PERGAMON

Phytochemistry 53 (2000) 55–58

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Antifungal nitro compounds from Skunk Cabbage (*Lysichitum americanum*) leaves treated with cupric chloride

Fujinori Hanawa[a,b,*], Satoshi Tahara[c], G.H. Neil Towers[a]

[a]*Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver B.C., Canada V6T 1Z4*
[b]*Forestry and Forest Production Research Institute (FFPRI), P.O. Box 16, Tsukuba, Ibaraki 305-8687, Japan*
[c]*Department of Applied Bioscience, Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan*

Received 23 March 1999; received in revised form 8 July 1999

Abstract

Two nitro compounds, 2-(4-methoxyphenyl)-1-nitroethane named as lysichitalexin and 2-(4-hydroxyphenyl)-1-nitroethane were isolated as stress metabolites from the leaves of *Lysichitum americanum* Hultén and St. John treated with cupric chloride. Their structures were determined by spectroscopic methods and chemical reactions. The former compound showed antifungal activities against *Fusarium oxysporum* and *Cladosporium herbarum*. Both compounds were isolated for the first time from this species and the former was isolated from natural sources for the first time. This is the first report on stress metabolites from a member of the Araceae.

Keywords: *Lysichitum americanum*; Skunk cabbage; Araceae; Stress metabolites; Phytoalexin; Fungitoxin; Nitro compounds

1. Introduction

Many plants produce stress metabolites when they are exposed to pathogens or abiotic agents such as heavy metal ions or UV light (Kuć, 1972). Some of these metabolites show fungitoxic activities and are thought to contribute to the defense mechanisms of plants as phytoalexins. In our research on stress metabolites of wild plants, cupric chloride has proven to be a good stressing agent for species such as dandelion, *Taraxacum officinale* (Tahara, Hanawa, Harada & Mizutani, 1988), *Iris pseudacorus* (Hanawa, Tahara & Mizutani, 1991a, 1991b), and *Veratrum grandiflorum* (Hanawa, Tahara & Mizutani, 1992). Because investigations of stress metabolites gives us unusual opportunities to encounter new compounds with new biological activities, and because there have been no reports about the chemical constituents of *Lysichitum americanum* (or *Lysichitum americanus*) except for the detection of indole from the spathe in the flowering season (Chen & Meeuse, 1971), we have investigated the production of stress metabolites in the leaves of this species treated with cupric chloride. Two nitro compounds, which are not common as stress metabolites, were identified. As for the chemical constituents from plants in the genus *Lysichitum* so far three isoquinoline-type alkaloids have been identified in *L. camtschatcense* (Katsui, Sato, Tobinaga & Takeuchi, 1966). Here, we report the structural determination of the nitro compounds and their antibiotic activities.

2. Results and discussion

During the initial screening of antimicrobial and antifungal extracts from wild British Columbia plants stressed with 3 mM of cupric chloride solution, *L. americanum* was found to produce antifungal activities against the well known plant pathogens, *Fusarium oxysporum* and *Cladosporium herbarum*. Active bands which were detected by a TLC overlay method (see Section 3) were found only in the EtOAc extract of the

* Corresponding author.
E-mail address: fujinori@ffpri.affrc.go.jp (F. Hanawa).

0031-9422/00/$ - see front matter
PII: S0031-9422(99)00443-4

cupric chloride solution in which the leaves had been soaked for 2 days. There was no activity in the EtOAc extract of the water used as substitute for cupric chloride solution in the control experiment. In addition, because antifungal activity was not detected in the Me_2CO extract obtained by rinsing the treated leaves, these compounds must have been released into the cupric chloride solution. Neither was there antifungal activity in the Me_2CO extract obtained by rinsing the leaves which had been treated with water. Therefore, we focussed on the EtOAc extract of the cupric chloride solution of the treated leaves.

The experiment was scaled up to 20 litres of cupric chloride solution in which 2 kg of *L. americanum* leaves were soaked for 2 days. The cupric chloride solution was extracted with EtOAc to give 321.3 mg of a brown oil after concentration. Subjecting the crude extract to silica gel column chromatography and prep. TLC, 7.4 mg of compound **1** and 2.5 mg of compound **2** were isolated.

Compound **1** was isolated as a pale yellow oil. The molecular formula $C_9H_{11}O_3N$ was suggested by HR-EI MS ($[M]^+$ m/z 181.07360, requires 181.07390). The HR mass analysis for the base ion peak m/z 134.07316 suggested $C_9H_{10}O$ as the chemical formula which requires 134.07317 and is assignable for $[M\text{-}HNO_2]^+$. Four protons of two methylenes (3.24 and 4.74 ppm) coupled by 7.1 Hz, three protons of a methoxyl group (3.76 ppm) on a phenyl ring and four protons of a Para-substituted phenyl ring system (6.87 ppm and 7.21 ppm) showing AA′XX′ coupling which is typical for the ring system, were observed in the ^{1}H-NMR spectrum. ^{13}C-NMR spectra (APT) also indicated the existence of two methylenes, a methoxyl group and a Para-substituted phenyl ring. These data suggested that compound **1** is either 2-(4-methoxyphenyl)-1-nitroethane or 2-(4-methoxyphenyl)ethane-1-nitrite (**3**). The presence of a nitro group was verified because compound **1** gave a yellow color on reaction with diphenylamine and did not react with the Griess reagent which reacts with nitrite (Feigel & Anger, 1966). Therefore, compound **1** was determined to be 2-(4-methoxyphenyl)-1-nitroethane, a compound isolated for the first time from natural sources and named lysichitalexin.

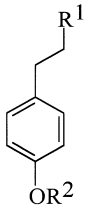
Compound **2** was isolated as a pale yellow oil. The molecular formula $C_8H_9O_3N$ was suggested by HR-EI MS ($[M]^+$ m/z 167.05810, requires 167.05824). The HR mass analysis for the base ion peak m/z 120.05751 suggested C_8H_8O as the chemical formula which requires 120.05752 and is assignable for $[M\text{-}HNO_2]^+$. Both the ^{1}H- and ^{13}C-NMR spectra were almost the same as those of compound **1**. Instead of a signal for a methoxyl group, however, a singlet for a proton which could be assigned to a hydroxyl group was detected at 8.28 ppm. Because compound **2** also gave a yellow color by reaction with diphenylamine and did not give a purple color with Griess reagent it was determined to be 2-(4-hydroxyphenyl)-1-nitroethane. This compound has previously been detected from cell suspension cultures of *Eschscholzia californica* (Höesel, Berlin, Hanzlik & Conn, 1985) under osmotic stress and from the microsomal reaction mixture of *Sorghum bicolor* (Halkier & Møeller, 1990) as a by-product of dhurrin biosynthesis from L-tyrosine. Thalictoside, a glucoside of compound **2**, was reported from *Thalictrum aquilegifolium* (Ina & Iida, 1986) and several other species (*Parabenzoin praecox* (Shimomura, Sashida, Oohara & Tenma, 1988), *Epimedium diphyllum* (Miyase, Ueno, Takizawa, Kobayashi & Oguchi, 1989), *Ocotea vellosiana* (Garcez, Yoshida & Gottlieb, 1995) and *Epimedium hunanense* (Liang, Vuorela, Vuorela & Hiltunen, 1997). Caffeoylthalictoside from *Parabenzoin praecox* (Shimomura et al., 1988) and arabinothalictoside from *Sagittaria trifolia* (Yoshikawa, Yoshizumi, Murakami, Matsuda, Yamahara & Murakami, 1996) are also known.

Table 1
Antimicrobial activities of nitro compounds from *Lysichitum americanum* by TLC overlay method

Concentrations (μg/cm^2)	Compound 1			Compound 2								
	31.4	15.7	7.9	3.9	2.0	1.0	31.4	15.7	7.9	3.9	2.0	1.0
B. subtilis	–	–	–	–	–	–	–	–	–	–	–	–
S. aureus	–	–	–	–	–	–	–	–	–	–	–	–
E. coli	–	–	–	–	–	–	–	–	–	–	–	–
C. albicans	–	–	–	–	–	–	–	–	–	–	–	–
F. oxysporum	++[a]	++	+/–[c]	–	–	–	–	–	–	–	–	–
C. herbarum	+[b]	+	+/–	–	–	–	–	–	–	–	–	–

[a] 9 mm diameter of inhibition zone.
[b] Inhibition zone < 9 mm diameter or not clear.
[c] Slight inhibition.

Antimicrobial activities of compounds **1** and **2** against several microorganisms are summarized in Table 1. It is worth noting that the compounds showed antifungal activities only for plant pathogens (*Fusarium oxysporum* and *Cladosporium herbarum*) but not for the animal pathogen *Candida albicans* or bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* which infect humans. These compounds could therefore contribute to the defense mechanisms of *Lysichitum americanum* as phytoalexins. They fit the recent definition of phytoalexin proposed by Purkayastha (1995), according to whom, a phytoalexin may be defined as an antimicrobial, low molecular weight, secondary metabolite formed de novo as a result of physical, chemical, or biological stress which resists or suppresses the activity of invaders.



1 R^1=NO_2, R^2=Me
2 R^1=NO_2, R^2=H
3 R^1=ONO, R^2=Me

3. Experimental

3.1. General

TLC was carried out using Merck pre-coated silica gel 60 plates (F254; layer thickness 0.20 mm for analytical TLC and 0.25 mm for prep. TLC). Compounds on the TLC plates were detected under UV light (wave length 365 and 254 nm) and with Gibbs reagent. EIMS spectrometry was carried out on a Kratos MS 50 instrument (direct insertion probe, 70 eV ionization potential). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AV-500, using TMS as an internal standard. Chemical shifts are given in δ ppm.

3.2. Plant materials and induction and isolation of stress metabolites

For the initial screening, *L. americanum* leaves, (voucher specimens deposited in the UBC herbarium) collected in Squamish, British Columbia were cut into 1 cm width strips at right angles to the long axis of the leaf, and each 50 g sample was soaked in 500 ml of 3 mM cupric chloride solution in a shallow container (ca. 25 W × 30 L × 10 H cm) at room temperature As a control, water was used instead of cupric chloride solution In total 100 g of leaves were used for each treatment. After 2 days, the solution was extracted with EtOAc twice and the extract was concentrated to dryness under reduced pressure and re-dissolved in 2 ml of EtOAc. The plant materials treated with both cupric chloride and water were further extracted with 500 ml of Me_2CO for 1 min to extract the compounds remaining in the material. The Me_2CO extracts were concentrated and subjected to organic solvent fractionation by EtOAc and water, and the EtOAc extracts were dissolved in 2 ml of a mixture of EtOAc and MeOH after concentration. These extracts were subjected to bioassay. For the isolation of stress metabolites, larger quantities of *L. americanum* leaves (2.0 kg) were collected in Whistler, British Columbia in July 1998. The materials were also cut into 1 cm width strips as before and each 200 g of leaves were soaked in 2 l of 3 mM cupric chloride solution in a shallow container (ca. 40 W × 80 L × 15 H cm) at room temperature. After 2 days, the solution was extracted with EtOAc twice and the extract was concentrated under reduced pressure to give 321.3 mg of brown oil. The crude extract (321.3 mg) was chromatographed over 15 g of silica gel as follows: frs. 1–14 (solvents A:B, [hexane:EtOAc], 30:1, each 15 ml), frs. 15–27 (A:B, 20:1, each 15 ml), frs. 28–69 (A:B, 10:1, each 15 ml), fr. 70 (A:B, 1:1, 200 ml), fr. 71 (EtOAc, 200 ml) and fr. 72 (MeOH, 100 ml). Compound **1** (7.4 mg) in frs. 11–16 and compound **2** (2.5 mg) in frs. 47–64 were purified with prep. TLC (*Rf* 0.57 and 0.46 respectively, A:B, 1:1).

3.3. Physicochemical properties

Lysichitalexin, 2-(4-methoxyphenyl)-1-nitroethane(**1**). Gibbs test: Dark brown after one day. IR ν_{max} (neat) cm^{-1}: 2935, 2838, 1613, 1553, 1515, 1435, 1380, 1250, 1180. HR-EIMS: $[M]^+$ 181.07360 ($C_9H_{11}O_3N$ requires 181.07390), $[M\text{-}NO_2]^+$ 134.07316 ($C_9H_{10}O$ requires 134.07317). EIMS *m/z* (rel. int.): 181 $[M]^+$ (23), 151 (11), 149 (20), 135 (47), 134 $[M\text{-}NO_2]^+$ (100), 129 (12), 121 (26), 120 (13), 119 (26). ^{1}H-NMR spectral data (500 MHz, Me_2CO-d_6): δ 3.24 (2H, *t*, *J* = 7.1 Hz, H-7), 3.76 (3H, *s*, OMe), 4.74 (2H, *t*, *J* = 7.1 Hz, H-8), 6.87 (2H, *dt*, *J* = 8.8, 2.6 Hz, H-3, H-5), 7.21 (2H, *dt-like*, *J* = 8.8, 2.4, 0.5 Hz, H-2, H-6). ^{13}C-NMR (125 MHz, Me_2CO-d_6): δ 33.0 (C-7, –CH_2–), 55.4 (4-O-Me), 77.3 (C-8, –CH_2–), 114.9 (C-3, 5), 129.3 (C-1), 130.6 (C-2, 6), 159.8 (C-4).

2-(4-Hydroxyphenyl)-1-nitroethane (**2**). Gibbs test: Dark brown after one day. IR ν_{max} ($CHCl_3$) cm^{-1}: 3598, 2926, 1710, 1614, 1590, 1554, 1515, 1435, 1380, 1260. HR-EIMS: $[M]^+$ 167.05810 ($C_8H_9O_3N$ requires 167.05824), $[M\text{-}NO_2]+$ 120.05751 (C_8H_8O requires 120.05752). EIMS *m/z* (rel. int.): 167 $[M]^+$ (12), 135 (11), 121 (38), 120 $[M\text{-}NO_2]^+$ (100), 119 (14). ^{1}H-NMR spectral data (500 MHz, Me_2CO-d_6): δ 3.21 (2H, *t*, *J* = 7.1 Hz, H-7), 4.71 (2H, *t*, *J* = 7.1 Hz, H-8), 6.77 (2H, *dt*, *J* = 8.7, 2.5 Hz, H-2, H-6), 7.11 (2H, *dt-like*, *J* = 8.7, 2.5, 0.5 Hz, H-3, H-5) 8.28 (1H, *s*). ^{13}C-NMR (125 MHz, Me_2CO-d_6): δ 33.1 (C-7, –CH_2–), 77.4 (C-8, –CH_2–), 116.3 (C-3, 5), 128.1 (C-1), 130.6 (C-2, 6), 157.4 (C-4).

3.3.1. Bioassay

TLC overlay assays for the isolated compounds against *B. subtilis*, *S. aureus*, *E. coli* and *C. albicans* were carried out as previously reported (Saxena, Farmer, Towers & Hancock, 1995). The assay for *F. oxysporum* (ATCC No. 48112) and *C. herbarum* (ATCC No. 28987) was modified slightly as follows. Both fungi were initially incubated on 20 ml of a Sabouraud medium containing 1.5% of agar in a 10 cm diameter petri dish for 3–4 days at 27°C in darkness. The spores formed over the media were sus-

pended in 10 ml of sterile 0.05% Tween 20 solution in distilled water. The spore suspension (200 μl) was added to 20 ml of Sabouraud broth containing 0.6% agar and subjected to the TLC overlay assay in the same way as reported previously, except the temperature 27°C.

Acknowledgements

We thank Dr. G.K. Eigendorf of the UBC Mass Spectrometer Center and Ms. Liane Darge of the UBC NMR lab. for the acquisition of MS and NMR spectral data. We also thank Zyta Abramowski for microbiological results and the Japan Science and Technology Corporation and the Natural Sciences and Engineering Research Council of Canada for financial support of this research.

References

Chen, J., & Meeuse, B. J. D. (1971). Production of free indole by some aroids. *Acta. Bot. Neer*, *20*(6), 627–635.

Feigel, F., & Anger, V. (1966). *Spot tests in organic analysis*. Amsterdam: Elsevier.

Garcez, W. S., Yoshida, M., & Gottlieb, O. R. (1995). Benzylisoquinoline alkaloids and flavonols from *Ocotea vellosiana*. *Phytochemistry*, *39*(4), 815–816.

Halkier, B. A., & Møeller, B. L. (1990). The biosynthesis of cyanogenic glucosides in higher plants. Identification of three hydroxylation steps in the biosynthesis of dhurrin in *Sorghum bicolor* (L.) Moench and the involvement of 1-aci-nitro-2-(p-hydroxyphenyl)ethane as an intermediate. *J. Biol. Chem*, *265*(34), 21114–21121.

Hanawa, F., Tahara, S., & Mizutani, J. (1991a). Flavonoids produced by *Iris pseudacorus* leaves treated with cupric chloride. *Phytochemistry*, *30*(7), 2197–2198.

Hanawa, F., Tahara, S., & Mizutani, J. (1991b). Isoflavonoids produced by *Iris pseudacorus* leaves treated with cupric chloride. *Phytochemistry*, *30*(1), 157–163.

Hanawa, F., Tahara, S., & Mizutani, J. (1992). Antifungal stress compounds from *Veratrum grandiflorum* Leaves treated with cupric chloride. *Phytochemistry*, *31*, 3005–3007.

Hesel, W., Berlin, J., Hanzlik, T. N., & Conn, E. E. (1985). In vitro biosynthesis of 1-(4′-hydroxyphenyl)-2-nitroethane and production of cyanogenic compounds in osmotically stressed cell suspension cultures of *Eschscholtzia californica* Cham. *Planta*, *166*(2), 176–181.

Ina, H., & Iida, H. (1986). Isolation and structure of thalictoside. *Chem. Pharm. Bull*, *34*(2), 726–729.

Katsui, N., Sato, K., Tobinaga, S., & Takeuchi, N. (1966). Alkaloids of *Lysichiton camtschatcense* Schott var. japonicum Makino. *Tetrahedron Lett*, *50*, 6257–6261.

Kuć, J. (1972). Compounds accumulating in plants after infection. *Microbial Toxins*, *8* (pp. 211–247). New York: Academic Press.

Liang, H. R., Vuorela, P., Vuorela, H., & Hiltunen, R. (1997). Isolation and immunomodulatory effect of flavonol glycosides from *Epimedium hunanense*. *Planta Med*, *63*(4), 316–319.

Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H., & Oguchi, H. (1989). Ionone and lignan glycosides from *Epimedium diphyllum*. *Phytochemistry*, *28*(12), 3483–3485.

Purkayastha, R. P. (1995). In M. Daniel, & R. P. Purkayastha, *Handbook of phytoalexin metabolism and action*. New York: Marcel Dekker.

Saxena, G., Farmer, S., Towers, G. H. N., & Hancock, R. E. W. (1995). Use of specific dyes in the detection of antimicrobial compounds from crude plant extracts using a thin layer chromatography agar overlay technique. *Phytochemical Analysis*, *6*, 125–129.

Shimomura, H., Sashida, Y., Oohara, M., & Tenma, H. (1988). Phenolic glucosides from *Parabenzoin praecox*. *Phytochemistry*, *27*(2), 644–646.

Tahara, S., Hanawa, F., Harada, Y., & Mizutani, J. (1988). A fungitoxin inducibly produced by dandelion leaves treated with cupric chloride. *Agric. Biol. Chem*, *52*(11), 2947–2948.

Yoshikawa, M., Yoshizumi, S., Murakami, T., Matsuda, H., Yamahara, J., & Murakami, N. (1996). Medicinal foodstuffs. Part II: On the bioactive constituents of the tuber of *Sagittaria trifolia* L. (Kuwai, Alismataceae): absolute stereostructures of trifoliones A, B, C, and D, sagittariosides a and b, and arabinothalictoside. *Chem. Pharm. Bull*, *44*(3), 492–499.