Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 3015–3020

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

WASALEXINS A AND B, NEW PHYTOALEXINS FROM WASABI: ISOLATION, SYNTHESIS, AND ANTIFUNGAL ACTIVITY

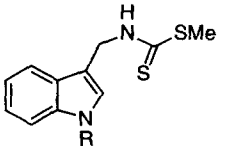
M. Soledade C. Pedras,* John L. Sorensen, Francis I. Okanga, and Irina L. Zaharia
Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK S7N 5C9, Canada

Received 23 July 1999; accepted 13 September 1999

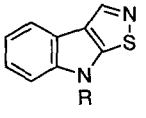
Abstract: The chemical structure determination of two phytoalexins from wasabi (*Wasabia japonica*, syn. *Eutrema wasabi)*, a plant resistant to virulent isolates of the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], as well as their synthesis and antifungal activity towards isolates of *P. lingam* and *P. wasabiae* is reported.

Phytoalexins, plant secondary metabolites synthesized de novo in response to diverse forms of stress, including fungal infection, are part of the plants' chemical and biochemical defense mechanisms.[1,2] The phytoalexins from cruciferous plants (family *Cruciferae*, e.g. broccoli, cauliflower, cabbage, rapeseed, mustard, wasabi),[3] as for example **1-6**, contain an indole or related ring and at least one sulfur atom.

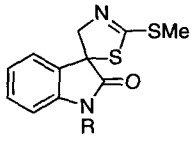
Recent results established that wasabi (*Wasabia japonica*, syn. *Eutrema wasabi*) is resistant to the blackleg fungus (*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.), the causative agent of one of the most damaging diseases of the agronomically important oilseed crop, canola (*Brassica napus* and *B. rapa*).[4] Consequently, it became worthwhile to establish the chemical traits responsible for the resistance of wasabi to *P. lingam*, as such traits are potentially transferable to canola. Subsequently, we have investigated and reported the structures of several constitutive antifungal compounds produced by wasabi, as well as the phytoalexin methyl 1-methoxyindole-3-carboxylate (**8**), and their activity towards isolates of *P. lingam* and *P. wasabiae*.[5] Herein we wish to communicate studies carried out to establish the structure of two new phytoalexins, which we named wasalexins A (**10a**) and B (**10b**), produced by foliar tissue of wasabi under elicitation by *P. lingam, P. wasabiae* or $CuCl_2$.



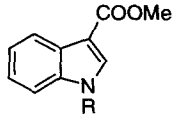
1 = H
2 = OMe



3 = H
4 = OMe



5 = H
6 = OMe



7 = Me
8 = OMe

In preliminary experiments, a time-course response of wasabi plants to abiotic elicitation was investigated. After eliciting and incubating plants, leaves were excised at intervals and extracted as previously reported;[5] control leaves were collected and treated in similar manner. Each extract was bioassayed for

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00523-5

antifungal activity against *Cladosporium cucumerinum* (biodetection on TLC plates employing spores of *C. cucumerinum*) and analyzed by HPLC.[6] The extracts of both elicited and control leaves showed similar zones of antifungal activity; however, the HPLC chromatograms of hexane extracts of elicited leaf tissue displayed two peaks with r_T = 18.0 and 23.8 min, which were not detectable on the chromatograms of extracts of control tissues. The structure of the compound with r_T = 18.0 was readily determined to be phytoalexin **8**, as previously reported.[5] It is worthy to note that the related 1-methylindole **7** was found to be a constitutive chemical defense present in roots of a cruciferous weed (*Camelina sativa*).[7]

Nonetheless, the structure of the compound with r_T = 23.8 min, a bright yellow metabolite, could not be unambiguously assigned. The HRMS spectroscopic data indicated a molecular formula of $C_{13}H_{14}N_2O_2S_2$, further corroborated by analysis of the NMR spectroscopic data. As in compound **8**, the NMR spectra of the yellow metabolite displayed five aromatic signals (δ_H 8.03, s, 7.86, d, 7.27, dd, 7.04, dd, 6.96, d; δ_C 139.1, 128.8, 125.0, 122.9, 107.1), as well as signals due to a MeO group (δ_H 4.01; δ_C 64.2), two overlapping MeS groups (δ_H 2.70; δ_C 16.2), and two carbonyl or equivalent carbons (δ_C 164.8 and 177.5).[8]

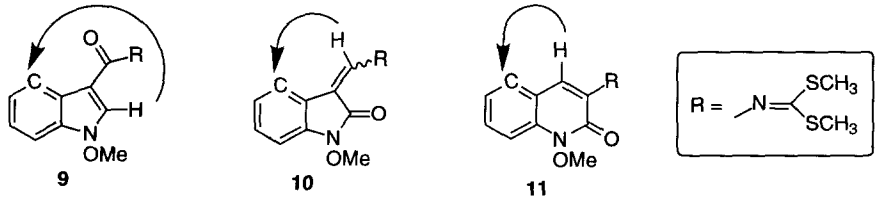
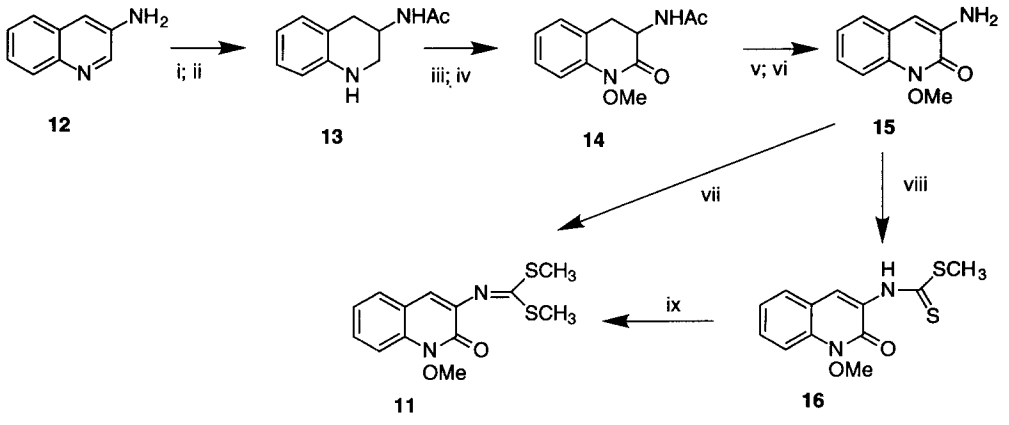


Figure 1. Proposed structures of wasabi yellow metabolite and selected ^{13}C-H long-range correlation observed in the HMBC spectrum.

Analysis of HMQC and HMBC data of the yellow compound established several C-H correlations reminiscent of an indole nucleus such as **9** or **10** (Figure 1); however, structure **9** corresponded to methoxybrassenin B, a phytoalexin previously isolated from *B. oleracea*,[9] whose NMR data did not correspond to those obtained for the yellow wasabi metabolite. Furthermore, the C-H correlation observed on the HMBC spectrum was thought more likely to correspond to a three-bond correlation ($^3J_{CH}$) than to a four-bond correlation ($^4J_{CH}$) and thus structure **11** appeared more plausible than **10**.[10] To prove the proposed structure, synthesis of the methoxyquinolone derivative **11** was undertaken.

The synthesis of compound **11** was achieved in seven (or eight) steps from 3-aminoquinoline (**12**), following the route shown in Scheme 1. After protection of the amino group of 3-aminoquinoline (**12**), the pyridine ring was reduced using sodium cyanoborohydride[11] to yield tetrahydroquinoline **13**, which upon treatment with hydrogen peroxide employing sodium tungstate as a catalyst,[12] followed by methylation with diazomethane yielded methyl hydroxamate **14**. Methyl hydroxamate **14** was then dehydrogenated with DDQ, hydrolyzed to the free amine **15**, which was transformed into the desired quinoline derivative **11** upon treatment with carbon disulfide followed by methyl iodide. The spectroscopic data of compound **11**, an almost white compound, were not identical to those of the bright yellow wasabi metabolite.[13]

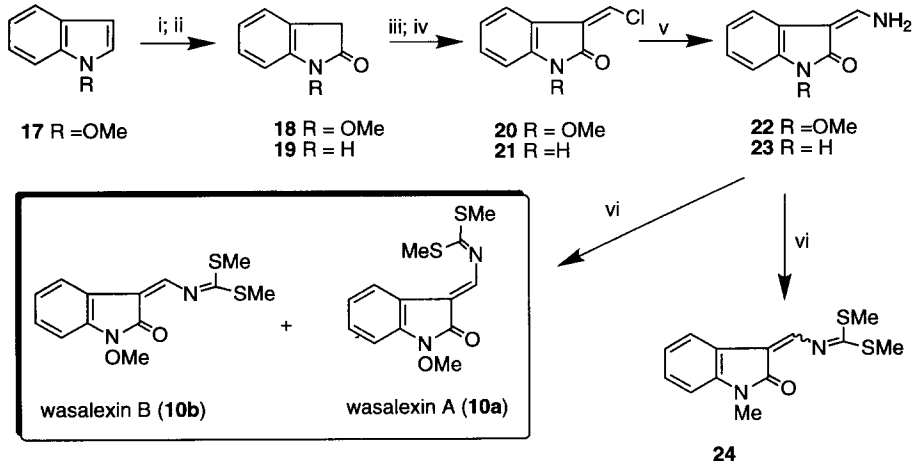
Scheme 1



(i) $(Ac)_2O$/py, 92%; (ii) $NaCNBH_3$/AcOH, 91%; (iii) $Na_2WO_4 \cdot 2H_2O/H_2O_2$; (iv) CH_2N_2, 62%; (v) DDQ, 100°C, 14%; (vi) HCl/MeOH, 80%; (vii) NaH, CS_2 ; CH_3I, 30%; (viii) CS_2, Et_3N; CH_3I, 24%; (ix) NaH, CH_3I, 72%

Next, the synthesis of compound **10** was sought; however, the synthesis of analog **24** was carried out first, since the required starting material 2-oxindole (**19**) was commercially available. Synthesis of **24** was carried out as summarized in Scheme 2; thus, **19** was allowed to react with ethyl formate,[14] the resulting enol transformed into enamine **23** via 3-chloromethyleneoxindole (**21**),[15] which in turn yielded desired product **24** upon treatment with carbon disulfide and methyl iodide.

Scheme 2



(i) NBS/t-BuOH; (ii) Zn/AcOH, 60%; (iii) HCO_2Et/NaOEt, R = OMe 87%, R = H 81%; (iv) $SOCl_2$, R = OMe = H 92 %; (v) NH_4OH, R = OMe 94%, R = H 55%; (vi) THF/NaH/CS_2; MeI, R = OMe 53%, R = H 24%.

Compound **24** had a bright-yellow color and spectroscopic data similar to those of the wasabi metabolite.[16] Consequently, compound **10** was then prepared; methoxyindole **17**[5] was converted to the desired starting material 1-methoxy-2-oxindole (**18**), upon bromination with NBS followed by reduction.[17] Synthesis of **10** from **18** followed a route similar to that described for **24**, as shown in Scheme 2. While compound **24** was obtained as a single isomer according to NMR and HPLC data, **10** was obtained as a mixture of *E/Z* isomers (1:2), which could be separated by reversed-phase TLC.[18] The spectroscopic data and HPLC retention time of the minor synthetic isomer **10** were identical to those of the natural yellow metabolite which we named wasalexin A. The spectroscopic and HPLC data obtained for the major synthetic isomer were identical to a minor compound isolated together with wasalexin A, which was named wasalexin B.[18]

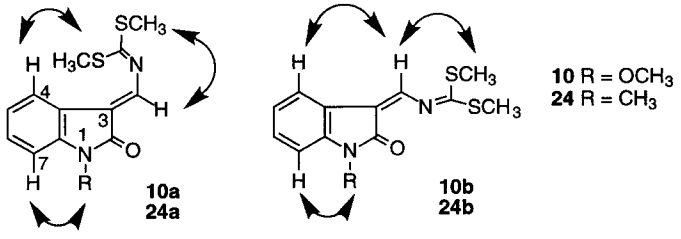


Figure 2. Selected NOE correlations observed for compounds **10a** and **24a**.

To complete the structure elucidation of wasalexins, assignment of the configuration of the exocyclic double bond was required. Thus, NOE experiments with wasalexin A and the analog **24a** were carried out; as expected, irradiation of the (O)CH_3 and CH_3 groups of **10a** and **24a**, respectively, caused an NOE enhancement of H-7, while irradiation of H-4 caused an enhancement of the (S)CH_3 signals and vice-versa. Conversely, no enhancements were observed for H-1' upon irradiation of H-4 and vice-versa, as indicated in Figure 2. These results allowed the unambiguous assignment of the structures of wasalexin A as **10a**, the major compound produced by elicited wasabi leaves, and wasalexin B as **10b**.[19]

Finally, antifungal bioassays established that wasalexin A (**10a**), similar to phytoalexin **8**, was active against *P. lingam* utilizing a spore germination and growth assay, causing 40–50% germination inhibition relative to controls, after 24 h, whereas compound **24a** had no detectable inhibitory effect.[20] Interestingly, the spore germination of *P. wasabiae* was not affected significantly by wasalexin A, although the mycelial growth of cultures incubated with wasalexin A was slower. Further bioactivity studies are underway to better understand the role of these compounds in the resistance of wasabi to *P. lingam*, the major pathogen responsible for the blackleg disease of canola.

Acknowledgments: Support for the authors' work was obtained in part from: the Natural Sciences and Engineering Research Council of Canada and the University of Saskatchewan. We would like to thank G. Séguin-Swartz, Agriculture and Agri-Food Canada, Saskatoon Research Station, SK, for a generous gift of wasabi plants, M. Takasugi, Hokkaido University, Sapporo, for providing spectra for brassenin B, Soongnyeong Lee, Department of Chemistry, University of Saskatchewan, for technical assistance in antifungal activity determinations, and K. Brown for 2D-NMR acquisitions.

References and Notes

1. Brooks, C. J.; Watson, D. G. *Nat. Prod. Rep.* **1985**, 427, and references therein.
2. (a) Dixon, R. A. *Biol. Rev.* **1986**, *61*, 239; (b) Dixon, R. A.; Harrison, M. J.; Lamb, C. J. *Annual Rev. Plant Phytopathol.* **1994**, *32*, 479; (c) Kúc, J. *Annual Rev. Phytopathol.* **1995**, *33*, 275; (d) Smith, C. J. *New Phytol.* **1996**, *132*, 1.
3. For a recent review on brassica phytoalexins: Pedras, M. S. C.; Khan, A. Q.; Taylor, J. L. In *Phytochemicals for Pest Control*; Hedin, P. A.; Hollingworth, R. M.; Masler, E. P.; Miyamoto, J.; Thompson, D. G. Eds.; ACS Symposium Series 658, 1997; pp 155-166.
4. (a) Pedras, M. S. C.; Taylor, J. L.; Morales, V. M. *Phytochemistry* **1995**, *37*, 1215; (b) Taylor, J. L.; Pedras, M. S. C.; Morales, V. M. *Trends in Microbiology* **1995**, *3*, 202.
5. Pedras, M. S. C.; Sorensen, J. L., *Phytochemistry* **1998**, *49*, 1959.
6. HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode-array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 id × 200 mm), equipped with a low-dispersion column-inlet filter. Mobile phase was a linear gradient from 75% H_2O - 25% CH_3CN to 100% CH_3CN, at a flow rate 1.0 mL/min over 35 min.
7. Conn, K. L.; Browne, L. M.; Tewari, J. P.; Ayer, W. A. *J. Plant Biochem. Biotech.* **1994**, *3*, 125.
8. Spectroscopic data of yellow metabolite (**10a**): HPLC r_T = 23.8 min; ^1H NMR (CD_2Cl_2): δ 2.70 (s, 6H), 4.01 (s, 3H), 6.96 (d, *J* = 8 Hz, 1H), 7.04 (dd, *J* = 8, 7 Hz, 1H), 7.27 (dd, *J* = 8, 7 Hz, 1H), 7.86, (d, *J* = 7 Hz, 1H), 8.03 (s, 1H); ^{13}C NMR (CD_2Cl_2): δ 16.0 (2 × q), 64.2 (q), 107.1 (d), 116.8 (s), 119.6 (s), 122.9 (d), 125.0 (d), 128.7 (d), 139.0 (s), 139.1 (d), 164.8 (s), 177.5 (s); HREIMS *m/z* measured: 294.0499 (294.0497 calcd. for $C_{13}H_{14}N_2O_2S_2$); EIMS *m/z* (% relative abundance): 294 $[M]^+$ (100), 263 (30), 247 (35), 216 (49), 143 (60); CIMS (NH_3) *m/z* (% relative abundance): 295 $[M+1]^+$ (97), 265 (100), 163 (34), 146 (83), 122 (38); FTIR ν_{max}: 2924, 1708, 1625, 1481, 1095, 743 cm^{-1}.
9. Monde, K., Sasaki, K., Shirata, A. and Takasugi, M., *Phytochemistry* **1991**, *30*, 3921.
10. In the standard HMBC experiment a coupling over four bonds is usually not observed (Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093). Although long range couplings over four, or even five bonds can be detected by HMBC, either special modifications to the routine pulse sequence, (Furihata, K.; Seto, H. *Tetrahedron Lett.* **1995**, *36*, 2817) or newer pulsed field gradients (Wagner, R.; Berger, S. *Magn. Reson. Chem.* **1998**, *36*, S44) are required.
11. Gribble, G. W.; Heald, P. W. *Synthesis* **1975**, 650.
12. Murahashi, S.; Oda, T.; Sugahara, T.; Masui, Y. *J. Org. Chem.* **1990**, *55*, 1744.
13. Spectroscopic data of **11**: ^1H NMR ($CDCl_3$): δ 2.57 (s, 6H), 4.13 (s, 3H), 7.14 (s, 1H), 7.25 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.47 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.48 (d, *J* =7.5 Hz, 1H), 7.53 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 15.5 (2 × q), 63.0 (q), 111.8 (d), 119.5 (s), 123.1 (d), 123.2 (d), 127.7 (d), 129.2 (d), 135.7 (s), 141.0 (s), 153.6 (s), 168.4 (s); HREIMS *m/z* measured: 294.0506 (294.0497 calcd. for $C_{13}H_{14}N_2O_2S_2$); EIMS *m/z* (% relative abundance): 294 $[M]^+$ (45), 247 (100), 183 (15), 173 (32); FTIR ν_{max}: 2925, 1659, 1598, 1566, 1216, 753 cm^{-1}.
14. Wenkert, E.; Bhattacharyya, N. K.; Reid, T. L.; Stevens, T. E. *J. Am. Chem. Soc.* **1956**, *78*, 797.
15. Behringer, H.; Wessauer, H. *Chem. Ber.* **1952**, *85*, 743.
16. Spectroscopic data of **24a**: ^1H NMR ($CDCl_3$): δ 2.69 (s, 6H), 3.25 (s, 3H), 6.78 (d, *J* = 8 Hz, 1H), 7.00 (dd, *J* = 8, 7 Hz, 1H), 7.22 (dd, *J* = 8, 7 Hz, 1H), 7.84, (d, *J* = 7 Hz, 1H), 8.08 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 15.9 (2 × q), 26.1 (q), 107.7 (d), 116.8 (s), 119.2 (s), 122.0 (d), 124.5 (d), 128.3 (d), 138.5 (s), 142.8 (d), 169.5 (s), 175.4 (s); HREIMS *m/z* measured: 278.0549 (278.0548 calcd. for

$C_{13}H_{14}N_2OS_2$); EIMS *m/z* (% relative abundance): 278 $[M]^+$ (42), 231 (100), 216 (53), 1158 (24); CIMS (NH_3) *m/z* (% relative abundance): 279 $[M+1]^+$ (10), 162 (100), 148 (37); FTIR ν_{max}: 2921, 1693, 1608, 1484, 1363, 1331, 929, 746 cm^{-1}.

17. Kawasaki, T.; Kodama, A.; Noshida, T.; Shimizu, K.; Somei, M. *Heterocycles* **1991**, *32*, 221.
18. Preparative TLC: RP-8 plates, (20 × 20 cm, 0.25 mm; EM Science, NJ) acetonitrile:water (7:3). It is worthy to note that wasalexin A (**10a**) appeared to be stable in CH_2Cl_2 but a small amount of isomerization to **10b** occurred during NMR acquisitions in $CDCl_3$; however, **10b** could not be obtained as a single compound due to isomerization to **10a** (<10%) during purification.
19. It is possible that wasalexin B (**10b**) is an artifact of the isolation process; however, considering that it was present in preliminary fractionation of leaf hexane extracts, and that wasalexin A also is isomerized, it appears more likely that both isomers are naturally occurring. Spectroscopic data of wasalexin B (**10b**): HPLC r_T = 21.5 min; ^1H NMR (CD_2Cl_2): δ 2.69 (s, 6H), 3.99 (s, 3H), 6.96 (d, *J* = 8 Hz, 1H), 7.04 (dd, *J* = 8, 7 Hz, 1H), 7.27 (dd, *J* = 8, 7 Hz, 1H), 7.46, (d, *J* = 7 Hz, 1H), 7.91 (s, 1H); ^{13}C NMR (CD_2Cl_2): δ 15.8 (2 × q), 63.9 (q), 107.2 (d), 114.3 (s), 119.4 (s), 120.2 (d), 122.0 (d), 128.2 (d), 137.9 (d), 139.0 (s), 161.7 (s), 173.7 (s); HREIMS *m/z* measured: 294.0497 (294.0497 calcd. for $C_{13}H_{14}N_2O_2S_2$).
20. Bioassays to determine pycnidiospores germination were carried out after modification of a method previously reported (Pedras, M. S. C.; Biesenthal, C. J. *Can. J. Microbiol.* **1998**, *44*, 547). That is, Nunclon 4-well multidish plates containing 1×10^7 spores / mL in 500 μL solutions of compound to be tested (3% Tween 80 and 2% DMSO in minimal medium) were incubated still under constant fluorescent light, at 24 ± 1° C. The percentage of spore germination was determined by counting about 300 spores (in randomly selected fields) for each replicate.