DEHYDRO-4-METHOXYCYCLOBRASSININ, A SULFUR-CONTAINING PHYTOALEXIN ISOLATED FROM TURNIP *BRASSICA CAMPESTRIS* L. SSP. *RAPA*[1]

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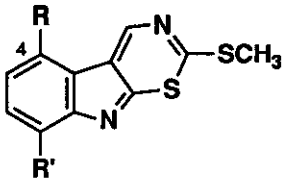
Abstract ——— Isolation, structure elucidation, and synthesis of dehydro-4-methoxycyclobrassinin (**1**), a turnip phytoalexin possessing a novel heterocyclic system, are described.

Phytoalexins are low molecular weight antimicrobial compounds synthesized *de novo* by plants after their exposure to micro-organisms.[2] These abnormal secondary metabolites are not usually detected in healthy plant tissues and their accumulation is considered to be one of the important disease resistance mechanisms.[3]

In a continuation of our studies on vegetable phytoalexins,[4] we have reported several indolic phytoalexins from crucifers such as Chinese cabbage,[5] cabbage,[6] and radish.[7] These studies indicated that crucifers are a rich source of novel bioactive heterocyclic compounds.

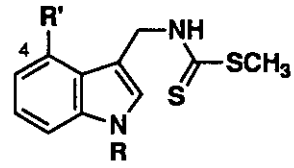
Recently, we have reported a high performance liquid chromatographic analysis (hplc)[8] of a standard mixture consisting of known cruciferous phytoalexins and related compounds. Using this method, an extract from the turnip roots *Brassica campestris* L. ssp. *rapa* inoculated with the bacterium *Pseudomonas cichorii* was examined. The hplc analysis of the extract indicated the presence of known phytoalexins: brassinin (**2**),[5] methoxybrassinin (**3**),[5] 4-methoxybrassinin (**4**),[6] cyclobrassinin (**5**),[5] and spirobrassinin (**6**).[7] In addition to these compounds, the hplc chromatogram showed several unidentified peaks not detected in the standard phytoalexin mixture or in a healthy turnip extract. These unidentified compounds were followed by the hplc analysis and tlc bioassay[5] using *Bipolaris leersiae* as the test fungus. We wish to report here the isolation, structure elucidation, and synthesis of a novel turnip phytoalexin (**1**), which was named dehydro-4-methoxycyclobrassinin.



1: R=OCH_3, R'=H

1': R=H, R'=OCH_3

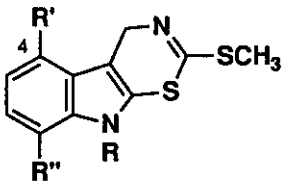
7: R=R'=H



2: R=R'=H

3: R=OCH_3, R'=H

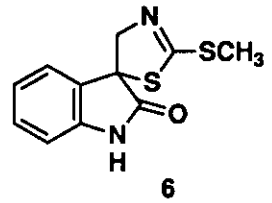
4: R=H, R'=OCH_3



5: R=R'=R''=H

5': R=OCH_3, R'=R''=H

8: R=R''=H, R'=OCH_3



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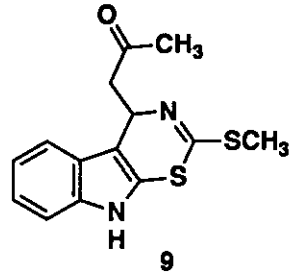

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Sixty turnip roots were sliced (2 mm thick) and aged at 20 °C for 1 day, and then inoculated with *P. cichorii*. After being incubated for 3 days at 20 °C, the tissue (5.4 kg) was frozen in liquid nitrogen, homogenized in ethyl acetate, and then centrifuged. The combined ethyl acetate layer was evaporated and the extract was separated successively by chromatography on silica gel (EtOAc), Sephadex LH-20 (MeOH), silica gel (5% MeOH in CH_2Cl_2), silica gel (benzene-EtOAc), and finally by preparative hplc[8] to yield an antifungal compound (**1**) (2.6 mg). The antifungal activity against *B. leersiae* was moderate and required a concentration of 350 ppm for 50% inhibition of the conidial germination.

High resolution mass spectrum and ^1H nmr spectral data ($CDCl_3$) of the compound (**1**) established its molecular formula as $C_{12}H_{10}N_2OS_2$. The ^1H nmr spectrum showed signals due to one methylthio (δ 2.80, 3H, s) and one methoxy (δ 4.08, 3H, s) groups, and one proton singlet signal at δ 9.00, which did not disappear on D_2O treatment. Irradiation of the methoxy protons resulted in signal enhancement of an aromatic proton at δ 6.86 (d, J = 8 Hz). Consecutive spin decoupling experiments assigned the sequence of the other two adjacent protons (δ 7.51, dd, J = 8 and 8 Hz; 7.41, d, J = 8 Hz).[9] Nine degrees of unsaturation calculated from the molecular formula and the uv-vis spectrum (λ_{max} 270 and 400 nm) suggested the presence of a highly conjugated system in **1**. Dehydrogenation of cyclobrassinin (**5**) with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) gave an unstable dehydrogenated product (**7**), $C_{11}H_8N_2S_2$ (m/z 232.0133, M^+) in 17% yield, which showed a low field one proton singlet signal at δ 8.76 and a similar uv-vis spectrum[10] to that of **1**. Co-occurrence of **1** with 4-methoxybrassinin (**4**), cyclobrassinin (**5**), and 4-methoxy-3-formylindole in the turnip extract suggested that the compound (**1**) is dehydro-4-methoxycyclobrassinin rather than dehydro-7-methoxycyclobrassin (**1'**). Since no NOE was observed between the methoxy and the low field proton at δ 9.00 in **1**, the structure was unambiguously determined by the following synthesis.

Treatment of 4-methoxybrassinin (**4**)[6,11] in dichloromethane with *N*-bromosuccinimide (NBS) at 21 °C for 20 min, followed by dehydrobromination with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) afforded unstable 4-methoxycyclobrassinin (**8**), mp. 146-147 °C (decomp.), $C_{12}H_{12}N_2OS_2$,[12] in 20% yield. This compound has not been isolated as a natural product to date. Refluxing of the benzene solution of **8** with DDQ for 1 h gave a dehydrogenated product, mp. 142-143 °C, $C_{12}H_{10}N_2OS_2$,[13] in 34% yield, which was identified as the compound (**1**) by direct hplc comparison

and by comparing their spectral data. Dehydro-4-methoxycyclobrassinin (**1**) and dehydrocyclobrassinin (**7**) have a common novel heterocyclic system and wait further investigation. Previous isolation[5b] of a cyclobrassinin with a 2-oxopropyl substituent (**9**) was explained as an artifact formed during acetone extraction of the inoculated Chinese cabbage since the compound (**9**) showed no optical activity. If dehydrocyclobrassinin (**7**) was present in the inoculated Chinese cabbage, nucleophilic addition of acetone to **7** would yield the compound (**9**). Instability of the synthesized compound (**7**) in protic solvent supports this view. Formation of dehydrocyclobrassinin (**7**) could be explained by either enzymatic dehydrogenation of cyclobrassinin (**5**) or by elimination of methanol from unknown *N*-methoxycyclobrassinin (**5'**) since methoxybrassinin (**3**) and cyclobrassinin (**5**) are main phytoalexins of *Brassica* species.[5,6,14]

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9. Additional data of **1**. Fdms m/z: 262. Hrms: m/z 262.0248. Calcd for $C_{12}H_{10}N_2OS_2$, 262.0234. Ms m/z (rel intensity): 262 (100, M^+) 247 (12), 229 (26), 219 (22), 159 (22), 146 (28). Ir ($CHCl_3$) ν_{max}: 3000, 2928, 2838, 1613, 1590, 1480, 1450, 1399, 1329, 1267, 1085 cm^{-1}. ^{13}C Nmr ($CDCl_3$) δ: 15.1 (-SMe), 55.6 (-OMe), 104.6, 112.2, 130.2, 140.7, 156.5. Other carbon signals were not detected due to low concentration.

10. Additional data of **7**. Ms m/z (rel intensity): 232 (100, M^+), 159 (81). Uv ($CHCl_3$) λ_{max}: 269 (13000) , 385 nm (7300). ^1H Nmr ($CDCl_3$) δ : 2.80 (3H, s -SMe), 7.35 (1H, ddd, J = 7, 7, and 2 Hz), 7.56 (1H, ddd, J = 7, 7, and 2 Hz), 7.77 (1H, ddd, J = 7, 2, and 1 Hz), 8.00 (1H, ddd, J = 7, 7, and 1 Hz). ^{13}C Nmr ($CDCl_3$) δ : 15.2 (-SMe), 119.1, 120.5, 123.2, 129.4.

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12. Additional data of **8**. Ms m/z (rel intensity): 264 (23, M^+), 191 (100), 161 (13). Ir (KBr) ν_{max}: 3160, 1605, 1590, 1442, 1427, 1357, 1254, 1097, 925, 771, 733 cm^{-1}. ^1H-Nmr ($CDCl_3$) δ : 2.54 (3H, s), 3.91 (3H, s), 5.28 (2H, s), 6.51 (1H, d, J = 7.7 Hz), 6.91 (1H, d, J = 7.7 Hz), 7.05 (1H, t, J = 7.7 Hz), 7.89 (1H, br s, NH). *Anal.* Calcd for $C_{12}H_{12}N_2OS_2$: C, 54.52; H, 4.58; N,10.60. Found: C, 54.62; H, 4.53; N, 10.56.

13. Additional data of synthetic **1**. Ir (KBr) ν_{max} : 3000, 2920, 1592, 1474, 1449, 1401, 1326, 1263, 1082 cm^{-1} Uv ($CHCl_3$) λ_{max}: 270 (18700), 400 nm (11600). *Anal.* Calcd for $C_{12}H_{10}N_2OS_2$: C, 54.94; H, 3.84; N, 10.68. Found: C,54.72; H, 3.80; N, 10.44.

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