Research Article

Mass Spectra Analyses of Amides and Amide Dimers of Steviol, Isosteviol, and Steviolbioside

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The mass spectra of a series of stevioside analogues including the amide and dimer compounds of steviol, isosteviol, and steviolbioside were examined. Positive ion mass spectral fragmentation of new steviol, isosteviol, and steviolbioside amides and the amide dimers are reported and discussed. The techniques included their synthesis procedures, fast-atom bombardment (FAB), and LC/MS/MS mass spectra. Intense $[M+H]^+$ and $[M+Na]^+$ ion peaks were observed on the FAB and ESI spectra. LC/MS/MS also yielded ES⁺ and ES[−] ion peaks that fairly agreed with the results of the FAB and ESI studies. Mass spectral analysis of compounds **4p-q**, **5a-g**, **6**, and **7** revealed the different cleavage pathway patterns that can help in identifying the structures of steviolbioside and its amide derivatives.

1. Introduction

1.1. Fragmentation Pattern of Steviolbioside Amide Derivatives. The mass spectrometry of these safe and sweet compounds such as steviol, isosteviol, and steviolbioside and their amides and amide dimer derivatives are an interesting current subject. Some reports [1] indicated that more than 50 of kaurane derivatives had been reviewed and presented in terms of specific activities which are antiparasitic, antimicrobial, antifertility, anti-inflammatory, and steroidogenesis. Bruno et al. [2] indicated the semisynthetic of ent-kauranes and the ester form displayed the antifeedant activity on insects. Compadre et al. [3] reported that the mass spectra analysis of this diterpenoid and its analogs revealed differences in stereochemistry, and Hussain et al. [4] used chemical ionization mass spectra to examine steviol and its aglycone. There were many reports to mention the toxicities [5], metabolism [6], bioactivities [7], microbial transformations [8], anti-HIV effects [9], genotoxicity [10], anti-

inflammatory effects [11], and synthesis [12–14] of steviol, stevioside, isosteviol, and their derivatives dimers [15]. In this paper, we report our works on steviol derivatives, steviolbioside, and their synthetic compounds which were examined by a number of mass spectrometric techniques including electron impact (EI), fast atom bombardment (FAB), and electrospray with tandem mass spectrometry LC/MS/MS ESI.

2. Experimental

For the synthetic purpose, steviol, isosteviol, and steviolbioside were prepared from stevioside, which was as obtained parts from plant extracts in China and purchased from Kyowa Foods Co. (Japan) as commercial food additives, via hydrolysis and purification by chromatography. The IR and NMR spectra were identified with those of the authentic sample [8, 12].

In the typical synthesis of amide reactions, alkylamines (1.1 eq) or alkyldiamines (2.1 eq) were added to a solution of

TABLE 1: Main ions observed in FAB mass spectra of compounds 5, 6, and 7.

Compd.	FAB ions: <i>m/z</i> (rel. abundance %)	[M + Na] ⁺ [M+H] ⁺ Cald./Measd.	
5a	<i>n</i> = 2	1331(10), 1332(7), 775(2), 661(10), 643(6), 307(12), 154(100), 137(56), 136(90), 107(39), 95(37), 73(51), 55(81)	1309.6 1331.0
5b	<i>n</i> = 4	1360(4), 1359(5), 690(7), 689(13), 671(6) 625(5), 581(6), 537(7), 493(6), 449(5) 391(6), 308(7), 307(32), 289(22), 255(10) 154(100), 136(95)	1337.6 1360.0
5c	<i>n</i> = 5	1373(32), 1374(24), 1375(9), 1239(3), 1049(4), 817(6), 703(9), 685(7), 619(8), 493(6), 363(5), 154(73), 55(100)	1351.6 1373.0
5d	<i>n</i> = 7	1401(4), 1379(1), 876(2), 731(67), 713(31), 714(18), 391(85), 392(38), 391(85), 308(22), 307(72), 289(63), 279(27), 222(35), 219(21), 154(100), 136(98)	1379.7 1401.0
5e	<i>n</i> = 8	1415(8), 1416(4), 1091(3), 859(3), 745(17), 727(9), 329(12), 307(36), 289(26), 255(19), 154(100), 136(97)	1393.7 1415.0
5f	<i>n</i> = 10	1444(9), 1443(12), 1309(2), 773(5), 619(4), 338(11), 155(20), 154(75), 152(12), 138(25), 137(43), 136(67), 109(27), 105(35), 91(68), 81(52), 77(61), 67(51), 55(100)	1421.7 1443.0
5g	<i>n</i> = 12	1471(4), 1472(2), 1175(1), 1148(2), 915(2), 801(14), 783(7), 619(4), 338(13), 307(14), 155(38), 154(100), 137(67), 136(92), 109(48), 105(55), 77(69), 67(54), 55(82)	1449.8 1471.0
6	<i>n</i> = 2	661 (23), 600(12), 579(9), 359(27), 341(47), 267(32), 154(98), 57(100), 55(85)	660.5 661.5
7	<i>n</i> = 2	661(100), 344(25), 273(52), 177(15)	661.0 661.5

title compounds (1.0 eq) in dried DMF (6 mL) at room temperature. The mixture was cooled to 0°C, and 1-benzotriazolyoxytri (pyrrolidino)phosphonium hexafluorophosphate (PyBOP) (1.1 eq or 2.2 eq) in dimethyl formamide (DMF) was added followed by 5.0 eq of diisopropylethylamine (DIEA). After 12~72 h at room temperature or 60°C, the reaction mixture was evaporated and purified by using silica column chromatography and eluted with a gradient solvent system containing CHCl₃, MeOH, and H₂O. The structures of all derivatives were recorded on 500 MHz NMR, IR, FAB-MS, and LC/MS/MS ESI [16].

High-resolution measurements were obtained by using a High-Resolution Mass Spectrometer (Finnigan/Thermo Quest MAT 95XL). The other resolution experiments were performed on Mass-Spectrometer (JEOL JMX-HX 110 and JEOL JMX-DX 300). For both low- and high-resolution FAB mass spectra were obtained using a JEOL JMX SX/SX 102 A spectrometer. Computerized peak matching was employed with measured masses being within 10 ppm of all calculated values. All the experiments were performed at 70 eV (electron spray ionization, ESI). LC/MS/MS spectra were recorded

using a micromass Quattro II triple-quadrupole mass spectrometer by injection 5 µL of each sample. Argon was used as the collision gas and the collision energy was in the range of 2~9 eV. The samples were dissolved in methanol to proper solutions with concentrations of 5 µg/mL, in 5 µL 50% MeOH) for analysis.

3. Test Compounds

As shown in Scheme 3, sample of compounds 4, 5, 6, and 7 was prepared by previous method.

4. Results and Discussion

A series of substituted steviolbioside amides and amide dimers obtained from the synthesis of title compounds have been investigated by FAB-MS in our laboratory. Some steviolbioside derivative molecules exhibited molecular ion of low intensity and showed the main fragmentation corresponded to the loss of their side chains, such as the sugar adjacent to the nitrogen atom. Steviolbioside 3 has a free carboxylic acid

SCHEME 1: Proposed fragmentation pathway leading to the generation of fragment peaks from steviolbioside-intermediate (linkage with benzotriazolyl).

at the carbon-4 position of skeleton. In order to facilitate the recognition of these compounds, we produced its indolyl amide **4p**, stearyl amide **4q**, and oleyl amide **4r** in good yields (81%–86%). In the same way, the steviolbioside amide dimers **5a–5g**, **6**, and **7** were prepared by using various alkyl-diamines ($n = 2, 4, 5, 7, 8, 10$, and 12) (Scheme 3). Among these diamine derivatives, aromatic diamines did not seem to react with bulky steviolbioside even in their amination reactions. We did not obtain the aromatic amides from this work that might be explained by the weak basicity of the aniline derivatives; the pKa values of the anilines or rather anilinium ions were around 4.8–5.4. In this work, the elec-

tron donor effects seemed to enhance the resultant amination reactions, and we observed that these glycoside sugars did not perturb amide reactions or amide dimer couplings.

Based on the mass spectra, the proposed general fragmentation patterns were elucidated. The parent derivatives, compounds **4p–4r**, **5a–5g**, **6**, and **7** all gave the different spectra FAB $[M+Na]^+$, $[M+H]^+$, LC/MS/MS $[M+Na]^+$ ES⁺, and ES⁻, as shown in Tables 1 and 2.

In the case of the 2-indolyethyl steviolbioside amide **4p**, the fragmentation pathways proposed for the molecule ion $[M+Na]^+$, $[C_{42}H_{58}N_2O_{12}Na]^+$ are shown in Figure 1 and Scheme 2. The main fragmentation pathway can be

SCHEME 2: Proposed fragmentation pathway leading to the generation of Fragment Peaks from steviolbioside amide 4p.

described by two major events. (1) α -Cleavage at the C–C bond resulted in the loss of ethylindolyl, and β -cleavage resulted in the loss of methylindolyl moieties producing the formation of molecules and the simultaneous rearrangement of the resulting fragment-formed ion generation at m/z 664.5 and m/z 347.1. (2) The main peaks at m/z 459.4, 621.5 and m/z 603.4 were, respectively, found to eliminate 2 glucose, 1 glucose, and 1 o-glucose molecules or indolyl from the parent peaks (Figure 1). In the FAB mass spectra of compound 4q, we observed that the main peak was formed by the molecule, $[C_{50}H_{87}NO_{12}]^+$ at m/z 893 and fragment species at m/z 731 and 556. This could have been due to the lost of 1 and 2 glucose molecules. In its LC/MS/MS spectra, we observed an intense peak at m/z 916.6, which was found to be characteristic of the N-stearyl steviolbioside amide 4q,

with $[M+Na]^+$ and the elimination of 1 glucose specie at m/z 754.3. Further loss of the second glucose was occurred mainly through C–C bond β -cleavage with the loss of the branching part of $C_{16}H_{33}$ in the stearyl amide at m/z 365.1. Followed by elimination of water at position C-13, the fragment ion was formed at m/z 347.1 (Table 2). In compound 4r, we observed that the base peak was the 4r molecule $[C_{50}H_{85}NO_{12}]^+$ at m/z 891 in the FAB mass spectra. With the further loss of 1, and 2 glucoses molecules, two product ions at m/z 729 and m/z 567 were produced, respectively. In its LC/MS/MS spectra we observed the molecule peak with Na at m/z 914.5 and some relative intensities of fragment ion peaks at m/z 858.4 (loss of a butyl species of oleyl), at m/z 752.5 (loss of 1 glucose from the m/z 774.6 fragment ion), and at m/z 612.6 (loss of 2 glucoses molecules and Na from the molecule peak). We also

FIGURE 1: LC/MS/MS spectra of Steviolbioside **4p** $[M+Na]^+$ and $[M-H]^-$.

observed a small fragment species at m/z 365.2; this could have been due to the loss of 2 glucose molecules and the C–C bond β -cleaved to amine nitrogen with loss of the $C_{16}H_{31}$ moiety from the oleyl group followed by loss of water (at m/z 347.2). An interesting fragment was observed at m/z 207.0

that was assumed to occur through γ -cleavage from the side chain of oleyl. In its ES^- fragmentation pattern we observed some intense fragment species at m/z 864.4 and m/z 836.4; and it was assumed that they were the result of the loss of ethyl and butyl molecules of side chains of oleyl.

FIGURE 2: LC/MS/MS spectra of Steviolbioside dimer **5a** [M+Na], steviol amide dimer **6** [M+Na], and Isosteviol amide dimer **7** [M+Na].

TABLE 2: LC/MS/MS/ESI determination of main fragmentation pathway for compounds 1–7.

Compds	Precursor ion mass [M+Na] ⁺		Product ion masses (intensity%)
	ESI+	ESI–	
1	357.3		357.2(100), 342.6(3), 299.5(2), 224.6(2), 197.1(4)
2		317.3, 318.3	317.3(100), 273.2(63), 271.1(43), 119.9(6), 109.0(8), 93.9(5)
3	665.4, 666.4		665.3(100), 547.3(3), 507.3(5), 363.3(2), 347.2(3), 151.0(21), 104.9(12)
		641.4, 642.4, 643.4	641.3(30), 479.3(100), 461.3(10), 371.4(2), 338.9(12), 317.2(78), 118.9(18), 112.8(35), 100.8(90), 58.9(60)
4p	807.5, 808.5		807.4(100), 687.7(4), 645.5(6), 347.1(17), 251.3(3), 184.9(5)
		783.5, 785.4	783.4(46), 621.4(98), 603.4(7), 459.4(100), 220.9(2), 112.7(3), 100.8(5)
4q	916.6, 917.6,		916.4(100), 796.7(3), 754.3(4) 347.1(35), 185.0(5)
		892.5	892.6(100), 730.5(3), 568.5(5), 161.0(50), 100.8(35)
4r	914.5,		914.5(100), 794.5(6), 752.4(18), 347.1(28)
		890.5, 891.5	566.5(20), 161.0(35), 112.8(43), 100.8(100), 70.9(33)
5a	1331.7, 1332.7, 1333.7		1331.7(100), 1169.7(20), 1007.2(35) 998.1(5), 887.5(2), 619.1(4)
5b	1360.0, 1360.9, 1361.9		1359.7(100), 1197.5(3), 1035.6(4) 1335.6(100), 1173.6(40), 1011.5(21), 849.6(7), 687.5(100)
5c	1374.1, 1375.1, 1376.1 1349.6	1335.8, 1336.8, 1337.8	1373.6(100), 1211.5(5), 1049.6(7) 1349.6(38), 1187.5(25), 1025.7(20), 863.7(6), 701.6(100), 412.1(23)
5d	1402.2, 1403.1, 1404.1		1401.7(100), 1239.7(15), 1077.8(30), 915.6(22), 609.5(18)
		1377.9, 1378.9, 1379.9	1377.8(3), 1215.7(2), 729.7(100), 370.6(15), 112.9(18), 100.9(40)
6	683.5, 684.5		661.4(31), 643.5(10), 344.4(12) 273.2(48), 255.2(100)
7	683.6		683.5(100), 273.4(30), 255.0(20), 177.0(30),

4.1. Fragmentation Pattern of Amide Dimers of Steviolbioside.

The LC/MS/MS spectra produced from the steviolbioside derivatives **5a**–**5g** revealed the most favorable fragmentation processes with losing the initial 1, 2, 3, and 4 glucose molecules from the [M+Na]⁺ ion, followed by elimination of Na and water (at position C-13) and side chains to from the ion peak, such as for **5a** ($n = 2$) at m/z 1169.7, 1007.6, 845.6, 683.7, and 665.6 (loss of water) (Figure 2, Table 2); **5b** ($n = 4$) at m/z 1173.7, 1011.7, 849.7, and 687.7; **5c** ($n = 5$) at m/z 1212.0, 1049.9, 887.8, and 725.8, and **5d**

($n = 7$) at m/z 1215.8, 1053.8, 891.8, and 729.8. In the FAB⁺ mass spectra [M+Na][±] of **5e**, **5f**, and **5g**, was at m/z 1415, 1443, and 1471. Loss of 4 glucose molecules and Na occurred at m/z 745, 773, and 801. A similar fragmentation pathway of **5a**–**5g** has been proposed for the steviolbioside amide dimer in FAB⁺ spectra, such as **5a** at m/z 1164, 983, 822, and 660; **5b** at m/z 1196, 1034, 870, and 710, listed in Table 1. In these fragment peaks, we found that the elemental composition of C₉H₁₆NO was the base peak at m/z 154, and it can be assumed that the fragmentation of the C–C bond

SCHEME 3: Synthesis of amide analogues of steviol, steviolbioside, and isosteviol.

via α -cleavage of the side chains occurred. In the LC/MS/MS spectra of compound **6**, we observed an intense peak at m/z 683.5 that was the characteristic of steviol amide dimer ($n = 2$) molecule $[\text{M}+\text{Na}]^{\pm}$. Its base peak at m/z 600.5 was probably formed by the loss of two products $[\text{CH}_2\text{CO}]^{\pm}$ and $[\text{CH}_2\text{CCH}_2]^{\pm}$, which were produced by the opening of the C/D ring of steviolbioside. We also observed in the daughter spectrum that the molecular peak was at 661.4 and loss of 1 H_2O molecule was at m/z 643.5. In the parent peak at m/z 273.2 and the base peak there was the loss of a water molecule at m/z 255.2. In compound **7**, we checked the FAB^+ mass spectrum at m/z 661 as a base peak $[\text{M}+\text{H}]^+$ and the parent peak at m/z 273 and its LC/MS/MS spectra of the isosteviol amide dimer ($n = 2$). It seemed to be the same fragmentation pattern as compound **6**. $[\text{M}+\text{Na}]^{\pm}$ peak was at m/z 683.6 and there was a loss of Na at m/z 661.6, followed by losing of $[\text{CH}_2\text{CO}]^{\pm}$ at the D ring to form the fragment species at m/z 641.6 and to produce an intense second-generation ion at m/z 451.4. The C–N bond was then cleaved to form the peaks at m/z 344.3 and 273.2. It probably was formed by the cleavage of the C–C bond resulting in the formation of species “d” (Scheme 1) at m/z 121.2 and 151.0 followed by the loss of Na to form the base peak at m/z 128.8. (Scheme 1).

In conclusion, due to the observed fragmentation pattern pathways, cleavage formed and their generated peaks further encourage us to check the synthetic products in biological

modification that will be useful in future studies on natural products.

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