Electrospray Mass Spectrometry with Consecutive Fragmentation Steps (ESI-MS^n) as a Tool for Rapid and Sensitive Analysis of Ginsenosides and Their Galactosyl Derivatives

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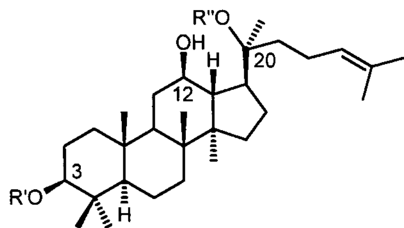
The ginsenosides Rb_1 (**3**) and Rg_1 (**4**) isolated from *Panax ginseng* were enzymatically modified with galactosyltransferase to furnish new derivatives carrying galactose units in one or both sugar chains at position C(20) and/or C(3) or C(6) of the protopanaxadiol and protopanaxatriol aglycones **1** and **2**, respectively. To determine the linkage position(s) of the introduced galactose unit(s), an electrospray-ionization MS analysis with consecutive fragmentation steps (ESI-MS^n) was carried out using an ion-trap mass spectrometer (*Figs. 2* and *3*). It was shown that both sugar moieties, located at different positions of the protopanaxadiol and protopanaxatriol aglycone, can be easily differentiated and analyzed in the subsequent fragmentation steps. Collision-induced dissociation (CID) of the Na^+-ionized molecule (MS^2) leads to cleavage of the most labile O–C(20) glycosidic bond, liberating the C(20) oligosaccharide fragment ion that can be analyzed in a subsequent fragmentation step (MS^3). MS^3 of the C(20) monodeglycosylated ginsenoside leads to cleavage of the second sugar moiety, allowing structure analysis of this fragment ion (MS^4). By this method, the linkages of the monosaccharides and branching positions can be rapidly determined using only a few µl of a 10^{-5} M sample solution.

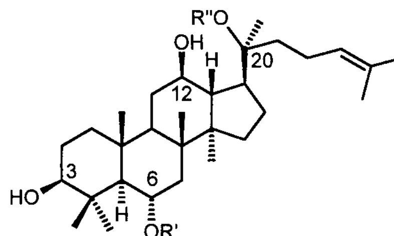
Introduction. – In many European countries, ginseng products (as well as other herbal medicines) are gaining popularity in both self medication and physician-directed therapy. In Commission E Monographs, where German authorities set standards for safety and efficacy of phytomedicines, ginseng is attributed with tonic-type properties 'for invigoration and fortification during times of fatigue and debility' [1].

The consensus opinion is that the major active principles of *Panax ginseng* are *O*-glycosides of the triterpene dammarane structure, known as ginsenosides. Over 20 ginsenosides have been extracted from the roots, leaves, and flower buds of ginseng [2][3]. Aglycones of the common ginsenosides include (20*S*-protopanaxadiol = (3β,12β,20*S*)-dammar-24-ene-3,12,20-triol; **1**) (Rg_1, Rb_2, Rc, and Rd) and (20*S*-protopanaxatriol = (3β,6α,12β,20*S*)-dammar-24-ene-3,6,12,20-tetrol; **2**) (Re, Rf, Rg_1, and Rg_2). The bisdesmosidic ginsenosides Rb_1 and Rg_1, with sugar moieties consisting exclusively of glucose units, are found in the largest amounts.

Recently, we have started a project aimed at exploiting glycosyltransferases for the introduction of sugar residues into glycosides [4] in order to enrich the structural



1 R' = R" = H; (20*S*)-protopanaxadiol
3 R' = sophorose, R" = gentobiose; Rb_1



2 R' = R" = H; (20*S*)-protopanaxatriol
4 R' = R" = glucose; Rg_1

informations for future studies on the structure-activity relationship of ginsenosides Rb_1 (**3**) and Rg_1 (**4**). Both ginsenosides were enzymatically treated with β-1,4-galactosyltransferase from bovine colostrum, which regio- and stereoselectively transfers galactose to glucose in position 4 [5][6], affording the corresponding galactosyl derivatives. Details of the synthetic methods, the isolation of the galactosyl derivatives, and the corresponding NMR analysis will be published elsewhere.

This paper focuses on the use of electrospray-ionization (ESI) mass spectrometry (MS) with collision-induced dissociation (CID) in a quadrupole ion trap for the rapid and efficient characterization of native ginsenosides and their biocatalytically generated galactosyl derivatives. The first use of ESI-MS in combination with liquid chromatography (LC) for analysis of ginsenosides was reported in 1995 [7] using standard references as well as plant extracts. MS/MS has been used for differentiation of isomeric ginsenosides after LC separation, for identification of the terpene core [8a], and for determination of ginsenosides in plant extracts [8b]. *Wang et al.* [9] applied HPLC/MS/MS for the identification and quantification of the ginsenosides in *Panax ginseng* and in *Panax quinquefolius*. While CID of $[M + H]^+$ as precursor ion in a triple-quadrupole mass spectrometer allowed the determination of the aglycones and the attached sugar(s), no cross-ring fragments were obtained to allow characterization of the linkages of the monosaccharides [10][11].

We report here ESI mass analyses with consecutive fragmentation steps (MS^n) in an ion-trap mass spectrometer [12] to identify the ginsenosides, to determine the sequence and linkages of the monosaccharides in both carbohydrate moieties in native bisdesmosidic ginsenosides, and to elucidate the structures of products derived from enzymatical galactosylation of these natural products. This analysis was carried out with the Nano-ESI technique [13], which employs small sample flow rates (*ca.* 30 nl/min). This is advantageous because of the low sample consumption and the higher ionization efficiency compared to ESI, which has flow rates in the µl/min range [14]. For that reason, nano-ESI-MS^n is clearly the most sensitive method for the determination of the sequence, linkages, and branching of sugars in picomol amounts.

Results and Discussion. – *Sequential Elimination of Saccharides from the Aglycone.* While the native ginsenoside Rb_1 (**3**) of the protopanaxadiol type **1** possesses a C(20) gentobiose and a C(3) sophorose unit, only one glucose moiety is attached to C(20) and C(6) of the protopanaxatriol-type aglycone **2** of the native ginsenoside Rg_1 (**4**), respectively. Compounds **3** and **4** were enzymatically galactosylated, and seven

derivatives were isolated. The structures of both native and derivatized ginsenosides (see *Table 1*) were analyzed by ESI and fragmentation in a quadrupole ion-trap mass analyzer. In all cases, CID of the positively ionized molecules in the ion trap led to sequential elimination of both sugar moieties. In the first fragmentation step (MS^2 of the molecular cation), the sugar moiety at position 20, and in the following fragmentation step (MS^3 of the monodeglycosylated ion), the sugar moiety at either position 3 or 6 was cleaved. The higher lability of the C(20) compared to the C(3) or C(6) glycosidic bond is probably due to a steric hindrance of OH−C(12), which triggers the cleavage of the intact oligosaccharide at C(20) by β-elimination.

Table 1. *Elucidated Structures of the Common Ginsenosides Rb₁* (**3**) *and Rg₁* (**4**) *and of Their Galactosyl Derivatives of the Protopanaxadiol and Protopanaxatriol Type*[a])

	Protopanaxadiol type			Protopanaxatriol type	
	R^I	R^{II}		R^I	R^{II}
Rb_1 (**3**)	β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→*O*-3)	β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→*O*-20)-	Rg_1 (**4**)	β-D-Glc*p*-(1→*O*-6)	β-D-Glc*p*-(1→*O*-20)-
Rb_1P1	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→*O*-3)	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→*O*-20)-	Rg_1P1	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→*O*-6)	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→*O*-20)-
Rb_1P2	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→*O*-3)	β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→*O*-20)-	Rg_1P2	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→*O*-6)	β-D-Glc*p*-(1→*O*-20)-
Rb_1P3	β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→*O*-3)	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*-(1→*O*-20)-			
Rb_1P4	β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→*O*-3)	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→*O*-20)-			
Rb_1P5	β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→*O*-3)	β-D-Gal*p*-(1→4)-[β-D-Glc*p*-(1→6)]-β-D-Glc*p*-(1→*O*-20)-			

[a]) Glc*p*: glucopyranosyl; Gal*p*: galactopyranosyl.

Because of the differences in the linkage type within the carbohydrate moieties attached to the aglycone, each di- or oligosaccharide eliminated can be clearly identified. This is illustrated in *Fig. 1* with the native ginsenoside Rb_1 (**3**) as example. The ESI mass spectrum in *Fig. 1,a* shows the Na^+-ionized molecule $[M+Na]^+$ at *m/z* 1131.6, accompanied by signals from salt adducts and some minor peaks from impurities. Upon fragmentation of the precursor ion (*m/z* 1131.6), only two fragment ions were produced by bond cleavage between the aglycone and the sugar moiety at C(20); *i.e.* by H-transfer, the intact disaccharide and the dehydrogenated singly deglycosylated molecule were generated, and both were detected as Na^+-attached ions at *m/z* 365 and 789, respectively (*Fig. 1,b*). Applying MS^3 to the singly deglycosylated molecule (*Fig. 1,c*) gives one high-intensity fragment at *m/z* 365. In this case, the C(3) sugar chain is cleaved as intact Na^+-attached disaccharide; the aglycone was not registered because of its low ability to attach a Na^+ or H^+ ion. Although both sugar chains of **3** have the same mass, they can be differentiated by applying MS^3 (*Fig. 1,d*) to

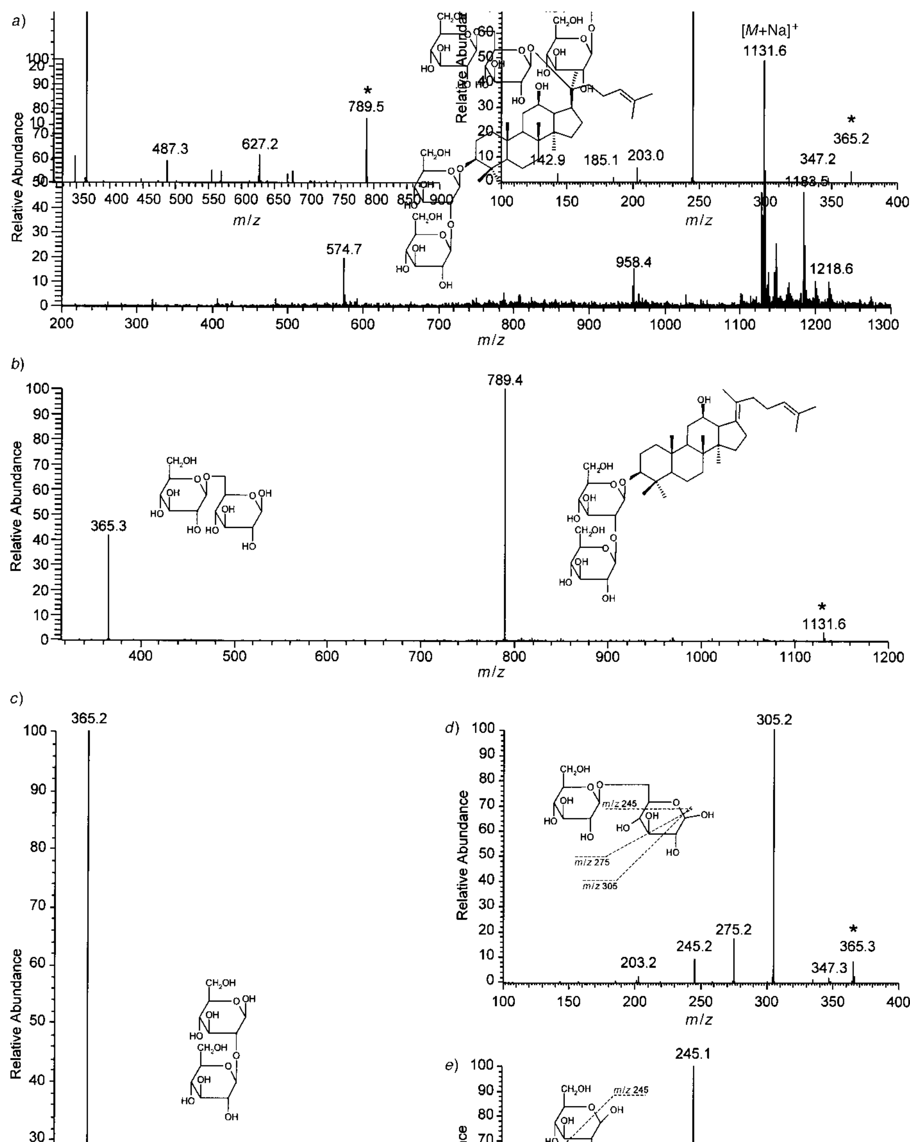


Fig. 1. a) *Positive-ion mass spectrum of ginsenoside* Rb_1 (**3**); b) MS^2 *of the [*M + *Na]*$^+$ *ion* (*m/z* 1131.6); c) MS^3 *of the monodeglycosylated ginsenoside* (*m/z* 789.5); d) MS^3 *of the disaccharide from position 20* (*m/z* 365.3); e) MS^4 *of the disaccharide from position 3* (*m/z* 365.2). Starred ions (*) indicate precursor ions for fragmentation.

the C(20) gentobiose unit and MS^4 to the C(3) sophorose unit (*Fig. 1,e*), respectively. While fragments produced by cleavages of the glycosidic bonds (*m/z* 203) are identical for both sugars, fragment ions from cross ring cleavages which reflect the linkage types between the sugar units, are different. The peaks at *m/z* 305, 275, and 245 for the C(20) gentobiose in *Fig. 1,d* correspond to losses of 60, 90, and 120 Da, respectively, which are characteristic for $1 \rightarrow 6$-linked hexoses [10][11]. By contrast, fragmentation of the C(3) sophorose leads to only one ring fragment ion at *m/z* 245, resulting from the loss of 120 Da, which is typical for $1 \rightarrow 2$-linked hexoses [10][11].

Analysis of Galactosylated Derivatives of Ginsenosides. Following the above described procedure, all structures of the galactosyl derivatives of ginsenosides could be analyzed. This is exemplified for the two products Rb_1P1 and Rb_1P3 (*Figs. 2* and *3*). The mass spectrum from Rb_1P1 in *Fig. 2,a* shows the ionized molecule peak $[M + Na]^+$ at *m/z* 1455.4 and a doubly charged ion $[M + 2Na]^{2+}$ at *m/z* 739.6 suggesting the introduction of two galactose molecules. Four possible attachment sites are indicated. MS^2 of the singly charged ginsenoside molecular ion (*Fig. 2,b*) affords two intense peaks, one at *m/z* 951, from the loss of a trisaccharide $[Rb_1P1 + Na - \text{trisaccharide}]^+$ and one at *m/z* 527, resulting from an intact $[\text{trisaccharide} + Na]^+$ ion. Based on the successive elimination of oligosaccharides described above, the intact trisaccharide (*m/z* 527) can be attributed to the C(20) sugar. MS^3 of the singly deglycosylated ion (spectrum not shown) reveals another trisaccharide from position 3. The next step in the analysis is to determine for both trisaccharides whether the galactose is $1 \rightarrow 4$-linked to the glucose at the non-reducing end to give a linear chain, or to the reducing glucose to give a branched molecule. MS^3 of the C(20) trisaccharide (*Fig. 2,c*) results in ring fragmentation, typical for $1 \rightarrow 6$-linked hexoses, from losses of 60, 90, and 120 Da, indicating the terminal attachment of galactose. In the case of branching at position 4 of the reducing sugar, a loss of 90 Da would not be possible. Indeed, it has been shown that $1 \rightarrow 4$-linked sugars under fragmentation produce ions from losses of 60 and 120 Da, but not of 90 Da [10][11]. The fragmentation of the trisaccharide from position 3 (*Fig. 2,d*) leads to one ring fragment (−120 Da) typical for a $1 \rightarrow 2$-linkage and is thus also a linear molecule. This fragment ion can not be produced in the case of branching at position 4 at the reducing sugar. Therefore, it could be established for the galactosylated ginsenoside derivative Rb_1P1 that both sugar moieties consist of linear trisaccharides.

Fig. 3 shows the spectra from the structure elucidation of product Rb_1P3 that, according to the positive ion molecule masses at *m/z* 1455.4 and 739.8 (*Fig. 3,a*), is again digalactosylated. Here, the MS^2 (*Fig. 3,b*) differs from that of the other derivatives in that there is only one intense peak (at *m/z* 689), which appears to be a tetrasaccharide. In this case, both galactose molecules must be present at the C(20) sugar chain. The ion corresponding to $[Rb_1P3 + Na - \text{tetrasaccharide}]^+$ is completely absent in the spectrum, suggesting that all the positive charge is retained on the tetrasaccharide. An explanation for this may be the higher affinity of the tetrasaccharide compared to the disaccharide to complex an alkali ion. In case of the doubly charged molecule at *m/z* 739, the positive charge due to the presence of a second Na^+ ion can be distributed among both carbohydrate moieties. MS^2 of the disodium ion (*Fig. 3,c*) shows the expected spectrum with two peaks from cleavage of the glycosidic bond. The peak at *m/z* 689 indicates the presence of a tetrasaccharide resulting from transfer of

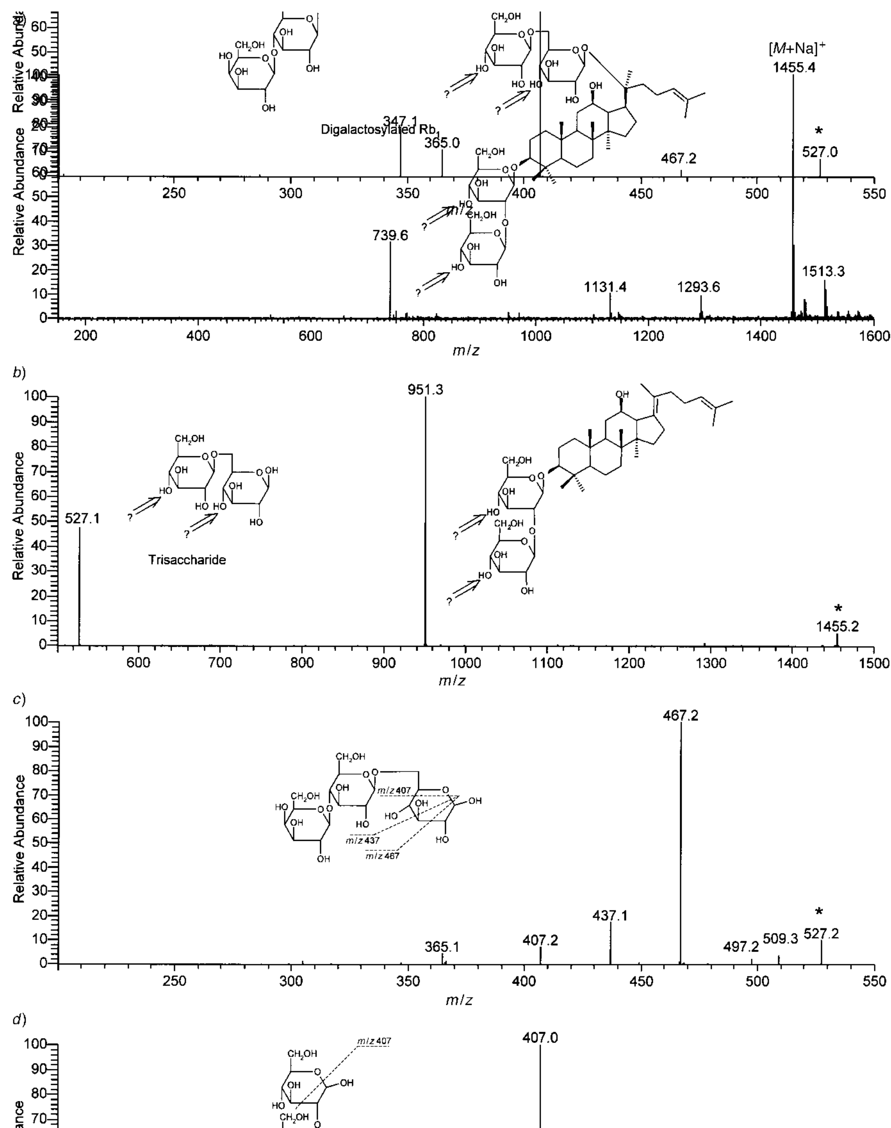


Fig. 2. a) *Positive-ion mass spectrum of enzymatically treated ginsenoside Rb_1 showing the digalactosylated product Rb_1P1* (possible attachment sites are indicated by ⇒); b) *MS^2 of the [M+Na]$^+$ ion* (*m/z* 1455.4); c) *MS^3 of the trisaccharide from position 20* (*m/z* 527.2); d) *MS^4 of the trisaccharide from position 3* (*m/z* 527.0). Starred ions (*) indicate precursor ions for fragmentation.

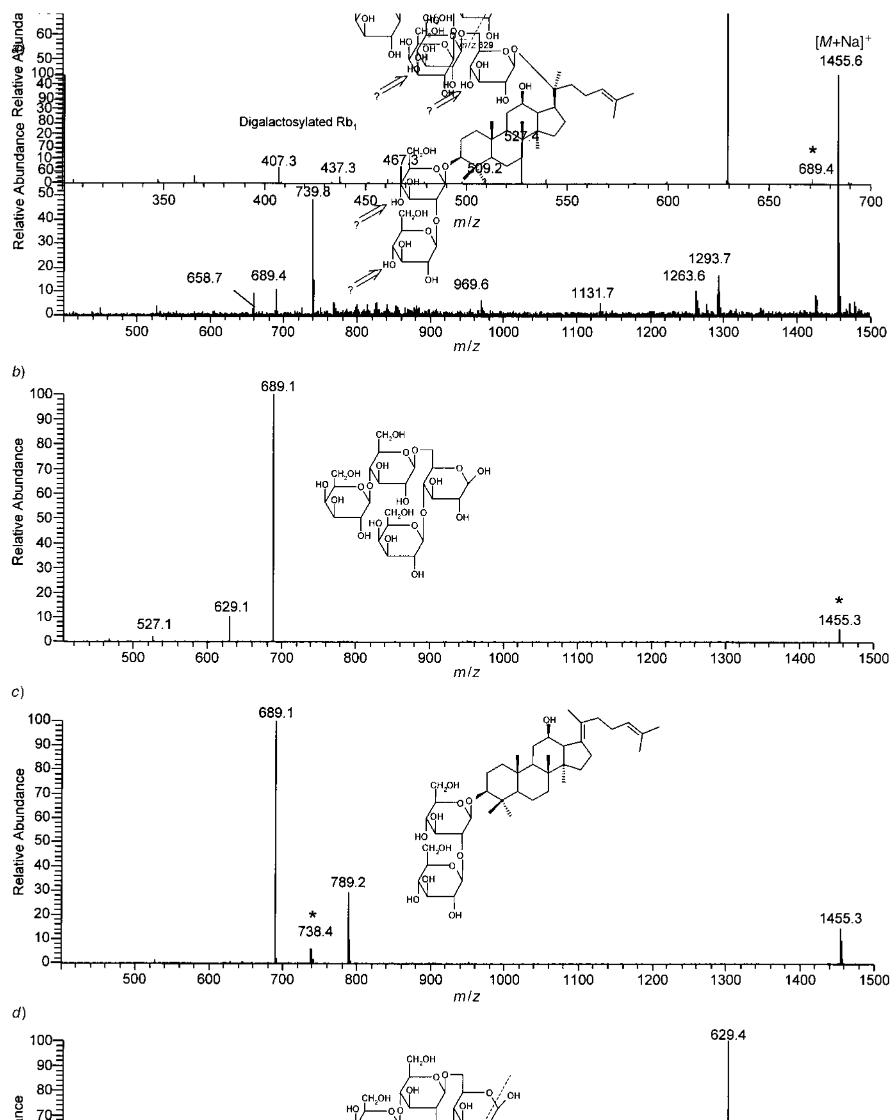


Fig. 3. a) *Positive-ion mass spectrum of enzymatically treated ginsenoside* Rb_1 *showing the digalactosylated product* Rb_1P3 (possible attachment sites are indicated by $\Rightarrow$); b) MS^2 *of the* $[M+Na]^+$ *ion* (*m/z* 1455.3); c) MS^2 *of the disodium* $[M+2Na]^{2+}$ *ion* (*m/z* 738.4); d) MS^3 *of the tetrasaccharide from position 20* (*m/z* 689.4). Starred ions (*) indicate precursor ions for fragmentation.

two galactose molecules to the disaccharide at position 20, and there is a further peak at *m/z* 789 [$Rb_1P3 + Na -$ tetrasaccharide]$^+$ with the original disaccharide at position 3. Applying MS^3 to the tetrasaccharide (*m/z* 689) (*Fig. 3,d*) results in only one cross-ring fragment from the reducing sugar, as indicated by the intense peak at *m/z* 629 from loss of 60 Da. Since there are no losses of 90 and 120 Da, as would be expected for $1 \rightarrow 6$-linked hexoses [10][11], it can be concluded that the OH group at C(4) of the reducing sugar is connected to a galactose unit, as expected. Thus, the second galactose must be connected to glucose at the nonreducing end, as shown by the structure in *Fig. 3,b*.

In the same way as described for these examples, it was possible to elucidate and verify the structures of all galactosyl derivatives listed in *Table 1* with nano-ESI-MS^n. The results are summarized in *Table 2*.

Table 2. *Cross-Ring Fragments Characterizing the Position of Galactose at the C(20) Sugar Moiety of Derivatized Rb_1 and Rg_1 Ginsenosides; at the C(3) Sugar Moiety of Derivatized Rb_1 Ginsenosides, and at the C(6) Sugar Moiety of Derivatized Rg_1 Ginsenosides*

	Sugar moiety at C(20)					Sugar moiety at C(3) (Rb_1) or C(6) (Rg_1)			
	m/z	N° of galactose introduced	Cross-ring fragments	Position of galactose		*m/z*	N° of galactose introduced	Cross-ring fragments	Position of galactose
Rb_1P1	527	1	– 60, – 90, – 120 Da	terminal	Rb_1P1	527	1	– 120 Da	terminal
Rb_1P3	689	2	– 60 Da	one galactose at each glucose	Rb_1P2	527	1	– 120 Da	terminal
Rb_1P4	527	1	– 60, – 90, – 120 Da	terminal	Rg_1P1	365	1	– 60, – 120 Da	C(4) of glucose
Rb_1P5	527	1	– 60 Da	not terminal	Rg_1P2	365	1	– 60, – 120 Da	C(4) of glucose
Rg_1P1	365	1	– 60, – 120 Da	C(4) of glucose					

Conclusion. – Nano-ESI-MS with CID in a quadrupole ion trap was shown to be an efficient and rapid method to analyze the structures of both native and galactosylated compounds. The high sensitivity of the method and the capability of carrying out several consecutive MS^n experiments allowed the determination of the different positions of the carbohydrate moieties at the protopanaxadiol and protopanaxatriol aglycone, the linkage position within the sugar moieties, and the exact point of attachment of galactose in ginsenoside derivatives obtained by treatment of the native RB_1 (**3**) and Rg_1 (**4**) with β-1,4-galactosyltransferase.

It should be mentioned that all MS^n results obtained were in agreement with the spectral data from NMR experiments carried out with the ginsenosides. Compared to NMR spectroscopy, ESI-MS combined with CID represents a simple, reliable, and efficient technique, making it an attractive and powerful tool for the analysis of ginsenosides and related compounds.

Experimental Part

Materials. Native ginsenosides Rb_1 (**3**) and Rg_1 (**4**) were kindly donated by *Indena*, Milano, Italy. Galactosyl derivatives of **3** and **4** were synthesized according to the method of *Whitesides* and co-workers [5] and *Paulson* and co-workers [6]. For ESI-MS, the samples were dissolved in MeOH at a concentration of 10^{-5} M.

Instrumentation. All ESI-MS and ESI-MS^n experiments were performed with a *LCQ*™ quadrupole ion-trap mass spectrometer (*Finnigan MAT*, San Jose, CA, USA) equipped with a Nano-ESI source (*Protana*, Odense, Denmark). Laboratory, gold-coated glass capillaries were filled with 3 µl of sample soln. and used for electrospray by application of a voltage between 700 – 1000 V. The transfer capillary was held at 200°. The capillary and the tube lens were held at the same potential (48 V). Positive-ion spectra were averaged over 20 scans, each scan consisting of 3 microscans. For CID, the relative collision energy in the trap, depending on the structure of the precursor ion, was set between 30 – 70% (corresponding to the *LCQ* software settings and defining the amplitude of the resonance excitation AC voltage), so that the relative abundance of the precursor ion did not exceed 15%. Due to the unavoidable presence of Na^+ ions during sample preparation and the high alkali-cation affinity of oligosaccharides, all positively charged ions produced were ionized with Na^+.

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