

NMR Spectral Analysis of Rebaudioside A, a Major Sweet Diterpene Glycoside of *Stevia rebaudiana* Bertoni at Various Temperatures

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ABSTRACT: Nuclear Magnetic Resonance (NMR) studies of rebaudioside A, one of the major sweet principles isolated from the leaves of *Stevia rebaudiana* Bertoni was performed at four different temperatures -10° C, 5° C, 40° C, and 80° C. The experiments were performed by dissolving the diterpene glycoside rebaudioside A in d5-pyridine and left at respective temperatures for 24 hrs before acquiring NMR spectral data. Experimental results indicated that there were not any notable differences in the ¹³C NMR chemical shift values, though there are few differences in the ¹H NMR chemical shift values of steviol, the aglycone part of rebaudioside A and anomeric protons of its four sugar units.

KEYWORDS: *Stevia rebaudiana*, Rebaudioside A, Diterpenoid glycoside, ¹H and ¹³C NMR spectral data, Temperature studies.

I. INTRODUCTION

The diterpene glycoside 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-β-D-glucopyranosyl ester (**1**) which is also known as rebaudioside A is one of the main steviol glycosides isolated from the leaves of *Stevia rebaudiana* (Bertoni). *S. rebaudiana* is a perennial shrub belonging to the family of Asteraceae (Compositae) native to Brazil and Paraguay, but now grown commercially in a number of countries, particularly in China, Japan, Taiwan, Korea, Thailand and Indonesia¹⁻². Extracts of the leaves of *S. rebaudiana* have been used for decades to sweeten food and beverages in Japan, South America and China, and the constituents in the leaves of *S. rebaudiana* are the potently sweet glycosides which are glycosides of the diterpene steviol, *ent*-13-hydroxykaur-16-en-19-oic acid³. These compounds are also known as Stevia sweeteners of which rebaudioside A tastes about 200-300 times sweeter than sucrose and is non-caloric.

As a part of our continuing research to discover natural sweeteners, we have isolated several diterpene glycosides from the commercial extracts of the leaves of *S. rebaudiana* and their structures were characterized on the basis of extensive 1D (¹H and ¹³C) and 2D (COSY, HSQC and HMBC) NMR as well as high resolution mass spectroscopic data and chemical modifications⁴⁻⁶. In this article, we are describing the ¹H and ¹³C spectral studies of rebaudioside A (**1**, Figure 1) at various temperatures -10° C, 5° C, 40° C, and 80° C in order to understand the stability of **1**. The complete ¹H and ¹³C NMR assignments at all four temperatures were achieved on the basis of 1D (¹H and ¹³C) and 2D (COSY, HSQC and HMBC) NMR as well as high resolution mass spectroscopic data.

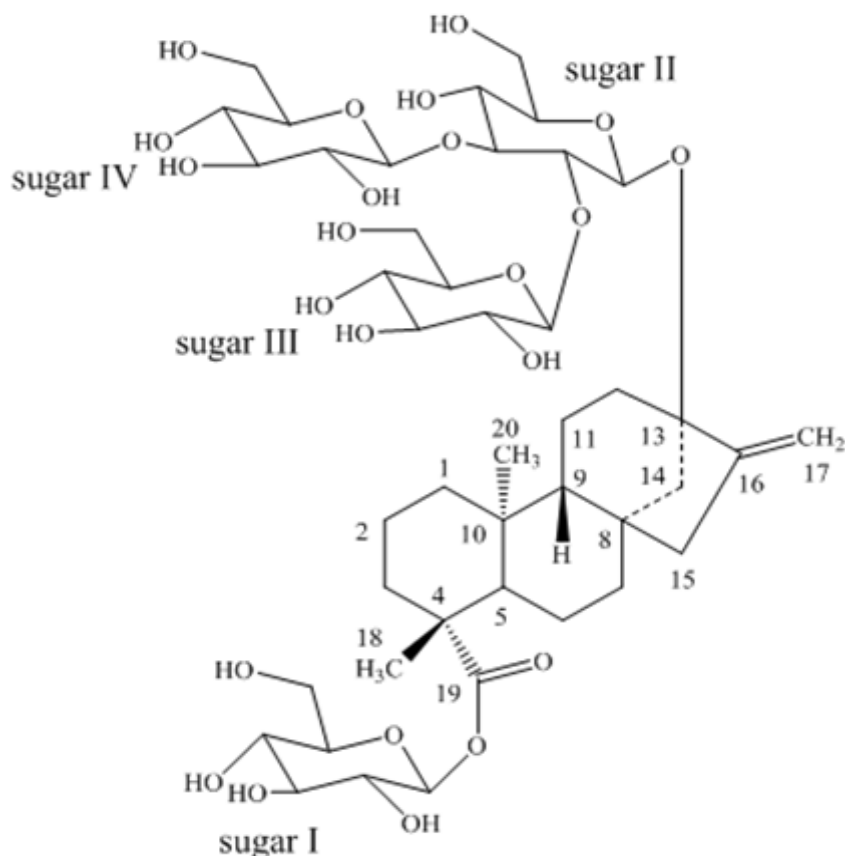


Figure 1: Structure of rebaudioside A (**1**)

II. MATERIALS AND METHODS

General Instrumentation Procedures

Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. Optical rotation was performed using Rudolph Autopol V at 25° C and IR spectral data was acquired using a Perkin Elmer 400 Fourier Transform Infrared (FT-IR) Spectrometer with Universal attenuated total reflectance (UATR) polarization accessory. HPLC analysis was performed using a Dionex UPLC ultimate 3000 system (Sunnyvale, CA), including a quaternary pump, a temperature controlled column compartment, an auto sampler and a UV absorbance detector. Phenomenex Luna NH₂ with guard column, 150x3.0 mm, 3μm (100A) were used for the characterization of rebaudioside A (**1**). NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instrument instruments using standard pulse sequences. The NMR spectra were performed in C₅D₅N; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS data were generated with a Thermo LTQ-FTMS mass spectrometer (100,000 resolutions) equipped with a Nano spray ionization source. Sample was diluted with methanol and introduced via infusion using the onboard syringe pump.

Isolation

Compound **1** was purified by the repeated isocratic elution (72% acetonitrile in water) of the commercial extract of *S. rebaudiana* Bertoni using Dionex Ultra Performance Liquid Chromatography (UPLC) ultimate 3000 system with Phenomenex Luna NH₂ guard column. Collected the peak eluting at *t_R* 3.89 min and dried the aqueous organic layer under nitrogen yielded pure **1**. Comparison of the 1D and 2D NMR, and high resolution mass spectral data of **1** with the reported values confirmed the structure as rebaudioside A⁷⁻⁹.

Identification of 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (Rebaudioside A, **1**)

White powder; mp 240-242 °C; [α]_D²⁵ -31.6 (c 0.5, H₂O); IR ν_{max}: 3355, 2912, 1734, 1125, 1026, 962, 882 cm⁻¹; ¹H NMR (600 MHz, C₅D₅N, δ ppm) spectroscopic data see Table 1; and ¹³C NMR (150 MHz, C₅D₅N, δ ppm) spectroscopic data see Table 2; HRMS: (M+Na)⁺ *m/z* 989.4214 (calcd. for C₄₄H₇₀O₂₄Na: 989.4206).

III. RESULTS AND DISCUSSION

The primary objective of this study is to utilize NMR spectroscopy to find variations in ^1H and ^{13}C NMR spectral values of the steviol glycoside rebaudioside A (**1**) isolated from the leaves of *S. rebaudiana* at various beverage storage temperatures to understand its stability at -10°C , 5°C , and 40°C , and an extreme harsh condition at 80°C . Compound **1** was isolated as a white powder and its molecular formula has been deduced as $\text{C}_{44}\text{H}_{70}\text{O}_{23}$ on the basis of its positive HR mass spectrum which showed an $[\text{M}+\text{Na}]^+$ adduct at m/z 989.4214; this composition was supported by ^{13}C NMR chemical shift values obtained from COSY, HQSC, and HMBC spectral data. The structure of **1** was assigned as 13-[(2-*O*- β -D-glucopyranosyl-3-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid β -D-glucopyranosyl ester based on the spectral and chemical studies that are consistent with the literature data of rebaudioside A⁷⁻⁹.

The 1D (^1H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR of rebaudioside A (**1**) at all four temperatures -10°C , 5°C , 40°C , and 80°C were achieved by dissolving four samples of **1** in 0.75 μL of *d*5-pyridine each. The solutions were kept for 24 hrs at respective temperatures before performing their NMR experiments. The ^1H and ^{13}C NMR values for all the protons and carbons in **1** at four -10°C , 5°C , 40°C , and 80°C were assigned on their respective COSY, HSQC and HMBC correlations and are given in Tables 1 and 2.

Table 1. ^1H NMR spectral data (chemical shifts and coupling constants) of rebaudioside A (**1**) in *d*5-pyridine at various temperatures^{a-c}.

Position	-10°C	5°C	40°C	80°C
1	0.67 (br t, $J=13.2$, 1H), 1.88 (m, 1H)	0.70 (br t, $J=13.6$, 1H), 1.90 (m, 1H)	0.79 (br t, $J=13.4$, 1H), 1.96 (m, 1H)	0.86 (br t, $J=13.2$, 1H), 1.98 (m, 1H)
2	1.42 (m, 1H), 2.23 (m, 1H)	1.43 (m, 1H), 2.21 (m, 1H)	1.46 (m, 1H), 2.25 (m, 1H)	1.44 (m, 1H), 2.23 (m, 1H)
3	1.02 (m, 1H), 1.98 (m, 1H)	1.04 (m, 1H), 1.99 (m, 1H)	1.03 (m, 1H), 1.98 (m, 1H)	1.07 (m, 1H), 2.02 (m, 1H)
5	0.96 (d, $J=12.8$, 1H)	1.01 (d, $J=12.6$, 1H)	1.05 (d, $J=12.8$, 1H)	1.01 (d, $J=12.8$, 1H)
6	2.08 (m, 1H), 2.50 (m, 1H)	2.05 (m, 1H), 2.48 (m, 1H)	2.07 (m, 1H), 2.49 (m, 1H)	2.10 (m, 1H), 2.46 (m, 1H)
7	1.41 (m, 1H), 1.84 (m, 1H)	1.42 (m, 1H), 1.83 (m, 1H)	1.47 (m, 1H), 1.82 (m, 1H)	1.43 (m, 1H), 1.85 (m, 1H)
9	0.78 (d, $J=12.8$, 1H)	0.82 (d, $J=12.6$, 1H)	0.93 (d, $J=12.4$, 1H)	1.10 (d, $J=12.8$, 1H)
11	1.65 (m, 1H), 1.80 (m, 1H)	1.62 (m, 1H), 1.84 (m, 1H)	1.69 (m, 1H), 1.82 (m, 1H)	1.72 (m, 1H), 1.88 (m, 1H)
12	1.58 (m, 1H), 2.53 (m, 1H)	1.60 (m, 1H), 2.51 (m, 1H)	1.65 (m, 1H), 2.46 (m, 1H)	1.52 (m, 1H), 2.45 (m, 1H)
14	1.92 (m, 1H), 2.63 (d, $J=12.6$, 1H)	1.90 (m, 1H), 2.65 (d, $J=12.4$, 1H)	1.94 (m, 1H), 2.65 (d, $J=12.8$, 1H)	1.93 (m, 1H), 2.66 (d, $J=12.4$, 1H)
15	1.65 (m, 1H), 2.18 (m, 1H)	1.68 (m, 1H), 2.23 (m, 1H)	1.72 (m, 1H), 2.25 (m, 1H)	1.76 (m, 1H), 2.26 (m, 1H)
17	4.93 (s, 1H), 5.85 (s, 1H)	4.97 (s, 1H), 5.75 (s, 1H)	5.03 (s, 1H), 5.65 (s, 1H)	5.06 (s, 1H), 5.66 (s, 1H)
18	1.29 (s, 3H)	1.30 (s, 3H)	1.33 (s, 3H)	1.34 (s, 3H)
20	1.19 (s, 3H)	1.21 (s, 3H)	1.27 (s, 3H)	1.32 (s, 3H)
1'	6.16 (d, $J=7.8$, 1H)	6.15 (d, $J=7.6$, 1H)	6.11 (d, $J=7.8$, 1H)	6.09 (d, $J=7.8$, 1H)
2'	3.86 (m, 1H), 4.06 (m, 1H)	3.87 (m, 1H), 4.04 (m, 1H)	3.85 (m, 1H), 4.04 (m, 1H)	3.86 (m, 1H), 4.03 (m, 1H)
3'	3.92 (m, 1H), 4.08 (m, 1H)	3.94 (m, 1H), 4.06 (m, 1H)	3.93 (m, 1H), 4.05 (m, 1H)	3.92 (m, 1H), 4.04 (m, 1H)
4'	4.12 (m, 1H)	4.14 (m, 1H)	4.14 (m, 1H)	4.12 (m, 1H)
5'	3.99 (m, 1H)	3.98 (m, 1H)	4.02 (m, 1H)	3.99 (m, 1H)
6'	4.20 (m, 1H), 4.42 (m, 1H)	4.21 (m, 1H), 4.40 (m, 1H)	4.18 (m, 1H), 4.45 (m, 1H)	4.20 (m, 1H), 4.32 (m, 1H)
1''	5.43 (d, $J=7.4$, 1H)	5.38 (d, $J=7.8$, 1H)	5.30 (d, $J=7.6$, 1H)	5.25 (d, $J=7.6$, 1H)
2''	4.33 (m, 1H)	4.36 (m, 1H)	4.35 (m, 1H)	4.34 (m, 1H)

3''	4.62 (m, 1H)	4.60 (m, 1H)	4.56 (m, 1H)	4.62 (m, 1H)
4''	4.01 (m, 1H)	3.99 (m, 1H)	4.03 (m, 1H)	4.01 (m, 1H)
5''	3.88 (m, 1H)	3.87 (m, 1H)	3.86 (m, 1H)	3.85 (m, 1H)
6''	4.22 (m, 1H), 4.30 (m, 1H)	4.21 (m, 1H), 4.34 (m, 1H)	4.23 (m, 1H), 4.37 (m, 1H)	4.22 (m, 1H), 4.30 (m, 1H)
1'''	5.13 (d, $J=7.6$, 1H)	5.10 (d, $J=7.4$, 1H)	5.05 (d, $J=7.8$, 1H)	5.03 (d, $J=7.6$, 1H)
2'''	4.08 (m, 1H)	4.10 (m, 1H)	4.07 (m, 1H)	4.10 (m, 1H)
3'''	4.22 (m, 1H)	4.20 (m, 1H)	4.24 (m, 1H)	4.22 (m, 1H)
4'''	4.01 (m, 1H)	3.97 (m, 1H)	3.97 (m, 1H)	4.01 (m, 1H)
5'''	3.91 (m, 1H)	3.86 (m, 1H)	3.76 dt (6.6, 12.6)	3.70 dt (6.4, 12.9)
6'''	4.29 (m, 1H), 4.43 (m, 1H)	4.26 (m, 1H), 4.45 (m, 1H)	4.25 (m, 1H), 4.45 (m, 1H)	4.29 (m, 1H), 4.43 (m, 1H)
1''''	5.62 (d, $J=7.8$, 1H)	5.60 (d, $J=7.4$, 1H)	5.55 (d, $J=7.6$, 1H)	5.50 (d, $J=7.8$, 1H)
2''''	4.13 (m, 1H)	4.16 (m, 1H)	4.10 (m, 1H)	4.12 (m, 1H)
3''''	4.47 (m, 1H)	4.45 (m, 1H)	4.47 (m, 1H)	4.47 (m, 1H)
4''''	4.34 (m, 1H)	4.36 (m, 1H)	4.31 (m, 1H)	4.34 (m, 1H)
5''''	4.11 (m, 1H)	4.13 (m, 1H)	4.06 (m, 1H)	4.09 (m, 1H)
6''''	4.23 (m, 1H), 4.52 (m, 1H)	4.24 (m, 1H), 4.53 (m, 1H)	4.22 (m, 1H), 4.48 (m, 1H)	4.33 (m, 1H), 4.49 (m, 1H)

^a assignments made on the basis of COSY, HSQC and HMBC correlations; ^b Chemical shift values are in δ (ppm); ^c Coupling constants are in Hz.

Table 2. ¹³C NMR chemical shift values of rebaudioside A (**1**) in d5-pyridine at various temperatures ^a.

Position	-10° C	5° C	40° C	80° C
1	40.9	41.0	41.3	41.5
2	19.7	19.8	19.9	20.0
3	38.7	38.8	39.0	39.1
4	44.3	44.4	44.5	44.7
5	57.5	57.7	57.9	58.2
6	22.5	22.6	22.7	22.8
7	41.9	42.1	42.3	42.5
8	40.3	40.3	40.4	40.5
9	53.9	54.2	54.6	54.9
10	40.2	40.2	40.3	40.4
11	21.0	21.1	21.2	21.2
12	38.7	38.8	38.9	39.1
13	87.6	88.0	88.7	88.9
14	44.6	44.8	45.0	45.2
15	47.7	47.9	48.3	48.6
16	155.1	154.9	154.7	154.5
17	105.9	105.7	105.4	105.3
18	28.6	28.7	28.9	29.0
19	177.9	177.8	177.5	177.2
20	15.7	15.8	16.1	16.3
1'	96.4	96.4	96.3	96.2
2'	74.2	74.3	74.4	74.6
3'	79.6	79.6	79.6	79.5
4'	71.0	71.2	71.2	71.2
5'	78.9	78.9	78.9	78.9
6'	62.0	62.3	62.6	63.0
1''	99.5	99.2	98.7	98.4
2''	81.8	81.6	81.3	81.1
3''	86.7	86.8	87.0	87.2
4''	71.4	71.4	71.5	71.8
5''	77.3	77.1	77.3	77.8
6''	62.5	62.7	63.3	63.4
1'''	105.2	105.5	105.3	105.3

2'''	75.6	75.7	75.8	75.9
3'''	78.5	78.6	78.6	78.9
4'''	71.6	71.8	72.0	72.0
5'''	79.2	79.3	79.5	79.5
6'''	63.2	63.2	63.5	63.9
1''''	104.7	104.9	105.0	104.9
2''''	79.6	79.5	79.7	79.5
3''''	77.8	77.8	77.9	78.0
4''''	71.5	71.7	71.6	71.9
5''''	80.0	79.9	79.8	79.5
6''''	62.5	62.8	62.5	63.2

^a assignments made on the basis of COSY, HSQC and HMBC correlations

From the above tables 1 and 2, it was evident that though there are differences in the proton chemical shift values at certain positions of rebaudioside A (**1**), whereas not any notable differences in the ¹³C NMR values. The H-1 proton which appeared as a broad triplet showed a low field chemical shift as the temperature increases from -10° C to 80° C (δ 0.67 to 0.86). Similar trend has been observed for the H-9 protons which appeared as doublets (δ 0.78 to 1.10), one of the exocyclic double bonds at C-17 (δ 4.93 to 5.06), and the two methyl signlets at C-18 (δ 1.29 to 1.34) and C-20 (δ 1.19 to 1.32) positions. A reverse trend of high field chemical shift has been observed for the other exocyclic double bond at C-17 (δ 5.85 to 5.66) and all the four anomeric protons of sugar units I (δ 6.16 to 6.09), II (δ 5.43 to 5.25), III (δ 5.13 to 5.03), and IV (δ 5.62 to 5.50).

IV. CONCLUSIONS

We are herewith reporting the complete ¹H and ¹³C NMR spectral assignments for 13-[(2-*O*- β -D-glucopyranosyl-3-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid- β -D-glucopyranosyl ester (rebaudioside A, **1**) that were made on the basis of extensive 1D and 2D NMR at four different temperatures for the first time. Results indicated that there are not any notable differences in the ¹³C NMR values of rebaudioside A at the four temperatures -10° C, 5° C, 40° C, and 80° C, whereas downfield chemical shift has been observed for the H-1, H-9 and one of the exocyclic double bonds of H-17 protons while high field chemical shift is observed for the other exocyclic double bonds of H-17 proton, and the four anomeric protons of sugars I to IV as the temperature increases from -10° C to 80° C.

V. ACKNOWLEDGEMENTS

We wish to thank Dr Wu Shaoxiong, and Dr Bing Wang of Emory University, Atlanta, USA for obtaining some selected spectral data and other chemistry related help.

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