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STRUCTURE ELUCIDATION OF THREE TRITERPENOID SAPONINS FROM *ALPHITONIA ZIZYPHOIDES* USING 2D NMR TECHNIQUES

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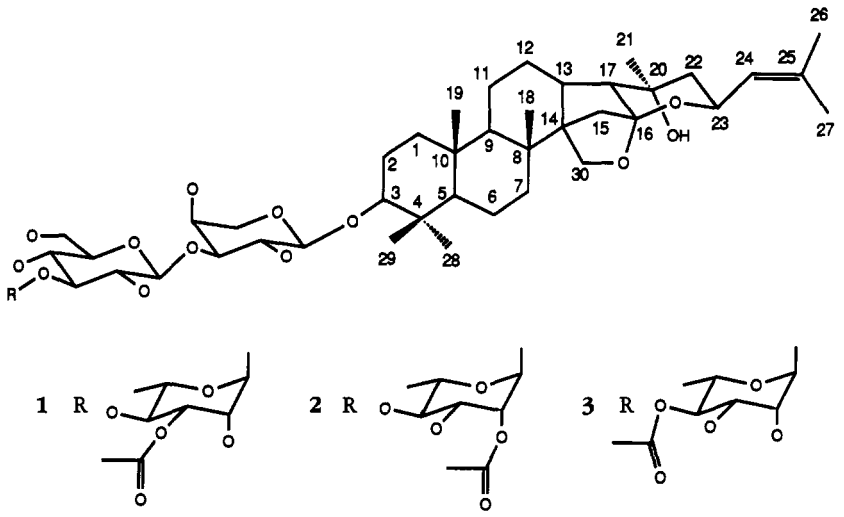
ABSTRACT.—High-field nmr experiments have been used to determine the structures of three new saponins, zizyphoisides C, D, and E [**1–3**], isolated from *Alphitonia zizyphoides*, a medicinal plant found in the Samoan rain forest. The saponins consist of the aglycone, jujubogenin, to which are attached three sugar units and an unsaturated side-chain. The three compounds are similar except for the location of an acetyl group on one of the sugar units.

Currently we are studying the active chemical components isolated from Samoan medicinal plants. In this paper we wish to report on the molecular structures of three novel triterpenoid saponins, zizyphoisides C, D and E [**1–3**]. These compounds were obtained from *Alphitonia zizyphoides* (Rhamnaceae), found in the rain forests of the Samoan islands. The indigenous Samoan people have used this plant for centuries as a tonic and as a treatment for a variety of abdominal disorders (1,2). An extract from this plant was found to be highly active in in vivo and in vitro bioassays (for example, 2 mg/ml produced 100% inhibition of electrically induced contractions on guinea-pig ileum). Detailed results of the pharmacological tests carried out on these compounds are described



elsewhere (3,4). The structures of these compounds were determined using mass spectrometry and by 1D and 2D nmr techniques.

RESULTS AND DISCUSSION

The 1D and 2D nmr spectra of all three saponins were obtained using 10–15 mg of material dissolved in 0.7 ml of deuterated pyridine. By running a quantitative ^{13}C-nmr experiment, with a long pre-pulse delay to allow for full relaxation and gated decoupling to eliminate nOe effects, forty-nine carbon signals were observed for each of the three unknown compounds (for **1**, two of the carbon signals were found to be coincident at 105.28 ppm). A molecular weight value of 954.5130 for all three compounds was determined using hrfabms. These data enabled us to establish the molecular formula as $C_{49}H_{78}O_{18}$ (theoretical molecular mass: 954.5184 daltons). The ^{1}H-nmr spectra of all three compounds were very similar. In the ^{1}H-nmr spectrum of **1** it was seen clearly that there were nine methyl groups, one of which (a doublet at 1.36 ppm) must have an adjacent methine group, while the remaining eight were connected to quaternary carbons. A singlet occurring at 2.15 ppm was typical of an acetyl methyl group. All these conclusions were confirmed by ^{13}C- and DEPT nmr experiments, and from the latter the presence of nine CH_3 groups, eleven CH_2 groups, twenty-one CHs, and eight quaternary carbon atoms was established. The carbon resonances were associated with the corresponding proton signals using a HETCOR experiment, and the results are provided in Tables 1 and 2.

Sugar analysis of all three samples gave the same result, namely, D-glucose, L-rhamnose and L-arabinose in a 1:1:1 ratio. In addition to the molecular ion, the most important mass spectral fragments for all three samples were observed at *m/z* 911, 765, and 603. This pattern supplied useful clues to the overall molecular structure, since *m/z* 911 represents a loss of an acetyl group (43 mass units) from the parent molecular ion, *m/z* 765 indicates the loss of a rhamnose unit with an attached acetyl group (189 mass units), and *m/z* 603 represents the loss of a glucose molecule together with an acetylated rhamnose unit. With the acetyl group attached to one of the sugar units, the oligosaccharide moiety was calculated to have an elemental formula of $C_{19}H_{30}O_{14}$. This left a unit represented by $C_{30}H_{48}O_4$ for the remaining aglycone segment of the molecule and the proposed structure for the whole molecule is shown as **1**.

An alkylidene carbon signal at 127.137 ppm and a quaternary signal at 134.184 ppm indicated a double bond between C-24 and C-25. These data, together with the aglycone formula, and its hydrogen deficiency of 7, suggested a six-ring triterpenoid structure similar to that found for another compound (code-name PT-2) from this plant (5). A careful comparison of both ^{1}H- and ^{13}C-nmr spectra for the aglycone moiety of PT-2 showed that they were very similar to those for **1–3** for carbons C-1 to C-19 and for C-28 to C-30. The structure of the remaining part of the aglycone of **1–3** was established using a combination of COSY and long-range HETCOR nmr experiments. These results are shown in Figure 1, where the dashed lines indicate the observed ^{1}H-^{1}H couplings and the solid arrows indicate the ^{1}H-^{13}C couplings.

The structure of the aglycone was found to be identical with that of a known compound, jujubogenin, which has been isolated from *Bacopa monniera* (6), for which the structure of a derivative has been confirmed by chemical evidence as well as by X-ray crystallography (6,7). Table 1 includes complete assignments of the ^{1}H-nmr spectrum and a comparison of the literature ^{13}C-nmr chemical shifts with the present values. The agreement was acceptable once the literature uncertainty in assigning C-8, C-10, C-15, C-18, C-19, C-27, and C-29 was clarified. At this point it was obvious that the three compounds [**1–3**] were isomers with differences only in their sugar side-chain. Compari-

TABLE 1. ^{1}H- and ^{13}C-Nmr Assignments of the Aglycone Moiety of Compound **1**.[a]

Position	Type (DEPT)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm) jujubogenin[b]
1	CH_2	39.08	0.80,1.54	38.8
2	CH_2	26.85	1.86, 2.13	28.1
3	CH	88.17	3.30 dd	77.8
4	C	39.74		39.5
5	CH	56.44	0.73 d	55.9
6	CH_2	18.35	1.68, 2.10	18.3
7	CH_2	36.11	1.33, 1.49	36.1
8	C	37.60		37.5
9	CH	53.09	0.88	53.0
10	C	37.34		36.7
11	CH_2	21.80	1.34, 1.54	21.7
12	CH_2	28.56	1.79, 1.92	28.6
13	CH	37.18	2.68 br	37.0
14	C	53.79		53.8
15	CH_2	36.91	1.54, 2.50 d	36.1
16	O-C-O	110.62		110.4
17	CH	54.03	1.43	53.9
18	CH_3	18.94	1.11 s,	18.5
19	CH_3	16.45	0.78 s	16.3
20	C-O	68.55		68.4
21	CH_3	30.07	1.39 s	30.0
22	CH_2	45.48	1.38, 1.75	45.3
23	CH-O	68.65	5.24 t	68.5
24	CH=	127.14	5.57 d	126.9
25	C=	134.18		134.0
26	CH_3	25.58	1.68 s	25.5
27	CH_3	18.34	1.71 s	18.9
28	CH_3	27.99	1.25 s	28.6
29	CH_3	16.95	1.13 s	16.3
30	CH_2-O	65.87	4.20, 4.30 d	65.8

[a]Assignments were aided by DEPT, COSY, and HETCOR experiments. The chemical shift values are presented in ppm and referenced to TMS.

[b]Data taken from Kawai and Shibata (6).

son of the spectra of these three saponins with that of PT-2 indicated, in each instance, that the sugars are attached to the C-3 position of the aglycone(s). The overlapping of proton signals within the sugar prevented the proton coupling network from being traced out by a COSY or a TOCSY nmr experiment. In order to facilitate unambiguous assignments, spectra of the following reference compounds (in pyridine-d_5) were taken and used: (1) methyl-α-L-rhamnopyranoside; (2) methyl-α-L-arabinopyranoside, and (3) methyl-β-D-glucopyranoside. The proton and carbon signals for all the sugar units in **1**–**3** were compared with the data for the model systems. The complete assignments are summarized in Table 2.

To establish the nature of the sugar sequences required analysis of data from several different experiments. The average ^{13}C-nmr longitudinal relaxation times (T_1) of the methine carbons of each sugar unit of compound **1** are shown in Table 2. Rhamnose has the largest T_1 value (0.31 sec), whereas arabinose has the shortest value (0.25 sec). Because longer T_1 relaxation times are generally associated with greater segmental motion, this suggests that the rhamnose is probably the farthest away from the more rigid aglycone entity while arabinose is the closest. The latter deduction was supported by the absence of a mass spectral peak corresponding to the single loss of an arabinose

TABLE 2. ^{1}H- and ^{13}C-Nmr assignments of the Sugar Units of **1–3** and T_1 Relaxation Times of Specific Carbon Nuclei.[a,b]

Compound	1			2		3		Reference[c]		ΔC13[d]
	^{13}C	^{1}H	T_1	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Arabinose										
A1 ...	105.3	(4.83, d)	0.25	104.8	(4.84)	105.0	(4.83)	104.6	(4.69)	+0.8
A2 ...	70.0	(4.76)	0.26	70.3	(4.74)	70.1	(4.74)	72.6	(4.66)	−2.6
A3 ...	74.9	(4.70)	0.25	75.0	(4.69)	74.6	(4.69)	70.8	(4.57)	+4.1
A4 ...	67.0	(4.76)	0.23	68.6	(4.50)	68.5	(4.51)	68.4	(4.49)	−1.4
A5 ...	66.0	(3.79, 4.25)		66.4	(3.74, 4.44)	66.1	(3.73, 4.26)	66.2	(3.54, 4.17)	−0.2
Glucose										
G1 ...	105.3	(5.11, d)	0.27	105.3	(5.11)	105.6	(5.10)	105.5	(5.01)	−0.2
G2 ...	78.6	(3.97)	0.23	78.5	(3.98)	78.5	(3.97)	75.0	(3.92)	+3.4
G3 ...	83.9	(4.32)	0.31	83.4	(4.28)	83.3	(4.26)	78.9	(4.11)	+5.0
G4 ...	71.6	(4.18)	0.28	71.6	(4.16)	71.5	(4.14)	71.5	(3.87)	+0.1
G5 ...	78.5	(4.22)	0.29	78.1	(4.26)	78.3	(4.19)	78.3	(4.03)	+0.2
G6 ...	62.8	(4.37, 4.50)		62.7	(4.32, 4.51)	62.6	(4.33, 4.51)	62.7	(3.97, 4.84)	+0.1
Rhamnose										
R1 ...	101.3	(6.40, s)	0.35	101.8	(6.32)	101.7	(6.32)	101.8	(6.45)	−0.5
R2 ...	72.3	(4.59)	0.30	71.7	(6.08)	74.9	(4.77)	72.4	(4.76)	−0.1
R3 ...	76.0	(5.79)	0.31	73.9	(4.67)	73.7	(4.67)	72.3	(4.60)	+3.7
R4 ...	74.1	(4.18)	0.34	74.0	(4.18)	76.1	(5.89)	74.1	(4.20)	0.0
R5 ...	69.1	(4.61)	0.28	69.0	(4.58)	68.8	(4.49)	69.5	(4.71)	−0.4
R6 ...	17.9	(1.36)		18.5	(1.63)	18.5	(1.64)	18.8	(1.61)	−0.9

[a]Chemical shift values are presented in ppm and referenced to TMS.
[b]T_1 data are expressed in sec.
[c]Reference data were obtained for model sugars (see text).
[d]Data represent ^{13}C-nmr chemical shift difference between **1** and reference sugar.

unit from the parent ion. The downfield ^{13}C-nmr shifts of the C-3 positions of the sugars (e.g., rhamnose, 76.0 ppm compared with the expected 72.3 ppm shift in the reference compound; arabinose, 74.90 ppm compared with 70.8 ppm; and glucose, 83.93 ppm compared with 78.9 ppm) indicate the probable points of glycosidic linkages in the oligosaccharide. Direct evidence for the sugar sequence and their linkage sites was further obtained by observing the through-space coupling between two protons residing one on each side of the respective linkages. This was carried out using both ROESY (8) and TOCSY (9) experiments. The genuine nOe coupling peaks were obtained by taking differences between the TOCSY and ROESY data. The sugar portion of a ROESY spectrum is illustrated in Figure 2, and the nOe peaks critical to the structural assignments are marked on the spectrum with corresponding sugar linkages indicated on the accompanying scheme.

A reliable and relatively easy method (10) for determining the location of *O*-acetyl groups on a monosaccharide is to measure ^{1}H-nmr chemical shifts of the protons attached at the possible substitution sites. Significant downfield shifts (by more than 1 ppm) are associated with acetyl substitution. Table 2 shows that, for **1**, the acetyl group is attached

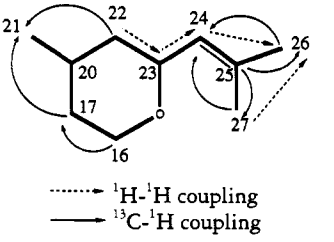


FIGURE 1. COSY and Long-Range HETCOR Observations for a Portion of the Aglycone of **1–3**.

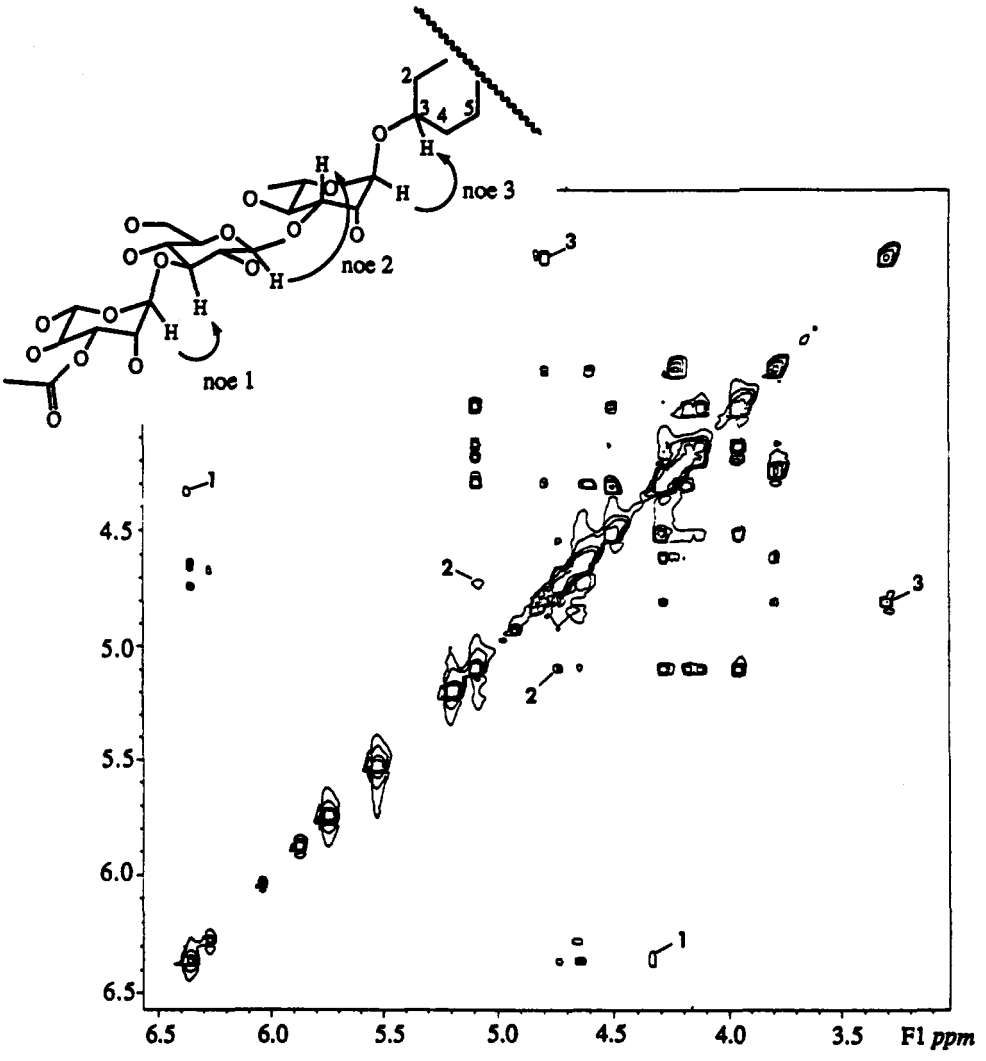


FIGURE 2. Through-space ^{1}H-^{1}H NOe Responses in the ROESY Nmr Spectrum and the Corresponding Glycosidic Bonding Locations in **1**.

to C-3 of the rhamnose unit; for **2**, the acetyl attachment is on C-2 of rhamnose; and in **3**, the acetyl group is located on C-4 of rhamnose. The configuration at the anomeric carbon atoms of the sugars (β for glucose; α for the other two sugars) was established from the chemical shifts of the anomeric carbons as well as the multiplicities of the anomeric protons (see Table 2). Thus, these three saponins are shown to be isomers with identical elemental formulae, differing only in the location of the acetyl group on the terminal rhamnose unit. Compound **1** is 3-*O*-acetyl-α-L-rhamnopyranosyl (1→3)-β-D-glucopyranosyl (1→3)-α-L-arabinopyranosyl (1→3) jujubogenin, and **2** and **3** are the 2-*O*-acetyl and 4-*O*-acetyl isomers of **1**, respectively. These structures are similar to that of colubrin (11), which was isolated from *Colubrina asiatica*, another plant found in many of the Pacific islands. The structural differences involve a different sugar in the trisaccharide moiety, the glycosidic linkages, and the location of the acetyl group (11).

Whether compounds **1–3** are all biosynthesized by their plant species of origin or

whether these isomers are generated from one of the compounds during isolation and purification still remains unclear. Such migration of an acetyl group from one carbon atom to another within the sugar unit has been suggested by other investigators (12).

EXPERIMENTAL

MASS SPECTRA.—The primary negative-ion fabms were obtained using a MAT 731 mass spectrometer and xenon gas. The high-resolution negative-ion fabms peaks were obtained on a VG 70-SEQ (EBqQ) spectrometer using angiotensin 11 inhibitor (purchased from Chemlog, South Plainfield, NJ) as the internal reference compound ($[M-H]^-=1030.51102$). A few hundred micrograms of the unknown compounds were dissolved in 100 μl of MeOH. One microliter of each solution was mixed with an equal volume of glycerol matrix in preparation for the fabms measurements.

NMR SPECTRA.—All nmr spectra were obtained with a Varian VXR-500 spectrometer equipped with a Sun 4/360 work-station. Samples were run at 26° in 5 mm tubes at concentrations of between 10–15 mg/0.7 ml of pyridine-d_5 with a trace of TMS as a reference. ^{1}H-Nmr chemical shifts were measured with respect to TMS whereas ^{13}C-nmr chemical shifts were reported with reference to the pyridine signal at 123.5 ppm. ^{1}H- and ^{13}C-nmr spectra were acquired using 90° pulse times of 11.2 and 10.8 μsec, respectively, with corresponding pulse recycle times of 2.5 and 3.0 sec. Quantitative ^{13}C-nmr spectra were obtained with gated decoupling to suppress the nOe effects.

The T_1 relaxation times of all carbon nuclei were measured with the inversion-recovery method. A pre-pulse delay of 100 sec was used to guarantee full magnetization recovery between acquisitions. The samples were purged with dry N_2 gas before measurement to eliminate O_2.

In COSY experiments, the f_1 and f_2 spectral width was 4 kHz, and the (t_1, t_2) data matrix was zero-filled to 2048 by 2048 to give a final resolution of 4 Hz per point. Sine-bell and Gaussian apodization were used in the f_1 and f_2 dimensions respectively, to improve line shapes.

For HETCOR and long-range HETCOR experiments, the spectral widths were typically 4 kHz in the f_1 (^{1}H) dimension and 20 kHz in f_2 (^{13}C). The (t_1, t_2) data matrix was zero-filled to 1024 by 2048 data points to give a digital resolution of 8 Hz per point in the f_1 dimension and 20 Hz per point in the f_2 dimension. Processing for all these experiments was done using Gaussian apodization. For the long-range HETCOR experiment, a coupling constant of 6 Hz was used for J_{nh}.

For ROESY and TOCSY experiments, the spectral width in both the f_1 and f_2 domains was 5 kHz. For ROESY experiments acquisitions were performed with mixing times of 100, 300 and 600 ms. The digital resolution for both ROESY and TOCSY spectra was 5 Hz per point in both dimensions.

PLANT MATERIAL.—*Alphitonia zizyphoides* (Spreng.) A. Gray was found near Aleisa village, Upolu island, Western Samoa. The native name is "toi," and the plant is used as an anti-inflammatory tonic. Voucher specimens (voucher number COX 840) are deposited in the BYU herbarium (BRY) and in the Gray herbarium at Harvard (GH).

ISOLATION PROCEDURES.—Dried EtOH extracts (4 g) of the bark of *A. zizyphoides* were dissolved in H_2O (500 ml), and the H_2O-soluble fraction was separated by centrifugation and extracted three times with EtOAc. The H_2O-insoluble fraction (800 mg) of the EtOH extract was dissolved in MeOH. It was then separated using flash chromatography (Si gel), and eluted with $CHCl_3$ followed by stepwise addition of MeOH (in 5% increments up to 50%, then in 10% increments) up to 100% MeOH. Fractions (200 ml) were monitored by tlc [Si-gel; $CHCl_3$-MeOH-H_2O (9:6:1) v/v]; the plates were visualized by spraying with vanillin/H_2SO_4 followed by heating. The fractions were further purified by prep. tlc and reversed-phase hplc [Polygosil RP-18, MeOH:H_2O:MeCN (5:2:3) v/v]. Because of their very similar structures, zizyphoides C, D and E [**1–3**] were only partially separated from one another. The whole procedure was repeated several times to produce about 20 mg of each component at a purity level of about 80%.

SUGAR ANALYSIS.—A purified sugar sample (0.1 mg) was dissolved in a few drops of 2M HCl and heated at 100° in a stoppered vial for 30 min. Into this reaction mixture, two drops of DMF and *bis*-trimethyltrifluoroacetamide (BSTFA) were added and the vial kept at 70° for 15 min. The derivatized sugars were then analyzed by gc.

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