acid concentration to more than

353 10% solutions, the aftertaste appeared to be a mixed taste of sweetness and
354 medicine-like tastes.
355
356 **4. Conclusion**
357 New stevia amino acid sweeteners, stevia glycine ethyl ester (ST-GL) **3** and
358 stevia L-alanine methyl ester (ST-GL) **4** were synthesized. Melting point,
359 solubility and heat stability of the new sweeteners were determined. The new
360 stevia amino acid sweeteners are stable in acidic, neutral and alkaline aqueous
361 solutions (at pH range of 2.5 to 9) at 100°C for 2 h. They are soluble in water,
362 ethanol and slightly soluble in methanol. Sensory properties of the new
363 sweeteners were evaluated relative to sucrose in an aqueous system. The
364 sweetness intensity rate of each sweetener was higher than sucrose. Sweetness
365 intensity of ST-GL and ST-AL was about 67 and 111 times more than 10%
366 sucrose solution, respectively. The sweetness intensity of the hydrolysed
367 stevioside (Hyd. ST) **2** was about 12 times more than 10% sucrose, which
368 indicates an improvement in the sweetness intensity due to the introduction of
369 glycine and L-alanine. Stevia amino acid sweeteners (ST-GL and ST-AL)
370 solutions had clean sweetness taste and absence of bitterness compared with
371 stevioside. Therefore, stevia amino acid sweeteners can be utilized as non-caloric
372 sweeteners in the production of low-calorie food for obese and diabetic people, in
373 order to promote caloric balance and to be beneficial for a healthy dietary
374 lifestyle.
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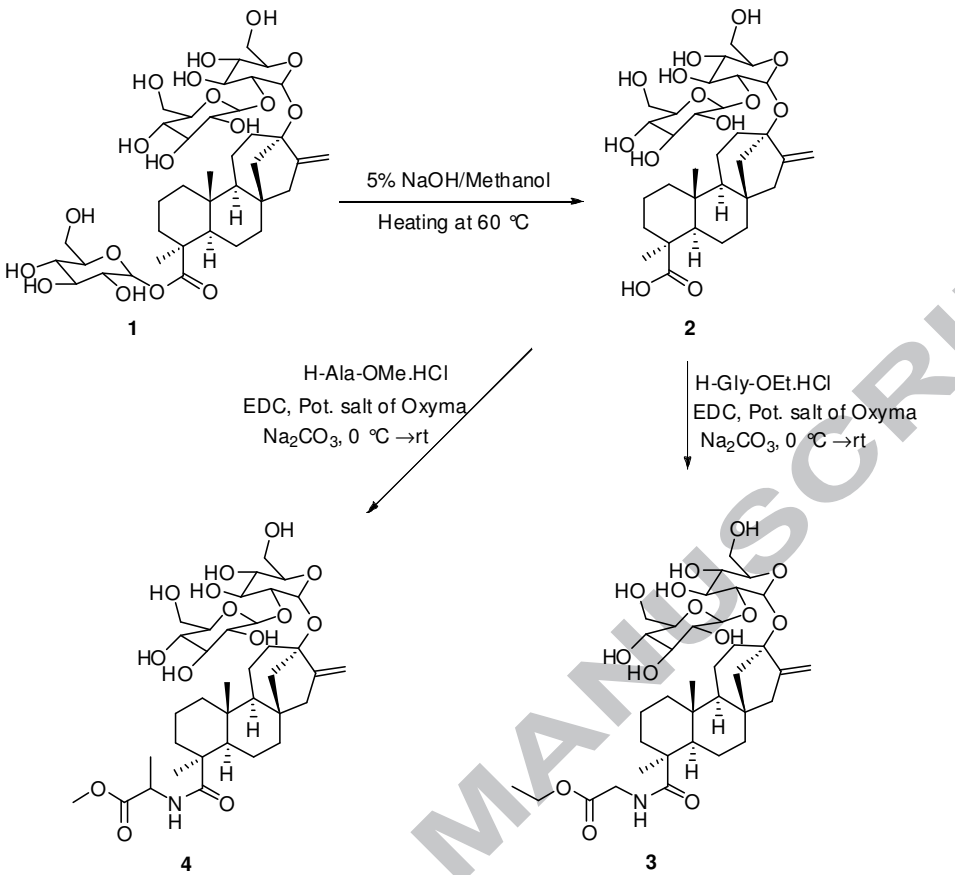

Scheme 1: Hydrolysis of stevioside (St) **1** to the corresponding acid **2**; synthesis of stevia glycine ethyl ester **3** and stevia L-alanine methyl ester **4**

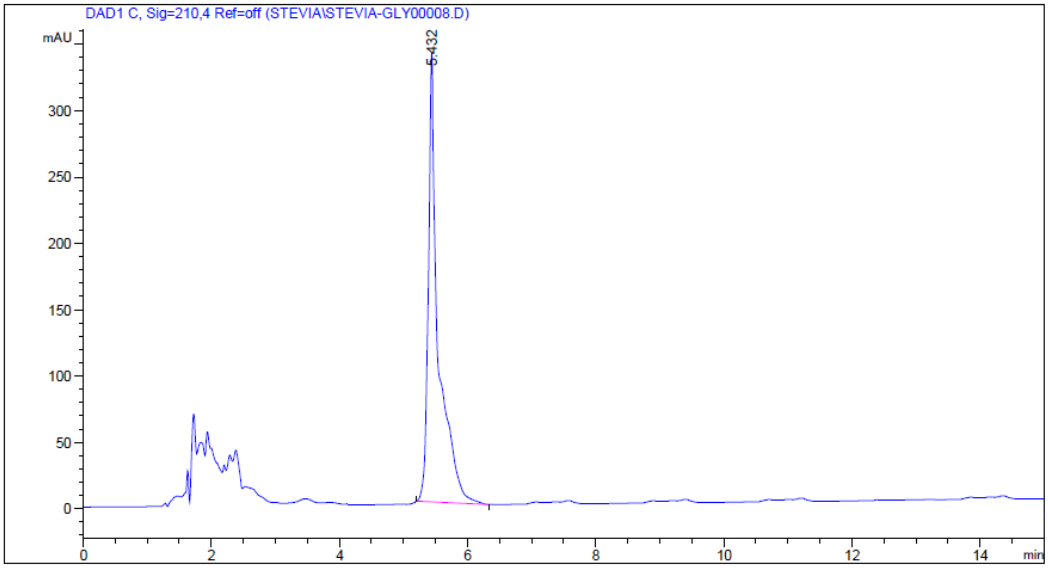

Figure 1: HPLC analysis of the sample of St-Gly-OEt **3**. Conditions: HPLC system linear gradient 84 to 55% CH_3CN/H_2O, H_3PO_4, pH = 5), over 15 min, detection at λ = 210 nm, Agilent 1200 PDA detector, Eclipse plus C18 column (3.5 µm 4.6x250 mm); flow rate 2.0 ml/min. Injection volume: 70 µl. t_R [St-Gly-OEt] = 5.43 min (100%)

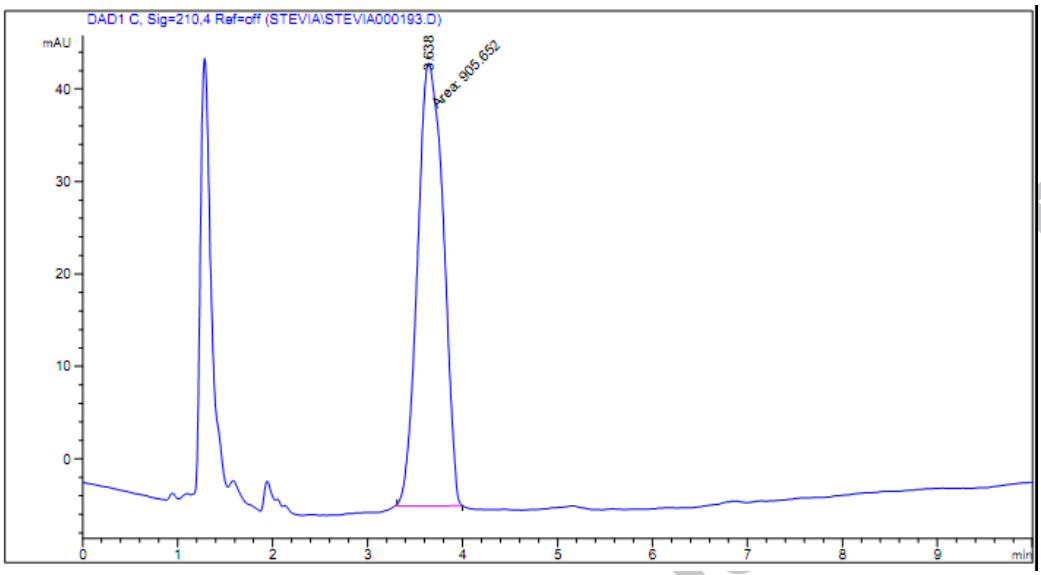

Figure 2: HPLC analysis of the sample of St-Ala-OMe **4**. Conditions: HPLC system linear gradient 84 to 55% CH_3CN/H_2O, H_3PO_4, pH = 5), over 15 min, detection at λ = 210 nm, Agilent 1200 PDA detector, Eclipse plus C18 column (3.5 µm 4.6x250 mm); flow rate 2.0 ml/min. Injection volume: 70 µl. t_R [St-Ala-OMe] = 3.64 min (100%).

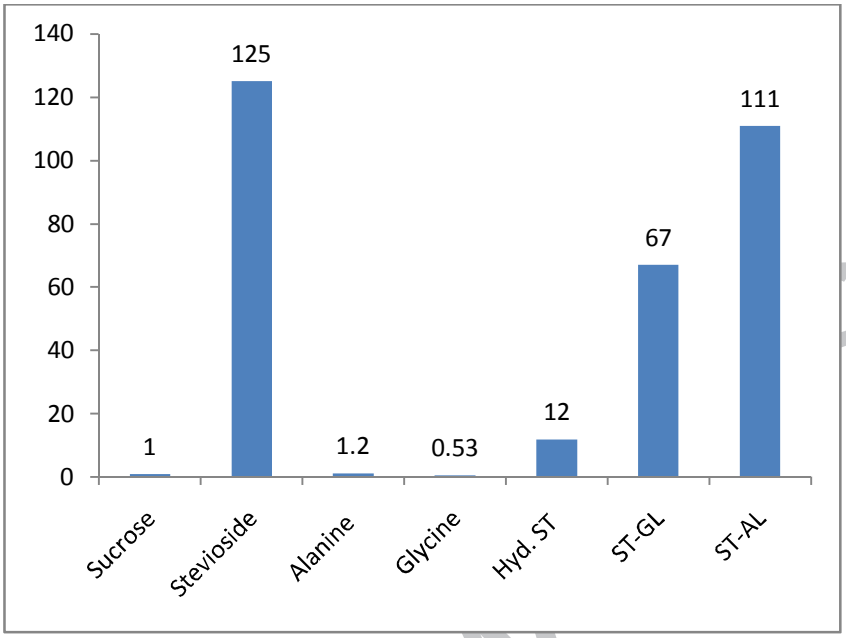

Figure 3. Potency of Stevioside, L-Alanine, Glycine, Hydrolysed Stevioside (Hyd. ST), Stevia-glycine (ST-GL) and Stevia-L-alanine (ST-AL) equivalent to the concentration of 10% sucrose solution.

535 **Table 1.** Equi-sweet concentrations (%) of stevioside (St), L-alanine, glycine,
536 hydrolysed stevioside (Hyd. ST), stevia-glycine (ST-GL) and stevia-L-alanine
537 (ST-AL) tested relative to sucrose equivalent sweetness
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Sweeteners	Equi-sweet concentrations (%)			
539				
Sucrose	2	5	10	20
540 Stevioside	0.01	0.04	0.08	0.12
541 L-Alanine	1.65	3.89	8.15	16.00
Glycine	3.25	8.25	19.50	37.50
542 Hyd. ST **2**	0.09	0.33	0.83	-----
543 ST-GL	0.02	0.07	0.15	0.39
ST-AL	0.016	0.05	0.09	0.24

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560 **Table 2.** Means for the descriptive sensory attributes of taste profile

Sweetener	Sweetness	Bitterness	Aftertaste		
			Sweetness	Bitterness	Other
Concentrations for equivalency at 5%					
Sucrose	6.00	0.0	0.0	0.0	0.0
Stevioside	7.00	0.0	2.83	0.0	Black liquorice
ST-GL	5.88	0.0	0.00	0.0	0.0
ST-AL	6.13	0.0	0.0	0.0	0.0
Concentrations for equivalency at 10%					
Sucrose	8.00	0.0	0.75	0.0	0.0
Stevioside	8.87	4.08	4.83	2.17	Astringency
ST-GL	8.00	0.0	0.00	0.0	0.0
ST-AL	8.13	0.0	0.5	0.0	0.0
Concentrations for equivalency at 20%					
Sucrose	9.50	0.0	2.6	0.0	0.0
Stevioside	5.07	4.08	0.0	5.17	Astringency
ST-GL	7.82	0	0	0	Medicine
ST-AL	8.09	0	1.7	0.0	Medicine

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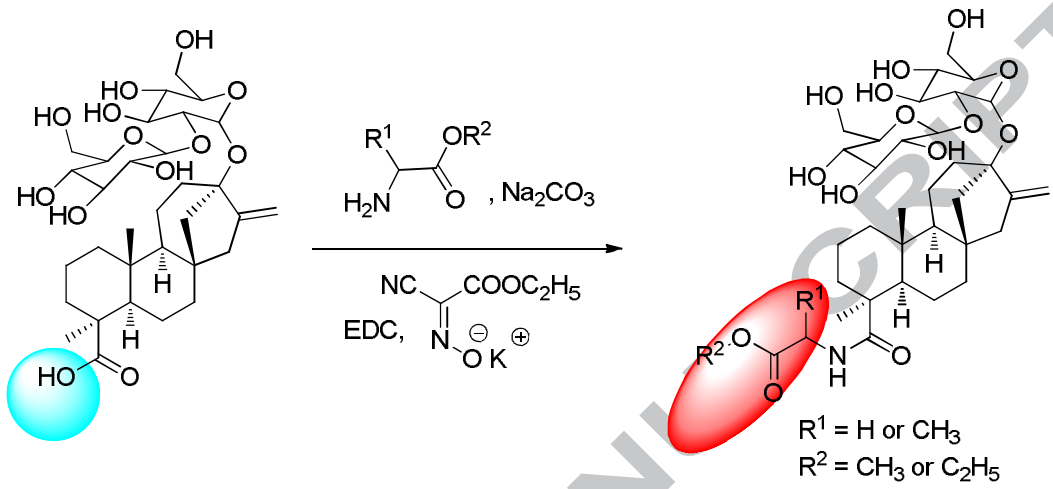
Graphical Absract

Mona I. Massoud*, Sherine N. Khattab*, Yahya El-Sayed Jad, Adnan A. Bekhit, Ayman El-Faham



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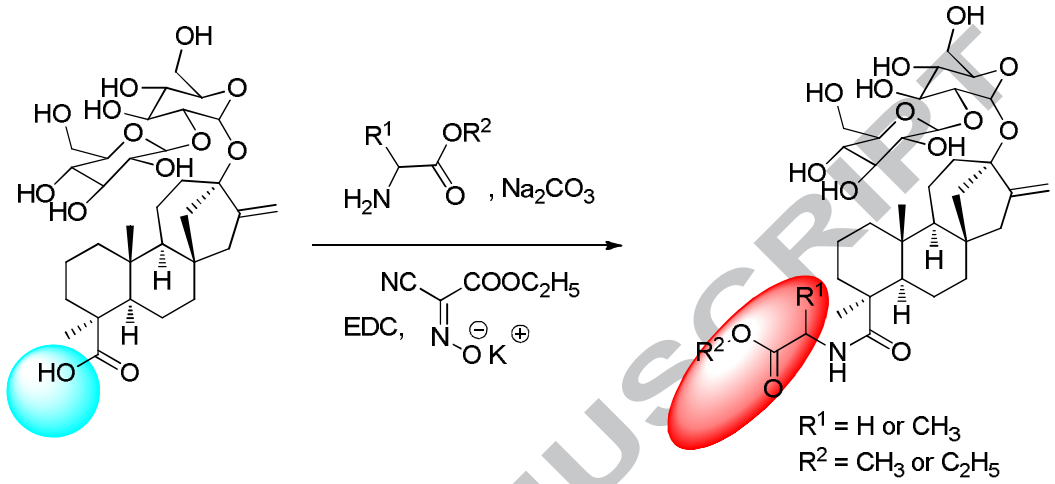

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