The first naturally occurring aromatic isothiocyanates, rapalexins A and B, are cruciferous phytoalexins†

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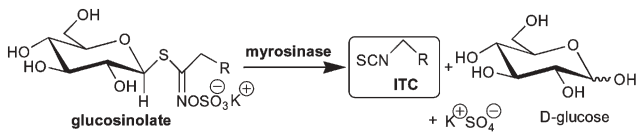
The discovery of the first naturally occurring aromatic isothiocyanates, indole-3-isothiocyanates, their first synthesis, antimicrobial activity and proposed biogenetic origin in canola plants are reported.

Isothiocyanates (ITCs) are a remarkable class of natural products produced by plants of various families, including Brassicaceae (syn. Cruciferae). Plant ITCs are enzymatic products resulting from hydrolysis of glucosinolates by plant enzymes trivially known as myrosinases (thioglucoside glucohydrolases, EC 3.2.3.1, Scheme 1).[1] ITCs are known to have crucial ecological roles in protecting plants against various pests, including insects and microbial systems.[2] Hence, ITCs are part of a group of constitutive plant chemical defences known as phytoanticipins. In addition to phytoanticipins, plants under stress biosynthesise *de novo* metabolites known as phytoalexins, which have selective antimicrobial activity. Both phytoanticipins and phytoalexins are involved in the chemical warfare between plants and their invaders.[3]

The phytoalexin arsenal of crucifers includes an unusual range of functional groups and indolyl structures that have sulfur and nitrogen, in addition to the most common organic elements (C, H, O).[4] The phytoalexins brassinin (**1**), 1-methoxybrassinin (**2**) and cyclobrassinin (**3**) were the first members of this unique group,[5] whereas erucalexin (**4**)[6] and caulilexin A (**5**)[7] were isolated very recently. Notwithstanding the large number of crucifers surveyed for ITC production, to date no cruciferous phytoalexins containing an ITC group have been reported, nor have aromatic ITCs.[2]

Furthermore, the significant ITC contents of cruciferous vegetables appear to make a remarkable contribution to their highly desirable anti-carcinogenic activity.[8] In fact, mechanisms through which ITCs and indoles may protect mammals against diverse cancers have been identified (*e.g.* induction of phase II enzymes such as glutathione transferases, NAD(P)H:quinone reductase, epoxide hydrolase).[9,10]



Scheme 1 Enzymatic formation of isothiocyanates (ITCs) from glucosinolates.[1]

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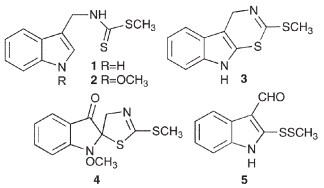
† Electronic supplementary information (ESI) available: Experimental details, synthesis and spectroscopic data of all new compounds **7–10** and **12–18**. See DOI: 10.1039/b615424g



Biotrophic fungi and oomycetes are capable of colonizing plant tissues in ways that prevent immediate recognition by the host,[11] which favour the invaders by avoiding the counter-chemical warfare. To understand the interaction of crucifers with such "disguised" microbial pathogens, we have analysed canola plants (*Brassica rapa*) infected with the white rust oomycete (*Albugo candida*, Pers. ex Chev., Kuntze). Gratifyingly, from infected leaf tissues of canola, two unique indole-3-isothiocyanates were isolated and demonstrated to be phytoalexins. The strong inhibitory activity of these isothiocyanates suggests a defensive role against the white rust oomycete, an economically important pathogen of many crucifers.

Leaves of canola were inoculated with *A. candida* for various periods, as described in ESI. HPLC chromatograms of extracts of infected leaves indicated the presence of several peaks not present in control leaves (non-inoculated). Several of these peaks corresponded to known phytoalexins; however, two of the peaks found no match in our libraries (DAD and MS).[12] To obtain sufficient amounts of these unidentified compounds, additional plants were infected and the infected leaves were incubated, excised, frozen in liquid nitrogen, and extracted with methanol, *cf.* ESI. The organic residue was separated by multiple chromatography to yield the two unidentified compounds, A (0.5 mg) and B (1.4 mg). The HREI-MS of compound A indicated the molecular formula $C_{10}H_8N_2OS$; the NMR spectroscopic data‡ indicated a disubstituted indole at C-3 and C-4 or at C-3 and C-7, an exchangeable proton attributable to N-1, and a methoxy substituent, accounting for C_9H_8NO. On biogenetic grounds[4] and HMBC spectroscopic data,‡ the methoxy substituent was placed at C-4. The three remaining atoms (CNS) required to fulfil the molecular formula were located at C-3; two arrangements could be suggested for the substituent at C-3, isothiocyanate (–N=C=S) or thiocyanate (–S–C≡N). The very strong absorption at *ca.* 2100 cm^{-1} in the FTIR spectrum suggested an isothiocyanate group. Based on this evidence, compound A was deduced to be 4-methoxyindole-3-isothiocyanate (**9**).

The HREI-MS spectroscopic data of compound B indicated the molecular formula $C_{10}H_8N_2O_2S$, *i.e.* an additional oxygen relative to compound A (**9**). The NMR spectra‡ were consistent with the molecular formula and, relative to compound A, displayed an additional exchangeable proton (δ_H 6.53) and an identical number

of carbons (with a lower field carbon at δ_C 143.2). The strong FTIR absorption characteristic of isothiocyanates (2139 cm^{-1}) was observed as well. Thus, compound B was deduced to be 5-hydroxy-4-methoxyindole-3-isothiocyanate (**18**). The structures of compounds A and B were confirmed by synthesis to be isothiocyanates **9** and **18**, respectively, as described below. The corresponding thiocyanates **10** and **14** were synthesised as well, to compare the antimicrobial activity of both functional groups.

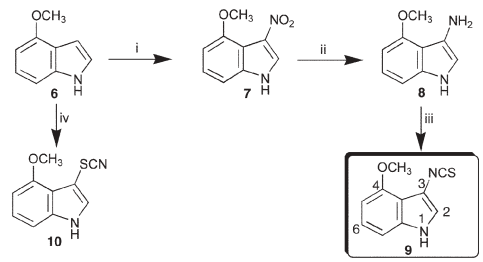
Facile syntheses of aromatic isothiocyanates employ treatment of the corresponding aromatic amines with thiophosgene.[13] However, because the required amines (4-methoxyindole-3-amine (**8**) and 5-hydroxy-4-methoxyindole-3-amine (**17**)) were unknown, procedures for their preparation were developed. The preparation of indole-3-amines by reduction of 3-nitroindoles[14] appeared an attractive strategy, although such nitrations do not appear to have been reported for 4-methoxyindoles. Thus, 4-methoxyindole[15] was nitrated using silver nitrate and benzoyl chloride in 30% yield, followed by hydrogenation to yield 4-methoxyindole-3-amine (**8**) in modest yield. In attempts to improve the yield of this conversion, the indole nitrogen was protected with benzyl and tosyl groups.[16] Whereas the nitration of 1-benzyl-4-methoxyindole proceeded in yields similar to those of non-protected indole **6**, nitration of 4-methoxy-1-tosylindole yielded the 7-nitro derivative almost exclusively. In addition, changing the nitration reagent to HNO_3–Ac_2O yielded 3-nitroindole **7** in substantially lower yield (11%). Finally, reaction of amine **8** with thiophosgene[13] in CH_2Cl_2–water yielded 4-methoxyindole-3-isothiocyanate (**9**) (Scheme 2). In addition, reaction of 4-methoxyindole with iodine–ammonium thiocyanate yielded 4-methoxyindole-3-thiocyanate (**10**, Scheme 2).[17] The spectroscopic and chromatographic properties of synthetic 4-methoxyindole-3-isothiocyanate (**9**) were identical in all respects to those of compound A isolated from canola leaves.

To prepare 5-hydroxy-4-methoxyindole-3-amine (**17**), first the corresponding indole (5-benzyloxy-4-methoxyindole) was prepared from 3-benzyloxy-2-methoxy-6-nitrobenzaldehyde (**11**) by condensation with nitromethane to yield dinitrostyrene **12**, followed by reduction with Fe and silica gel in acetic acid, in 60% yield over three steps.[18] Nitration of 5-benzyloxy-4-methoxyindole using benzoyl nitrate to produce 5-benzyloxy-4-methoxy-3-nitroindole occurred in very poor yield (5%). However, protection of *N*-1 with a benzyl group yielded the desirable 3-nitroindole **16** in a more acceptable yield (20%). Reduction of 3-nitroindole **16** (H_2, 40 psi) using Pd/C, AcOH, yielded the corresponding indole-3-amine **17** (lower H_2 pressures did not reduce the *N*–Bn bond). As in the case of isothiocyanate **9**, reaction of amine **17** with thiophosgene yielded 5-hydroxy-4-methoxyindole-3-isothiocyanate (**18**). The spectroscopic and chromatographic properties of synthetic 5-hydroxy-4-methoxyindole-3-isothiocyanate (**18**) were identical in all respects to those of compound B isolated from canola. To prepare thiocyanate **14**, reduction of dinitrostyrene **12** with Pd/C in methanol and AcOH afforded indole **13** in 29% yield (Scheme 3).[19] Treatment of 5-hydroxy-4-methoxyindole (**13**) with ammonium thiocyanate afforded 5-hydroxy-4-methoxyindole-3-thiocyanate (**14**) in 49% yield.

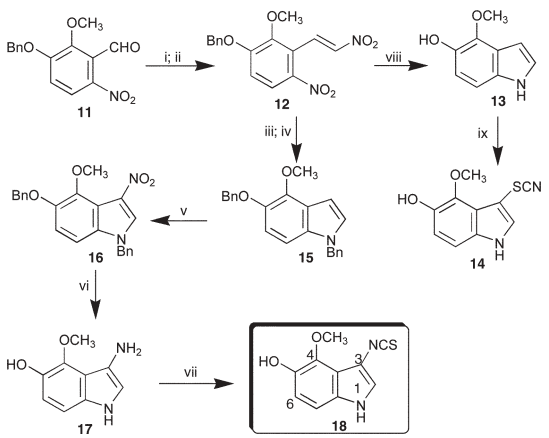
Finally, with compounds A (**9**) and B (**18**) available in sufficient amounts, their effect on the white rust pathogen was determined. A bioassay using impregnated cellulose membranes[20] established that compound A (**9**) displayed higher inhibitory effect on zoospore germination than compound B (**18**), and thiocyanates **10** and **14**. Compound A (**9**) caused complete germination inhibition at 5.0×10^{-6} M, whereas **10** and **18** caused complete inhibition at ten times higher concentration (5.0×10^{-5} M), and **14** caused only 75% inhibition at similar concentration (5.0×10^{-5} M).

Compounds A (**9**) and B (**18**) are plant metabolites biosynthesised in response to biotic (*A. candida*) and abiotic stress (UV), display antimicrobial activity, and are not detectable in non-stressed plants. Consequently, these metabolites are new phytoalexins for which we propose the names rapalexin A (**9**) and rapalexin B (**18**).

As shown in Table 1, rapalexin A (**9**), the most potent of both phytoalexins, was detected only eight days after inoculation whereas rapalexin B (**18**) was detected five days after inoculation in substantially larger amounts.§ Because rapalexins A (**9**) and B (**18**) are the first crucifer phytoalexins reported to display strong activity against a biotroph, particularly rapalexin A, their biogenetic origin and pathway are of great importance. Interestingly, the direct attachment of the isothiocyanate functional group to C-3 of indole has not been observed in naturally



Scheme 2 Synthesis of rapalexin A (**9**) and thiocyanate **10**. *Reagents and conditions*: i, $AgNO_3$, benzoyl chloride, CH_3CN, 30%; ii, Pd/C, AcOH, H_2; iii, CH_2Cl_2, $CaCO_3$, $CSCl_2$, 20% over two steps; iv, NH_4SCN, I_2, MeOH, r.t., 75%.

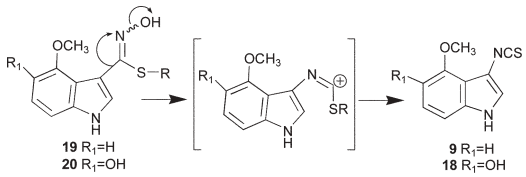


Scheme 3 Synthesis of rapalexin B (**18**) and thiocyanate **14**. *Reagents and conditions*: i, CH_3NO_2, 18-crown-6, KF, 4-methylmorpholine, r.t.; ii, NaOAc, Ac_2O, 60 °C, 88% over two steps; iii, Fe, SiO_2, toluene–AcOH, 90 °C, 75%; iv, NaH, THF, $PhCH_2Br$, 93%; v, $AgNO_3$, benzoyl chloride, CH_3CN, 20%; vi, Pd/C, AcOH, H_2/40 psi; vii, CH_2Cl_2, $CaCO_3$, $CSCl_2$, 30% over two steps; viii, Pd/C, H_2, MeOH, AcOH, 29%; ix, NH_4SCN, I_2, MeOH, 0 °C, 49%.

Table 1 Production of rapalexins A (**9**) and B (**18**) in leaves of canola (*Brassica rapa*) infected with *Albugo candida*

Days after inoculation	Rapalexin A (**9**) (nmol g^{-1} fresh leaves)	Rapalexin B (**18**) (nmol g^{-1} fresh leaves)
5	not detected	2.3–3.3
6	not detected	4.1–8.1
7	not detected	4.3–8.3
8	0.4–0.6	5.3–14.7
9	0.7–1.1	3.9–9.7
10	0.7–0.9	7.5–9.1

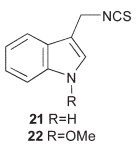
[a] Amounts of rapalexins A and B in leaves were determined by HPLC using calibration curves; the correlation coefficients of phytoalexin calibration curves were $\geqslant 0.9998$.



Scheme 4 Proposed biosynthetic precursors **19** and **20** of rapalexins A (**9**) and B (**18**), respectively.

occurring isothiocyanates.[2] Nevertheless, considering that all crucifer isothiocyanates reported so far derive from the corresponding glucosinolates,[2] rapalexins A and B may derive from yet to be discovered indolyl glucosinolates or from closely related indolyl thiohydroximates such as **19** and **20**, *via* a Lossen type rearrangement,[21] as depicted in Scheme 4. Clearly, this hypothesis requires further work with isotopically labelled precursors. That is, the discovery of these unique isothiocyanates **9** and **18** reveals additional pathways that need to be integrated in the biosynthetic puzzle of crucifer phytoalexins.[22]

Finally, it is noteworthy that both isothiocyanates **9** and **18** are stable compounds under the various conditions used for their isolation, purification and analysis, in contrast to indolyl-3-methyl isothiocyanates such as **21** and **22**.[23] It would be of interest to determine the potential anti-carcinogenic activity of rapalexins in mammalian systems. Further work to improve the syntheses and establish the biosynthetic precursors of rapalexins A and B is in progress.



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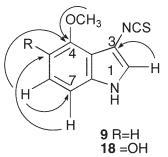
Notes and references

‡ NMR spectroscopic data for rapalexin A (**9**): ^{1}H-NMR (500.1 MHz, $CDCl_3$): δ 7.98 (brs, N–H), 7.18 (t, J = 8.0 Hz, 1H), 7.09 (d, J = 2.2 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 7.9 Hz, 1H), 4.00 (s, 3H). ^{13}C-NMR (125.8 MHz, $CDCl_3$): δ 154.3 (s), 136.9 (–N=C=S), 136.1 (s), 125.3 (d), 118.5 (d), 114.4 (s), 108.1 (s), 105.1 (d), 101.2 (d), 55.7 (q). For additional data see ESI. NMR spectroscopic data for rapalexin B (**18**): ^{1}H-NMR (500.1 MHz, CD_3CN): δ 9.31 (brs, 1H), 7.34 (d, J = 2.9 Hz, 1H), 7.09 (d, J = 8.7 Hz, 1H), 6.86 (d, J = 8.7 Hz, 1H), 6.53 (s, 1H), 3.94 (s, 3H). ^{1}H-NMR (500.1 MHz, CD_3OD): δ 7.29 (s, 1H), 7.01 (d, J = 8.7 Hz, 1H), 6.82 (d, J = 8.7 Hz, 1H), 3.97 (s, 3H). ^{13}C-NMR (CD_3OD): δ 143.2 (s), 138.7 (s), 133.7 (–N=C=S), 131.1 (s), 121.7 (d), 117.8 (s), 114.8 (d), 108.3 (d), 104.5 (s), 61.2 (q). For additional data see ESI.



Selected HMBC correlations of rapalexins A (**9**) and B (**18**)

§ The production of rapalexins A and B is localised and restricted to areas of infected tissues where the concentration can be around 0.1 mM (like any other phytoalexins).

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23 (*a*) Both compounds **21** and **22** remain to be isolated or prepared. See, P. Kutschy, M. Dzurilla, M. Takasugi, M. Torok, I. Achbergerova, R. Homzova and M. Racova, *Tetrahedron*, 1998, **54**, 3549–3566; (*b*) A. B. Hanley and K. R. Parsley, *Phytochemistry*, 1990, **29**, 769–771.

