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# Phytoalexins from the Crucifer Rutabaga: Structures, Syntheses, Biosyntheses, and Antifungal Activity

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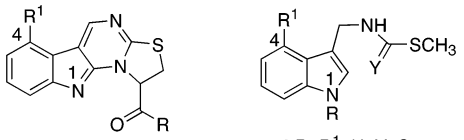
Phytoalexins are inducible chemical defenses produced de novo by plants in response to diverse forms of stress, including microbial attack. Our search for phytoalexins from economically important crucifers lead us to examine rutabaga tubers (*Brassica napus* L. ssp. *rapifera*). Three new phytoalexins, named isalexin (**9**), brassicanate A (**10**), and rutalexin (**11**), were isolated together with five known phytoalexins, brassinin (**4**), 1-methoxybrassinin (**5**), spirobrassinin (**13**), brassicanal A (**14**), and brassilexin (**15**). The chemical structures of the new phytoalexins were proven by syntheses, and their biological activity against four plant pathogens were determined. Biosynthetic studies using tetra- and pentadeuterated precursors established that indolyl-3-acetaldoxime (**22**) and brassinin (**4**) are precursors of brassicanate A (**10**) and rutalexin (**11**) and that cyclobrassinin (**23**) is a biosynthetic precursor of rutalexin (**11**), whereas tryptamine (**24**) is not a precursor of rutabaga phytoalexins.

## Introduction

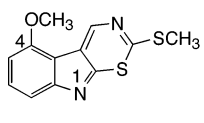
Although the metabolites produced by rutabaga (*Brassica napus* L. ssp. *rapifera*) tubers include a variety of biologically active compounds such as carotenoids,[1] glucosinolates,[2] and fluorenes,[3] no phytoalexins have been reported to date. Phytoalexins are essential secondary metabolites produced de novo by plants in response to diverse forms of stress, including microbial attack.[4] The fluorenes identified recently[3] in rutabaga tubers are oviposition stimulants of the cabbage root fly,[5] the so-called CIFs (cabbage identification factors): CIF1 (**1**) and its glycine conjugate CIF2 (**2**), and CIF3 (**3**).[3,5] Structural similarities between CIFs (**1**−**3**) and cruciferous phytoalexins such as brassinins **4**−**6**, brassitin (**7**), and 4-methoxydehydrocyclobrassinin (**8**) suggest common biogenetic precursors. Interestingly, some cruciferous phytoalexins (**4**, **5**, and **7**) are also active as oviposition stimulants for the cabbage root fly, albeit far less active than CIFs.[6] Crucifers (family Cruciferae or Brassicaceae) comprise a number of economically important oilseed and condiment crops such as canola (*Brassica napus* and *Brassica rapa*), rapeseed (*B. napus* and *B. rapa*), and mustards (*Brassica juncea*, *Brassica carinata*, and *Sinapis alba*) and many vegetables including rutabaga, turnip (*B. rapa*), and cabbages (*Brassica oleracea*). In addition, the wild crucifer *Arabidopsis thaliana* is an important model plant and the first flowering plant to have its genome sequenced.[7] Our ongoing search for phytoalexins from economically important brassicas[8] as well as wild crucifers[9] led us to examine rutabaga tubers. We report here the isolation, structure determination, synthesis, biosynthesis, and antifungal activity of three new phytoalexins



**1** R=OH; $R^1$=H
**2** R=NHCH$_2$COOH; $R^1$=H
**3** R=OH; $R^1$=OCH$_3$



**4** R=$R^1$=H; Y=S
**5** R=OCH$_3$; $R^1$=H; Y=S
**6** R=H; $R^1$=OCH$_3$; Y=S
**7** R=$R^1$=H; Y=O



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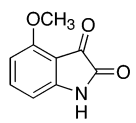

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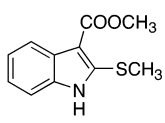

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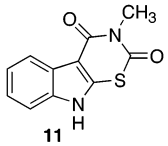
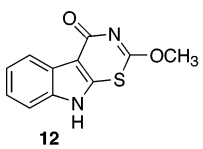


**11**



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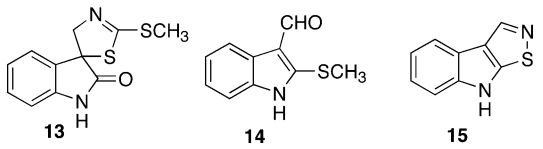
from rutabaga tubers (*B. napus* ssp. *rapifera*) which we named isalexin (**9**), brassicanate A (**10**), and rutalexin (**11**).

## Results and Discussion

**Phytoalexin Elicitation, Isolation, and Structure Determination.** To establish the production of inducible metabolites, the response of rutabaga (*B. napus* ssp. *rapifera)* tubers to abiotic elicitation (UV irradiation) was analyzed over a period of time. After elicitation and incubation of freshly cut rutabaga slices, the aqueous solutions contained in wells were collected every 24 h up to 120 h. Extraction of the aqueous solutions with EtOAc and of the tissues with MeOH was followed by HPLC analysis of the extracts (photodiode array detection); control roots were treated in a similar manner but without UV irradiation. The HPLC chromatograms of the various extracts showed that no elicited components were detected in either aqueous or methanolic extracts, whereas several components were detected in the EtOAc extracts of irradiated tubers. Comparison of the HPLC retention times ($t_R$) and UV spectra of these components with those of known compounds available in our library indicated the presence of three unknown components, with $t_R$ 3.7, 13.3, and 14.9 min, in the extracts of irradiated slices. Additional experiments to analyze the response of *B. napus* ssp. *rapifera* to biotic elicitation were carried out using the phytopathogenic fungus *Rhizoctonia solani* Kuhn. Similar to experiments using abiotic elicitation, extraction of the aqueous solutions with EtOAc was followed by HPLC analysis of the extracts (photodiode array detection). Comparison of the HPLC chromatograms of the various extracts showed the presence of elicited components similar to those obtained by UV irradiation, namely three unknown components with $t_R$ 3.7, 13.3, and 14.9 min. The elicited components were present in lower amounts in tissues elicited with *R. solani* than in UV-irradiated tissues. Subsequently, large- scale experiments using UV-irradiated rutabaga tubers (13 kg) were used to isolate the unknown components. As expected, the HPLC chromatograms of EtOAc extracts obtained from these large-scale experiments showed the presence of known phytoalexins and three unknown metabolites. The EtOAc extracts were combined and separated by FCC (reversed-phase C-18) and the fractions analyzed by HPLC. The fractions containing the unknown metabolites were pooled together and further separated by a combination of preparative TLC on silica gel and C-18 reversed-phase to afford metabolites **9** (1.3 mg), **10** (1 mg), and **11** (1 mg), as well as the known phytoalexins brassinin (**4**, 0.5 mg), 1-methoxybrassinin (**5**, 1 mg), spirobrassinin (**13**, 6.4 mg), brassicanal A (**14**, 1.3 mg), and brassilexin (**15**, 1.3 mg). The phytoalexins **5** and **13**−**15** were identified by comparison with synthetic samples available in our library.




**13** **14** **15**

The HRMS spectrum of the compound with $t_R$ at 3.7 min (**9**) indicated the molecular formula of $C_9H_7NO_3$. The NMR spectra of this compound displayed aromatic signals plus a methoxy group ($\delta_H$ 3.91 and $\delta_C$ 57.2). Analysis of the $^1H$ and $^{13}C$ NMR data suggested the presence of a 2,3-dioxoindole system with a methoxy group located at either C-4 or C-7. The structure of this metabolite was finally confirmed to be 4-methoxyisatin (**9**) by synthesis.[10] This appears to be the first time that 4-methoxyisatin is reported as a naturally occurring compound; this new metabolite was named isalexin (**9**).

The HRMS spectrum of the compound with HPLC $t_R$ at 14.9 min (**10**) indicated a molecular formula of $C_{11}H_{11}NO_2S$. The NMR spectra displayed aromatic signals attributable to an indole moiety, a signal due to a methoxy group ($\delta_H$ 3.98 and $\delta_C$ 51.3), and another one likely due to a thiomethyl group ($\delta_H$ 2.64 and $\delta_C$ 15.0). The structure of this metabolite was deduced to be methyl 2-methylthioindole-3-carboxylate (**10**) and was confirmed by synthesis, as described below. Although this compound was obtained previously as a side product in the reaction of sodium thiomethoxide with methyl 1-methoxyindole-3-carboxylate,[11] this is the first report of its isolation from natural sources. Due to its structural resemblance with brassicanal A (**14**) we named this metabolite brassicanate A (**10**).

The HRMS spectrum of the compound with $t_R$ at 13.3 min (**11**) indicated the molecular formula of $C_{11}H_8N_2O_2S$. The $^1H$ NMR spectrum (in DMSO-$d_6$) displayed four signals in the aromatic area, a $D_2O$ exchangeable proton and a methyl singlet at $\delta_H$ 3.37 ppm. The $^1H$ NMR spectroscopic data were similar to those reported for cyclobrassinone (**12**), a known phytoalexin isolated previously from UV-irradiated tubers of kohlrabi (*B. oleracea* var. *gongylodes*).[12] However, the $^{13}C$ NMR data of our metabolite **11** suggested that its methyl group was located at a nitrogen ($\delta_C$ 28.2 ppm) rather than at an oxygen, as in cyclobrassinone (**12**, $\delta_H$ 4.17 and $\delta_C$ 57.5).[13] That is, the compound we isolated with $t_R$ at 13.3 min (**11**) was not cyclobrassinone (**12**) but its N-Me isomer **11**. Subsequently, we investigated the phytoalexins produced in kohlrabi (*B. oleracea* var. *gongylodes*) in an attempt to isolate cyclobrassinone (**12**); we isolated and identified isalexin (**9**), brassicanate A (**10**), and metabolite **11**, together with several known phytoalexins, but have not isolated or detected cyclobrassinone (**12**).[14] To assign the structures unambiguously, both **11** and cyclobrassinone (**12**)[13] were synthesized and their $^1H$ and $^{13}C$ NMR

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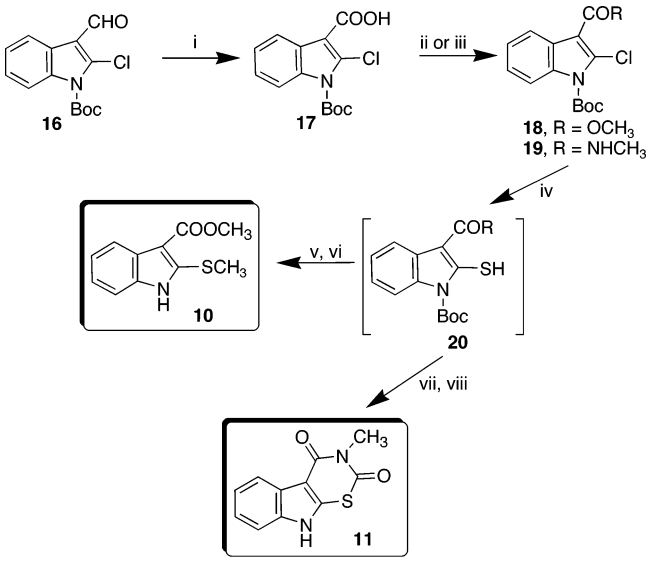
(14) A personal communication with D. Gross informed us that the natural product isolated from kohlrabi was not available.

spectra obtained under similar conditions.[15] The data obtained for synthetic **11** was identical in all respects to the natural product, including the methyl singlet at $\delta_H$ 3.37 ppm ($\delta_C$ 28.2).[16] As expected, the resonance of the methyl group of synthetic cyclobrassinone (**12**) at $\delta_H$ 4.17 ppm ($\delta_C$ 57.5) was consistent with its location at the oxygen. These results indicate that the structure of the natural product first isolated from kohlrabi and named cyclobrassinone (**12**) is identical to **11** and that the reported cyclobrassinone is not a natural product. Although later on cyclobrassinone (**12**) was synthesized by Kutchy's group, the authors[13] did not have the natural product or $^{13}C$ NMR data to establish unambiguously the identity of the natural product. Metabolite **11**, which we named rutalexin, is produced by both *B. napus* ssp. *rapifera* and *B. oleracea* var. *gongylodes*.

**Phytoalexin Synthesis.** The syntheses of metabolites **9**−**11** were essential to prove their structures as well as to provide sufficient quantities of these compounds to determine their antifungal activities. Isalexin (**9**) was prepared by directed *o*-lithiation of *N*-Boc-*m*-anisidine with *n*-BuLi, as previously reported.[10] In addition, we developed a concise route to synthesize brassicanate A (**10**) and rutalexin (**11**), using as key step the well-known[13] nucleophilic substitution reaction of 2-chloroindole derivatives with sulfides. Thus, the methyl carboxylate **18**, obtained from $NaClO_2$-mediated oxidation[11] of aldehyde **16**[13] followed by methylation with $CH_2N_2$, was allowed to react with NaSH in aqueous MeOH to yield intermediate **20** (R = $OCH_3$). After methylation ($K_2CO_3$/MeI) followed by deprotection using TFA, brassicanate A (**10**) was obtained in 51% overall yield (Scheme 1). To prepare rutalexin (**11**), Boc-2-chloroindole-3-carboxylic acid (**17**) was converted into the corresponding amide **19** upon treatment with thionyl chloride in dry THF followed by addition of methanamine. The carboxamide **19** was allowed to react with NaSH in DMF/$H_2O$ to yield intermediate **20** (R = $NHCH_3$) after extraction and concentration of the reaction mixture. The crude product **20** was allowed to react with NaH followed by phosgene,[17] and the resulting product was deprotected[13] to yield rutalexin (**11**) in 24% overall yield (Scheme 1). The spectroscopic data of synthetic compounds **10** and **11** were identical in all respects to those of the natural products brassicanate A and rutalexin, respectively, isolated from rutabaga and kohlrabi tubers.

**Antifungal Activity.** To establish if metabolites **9**−**11** were phytoalexins, antifungal bioassays were carried out against three of the major cruciferous fungal pathogens: *Phoma lingam* (Tode ex Fr.) Desm. (perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.), *Sclerotinia sclerotiorum* de Bary, and *Rhizoctonia solani* Khun, as shown in Table 1. Because neither rutalexin (**11**) nor cyclobrassinone (**12**) was sufficiently soluble in culture media, a TLC bioassay using *Cladosporium cucumerinum* was also used.[18] Brassicanate A (**10**) was the most inhibitory of the compounds shown in Table 1, displaying complete inhibition of the three fungal species at $5 \times 10^{-4}$ M. In general, brassicanate A (**10**) is a stronger mycelial growth inhibitor against *R. solani* and *S. sclerotiorum* than brassicanal A (**14**), but both compounds displayed similar inhibitory effect against *P. lingam*. Furthermore, brassicanate A (**10**) was a stronger inhibitor than brassicanal A (**14**) against *C. cucumerinum*. Complete inhibition was observed at the minimal amount of $10^{-8}$ mol for brassicanate A (**10**) and $10^{-6}$ mol for brassicanal A (**14**). Isalexin (**9**) showed selective antifungal properties (Table 1) giving 48% of inhibition at $5 \times 10^{-4}$ M against *P. lingam* whereas no inhibitory activity was observed against *R. solani* and *S. sclerotiorum*. In addition, isalexin (**9**) showed complete inhibition against *C. cucumerinum* at a minimal amount of $10^{-6}$ mol, whereas rutalexin (**11**) and cyclobrassinone (**12**) showed antifungal activity at a minimal amount of $2 \times 10^{-6}$ mol.

**SCHEME 1. Syntheses of *Brassica napus* ssp. *Rapifera* (Rutabaga) Phytoalexins**[a]




[a] Reagents and conditions: (i) $NaClO_2$, 2-methylbut-2-ene, *tert*-butyl alcohol/$H_2O$, rt, 2 h, 96%; (ii) $CH_2N_2$, $Et_2O$, rt, 5 min, 100%; (iii) $SOCl_2$, THF, rt, 3 h, then $CH_3NH_2$, THF, 0 °C, 20 min, 85% (based on acid **17**); (iv) NaSH, MeOH/$H_2O$, rt, 1 h (synthesis of **10**) or NaSH, DMF/$H_2O$, 0 °C, 2 h (synthesis of **11**); (v) $CH_3I$, $K_2CO_3$, MeOH/$H_2O$, rt, 10 min, 66% (based on **18**); (vi) TFA, $CH_2Cl_2$, rt, 1 h, 80%; (vii) NaH, THF, 0 °C, 10 min then $COCl_2$ (20% in toluene), −78 °C to rt, 4 h, 30% (based on **19**); (viii) 165−170 °C, 30 min, 100%.

**Phytoalexin Biosynthesis.** A number of biosynthetic studies have demonstrated that *S*-tryptophan is the precursor of most cruciferous phytoalexins[8] via indolyl-3-acetaldehyde oxime[19] (**22**, Scheme 2). We have established previously that biosynthetic incorporation of intermediates/precursors into phytoalexins is more effective in turnip root tubers than in leaves or stems, likely due to faster intake of the solutions (i.e., no uptake of the substrate solution via the plant vascular system is

(15) Because both compounds **11** and **12** showed low solubility in $CDCl_3$ or $CD_2Cl_2$ but reasonable solubility in DMSO-$d_6$, complete NMR data were acquired in DMSO-$d_6$.

(16) $^1H$ NMR: rutalexin (**11**) (500 MHz, $CDCl_3$) $\delta_H$ 8.52 (br s, 1H, $D_2O$ exchangeable), 8.28 (m, 1H), 7.40 (m, 3H), 3.57 (s, 3H); cyclobrassinone (**12**) (500 MHz, $CDCl_3$) $\delta_H$ 8.70 (br s, 1H, $D_2O$ exchangeable), 8.48 (m, 1H), 7.39 (m, 3H), 4.22 (s, 3H); data published for **12** (ref 12) ($CDCl_3$) $\delta_H$ 8.56 (s, 1H) 8.31 (m, 1H) 7.41 (m, 1H) 7.37−7.32 (m, 2H) 3.55 (s, 3H).

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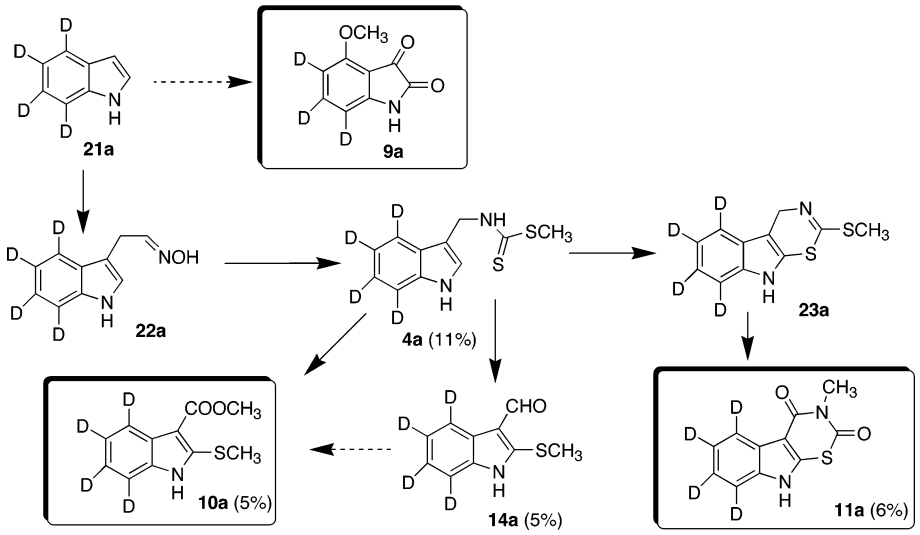
**TABLE 1. Antifungal Activity[a] of Brassicanate A (10), Brassicanal A (14), Isalexin (9) Rutalexin (11), and Cyclobrassinone (12) against Pathogens of Crucifers: *Phoma lingam* (Perfect Stage = *Leptosphaeria maculans*) (90 h Incubation), *Rhizoctonia solani* (72 h Incubation), *Sclerotinia sclerotiorum* (24 h Incubation), and *Cladosporium cucumerinum* (36 h Incubation)**

| compd | concn (M) | *R. solani* (%) | *S. sclerotiorum* (%) | *P. lingam* (%) | *C. cucumerinum* (mol) |
|---|---|---|---|---|---|
| brassicanal A (**14**) | $5.0 \times 10^{-4}$ | 28 ± 5 | 80 ± 3 | c.i.[b] | $10^{-6}$ |
| | $2.5 \times 10^{-4}$ | n.i.[c] | 37 ± 7 | 73 ± 9 | |
| | $1.0 \times 10^{-4}$ | n.i.[c] | n.i.[c] | n.i.[c] | |
| brassicanate A (**10**) | $5.0 \times 10^{-4}$ | c.i.[b] | c.i.[b] | c.i.[b] | $10^{-8}$ |
| | $2.5 \times 10^{-4}$ | 23 ± 6 | 81 ± 4 | 71 ± 5 | |
| | $1.0 \times 10^{-4}$ | n.i.[c] | 56 ± 3 | n.i. | |
| isalexin (**9**) | $5.0 \times 10^{-4}$ | n.i.[c] | n.i.[c] | 48 ± 6 | $10^{-6}$ |
| | $2.5 \times 10^{-4}$ | n.i.[c] | n.i.[c] | 35 ± 9 | |
| | $1.0 \times 10^{-4}$ | n.i.[c] | n.i.[c] | n.i.[c] | |
| rutalexin (**11**) | not soluble in medium | | | | $2.0 \times 10^{-6}$ |
| cyclobrassinone (**12**) | not soluble in medium | | | | $2.0 \times 10^{-6}$ |

[a] Percent of inhibition = 100 − [(growth on medium containing compound/growth on control medium) × 100)] ± standard deviation; incubation time (fungal growth on control medium) depends on each species. [b] c.i. = complete inhibition. [c] n.i. = no inhibition (growth on control medium and on medium containing compound is similar).

**SCHEME 2. Biosynthetic Relationship of Indolyl-3-acetaldoxime (22a) and Phytoalexins[a] Brassinin (4a), Isalexin (9a), Brassicanate A (10a), Rutalexin (11a), Brassicanal A (14a), and Cyclobrassinin (23a) in *Brassica napus* ssp. *Rapifera* (Rutabaga) (Dashed Arrows Indicate Hypothesis Not Demonstrated)**




[a] Percentages in brackets represent deuterium incorporation resulting from feeding experiments with indolyl-3-acetaldoxime (**22a**).

necessary).[19] Hence, rutabaga tubers were anticipated to be useful to establish biosynthetic relationships among the various phytoalexins. Toward this end, rutabaga tubers were incubated with tetradeuterated precursors **21a**−**23a** and **4a**,[20] which were synthesized as previously reported (percentage of deuterium >98%).[19] In addition, because tryptamine (**24**) was transformed to $N_b$-hydroxytryptamine in an Arabidopsis mutant (YUCCA),[21] and suggested to be a precursor of indolyl-3-acetaldoxime (**22**), we tested this hypothesis in rutabaga tubers. [2,4,5,6,7-$d_5$]-Tryptamine (**24a**) was synthesized from [2,4,5,6,7-$d_5$]-tryptophan as described in the Supporting Information and added to elicited rutabaga tubers.[19] After appropriate incubation periods, compounds were extracted and separated by multiple chromatography, and the resulting products were analyzed by HPLC-DAD, HRMS-EI, and $^1$H NMR.

Results of the incorporation of deuterium measured by HRMS are shown in Table 2 and summarized in Schemes 2 and 3. As expected,[19] [4,5,6,7-$d_4$]-indolyl-3-acetaldoxime (**22a**) was incorporated into all phytoalexins isolated from rutabaga, except for isalexin (**9**), which was detected but not isolated. By contrast, none of the phytoalexins isolated in the experiments using [2,4,5,6,7-$d_5$]-tryptamine (**24a**) contained deuterium (Table 2, Scheme 3); however, tryptophol (**25a**) was isolated containing a high level of deuterium (96%). Because tryptophol (**25**) is not usually present in rutabaga tubers, it is clear that it results from the biotransformation of $d_5$-tryptamine (**24a**) by rutabaga enzymes (tryptamine is stable in control solutions). Importantly, incorporation of [4,5,6,7-$d_4$]-brassinin (**4a**) into brassicanate A (**10a**) and brassicanal A (**14a**) demonstrates that **4a** is a precursor of both **10a** and **14a**. Similarly, incorporation of [4,5,6,7-$d_4$]-indolyl-3-acetaldoxime (**22a**), $d_4$-brassinin (**4a**), and [4,5,6,7-$d_4$]-cyclo-

(20) To prevent confusion, all deuterium-labeled compounds are numbered with the number corresponding to the nonlabeled compound followed by the letter **a**, e.g., brassinin (**4**) and [4,5,6,7-$d_4$]-brassinin (**4a**).

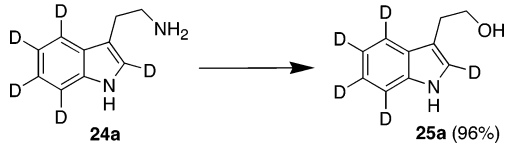
(21) Zhao, Y.; Christensen, S. K.; Fankhauser, C.; Cashman, J. R.; Cohen, J. D. *Science* **2001**, *291*, 306−309.

**TABLE 2. Metabolism of [4,5,6,7-$d_4$]-Brassinin (4a), [4,5,6,7-$d_4$]-Cyclobrassinin (23a), [4,5,6,7-$d_4$]-Indole (21a), [4,5,6,7-$d_4$]-Indolyl-3-acetaldoxime (22a), and [2,4,5,6,7-$d_5$]-Tryptamine (24a) in Rutabaga (*Brassica napus*) and Kohlrabi (*B. oleracea*) Tubers**

| precursor | plant, incubation time | isolated metabolites (total amount of *d*) |
|---|---|---|
| [4,5,6,7-$d_4$]-brassinin (**4a**) | rutabaga, 3 days | rutalexin (**11**) (20% of $d_4$-incorporation), brassicanate A (**10**) (37% of $d_4$-incorporation), spirobrassinin (**13**) (76% of $d_4$-incorporation) brassicanal A (**14**) (21% of $d_4$-incorporation) |
| [4,5,6,7-$d_4$]-brassinin (**4a**) | kohlrabi, 4 days | rutalexin (**11**) (48% of $d_4$-incorporation), spirobrassinin (**13**) (89% of $d_4$-incorporation) |
| [4,5,6,7-$d_4$]-cyclobrassinin (**23a**) | rutabaga, 3 days | rutalexin (**11**) (16% of $d_4$-incorporation) |
| [4,5,6,7-$d_4$]-indole (**21a**) | rutabaga, 3 days | rutalexin (**11**) (6% of $d_4$-incorporation), spirobrassinin (**13**) (5% of $d_4$-incorporation), brassicanal A (**14**) (5% of $d_4$-incorporation), brassinin (**4**) (6% of $d_4$-incorporation), 1-methoxybrassinin (**5**) (15% of $d_4$-incorporation), brassilexin (**15**) (2% of $d_4$-incorporation) |
| [4,5,6,7-$d_4$]-indolyl-3-acetaldoxime (**22a**) | rutabaga, 3 days | rutalexin (**11**) (6% of $d_4$-incorporation), brassicanate A (**10**) (5% of $d_4$-incorporation), spirobrassinin (**13**) (6% of $d_4$-incorporation), brassicanal A (**14**) (5% of $d_4$-incorporation), brassinin (**4**) (11% of $d_4$-incorporation), 1-methoxybrassinin (**5**) (11% of $d_4$-incorporation) |
| [4,5,6,7-$d_4$]-Indolyl-3-acetaldoxime (**22a**) | kohlrabi, 4 days | rutalexin (**11**) (16% of $d_4$-incorporation) |
| [2,4,5,6,7-$d_5$]-Tryptamine (**24a**) | rutabaga, 3 days | tryptophol (**25**) (96% of $d_5$-incorporation), brassinin (**4**) (no *d* incorporation), 1-methoxybrassinin (**5**) (no *d* incorporation), spirobrassinin (**13**) (no *d* incorporation), brassilexin (**15**) (no *d*incorporation) |

[a] The % of $d_n = [M + n]^+/[M]^+ + [M + n]^+ \times 100$, $n = 4$ or 5; HRMS data indicated that $[M + 4]^+$ and $[M + 5]^+$ are not present in natural abundance samples.

**SCHEME 3. Metabolism of [2,4,5,6,7-$d_5$]-Tryptamine (24a)[a] in *Brassica napus* ssp. *Rapifera* (Rutabaga)**




[a] Percentage in parentheses represents deuterium incorporation resulting from feeding experiments with tryptamine (**24a**).

brassinin (**23a**) into rutalexin (**11**) indicated the sequence of biosynthetic of steps shown in Scheme 2. This biosynthetic relationship was also observed in *B. oleracea* var. *gongylodes* (kohlrabi). As expected, [4,5,6,7-$d_4$]-indole (**21a**) was confirmed to be a precursor of brassinin (**4a**), 1-methoxybrassinin (**5a**), spirobrassinin (**13a**), and brassilexin (**15a**). Isalexin (**9**) could never be isolated from feeding experiments, but it is likely to derive from oxidation and methylation of indole, as previously shown[22] for isatin (2,3-dioxoindole), or from degradation of tryptophan. These experiments demonstrate for the first time that brassicanate A (**10**), rutalexin (**11**), and brassicanal A (**14**) are biosynthetically derived from brassinin (**4**). Although **14** could be the immediate precursor of brassicanate A (**10**), since incorporation of deuterated brassinin into **14** was lower than its incorporation into brassicanate A (**10**) (Table 2, entry 1, 37% vs 21%) this potential biosynthetic relationship cannot be confirmed. The biosynthetic relationships established in this work confirm once more the central role of brassinin (**4**) in the biosynthesis of a large number of the currently known cruciferous phytoalexins.

## Conclusion

We have isolated from *Brassica napus* ssp. *rapifera* (rutabaga) three new metabolites, isalexin (**9**), brassicanate A (**10**), and rutalexin (**11**), and the five known phytoalexins brassinin (**4**), 1-methoxybrassinin (**5**), spirobrassinin (**13**), brassicanal A (**14**), and brassilexin (**15**). These phytoalexins were produced after abiotic (UV irradiation) or biotic (fungal infection) elicitation. In addition, from *B. oleracea* var. *gongylodes* (kohlrabi) we isolated **9**−**11** and the known phytoalexins 1-methoxybrassinin (**5**), spirobrassinin (**13**), and brassilexin (**15**); however, we did not detect cyclobrassinone (**12**). These results suggest that the compound previously isolated from kohlrabi and reported to have structure **12** does not appear to be naturally occurring. We devised efficient syntheses for brassicanate A (**10**) and rutalexin (**11**) and tested the biological activity of the new metabolites against four fungal plant pathogens. It was established that these metabolites are new phytoalexins; brassicanate A (**10**) showed the strongest antifungal activity. Finally, biosynthetic studies established that indolyl-3-acetaldoxime (**22**) and brassinin (**4**) are biosynthetic precursors of brassicanate A (**10**) and rutalexin (**11**) and that cyclobrassinin (**23**) is an intermediate between brassinin (**4**) and rutalexin (**11**). We demonstrated that tryptamine (**24**) is not a precursor of rutabaga phytoalexins. The percentages of deuterium incorporation from indolyl-3-acetaldoxime (**22**) and brassinin (**4**) into the previously known phytoalexins brassinin (**4**), 1-methoxybrassinin (**5**), spirobrassinin (**13**), and brassilexin (**15**) are consistent with our previous studies.[19,23]

## Experimental Section

**Plant Material and Antifungal Bioassays.** Tubers of rutabaga (*B. napus* ssp. *rapifera*) and kohlrabi (*B. oleracea* var.

(22) Maugard, T.; Enaud, E.; Choisy, P.; Legoy, M.-D. *Phytochemistry* **2001**, *58*, 897−904.

(23) Pedras, M. S. C.; Montaut, S. *Chem. Commun.* **2004**, 452−453.

*gongylodes*) were purchased from local stores. Bioassays on *S. sclerotiorum* clone #33, *R. solani* AG2-1, and *P. lingam* BJ-125 were performed as previously described.[24] Antifungal activity bioassays against *C. cucumerinun* were conducted in duplicate as previously described.[18] Isalexin (**9**), brassicanal A (**14**), and brassicanate A (**10**) were dissolved in $CH_3CN$ and rutalexin (**11**) and cyclobrassinone (**12**) in THF.

**Phytoalexin Elicitation and Isolation.** Rutabaga tubers and kohlrabi roots were sliced horizontally (15 mm thick), and cylindrical wells (1.6 cm in diameter) were made on one surface with a cork-borer, as previously described.[19] The aqueous solution from each slice was harvested and extracted with EtOAc, the extracts were dried over $Na_2SO_4$, concentrated, and analyzed by HPLC. The aqueous solution was also concentrated and analyzed by HPLC. For each slice, the tissue around the wells was cut, homogenized, and extracted with MeOH. After filtration, the extracts were concentrated to dryness and analyzed by HPLC.

To obtain larger amounts of extracts to isolate new metabolites, experiments were carried out with ca. 13 kg of rutabaga tubers. The tubers were sliced, UV-irradiated, and incubated in the dark, and after 3 days the water from each well was collected (3 L), freeze-dried to reduce the volume (200 mL), and extracted with EtOAc. The organic extracts were combined, dried over $Na_2SO_4$, concentrated to dryness (ca. 100 mg), and separated FCC (reversed-phase C-18, gradient $H_2O-CH_3OH$ from 100:0 to 0:100). After HPLC analysis of each fraction, F5 to F10 were combined (40 mg) and fractionated on a silica gel preparative TLC (0.5 mm of thickness) developing twice with $CH_2Cl_2-CH_3OH$ 98:2 (v/v) to yield in order of decreasing polarity **9** (1.3 mg), **13** (6.4 mg, $[\alpha]_D^{20} = -53$ (*c* 0.30, $CHCl_3$); lit. $[\alpha]_D^{20} = -69.5$ (*c* 1.14, $CHCl_3$)),[25] **14** (1.3 mg), **15** (1.3 mg), **11** (1 mg), **10** (1 mg), **4** (0.5 mg), and **5** (1 mg).

**Root Elicitation with *R. solani* AG 2-1 Mycelium.** Rutabaga tubers were prepared as above, and each well was inoculated with mycelium and filled with water. After 2, 3, 4, and 5 days of incubation, in moistened covered plastic boxes, at 20 °C, in the dark, the aqueous solution from each slice (control and fungus infected) was harvested and extracted with EtOAc, and the extract was dried over $Na_2SO_4$, concentrated, and analyzed by HPLC. The aqueous solution was also concentrated and analyzed by HPLC.

**Feeding of Deuterated Compounds to Tubers.** Slices were prepared as described above and were incubated with deuterated compounds (ca. $10^{-3}$ M in 0.2% of DMSO and 0.1% aq Tween-80) in the dark at 20 °C (3 days for rutabaga and 4 days for kohlrabi). The aqueous solution was collected, extracted with EtOAc, and dried over $Na_2SO_4$ and the extract was analyzed by HPLC. The aqueous solution was concentrated and analyzed by HPLC. The EtOAc extracts were separated by multiple preparative TLC ($CH_2Cl_2-MeOH$, 98:2 v/v) and preparative HPLC.

**Methyl 2-Methylthiolindole-3-carboxylate (Brassicanate A, 10).** Methyl 1-Boc-2-chloroindole-3-carboxylate (**18**) was dissolved in MeOH (2 mL) and a solution of NaSH (42 mg, 0.75 mmol) in water (0.25 mL) was added dropwise at rt. After the mixture was stirred for 1 h at rt, $K_2CO_3$ (69 mg, 0.5 mmol) and iodomethane (30 $\mu$L, 0.5 mmol) were added and the stirring continued for further 10 min. The reaction mixture was diluted with $H_2O$ (20 mL), the product was extracted with $CH_2Cl_2$, the combined extracts were dried ($Na_2SO_4$), and the solvent was evaporated under reduced pressure. The residue was chromatographed on silica gel (hexanes−$Et_2O$, 5:1) and concentrated to afford a colorless oil (53 mg, 66%). The 1-Boc-protected brassicanate A (47 mg, 0.15 mmol) was dissolved in $CH_2Cl_2$ (2 mL), and TFA (225 $\mu$L, 2.9 mmol) was added dropwise at rt. After the mixture was stirred for 1 h at rt, the solvent was evaporated and the residue was chromatographed on silica gel (hexanes−EtOAc, 4:1), the solvent was again evaporated, and the residue was crystallized from $CH_2Cl_2$−hexane to afford **10** as colorless needles (26 mg, 80%): mp 109−110 °C (lit.[11] mp 105−107 °C, $CHCl_3$−hexane); HPLC $t_R$ = 14.9 min; $^1$H NMR and IR as in ref 11; $^{13}$C NMR (125 MHz, $CDCl_3$) 166.1 (s), 143.4 (s), 136.1 (s), 127.5 (s), 122.5 (d), 122.3 (d), 120.8 (d), 110.6 (d), 105.5 (d), 51.3 (q), 15.0 (q); HREIMS *m*/*z* (rel abundance) measured 221.0506 (221.0511 calcd for $C_{11}H_{11}NO_2S$); EIMS *m*/*z* (rel abundance) 221 $[M]^+$ (100), 206 (5), 190 (32), 175 (18), 161 (9), 120 (11), 89 (5); UV $\lambda_{max}$ (log $\epsilon$) 206 (4.4), 238 (4.4), 268 (3.9), 301 (4.0).

**Methyl-1,3-thiazino[6,5-*b*]indole-2,4-dione (Rutalexin, 11).** A solution of NaSH (35 mg, 0.65 mmol) in water (0.3 mL) was added dropwise with stirring to a solution of amide **19** (39 mg, 0.13 mmol) in DMF (0.5 mL) cooled to 0 °C. After being stirred for 2 h at 0 °C, the reaction mixture was diluted with brine (30 mL) and the pH was adjusted to 5 with 1 M HCl. The reaction mixture was extracted with EtOAc, and the combined extract was dried ($Na_2SO_4$) and concentrated to dryness under vacuum. NaH (60% suspension in mineral oil, 16 mg, 0.52 mmol) was added to the cooled residue dissolved in dry THF (2 mL), and the reaction mixture was stirred at 0 °C. After 10 min, the reaction mixture was cooled to −78 °C and phosgene (20% solution in toluene, 125 $\mu$L, 0.25 mmol) was added. The mixture was gradually warmed to rt (4 h), the solvent was evaporated, and the residue was chromatographed on silica gel ($CH_2Cl_2$−hexane, 1:1). Evaporation of the solvent afforded 13 mg (30%, based on amide **19**) of *N*-Boc-rutalexin as a colorless solid. *N*-Boc-rutalexin (27 mg, 0.08 mmol) was heated without solvent for 30 min at 165−170 °C (oil bath) to yield rutalexin (**11**) as a white solid (19 mg, quantitative): mp 313−315 °C; HPLC $t_R$ = 13.3 min; $^1$H NMR (500 MHz, DMSO-$d_6$) 12.57 (br s, 1H, $D_2O$ exchangeable), 8.09 (m, 1H), 7.54 (m, 1H), 7.28 (m, 2H), 3.37 (s, 3H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) 162.8 (s), 160.0 (s), 136.9 (s), 135.0 (s), 125.1 (s), 123.6 (d), 122.1 (d), 119.5 (d), 111.8 (d), 101.5 (s), 28.2 (q); HREIMS *m*/*z* (relative abundance) measured 232.0309 (232.0306 calcd for $C_{11}H_8N_2O_2S$); EIMS *m*/*z* (relative abundance) 232 $[M]^+$ (26), 175 (100), 147 (18), 120 (21), 103 (5); FTIR $\nu_{max}$ 3215, 2925, 1728, 1668, 1633, 1463, 1232 cm$^{-1}$; UV $\lambda_{max}$ (log $\epsilon$) 213 (4.0), 242 (3.7), 275 (3.6).

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**Supporting Information Available:** General experimental procedures and characterization data for compounds **17**, **18**, **19**, and **24a** and spectral data for compound **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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