Structure Elucidation of Triterpenoid Saponins Found in an Immunoadjuvant Preparation of *Quillaja brasiliensis* Using Mass Spectrometry and $^1$H and $^{13}$C NMR Spectroscopy

Federico Wallace 1, Carolina Fontana 2,* C, Fernando Ferreira 1,3,* and Cristina Olivaro 1,*

**Abstract:** An immunoadjuvant preparation (named Fraction B) was obtained from the aqueous extract of *Quillaja brasiliensis* leaves, and further fractionated by consecutive separations with silica flash MPLC and reverse phase HPLC. Two compounds were isolated, and their structures elucidated using a combination of NMR spectroscopy and mass spectrometry. One of these compounds is a previously undescribed triterpene saponin (Qb1), which is an isomer of QS-21, the unique adjuvant saponin employed in human vaccines. The other compound is a triterpene saponin previously isolated from *Quillaja saponaria* bark, known as S13. The structure of Qb1 consists of a quillaic acid residue substituted with a $\beta$-D-Galp-(1→2)[$\beta$-D-Xylp-(1→3)]-$\beta$-D-GlcpA trisaccharide at C3, and a $\beta$-D-Xylp-(1→4)-$\alpha$-L-Rhap-(1→2)-[$\alpha$-L-Arap-(1→3)]-$\beta$-D-Fucp moiety at C28. The oligosaccharide at C28 was further substituted at O4 of the fucosyl residue with an acyl group capped with a $\beta$-D-Xylp residue.

**Keywords:** *Quillaja brasiliensis*; immunoadjuvant saponins; structural analysis; NMR spectroscopy; QS-21 isomer

1. Introduction

*Quillaja brasiliensis* (A. St.-Hill. & Tul.) Mart. (*Quillajaceae*) (Qb) is an endemic tree species of South America. It belongs to a very restricted botanical family that includes a single genus (*Quillaja Molina*) with only two currently accepted species, with the other one being *Quillaja saponaria* Molina (Qs) [1].

Saponins from *Quillaja* plants present similar chemical and biological properties, being the immunoadjuvant and immunoestimulant activities the most relevant. These saponins, either alone or in colloidal formulations, have proved effective to generate both humoral and cellular response against the co-administered antigens, thus becoming important compounds for vaccine development [2–9].

Qs saponins are employed in the manufacture of vaccines for human and veterinary use, and the bark of this tree is one of the main sources of triterpene saponins worldwide [10,11]. Quil-A®, a commercial mixture of various saponins obtained from the bark extract of Qs, is used in veterinary vaccines, but it has not been considered for inclusion in human formulations due to its high reactogenicity. However, QS-21, a mixture of two isomeric saponins (QS-21Xyl and QS-21Api) that are present in Quil-A® (Figure 1), has been shown to be less reactogenetic than the latter while maintaining adjuvant properties. It has been tested as adjuvant in human vaccines either for the prevention or treatment of diseases such as cancer, HIV, tuberculosis, Alzheimer’s, and COVID-19 [12–15]. Currently,
there are two licensed vaccines for human use that contain the AS01 adjuvant, which is a combination of QS-21 with monophosphoryl lipid A and liposomes: MosqirixTM, a malaria vaccine approved in 2015 for use in children living in areas where this disease is endemic, and ShingrixTM, an herpes zoster vaccine approved in 2018 [16,17]. It is worth pointing out that QS-21 induces a strong Th1/Th2 immune response with cytotoxic T lymphocytes (CTL) production in a relatively short time, as compared with other adjuvants [18].

Figure 1. Chemical structures of saponins Qb1 (top) and S13 (bottom) isolated from Fraction B of Quillaja brasiliensis. QS-21Xyl and QS-21Api (top) isolated from Quillaja saponaria.

Even though the study of Qb saponins started relatively later, Qb represents a natural renewable alternative source of these products, since saponins are also abundant in the leaves. It has been previously shown that the aqueous extract and some purified fractions obtained from leaves of Qb have immunoadjuvant activity comparable to that of Quil-A® [19,20]. The adjuvant potential of Qb saponins has been confirmed in experimental vaccines against different viruses in murine models. These studies have been performed with the aqueous extract, purified fractions, and nanoparticles derived from purified fractions, which have been formulated with and without the presence of antigen [2,3,19–24]. Recently, we presented the first structural studies of an immunoadjuvant fraction of saponins obtained from leaves, named Fraction B (FB), using direct infusion and liquid chromatography/electrospray ionization ion trap multiple stage mass spec-
trometry (DI-ESI-IT-MS and LC-ESI-IT-MS) in combination with classical methods of monosaccharide and methylation analysis [25,26]. Forty-eight bidesmosidic saponins, bearing five types of triterpenic aglycones including quillaic acid, hydroxylated quillaic acid (22β), gypsogenin, phytolaccinic acid and its O-23 acetate, were preliminarily characterized. In a continuation of the characterization of saponins from FB, we performed the isolation and structure elucidation of one undescribed triterpene saponin, which is isomer of QS-21, named Qb1, and other saponin previously reported in Qs, known as S13 [27]. Their structures were elucidated using a combination of mass spectrometry (ESI-MS) and NMR spectroscopy methods.

2. Results and Discussion
2.1. Saponin Qb1

Three enriched saponins fractions (B1, B2, and B3) were collected after chromatographic separation of FB [25,26] on a silica flash medium-pressure liquid chromatography (MPLC) column. Fraction B3 was further purified by semi-preparative-high performance liquid chromatography (HPLC) on a reverse phase column, affording a previously undescribed triterpenic saponin named Qb1. This compound was analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) and showed a chromatographic peak with a retention time of 24.8 min, while Qs saponin QS-21 showed a peak at 28.1 min, demonstrating that the commercial standard of QS-21 used herein is not a mixture of the two isomers (QS-21Xyl and QS-21Api) but one of them. The multiplicity-edited 1H,13C-HSQC spectrum of QS-21 showed in the region of the anomeric resonances seven cross-peaks corresponding to pyranosyl residues (δH/δC 5.37/94.9, 5.18/100.8, 4.79/103.8, 4.61/104.9, 4.52/105.7, 4.47/107.1 and 4.38/104.5) and one cross-peak corresponding to a furanosyl residue (δH/δC 4.98/108.7); the latter is in accordance with the presence of a α-L-Araf residue [28]. The lack of another residue in the form of furanose (i.e., the anomeric carbon resonance of the β-D-Api residue of QS-21Api isomer is expected at δC~112 ppm) implies that the commercial standard contains QS-21Xyl as a major component. Qb1 and QS-21Xyl exhibited not only the same molecular mass but also remarkable similar MS2 spectra (Figure 2B,C, respectively), obtained using identical experimental conditions. In a preliminary study [25], this saponin (Qb1) was characterized using LC-MS and corresponded to the saponin 14 mentioned in the original work. The MS spectrum of Qb1 showed a deprotonated pseudomolecular ion at m/z 1988.0 [M-H]-. A general structure of Qb1 is depicted in Figure 2A, which takes into consideration the most conserved structural features of Qs saponins reported previously. The MS2 spectra of the precursor ions [M-H]- of Qb1 and QS-21Xyl are shown in Figure 2B,C, respectively. In the case of Qb1, the daughter ion m/z 955.6 (fragment a) is consistent with a saponin composed of a quillaic acid residue substituted with a trisaccharide moiety at C3, in which X0 is a pentose residue. Furthermore, the daughter ion m/z 1511.7 (fragment b) indicates that the α-L-Rhap-(1→2)-β-D-Fucp moiety is substituted with two pentose residues, but their exact location could not be determined using solely MS spectrometry (all the possible substitution positions are indicated as X1, X2, X3 and X4). Finally, the daughter ions m/z 1553.6 and 1725.8 (fragments c and d, respectively) are consistent with fragmentations of the Fa(I) and Fa(II) acyl chains, whereas the pseudomolecular ion m/z 1988.0 reveals that the acyl chain is capped with a pentose residue (X5). The above data are for Qb1 (Figure 2B), and a similar analysis can be carried out for QS-21Xyl (Figure 2C).
Figure 2. General structure of QS-21 and isomers (A). Negative mode MS$^2$ spectra of Qb1 and QS-21Xyl (B, C, respectively) obtained from the precursor ion [M-H]$^-$ at m/z 1988.0 and 1988.1, respectively. The most relevant daughter ions are assigned in the generic structure shown in A. The $X_0$ and $X_5$ substituents correspond to pentose residues in both Qb1 and QS-21Xyl. Two additional pentose residues are substituting either the Rha or Fuc residues in both compounds, but their exact positions could not be determined solely by the MS$^2$ data (note that in this case all the possible substitution positions are indicated as $X_1$–$X_4$).

A combination of 1D and 2D NMR experiments, such as $^1$H, $^1$H-TOCSY, $^1$H, $^1$H-NOESY, multiplicity-edited $^1$H, $^13$C-HSQC, $^1$H, $^13$C-H2BC, $^1$H, $^13$C-HSQC-TOCSY, $^1$H, $^13$C-HSQC-NOESY and $^1$H, $^13$C-HMBC, were performed and permitted the total assignment of all the $^1$H and $^13$C signals of Qb1 in methanol-$d_4$ solution. The proton spin systems were determined
by analysis of $^1$H,$^1$H-TOCSY spectra with increasing mixing times, using the anomic proton signals as a starting point for the assignments. In some cases, $^1$H,$^1$H-NOESY spectra were also used to establish intra-residue connectivities in residues with the galacto-configuration (i.e., assignment of H5 protons). The $^{13}$C signals were assigned using one-bond proton–carbon correlations from multiplicity-edited $^1$H,$^{13}$C-HSQC spectra and two-bond heteronuclear correlations from the $^1$H,$^{13}$C-H2BC spectra. The assignments carried out using homonuclear experiments ($^1$H,$^1$H-TOCSY and $^1$H,$^1$H-NOESY) were also confirmed using $^1$H,$^{13}$C-HSQC-TOCSY and $^1$H,$^{13}$C-HSQC-NOESY spectra. The anomic configurations of the sugar residues were established using $^3$$^J_{H1,H2}$ values measured directly from the $^1$H NMR spectrum anomic proton signals [29,30] or $^1$$J_{C1,H1}$ values measured from the residual coupled C1/H1 crosspeaks in the $^1$H,$^{13}$C-HMB-C spectra. The inter-residue correlations were determined using $^1$H,$^1$H-NOESY and/or $^1$H,$^{13}$C-HMB-C spectra.

Quillaic acid was identified as the triterpene aglycone of the saponin by NMR spectroscopic data (Table S1) and by comparison with literature data [28,31]. The $\delta_c$ values observed for C3 (86.4) and C28 (177.3) were also consistent with the bidesmosidic nature of this saponin and with our previous preliminary characterization by mass spectrometry [26,27].

The multiplicity-edited $^1$H,$^{13}$C-HSQC spectrum of Qb1 revealed eight resonances in the anomic region (Figure 3B). The anomic protons resonated as doublets at $\delta$ 5.41 ($J = 8.1$ Hz), 5.29 ($J = 1.9$ Hz), 4.80 ($J = 7.1$ Hz), 4.59 ($J = 7.7$ Hz), 4.47 ($J = 7.7$ Hz), 4.43 ($J = 8.6$ Hz), 4.41 ($J = 6.7$ Hz), and 4.27 ($J = 7.7$ Hz) in the $^1$H NMR spectrum. In the multiplicity-edited $^1$H,$^{13}$C HSQC spectrum, these protons correlated to the carbon signals at $\delta_c$ 95.1, 101.8, 103.8, 104.9, 107.0, 104.6, 105.6 and 104.2, respectively.

In the $^1$H,$^1$H-TOCSY spectrum recorded with the longest mixing time (Figure S1), the residues with the anomic resonances at 5.41 and 4.80 ppm showed correlations from H1 to H2–H4, revealing that they have the galacto-configuration (i.e., the correlations from H1 to H5 and H6 are not observed due to the small $^3$$J_{H1,H5}$ value). Both monosaccharides showed intra-residue $^1$H,$^1$H-NOESY correlations between H1 and H5, as well as large $^3$$J_{H1,H2}$ values (8.1 and 7.1 Hz, respectively), indicating that they are found in a $\beta$-pyranose form. In the former monosaccharide, two intra-residue correlations could be observed in the $^1$H,$^1$H-NOESY spectrum from H4 (5.29 ppm) to H5 (3.85 ppm) and H6 (1.08 ppm); the chemical shift of H6 revealed that this is a 6-deoxyhexose, and thus it is the $\beta$-D-Fucp residue. The other monosaccharide showed a $^1$H,$^1$H-TOCSY correlation from H5 to the H6a and H6b protons (3.73 and 3.76 ppm, respectively) and thus it can be assigned to the $\beta$-D-Galp residue. The H1 resonance at 5.29 ppm showed a single correlation to H2 (3.96 ppm) in the aforementioned $^1$H,$^1$H-TOCSY spectrum, revealing that the monosaccharide has the manno-configuration (i.e., the small $^3$$J_{H1,H2}$ and medium $^3$$J_{H2,H3}$ values hamper the magnetization transference from H1 to protons beyond H2); however, the remaining protons in this spin system (H3–H6) could be assigned using the correlations from H2 observed in the same spectrum. In this case, the low chemical shift of H6 (1.30 ppm) is consistent with a 6-deoxyhexose, and thus this is the $\alpha$-L-Rhap residue. In addition, the chemical shifts of this residue are remarkably similar to those reported previously for the 4-O-substituted $\alpha$-L-Rhap residue of QS-21Xyl [30]. The $^1$$J_{C1,H1}$ value (172 Hz), determined from the residual coupled C1/H1 crosspeak in the $^1$H,$^{13}$C-HMB-C spectrum, is consistent with this monosaccharide adopting an $\alpha$-pyranose configuration [32]. The monosaccharide with H1 at 4.43 ppm showed $^1$H,$^1$H-TOCSY correlations from the anomic proton to H2–H5, revealing that this monosaccharide has a gluco-configuration. The large H1–H2 coupling constant ($J = 8.6$ Hz) is then consistent with a $\beta$-pyranose form and thus this residue can be assigned to the $\beta$-D-GlcPA. According to the MS data (Figure 1A), the remaining residues should all be pentoses. In the $^1$H,$^1$H-TOCSY spectrum (Figure S1), the residues with the anomeric protons at 4.59, 4.47, and 4.27 ppm showed patterns consistent with Xylp residues (i.e., all protons correlations from H1 to H2–H5 could be traced in the spectrum recorded with $\tau_m$ 100 ms). In all three cases, intra-residue correlations from C5 to H1 were observed in the $^1$H,$^{13}$C-HMB-C spectrum, confirming that the monosaccharides are in the pyranose form; the large $^3$$J_{H1,H2}$ values (7.7 Hz) indicate that these are all $\beta$-D-Xylp residues. Finally,
in the residue with the anomeric proton resonance at 4.41 ppm, $^1$H,$^1$H-TOCSY correlations from H1 to H2–H4 could be identified, but no correlations were observed from H1 to $H_5^{eq}$ in the spectrum recorded with the longest mixing time (Figure S1); the H4 proton of this residue shows a sharp resonance, comparable to that of H4 of Fuc$p$ and Gal$p$, which is consistent with this pentose being an Ara$p$ residue (i.e., it is expected that $^3J_{H3,H4}$, $^3J_{H4,H6^{ax}}$, and $^3J_{H4,H6^{eq}}$ have medium to small values [33]). Furthermore, key correlations observed in the $^1$H,$^1$H-NOESY spectrum between H1-H5, and three-bond heteronuclear correlations observed in the $^1$H,$^1$H-HMBC spectra from C5 to H1, confirmed that this residue is indeed adopting a pyranose form; based on the value of $^3J_{H1,H2}$ (6.7 Hz), this residue can be assigned to an $\alpha$-L-Ara$p$. It is worth pointing out that, in the saponins of Q. saponaria reported previously, the arabinose residues have always been found in furanose forms.

**Figure 3.** Selected regions of the multiplicity-edited $^1$H,$^1$C-HSQC spectrum of Qb1 showing the ring and hydroxymethyl groups (A) and the anomeric region (B). In the first figure (A), the CH$_2$ groups correlations appear in red.
The $^1$H and $^{13}$C chemical shifts of the two oligosaccharide chains from Qb1 are compiled in Table 1. All the monosaccharides identified herein are consistent with the monosaccharide analysis performed previously on FB [26]; the absolute configuration was assumed according to the saponins of the related species Q. saponaria. The substitution positions were deduced based on the high downfield chemical shifts at the substitution positions, in comparison to the respective free monosaccharides; thus, the C2, C3, and C4 chemical shifts of the Fuc residue (75.1, 81.9, and 74.7 ppm, respectively) are consistent with a trisubstituted monosaccharide $→$2,3,4-$\beta$-D-Fucp-$\text{→}$, the C2 and C3 chemical shifts of the GlcA residue (78.3 and 86.7 ppm, respectively) reveal a two-substituted monosaccharide $→$2,3-$\beta$-D-GlcPa-$\text{→}$, and C4 of the Rha residue (84.1 ppm) indicate that this monosaccharide is $\text{→}$4-$\alpha$-L-Rhap-$\text{→}$. All pentoses, as well as the galactose residue, are expected to be unsubstituted, since no significant glycosylations shifts were observed apart from C1. The sequence of the two oligosaccharide chains and their connection to C3 and C28 of the aglycone were obtained from $^1$H,$^{13}$C-HMBC and $^1$H,$^1$H-NOESY experiments (Table 1). The oligosaccharide linked to C3 of the quillaic acid moiety was characterized as $\beta$-$\text{D-Galp}$-$\text{→}$2-$[\beta$-$\text{D-Xylp}$-$\text{→}$3]-$\beta$-$\text{D-GlcpA}$, since three-bond heteronuclear correlations were observed in the $^1$H,$^{13}$C-HMBC spectrum from the anomeric protons of GlcA, Gal and Xyl(I) to the respective substitution positions (i.e., C3 of the quillaic acid moiety, and C2 and C3 of the GlcA residue, respectively). The chemical shifts of this trisaccharide moiety (Table 1) are consistent with those reported in bibliography for Q. saponaria saponins that share the same structural element [30,33]. In addition, the quillaic acid plus this trisaccharide were consistent with the MS$^2$ spectrum, which showed an ion at $m/z$ 955.6 (Figure 2A). The $^1$H,$^{13}$C-HMBC spectrum showed a cross peak at δ 5.41 (H1 of Fuc)/177.3 (C28 of Qa) confirming that Fuc residue is linked to C28 of the aglycone. Correlations observed in the $^1$H,$^{13}$C-HMBC spectrum (Figure S2) from the anomeric protons of the Rha and Ara residues to C2 and C3 of the fucosyl residue, and from H1 of Xyl(II) to C4 of the Rha residue are consistent with the following tetrasaccharide structure: $\beta$-$\text{D-Xylp}$-$\text{→}$4-$\alpha$-$\text{L-Rhap}$-$\text{→}$2-$\alpha$-$\text{L-Arap}$-$\text{→}$3]-$\beta$-$\text{D-Fucp}$. All these data are also consistent with the correlations observed in the same spectrum from C1 of the aforementioned monosaccharides to the respective protons at the substitution positions, as well as those observed in the $^1$H,$^1$H-NOESY spectrum (Table 1). The $\beta$-$\text{D-Fucp}$ residue is also substituted at O4 with a dimeric C9 acyl group capped with Xyl(III); the latter was demonstrated from the $^1$H,$^{13}$C-HMBC correlation from H4 of the fucosyl residue to the carbon signal at δC 172.9 (C1 of the acyl group Fa(I)). All $^1$H and $^{13}$C signals for the acyl group were assigned (Table S1) and compared with bibliographic data of Q. saponaria saponins [28,31], resulting in a 3,5-dihydroxy-6-methyloctanoic acid moiety. Unlike the Q. saponaria saponins (that have an $\alpha$-$\text{L-Araf}$ residue attached to this acyl group) (Figure 1), saponin Qb1 has a $\beta$-$\text{D-Xylp}$ residue, which was confirmed from the $^1$H,$^{13}$C-HMBC correlation from the anomeric proton at δ 4.27 (H1 of Xyl(III)) to the carbon signal at δC 80.2 (C5 of the acyl group Fa(II)). The loss of 476.3 Da in the MS$^2$ spectrum corresponds to the loss of the entire acyl group with an attached pentose, as described above (Figure 2). Consequently, the structure of Qb1 (Figure 1 top) consists of a quillaic acid moiety substituted with the trisaccharide $\beta$-$\text{D-Galp}$-$\text{→}$2-$[\beta$-$\text{D-Xylp}$-$\text{→}$3]-$\beta$-$\text{D-GlcpA}$ at C3, and the tetrasaccharide $\beta$-$\text{D-Xylp}$-$\text{→}$4-$\alpha$-$\text{L-Rhap}$-$\text{→}$2-$[\alpha$-$\text{L-Arap}$-$\text{→}$3]-$\beta$-$\text{D-Fucp}$ at C28. The latter oligosaccharide is further substituted at O4 of the fucosyl unit with a glycosylated acyl group terminated by a $\beta$-$\text{D-Xylp}$. 
Table 1. \(^1\)H and \(^{13}\)C NMR chemical shifts (ppm) of the two oligosaccharide chains from Qb1, and inter-residue correlations from \(^1\)H,\(^1\)H-NOESY and \(^1\)H,\(^{13}\)C-HMBC spectra. The spectra were recorded in CD\(_3\)OD at 25 °C on a Bruker Avance 500 MHz spectrometer.

| Residue     | Abbreviation | \(^1\)H/\(^{13}\)C | \(^1\)H,\(^{13}\)C-HMBC | \(^1\)H,\(^1\)H-NOESY |
|-------------|--------------|----------------|--------------------------|---------------------|
| Qa C3-O-glycan | →2,3)-β-D-GlcA-(1→ | GlcA | 4.43 [8.6] 3.65 3.67 3.49 3.65 | 62.2 | C3, Qa \(^{(a)}\) H3, Qa |
|             |              | 104.6 103.8 104.9 | 73.6 75.3 75.4 70.9 76.7 | n.d. | H3, Qa \(^{(a)}\) H2, GlcA |
| β-D-Galp-(1→ | Gal          | 4.80 [7.1] 3.44 3.43 3.81 3.48 3.73, 3.76 | 3.81 3.43 3.81 3.48 | C2, GlcA |
| β-D-Xylp-(1→ | Xyl(I)       | 4.59 [7.7] 3.23 3.30 3.49 3.18, 3.89 | 3.30 3.23 3.30 3.49 | C3, GlcA |
| Qa C28-O-glycan | →2,3,4)-β-D-Fucp-(1→ | Fuc | 5.41 [8.1] 3.89 4.00 5.29 3.85 1.08 | 16.9 | C28, Qa \(^{(a)}\) H2, Fuc |
|             |              | 95.1 [164] 101.8 [172] | 75.1 71.9 72.2 | 71.2 69.2 | H2, Fuc |
| →4)-α-L-Rhap-(1→ | Rha          | 5.29 [1.9] 3.96 3.80 3.56 3.77 1.30 | 3.80 3.96 3.80 | C4, Rha |
| β-D-Xylp-(1→ | Xyl(II)      | 4.47 [7.7] 3.20 3.30 3.49 3.18, 3.89 | 3.30 3.20 3.30 | C5, Fa(II) \(^{(a)}\) H4, Rha |
| β-D-Xylp-(1→ | Xyl(III)     | 4.27 [7.7] 3.18 3.30 3.49 3.18, 3.89 | 3.30 3.18 3.30 | C5, Fa(II) \(^{(a)}\) H5, Fa(II) \(^{(a)}\) |
| α-L-Arap-(1→ | Ara          | 4.41 [6.7] 3.54 3.51 3.78 3.49, 3.84 | 3.51 3.54 3.51 | C3, Fucp |
|             |              | 105.6 104.2 104.2 | 72.5 75.2 75.2 | 69.5 66.9 | H3, Fucp |

\(^3\)J\(_{\mathrm{H1,H2}}\) values are given in hertz in square brackets and \(^1\)J\(_{\mathrm{C1,H1}}\) are given in braces. \(^{(a)}\) Chemical shifts of these atoms are shown in Table S1 in the Supplementary Materials.
2.2. Saponin S13

Fraction B1 was further purified by semi-preparative HPLC on reverse phase column, yielding a pure saponin that was analyzed by LC-MS. This compound eluted at a retention time of 29.8 min and showed a deprotonated pseudomolecule ion [M-H]− at m/z 1559.7. We previously identified this saponin in the FB extract of Qb using LC-MS (cf. saponin 1 in the original work [25]) and tentatively assigned it to S13, a saponin previously reported in Qs [27] (Figure 1 bottom).

A combination of 1D and 2D NMR experiments, such as multiplicity-edited 1H,13C-HSQC, 1H,13C-TOCSY, 1H,13C-HMBC, 1H,13C-H2BC, and 1H,13C-NOESY were performed and permitted the total assignment of all the 1H and 13C signals in methanol-d4 solution. The 1H and 13C chemical shifts for the aglycone and acyl chain moieties are given in Table S2, whereas the chemical shifts of the oligosaccharide moieties are given in Table S3. As expected, these chemical shifts are comparable to those of S13, a saponin previously isolated by Nord and Kenne from Qs [27]. In this case, the aglycone corresponds to a 23-O-acetylphytolactic acid moiety.

The 1H NMR spectrum of S13 revealed five anomic protons that resonated as doublets at 5.57 (J = 1.5 Hz), 5.47 (J = 8.2 Hz), 4.55 (J = 7.7 Hz), 4.48 (J = 7.6 Hz), and 4.37 (J = 7.6 Hz). In the multiplicity-edited 1H,13C HSQC spectrum, these protons correlated to the carbon signals at δc 98.6, 94.8, 106.0, 105.2, and 104.9, respectively. The sugar residues were respectively assigned to α-L-Rhap, β-D-Fucp, β-D-Galp, β-D-Glcp and β-D-GlcPA. Key inter-residue correlations observed in the 1H,13C-HMBC and 1H,13C-NOESY spectra (Table S3) allowed to confirm that the disaccharide β-D-Galp-(1→2)-β-D-GlcPA is linked to C3 of the aglycone, and that the branched trisaccharide α-L-Rhap-(1→2)[β-D-GlcP-(1→3)]-β-D-Fucp is located at C28. The 1H,13C-HMBC spectrum showed a cross peak at δH/δC 4.48/82.56 (H1 β-D-Glcp/C3 β-D-Fucp) and the 1H,13C-NOESY spectrum showed a cross peak from the aforementioned H1 resonance to δH 4.12 (H1 β-D-Glcp/H3 β-D-Fucp), corroborating that β-D-Glcp was linked to C3 of the β-D-Fucp (Table S3). The trisaccharide is substituted with two five-carbon length aliphatic acyl chains (2-methylbutanoyl acid moieties) at O3 and O4 of the fucose and rhamnose residues, respectively; furthermore, an acetyl group is located at O2 of the Rha residue. The location of the acyl groups were confirmed by the analysis of the 1H,13C-HMBC spectrum, since three-bond heteronuclear correlations could be observed from the carbonyl carbons at δc 178.6, 172.2 and 177.7 (residues Fa(I), Ac(I) and Fa(II), respectively) to the respective protons at the substitution positions: δH 5.38 (H4 β-D-Fucp), 5.34 (H2 α-L-Rhap) and 4.98 (H3 α-L-Rhap), respectively.

3. Materials and Methods
3.1. Materials and Chemical Reagents

FB, an immunoadjuvant preparation obtained from the aqueous extract of Quillaja brasiliensis leaves, was produced by fractionation on a C18 SPE column as described previously [25,26]. QS-21 was purchased from Desert King Chile S.A (Valparaiso, Chile). HPLC grade acetonitrile and formic acid were purchased from J. T. Baker (Phillipsburg, NJ, USA). Distilled water was purified with a Milli-Q water purification system (Millipore, Bedford, MS, USA). Thin layer chromatography (TLC) plates ( precoated plates, silica gel 60, F254, 0.2 mm layer thickness) were purchased from Machery-Nagel (Duren, Germany).

3.2. Isolation of Saponins
3.2.1. Medium-Pressure Liquid Chromatography (MPLC)

The FB (100 mg) was dissolved in 2 mL of the eluent CH2Cl2/MeOH/H2O/CH3COOH (270:139:25:1) and loaded onto a MPLC column (Buchi Borosilicat 3.3, 460 mm × 15 mm i.d., Switzerland) equipped with a precolumn (Buchi Borosilicat 3.3, 110 mm × 15 mm i.d., Switzerland), both packed with flash silica gel as the stationary phase (0.040–0.063 mm, Merck, E. Merck, Darmstadt, Germany). Chromatography was performed isocratically using the eluent described above at a flow rate of 5 mL/min and beginning to collect after passing 50 mL of the eluent through the column. The chromatographic separation of FB
resulted in 46 fractions of 7 mL each, which were monitored by thin layer chromatography (TLC) for saponins. The saponin containing fractions were pooled out providing three major fractions: B1 (fractions 5–9), B2 (fractions 16–21), and B3 (fractions 26–37). The fractions were freeze-dried and kept at −20 °C until use.

3.2.2. Semi-Preparative High Performance Liquid Chromatography (HPLC)

Fractions B1 and B3 were subjected to further separations using a Shimadzu LC-20AR HPLC system (Shimadzu, Kyoto, Japan) equipped with a reverse phase column (Shim-pack PREP-ODS, 250 mm × 20 mm, 5 µm, Shimadzu, Kyoto, Japan), a binary pump, and a UV-Visible detector (SPD-20AV, Shimadzu, Kyoto, Japan). Fractions were redissolved in 0.1% formic acid in water and injected into the column. Compounds were eluted with a linear gradient with 0.1% formic acid in water (A) and 0.1% formic acid in CH$_3$CN (B) as the mobile phase at a flow rate of 10 mL/min. Eluent B was increased from 5–45% in 3 min, then from 45 to 53% in 20 min, then held at 100% for 6 min, and finally set back to 5% for 4 min. The detection wavelength was set at 214 nm. Purified compounds Qb1 (2 mg, retention time at 13.5 min) and S13 (1 mg, retention time 20.5 min) were obtained by manual collection from B3 and B1, respectively.

3.3. Analysis of Fractions and Purified Saponins

3.3.1. Thin Layer Chromatography (TLC)

Each fraction from the MPLC system was analyzed by TLC on silica gel plates using BuOH/H$_2$O/CH$_3$COOH (6:2:2) as mobile phase, and anisaldehyde-H$_2$SO$_4$/heating as detection reagent.

3.3.2. Liquid Chromatography Mass Spectrometry (LC-MS)

Fractions obtained from the MPLC (B1 and B3) and HPLC systems were monitored by LC-MS. The purity of the isolated compounds (Qb1 and S13) and commercial QS-21 was also analyzed by LC-MS. The chromatography step was performed on an Ultimate 3000 RSLC systems (Dionex, Germering, Germany) coupled to a linear ion trap mass spectrometer LTQ XL from Thermo Scientific (San José, CA, USA) with an ESI interface. The instrument control and data collection were done using Xcalibur software (v3.0.63) from Thermo Scientific (San José, CA, USA) The ESI and chromatographic conditions were performed as detailed before [25,26]. The MS analysis was carried out in negative ion mode and under Full scan, Full scan MS/MS, and SIM scan modes.

3.3.3. NMR Spectroscopy

Unless otherwise specified, the proton detected NMR experiments were acquired on a Bruker Avance III 500 MHz spectrometer equipped with a 5 mm Z-gradient TXI (1H/$^{13}$C/$^{15}$N) probe; the 1D $^{13}$C NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm Z-gradient BBO probe. The NMR samples were prepared by dissolving the isolated compounds (~2 for Qb1 and ~1 mg for S13) and the commercial QS-21 (~1 mg) in ~200 µL of deuterated methanol (CD$_3$OD; ≥99.8 atom% D, Sigma-Aldrich, St. Louis, MO, USA) and placed in 3 mm tubes. All experiments were performed at 298 K, and the $^1$H and $^{13}$C chemical shifts for Qb1 and QS-21 are reported in ppm using the residual solvent peak as reference ($^1$H; 5.32 and $^{13}$C 124.0, respectively) [27]. The assignments of the $^1$H and $^{13}$C resonances were obtained using 2D NMR spectra such as multiplicity-edited $^1$H,$^{13}$C-HSQC [34], $^1$H,$^1$H-TOCSY [35], and $^1$H,$^{13}$C-HSQC-TOCSY with mixing times of 20, 40, 60, and 100 ms, $^1$H,$^{13}$C-H2BC [36], and $^1$H,$^{13}$C-HMBC [37]. The $^1$H,$^1$H-NOESY [38] and $^1$H,$^{13}$C-HSQC-NOESY spectra were recorded with mixing times of 300 and 200 ms, respectively. The NMR data processing was carried out using the MestReNova (v 14.2.0) and Topspin (4.0.7) software. Considering the small amount of material recovered for S13, the $^1$H,$^{13}$C-HMBC of fraction B1 was used for the analysis.
4. Conclusions

Herein we reported the isolation, purification, and structural characterization of two triterpene saponins from the aqueous extract of *Q. brasiliensis* leaves, including a previously undescribed isomer of the QS-21 saponins. The chemical structure of this compound was established using a combination of mass spectrometry and 1D and 2D NMR spectroscopy. Considering the structural similarities of Qb1 with QS-21, it would be expected that this compound would also display immunoadjuvant potential. In particular, this novel molecule displays three key structural features that have been previously identified as being relevant to the adjuvant activity of QS-21: (a) the C23 aldehyde and (b) C16 hydroxyl groups in the quillaic acid moiety, and c) the fatty acyl side chain that extends from O4 of the fucosyl residue [39]. In this regard, the isolation of Qb1 from Qs leaves acquires a relevant importance, since there is an imperative need to find alternatives to the limited supply of QS-21, due to its low abundance in the Qs bark [40,41]. Further work will be necessary to determine the adjuvant activity and toxicity of Qb1.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27082402/s1, Table S1: The $^1$H and $^{13}$C NMR chemical shifts (ppm) of the triterpene and fatty acyl chain moieties of saponin Qb1; Table S2: The $^1$H and $^{13}$C NMR chemical shifts (ppm) for the triterpene and acyl chain moieties of saponin S13; Table S3: $^1$H and $^{13}$C NMR chemical shifts (ppm) of the two oligosaccharide chains of the S13 saponin, and inter-residue correlations from $^1$H,$^1$H-NOEY and $^1$H,$^{13}$C-HMBC spectra. The spectra were recorded in CD$_3$OD at 25 °C on a Bruker Avance 500 MHz spectrometer; Figure S1: Selected regions of the $^1$H,$^1$H-TOCSY spectrum of Qb1 showing correlations from anomeric protons; Figure S2: Selected region of the $^1$H,$^{13}$C-HMBC spectrum of Qb1 showing key correlations from anomeric protons. Only the inter-residue correlations are annotated.

Author Contributions: Conceptualization, F.W., F.F. and C.O.; formal analysis, F.W.; acquisition and design NMR experiments, C.F.; interpretation of data, F.W., C.F. and C.O.; investigation, F.W., C.F., F.F. and C.O.; writing—original draft preparation, F.W. and C.O.; writing—review and editing, F.W., C.F., F.F. and C.O.; resources and funding acquisition, F.F. and C.O.; project administration, C.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Agencia Nacional de Investigación e Innovación (ANII), Grant No. FMV_3_2018_1_149104, and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are available within the article and its Supplementary Materials.

Acknowledgments: The authors thank Guillermo de Souza from Espacio de Ciencia y Tecnología Química, Universidad de la República, for his assistance with the production of Fraction B (FB).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Sample Availability: Samples of the compounds are not available from the authors due to the limited amount of isolated material.

References
1. Luebert, F. Taxonomy and distribution of the genus *Quillaja* Molina (Quillajaceae). *Feddes Repert.* 2013, 124, 157–162. [CrossRef]
2. Cibulski, S.P.; Mouriglia-Éttlin, G.; Teixeira, T.F.; Quirici, L.; Roehe, P.M.; Ferreira, F.; Silveira, F. Novel ISCOMs from *Quillaja brasiliensis* saponins induce mucosal and systemic antibody production, T-cell responses and improved antigen uptake. *Vaccine* 2016, 34, 1162–1171. [CrossRef]
3. Cibulski, S.; Rivera-Patron, M.; Suárez, N.; Pirez, M.; Rossi, S.; Yendo, A.C.; de Costa, F.; Gosmann, G.; Fett-Neto, A.; Roehe, P.M.; et al. Leaf saponins of *Quillaja brasiliensis* enhance long-term specific immune responses and promote dose-sparing effect in BVDV experimental vaccines. *Vaccine* 2018, 36, 55–65. [CrossRef]
4. Garçon, N.; Chomez, P.; Van Mechelen, M. GlaxoSmithKline Adjuvant Systems in vaccines: Concepts, achievements and perspectives. Expert Rev. Vaccines 2007, 6, 723–739. [CrossRef]

5. Lacaille-Dubois, M.A.; Wagner, H. A review of the biological and pharmacological activities of saponins. Phytomedicine 1996, 2, 363–386. [CrossRef]

6. Marty-Roix, R.; Vladimir, G.I.; Pouliot, K.; Weng, D.; Buglione-Corbett, R.; West, K.; MacMicking, J.D.; Chee, J.D.; Wang, S.; Lu, S.; et al. Identification of QS-21 as an Inflammasome-activating Molecular Component of Saponin Adjuvants. J. Biol. Chem. 2016, 291, 1123–1136. [CrossRef]

7. Barbosa, A.D.P. Saponins as immunoadjuvant agent: A review. Afr. J. Pharm. Pharmacol. 2014, 8, 1049–1057. [CrossRef]

8. Silveira, F.; Cibulski, S.P.; Varela, A.P.; Marqués, J.M.; Chabalgoity, A.; de Costa, F.; Yendo, A.C.A.; Goessmann, G.; Roche, P.M.; Fernández, C.; et al. Quillaja brasiliensis saponins are less toxic than Quil A and have similar properties when used as an adjuvant for a viral antigen preparation. Vaccine 2011, 29, 9177–9182. [CrossRef]

9. de Costa, F.; Yendo, A.C.A.; Cibulski, S.P.; Fleck, J.D.; Roche, P.M.; Spilki, F.R.; Goessmann, G.; Fett-Neto, A.G. Alternative Inactivated Poliovirus Vaccines Adjuvanted with Quillaja brasiliensis or Quil-A Saponins Are Equally Effective in Inducing Specific Immune Responses. PLoS ONE 2014, 9, e105374. [CrossRef]

10. Fleck, J.D.; Kauffmann, C.; Spilki, F.; Lencina, C.L.; Roche, P.M.; Goessmann, G. Adjuvant activity of Quillaja brasiliensis saponins on the immune responses to bovine herpesvirus type 1 in mice. Vaccine 2006, 24, 7129–7134. [CrossRef]

11. Yendo, A.C.A.; de Costa, F.; Cibulski, S.P.; Fleck, J.D.; Roche, P.M.; Souza, D.O.; et al. IMXQB-80: A Quillaja brasiliensis saponin-based nanoadjuvant enhances Zika virus specific immune responses, including Neutralizing Antibodies and Splenocyte Proliferation. Inactivated Poliovirus Vaccine Adjuvanted with Quillaja brasiliensis Enhances Anti-Zika Immune Responses, Including Neutralizing Antibodies and Splenocyte Proliferation. Front. Immunol. 2021, 12, 515. [CrossRef]

12. Cibulski, S.; Teixeira, T.F.; Varela, A.P.M.; Teixeira, T.F.; Cancela, M.P.; Sesterheim, P.; Souza, D.O.; et al. Domain III Recombinant Protein Delivered With Saponin-Based Nanoadjuvant From Quillaja brasiliensis Enhances Anti-Zika Immune Responses. Molecules 2020, 25, 3916. [CrossRef]

13. Wallace, F.; Bennadji, Z.; Ferreira, F.; Olivaro, C. Analysis of an immunoadjuvant saponin fraction from Quillaja brasiliensis leaves by electrospray ionization ion trap multiple-stage mass spectrometry. Phytochem. Lett. 2017, 20, 228–233. [CrossRef]

14. Wallace, F.; Bennadji, Z.; Ferreira, F.; Olivaro, C. Structural characterisation of new immunoadjuvant saponins from leaves and the first study of saponins from the bark of Quillaja brasiliensis by liquid chromatography electrospray ionisation ion trap mass spectrometry. Phytochem. Anal. 2019, 30, 644–652. [CrossRef]

15. Nord, L.I.; Kenne, L. Novel acetylated triterpenoid saponins in a chromatographic fraction from Quillaja saponaria Molina. Carbohydr. Res. 2000, 329, 817–829. [CrossRef]

16. Jacobsen, N.E.; Fairbrother, W.J.; Kensil, C.R.; Lim, A.; Wheeler, D.A.; Powell, M.F. Structure of the saponin adjuvant QS-21 and its base-catalyzed isomerization product by 1H and natural abundance 13C NMR spectroscopy. Carbohydr. Res. 1996, 280, 1–14. [CrossRef]

17. Jansson, P.-E.; Kenne, L.; Widmalm, G. Computer-assisted structural analysis of regular polysaccharides. Pure Appl. Chem. 1989, 61, 1181–1192. [CrossRef]
30. Agrawal, P.K. NMR Spectroscopy in the Structural Elucidation of Oligosaccharides and Glycosides. *Phytochemistry* 1992, **31**, 3307–3330. [CrossRef]

31. Nord, L.I.; Kenne, L. Separation and Structural Analysis of Saponins in a Bark Extract from *Quillaja saponaria* Molina. *Carbohydr. Res.* 1999, **320**, 70–81. [CrossRef]

32. Bock, K.; Pedersen, C. A Study of 13CH Coupling Constants in Hexopyranoses. *J. Chem. Soc. Perkin Trans. 2* 1974, **3**, 293–297. [CrossRef]

33. Rao, V.S.R.; Qasba, P.K.; Balaji, P.V.; Chandrasekaran, R. *Conformation of Carbohydrates*, 1st ed.; CRC Press: London, UK, 1998; pp. 49–67. [CrossRef]

34. Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletzky, O.; Glaser, S.J.; Sørensen, O.W.; Griesinger, C. A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *J. Biomol. NMR* 1994, **4**, 301–306. [CrossRef]

35. Bax, A.D.; Donald, G.D. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* 1969, **65**, 355–360. [CrossRef]

36. Nyberg, N.T.; Duus, J.O.; Sørensen, O.W. Heteronuclear Two-Bond Correlation: Suppressing Heteronuclear Three-Bond or Higher NMR Correlations while Enhancing Two-Bond Correlations Even for Vanishing $^2$$^1$$^H$$^C$. *J. Am. Chem. Soc.* 2005, **127**, 6154–6155. [CrossRef] [PubMed]

37. Claridge, T.D.W.; Pérez-Victoria, I. Enhanced $^{13}$C resolution in semi-selective HMBC: A band-selective, constant-time HMBC for complex organic structure elucidation by NMR. *Org. Biomol. Chem.* 2003, **1**, 3632–3634. [CrossRef] [PubMed]

38. Kumar, A.; Ernst, R.R.; Wüthrich, K. A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. *Biochem. Biophys. Res. Commun.* 1980, **100**, 2229–2246. [CrossRef]

39. Wang, P. Natural and Synthetic Saponins as Vaccine Adjuvants. *Vaccines* 2021, **9**, 222. [CrossRef]

40. Kamstrup, S.; San Martin, R.; Doberti, A.; Grande, H.; Dalsgaard, K. Preparation and Characterisation of Quillaja Saponin with Less Heterogeneity than Quil-A. *Vaccine* 2000, **18**, 2244–2249. [CrossRef]

41. Harandi, A.M.; Medaglini, D.; Shattock, R.J. Vaccine Adjuvants: A Priority for Vaccine Research. *Vaccine* 2010, **28**, 2363–2366. [CrossRef]