PERGAMON

Phytochemistry 59 (2002) 603–609

PHYTOCHEMISTRY

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Nepenthes insignis uses a C_2-portion of the carbon skeleton of L-alanine acquired via its carnivorous organs, to build up the allelochemical plumbagin

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Received 12 October 2001; received in revised form 13 December 2001

Abstract

Tropical pitcher plants (*Nepenthes*) catch animals in their specialized cup-shaped leaves, digest the prey by secreting enzymes, and actively take up the resulting compounds. The benefit of this behaviour is the ability to grow and compete in nutrient-poor habitats. Our present in vitro study shows that not only the nitrogen of alanine fed to the carnivorous organs is used by the plant but that in addition intact C_2-units derived from C-2 and C-3 of stable isotope labelled L-alanine serve as building blocks, here exemplarily for the synthesis of the secondary metabolite plumbagin, a potent allelochemical. This result adds a new facet to the benefit of carnivory for plants. The availability of plumbagin by a de novo synthesis probably enhances the plants' fitness in their defence against phytophagous and pathogenic organisms. A missing specific uptake or CoA activation mechanism might be the reason that acetate fed to the pitchers was not incorporated into the naphthoquinone plumbagin. The dihydronaphthoquinone glucosides rossoliside and plumbaside A, here isolated for the first time from *Nepenthes*, by contrast, showed no incorporation after feeding of any of the two precursors, suggesting these compounds to be storage forms with probably very low turnover rates. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Nepenthes insignis*; Nepenthaceae; Carnivory; L-Alanine uptake; Naphthoquinones; Plumbagin; Phytoalexin; Secondary metabolism

1. Introduction

Nepenthes are carnivorous plants (Juniper et al., 1989) using their specialized leaves as passive pitfalls. Animal prey, consisting mostly of arthropods, are attracted to the pitcher opening by nectar secreted from glands situated at the lid and around the peristom. The foraging insects lose their footing while moving on the slippery collar and the inner side of the upper part of the pitcher, which is lined with small tiles of epicuticular wax. They are then trapped in the secrete accumulated at the base where they are soon drowned. Enzymes secreted by the plant into the pitcher fluid break down the quarry (Frazier, 2000)—in the wild with the additional assistance by the pitcher infauna (Greeney, 2001). In their dual role (Owen et al., 1999) the secretory glands also absorb some of the resulting small molecules including amino acids and ions (Lüttge, 1965).

Phytochemically, naphthoquinones are characteristic of *Nepenthes*. From the roots of *N. rafflesiana* Jack, plumbagin (**1**; Fig. 1), droserone, hydroxydroserone and the nepenthones A–C have been described (Cannon et al., 1980), while from the roots of *N. thorelii* Lecomte, again compound **1** and droserone, but also 2-methylnaphthazarine, and isoshinanolone are known (Likhitwitayawuid et al., 1998). Naphthoquinones are chemotaxonomic markers within the *Nepenthales* and their acetogenic biogenesis has, after an early work by Durand and Zenk (1971) on *Plumbago europaea*, recently been investigated in more detail in cell cultures of *Ancistrocladus heyneanus* (Ancistrocladaceae) (Bringmann et al., 1998) and Tri*phyophyllum peltatum* (Dioncophyllaceae) (Bringmann et al., 2000), both of which are closely related to *Nepenthes* (Meimberg et al., 2000).

In this communication we report on two glucosides that are new to *Nepenthes* and show that the carbon chain of alanine fed to the carnivorous organs of

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PII: S0031-9422(02)00003-1

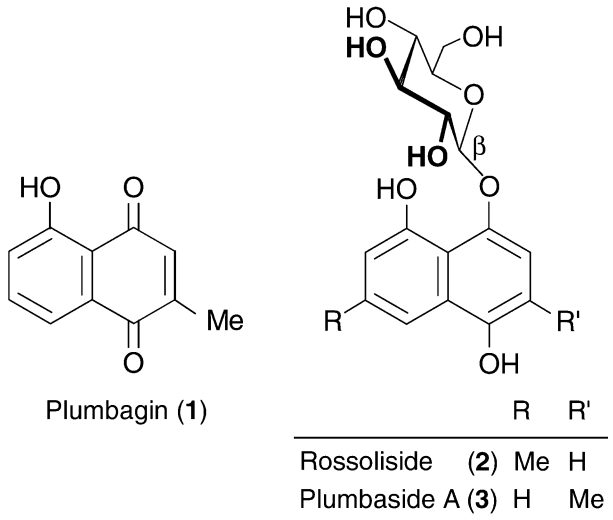

Fig. 1. Secondary metabolites isolated from *Nepenthes insignis*.

Nepenthes insignis Danser, a species occurring in the lowlands of New Guinea (Rischer, 1995), is utilized to build up the secondary metabolite **1**. This result implies practical, physiological, and ecological consequences with respect to carnivory in plants. Using an in vitro system excludes possible interference with microbial activities, which in nature contribute to the digestion, too (Frazier, 2000).

2. Results and discussion

2.1. Secondary metabolites from N. insignis

From in vitro cultivated *N. insignis*, three secondary metabolites (Fig. 1) were isolated and their structures were determined. Yields from individual experiments are given in Table 1. Rossoliside (7-methylhydrojuglone-4-*O*-ß-glucoside, **2**) and plumbaside A (hydroplumbagin-4-*O*-ß-glucoside, **3**) had not been found in *Nepenthes* before. Compound **2** had previously been detected in some Droseraceae e.g. *Drosera rotundifolia* (Sampara-Rumantir, 1971), *D. spathulata* (Budzianowski, 1995), and *D. intermedia* (Budzianowski, 1996). Compound **3** was already known from *Ceratostigma minus* (Plumbaginaceae) (Yue et al., 1994) and from Droseraceae like *Dionaea muscipula* (Kreher et al., 1989, 1990), *D. rotundifolia* (Vinkenborg et al., 1969), *D. intermedia* (Budzianowski, 1996), and *D. gigantea* (Budzianowski, 2000). The occurrence of both glucosides in *Nepenthes insignis* is a further hint at the close phylogenetic relationship of the mentioned families. Interestingly, plants treated with acetate or alanine contained the two glucosides in clearly lower concentrations than unfed plants.

Plumbagin (**1**), which is already known from *Nepenthes* (see above), is a relatively widespread metabolite in other plants, too: it is found in the related Ancistrocladaceae, Dioncophyllaceae, Droseraceae, and Plumbaginaceae, but has also been reported from Ebenaceae and interestingly even from monocots, viz Iridaceae (Thomson, 1971, 1987). The amount of **1** was slightly higher in the fed plants.

2.2. Precursor feeding to the pitchers

In two sets of experiments, either [U-$^{13}C_2$]sodium acetate or (L)-[U-$^{13}C_3$,^{15}N]alanine was fed to the pitchers of sterile grown *N. insignis* and the isolated secondary compounds were compared spectroscopically with those from unfed plants (Table 1).

The feeding experiments revealed that **1** unambiguously originates from L-alanine fed to the carnivorous organs of *N. insignis*. The ^{13}C NMR spectrum of **1** isolated after the alanine feeding experiment showed an incorporation rate of ca. 40% (given as intensity ratio of satellite peaks to central peak) and gave a coupling pattern typical of an incorporation of intact $^{13}C_2$-units (Table 2), except for C-3 (see below).

Coupling constants and patterns obtained from the 2D-INADEQUATE experiment (Fig. 2) showed pairwise coupling of the C atoms 2-CH_3/2, 1/9, 8/7, 6/5 and 10/4, whereas C-3 remained isolated, confirming the proposed folding of the polyketide and decarboxylation at C-3.

The high rate of the resorption of L-alanine via the roofed glands at the bottom of the pitcher was already described by Lüttge (1965). Alanine uptake is probably mediated by an energy dependent mechanism (An et al., 2001) similar to the suggested co-transport of H^+ ions

Table 1
Yields of isolated secondary metabolites after the feeding experiments

Experiment	Fr. wt (g)	Dry wt (g)	Raw extract (mg) (% dry wt)	Fraction 2 (mg) (% dry wt)	2 (mg) (% dry wt)	3 (mg) (% dry wt)	Fraction 4 (mg) (% dry wt)	1 (mg) (% dry wt)	1/3
Control	7.04	0.71	313 (44.7)	87.5 (12.3)	6.1 (0.9)	7.7 (1.1)	5.5 (0.8)	1.7 (0.2)	0.2
[U-$^{13}C_2$] sodium acetate	6.30	1.01	336 (33.3)	92.5 (9.1)	5.1 (0.5)	5.3 (0.5)	19.9 (2.0)	4.7 (0.3)	0.5
(L)-[U-$^{13}C_3$,^{15}N] alanine	1.66	1.51	529 (35.2)	65.7 (4.4)	1.7 (0.1)	2.8 (0.2)	11.3 (0.8)	5.2 (0.3)	1.9

Table 2
^{13}C NMR data of labelled plumbagin (**1**) isolated after application of L-[U-$^{13}C_3$,^{15}N]alanine to the pitchers

C-atom	2-CH_3	2	1	9	8	7	6	5	10	4	3
δ (ppm)	16.50	149.6	184.7	132.1	119.3	136.1	124.2	161.2	115.2	190.3	135.5
I_s[a] (%)	38.9	40.4	49.7	60.5	47.7	47.2	50.4	42.2	46.4	47.0	30.0
$^1J_{C-C}$ (Hz)	43.7	43.7	55.2	55.2	55.2	55.2	66.7	65.9	54.4	53.7	–

[a] Intensity ratio of satellite peaks compared to given central peak.

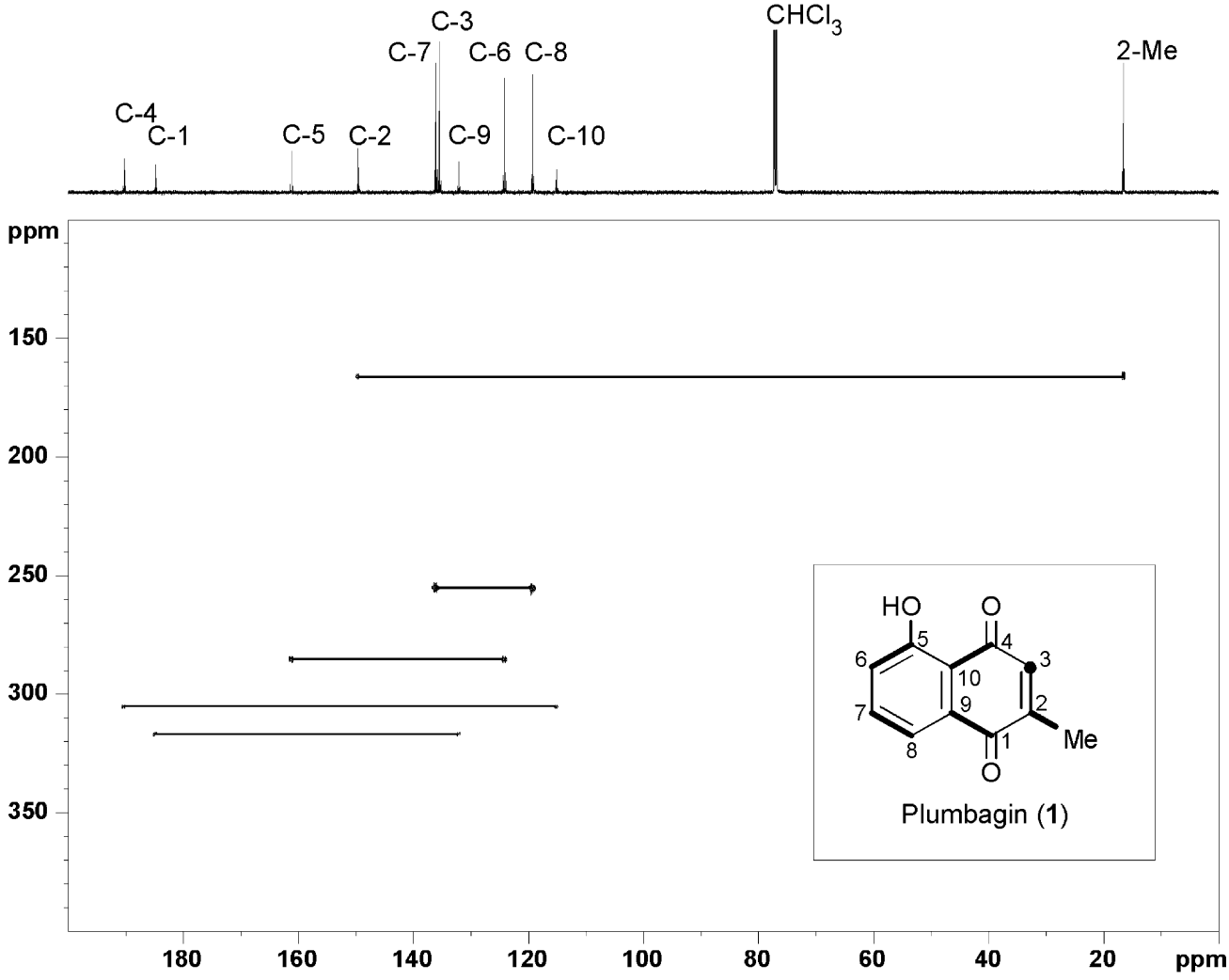

Fig. 2. 2D-INADEQUATE NMR spectrum of $^{13}C_2$-labelled **1** from the alanine feeding experiment; the pairwise ^{13}C–^{13}C correlations indicate [$^{13}C_2$] units incorporated intact.

and amino acids in *Dionaea muscipula* (Rea, 1984). Once the amino acid has been taken up by the plant, the biosynthetic pathway to plumbagin (**1**) seems rather straightforward (Fig. 3) and, after the expected transformation of L-alanine into acetyl-CoA via pyruvate by oxidative deamination and decarboxylation, it is obviously the same polyketide route as in *Ancistrocladus heyneanus* (Bringmann et al., 1998).

No incorporation, however, was found after feeding labelled acetate into the pitcher. Possible explanations for this are that either acetate was not activated to acetyl-CoA (which is not separately required within the thiolating decarboxylation step of pyruvate) or that it was not absorbed by the plant at all either due to the general absence of an uptake mechanism or because of the high buffer capacity of the applied solution. For alanine the elevation in uptake rates is, at least in *Dionaea*, correlated with a pH decrease to 4.4 (Rea and Whatley, 1983). In *Nepenthes* pitchers, this pH range is optimal for protease activity (Nakayama and Amagase, 1968)

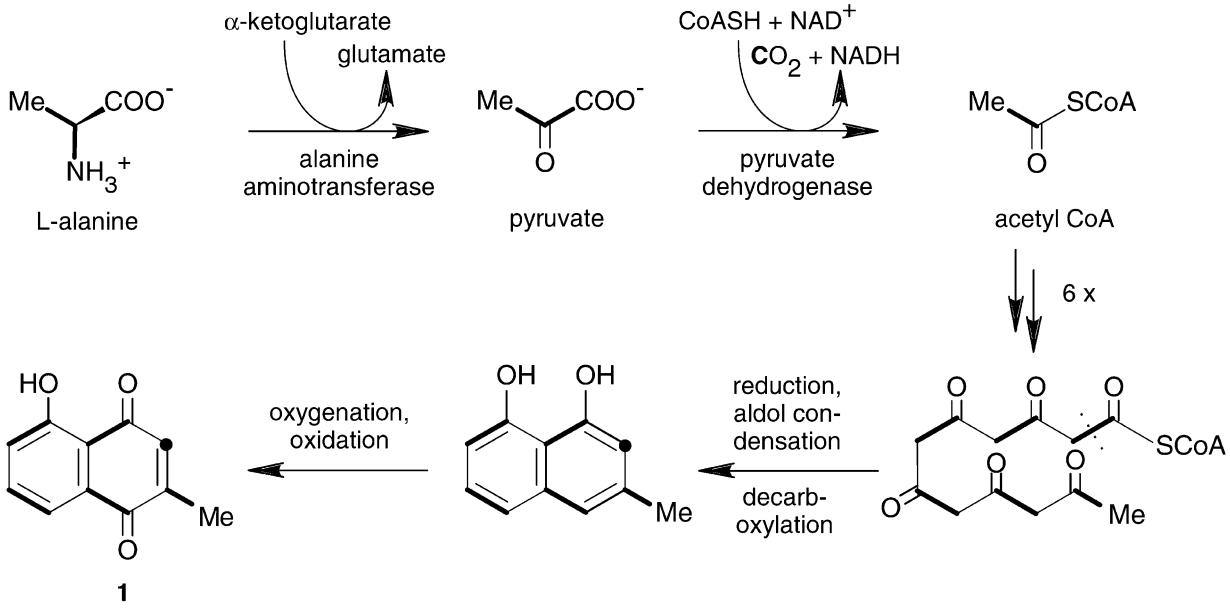

Fig. 3. Proposed biosynthetic pathway leading from L-alanine fed to the pitchers to **1**.

although the value may only be reached locally near the glands. On the other hand, the altered quantities of metabolites found after feeding acetate, compared to the control, may also indicate its uptake, despite the missing incorporation into the isolated compounds, possibly for the reason given above.

None of the two glucosides, **2** or **3**, were found to be labelled after any of the feeding experiments. They probably constitute storage forms of the respective naphthoquinones, and the turnover might still be too slow for a positive result even after 90 days of feeding.

2.3. Physiological implications

In the past, the benefit of carnivory for plants was exclusively attributed to the acquisition of nitrogen (Juniper et al., 1989). Undoubtedly the availability of nitrogen in most of their restricted habitats is a limiting factor and carnivorous plants growing completely heterotrophically are unknown. The present results show that intact C_2 units of the absorbed alanine molecules are directly channelled into secondary metabolism, here for the de novo synthesis of an allelochemical. The benefit of this carbon source is therefore obviously not a contribution to better growth per se but is likely to be an example of carnivory for an enhancement of the plants' ability to chemically defend themselves against other organisms.

2.4. Ecological implications

Even if the direct incorporation of acetate units derived from alanine into the carbon skeleton of **1** may be considered as a type of waste reprocessing, the result—a de novo synthesis and the resulting enhanced concentration of **1**, at least relative to the storage form **3**—may well be of ecological importance as it possibly improves the fitness of the plants.

1,4-Naphthoquinones, and plumbagin (**1**) in particular, are phytochemicals with high biological potency. Besides the effects of **1** on vertebrates, ranging from antifertility activity (Chowdhury et al., 1982) to cytotoxicity (Noda et al., 1997), there are many reports on its allelochemical effects on other organisms of greater interest in this context. Thus, **1** acts as an antifeedant agent on herbivorous insects. Depending on its concentration, it is a repellent even for adapted *Lepidoptera* (Villavicencio and Perez-Escandon, 1994). Insects feeding on plants containing a critical dose of **1** die either immediately or during the next ecdysis due to the inhibiton of ecdysteroid (Joshi and Sehnal, 1989) and chitin synthetase (Kubo et al., 1983) production. Plumbagin (**1**) is held responsible for fungicidal, including plant pathogenic species (Kubo et al., 1980), and microbicidal (Poul et al., 1999) activities, too. Furthermore it is a potent phytoalexin against parasitic plants (Bringmann et al., 1999) and inhibits the germination of seeds from other species (Spencer et al., 1986).

In the field, *Nepenthes* are only rarely injured by damages caused by herbivores (Rischer, pers. obs.). Only larvae of *Lepidoptera* are more frequently observed mining the leaves (Hering, 1931) or feeding on developing seed capsules (Clarke, 1997), but they may well represent highly specialized insects. These findings are additional indicators of the repelling function of **1** in *N. insignis*.

2.5. Practical implications

The results show that following a concept previously attempted with only limited success for the carnivorous glandular leaves of *Triphyophyllum peltatum* (Dioncophyllaceae) (Bringmann et al., 2001), *Nepenthes* pitchers are ideally suited for the use of L-alanine as an actively absorbed 'masked' precursor to acetyl-CoA and thus constitute a useful tool in investigations on the biosynthesis of acetogenic secondary metabolites. The 'concave' geometry of the leaves of *Nepenthes* (i.e. the fact that the compound administered is in their interior) allows far longer 'feeding' durations than the—moreover sensitive and fragile—'convex' glandular leaves of *T. peltatum* (with the compound only as a thin film *outside* the leaves) and therefore yields better incorporation rates.

3. Experimental

3.1. Plant material and growth conditions

Small sterile plants (ca. 6 cm Ø) of one clone of *N. insignis* obtained from seeds were grown in vitro on 1/5 MS (Murashige and Skoog, 1962) medium with full strength organics, 3% sucrose, and 0.2% Gelrite® (Carl Roth GmbH & Co, Karlsruhe, Germany) but without phytohormones.

For the feeding experiments, rooted plants were transferred under sterile conditions to small petridishes filled with sterilized Seramis® (Effem GmbH, Verden/Aller, Germany). Care was taken to remove all medium attached to the roots. The plants were placed in the middle of the petridishes so that the pitchers were hanging over the side (Fig. 4). This arrangement served as an additional safety precaution and made it impossible for fluid to drip out of old pitchers containing labelled precursors from the feeding experiments into the root ball. The petridishes with the plants were then placed in larger vessels which were lined with moistened filter paper circles and these containers were sealed with parafilm. All cultures were kept under fluorescent light with a 14-h photoperiod at 51 μmol m^{-2} s^{-1} photosynthetically active radiation.

Fig. 4. Arrangement of *Nepenthes insignis* in a Petri dish.

3.2. Feeding experiments

[U-$^{13}C_2$]Sodium acetate and (L)-[U-$^{13}C_3$,^{15}N]alanine were purchased from Promochem (Wesel, Germany). Aqueous solutions (4 mg/ml, in the case of sodium acetate adjusted to pH 5.8 with HCl) were prepared and filter-sterilized (0.22 μm Disposable Filter Holder No. FP030/3, Schleicher & Schuell GmbH, Dassel, Germany). In weekly intervals the absorbed pitcher fluid was replaced by the respective solution using a disposable syringe with cannula. For easier access to the pitchers the lids of the already opened pitchers had been removed beforehand.

3.3. Extraction

Complete plants previously unfed or fed with either labelled sodium acetate or alanine were harvested after 90 days. Their fresh weights (fr. wt) and their dry weights (dry wt) after lyophilization were determined. The material was ground and extracted with a 9:1 mixture of methanol:H_2O overnight at room temperature. The solvent was evaporated to dryness subsequent to filtering through filter paper. After lyophilization, the dry weight of the crude extract was determined. Using disposable columns packed with C_{18}-material (Bakerbond spe Octadecyl® No. 7020–03, J.T. Baker Inc., Phillipsburg, USA) a first fractionation was achieved and the chlorophyll was removed. For this purpose, the residue was redisolved in 6 ml of 1:1 methanol:H_2O and applied to the column conditioned according to the manufacturer's instructions. After collection of fraction 1, three further fractions were obtained by successively washing the column with 3 ml portions of 7:3 methanol:H_2O.

3.4. Isolation of secondary metabolites

The residue of fraction 2 was weighed and redissolved in 1 ml methanol. Further purification was achieved by preparative HPLC using a Waters Delta Pak C_{18} column (300 Å, 19×30 cm, 15 μm, No. 11804, Waters Corp., Milford, USA) with an isocratic mobile phase (4:1 H_2O:acetonitrile) at a flow rate of 6 ml/min. Peaks were detected at 254 nm and manually collected, yielding pure rossoliside (**2**) and plumbaside A (**3**). Compound **1** was isolated from fraction 4 by flash CC (silica gel 60, 100:5 petroleum ether:ethyl acetate).

3.5. General procedures for metabolite characterization

Melting points were determined on a Reichert-Jung Thermovar hot-plate and are uncorrected. Optical

rotations were taken on a Perkin-Elmer 241MC polarimeter (25 °C, 10 cm cell); IR spectra were measured on a Perkin-Elmer 1429 spectrophotometer. Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker DMX 600 (Bruker Analytik, Rheinstetten, Germany) at 600 and 150 MHz for ^{1}H and ^{13}C NMR respectively using $CDCl_3$ ($\delta = 7.26$ and 77.01) and CD_3OD ($\delta = 3.30$ and 49.01) as solvents and internal ^{1}H and ^{13}C standards. The 2D INADEQUATE experiment was optimized for $^{1}J(^{13}C,^{13}C) = 50$ Hz. EIMS and HRMS were determined on Finnigan MAT 8200 and MAT 90 instruments (70 eV). Organic solvents were dried and distilled prior to use.

3.6. Isolated metabolites

Plumbagin (**1**): orange powder (CH_2Cl_2): mp 77 °C; lit. 76–77 °C (Sampara-Rumantir, 1971); all physical and spectroscopic properties were identical to those of a reference sample (Product No. P7262) purchased from Sigma-Aldrich (Deisenhofen, Germany).

Rossoliside (**2**): amorphous powder; $[\alpha]_D^{20}$ −168.2° (MeOH, *c* 0.122); lit. $[\alpha]_D$ (i.a. Budzianowski, 1995) not reported; all spectroscopic properties matched those described in the literature (Sampara-Rumantir, 1971; Budzianowski, 1995).

Plumbaside A (**3**): amorphous powder; $[\alpha]_D^{20}$ −31.8° (MeOH, *c* 0.077); lit. $[\alpha]_D$ (i.a. Kreher et al., 1990) not reported; all spectroscopic properties matched those reported earlier (Yue et al., 1994).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 251 'Ökologie, Physiologie und Biochemie pflanzlicher und tierischer Leistung unter Stress'), the Max-Buchner-Forschungsstiftung (fellowship to H.R.) and the Fonds der Chemischen Industrie (fellowship and to A.H. and research funds). The authors thank M. Wohlfarth for helpful discussions on NMR techniques.

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