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Fortschritte der Chemie organischer Naturstoffe

Progress in the Chemistry of Organic Natural Products

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L. Zechmeister

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Triterpenoid Saponins

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1. Introduction

Saponins are complex molecules made up of sugars linked to a triterpenoid or a steroid or a steroidal alkaloid. These natural products are attracting much attention in recent years because of the host of biological activities they exhibit. The diversity of structural features, the challenges of isolation because of their occurrence as complex mixtures, the pharmacological and biological activities still to be discovered, and the prospect of commercialization – these all are driving the study of saponins. Triterpenoid saponins are dominating constituents of this class and occur widely throughout the plant kingdom including some human foods *e.g.* beans, spinach, tomatoes, and potatoes, and animal feed *e.g.* alfalfa and clover. Saponins were initially a rather neglected area of research primarily because of great difficulties in their isolation and characterization. With the advent of more sophisticated methods of isolation and structure elucidation through the last two decades, there has been increased interest in these natural products. Besides structure determination, research activities are now moving forward to clarify structure–activity relationships. Our previous reviews on triterpenoid saponins (*1*, *2*) covered literature from 1979 to mid-1989. The literature on triterpenoid saponins up to 1988 has also been covered by two reviews by HILLER *et al.* (*3*, *4*). This review incorporates newer trends in isolation and structure determination of triterpenoid saponins, new triterpenoid saponins isolated and biological properties of these products reported during the period late 1989–mid 1996.

2. Isolation

Saponins generally occur as complex mixtures and the usual methods of solvent extraction, column chromatography and preparative TLC are often found to be inadequate for isolation of the pure individual constituents. Special techniques are, therefore, employed to achieve the objective. As an example the saponins of the Chinese medicinal plant *Ardisia crenata* were successfully isolated as follows: The dried and powdered roots of the plant were first defatted with petrol and then extracted with $CHCl_3$ and MeOH under reflux. The MeOH extract was applied to a column of Diaion HP-20 and washed with water, 30, 50, 70 and 100% MeOH to give 50 fractions. The saponin-containing fractions were combined according to their TLC behavior. Each of the combined fractions was purified by an ODS column followed by further separation by HPLC (*5*). A similar procedure was adopted by XU *et al.* for the

separation of the new saponins from *Mussaenda pubescens* (*6*). Ground air dried whole plants were extracted by cold percolation with EtOH. The extract was concentrated and partitioned between water – petroleum ether, water – EtOAc and water – *n*-BuOH (saturated with water). The *n*-BuOH extract was applied to a DA-201 column and eluted successively with H_2O, 40% and 60–70% EtOH. The crude saponin obtained from the last fraction was repeatedly separated by silica gel column chromatography. MASSIOT *et al.* (*7*) isolated the saponins from aerial parts of Alfalfa (*Medicago sativa*) by ether precipitation of the saponin mixture from MeOH solution of the *n*-BuOH extract followed by purification with flash chromatography and thick layer chromatography. Dried and powdered leaves were boiled with a mixture of MeOH and water (4:1) for 3 h. After filtration MeOH was removed and the aqueous layer was extracted three times with *n*-BuOH. The organic layers were combined and evaporated. The residue was dissolved in MeOH, the volume of MeOH concentrated and then diluted with ether. The precipitate was filtered, dried and further purified by flash chromatography on silica gel (particle size: 40–63 μm) under a pressure of 2 bar and thick layer chromatography.

Holothurinosides, new antitumor triterpenoid glycosides from the sea cucumber *Holothuria forskalii*, were isolated (*8*) as follows: Body walls and Cuverium tubules of 19 specimens were collected and extracted with MeOH. The dried MeOH extract was partitioned between water and hexane and the water layer further partitioned between water and *n*-butanol. The *n*-butanol extract was concentrated and passed through a column of a Amberlite XAD-2 which was washed with water and MeOH. The MeOH elute was chromatographed on Sephadex LH-20 eluting with methanol-water (2:1). The fractions thus obtained were further purified by droplet counter current chromatography (DCCC) (ascending mode) and HPLC on a $C_{18}\mu$ Bondapack column.

A somewhat different procedure was adopted for separation and isolation of the bioactive saponins from the fruit pericarps of *Acacia auriculiformis* (*9*). The air dried and powdered fruit of the plant was extracted with petroleum ether, $CHCl_3$ and MeOH. The MeOH extract was partitioned between water and *n*-BuOH. The organic layer was concentrated at reduced pressure, adsorbed on silica gel, dried, and extracted successively in a soxhlet on a water bath with $CHCl_3$, ethyl acetate and a $CHCl_3$-MeOH (80:20) mixture. The ethyl acetate extract on chromatographic purification yielded three relatively non-polar saponins. The $CHCl_3$-MeOH extract was chromatographed on silica gel and a Sephadex LH-20 column followed by preparative TLC and preparative HPLC (S-10-ODS column) to yield three polar acylated saponins.

3. Structure Elucidation

Structures of the isolated pure saponins are generally investigated by a combination of chemical and spectroscopic methods. However, the present favorable trend is to determine structures by spectroscopic methods alone which have the advantage of allowing one to examine a small amount of the intact saponin prior to any treatment which might produce artifacts. The saponins are composed of an aglycone to which are attached one or more sugar chains. In the usual method acid and alkaline hydrolysis experiments are performed to liberate the sugars, acyl constituents and aglycones which are separately investigated for characterization. The sugar and acyl constituents are identified by GC analysis of suitable derivatives and aglycones are characterized by spectroscopic methods. If a saponin contains an acid labile aglycone milder hydrolysis techniques are needed which are described in the previous review (*2*).

3.1. Mass Spectroscopy

The molecular masses of saponins are conveniently determined by soft-ion mass spectroscopic methods such as fast-atom bombardment mass spectrometry (FAB-MS) (*10*, *11*) in the positive and/or negative mode. Other desorption ionization techniques are field desorption (*12*), plasma desorption (*13*) and laser desorption (*14*). More recently, liquid chromatography/mass spectrometry and collision-induced dissociation of doubly charged molecular ions have been employed for structural elucidation (*15*). The molecular masses of the triterpenoid saponins gymnemic acids and their congeners were determined by IMOTO *et al.* (*16*) by high performance liquid chromatography combined with atmospheric pressure ionization mass spectrometry (API-MS). The crude saponin isolated from the leaves was chromatographed on an ocatadecyl silica column and eluted with an aqueous methanol solution containing ammonium acetate. The fractions thus separated were directly introduced into an atmospheric pressure ionization mass spectrometer connected with a liquid chromatograph by an interface consisting of a nebulizer and a vaporizer through a PTFE tube (Hitachi, Japan). The vaporized sample and solvent molecules at 300 °C were introduced into the ion source of the API system. The drift voltage of the spectrometer was set at 160 V. Quasimolecular ions of gymnemic acids were detected as ammonium adduct ions and/or proton adduct ions. Molecular masses of 13 gymnemic acids and 5 compounds not containing glucuronic acid

as part of the structure were determined. Three pairs of geometrical isomers of gymnemic acids were also detected. Moreover, acyl residues such as acetyl, tiglyl and benzoyl in the gymnemic acids were identified by interpretation of the fragmentation patterns.

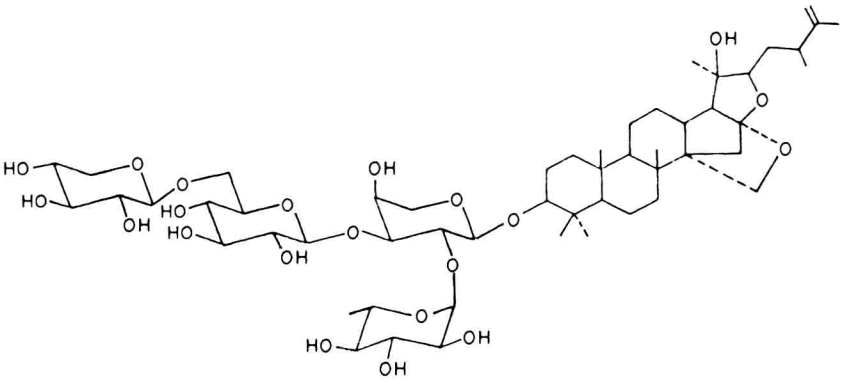
Several workers have presented interesting results of their use of mass on spectrometric techniques in structure elucidation of saponins in a symposium "Saponins: Chemistry and Biological Activity" recently held in Chicago. For example, papers on the application of tandem mass spectral approaches to structure determination of saponins (*17*), structure determination of saponins from mungbean sprouts by tandem mass spectrometry (*18*), saponins from alfalfa, clover and mungbeans analyzed by electrospray ionization mass spectrometry (ESI MS) compared with positive and negative FAB mass spectrometry (*19*) and structure confirmation of alfalfa saponins by LSIMS and B/E LSIMS/MS (*20*) demonstrated the great potential of these ionization techniques.

The usefulness of tandem mass spectrometry in the structure elucidation of oleanene-type triterpene bisdesmosides was discussed by Arao *et al.* (*21*). In the MS/MS of $[M\text{-}H]^-$, $[M+H]^+$ ions of the bisdesmosides, the ions which originated from the cleavage of glycosidic bonds, were mostly observed. On the other hand the MS/MS of an $[M+Na]^+$ ion displayed various fragment ions together with those given by glycosidic bond cleavage. The ion derived *via* an retro Diels-Alder fission also appeared. The ESI MS of bellidiastroside C_2 (see Table), a oleanene-type triterpene bisdesmoside generated $[M+H]^+$ and $[M+Na]^+$ and m/z 1091 and 1113 respectively (*22*). MS/MS of $[M+H]^+$ afforded ions which indicated that the saponin had a terminal pentose, a terminal hexose and two inner deoxyhexoses. Appearance of an ion at m/z 425 $[\text{pent}+\text{dhex}+\text{dhex}-H_2O+H]^+$ suggested that three of the sugars were present as a trisaccharide unit.

3.2. NMR Spectroscopy

Of all the physical methods, the NMR technique has changed most during the last two decades, first with the introduction of the Fourier transform (FT) method and more recently as a result of the growth of multiple pulse and 2D NMR. The developments consequent on the pulse technique permit enormously greater control and manipulation of the sample's magnetization. Consequently, the structure information which is gleaned through pulse NMR is probably greater and more readily obtained than by any other single technique.

High-field NMR experiments, *viz.* COSY, HETCOR, TOCSY, NOESY, ROESY and 2D INADEQUATE techniques, coupled with computerized spectral analysis were used for the determination of the complete structure of a novel triterpenoid tetrasaccharide zizyphoiside A (**1**) (code name PT-2) isolated from *Alphitonia zizyphoides* (Rham-



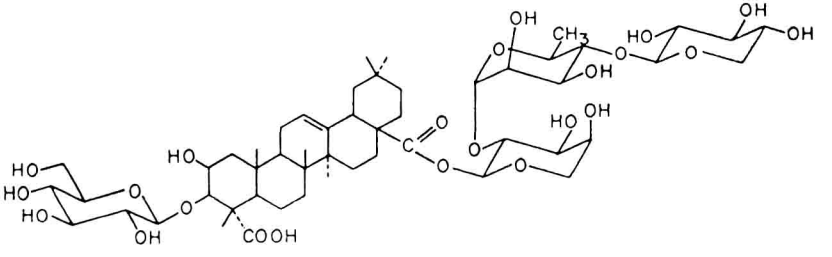
Zizyphoiside A (**1**)

naceae) (*23*). The 2D INADEQUATE technique (coupled with a computerized spectral analysis) was successfully employed to determine the structure of a fairly large saponin using a small amount of sample (60 mg). The 1D ^{13}C spectrum displayed 54 carbon signals. A DEPT experiment revealed 8-CH_3, 13-CH_2, 26-CH and 7 quaternary carbon atoms. Correlation of ^{13}C signals with those of directly bonded protons was achieved by means of 2D HETCOR experiment. The proton and carbon signals for the sugar units were assigned by means of HETCOR, COSY and TOCSY experiments. Starting from the anomeric carbon atom of each of the four sugar units all hydrogens within each sugar were identified using COSY and TOCSY data. Using HETCOR results, each assigned hydrogen was assigned to the corresponding carbon signal. The four sugars, rhamnose, xylose, glucose and galactose were identified by comparison with published chemical shift data for methyl glycosides. The FAB-MS fragmentation pattern indicated that both xylose and rhamnose are terminal sugars. However, the sugar-sugar and sugar-aglycone linkages were indicated by ROESY data which were used to obtain spatial correlations. The observed ROESY coupling between H-1 of the galactose and H-3 of the aglycone suggested that the galactose is linked to the aglycone at C-3. This was also confirmed by the downfield shift of C-3. A ROESY peak for H-1 of glucose and H-3 of galactose disclosed that the glucose and galactose were 1,3-linked. The other

intersugar linkages were also determined by the observation of ROESY coupling between H-1 of rhamnose and H-2 of galactose, and between H-1 of xylose and H-6 of glucose.

The 2D INADEQUATE spectral data required analysis by the program CC Bond because the signals were too weak to be identified visually. The computer analysis permitted identification of 35 of the 53 C–C bonds in the saponin from ^{13}C–^{13}C connectivities. The structure of the aglycone moiety was also revealed by standard HETCOR and long-range correlation experiments, COSY and TOCSY data as well as comparison of the ^{13}C chemical shift assignments with those of a similar reference compound, jujubogenin. The stereochemistry at the C-1 position of the sugars was deduced from a comparison of the ^{13}C values with those of sugars of known structure and from the magnitude of the corresponding anomeric ^{1}H–^{1}H coupling constants.

The structures of three medicagenic acid bisdesmosides, one monodesmoside of the same acid and one soyasapogenol B monodesmoside were elucidated on the peracetylated derivatives of the saponins using the techniques such as COSY, relayed COSY, HOHAHA, HMQC, HMBC and ROESY (*7*). For example the assignments of the ^{13}C signals of medicagenic acid in saponin (**2**) were made using ^{1}H–^{13}C correlation experiments in the reverse mode such as HMQC for ^{1}J and HMBC for ^{2}J and ^{3}J. These experiments permitted assignments of most of the

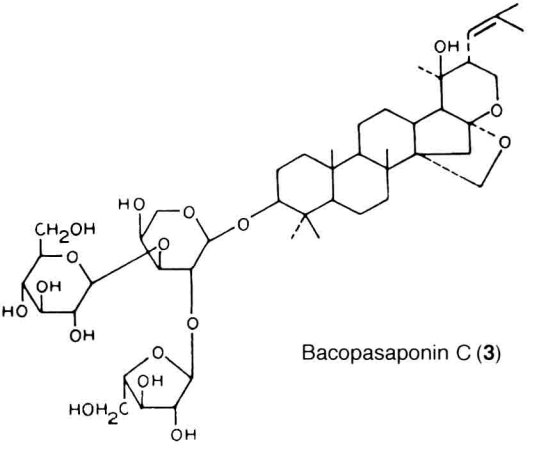


Saponin (**2**)

signals of the aglycone by observation of correlations with the angular methyl protons. The spin network of peracetylated (**2**) was identified by COSY, relayed COSY, HMQC and HMBC experiments. The HMBC experiment also allowed sequencing of all the elements of the molecule. The configuration and conformation of the arabinose unit were revealed from ^{3}J-H-1-H-2 which was found to be 6 Hz. The value indicated the α-L-configuration. The corresponding value for the β-L configuration in $^{4}C_{1}$ conformation is 2.3 Hz. The α-L configuration was also inferred

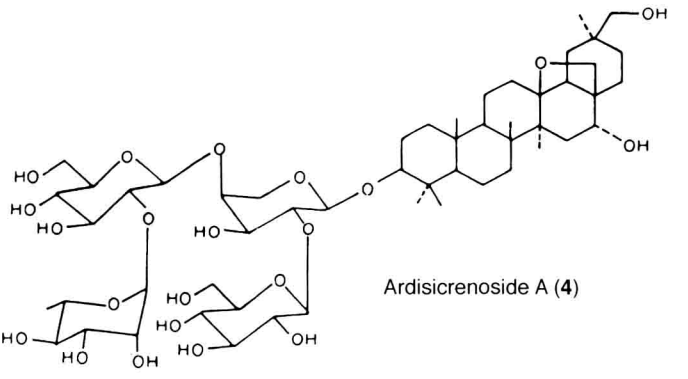
from ROESY experiments. ROEs were found between arabinose H-1 and H-3 indicating α-L-arabinose in $^4C_1$ conformation but not ruling out the presence of some $^1C_4$ conformation isomer. The ROESY experiment also disclosed the β-D-glucose and the β-D-xylose configuration by H-1-H-5 ROEs. The α-L-rhamnose configuration was deduced from long range H-1-H-5 coupling in LR COSY. The ROEs, aglycone H-3 to glucose H-1, rhamnose H-1 to arabinose H-2 and xylose H-1 to rhamnose H-4 provided sequential information.

The structures of three new dammarane-type triterpenoid saponins, bacopasaponins A, B and C isolated from the reputed Indian medicinal plant *Bacopa monniera*, were elucidated by spectroscopic methods and some chemical transformations (*24*). The ^1H and ^{13}C signals of all the saponins were assigned and ring sizes of the sugars determined by DEPT, ^1H–^1H COSY, HSQC and HMBC experiments. The configurations at C-20 and C-22 of the aglycone pseudojujubogenin in bacopasaponin C (**3**) were determined by phase-sensitive ROESY.



Bacopasaponin C (**3**)

The structures of two novel triterpenoid saponins, ardisicrenoside A and ardisicrenoside B, were determined by 2D NMR COSY, HOHAHA, HETCOR, HMBC and ROESY experiments (*5*). For example, ardisicrenoside A (**4**) showed in its ^{13}C NMR spectrum four anomeric carbon signals and its new aglycone displayed six sp^3 quaternary carbon atoms. The ^{13}C data of the aglycone part were similar to that of the known triterpene, cyclamiretin A (*25*). These data suggested that ardisicrenoside A is a triterpenoid tetrasaccharide. The assignments were confirmed by long-range coupling in HMBC and by spatial interaction in ROESY. The spatial proximities observed between H-3 and H-23, H-3 and H-5, H-16

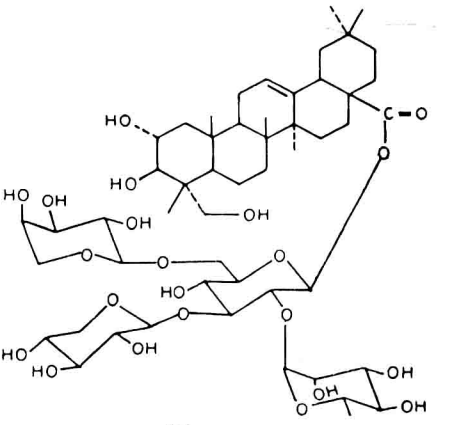


Ardisicrenoside A (**4**)

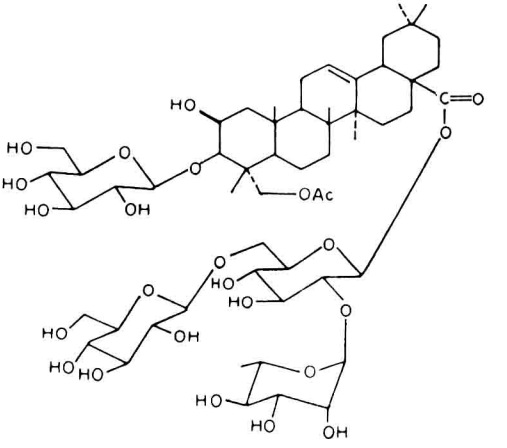
and H-28 suggested β and α configurations at C-3 and C-16 respectively. A correlation between H-18 and H-30 allowed assignment of the hydroxymethyl group to C-30.

The nature of the monosaccharides and their sequence were determined by means of H COSY, HOHAHA, HETCOR, HMBC and ROESY experiments. Starting from the anomeric protons of each sugar unit, all the hydrogens within each spin system were identified using COSY aided by the 2D HOHAHA spectrum. On the basis of the assigned hydrogens, the ^{13}C resonances of each sugar unit were assigned by HETCOR and further ascertained by an HMBC experiment.

A novel arjunolic acid tetrasaccharide (**5**) with an unusual carbohydrate chain was isolated from *Heteropappus biennis*. Its structure was established mainly by a combination of 1D selective and 2D NMR techniques such as COSY, TOCSY, ROESY, HMQC and HMBC. Molecular modelling calculations revealed that the oligosaccharide chain in the molecule is rather rigid (*26*). The structure of the complex carbohydrate chain was determined by NMR pulse experiments. The characteristic ^{13}C values of the anomeric carbons indicated four different monosaccharide units. The proton connectivities of the individual sugars were determined by H, H-COSY; 2D H,H-COSY and 1D TOCSY experiments were used to determine coupling constants. Using *Gaussian* pulses, the transitions of the anomeric protons of the individual monosaccharide units were selectively excited and then the magnetization was transferred within one monosaccharide residue to H-C(2), H-C(3), H-C(4), H-C(5) and in case of the glucose to $CH_2(6)$ depending on the mixing time used. The carbon atoms were identified by an HMQC spectrum. The HMBC technique was used to determine the sequence of the carbohydrate chain which was also confirmed by the ROESY spectra (1D, 2D).



(**5**)



Asterbatanoside F (**6**)

NMR techniques including COSY, HETCOR, COLOC, HOHAHA, ROESY and selective INEPT were used for elucidation of the structure of four novel triterpenoid saponins, asterbatanosides F, G, H and I, from the roots of *Aster batagensis* (*27*). For example the COLOC spectrum of asterbatanoside F (**6**) displayed a correlation contour between the H-23 signal and the carbonyl carbon signal of the acetyl group suggesting presence of an acetyl group at the C-23 position of the aglycone. The 2D COSY and HOHAHA spectra helped to assign all of the proton signals in each monosaccharide and the HETCOR spectrum permitted assignment of all carbon signals of the sugar units. In a selective INEPT experiment,

irradiation of the anomeric proton signal of the rhamnose at δ 6.47 enhanced the carbon resonance at δ 75.3 of C-2 of the inner glucose in the 28-O-sugar units suggesting a (1 → 2) linkage between the rhamnose and the 28-O-inner glucose unit. These conclusions were verified by a ROESY experiment which showed NOE correlations between H-1 of the rhamnosyl unit and H-2 of the inner glucosyl unit, and between H-1 of the outer glucosyl unit and H-6 of the inner glucosyl unit. Moreover, each glucose H-1 showed NOE with H-3 and H-5, and the rhamnose H-1 showed NOE with H-4 which confirmed the configuration of the sugar units.

4. Biological Activity

Triterpenoid saponins are widely distributed throughout the plant kingdom. Saponins in general have been in use as natural detergents, fish poisons, arrow poisons and foaming agents from the early stages of civilization. Earlier studies of the biological activities of saponins were limited to crude extracts containing saponins as well as other polar constituents. However, with the introduction of more and more sophisticated methods of isolation and structure determination, there has been increased interest in the study of structure-activity relationships among triterpenoid saponins. The results published so far provide a growing body of information about their diverse effects, particularly in health-related areas. Saponins are present in many animal feedstuffs and also in some human foods. Although many saponins are highly toxic when given intravenously to higher animals, the toxicity is very much lower when they are administered orally. This is because of their almost complete failure to cross the gut and enter the blood stream, and because the hemolytic effect is very much reduced in the presence of plasma.

4.1. Antifungal Activity

Many saponins exhibit antifungal activity under experimental conditions. The antifungal action of glycosides of polygalacic acid has been reported (*28*). The bisdesmosides virgaureasaponins 1 and 2, bellissaponin 1 and the corresponding mono-desmosides (prosapogenins) isolated from *Solidago virgaurea* and *Bellis perennis* inhibited the growth of *Candida* and *Cryptococcus* species *in vitro*. The bisdesmosides were more active than prosapogenins. Structure-activity relationships of α-hederin from *Hedera rhombea* was investigated by comparing its