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1. Introduction

1.1. Saponins are natural surfactants

Saponins are amphipathic glycosides that constitute a class of secondary metabolites found in natural sources, in particular abundance in various plant species and more recently found in marine organisms [1]. They were named by the soap-like foaming they produce when shaken in aqueous solutions, presenting surfactant properties, explained by their chemical and structural composition.

Saponins are constituted by one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. This aglycone part is termed sapogenin [2]. The number and length of oligosaccharide chains attached to the sapogenin core can vary. The chain lengths change from 1 to 11, with 2-5 residues of D-glucose, D-glucoronic acid or D-galactose being the most frequent, and with both linear and branched saccharides chain [3]. The lipophilic aglycone can be any one of a wide variety of polycyclic organic structures originating from the serial addition of 10-carbon (C10) terpene units to compose a C30 triterpene skeleton, often with subsequent alteration to produce a C27 steroidal skeleton [4].

2. Natural sources of saponins: Plants and marine animals

Many plants accumulate saponins in one or several organs, specifically in leaves, stems, roots, bulbs, blossom and fruit. The crushed leaves or roots of perennial herbs from the genus Saponaria were tradicionally used as soap. Particular types of saponins, like gypenosides and
ginsenosides, are heavily found in jiaogulan (a herbaceous climbing vine of the family Cucurbitaceae); and in ginseng (a slow-growing plant with fleshy roots, genus Panax), respectively [5].

There are several characteristics associated to saponins extracted from plants. These compounds may serve as anti-feedants to protect the plant against microbes and fungi [2]. In addition, some plant saponins may enhance nutrient absorption and aid in animal digestion [6] and tea saponins can improve daily weight gain and feed efficiency in goats [7]. Many plant steroidal saponins have also been reported to exhibit antimicrobial activities, in particular by weakening the virulence of \textit{C. albicans} and killing fungi by destroying the cell membrane [8]. Moreover, since some saponins are toxic to cold-blooded organisms, insects and fish, they were commonly used by indigenous tribes to obtain aquatic food sources [9].

Saponins were initially thought to be exclusive metabolites of plant origin but the world-wide development in the investigation of marine organisms as sources of new bioactive metabolites disclosed a wider distribution of these molecules also among marine animals [5]. Presently, saponins are recognized as the most common characteristic metabolites in two classes of the phylum Echinodermata (Holothuroidea and Asteroidea), where they occur as natural glycosidic surfactants. Furthermore, several steroid and triterpenoid oligoglycosides have been isolated from different species of marine sponges, more rarely \textit{Anthozoa}s, and also from fishes of the genus \textit{Pardachirus}, where they have been shown to act as shark repellents [10].

3. Biomedical and pharmacological applications of saponins

Due to the surfactant properties of saponins they can be used to enhance penetration of macromolecules, e.g. proteins through cell membranes, making them useful as adjuvants in vaccines [11]. A wide range of pharmacological applications, such as antiplatelet, hypocholesterolemic, antitumoral, anti-HIV, immunoadjuvant, anti-inflammatory, antibacterial,
insecticide, fungicide and anti-leishmanial agents have been also described for saponins [2]. Seven triterpenoid saponins from the plant Gypsophila paniculata have been shown to increase the cytotoxicity of immunotoxins and other targeted toxins directed against human cancer cells [12][13].

Holothurins, saponins isolated from sea cucumber, have displayed a wide spectrum of biological effects such as hemolytic, cytostatic, antineoplastic, anticancer and antitumor activities [14]. These glycosides are also frequently studied in the research of chemical constituents and activities of starfish, with considerable clinical interest, since they showed several physiological, pharmacological and immunological activities, such as cytotoxic, hemolytic, antifungal, antiviral, anti-inflammatory or ichthyotoxic. In particular, sulfated steroidal glycosides (asterosaponins) are one of the bioactive secondary metabolites from starfish, responsible for the toxicity of these marine organisms [15].

Concerning the commercial formulations of plant-derived saponins, these compounds are available via controlled manufacturing processes by Sigma-Aldrich (St. Louis, USA), which make them of use as chemical and biomedical reagents.

4. Saponins from asteroidea

The echinoderms (phylum Echinodermata) are exclusively marine invertebrates and, with some exceptions, are all benthic organisms (bottom-dwellers) and are one of the closest living relatives to vertebrates (phylum Chordata), since they both belong to the superphylum Deuterostomia. Asteroidea (starfishes) is one of the five classes of echinoderms, with about 1,500 living species [16]. The surface of their body is often brightly coloured and is generally spiny or warty. All starfishes possess five-part radial symmetry around a central disk. This means that each of their arms has an exact replica of all internal organs. They are also characterized by a unique water vascular system, consisting of a set of water-filled canals branching from a ring canal and leading to tube feet, involved in locomotion, respiration, sensation and feeding [17]. The body cavity of echinoderms is filled with coelomic fluid, which bathes the internal organs and forms the fluid medium, where the coelomocytes (the echinoderm immune cells) are suspended. The composition of coelomic fluid is similar to sea water in dissolved salts and other minerals [18]. Since the coelomic fluid bathes all the internal organs, it is extremely rich in secreted molecules, like growth factors, hormones, neuropeptides and glycosides, which are involved in cell signaling and immunity processes.

According to their most relevant structural features, glycosides from starfish were subdivided into three main groups: glycosides of polyhydroxy steroids, cyclic steroidal glycosides and asterosaponins.

The glycosides of polyhydroxy steroids consist of a polyhydroxylated steroidal aglycone and a carbohydrate portion that is usually composed of several monosaccharide units. The most common glycosylation position is C(24), but sometimes glycosides carry the sugar moiety at C(3) and C(26). In starfish they occur as sulfonlated and free hydroxy forms, with few
examples of phosphorylated ones. The OH groups are usually found in positions 3β,6α (or β), 8β,15α (or β), 16α (or β).

Figure 2. Main groups of glycosides of the polyhydroxy steroids from starfish. The first group (B1) are 3β-OH steroids with glycosylation on the side chain. The second group (B2) contains 3β-O-glycosylated steroids. The third group (B3) contains 3β-O-glycosylated steroids with additional glycosylation on the side chain. The fourth group (B4) are 6β-O-glycosylated steroids. G represents a glycosylation component. Adapted from [15].

The cyclic steroidal glycosides occur in few starfish. An anionic charge is due to the presence of a glucuronic acid unit. The 7,8-dehydro-3b,6b-dihydroxy steroidal nucleus is unprecedented, and the most remarkable feature is the trisaccharide chain, which is cyclized between C(3) and C(6) of the aglycone giving rise to a macrocyclic ring reminiscent of a crown ether.

Asterosaponins are constituted by an aglycone with 9,11-didehydro-3β,6α-dihydroxy steroidal nucleus; a sulfate group at C(3); and generally a side chain with the 20β-OH and 23-oxo functions. The oligosaccharide chain, commonly made up of five or six sugar units with β-oriented structure, and sometimes containing also three or four sugar units, is always glycosidically linked at C(6). The sugar units can be xyloses, galactoses, fucoses or quinovoses. Most of the structural diversity is confined to the substitution pattern of the side chain [15].

Four asterosaponins from *M. glacialis* have been scrutinized to date [15]. Their structures are illustrated in Figure 5 and Figure 6.
Figure 3. Structure of cyclic steroidal glycoside sepositoside A, found in two starfish species, *Echinaster sepositus* and *E. luzonicus*. Adapted from [15].

Figure 4. A sulfated steroidal glycoside (asterosaponin) typical structure. Adapted from [19].

Marthasterosides A1 and A2 (see Figure 5) differ only in the identity of one sugar residue, while marthasterosides B and C (see Figure 6) differed only in the steroidal side chain.
Figure 5. Asterosaponins marthasterosides A1 (A1) and A2 (A2) structures. Adapted from [15].

Figure 6. Asterosaponins marthasterosides B (B) and C (C) structures [15]. Adapted from [15].
So far, studies performed with asterosaponins from starfish indicated that they possess several bioactive properties such as: cytotoxic to tumor cells and viruses; hemolytic activity toward erythrocytes of various origins; and anti-inflammatory and antifungal activities [20]. Additionally, cytotoxic asterosaponins from the starfish *Culcita novaeguineae* were reported to promote polymerization of tubulin [21]. Agents that promote tubulin polymerization exhibit anticancer activity by disrupting normal mitotic spindle assembly and cell division as well as inducing apoptosis (programmed cell death) [22].

5. Methods to extract asterosaponins

Usually, the purification/isolation of steroidal glycosides from a sample mixture, extracted from starfish, is not an easy task [23]. The extraction of the crude sample material with organic solvents often takes several hours and is frequently followed by a series of preparation steps such as gel chromatography, counter current chromatography or preparative column chromatography [24].

Currently, it is common to collect steroidal glycosides from *n*-BuOH extracts of entire animals or arms and central disks of starfish [25]. The animals are chopped in small pieces, homogenized in EtOH, filtered and then the extraction is performed with *n*-BuOH. Each *n*-BuOH extract is chromatographed and the enriched fraction separated by HPLC on RP-C18 columns typically with MeOH:H$_2$O (2:1) and eluted to give fractions containing mixtures of sulfated saponins [21], [26]. These fractions are analyzed by ESI-MS/MS to infer about the molecular masses and structures of these compounds, as will be discussed in the next section.

The investigation conducted in our group with *Martasterias glacialis* showed that asterosaponins can be found in the coelomic fluid as well (unpublished results). The used method includes a first step of ultrafiltration, to obtain a low molecular mass fraction; and then a step of desalination/concentration in Solid Phase Extraction (SPE) cartridges, where the compounds can be eluted in an increasing order of acetonitrile with 5% (v/v) formic acid (unpublished results). Each SPE elution fraction was analyzed by direct infusion in ESI-MS/MS.

6. MS to characterize asterosaponins

Mass spectrometry has been playing an important role in the structural analysis of complex natural products mainly due to its high sensitivity, rapid analysis time and selectivity [27]. It has the potential ability to rapidly detect the bioactive compounds in mixtures and give information on their structures as well as their molecular masses [28]. Over the last decade, the development of the so-called soft ionization techniques such as electrospray ionization (ESI) allowed the transfer of the analyte into the gas phase without extensive degradation. This led to a rapid and direct analysis of polar, non-volatile and thermally labile classes of compounds [29], e.g., polypeptides, carbohydrates and natural glycosides.
Initially, previous studies of saponins were performed using electron impact (EI) MS but this technique requires derivatization. The development of desorption chemical ionization (DCI) MS allowed analysis of saponins without derivatization, but only for saponins with ether glycosidic linkages [30]. Field desorption was also employed to analyze native saponins. However, due to the instability of ion currents dependent on the temperature of the emitter, the mass spectra were not reproducible [31]. Fast atom bombardment (FAB) ionization combined with tandem mass spectrometry (MS/MS) was employed to analyze native saponins as well, and some useful structural information was obtained. Unfortunately, the sensitivity of FAB was not satisfactory due to the chemical noise from the matrix background [23]. As a consequence, the ESI technique replaced FAB by the end of the 1990s. Moreover, nuclear magnetic resonance (NMR) spectroscopy is generally used to provide detailed structural information for saponins (as described in Chapter 8), but milligram quantities of high-purity samples are usually needed [27].

There is a lack of information concerning the fragmentation pattern of asterosaponins in MALDI-TOF/TOF. In our experience, using α-cyano-4-hydroxycinnamic acid as a MALDI matrix (unpublished results), this fact is related with some ionization difficulties of these compounds by MALDI.

Recent studies have reported the use of ESI-MS for determination of saponins with higher sensitivity and better reproducibility than the other types of ionization [30]. Moreover, the possibility of using electrospray tandem mass spectrometry (ESI-MS/MS) presents great advantages for the characterization of these compounds, by providing more information of their structure. Based on each fragmentation spectra obtained from MSn experiments, the molecular structures of the compounds can be estimated by the identification of the mass losses between successive fragmentation peaks.

In fact, ESI-MS/MS has been shown to provide high sensitivity for steroidal glycoside analysis. In particular, this technique seems to be a fast and suitable screening method for the structural information of sulfated steroidal glycosides extracted from starfish. It can provide information about their molecular mass and oligossacharide sequence by cleavage of glycosidic bonds [31].

7. MS spectra characteristic for asterosaponins

Asterosaponins can be detected in the ESI-MS spectra in the form of [M+Na]+ (due to the presence of sodium ions during the process of sample preparation and the strong affinity of sugar to sodium ions in the gas phase) and [M-Na]- ions, in positive and negative ion modes, respectively (see an example of a full MS spectrum in Figure 7).

Full scan MS spectra in negative ion mode, usually show characteristic peak series with two or more peaks located in a higher mass range (m/z 1000-1400), increasing their value of the same mass difference, typically 14 Da. The mass difference of 14 Da can be attributed to the presence of a methoxy group in one of their sugar residues. This structural feature is very common in asterosaponins isolated from starfishes [19]. More structural information of these compounds can be easily achieved by ESI-MS/MS, as previously mentioned.
Figure 7. Example of a full ESI-MS spectrum, in negative mode, of the SPE fraction eluted with 75% acetonitrile extracted from coelomic fluid of the starfish *Marthasterias glacialis*, showing some major precursor ions. The precursor ion m/z 1389.6 [M-Na] is asterosaponin masthasterosides A1 (C_{62}H_{101}NaO_{32}S).

There are several typical mass losses between the precursor and the fragment ions, detected in the MS^n spectra, which are characteristic for asterosaponins. Specifically, the typical mass losses detected in the ESI-MS^n spectra are related to the aglycone, the sulphate group or the oligosaccharide chain losses, which are structural features of asterosaponins. The most common sugars found in asterosaponins are xyloses, fucoses, quinovoses, galactoses and glucoses.

In the ESI-MS/MS spectra, it is common to detect a mass loss of 100 Da between the precursor and the more intense fragment ion (see an example in Figure 8). This corresponds to the loss of a C_{6}H_{12}O molecule arising from C(20)-C(22) bond cleavage and 1H transfer (known as a retro aldol cleavage), characteristic for asterosaponins containing an aglycone with a 20-hydroxy-23-oxo side chain [19]. Additionally, it is possible to detect a fragment ion of m/z 97, which indicates the dissociation of the HSO_{4}^{-} group.

As mentioned before, it is possible to perform MS^n experiments to infer about the structure of compounds. As long as the signal intensity of the precursor ion is strong enough, the fragmentation process can be repeated. As so, in the ESI-MS^n spectra, ions arising from the cleavages of the glycosidic bonds from the terminal sugar moieties of the oligosaccharide chain of asterosaponins can be detected (see an example in Figure 9). In particular, the mass difference of 146 Da between two fragment ions indicates a loss of a terminal deoxyhexose residue (attributable to isomeric fucose or quinovose). In addition, the mass differences between two fragment ions of 132 and 162 Da are attributable to the losses of a pentose and of a hexose residue, respectively [25].
Figure 8. Example of a ESI-MS$^2$ spectrum, in negative mode, obtained for precursor ion $m/z$ 1389.6. In this figure it is possible to detect a mass difference of 100 Da between the precursor and the fragment ion $m/z$ 1289.6.

Figure 9. Example of a ESI-MS$^3$ spectrum, in negative mode, obtained for the precursor ion $m/z$ 1289.6 generated from the molecular ion $m/z$ 1389.6. In this figure it is possible to detect ions arising from the cleavages of glycosidic bonds. It can be detected a mass difference of 146 Da between precursor and fragment ion $m/z$ 1143.5, attributable to the loss of isomeric fucose (Fuc) or quinovose (Qui). The same mass difference of 146 Da can be detected between fragment ions $m/z$ 1143.5 and $m/z$ 997.4. Also, a mass difference of 162 Da can be detected between fragment ions $m/z$ 997.4 and $m/z$ 835.3, which is consistent with a loss of one unit of galactose (Gal).
Figure 8 and Figure 9 show the fragmentation spectra of precursor ion \( m/z \) 1390, illustrating the typical asterosaponin mass losses of 100 (loss of a \( \text{C}_6\text{H}_{12}\text{O} \) molecule), 146 and 162 Da. This precursor ion ([M-Na]) corresponds to masthasterosides A1 (\( \text{C}_{62}\text{H}_{101}\text{NaO}_{32}\text{S} \)), an asterosaponin that has been described for the starfish *Marthasterias glacialis*. Furthermore, the losses of three units of 146 Da (fucose or quinovose) and one unit of 162 Da (galactose) are consistent with the molecular structure of this asterosaponin (see Figure 10). An illustration of the fragmentation of masthasterosides A1 (precursor ion \( m/z \) 1390) can be seen in Figure 11, where the typical asterosaponin mass losses are represented.

![Figure 10](image)

**Figure 10.** Structure of asterosaponin masthasterosides A1 (\( \text{C}_{62}\text{H}_{101}\text{NaO}_{32}\text{S} \)), indicating the fucose (Fuc), quinovoses (Qui), galactoses (Gal), xyloses (Xyl) units. [32].

ESI-MS and ESI-MS\(^{n}\) seem to be essential techniques to study saponin mixtures. The isolation of pure saponins is a time and/or material consuming tasks, and for this reason, these techniques are important tools to obtain information on the complexity of a saponin mixture, with the objective of finding new structures. However, only a partial characterization of asterosa-
ponins is possible using ESI-MS approach since the information obtained is limited on the presence of sugar residues, the sulfate groups and the aglycone. As so, NMR spectroscopy and chemical reactions are required in order to complete characterize these molecules [19].

Figure 11. Scheme of the fragmentation of precursor ion m/z 1390, showing typical asterosaponin mass losses: $C_6H_{12}O$, fucose, quinovose and galactose.

8. Limitations of MS: Future NMR spectroscopy work (both $^1$H and $^{13}$C)

Nuclear Magnetic Resonance (NMR) is a very powerful tool in the study of natural compounds in general, insofar as this technique provides a wealth of structural information in the form of chemical shifts, coupling constants and coupling patterns. While the chemical shift values inform us of the nature of the chemical group in which a particular nucleus is involved, the coupling constants inform us about the structural relationship between pairs of atoms, their magnitude and splitting patterns depending on the shape and density of the electronic clouds surrounding them. To this information, two-dimensional correlation techniques, allow us to follow the resonating spins along the molecular structure, assigning them to confirm the identity of a known compound or establish the molecular structure of a new molecule. These general features of the NMR technique are perfectly applicable to the study of saponins and in particular to asterosaponins, and especially suited to determine the molecular structure of a newfound saponin or asterosaponin.
The general procedure is based on $^1$H and $^{13}$C NMR and consists in acquiring a one-dimensional $^1$H spectrum and a series of five 2D spectra: COSY, TOCSY, NOESY, HSQC and HMBC.

The COSY (or COrrelation SpectroscopY), is a proton-proton spectrum, which relies on the existence of coupling constants between pairs of nuclei, and so, correlates signals belonging to geminal or vicinal hydrogen nuclei, i.e. two or three bonds apart in the molecule. This is a very important tool, since it allows the sequential assignment of the signals within the molecule.

The TOCSY (or TOtal Correlated SpectroscopY), is a proton-proton spectrum that allows to detect the signals belonging to the same spin system, i.e. with sequential coupling constants between them. This spectrum can be of vital importance in overcoming signal overlap, its use being, not restricted, but most obvious, in the assignment of the sugar resonances (see below).

The NOESY (or Nuclear Overhauser Effect SpectroscopY), is a proton-proton spectrum that allows to establish proximity in space. This spectrum allows to overcome regions in the structure for which there is no proton-proton coupling (quaternary carbons, ester or ether bonds, glycosidic bonds, etc), and thus, among other features, establish acetilation or glycosilation sites.

The HSQC (or Heteronuclear Single Quantum Coherence spectrum), is a proton-carbon spectrum that correlates carbon signals with directly bond protons. It is a very robust experiment that relies on the existence of relatively large one bond $J$ coupling ($|J_{CH}|\sim145$ Hz) between $^{13}$C nuclei and directly bound $^1$H. As the signal acquisition in this spectrum is done on the $^1$H frequency, it is much more sensitive than the experiments that observe carbon directly, and allows obtaining $^{13}$C information with $^1$H sensitivity. One drawback of this experiment is that quaternary carbons are not detected.

The HMBC (Heteronuclear Multiple Bond Correlation spectrum), is a proton-carbon spectrum in which the $|J_{CH}|$ correlations (one bond) are suppressed and the sequence is optimized for long range smaller couplings, two, three and sometimes four bonds apart. Like the HSQC, the HMBC also provides $^{13}$C information with $^1$H sensitivity, but because it is optimized for smaller values of $J$ coupling (one order of magnitude smaller) it is a much more time consuming and less sensitive experiment. The HMBC and the HSQC experiments work as a pair in the sense that the HSQC carries very little structural information (each carbon is only correlated with its own hydrogen), while the HMBC can carry a large amount of information regarding neighboring chemical groups but it is very difficult to interpret without the HSQC information. Taken together, these experiments allow, in most cases, to follow the molecular structure through its carbon backbone.

The main drawback of NMR is its relative insensitivity, in fact, in order to record a $^1$H spectrum the compound of interest must be present in the milimolar concentration range. Using state of the art equipment, like high field spectrometers equipped with cryogenically cooled probes this value can be reduced to 0.1 mM or less, but in general a 1 mM (or higher) concentration sample is required to acquire all the necessary information. When available, the highest field machine is always the better choice. This is not only because of the increase in sensitivity provided by the higher field but also, and most importantly, because of the higher resolution
between signals that can be obtained. In fact, the main difficulty in analyzing NMR spectra is signal overlap. Because of the structural similarities between different saponins, this also means that when characterizing a new saponin, the sample has to be purified prior to analysis.

As previously described, asterosaponins, like saponins in general, are composed of an aglycone polycyclic structure and one or more sacharide or polisacharides with possible several modifications. The aglycone structures contain, in most of the cases, several chemical groups resonating in very different chemical shift ranges (see Table 1). This dispersion facilitates spectral interpretation and assignment. However, the carbon and hydrogen nuclei of the sacharide structures have chemical shifts in relatively narrow regions of the spectrum (65-75 ppm in $^{13}$C and 3.0-4.5 ppm in $^1$H, with the exception of position one of hexoses (the anomeric position), which resonate between 90 and 105 ppm in $^{13}$C and 4.0 to 5.5 ppm in $^1$H).

<table>
<thead>
<tr>
<th>Type of chemical group</th>
<th>$^{13}$C chemical shift range (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary alkane carbons</td>
<td>12-24</td>
</tr>
<tr>
<td>Secondary alkane carbons</td>
<td>20-41</td>
</tr>
<tr>
<td>Tertiary alkane carbons</td>
<td>35-57</td>
</tr>
<tr>
<td>Quaternary alkane carbons</td>
<td>27-43</td>
</tr>
<tr>
<td>Alcohol carbons</td>
<td>65-91</td>
</tr>
<tr>
<td>Olefinic carbons</td>
<td>119-172</td>
</tr>
<tr>
<td>Carbonyl carbons</td>
<td>177-220</td>
</tr>
<tr>
<td>Carbons bearing fluoride atoms</td>
<td>88-102</td>
</tr>
</tbody>
</table>

Table 1. Typical $^{13}$C chemical shift ranges of chemical groups found in saponins. Adapted from [33].

Since all sacharides have one anomeric position with characteristic carbon and proton resonances, these can be used as “handles” to discover the type of sacharide and its glycosidic linkage to the rest of the molecule. Starting on the anomeric position and using the COSY information one could follow the sequential assignment of each of the sugar signals, but because the rest of the sugar moiety is chemically very similar, this task becomes difficult due to signal overlap. If more than one sugar unit is present, the task becomes daunting. This problem, however, can be solved with the assistance of the TOCSY spectrum. Since all the hydrogen signals in the sugar ring are linked via J couplings to their vicinal partners, they constitute a spin-system, which will appear as a correlating unit in a TOCSY spectrum (see Figure 12).

With the sugar signals identified, their chemical shifts and J coupling patterns allow their identification. The value of the $^3J_{H1,2}$ (the coupling constant between protons at positions 1 and 2) of an hexose can be indicative of its configuration. In hexoses where the hydroxyl at position 2 is in the equatorial configuration (like glucose or galactose), values of $^3J_{H1,2}$ around 3 or 7 Hz
are indicative of the α or β configuration, respectively. In the case of hexoses where the hydroxyl at position 2 is in the axial configuration (like mannose or talose), both anomers produce couplings at position 1 of around 1.5 Hz, and the distinction can only be made by measuring the $^{1}J_{C,H1}$ (the one bond coupling constant between the carbon at position 1 and its directly attached proton); α anomers displaying values around 170 Hz, while β anomers display values around 160 Hz [34]. Since the anomeric position is involved in the glycosidic linkage, these signals can also be used to establish the glycosylation sites using NOESY (that detects proximity in space and therefore can correlate signals across the glycosidic bond) or using a combination of HSQC/HMBC to follow the carbon backbone resonances and the correlation of the anomeric carbon of the sugar and a proton resonance of the aglycone structure.

NMR cannot distinguish between optical isomers, but it can detect differences between diastereoisomers. These differences are not apparent in the 2D correlation maps, since the distance in terms of bonds between the several atoms of diastereoisomeric structures is always the same, but are manifested in different chemical shift and J coupling values. The most obvious example of how NMR can be used to solve diastereoisomeric structures is the identification of sugars and their configuration (see above) but, in certain cases, it can also be used to solve other chiral centers by comparison of the acquired data and the published literature.

Figure 12. COSY (on the left-hand side) and TOCSY (on the right) spectra of a trisaccharide (α-glucopyranosyl-(1-3)-β-glucopyranosyl-(1-1)-α-glucopyranose) acquired at 500 MHz. In the TOCSY, the spin systems of all the resonances belonging to each saccharide unit are clearly visible at the anomeric frequencies.
Also, in the case of other modifications, like phosphorylation or sulforilation, NMR can provide the site of those modifications. In the case of phosphorylation, the answer can be readily obtained by recording a $^{31}$P/$^1$H HSQC spectrum that will correlate the phosphorus resonances with the $^1$H signals closest in the molecule (3 or 4 bonds apart). In the case of sulfate groups, as sulfur is not very amenable to NMR studies, a different approach is taken that requires chemical modification. A desulfation procedure, like the one described in Tang et al. (2009), is applied to produce the desulfated saponin. A direct comparison of HSQC spectra before and after desulfation will detect a large chemical shift perturbation (usually a 10 ppm decrease in the $^{13}$C chemical shift values) in some signals providing the modification sites.

9. Concluding remarks

Saponins are glycosides isolated from plant and marine organisms with several promising biomedical and pharmacological applications. Due to their structural complexity and diversity their characterization requires combination of results from different methodologies, namely of mass spectrometry and NMR. ESI-MS/MS analysis appears to be more suitable mass spectrometry approach to study these compounds, since it allows some structural elucidation using MSn experiments. Although, there are several structural features like sugar identity, sugar linkage pattern or site of attachment of sulfate groups, that mass spectrometry cannot address readily and that NMR spectroscopy can solve unambiguously. So, in the full structural characterization of a new saponin or asterosaponin, studies on the pure compounds using several NMR techniques (eventually in combination with specific chemical modifications) are of fundamental importance.

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