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Brassicaceae contain nortropane alkaloids

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Abstract

The report of cochlearine, the 3-hydroxybenzoate ester of tropine found in *Cochlearia officinalis*, Brassicaceae, initiated a screening for tropane alkaloids in *Cochlearia* species and for calystegines in further Brassicaceae. All ten *Cochlearia* species investigated contained cochlearine, tropine, and pseudotropine. Calystegines, nortropane alkaloids deriving from pseudotropine, were also identified in all *Cochlearia* species and accumulated up to 0.5% dry mass in leaves. Brassicaceae species of all major lineages of the family were analysed for calystegines. Of the 43 species included in the study, 18 accumulated calystegines of various structures. This is the first screening of Brassicaceae for products of the tropane alkaloid pathway, which is known as characteristic for plants of Solanaceae family. The identification of calystegines in all branches of the Brassicaceae family including *Aethionema*, a species at the basis of the family, suggests tropane alkaloids as secondary compound typical for Brassicaceae.

Keywords: *Cochlearia officinalis*; Brassicaceae; Calystegine; Cochlearine; Tropane alkaloid

1. Introduction

Brassicaceae form a large plant family systematically placed in the eurosid II clade within the subclass of eudicotyledons. The cruciferous plant family, another name used for Brassicaceae, contains 338 genera and 3350 species that are distributed worldwide (Mitchell-Olds et al., 2005). Vegetables, condiments, and decorative plants belong to the Brassicaceae, and also the model plant for molecular biology *Arabidopsis thaliana*, Well-known secondary products in Brassicaceae are the glucosinolates and phenylpropanid derivatives like the seed-specific sinapine.(4-hydroxy-3,5-dimethoxy-cinnamic acid choline ester) (Regenbrecht and Strack, 1985). Alkaloids, in contrast, are not typical secondary compounds in Cruciferae, notwithstanding a few indole alkaloids with rather simple structures like camalexin in *Arabidopsis* (Glawischnig et al., 2004) or indigo and tryptanthrin in *Isatis tinctoria* (Oberthür et al., 2004). Further lunarine, an oxidation product of dicumaroylspermidine was identified in seeds of *Lunaria* species (Sagner et al., 1998).

Tropane alkaloids comprise the medicinally applied compounds hyoscyamine and scopolamine, and they are found in a number of Solanaceae, e.g. *Atropa, Hyoscyamus*, and *Duboisia* species. A few years ago, a novel group of hydroxylated nortropane alkaloids was identified, the calystegines (Fig. 1). They occur in all Solanaceae that contain medicinal tropane alkaloids, but are more widespread in the family. Potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicon*) for example contain calystegines (Dräger, 2004).The compounds' name derives from *Calystegia sepium*, indicating that this Convolvulaceae as well and many others also accumulate calystegines (Schimming et al., 1998, 2005). Solanaceae and Convolvulaceae are sister families in the order of Solanales, but other

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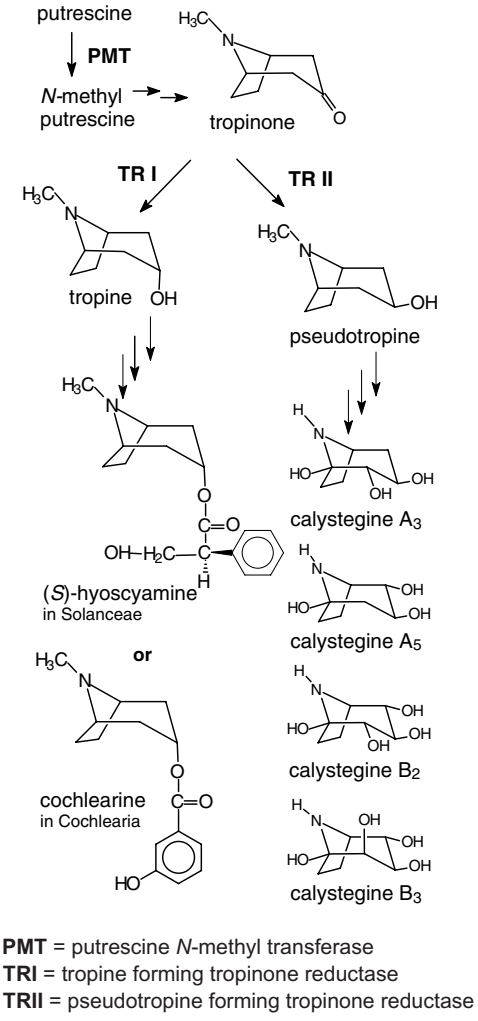

Fig. 1. Cochlearine and calystegines as products of tropane alkaloid biosynthesis. Enzymes indicated in bold were identified and the coding genes were isolated from Solanaceae.

angiosperms that are taxonomically remote also proved positive for calystegines, in particular, Moraceae (Asano et al., 1994) and Erythroxylaceae (Brock et al., 2005).

We are interested in the distribution and in the evolution of the tropane alkaloid biosynthesis in the plant kingdom. The understanding of taxonomical interrelationships and phylogeny within the Brassicaceae is probably most advanced among all plant families, certainly due to the model plant *A. thaliana* belonging to the family. Therefore, Brassicaceae are ideally suited for investigations on the molecular evolution of secondary product formation. Excellent studies were published on glucosinolate evolution in *A. thaliana* (Windsor et al., 2005; Kroymann and Mitchell-Olds, 2005). Cochlearine, the 3-hydroxybenzoate ester of tropine, was the first tropane alkaloid identified in *Cochlearia officinalis*, Brassicaceae (Liebisch et al., 1973). The authors based their investigation on a communication (Platonova and Kuzovkov, 1963), in which an ester of 3-hydroxybenzoic acid and tropine was mentioned as component of *Cochlearia arctica*. *C. arctica* is now considered as *C. groenlandica* L. The finding of cochlearine prompted us to test *Cochlearia* species and further Brassicaceae for calystegines.

2. Results and discussion

2.1. Tropane alkaloids in Cochlearia species

Cochlearine as reference compound was synthesised starting from tropine, the amino alcohol moiety and from 3-hydroxybenzoic acid (Ladenburg, 1883). *C. officinalis* and *C. arctica* plants revealed cochlearine in roots, approx. 0.05% dry mass; in aerial parts cochlearine reached 0.02% dry mass. Cochlearine was also identified in extracts of all other *Cochlearia* species investigated. The concentrations were always comparably low as in *C. officinalis*. Tropine, the alcohol moiety of cochlearine, and in addition pseudotropine were detected in most *Cochlearia* samples investigated, but in minor concentrations (Fig. 2). Appearance of both, tropine and pseudotropine, indicates that in *Cochlearia* tropinone acts as a metabolite for reduction by tropinone reductases to both amino alcohols as known from Solanaceae (Dräger, 2006). Pseudotropine is considered as the first specific metabolite on the tropane pathway branch to calystegines (Fig. 1). The metabolite was not detected in any Brassicaceae samples other than *Cochlearia*; in particular, *Camelina sativa*, *Carrichtera annua*, *Crambe cordifolia*, *C. kotschyana*, *C. orientalis*, *Diplotaxis murales*, *Iberis. amara*, and *Moricandia arvensis* were found to contain neither tropine nor pseudotropine. In these plants no or only little amounts of calystegines were detected (see below) suggesting that even lower amounts of pseudotropine may have escaped the analysis.

Calystegines were found in high concentrations in *C. officinalis* tissues (Fig. 3). Identification of individual calystegine structures in *C. officinalis* revealed a mixture characterised by calystegine A_5 as major compound. Calystegine patterns in Solanaceae and Convolvulaceae are dominated by other calystegines, calystegines A_3 and B_2 in Solanaceae (Dräger et al., 1995; Keiner and Dräger, 2000) and calystegines B_1 and B_2 in Convolvulaceae (Schimming et al., 1998, 2005). Erythroxylaceae possess calystegines A_3 and B_2 as major compounds (Brock et al., 2005). The biosynthesis of calystegines (Fig. 1) implies that carbon 3 in the tropane bicyclus carries a hydroxyl group arising by tropinone reductase II activity acting on tropinone. Enzymes and mechanisms of subsequent hydroxylations on the nortropane skeleton, however, are not elucidated by now. It may be conceived that basic steps of tropane biosynthesis are similar, but hydroxylating enzymes may differ between Solanaceae and Brassicaceae. *C. officinalis* is a biannual with a vegetative rosette stage in the first year and flowers in the second year of cultivation. The high calystegine content in flowers observed in *C. officinalis* is also typical for Solanaceae (Dräger et al.,

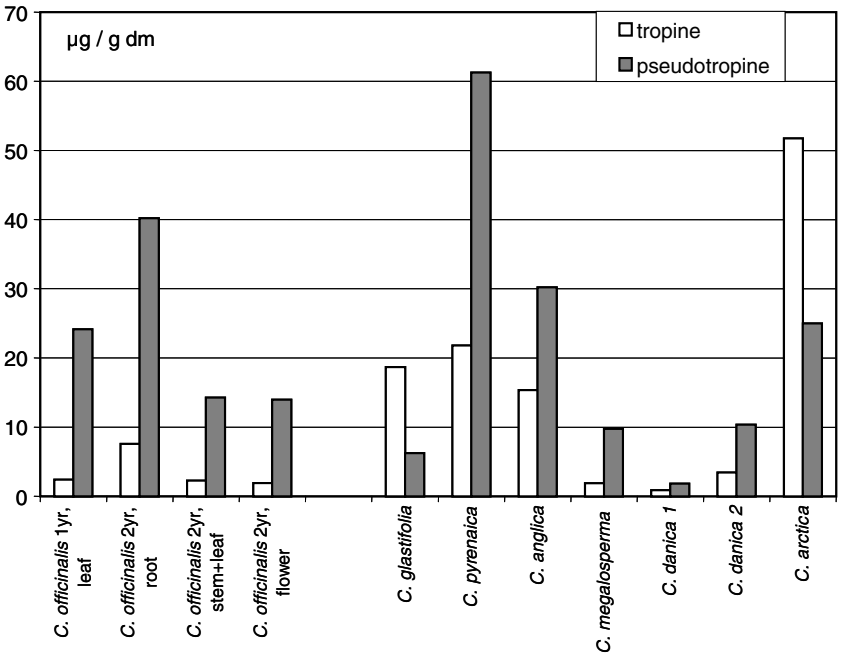

Fig. 2. Tropine and pseudotropine in *Cochlearia* species. *C. officinalis* samples were from 1 year old rosette leaves (1 yr) or 2 year old (2 yr) flowering plants. Concentrations indicated in microgram per gram dry mass (µg/g dm) from single samples.

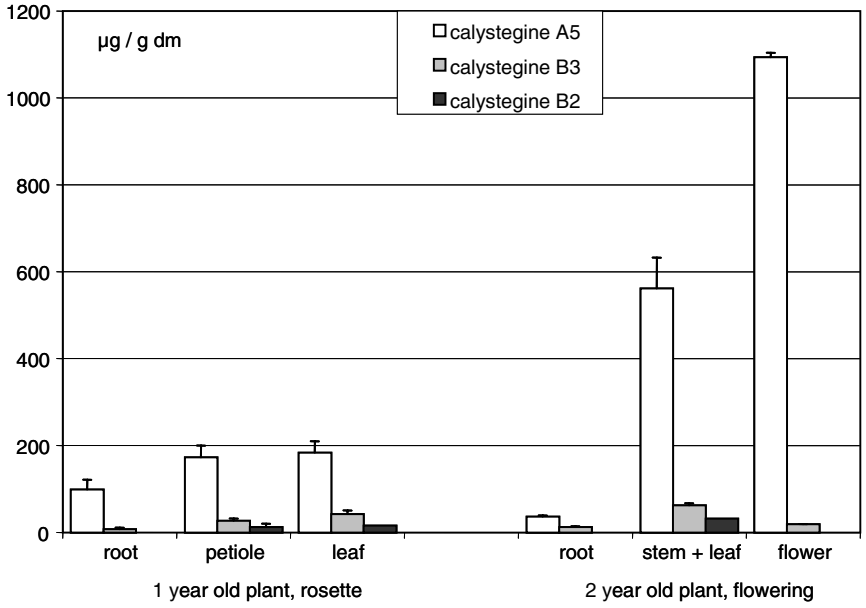

Fig. 3. Calystegine accumulation in *Cochlearia officinalis*. Concentrations indicated in microgram per gram dry mass (µg/g dm), vertical lines indicate standard deviation ($n = 3$–5).

1995) and reached a similar level as calystegines in flowers of potato plants (Keiner and Dräger, 2000).

As calystegines were present in the whole plant of *C. officinalis*, other *Cochlearia* species were investigated for calystegines. All species that were analysed contained calystegines, and calystegine A_5 prevailed in all plants. Calystegine concentrations in some species were higher than usually found in Solanaceae leaves (Dräger, 2004), however, they were highly variable, e.g. *C. danica* accessions contain between 0.7 and 5 mg/g dry mass calystegines (Fig. 4). Seasonal, climate and tissue-dependent variations in calystegine content were observed before in Solanaceae and Convolvulaceae, but *Cochlearia* samples used here were taken from plants in pots raised from seeds and kept under constant climate conditions. It remains to be examined in detail, whether high calystegine variations in *Cochlearia* species are maintained throughout the whole life cycle of plants. The striking differences between two

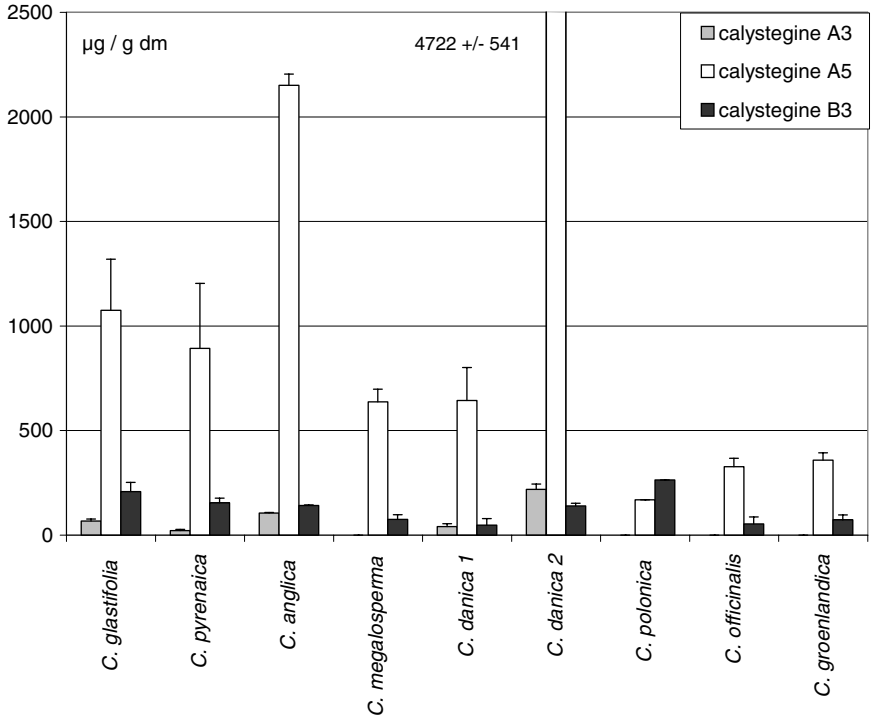

Fig. 4. Calystegines in rosette leaves of *Cochlearia* species. Concentrations indicated in microgram per gram dry mass (µg/g dm), vertical lines indicate standard deviation ($n = 3$–4).

provenances of *C. danica* demand for future genetic analysis of enzymes and regulators involved in calystegines biosynthesis.

2.2. Calystegines in Brassicaceae

After calystegines proved to be constituents throughout the genus *Cochlearia*, investigation of further Brassicaceae obviously became interesting. Plants were chosen from major evolutionary lineages of the family (Koch et al., 2006). Leaf samples from the majority of Brassicaceae contained calystegines (Fig. 5). Cochlearine or other tropine esters, however, were not found in Brassicaceae outside the genus *Cochlearia*. In the various Brassicaceae species the same calystegines were found as in *Cochlearia* and in addition calystegine B_2 could be detected.

In this first screening, concentrations of total calystegines in other Brassicaceae did not reach those of *Cochlearia* species, but again, calystegine accumulation throughout the whole life cycle of plants and in different plant organs remains to be determined. Some Brassicaceae species did now show calystegines in the leaf samples that were analysed: *A. thaliana* (L.) Heynh. L., *Barbarea vulgaris* R. Br., *Berteroa incana* DC, *Bunias erucago* L., *Bunias orientalis* L., *Coringia orientalis* Dum, *Cronopus squamatus* (Forssk.), Aschers., *Diplotaxis murales* (L) DC, *Isatis tinctoria* L., *Lunaria annua* L., *Lunaria rediviva* L., *Matthiola incana* (L.)R.Br. in Aiton, *Neslia paniculata* Dev., *Peltaria alliacea* Jacq., *Sisymbrium strictissimum* L. or only traces of calystegines were identified in *Carrichtera annua* (L.) DC. Several provenances of *A. thaliana* were analysed repeatedly and did not contain calystegines. In the Arabidopsis genome, however, 18 genes were annotated as "putative tropinone reductase". These sequences share about 50% identity to TRs of Solanaceae suggesting that they are phylogenetically related and possibly possess common ancestor genes. The annotation as tropinone reductases must be questioned after the results presented here, as the absence of tropine esters and other tropane alkaloids in this important species was confirmed by numerous repeated analyses. It will be interesting to unravel the function of the gene products in *Arabidopsis* metabolism.

3. Conclusions

In the sampling of Brassicaceae all tribes of the family were covered and calystegines were detected in species throughout the family (Fig. 6). All together 43 Brassicaceae species were analysed, among them 10 *Cochlearia* species, and 18 species contained calystegines. *Aethionema* is a genus placed at the basis of the Brassicaceae (Koch et al., 2003). The detection of calystegine in this genus points to existence of tropane alkaloid biosynthetic genes formation early in the evolution of the family. Losses (or gains) of a common trait within an angiosperms plant family during evolutionary speciation have often been described. Examples of secondary metabolites distribution patterns were shown for e.g. Fabaceae (Wink and Mohamed, 2003). The disappearance of a certain metabolite in a species

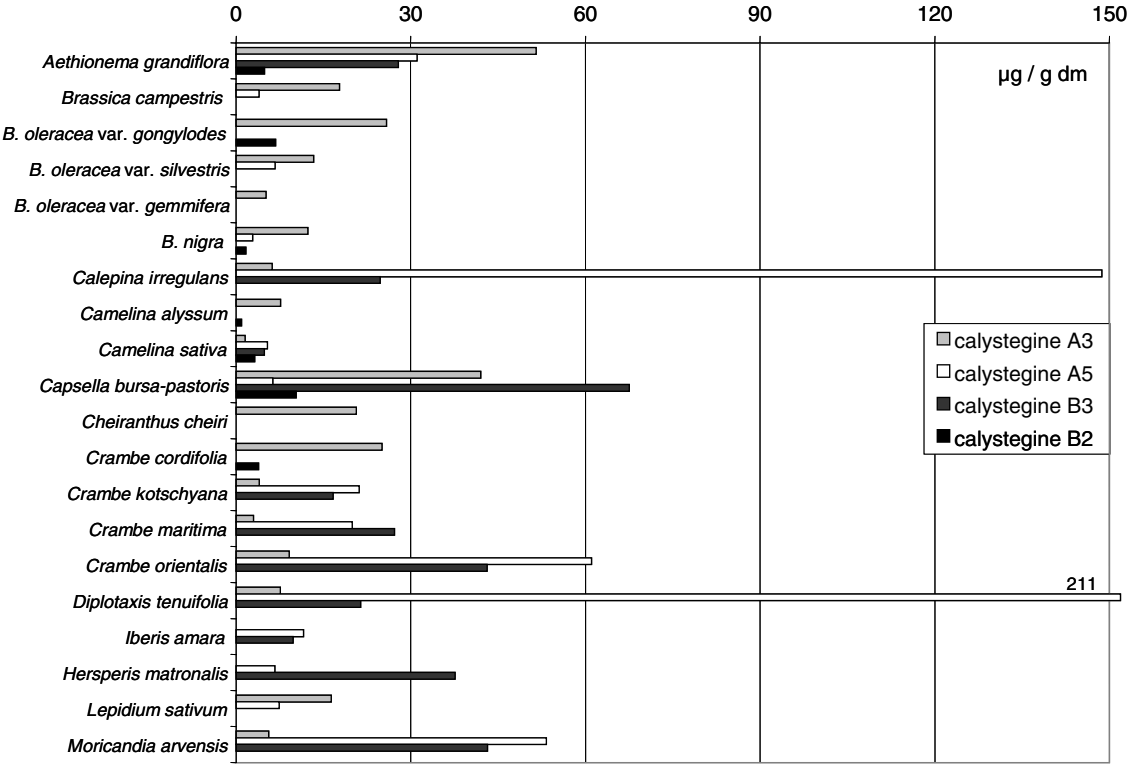

Fig. 5. Calystegines in leaves of flowering Brassicaeae plants. Concentrations indicated in microgram per gram dry mass (µg/g dm), vertical lines indicate standard deviation ($n = 2$).

within a plant lineage may be interpreted as the corresponding genes being turned off in the particular species or only in the developmental stages or organs that were investigated.

The finding of calystegines in *Cochlearia* and further Brassicaceae raises the questions for enzymes and metabolic steps involved in their biosynthesis in this plant family. For an understanding of tropane alkaloid evolution, it will be most interesting to investigate whether enzymes and genes involved in tropane alkaloid biosynthesis of Brassicaceae show similarity to those known from Solanaceae (Dräger, 2004) or whether they are completely different.

4. Experimental

4.1. Plants and seeds

Together 43 Brassicaceae species for investigation were obtained from the Botanical Garden of Martin Luther University Halle (BGH), purchased from Rühlemann seed trader, Germany (RS), or taken from our own collection (*Cochlearia* collection Marcus Koch, Heidelberg, CMK).

BGH: *Aethionema grandiflora* L., *A. thaliana* (L.) Heynh. L., *Barbarea vulgaris* R. Br., *Cochlearia groenlandica* L., C. *officinalis* L (BGH), *B. vulgaris* R. Br., *B. incana* DC, *B. erucago* L., *B. orientalis* L., *Calepina irregularis* (Asso) Thell., *Camelina alyssum* (Mill.) Thell., *Camelina sativa* (L.) Cantz, *Capsella bursa-pastoris* (L.) Medik., *Carrichtera annua* (L.) DC, *Cheiranthus cheiri* L., *Cochlearia groenlandica* L., *C. officinalis* L., *Coringia orientalis* Dum., *Crambe cordifolia* Stev., *Crambe kotschyana* Boiss., *Crambe maritima* L., *Crambe orientalis* Jacq., *Cronopus squamatus* (Forssk.), *Diplotaxis murales* (L), *Diplotaxis tenuifolia* (L.) DC, *Hesperis matronalis* L., *Iberis amara* L., *Isatis tinctoria* L., *Lunaria annua* L., *Lunaria rediviva* L., *M. incana* (L.)R.Br., *Moricandia arvensis* (L.) DC, *Neslia paniculata* Dev., *Peltaria alliacea* Jacq., *Sisymbrium strictissimum* L.

CMK: *C. anglica* L., *C. glastifolia* L., *C. danica* L., *C. megalosperma* (Maire) Vogt, *Cochlearia officinalis* L., *C. polonica* Fröhl., *C. pyrenaica* DC.

RS: *Brassica campestris* L. var. *Savannah, Brassica nigra* (L.) WDJ Koch, *Brassica oleracea* L. var *gongylodes, B. oleracea* L. var. *silvestris, B. oleracea* L. var. *gemmifera*, *Lepidium sativum* L.

Cochlearia seeds were germinated, and mature leaves were collected for analysis typically 6 weeks after germination from non-flowering plants. Only *C. officinalis* samples were additionally harvested from 2-year-old plants at flowering stage. Brassicaceae species from the Botanical Garden were collected as mature leaves from flowering plants. Some commercial cabbage species were raised from seeds, and mature leaves were collected typically 6–8 weeks after germination from non-flowering plants. Leaf samples (10–50 g fresh mass) were dried by lyophilisation directly after collection.

Fig. 6. Phylogeny of Brassicaceae species. Red names: calystegines identified; blue names: calystegines not detected. The systematic position of taxa not included in this tree were indicated by blue and red dots according to published data (Bailey et al., 2006; Beilstein et al., 2006).

4.2. Synthesis of cochlearine

The procedure for atropine synthesis was modified (Ladenburg, 1883). 2.07 g (15 mmol) of 3-hydroxy benzoic acid and 2.12 g (15 mmol) of tropine were suspended in 20 ml of water. 20 ml HCl conc. were added. The mixture was heated in an open vessel on a water bath under occasional stirring. Every 2 h, 20 ml HCl were added. After completion of the reaction the crude product was dried under vacuum and dissolved in an aq. KOH soln. to yield a weakly basic liquid. This was extracted with $CHCl_3$ for three times, the united organic layers were washed with water, dried over Na_2SO_4 and evaporated. The residue was purified over a Sephadex LH-20 column using MeOH/$HCCl_3$ 2:1 as eluent, Yield: 49%.

^{1}H-NMR (CD_3OD) [δ ppm; J Hz]: 7.46 (1 H, *ddd*, $J = 7.68$, 1.45, 1.04, C6′–H); 7.42 (1H, *dd*, $J = 2.49$, 1.45, C2′–H); 7.29 (1H, *dd*, $J = 8.09$, C5′–H); 7.01 (1H, *ddd*, $J = 8.09$, 2.49, 1.04, C4′-H); 5.16 (1H, *t*, $J = 5.2$ –OCH); 3.20 (2H, *br s*, C1/C5–H); 2.32 (3H, *s*, $-NCH_3$); 2.21 (2H, *dm*, $J = 14.94$, C2/C4–H_α); 2.16 (4H, *m*, C6/C7–H_2); 1.86 (2H, *br d*, $J = 14.94$, C2/C4–H_β). MS (EI, 70 eV) [*m/e*]: 261 (M^+), 140, 124 (B), 94, 82, 67.

4.3. Analysis of cochlearine, tropine, and pseudotropine

Dried leaves of *Cochlearia* species were powered and extracted with 80% methanol (3×10 ml/g dry mass). Methanol was reduced *in vacuo*; the residual extract was acidified to pH 3–4 (2% tartaric acid) and extracted successively by petrol ether, dichloromethane, and ethyl acetate. The organic extracts removed lipids and chlorophyll and were discarded. The pH of the aqueous solution was adjusted to 10 by ammonia and extracted by a mixture of chloroform and isopropanol (3:1). These extracts were reduced *in vacuo*, dissolved in ethyl acetate, and analysed for cochlearine, tropinone, tropine, and pseudotropine. Analysis was done by GC–MS (Richter et al., 2005) and by TLC as described (Dräger, 2002).

4.4. Analysis of calystegines

Extraction for calystegines and sample preparation by cation exchange columns was done as described (Brock et al., 2005). The extracts were used for identification of calystegines by TLC (Dräger, 2002). Silylation of lyophilised samples was performed in pyridine with hexamethyldisilazane and trimethylchlorosilane in a total volume of 60 µl, filled up to 100 µl with dry hexane and centrifuged after reaction for 30 min at 50 °C. The supernatants (90 µl) were transferred into microvials (200 µl), 9 µl azobenzene (1 mg/ml) were added as internal standard. GC analysis was performed as described (Brock et al., 2005). Identification was done with reference compounds, by retention time comparison and by GC–MS. Reference compounds were obtained by extraction from plant material provided by Prof. N. Asano, Hokuriku University, Kanazawa, Japan and by synthesis provided by Prof. R. Madsen, Technical University of Denmark, Lyngby, Denmark. Skaanderup and Madsen (2003) Calystegines with the same number of hydroxyl groups tend to show similar molecular fragments by GC–MS, but specific differences in fragmentation and different retention times enabled assignment of the signals to the individual calystegines (Schimming et al., 2005). The occurrence of a new calystegine in Brassicaceae with a retention time and fragmentation pattern exactly like one of the reference compounds, however, cannot be definitely excluded. Using 100–200 mg plant tissue per sample, the limit of detection for each individual calystegines was 3 µg/g dry mass by gas chromatographic analysis. For each analysis 2–6 independent samples were analysed.

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