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Cyanogenesis in *Aralia spinosa* (Araliaceae)#

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ABSTRACT

A systematic survey of *Aralia spinosa* (Araliaceae), covering an entire growing season and including aboveground organs at various developmental stages, revealed that only about half of all samples collected showed cyanogenesis. Cyanogenesis was detected in inflorescences and leaves but is apparently restricted to certain harvest times or developmental stages. The structurally unusual triglochinin, characterized by a hex-2-enedioic acid partial structure, was the only cyanogenic glycoside detected. This is the first description of triglochinin in this species and in the family of Araliaceae. Triglochinin is biogenetically derived from tyrosine, which is in good agreement with the few cyanogenic glycosides previously detected in members of the Araliaceae family. Triglochinin was identified, characterized, and quantified by modern chromatographic methods, and the amount of enzymatically releasable hydrocyanic acid was determined qualitatively and quantitatively. Two isomers of triglochinin were detected chromatographically at minor levels. The isomeric pattern agreed well with literature data from other triglochinin-containing plants. This was confirmed in the two species, *Triglochin maritima* and *Thalictrum aquilegiifolium*, which were comparatively studied. In the case of *A. spinosa*, inflorescence buds harvested in July showed the highest content of triglochinin, just under 0.2% on a dry weight basis. The detection of triglochinin adds to the knowledge of toxicological properties and the dereplication of U(H)PLC/MS² data provides a comprehensive phytochemical profile of *A. spinosa*.

ABBREVIATIONS

BPC	base peak chromatogram
CNglc(s)	cyanogenic glycoside(s)
d. w.	dry weight
ESI	electrospray ionization
f. w.	fresh weight
HCN	hydrocyanic acid
M_{mi}	monoisotopic mass
SAX-SPE	strong anion exchange solid phase extraction
SIR	single ion recording
TIC	total ion current chromatogram

Introduction

Cyanogenesis is the ability of organisms to release HCN after the enzymatic degradation of CNglcs. It has been described for more than 3000 species of higher plants. CNglcs are derivatives of α-hydroxynitriles (cyanohydrins) stabilized by β-glycosidically bound sugars or sugar chains. They are stored in plant tissues in sometimes large amounts, usually together with (but spatially separated from) their degrading enzymes, β-glucosidases, and in some cases α-hydroxynitrile lyases. Upon tissue injury (e.g., by feeding insects), decompartmentation occurs, and the action of the degrading enzymes causes the degradation of the CNglcs

Dedicated to Prof. Dr. A. Nahrstedt (*1940 †2016) on the 5th anniversary of his death. He made a major scientific contribution to the knowledge of cyanogenic glycosides in plants and insects.

(via cyanohydrin intermediates) to the corresponding carbonyl compound and free HCN. CNglcs are a well-characterized group of natural products. Including the biogenetically related β- and γ-hydroxynitrile glucosides, more than 100 different structures are known from natural sources [1–3]. Cyanogenesis can also be found in some arthropods, especially within Lepidoptera (Insecta). Herbivorous insects can synthesize CNglcs *de novo* and/or sequestrate them from their host plants. Today, the biosynthetic pathway of CNglcs is thought to have evolved convergently in plants and animals [4, 5].

Cyanogenesis is widespread in the plant kingdom and found in many different taxa, but reports dealing with cyanogenesis within the Araliaceae family are rare and contradictory. Gibbs mentions doubtful positive results in a few genera [6]. Some years ago, two CNglcs that are biogenetically derived from tyrosine have been isolated from the tropical Araliaceae *Polyscias australiana* L. [7]. To our knowledge, this is the only report to date of structural evidence for CNglcs in a member of this family.

Cyanogenesis is also described for *Aralia spinosa* L., commonly known as devil's walking stick, angelica tree, or prickly ash. *A. spinosa* is a deciduous arborescent shrub or small tree native to the eastern United States, from western Pennsylvania south to Florida, and southwestward to Texas [8, 9]. Putative cyanogenesis is described to be restricted only to certain developmental stages. A single plant from the Missouri Botanical Gardens sampled over a 1-year period gave only 1 positive test [10–12]. The nature of the responsible CNglc(s) remains unknown to date.

Little is known about the medicinal uses of *A. spinosa*. The related species *Aralia continentalis* Kitag., *Aralia echinocaulis* Hand.-Mazz., and *Aralia taibaiensis* Z. Z.Wang & H. C.Zheng are used in traditional Korean (*A. continentalis*) and Chinese medicine, respectively [13–15]. Among the Araliaceae, ginseng root (*Panax ginseng* C. A.Mey.) and ivy leaves (*Hedera helix* L.) are worth mentioning, as both are well-studied herbal medicines that have an established place in the pharmacopoeia of European medicine [16, 17].

Our own preliminary studies on the cyanogenesis of *A. spinosa* supported previous findings on its temporary occurrence [10]. A single plant from the Botanical Garden in Münster sampled at different times of the year showed clear positive results only in a few cases. Material taken from the herbarium of the institute (collected in 1986) was still cyanogenic, but it was impossible to identify the responsible cyanogenic compounds out of this material due to degradation. Hence, it was decided to collect fresh plant material from *A. spinosa* in a systematic approach lasting for one complete growing season. The aim was to identify the cyanogenic principle and to quantify the putative CNglcs by means of U(H)PLC/MS in combination with the measurement of releasable HCN.

Results and Discussion

Samples of aboveground parts of *A. spinosa* were collected throughout one complete growing season of the plant from a single site in the Botanical Garden of Münster (▶ **Fig. 1** and **Fig. 1Sa/1Sb**, Supporting Information). During this time, samples were taken every 14 days from the respective plant parts. All samples were promptly shock frozen in liquid nitrogen, pulverized, and freeze-dried directly, avoiding thawing of the sample. Stems, shoot axils, terminal buds, leaf buds, young and older pinnate leaves, complete young shoots, flower buds, flowers, fruits, and senescent leaves were collected and immediately tested for cyanogenesis by use of Feigl-Anger test papers [18, 19]. Positive tests were observed only on 10 out of a total of 17 harvest days. Especially in spring and early summer (collections between March 11-June 3), not a single positive detection was made.

▶ **Table 1** shows the complete list of all samples of the annual cycle harvest with a detailed description of the development stages including the respective d. w. After a total of 32 samples was obtained, all freeze-dried and powdered plant materials were again systematically analyzed for their content of releasable HCN by means of a Feigl-Anger test (**Fig. 2S**, Supporting Information) evaluated in a semiquantitative manner. This resulted in Feigl-Anger score values ranging from 0 to a maximum of 4. It was not until mid-June that one single leaf sample (#08) with positive evidence of HCN could be detected. In July, all leaf samples remained negative. Only one single July sample, young inflorescence buds (#10B), was positive. After that, in August, all samples (mostly blended samples of complete pinnate leaves; #08, 10B, 12A/B, 13A–C) were clearly positive. One young shoot harvested directly from the ground (#13A), which had a particularly high HCN content (score: 4), should be highlighted. In September, all green leaf samples were positive, but chlorotic (#14A) and yellow-red senescent leaves (#14C) showed negative results. Also, samples harvested at the end of the month of September (#15A–C) showed only low amounts of HCN. All fruit samples (#14D/E) were negative, too. Now, one would expect this trend to continue in the following month, but in October, unexpectedly, all samples including green leaves (#16A, 17A) and senescent leaves (#16B, 17B) were clearly positive again.

To confirm the semiquantitative results, all samples were re-investigated using a quantitative HCN assay by Conway's microdiffusional apparatus [20] in combination with a colorimetric method according to Aldridge [21] modified by Nahrstedt [22]. All results are reported in ▶ **Table 1** as mean values of at least duplicate determinations (in case of doubt, the measurements were repeated). Essentially, the Feigl-Anger results could be confirmed, which shows that the semiquantitative estimation provides, in principle, a well-suited method. However, it must also be noted that this assay is obviously susceptible to error, especially for samples with a higher cyanide content (e.g., #12A/B, 13A, 14B). In our experience, the visual estimation of the Feigl-Anger paper's color depth is linear only to a limited extent. The precision and repeatability expected from modern chromatographic methods cannot be achieved with either the diffusion method or the Feigl-Anger test. Nevertheless, the detection of enzymatically releasable HCN is an important parameter for the characterization of cyanogenic plants. In summary, the contradictory literature reports on cyanogenesis of *A. spinosa* were clearly confirmed. Cyanogenesis seems to depend strongly on the time of sampling and on the investigated organ.

In a next step, the CNglcs responsible for cyanogenesis were to be identified. Some preliminary TLC experiments showed that one CNglc with a relative high polarity was detectable as a weak HCN-positive spot at $R_f = 0.21$ in the sandwich picrate test [23] and a weak green spot by detection with anisaldehyde sulfuric acid re-

▶ **Fig. 1** *A. spinosa*. Different stages of development (date of photo taken). **a** Spring buds at a shoot axis that was harvested in autumn of the previous year (February 16). **b** Axillary bud at the stem with prickles (March 25). **c** Young leaflets (April 8). **d** Pinnate leaves (May 6). **e** Inflorescence buds from the apical stem (July 16). **f** Bloomed inflorescence (August 24). **g** Immature fruits (August 24). **h** Ripe fruits (September 10). **i** Senescent leaves (October 7). A colored version of this illustration can be found in the Supporting Information.

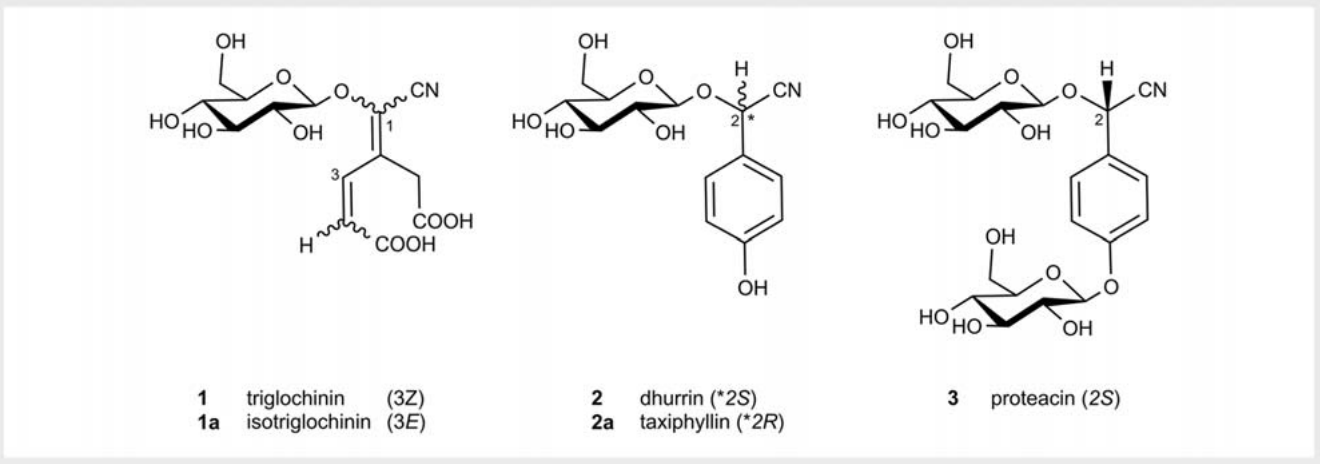
agent (**Fig. 3S**, Supporting Information). Of all reference substances that were co-applied, only triglochinin showed an identical R_f value under the given conditions. The structure of triglochinin (**1**), a very unusual CNglc with a dicarboxylic acid moiety, is shown in ▶ **Fig. 2**.

This preliminary result was to be confirmed by use of modern chromatographic and mass spectrometric techniques. For the identification of **1**, we used two U(H)PLC systems differing in stationary phases and detection modes. ▶ **Fig. 3** illustrates the chromatograms of a typical HCN-positive sample as an example and **Fig. 4S**, Supporting Information, shows UV chromatograms of all investigated samples (system 1). The upper part of ▶ **Fig. 3** shows the UV chromatogram obtained by U(H)PLC system 1 at $\lambda = 275$ nm (▶ **Fig. 3 a**). The identity of **1** was confirmed by coelution with reference compound **1** ($R_t = 2.25$ min) and comparison to the respective UV spectra (▶ **Fig. 3 a1**). The mass spectrometric

▶ **Table 1** *A. spinosa* samples (annual cycle). Harvest at 14-day intervals during 1 growing season. Numbering, date of harvest, description of samples and development stages, d. w. (after lyophilization), and cyanogenic potential (semiquantitative and quantitative). The Feigl-Anger score values are defined by the intensity of blue coloration: negative (0), weak (1), medium (2), clear (3), and very intense (4); quantitative HCN determination according to [20–22].

Sample Nr.	Harvest date	Sample description and development stage	Dry weight	HCN (semiquantitative score)					HCN (quantitative)
			[g/100 g]	0	1	2	3	4	[mg/kg] [a]
#01	2014–03–11	terminal buds on the shoot axis (cut was made in the fall of the previous year), ▶ **Fig. 1 a**	18.3	0					nd [b]
#02	2014–03–25	stems, terminal buds, axillary buds, ▶ **Fig. 1 b**	20.3	0					nd
#03	2014–04–08	very young leaflets (terminal and lateral), ▶ **Fig. 1 c**	11.8	0					nd
#04	2014–04–22	young leaflets (terminal and lateral)	18.3	0					nd
#05 A	2014–05–06	pinnate leaves (terminal and lateral), ▶ **Fig. 1 d**, young stems	17.3	0					nd
#05 B	2014–05–06	very young leaflets	–	0					nd
#06 A	2014–05–20	young leaflets (terminal and lateral)	22.7	0					1.1
#06 B	2014–05–20	very young leaflets, buds	14.9	0					nd
#07	2014–06–03	pinnate leaves (blended sample)	29.0	0					nd
#08	2014–06–17	pinnate leaves (blended sample)	17.9				3.2		70.8
#09	2014–07–01	pinnate leaves (blended sample)	27.1	0					1.9
#10 A	2014–07–16	pinnate leaves (blended sample)	20.6	0					2.1
#10 B	2014–07–16	flower buds (green) from inflorescence, ▶ **Fig. 1 e**	20.7			1.9			87.4
#11	2014–07–30	pinnate leaves (blended sample)	16.1	0					0.1
#12 A	2014–08–12	pinnate leaves (blended sample)	15.4			1.9			0.2
#12 B	2014–08–12	pinnate leaves	19.6			2.3			121.8
#13 A	2014–08–27	whole young plants, harvested close to the ground	12.7					4.0	23.0
#13 B	2014–08–27	terminal pinnate leaves	25.2			2.2			92.8
#13 C	2014–08–27	pinnate leaves, blended sample, harvested at a height of 1 m	16.4			2.0			69.1
#14 A	2014–09–10	chlorotic leaves	22.7	0					0.5
#14 B	2014–09–10	green leaves, blended sample, harvested at a height of 1 m	13.8				2.8		12.9
#14 C	2014–09–10	senescent leaves (yellow-red)	20.8	0					0.2
#14 D	2014–09–10	fruits, completely ripe (purple), ▶ **Fig. 1 h**	22.4	0					0.2
#14 E	2014–09–10	fruits, mid-ripe (green)	20.6	0					1.2
#15 A	2014–09–24	pinnate leaves	20.3	0.3					0.4
#15 B	2014–09–24	senescent leaves (yellow-red)	21.3	0.3					1.2
#15 C	2014–09–24	pinnate leaves	25.8	0.4					0.7
#16 A	2014–10–07	green pinnate leaves	23.7			1.9			0.2
#16 B	2014–10–07	senescent leaves, ▶ **Fig. 1 i**	23.0		1.3				0.2
#17 A	2014–10–22	green leaves	20.6			2.0			0.5
#17 B	2014–10–22	senescent leaves	20.4				3.2		17.5

[a] d. w., [b] not detectable



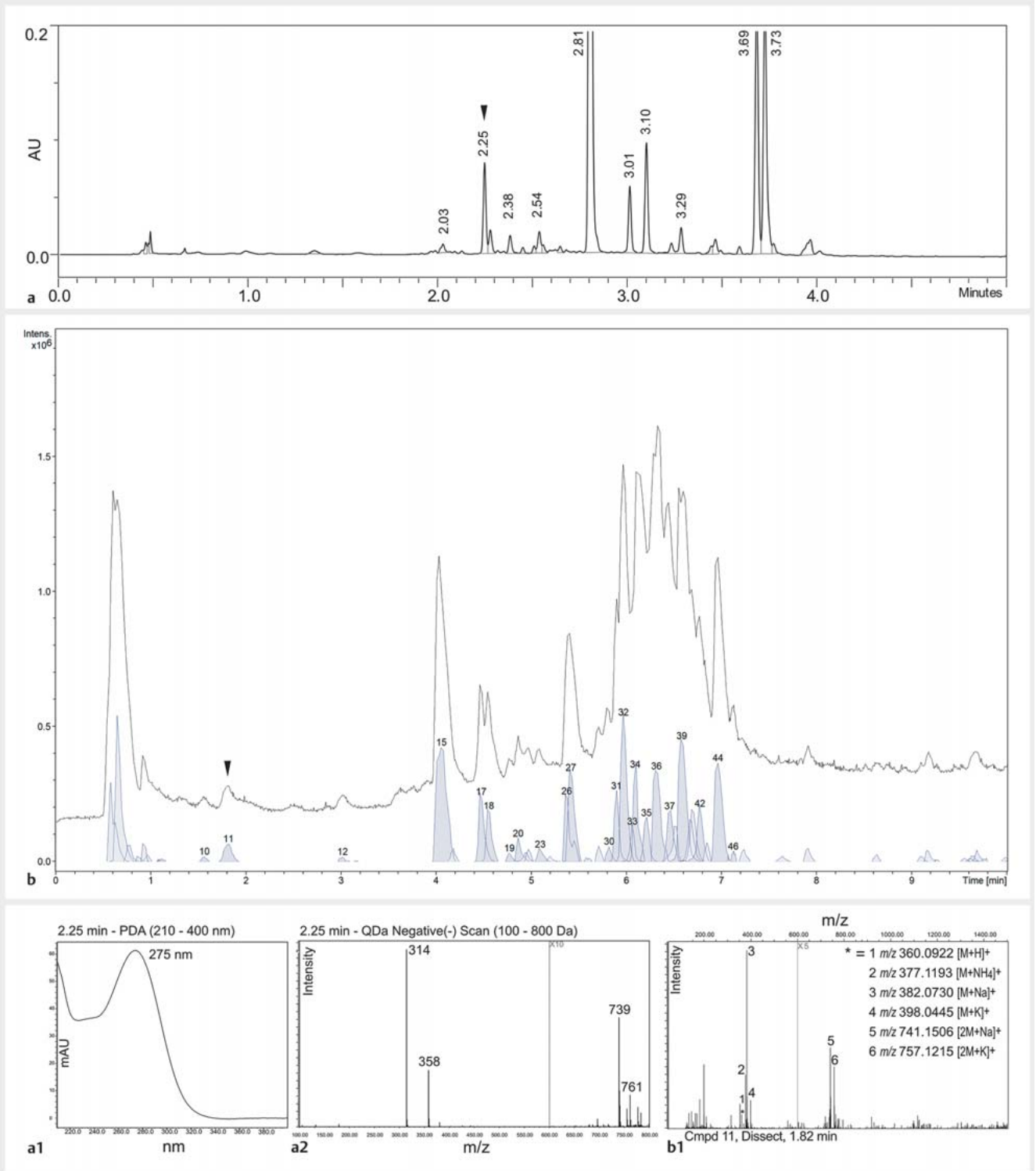
▶ **Fig. 2** Structural formulas of triglochinin and biogenetically related CNglcs.

data (▶ **Fig. 3 a2**) (QDa-detector, ESI negative mode) also confirmed the identity of **1**. All data fit perfectly with the structure of **1** and included the deprotonated molecule ($[M - H]^-$ at m/z 358), two dimeric ions at m/z 739 and m/z 761 (probably $[2\,M - 2H + Na]^-$ and $2\,M - 3H + 2Na]^-$, resp.), and a base peak at m/z 314, likely due to a decarboxylation reaction ($[M - CO_2\text{-}H]^-$). The chromatogram shown in ▶ **Fig. 3 b** was recorded by U(H)PLC system 2. It shows a TIC recorded in the ESI positive mode with a micrOTOF-QII detector. A software-based evaluation of the 3D data (= "dissect compound" algorithm of the DataAnalysis software, version 4.1 SP1; Bruker) revealed a variety of "dissect compounds", each characterized by HRMS. "Dissect compound" 11 (R_t = 1.82 min) was shown to be identical to **1**. All m/z values (mass spectrum) (▶ **Fig. 3 b1**) fit perfectly with a molecule showing an M_{mi} of 359.0838 Da, leading to the molecular formula of $C_{14}H_{17}NO_{10}$, which again confirms the presence of **1** in the investigated sample. To corroborate these results, all collected samples (cf. ▶ **Table 1**) were subsequently analyzed by the described methods, as illustrated in **Fig. 4S**, Supporting Information. The triglochinin peak was found in all cyanogenic samples but was hardly detectable in the acyanogenic samples. Thus, the presence of **1** in all cyanogenic (= Feigl-Anger positive) samples of *A. spinosa* was confirmed by coelution in two U(H)PLC systems with different selectivity and equipped with different UV spectroscopic and mass spectrometric detection techniques.

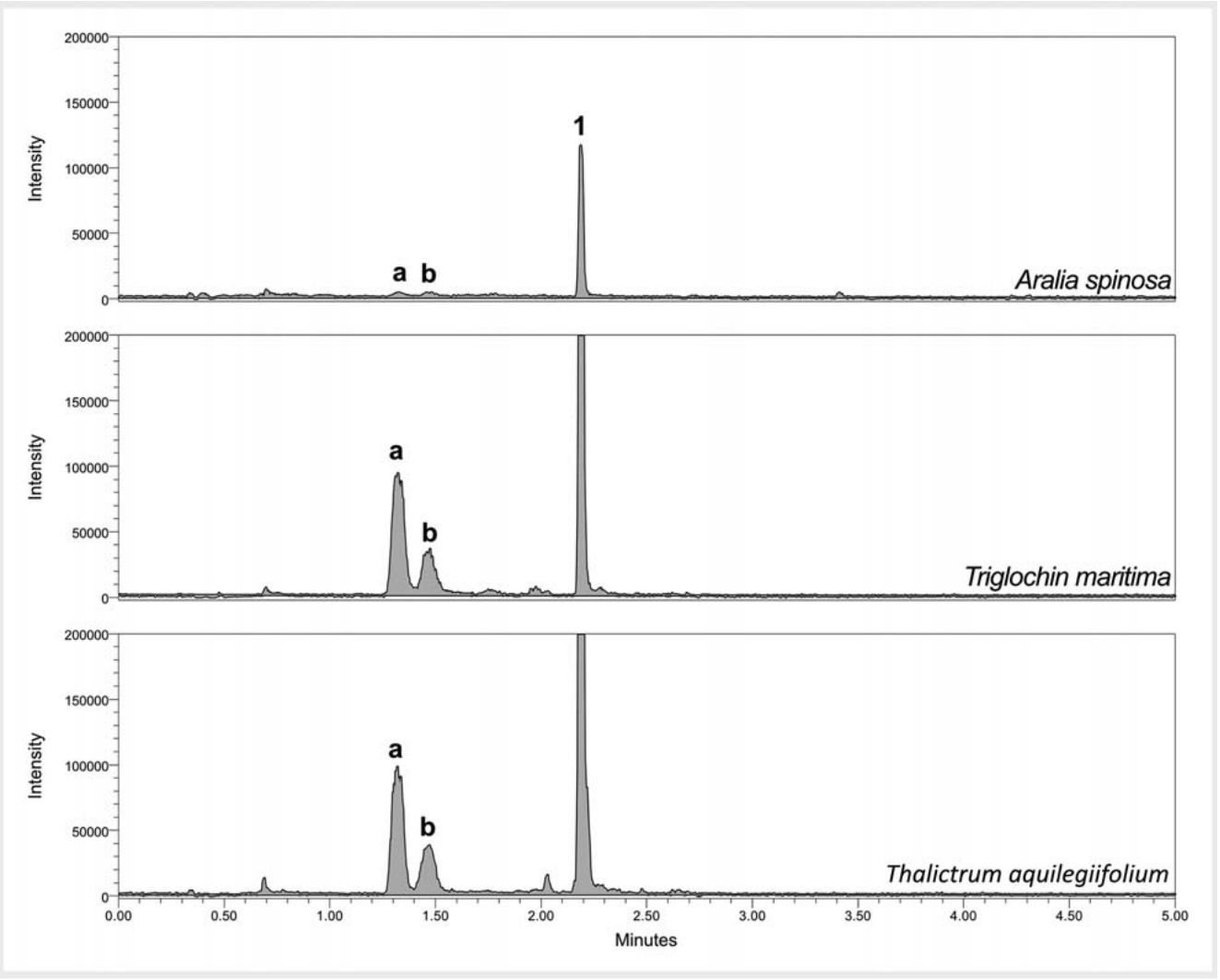
To further confirm the presence and identity of **1**, an enrichment of this compound was attempted in the extract. After the preparation of a dry extract obtained by methanol-water extraction, the compound was purified by hydrostatic column chromatography on a styrene-divinylbenzene gel (MCI Gel CHP20P) as the stationary phase. The fractionation was performed by descending polarity with an increasing proportion of methanol in the mobile phase. All fractions containing **1** were combined and separated from the nonacidic accompanying substances using SAX-SPE cartridges. This resulted in a fraction highly enriched in **1**, which was identical to the reference substance in all methods described above. This successful enrichment from a crude extract provides further evidence for the presence of **1** in *A. spinosa*. At this stage of the investigation, due to the described enrichment process, at least one putative isomer of **1** was detected.

Reports on the occurrence of isomeric compounds are known since the first isolation of **1** itself from seaside arrowgrass (*Triglochin maritima* L., Juncaginaceae). In 1970, Eyjólfsson isolated a CNglc from the flowers of this monocotyledonous plant and named it triglochinin. The initially proposed structure was corrected shortly thereafter to be **1** [24, 25]. Even in these earliest studies on **1**, the presence of small amounts of an isomeric compound has been mentioned and the corresponding compound was named isotriglochinin (**1a**). Ettlinger and Eyjólfsson showed that the UV spectrum of **1** had a maximum at λ = 275 nm compared to λ = 283 nm in case of the isomer [24, 25]. In addition, young seedlings of *T. maritima* also contain the biogenetically related taxiphyllin (**2a**) [26]. Nahrstedt was able to isolate a mixture of **1** and **1a** from *Alocasia macrorrhizos* (L.) G.DON [27] and *Arum maculatum* L. [28], both from the family Araceae. Regarding **1**, the configuration at the 3,4-double bond was determined to be (*Z*) while in the case of **1a**, a (3*E*)-configuration has been proven [27]. In a systematic study within this plant family, **1** was also shown to be the dominant CNglc in a further four species. It was also shown that a gentle cold methanolic extraction followed by careful purification steps (e.g., freeze-drying of fractions instead of rotary evaporation) largely prevents the formation of isomeric compounds [29]. This suggests that the isomers of **1** should be considered as artifacts of isolation. Nahrstedt found that the isomers elute prior to **1** in HPLC separation on reversed-phase stationary phases [30], which is in accordance with our own observations. According to Nahrstedt, the literature data show evidence only for the configuration of the double bond at the C3 position. For an informative summary of the structures and spectroscopic properties of triglochinin and its possible isomers (collectively often referred to as "triglochinins"), see [31].

We decided to systematically study the occurrence of isomers of **1** in *A. spinosa* by comparison with two other species that are well-known sources of this unusual CNglc: *T. maritima* (mentioned above) and *Thalictrum aquilegiifolium* L., commonly known as columbine meadow-rue. *T. aquilegiifolium* is a plant species from the



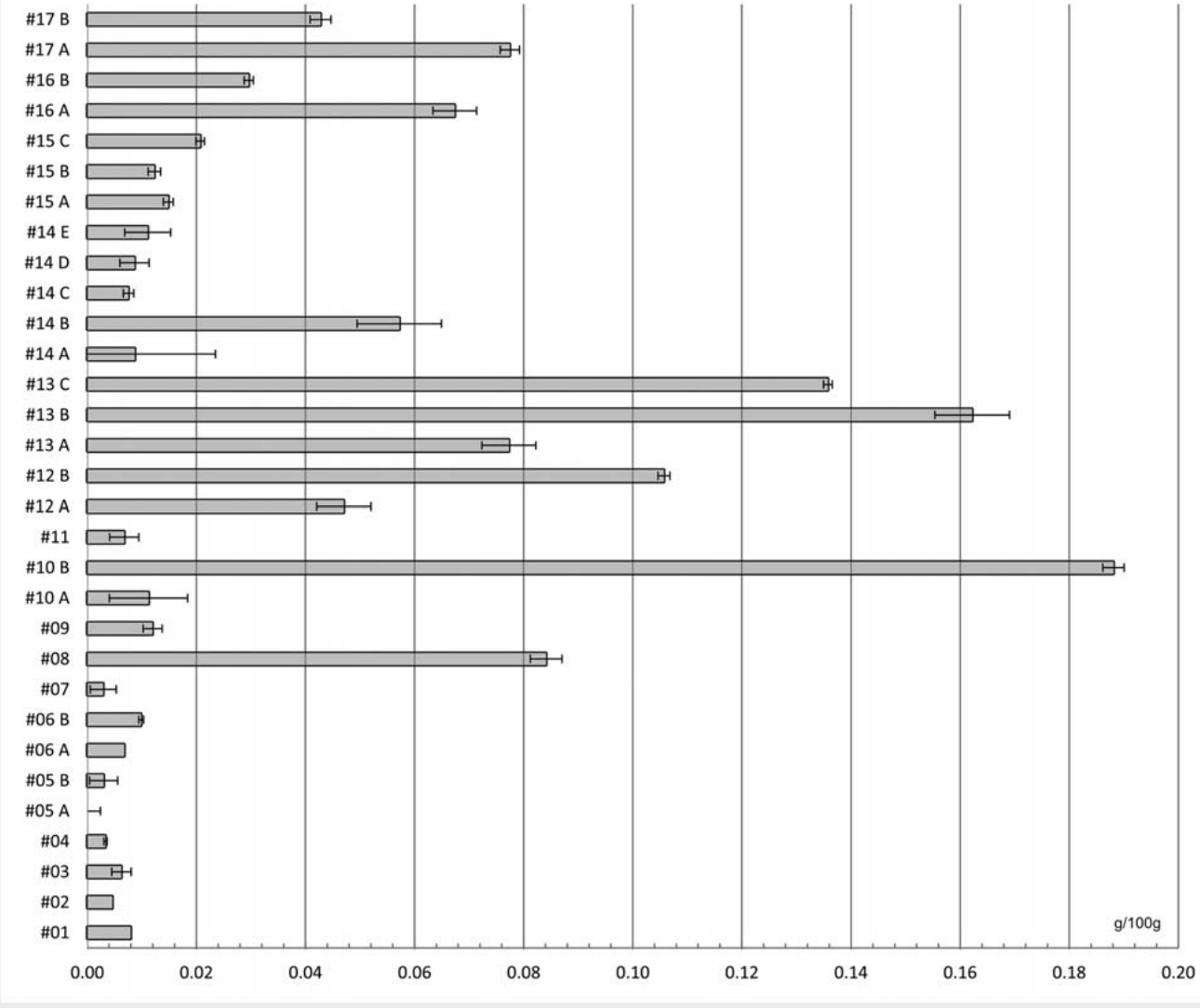
▶ **Fig. 3** UV (**a**) and MS (**b**) U(H)PLC chromatograms of a typical *A. spinosa* sample (sample #10B, conc. 20 mg/mL extracted with MeOH-H_2O (60 : 40, v/v). The triglochinin peaks in **a** (R_t = 2.25 min) and **b** (1.82 min, "dissect compound" 11) are marked by a black tip. **a** System 1 (HSS T3, 0.6 mL/min): UV chromatogram at λ = 275 nm with UV spectrum (**a1**, PDA, 210–400 nm) and mass spectrum (**a2**, QDa, ESI-, 100–800 Da, m/z ≥ 600: intensity factor 10) of peak at 2.25 min. Further UV and mass spectra can also be found in **Fig. 5S**, Supporting Information. **b** System 2: TIC chromatogram (black line) and "dissect compounds" (peaks 10–46) with mass spectrum (**b1**, qTOF, ESI+, 100–1500 Da, m/z ≥ 600: intensity factor 5) of compound **11** at 1.82 min.



▶ **Fig. 4** Comparison of the chromatogram of *A. spinosa* (sample #10B) with those of *T. maritima* (leaves) and *T. aquilegiifolium* (leaves). The two pre-eluting peaks (**a** and **b**) are isomeric compounds of **1**. U(H)PLC system 1 (HSS T3, 0.5 mL/min). Extracts: 150 mg freeze-dried material extracted with 10 mL MeOH-H_2O (60 : 40, v/v). Detection: QDa, ESI-, SIR mode (m/z = 358).

Ranunculaceae family and is known to contain **1** as the major CNglc together with smaller amounts of proteacin (**3**) and *p*-glucosyloxy-mandelonitrile [32]. ▶ **Fig. 4** shows three chromatograms that had been recorded in the sensitive and selective SIR (m/z = 358) of the QDa detector (an ESI/single-quadrupole) in the negative mode. Triglochinin (**1**) can be seen in the chromatogram of an extract of *A. spinosa* as well as in the two reference extracts of *T. maritima* and *T. aquilegiifolium*. Since all extracts had the same concentration, a semiquantitative comparison of the peak areas is possible. It is obvious that the content of **1** in the two reference extracts is significantly higher (*T. maritima*: 4.5-fold, *T. aquilegiifolium*: 7.4-fold). That the two pre-eluting peaks (**a** and **b**) in the reference extracts are truly isomeric compounds of **1** and can be deduced from their mass spectra, which in all cases, are nearly congruent in the negative mode (m/z = 314, 358) (cf. ▶ **Fig. 3 a2**). Both isomers can also be detected in *A. spinosa*, albeit to a less significant extent in comparison to the reference species. Despite all quantitative differences, a comparable isomeric pattern in all three plants studied is obvious. With some caution, it may be reasonable to assume that the detected isomers of **1** perhaps occur in all plants in which **1** can be detected. This is supported by the works cited above and also by a more recent paper in which a comprehensive metabolite profiling of *Arum palaestinum* Boiss. was performed [33]. Again, the pattern of **1** and two previously eluting isomers were found. The authors named the compounds triglochinins I, II, and III. The contribution of extraction and subsequent cleanup to isomer formation remains unclear. However, according to the literature and to our results, the occurrence of isomers of **1** seems to be a regularly observed phenomenon.

The structure of **1** is biochemically related to the *p*-hydroxy-mandelonitrile glucosides and putatively belongs to the biogenetic group of tyrosine-derived CNglcs (like **2**, **2a**, or the rare proteacin **3**). The final biogenetic formation of **1** most likely occurs



▶ **Fig. 5** U(H)PLC quantification of triglochinin in *A. spinosa* samples (annual cycle harvest). The data are mean values (n = 5) given in g/100 g d.w. The error bars indicate the standard deviation. For details of the sample numbers (#01 – #17 B), see ▶ **Table 1**.

through the ring opening of phenolic precursors [34, 35]. Recent studies on the biogenesis of CNglcs in *T. maritima* have shown that there is apparently a branch point between the biosynthesis of **1** and **2a** [36, 37]. As mentioned, **1** and **3** co-occur in *T. aqulilegiifolium* [32]. In a study on the distribution of CNglcs in the Araliaceae family, **2** and **3** were shown to be constituents of *P. australiana* [7]. As mentioned, both compounds (including **1**) are biogenetically derived from tyrosine. This may be a tentative indication of a chemotaxonomic feature of the family Araliaceae. In order not to overlook the presence of further biogenetically related CNglcs in *A. spinosa* extracts, both compounds were also specifically searched for in all samples examined. As a result, compounds **2**, **2a**, and **3** were absent within the detection limit.

The described U(H)PLC methods allowed us to determine the content of **1** in all samples collected (▶ **Fig. 5**). In general, it was possible to confirm the results obtained in the determination of the releasable HCN. If the content of **1** is determined indirectly via the amount of released HCN, values similar in magnitude to those obtained by direct quantification should result. For example, sample #10B contains 0.19% triglochinin according to the U(H)PLC analysis. Assuming a complete release of HCN from **1**, the quantified amount of HCN (87.4 mg/kg) leads to a calculated value of 0.12% triglochinin. In the case of sample #08, it is 0.084% (determined quantitatively) vs. 0.094% (calculated via HCN). Thus, it can be assumed that triglochinin is the only, or at least dominant, cyanogenic principle of *A. spinosa*. This fits with the result that further CNglcs were not found in the course of our investigations, with the exception of the isomers of **1**, which, however, were detectable only in very small amounts.

In order to tentatively characterize the extract composition beyond triglochinin and its isomers, chromatographic peaks were detected by the "dissect compound" algorithm of the DataAnalysis software (version 4.1 SP1; Bruker). The chromatogram is shown in ▶ **Fig. 3b**. Tentative identities were assigned on the basis

of data interpretation, where possible, library comparison [38], and known constituents of the genus *Aralia* as retrieved from the Reaxys database [39]. Diagnostically important data are compiled in ▶ **Table 2** and comprehensive data can be found in Supporting Information (**Suppl. 6** [List of Mass Fragments], Supporting Information). Detected secondary metabolites divide into phenolics eluting after 4–6 mins and saponins eluting after 6–7 min. The phenolics are, for the main part, quinic acid conjugates with caffeic, coumaric, or ferulic acid while two quercetin monoglycosides are the only observed flavonoids. The 12 detected saponins feature aglyca of 456 Da ($C_{30}H_{48}O_3$), 472 Da ($C_{30}H_{48}O_4$), and 488 Da ($C_{30}H_{48}O_5$), all of which can be attributed to sapogenins known from the genus *Aralia*, e.g., oleanolic acid, hederagenin, and caulophyllogenin [40]. Except for two minor congeners, the saponins possess 4–6 sugars, including one desoxyhexosyl, one pentosyl, and two hexosyl moieties. Of note is the presence of a hexuronic acid-containing saponin and two saponins malonylated on the sugar chain. Malonylated saponins are known from the Araliaceae species *P. ginseng* [41]. As expected, the phenolic compounds can also be detected in the UV chromatogram recorded by U(H)PLC system 1, as shown in ▶ **Fig. 3 a** and **Fig. 5S**, Supporting Information. The evaluation of the respective UV and MS spectra of the main peaks (labelled with small letters **a**–**g** in **Fig. 5S**, Supporting Information) agrees well with the results described above. Five hydroxycinnamic acid derivatives of quinic acid (**a** to **f**) and a quercetin deoxyhexoside (**g**) were detected. Detailed information can be found in Supporting Information (**Fig. 5S**, Supporting Information). The prevailing compound (peak **a**) was clearly assigned to 5-caffeoylquinic acid (chlorogenic acid).

As a conclusion, cyanogenesis was detected in *A. spinosa* in less than half of all cases during a complete annual harvesting cycle. Especially during springtime and early summer, HCN was not detectable in any sample. This is worth mentioning because in many other cases of cyanogenic plants, it is mainly the young shoots and leaves that have the highest content of CNglcs. The unusual CNGlc triglochinin (**1**) is responsible for the observed weak cyanogenesis. Despite a targeted search, no other CNglcs could be detected. Chromatographically, very small amounts of two isomers were detectable, which agrees with observations made in the study of other plants containing **1**.

According to Kingsbury, plants containing more than 20 mg potential cyanide per 100 g f. w. are considered toxic and may be poisonous to both humans and livestock [42]. In comparison, the *A. spinosa* samples show a maximum content of only 3 mg HCN/100 g f. w. We can only speculate here about the ecological functions of those small amounts of CNglcs. Probably, it is not protection from herbivores, against which the plant has already developed effective mechanical protection by being covered with prickles. Today, multiple roles for CNglcs are known and some aspects are discussed in [1, 2, 43, 44]. Among other secondary metabolites, CNglcs can improve adaptation to environmental challenges and therefore contribute to the phenotypic plasticity of plants [1]

The knowledge on CNglcs in the family Araliaceae remains scarce. To date, only a few species have been studied and, so far, all detected CNglcs appear to be tyrosine derived [7, 45]. However, extensive studies in other representatives of this family are needed before a general chemotaxonomic rule can be derived from these data.

Materials and Methods

Chemicals

If not otherwise stated all chemicals were purchased from Merck. The CNglc reference substances were obtained from the chemical collection of the Institute for Pharmaceutical Biology and Phytochemistry, University of Münster. In particular, triglochinin (**1**) was previously isolated from *T. maritima* by A. Nahrstedt.

Plant material

Aralia spinosa

Dried leaf material from 1986 was obtained from the institute's herbal drug collection. The material has been identified and was collected in 1986 by David A. Jones (Dept. Botany, University of Gainesville, Florida, USA) and originates from the collection of Adolf Nahrstedt.

Annual cycle harvest: aboveground parts were collected throughout the complete growing season of 2014 from one single site (coordinates: 51°57′48.0′′N 7°36′38.2′′E) in the Botanical Garden of Münster, Germany (**Fig. 1S**, Supporting Information). The plant was sown in 2002 and planted in 2003. The official identification was done by the Botanical Garden of Münster. The accession number is 02–01–11 798–1–0. All samples were taken from a limited garden area. The different shoots most likely result from the activity of underground runners. Thus, we assume that these are clones, but we cannot prove this beyond a doubt.

During this time, samples were taken every 14 days from the respective plant parts: stems, shoot axils, terminal buds, leaf buds, pinnate leaves (young and old), complete young shoots, flower buds, inflorescences, immature fruits, ripe fruits, and senescent leaves. Back in the lab, the plant material was frozen immediately and stored at − 25 °C. Prior to freeze-drying, all samples were shock frozen with liquid nitrogen and pulverized finely in a mortar. Careful attention was paid to ensure that the samples did not thaw during all steps until the end of the freeze-drying process. The average dry matter determined after freeze-drying was 20.0 % (▶ **Table 1**).

Thalictrum aquilegiifolium

Origin of seed: Lublin Botanical Garden, Poland, accession number 1–3105–15. Six specimens of this plant were grown in the Medicinal Plant Garden at the IPBP (sown on April 7, 2015, pricked out on July 30, 2015). All aboveground parts were harvested on September 21, 2015. The freeze-dried plant powder was prepared as described above (dry matter: 20.9 %).

Triglochin maritima

Unripe green and ripe (dry) fruits as well as fresh leaves were collected from NSG Graswarder, Heiligenhafen, Germany (courtesy of NABU Schleswig-Holstein). Freeze-dried plant powders were prepared as described above (dry matter of ripe fruits: approx. 65 %, dry matter of leaves: approx. 15 %). The freeze-dried leaf material was used for the testing on isomers. Voucher specimens

▶ **Table 2** Compounds detected in a typical *A. spinosa* sample [sample #10B, conc. 20 mg/mL extracted with MeOH-H_2O (60 : 40, v/v)], as tentatively identified by U(H)PLC system 2.

No.	R_t/min	Area	m/z	Ion formula	Err/mDa	mΣ^a	Fragments m/z[b]	Identity[c]
10	1.56	60938	166.0855	$[C_9H_{12}NO_2]^+$	0.8	23	120	phenylalanine
11	1.82	375134	382.0730	$[C_{14}H_{17}NNaO_{10}]^+$	1.5	9	198, 180, 162, 152, 145, 134, 127	triglochinin
12	3.02	53033	205.0968	$[C_{11}H_{13}N_2O_2]^+$	− 0.3	29	188	tryptophane
15	4.06	3282758	355.1015	$[C_{16}H_{19}O_9]^+$	0.9	1	163, 145, 135, 117, 107, 89	monocaffeoyl quinic acid
17	4.47	1038334	355.1014	$[C_{16}H_{19}O_9]^+$	0.9	7	163, 145	monocaffeoyl quinic acid
18	4.55	793588	339.1051	$[C_{16}H_{19}O_8]^+$	2.4	3	147, 119, 91	monocoumaroyl quinic acid
19	4.77	109066	369.1178	$[C_{17}H_{21}O_9]^+$	− 0.2	29	177	monoferuloyl quinic acid
20	4.86	307711	339.1041	$[C_{16}H_{19}O_8]^+$	− 3.3	16	147	monocoumaroyl quinic acid
23	5.09	198709	465.1015	$[C_{21}H_{21}O_{12}]^+$	− 1.2	26	303, 201, 153	quercetin hexoside
26	5.37	1106488	517.1337	$[C_{25}H_{25}O_{12}]^+$	0.4	8	499, 319, 163, 145	dicaffeoylquinic acid
27	5.42	1618191	449.1066	$[C_{21}H_{21}O_{11}]^+$	1.3	5	303, 285, 257, 229, 165, 163, 153, 121	quercetin deoxy-hexoside
30	5.82	191280	1267.6269	$[C_{60}H_{99}O_{28}]^+$	− 4.9	103	795, 779, 633, 601, 487, 473, 471, 455, 437, 325, 309, 289, 273, 191, 165, 163, 147	472-dhex1 hex4
31	5.90	912866	1399.6709	$[C_{65}H_{107}O_{32}]^+$	3.1	69	1253, 1223, 1105, 1075, 943, 927, 781, 765, 729, 633, 619, 603, 487, 473, 471, 457, 455, 441, 437, 405, 325, 309, 295, 279, 273, 259, 243, 177, 163, 147, 132	472-dhex1 hex4 pen1
32	5.97	2404921	1091.5599	$[C_{53}H_{57}O_{23}]^+$	3.3	21	959, 945, 929, 813, 797, 767, 651, 635, 621, 617, 603, 599, 581, 489, 471.3, 471.2, 453, 441.3, 441.2, 435, 427, 425, 413, 407, 325, 309, 295, 279, 273, 259, 255, 243, 225, 207, 163, 147	488-dhex1 hex2 pen1
33	6.06	446540	1485.6657	$[C_{68}H_{109}O_{35}]^+$	− 8.7	53	1381, 1339, 1323, 1177, 1161, 1013, 851, 761, 719, 705, 689, 671, 653, 633, 617, 603, 573, 557, 543, 527, 509, 491, 471, 455, 441, 411, 395, 381, 363, 345, 325, 309, 293, 279, 273, 249, 231	472-dhex1 hex4 pen1 mal1
34	6.09	1303922	1237.6172	$[C_{59}H_{97}O_{27}]^+$	− 3.9	92	1075, 943, 913, 795, 767, 765, 763, 749, 649, 633, 617, 603, 601, 587, 573, 487, 471, 457, 455, 441, 325, 309, 295, 279, 163, 147, 133	472-dhex1 hex3 pen1
35	6.21	670107	1323.6242	$[C_{62}H_{99}O_{30}]^+$	2.6	41	1219, 1161, 1015, 853, 851, 689, 601, 543, 527, 471, 455, 439, 411, 395, 381, 325, 309, 279, 249, 231, 147	472-dhex1 hex3 pen1 mal1

continued

▶ **Table 2** *Continued*

No.	R_t/min	Area	m/z	Ion formula	Err/mDa	mΣ[a]	Fragments m/z[b]	Identity[c]
36	6.32	1939727	1075.5639	$[C_{53}H_{87}O_{22}]^+$	−4.4	67	929, 913, 797, 767, 751, 619, 605, 473, 455, 437, 409, 325, 309, 295, 279, 163, 147, 133	472-dhex1 hex2 pen1
37	6.45	897079	1075.5633	$[C_{53}H_{87}O_{22}]^+$	5.0	97	943, 797, 781, 635, 619, 473, 471, 455, 437, 409, 325, 309, 295, 279, 163, 147, 133	472-dhex1 hex2 pen1
39	6.58	2621839	1221.6253	$[C_{59}H_{97}O_{26}]^+$	0.9	60	897, 751, 633, 619, 603, 487, 471, 457.4, 457.2, 439, 427, 325, 309, 295, 279, 163, 147, 133	456-dhex1 hex3 pen1
42	6.77	901062	1191.6119	$[C_{58}H_{95}O_{25}]^+$	3.8	54	1059, 1045, 1029, 913, 897, 781, 721, 603, 457, 471, 457.4, 457.2, 441, 439, 427, 339, 309, 295, 279, 163, 147, 133	456-dhex1 hex2 pen2
44	6.96	2100408	1059.5688	$[C_{53}H_{87}O_{21}]^+$	−4.6	19	927, 913, 897, 781, 765, 751, 735, 603.4, 603.2, 589, 471, 457.4, 457.2, 439, 325, 309, 295, 279, 163, 147, 133	456-dhex1 hex2 pen1
46	7.12	90715	949.4775	$[C_{47}H_{74}NaO_{18}]^+$	0.8	136	927, 765, 457, 439, 325, 295, 133	456-hex1 hexU1 pen1

[a] mΣ ≤ 50 is considered in good agreement. The values of saponins usually are worse because of interference with $[2\,M + 2H]^{2+}$ ions; [b]If two isobaric fragments occur, the corresponding m/z values are given with one decimal place; [c]The entries on saponins consist of the molecular weight of the aglycon and the number of linked moieties; dhex: Desoxyhexose, hex: hexose, hexU: hexuronic acid, pen: pentose, mal: malonic acid

have been deposited in the institute's herbarium under numbers #529 (*A. spinosa*), #530 (*T. maritima*), and #531 (*T. aquilegiifolium*).

TLC

TLC plates (silica gel 60 F_{254}; Merck) were used with butan-2-one/ethyl acetate/formic acid/water (volume parts 5/5/0.5/1) as the mobile phase. Detection was performed by the sandwich picrate test [23] or with anisaldehyde-sulfuric acid reagent [46]. R_f values of selected CNglcs are as follows: linustatin 0.03, amygdalin 0.10, triglochinin 0.21, linamarin 0.35, lotaustralin 0.40, prunasin 0.52 (**Fig. 3S**, Supporting Information).

U(H)PLC

System 1: Waters Acquity UPLC with PDA and QDa detectors.

Fast UPLC analysis was performed using an Acquity UPLC (Waters) system equipped with a PDA eλ detector (210–400 nm) and QDa detector (ESI, pos. and neg. mode, single quadrupole, 100–800 Da). Further components included a binary solvent manager (flow rate: 0.5–0.6 mL/min, mobile phase: A: H_2O + 0.1 % formic acid, B: CH_3CN + 0.1 % formic acid), sample manager (inj. vol.: 1–2 µL), column heater (40 °C), and Waters Empower 3 software. The gradient was minute/%A:%B → 0/98:2, 1/98:2, 9/0:100, 9.5/0:100, 10/98:2. Two reversed-phase stationary phases with known good retention of polar compounds but different selectivity were used:

- Column 1: Waters Acquity UPLC HSS T3, 1.8 µm, 2.1 × 100 mm;
- Column 2: Waters Acquity UPLC BEH Shield RP18, 1.7 µm, 2.1 × 100 mm.

System 2: Dionex Ultimate with Bruker Daltonics micrOTOF-QII

Chromatographic separations were performed on a Dionex Ultimate 3000 RS Liquid Chromatography System on a Dionex Acclaim RSLC 120, C18 column (2.1 × 100 mm, 2.2 µm) with a binary gradient (A: water + 0.1 % formic acid; B: CH_3CN + 0.1 % formic acid) at 0.4 mL/min. The following gradient was applied: minute/%A:%B → 0/95:5, 0.4/95:5, 9.9/0:100, 15.0/0:100, 15.1/95:5, 20/95:5. The injection volume was 2 µL. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS over a wavelength range of 200–400 nm and a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer equipped with an Apollo ESI source in the positive mode at 3 Hz over a mass range of m/z 50–1500. Data-dependent MS/MS scans (40 eV) were included.

Hydrocyanic acid determination assays

Qualitative and semiquantitative assay

A qualitative and semiquantitative determination of HCN was performed using Feigl-Anger test paper strips [18, 23]. All freeze-dried samples were finely ground in a laboratory mill. In each case, 40–80 mg of the plant powder were weighed into a sample vial. After addition of 200 µL of an aqueous enzyme preparation (see

below), the test strip was placed into the gas space above the moistened sample. The test tube was then tightly closed. The evaluation of the intensity of the blue coloration was performed visually after 2 h: range from 0 (= negative) to 4 (= very intense blue coloration). All values obtained were corrected by the weight of the sample to obtain comparable semiquantitative Feigl-Anger score values: negative (0), weak (1), medium (2), clear (3), and very intense (4).

Enzyme preparation (degradation test)

An aqueous solution (c = 2 mg/mL) of "emulsin" (β-glucosidase from almonds, lyophilized powder; Sigma-Aldrich G4511) was used as enzyme preparation. The efficiency of the enzymatic degradation of **1** was tested with sample #10B. Of the dried plant powder, 400 mg were extracted with 10.0 mL MeOH 60 % (v/v) in a 15-mL Falcon tube using an ultrasonic bath and centrifuged at 8000 rpm. An aliquot of 1.0 mL was pipetted into an Eppendorf tube, brought to dryness with a vacuum concentrator ("Speed-Vac"), and resuspended in 1.0 mL of the enzyme solution (or pure water as a control experiment). All samples were filtered through 0.45 µm membrane filters prior to U(H)PLC analysis (system 1). Result: the peak at 2.25 min had completely disappeared after overnight incubation in the aqueous enzyme preparation at 36 °C. In the control experiment, **1** was shown to be stable under the given conditions.

Quantitative assay

The content of enzymatically releasable HCN was quantitatively determined using Conway's microdiffusion apparatus [20]. An alkaline trap solution (2 mL, 1 N NaOH) was pipetted into the outer circle of the "Conway chamber". Of the plant powder, 25–100 mg were accurately weighed and positioned in the inner circle and 1 mL of an enzyme preparation (see above) was added. The chamber was immediately sealed tightly and incubated overnight at 37 °C. The trap solution was quantitatively transferred to a 10-mL volumetric flask while rinsing with 3 × 300 µL of water. For the colorimetric cyanide determination, the Aldridge cyanide test [21] (in a modified form according to Nahrstedt [22]) was used. The color product was measured at 520 nm against a reagent blank. For calibration, a cyanide standard solution (6.80 µg/mL KCN in water) was used. The calibration was checked daily. Quantitation limit: 1 µM HCN in test solution; resp. 2.7–10.8 µg HCN/g plant material.

Triglochinin enrichment

Of the freeze-dried immature flowers (sample #10B) (▶ **Table 1**), 20 g were finely ground in a mortar and suspended in methanol-water (60 : 40, v/v). The extraction was performed in an ultrasonic bath (30 min) and the suspension was filtrated with a Gooch crucible. The residue was reextracted twice as described. The combined filtrates were evaporated on a vacuum rotary evaporator until the methanol was completely removed. The aqueous residue was freeze-dried to yield 9.5 g of dry extract (drug-extract ratio: 2.1 : 1).

A chromatography column with frit (ø ID = 5.5 cm) was filled with a styrene-divinylbenzene gel (MCI GEL, particle size: 75–150 µm suspended in methanol; Sigma-Aldrich) at a bed height of 17 cm. The stationary phase was prewashed with methanol and equilibrated with methanol-water (10 : 90, v/v). Of the dry extract, 5 g were suspended in methanol-water (10 : 90, v/v) and the suspension was added to the top of the column. The elution was performed by a methanol-water step gradient (10 % → 100 % methanol, 10 steps à 200 mL). One hundred and thirty fractions were collected in intervals of 2 min (ca. 12 mL). All fractions were analyzed by U(H)PLC system 1 with a special focus on the peaks that showed the typical UV spectrum (λ_{max} = 275 nm) and mass spectrum ($[M - H]^-$; m/z = 358) of triglochinin. Fractions 18–21 (elution volume 212–248 mL) were combined and yielded 1.35 g after freeze-drying. The described purification was performed in an analogous manner with 3.6 g dry extract. The two purification steps yielded a total of 2.53 g of a triglochinin-enriched dry extract.

For further purification, SAX-SPE cartridges (Waters Oasis MAX SPE) were used. Of the triglochinin-enriched dry extract, 1.6 g were dissolved in 24 mL water. The clear solution was put on top of SPE cartridges in portions of each 3 mL. The elution was as follows: (A) fixation of anions with 10 mL 10 % NH_3, (B) elution of lipophilic neutrals with 9 mL methanol, (C) elution of anions with 4 mL (C1) + 5 mL (C2) HCOOH 2 % (in MeOH-CH_3CN 30 : 70, v/v), and (D) washing/conditioning with 10 mL methanol. All fractions were monitored by U(H)PLC as described above. Only eluate C2 was shown to contain triglochinin, and after combining and freeze-drying, yielded 22 mg.

Chromatography data of triglochinin in *Aralia spinosa* extracts

TLC. The sandwich picrate test [23] in Feigl-Anger-positive *A. spinosa* extracts showed a positive band at the same R_f value of the triglochinin reference: R_f = 0.21.

U(H)PLC (system 1). All chromatograms of Feigl-Anger-positive plant material (sample #08, 10B, 12–13C, 14B, 15A-17B) showed a peak at R_t = 2.25 min (column 1, flow rate 0.6 mL/min) and R_t = 0.85 min (column 2, flow rate 0.5 mL/min). The peaks showed coelution with **1**, identical UV spectra (λ_{max} = 275) and MS spectra (m/z = 358 $[M - H]^-$, 314). All chromatograms of HCN-negative plants (sample #01–07, 09, 10A, 11, 14A, 14C, 14D, 14E) showed no or only a very small peak at the respective R_t value.

U(H)PLC (system 2). Chromatograms of cyanogenic *A. spinosa* samples (e.g., #10B) and of reference **1** showed a peak at R_t = 1.82 min, mass data (m/z): 382.0730 $[M + Na]^+$, 377.1193 $[M + NH_4]^+$, 360.0922 $[M + H]^+$, 741.1506 $[2M + Na]^+$; leading to an M_{mi} of 359.0838 Da. This M_{mi} fits perfectly to the molecular formula of triglochinin $C_{14}H_{17}NO_{10}$ [$M_{mi\ (calculated)}$ = 359.0852 Da]. Non-cyanogenic samples (e.g., #07) showed no peak at R_t = 1.82 min in TIC MS full scan and BPC m/z 382 ± 0.2.

Quantification of triglochinin

Extraction and sample preparation

In a systematic approach with different solvent combinations, a mixture of methanol-water (60 : 40, v/v) was shown to be the optimal extraction solvent mixture. A maximum yield of **1** was obtained in the absence of β-glucosidase activity. The freeze-dried plant material was ground in a laboratory mill and about 200 mg were weighted exactly into a 15-mL Falcon tube. Of the extraction

solvent mixture, 10.0 mL were added. After intensive extraction (30 min) on a roller shaker (IKA ROLLER 10 digital), the tubes were put in an ultrasonic bath (Bandelin Sonorex) for another 30 min. All samples were filtered through membrane filters (0.2 µm pore size) prior to injection. The first 500 µL were discarded.

All samples were analyzed using U(H)PLC system 1 as part of three sequence runs with two different stationary phases: HSS T3 (**Fig. 4S**, Supporting Information) and BEH Shield (data not shown). The area values for **1** were obtained by suitable extraction from the 3D data of the PDA detector ($\lambda = 275$ nm) and the QDa detector (SIR at $m/z = 358$) and evaluated with the respective calibration curves (see validation).

Validation (basic parameters)

Quantification of **1** in *A. spinosa* samples was performed by means of U(H)PLC system 1 with the PDA detector at $\lambda = 275$ nm and with the QDa detector in the negative SIR mode at $m/z = 358$. The calibration curves were recorded at 7 concentration levels of **1**: 0.312, 0.78, 1.56, 3.12, 7.80, 15.6, and 31.2 µg/mL. The regression curve of the UV calibration showed perfect linearity (linear equation: $y = 2158.5\,x - 264.63$; $R^2 = 0.999$) while calibration with the mass detector fitted very well with a polynomial regression model (quadratic equation: $y = 522.49\,x^2 + 16\,816\,x + 11\,808$; $R^2 = 0.9976$). Further parameters: limit of quantification: 0.5 µg/mL; limit of detection [PDA ($\lambda = 275$ nm): 0.1 µg/mL, QDA (SIR): < 0.1 µg/mL].

Supporting information

Color photographs of *A. spinosa* (**Fig. 1Sa/1Sb**), Feigl-Anger tests of *A. spinosa* samples (**Fig. 2S**), TLC of CNglcs and *A. spinosa*, (**Fig. 3S**), UV chromatograms of all *A. spinosa* samples (**Fig. 4S**), UV and MS spectra of *A. spinosa* sample #10B (**Fig. 5S**), and a mass list of "dissect compounds" of sample #10B are available as Supporting Information.

Contributors' Statement

Conception and design of the study: M. Lechtenberg, A. Hensel; collecting of plant material: M. Lechtenberg, L. Kastner; data collection: M. Lechtenberg, J. Sendker, L. Kastner; analysis and interpretation of the data: M. Lechtenberg, J. Sendker, A. Hensel; statistical analysis: M. Lechtenberg, J. Sendker, A. Hensel; drafting the manuscript: M. Lechtenberg, J. Sendker; critical revision of the manuscript: A. Hensel.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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