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BIOLOGICAL ACTIVITIES OF FUROSTANOL SAPONINS FROM *NICOTIANA TABACUM*

SABINE GRÜNWELLER, ERNST SCHRÖDER* and JÜRGEN KESSELMEIER† ‡

Botanical Institute, University of Cologne, Gyrhofstrasse 15, D-5000 Köln 41, F.R.G.; *Finnigan M.A.T. GmbH, Barkhausenstrasse 2, D-2800 Bremen 14, F.R.G.

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; furostanol saponins; biological activity; saponin accumulation; saponin degradation.

Abstract—Two new furostanol saponins, tobacco saponin A and B were isolated from *Nicotiana tabacum* seeds. The 26-desgluco derivative of saponin B showed hemolytic and fungicidal activity, whereas the monodesmosidic derivative of the minor saponin A had no such effects. Both saponins accumulate during ripening and are degraded during germination of the seeds. They were not found in any other part of the plant. Several *Nicotiana* species possess the same saponin pattern as recorded for *N. tabacum*.

INTRODUCTION

Many members of the nightshade family are known to contain either steroidal or alkaloid saponins or both. However, to our knowledge, the occurrence of saponins has not been reported from the tobacco plant, although several experiments have been carried out especially to examine the synthesis and metabolism of tobacco sterols [1–4].

In 1980, Kesselmeier analysed methanol extracts from tobacco tissue cultures by TLC [5]. These extracts contained substances, which showed a positive reaction with orcinol (sugar residues) and with stannic chloride (red colour, indicating triterpenoid skeleton). These observations suggested the presence of saponins in the tobacco plant. The aim of the present investigation was to locate and characterise the glycosides.

RESULTS AND DISCUSSION

Occurrence

To answer the question whether the occurrence and detection of tobacco saponins depends on the developmental stage of the plants, seeds, young plants of different height and parts of adult plants of *Nicotiana tabacum* var. Hanica were extracted with methanol, purified and analysed by TLC. Only the seed extract contains substances which showed a positive reaction both with orcinol and stannic chloride (Table 1). Such compounds could not be found in any other samples analysed.

The occurrence of saponins in the seeds could be due to *de novo* synthesis or to transport into the seeds. In the latter case, other parts of the plant should be a source of the glycosides. As shown in Table 1, young leaves, petals and carpels contain substances which also gave a positive reaction with orcinol and stannic chloride, although the colour resulting from stannic chloride spraying was not red but brown. Possibly the substances detected are saponins too, but their reaction with stannic chloride proves them to be different from the glycosides in the seeds. Therefore, transport of saponins into the ripening seeds seems unlikely. A similar observation was reported by Willuhn [6] who failed to find any transport of alkaloid saponins from the shoots to the ripening fruits of *Solanum dulcamara*. However, the glycosides found in tobacco petals, carpels and young leaves, could be precursors of the seed saponins. This question therefore cannot be answered at the moment, but because of their unspecific reaction with stannic chloride, these glycosides were not included in our investigations. Only the so-called 'positively reacting substances' showing a red colour after spraying with stannic chloride were investigated here. There were several positively reacting substances detected in the seed extract: three main glycosides and at least two more polar glycosides in only trace amount. The three main glycosides were named tobacco saponin A, B and C (R_f values between 0.13 and 0.22), A being the most polar and C the least polar compound.

Structure

In order to get sufficient quantities of the different saponins, a partly purified methanol extract of 200 g seeds was chromatographed on silica gel with chloroform–methanol–water, (35:15:2) to yield the three components. After acid hydrolysis their aglycones were analysed by GC and mass spectrometry (EI). This revealed the spirostanol tigogenin or one of its isomers as the aglycone of each saponin. The sugar residues were analysed by gas and paper chromatography, showing that glucose and rhamnose were present in each saponin. It is likely that the sugars are linked to the hydroxyl group at C-3 of the aglycone, in which case the saponins should be typical monodesmosidic saponins. Surprising-

Dedicated to Prof. Dr. L. Bergmann on the occasion of his 63rd birthday.

†Author to whom correspondence should be addressed.

‡Present address: Max-Planck-Institut für Chemie, Department of Biogeochemistry, Saarstrasse 23, D-6500 Mainz, F.R.G.

Table 1. TLC analysis of extracts from different parts of tobacco plants

	Number of compounds with comparatively high polarity (R_f 0–0.5) and reaction with detecting agents		
	Number	$SbCl_3$	Orcinol
Adult plant			
young leaves	7	(+)*	+
mature leaves	—	—	—
old leaves	—	—	—
roots	—	—	—
Plant, 25 cm			
leaves	—	—	—
stems	—	—	—
Plant, 18 cm			
shoots	—	—	—
Plant, 8 cm			
shoots	—	—	—
Seeds	3 + traces (2)	+	+
Blossom			
stem	—	—	—
sepals	—	—	—
petals	7	(+)*	+
Fruit			
stem			
sepals	—	—	—
carpels	6	(+)*	+
placenta	—	—	—

*The reaction of the detected compounds with $SbCl_3$ resulted in a brown spot. The typical colour observed with triterpenoids and steroids is red.

ly, when added to a suspension of red blood cells, the three glycosides showed no hemolytic activity, which is a characteristic of monodesmosidic spirostanol saponins [7]. This could be explained by an original bisdesmosidic and biologically inactive furostanol structure of the saponins. When furostanols are hydrolysed by acid the open side chain at C-22 rearranges to form a ring after abstraction of water and thus becomes a spirostanol. The furostanol character can be confirmed by spraying with Ehrlich's reagent. With this procedure all the tobacco saponins gave a positive reaction. Additionally their IR spectra lacked the four typical spiroketal bands. To verify the furostanol structure, the saponins were analysed by Fast-Atom-Bombardment mass spectrometry (FABMS). The molecular ions detected (*m/z* 1196, 1050 and 1064) are consistent with furostanol glycosides linked with a sugar moiety consisting of glucose and rhamnose; saponin A containing two glucose and three rhamnose and saponin B two glucose and two rhamnose. Saponin C (*m/z* 1064), however, represents the 22-methoxy derivative of saponin B. This is in accordance with Tschesche *et al.* [8] who showed that 22-hydroxyfurostanols are partly methylated when extracted with methanol, so that saponin C is probably an artifact formed during the extraction and purification procedure. Corresponding to the relatively small quantity of 22-hydroxy-A, there were only trace amounts of 22-methoxy-A, which co-migrated with saponin B during chromatography and caused a weak signal (*m/z* 1210) within the mass spectrum of B.

In order to gain more structural information, the tobacco saponins were incubated with avenacosidase, a β-glucosidase from oat, which specifically splits off the glucose units at C-26 of the oat nuatigenin saponins, avenacosides A and B [9–12]. TLC analysis of the reaction products revealed one single product from both saponins A and B. Their FAB mass spectra showed molecular ions at *m/z* 1034 and 888 indicating the loss of one glucose molecule. It can be concluded that this glucose is linked to C-26 of the furostanol skeleton. The molecular ions detected also indicated the persistence of the open side chain because no loss of water, involving the OH-function at C-22 that accompanies the closure of the spiro ring system, could be observed. Additionally, the 26-desgluco saponins still showed a positive reaction to Ehrlich's reagent. This is in contrast to the results of several authors using β-glucosidases of various sources which led to spirostanol glycosides being produced [8, 13–15]. It may be due to a different mode of action of the avenacosidase enzyme. With the FAB mass spectra further structural information was achieved. Among the signals indicating the subsequent loss of sugar molecules there was only a peak for the aglycone + glucose but none for the aglycone + rhamnose. Therefore glucose is probably the carbohydrate attached to C-3 of the aglycone whereas the rhamnose molecules occupy terminal positions. Summarizing the present information saponin A is tentatively identified as 3-*O*-[(rhamnopyranosyl)$_3$-glucopyranosyl]-26-*O*-(glucopyranosyl)-furostanol-3,22,26-triol and saponin B is 3-*O*-[(rhamnopyranosyl)$_2$-glucopyranosyl]-26-*O*-(glucopyranosyl)-furostanol-3,22,26-triol.

Biological activity

All saponins were examined for hemolytic and fungicidal activity (Table 2). As already mentioned above, saponins A, B and artifact C showed no hemolytic activity. 26-Desgluco-B revealed a strong hemolytic activity: total hemolysis of red blood cells occurred at 8 μg ml^{-1}. In contrast there was no disruption of red blood cells with 26-desgluco-A up to 100 μg ml^{-1}. Similar behaviour was registered in a fungicidal test when, after TLC, both saponins were sprayed with spores of *Cladosporium cucumerinum* and then with agarose. Desgluco-B inhibited fungal growth, whereas desgluco-A had no effect. The bisdesmosidic saponins had no influence on fungal development. In a further fungicidal test, the saponins were examined as 'plant protective agents', in the following 'spray-test'. Six different plant species were sprayed with a saponin solution and then with spores of a pathogenic fungus (see Experimental). After a

Table 2. Biological activities of tobacco saponins

	Hemolytic activity	Fungicidal activity against *Puccinia recondita* on wheat	Fungicidal activity against *Cladosporium cucumerinum* on agarose
Saponin A	—	—	—
26-Desgluco A	—	—	—
Saponin B	—	+	—
26-Desgluco B	+	+	+

few days the spread of the fungus was compared with non-saponin-sprayed plants. Saponin caused 80% growth reduction of *Puccinia recondita* on wheat. Desgluco-B reduced growth of the same fungus with a 60%-rate. There was no influence on the development of other fungi used in this investigation. Saponin A and desgluco-A had no effect at all. From the literature, it is clear that monodesmosidic spirostanol glycosides vary in their biological activities [7]. As shown by the present results, hemolytic and fungicidal activity can be achieved with an open side chain at C-22 of the steroidal skeleton. However, the biological inactivity of the monodesmosidic derivative of saponin A, which contains one more rhamnose in the sugar chain at C-3 than saponin B, is puzzling, since Dimoglo *et al.* [16] found that 24 out of 25 monodesmosidic steroid saponins with a sugar chain consisting of four or more carbohydrates exhibited fungicidal activity. Surprisingly the bisdesmosidic saponin B showed fungicidal activity too, when used in the 'spray-test'. This may be due to degradation of the bisdesmosidic structure by fungal glycosidases leading to a fungitoxic monodesmoside. Several fungi contain enzymes which enable them to totally degrade saponin sugar chains producing biologically inactive aglycones. In the case of saponin B, in the absence of rhamnosidases or an enzyme which is able to hydrolyse the sugar chain at C-3 of the aglycone, the action of fungal β-glucosidases will result in an activation of the bisdesmosidic saponin. Such an effect was observed by Sieber [17] who investigated the activity of avenacosides against *Erysiphe graminis* varieties.

The possible activation of saponin B by fungi leads to the question of whether tobacco plants possess an activating enzyme. It is known that plants, such as ivy and oat, which contain bisdesmosidic saponins, possess a specific enzyme which transforms the glycosides to their monodesmosidic, biologically active derivatives [9, 18]. However, we could find no evidence of an enzyme converting the bisdesmosidic saponins to their biologically active, monodismosidic derivatives in the tobacco seeds and plant.

Accumulation in ripening seeds and decrease during germination

To investigate the saponin content of ripening seeds and germinating seedlings, quantitative analyses were carried out using TLC-separation of purified methanol extracts, densitometric measurement of orcinol-coloured spots and reference to known amounts of oat saponins (avenacosides). Assuming that M_rs (avenacosides: m/z 1064 and 1196) and sugar composition (avenacosides: glucose:rhamnose = 3:1 and 4:1) were comparable, the absolute amount of the seed glycosides was estimated. The stage of growth of ripening tobacco seeds was determined by optical criteria (1 = white, transparent; 2 = brown, transparent; 3 = brown, hard; 4 = dark-brown, attached to placenta; 5 = dark-brown, dry, no longer attached to placenta). The saponin content of the young seeds is very low, but increases during maturation with respect to both dry weight and number of seeds (Fig. 1). From the fourth to the fifth stage of growth, the saponin content of a single seed does not markedly change while its dry weight still increases (Fig. 2).

In contrast to the accumulation in ripening seeds, saponin content of the seedlings decreases continuously during germination (Fig. 3). A 21-day-old seedling contains only a quarter of the saponin in the ripe seed. TLC analysis of purified seedling extracts showed several substances with a positive reaction to stannic chloride (red) and orcinol. With increasing age of the seedlings, the substances present became more and more lipophilic. These components are probably decomposition products of the seed glycosides. The absence of seed saponins in adult tobacco plants agrees with the observation of Hauser [19] that the furostanol saponin of tomato seeds is not present in the adult plant. However, it is not known whether the tomato saponin is degraded as quickly as the tobacco glycosides. There is a suggestion that 10-day-old tomato seedlings contain both a furostanol and alkaloid saponin, probably tomatine [19]. In contrast to the furostanol glycoside, tomatine is not present in the seed [20]. Its synthesis only begins with germination [21]. Different saponins are reported in seed and plant of *Nicotiana plumbaginifolia*: the green parts of the plant contain a solasodine glycoside, solaplumbin [22], whereas two furostanol saponins (Table 4) are found in the seeds.

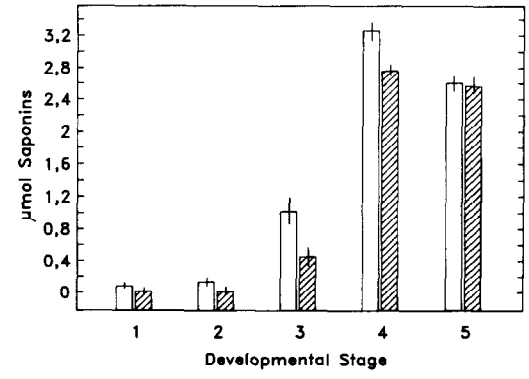

Fig.1. Changes in saponin content of seeds during ripening (mean values obtained from three experiments with three samples per stage ± s.d.); □ µmol equivalents of avenacosides g^{-1} seeds, ▨ µmol equivalents of avenacosides per 10000 seeds. Determination of developmental stages by optical criteria: 1 = white, transparent; 2 = brown, transparent; 3 = brown, hard; 4 = dark-brown, attached to placenta; 5 = dark-brown, dry, no longer attached to placenta.

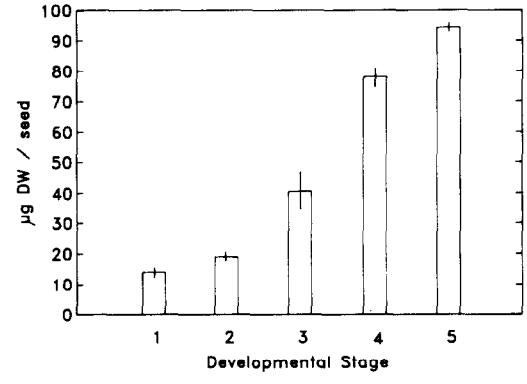

Fig. 2. Increase in dry weight during seed ripening (mean values of three evaluations ± s.d.). Developmental stages as described in Fig. 1.

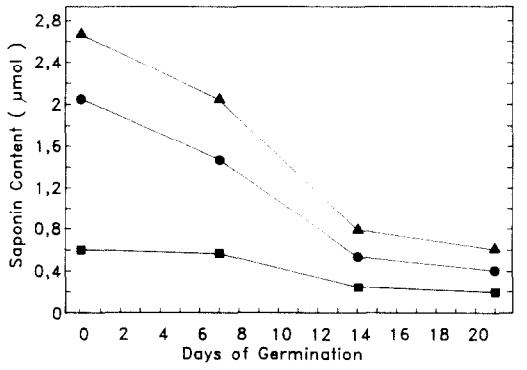

Fig. 3. Changes in saponin content of seeds during germination. Age of seedlings is referred to the amount of saponins (µmol avenacoside equivalents) per 10280 seeds (=1 g seeds) and seedlings, respectively (mean values of two experiments). ■ Saponin A, ● saponin B, ▲ total saponins.

Occurrence in tobacco varieties and other Nicotiana *species*

To examine the presence of saponins in other tobacco varieties, the seeds of 10 varieties were extracted with methanol and the purified extracts were analysed by TLC. As shown in Table 3, the same saponin pattern was found in all varieties examined, saponin B being the major and saponin A the minor component. In all cases, the values calculated revealed 0.25–0.31% of saponin per seed dry weight.

Nine of 11 *Nicotiana* species analysed showed the same pattern as tobacco glycoside B being again the major component (Table 4). Total saponin content and per cent of dry weight were more variable than in tobacco. Two species had a different saponin pattern, *N. rustica* and *N. paniculata*; their seed extracts also contain stannic chloride (red) and orcinol-positive compounds, but of a more lipophilic character. Both species belong to the subgenus *Rustica*. In contrast, another member of this group, *N. glauca*, has the same saponin pattern as the other eight species belonging to the subgenus *Tabacum* and the subgenus *Petunioides*. Therefore the different patterns do not correlate with the classification of the *Nicotiana* species.

In contrast to monodesmosidic alkaloid and spirostanol saponins, only a few bisdesmosidic furostanol glycosides have been found in other solanaceous plants. However, like the tobacco saponins, they were identified mainly in seed extracts of e.g. *Capsicum annuum* [13], *Solanum melongena* [23] and *Lycopersicon esculentum* [24]. The saponins in the seeds of *Capsicum annuum* have a 0.3% share of seed dry weight. This is within the range calculated for tobacco glycosides.

EXPERIMENTAL

General. Normal phase CC: silica gel 60 (25 × 3 cm, 70–230 mesh). Reversed phase chromatography: octyl-Si-100-Polyol (Serva, FRG) RP8 column (30 µm, 3.5 × 1.8 cm). Gas chromatography: Carlo Erba 2900 FID using a 0.3 mm × 50 m fused silica column packed with OV 101, 100–300° (10° min^{-1}) for sugar-analysis and 50–150° (20° min^{-1}), 150–300° (5° min^{-1}) for aglycone-analysis, H_2 as carrier gas. EIMS was recorded at 70 eV. FABMS: positive ion mode, target was bombarded with 6 keV Xe-atoms, samples were suspended in glycerol. IR spectra were recorded in KBr discs. Whatman No. 1 paper was used for PC and precoated silica gel plates with a combination layer Kieselguhr/silica gel (Macherey & Nagel, F.R.G.) for analytical as well as prep. TLC. The following solvents were employed: PC: *n*-BuOH–C_6H_6–pyridine–H_2O (5:1:3:3) (descending). TLC: $CHCl_3$–MeOH–1% NH_3 (2:1:1) (lower phase). Spraying reagents: orcinol (according to ref. [25]), Ehrlich's reagent and $SbCl_3$-saturated $CHCl_3$ solution for TLC; aniline hydrogen phthalate solution for PC.

Origin of plant seeds and growth conditions. Seeds of *Nicotiana tabacum* var. Samsun were obtained from the Max-Planck Institut für Biologie, Tübingen, all other tobacco varieties from the Landesanstalt für Pflanzenbau, Forchheim. *Nicotiana* species were received from Garten des Botanischen Instituts der Universität Köln; Botanischer Garten St Gallen; Botanischer Garten der Karl-Marx-Universität, Leipzig; Botanischer Garten der Stadt Wuppertal; Landesanstalt für Pflanzenbau, Forchheim; Botanischer Garten der Universität Hohenheim. Tobacco plants were grown in the greenhouse with additional, permanent illumination by high pressure mercury lights (200 µE min^{-1} m^{-2}). Germination of tobacco seeds took place in Petri dishes with humidified filter paper exposed to natural day-light. Oat seedlings were grown as described earlier [26].

Extraction procedures. Young tobacco plants, leaves and roots (30 g fr. wt) as well as petals, sepals and carpels of 15 blossoms were extracted with 150 ml of boiling MeOH. Stems

Table 3. Saponin content of seeds of different tobacco varieties

Variety	nmol saponins* per 10000 seeds B	A	Total	% of dry weight
Burlina 183	2140	450	2590	0.29
Hanica	2130	410	2540	0.25
Bashi Bagli	2250	270	2520	0.30
Baffra Bassma	2070	440	2510	0.26
Samsun	1990	310	2300	0.28
Xanthic	1940	290	2230	0.31
Geudertheimer III	1840	380	2220	0.29
Calcar × Samsun dere	1780	380	2160	0.24
Bursanica 217	1840	280	2120	0.27
Perega	1780	240	2020	0.26
Aurea	1570	240	1810	0.25

*Avenacoside equivalent.

Table 4. Saponin content of seeds of different *Nicotiana* species

Species	nmol saponins* per 10000 seeds B	A	Total	% of dry weight
N. suaveolens	3400	660	4060	0.29
N. alata	2910	390	3300	0.25
N. plumbaginifolia	2880	230	3110	0.26
N. longifolia	1280	520	1800	0.15
N. langsdorfii	1400	80	1480	0.21
N. glutinosa	850	20	870	0.23
N. glauca	320	310	630	0.13
N. silvestris	400	190	590	0.16
N. longiflora	330	110	440	0.13

*Avenacoside equivalent.

and placentas of 15 blossoms and 20 g seeds were pulverized in liquid N_2 and extracted for 30 min with 150 ml boiling MeOH. The volume of extracts containing large amounts of chlorophyll was reduced to 15 ml under low pressure, diluted with 15 ml H_2O and kept at 0° for 30 min. The pptd chlorophyll was centrifuged at 40000 *g* for 20 min. The supernatant as well as the chlorophyll-free extracts were evapd to dryness and dissolved in BuOH. To remove polar substances the BuOH-solution was extracted with H_2O and the organic layer again evapd. The residue was dissolved in small amounts of MeOH and then diluted with H_2O to obtain a 5% MeOH soln which was submitted to reversed phase CC. Separation was accomplished by stepwise elution with 40% MeOH (40 ml), 70% MeOH (40 ml) and 100% MeOH (40 ml). The 70% fraction (saponins) was evapd to dryness, dissolved in MeOH and analysed by TLC. Empty seed coats were extracted and purified like ripe seeds.

Preparative isolation of seed saponins. Seeds (100 g) were pulverized in a coffee grinder and extracted × 3 with 1 l MeOH for 12 hr. The extracts were evapd and a remaining vol. of 15–20 ml was diluted with a 10-fold vol. of ether. The pptd saponins, sugars and other polar substances were centrifuged at 1500 *g* for 10 min, the pellet was allowed to dry overnight, redissolved in BuOH and extracted with H_2O. The organic layer was evapd to dryness, the residue dissolved in MeOH and purified by reversed phase chromatography as described above. The combined saponin fractions were evapd and redissolved in MeOH. For separation of the saponins the MeOH soln was chromatographed on a silica gel column with $CHCl_3$–MeOH–H_2O, (35:15:2) as eluant.

Acid hydrolysis of saponins. For sugar hydrolysis, each saponin was incubated with 0.5 M H_2SO_4 at 100° for 6 hr. The solutions were neutralized with resin (Dowex 1 × 2, $HCOO^-$ form) and evapd to dryness. Sugars were examined by PC and GC. Since acid hydrolysis of glycosides gives a mixture of different anomeric and isomeric carbohydrates, sugars were reduced with $NaBH_4$ (1 mg mg^{-1} sugar) prior to GC-analysis to obtain the corresponding alcohols. After stirring for 4 hr at room temp., the reaction was stopped by adding a double vol. of HOAc. The acid solution was cautiously evapd while MeOH was added several times. After total evapn the samples were dried in a desiccator. For aglycone hydrolysis each saponin was dissolved in MeOH containing 2% HCl and kept at 37° for eight days. The aglycones were pptd by adding H_2O, dried and analysed by GC and EIMS.

Isolation of avenacosidase and avenacosides. Avenacosidase was isolated from oat seedlings according to ref. [12]. Avenacosides were extracted, purified and determined according to ref. [27].

Preparation of raw enzyme extracts from tobacco. Leaf extract: 6 green leaves of a flowering tobacco plant (25 g fr.wt) were homogenized in 300 ml of citrate buffer (pH 6.5), filtered through a nylon net (25 μm pore size) and centrifuged for 20 min at 40000 *g*. The sediment was dissolved in 14 ml of citrate buffer to give a crude membrane fraction. The 40000 *g* supernatant was submitted to $(NH_4)_2SO_4$ pptn (70%) and centrifuged at 40000 *g* (20 min). The protein sediment was dissolved in 12 ml buffer (soluble protein fraction).

Extracts of seedlings. 12-day-old seedlings. (0.8 cm high, grown from 6 g of seeds) were homogenized in 300 ml of citrate buffer, filtered through a nylon net and treated as described above. The membrane sediment was dissolved in 6 ml, the protein sediment of the soluble fraction in 8 ml of buffer. Seeds: 30 g of tobacco seeds were pulverized in a coffee grinder, then immediately poured into 250 ml citrate buffer and gently stirred for 10 min. To remove the seed coats the homogenate was centrifuged for 10 min at 10000 *g*. The supernatant was centrifuged again at 40000 *g* (20 min). The membrane sediment was dissolved in 2 ml, the pptd protein (see above) of the supernatant in 10 ml of buffer.

Enzymatic incubation of tobacco saponins. The substrate solution contained a mixture of saponin A, B and 22-MeO-B at a concentration of 1 mg per 100 μl. Incubation was carried out at room temp. for 1 and 24 hr. The reaction was stopped by adding 20 ml of MeOH. After centrifugation (5 min, 40000 *g*) the supernatant was evapd, the residue was dissolved in 1 ml MeOH and analysed by TLC.

Incubation with raw enzyme extracts of tobacco seeds: soluble fraction: 2 ml enzyme extract, 1 ml citrate buffer (0.2 M, pH 6.5), 100 μl substrate solution. Membrane fraction: 750 μl of dissolved membrane sediment, 1 ml of buffer, 100 μl of substrate solution. Incubation with crude enzyme extracts of seedlings: soluble fraction and membrane fraction: 1.5 ml enzyme extract, 100 μl substrate solution. Incubation with crude enzyme extracts of tobacco leaves: soluble fraction: 2 ml enzyme extract, 100 μl substrate solution. Membrane fraction: 2.5 ml of dissolved membrane sediment, 100 μl of substrate solution.

Incubation with avenacosidase from oat: 0.5 ml enzyme solution, 2 ml citrate buffer, 100 μl substrate solution, containing either 1 mg saponin A or B.

Preparation of 26-desgluco saponins by enzymatic degradation. Saponins (30 mg) (either A or B, dissolved in 0.5 ml H_2O) per ml of avenacosidase solution were incubated at room temp. for 12 hr. After stopping the reaction and centrifugation (see above), the supernatant was evapd, the residue redissolved in MeOH and analysed by TLC. If further purification was needed the saponin was submitted to prep. TLC. 2 cm of each side of the

chromatographed plate and a co-chromatographed saponin standard were sprayed with ANS (0.2 g 8-anilinonaphthalene-sulphonic acid in 100 ml MeOH), so the saponin band could be identified under UV-light (254 nm). The marked saponin section was scraped off, mixed with MeOH, and silica gel was removed by centrifugation (5 min, 1500 *g*). The supernatant was evapd to dryness and the saponin was weighed.

Spectral data. Aglycone after acid hydrolysis: EIMS *m/z* 416 $[M]^+$, 401, 386, 357, 347, 344, 302, 273, 139 (basic ion), 122, 115. Tobacco saponin A: FABMS *m/z* 1219 $[M+Na]^+$, 1179 $[(M+H)-18]^+$, 1033 $[(M+H)-146-18]^+$, 1017 $[(M+H)-162-18]^+$, 871 $[(M+H)-162-146-18]^+$, 725 $[(M+H)-162-146\times2-18]^+$, 579 $[(M+H)-162-146\times3-18]^+$, 417 $[(M+H)-162\times2-146\times3-18]^+$. IR ν_{max}^{KBr} cm^{-1}: 3650–3250 (OH), no spiroketal bands. 26-desgluco saponin A: FABMS *m/z* 1057 $[M+Na]^+$. Tobacco saponin B: FABMS *m/z* 1073 $[M+Na]^+$, 1033 $[(M+H)-18]^+$, 871 $[(M+H)-162-18]^+$, 725 $[(M+H)-162-146-18]^+$, 579 $[(M+H)-162-146\times2-18]^+$, 417 $[(M+H)-162\times2-146\times2-18]^+$. IR ν_{max}^{KBr} cm^{-1}: 3650–3250 (OH), no spiroketal bands. 22-MeO–B: FABMS *m/z* 1087 $[M+Na]^+$, 1033 $[(M+H)-32]^+$, 871 $[(M+H)-162-32]^+$, 725 $[(M+H)-162-146-32]$, 579 $[(M+H)-162-146\times2-32]^+$, 417 $[(M+H)-162\times2-146\times2-32]^+$. IR ν_{max}^{KBr} $\times cm^{-1}$: 3650 – 3250 (OH), no spiroketal bands. 26-desgluco saponin B: FABMS *m/z* 911 $[M+NA]^+$.

Quantitative analysis of saponins. Quantitative extraction of seeds and seedlings: seeds at different stages of ripening were collected, freeze-dried and weighed. An aliquot was counted to determine the number of seeds. They were pulverized in liquid N_2 and extracted while continually stirring with 100 ml MeOH per g dry weight overnight × 3. The combined extracts were evapd and the residue was extracted with $CHCl_3$ to remove apolar substances. To regain the saponins dissolved with $CHCl_3$ the extract was chromatographed over silica gel and apolar compounds were separated off with $CHCl_3$–MeOH (9:1). Then saponins were eluted with 100% MeOH, re-combined with the defatted extract and evapd. The residue was dissolved in BuOH. Further purification steps (extraction with H_2O, RP-8 column chromatography) followed as described above. For determination of saponin quantities in different tobacco varieties and *Nicotiana* species 1 g of seeds was counted, extracted and purified like the ripening seeds. Germinating seeds were pulverized in liquid N_2 and extracted with 100 ml MeOH per g overnight × 3. Seedlings with developed cotyledons were extracted by pouring 100 ml MeOH into the Petri dish (overnight extraction, × 3). Purification of these extracts was performed as described above. Here extraction with $CHCl_3$ was used to remove photosynthetic pigments.

Densitometric determination of saponins: Aliquots of samples and increasing concentrations of an avenacoside standard solution were chromatographed as described for analytical TLC. Plates were sprayed with orcinol and photographed. Spots on the negative were measured densitometrically. The amounts of avenacosides showed linearity from 0.27 to 2.2 nmol per spot.

Test for hemolytic activity. Tobacco saponins were tested for hemolytic activity according to ref. [28]. Compounds added to the erythrocyte suspension were dissolved in dimethylformamide. After incubation for 30 min, the suspensions turned clear if total hemolysis had occurred.

Test for fungicidal activity with Cladosporium cucumerinum. Tobacco saponins were tested according to ref. [29]. TLC plates and systems used were similar to those for analytical TLC. After chromatography the plates were dried for two days, so that organic compounds of the solvent were totally removed and could not interfere with fungal growth.

Test with tobacco saponins as 'plant protective agents' (spray-test). Tobacco saponins were dissolved in a small volume of EtOH and then diluted with H_2O to a concn of 250 ppm. After spraying with saponins, various test plants (see below) were sprayed with spores of a fungus pathogenic to that plant. The fungi (and plants) tested were: *Puccinia recondita* (wheat), *Pyricularia oryzae* (rice), *Phytophthora infestans* (tomato), *Erysiphe graminis hordei* (barley), *Venturia inaequalis* (apple), *Botrytis cinerea* (pepper). After 4–8 days, fungal growth was compared to the amount determined for plants untreated with saponins.

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