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COMMUNICATION

The first isocyanide of plant origin expands functional group diversity in cruciferous phytoalexins: synthesis, structure and bioactivity of isocyalexin A†

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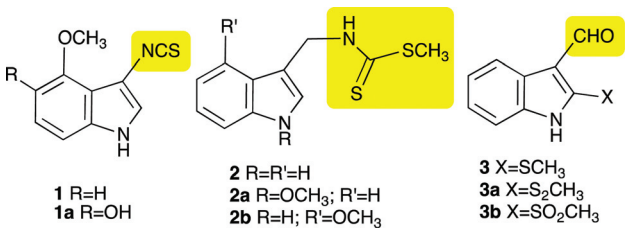
Although isocyanides are not rare amongst terrestrial microbes and marine organisms, despite tens of thousands of natural products isolated from plants, isocyanides are still missing. Isocyalexin A is the first isocyanide of plant origin. Isocyalexin A was isolated from UV-irradiated rutabaga roots and shown to be a new cruciferous phytoalexin. Its chemical structure was proven by analysis of NMR spectroscopic data and chemical synthesis.

Naturally occurring isocyanides (ICs) comprise a diverse range of metabolites produced by terrestrial microorganisms and marine organisms.[1–3] Remarkably, to date no metabolites containing an IC (or isonitrile) group appear to have been reported from plant sources. Unlike marine organisms such as sponges, where both ICs and isothiocyanides (ITCs) are known to co-occur (Fig. 1),[2] ICs from the plant kingdom are unknown,‡ but ITCs are common.[4,5] On the other hand, cyanide (or nitrile, CN) containing metabolites are common in plants and other terrestrial organisms, as well as marine organisms.[6] Natural ICs,[2] ITCs[4] and CNs[6] are known to have biological roles related with defence of the producing organisms.

The first naturally occurring aromatic ITC, rapalexin A (**1**), was isolated from canola plants (*Brassica rapa* L.)[4] and later on shown to be produced by other cruciferous species subjected to stress, including UV-irradiation and pathogen attack.[7] These plant defence compounds, known as phytoalexins, are essential to protect plants, but are only biosynthesized in response to stress. To date, there is no reasonable explanation for the absence of ICs from plants, particularly considering that they are derived from the metabolism of protein amino acids in terrestrial microorganisms such as fungi and bacteria.[3,8] Metabolism of amino acids in plants is known to afford numerous secondary metabolites with defensive roles.[4,6,7] Specifically, ITCs from cruciferous species are derived from various amino acids, including methionine, valine, leucine, phenylalanine and tyrosine.[5,9] By contrast, it is relevant to note that the molecular diversity of cruciferous phytoalexins, most of which are derived from (*S*)-tryptophan (Trp), is imparted by different functional groups, including isothiocyanate (**1**, **1a**), dithiocarbamate (**2**, **2a**, **2b**) and aldehyde (**3**, **3a**, **3b**).[7]



The IC group has ambident character, as implied by its resonance structures shown in Fig. 1, a versatility widely appreciated in synthetic chemistry.[10,11] Due to their unique reactivity, ICs are very popular components of a huge number of organic reactions, particularly since the Passerini and Ugi reactions were discovered.[12,13]

During our previous work related with metabolic pathways of phytoalexins in rutabaga roots,[14,15] inconsistent production of an indolyl-containing metabolite **X** was detected by HPLC using diode array (DAD) and mass (MS) detectors. The UV spectrum obtained for this component was not available in our UV spectral

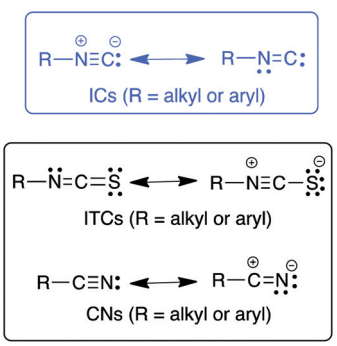


Fig. 1 Resonance structures of isocyanides (ICs), isothiocyanides (ITCs) and cyanides (CNs).

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† Electronic supplementary information (ESI) available: General experimental and detailed experimental procedures for: antifungal bioassays and syntheses and spectroscopic characterization of new compounds **4**, **7**, **9** and **13**. See DOI: 10.1039/c2ob25492a

libraries, suggesting it was a new metabolite. Subsequently, work to optimize elicitation conditions of rutabaga root slices led to consistent production of this compound (Fig. 2).

After UV-irradiation of rutabaga root slices for 90 min (*vs.* 60 min) and further incubation, the amounts of metabolite **X** increased dramatically (5–10-fold). The consistent and increased production facilitated the isolation and chemical structure elucidation of **X**. Rutabaga root slices UV-irradiated for 90 min and incubated in the dark,[14] were extracted and the organic extract was fractionated to yield **X** in sufficient amount to obtain ^{1}H NMR, HRMS-EI and FTIR spectroscopic data. The molecular formula suggested by HRMS ($C_{10}H_8N_2O$) was consistent with the ^{1}H NMR spectrum¶ of 3-substituted-4-methoxyindole (C_9H_8NO); the missing carbon and nitrogen atoms detected by HRMS suggested the presence of either a CN or an IC group at C-3. Since the ^{1}H NMR spectral data did not match those of 4-methoxyindole-3-carbonitrile,‖ a synthetic compound available in our library, the structure of **X** was assigned as 4-methoxyindole-3-isonitrile (**4**). A literature search revealed that neither this compound nor any other substituted indole-3-isonitrile had been previously reported. In addition, to date no ICs from plant origin have been reported. The chemical structure of metabolite **4** was confirmed by synthesis, as described below.

One of the most common syntheses of ICs involves formylation of the corresponding amines followed by dehydration.[16] 3-Aminoindoles are unstable compounds that readily oxidise to undetermined products upon exposure to oxygen;[4,17] hence *in situ* transformation of 3-aminoindoles is necessary to obtain reasonable yields of products. Initially, a route to indole-3-isonitriles was investigated using 3-nitroindole (**5**), a substrate simpler to make than 4-methoxy-3-aminoindole (**11**).[18] Following hydrogenation of 3-nitroindole (**5**) in ethanol,[4] the resulting amine was immediately quenched with EtOCHO, without filtering off the catalyst. After 24 h only a very small amount of the corresponding formamide **7** was isolated (*ca.* 4%). Heating 3-aminoindole (**6**) in EtOCHO and Et_3N in a sealed vial at 60 °C did not improve formamide yields.[19]

Next, it was reasoned that instead of dehydration of formamide, reduction of the corresponding carbamate could potentially yield an isocyanide. Thus, after hydrogenation of **5** in ethanol, the resulting amine **6** was quenched at −15 °C with phosgene to yield, after 5 h, ethyl carbamate **8** in low yield (*ca.* 32%). However, in our hands reduction of carbamate **8** to the corresponding formamide under a variety of conditions (*e.g.* Et_3SiH, $NaBH_4$, $NABH_4$–$NiCl_2$) could not be accomplished. Thus, direct formylation of 3-aminoindole (**6**) was attempted by adding a mixture of HCO_2H–Ac_2O to the hydrogenated reaction mixture; under these conditions, (*N*′-formyl)-3-aminoindole (**7**) was obtained albeit in poor yield (*ca.* 25%). Subsequently, hydrogenation–formylation of 3-nitroindole (**5**) was carried out in MeOH–HCO_2H–AcOCHO; satisfactory yields of formamide **7** were obtained consistently. Dehydration of **7** provided a short route to the first ever reported 3-indoleisonitrile (**9**, Scheme 1). The hydrogenation of *N*-protected-3-nitroindoles has been previously reported in Ac_2O,[17] however this is the first time that a non-protected nitroindole has been hydrogenated and formylated in a one-pot procedure using Pd/C–H_2.

Subsequently, to obtain metabolite **4**, it was necessary to prepare the starting material 4-methoxy-3-nitroindole (**10**). This compound was previously synthesised by nitration of 4-methoxyindole in 30% yield.[4] Thallation–iodination of 3-

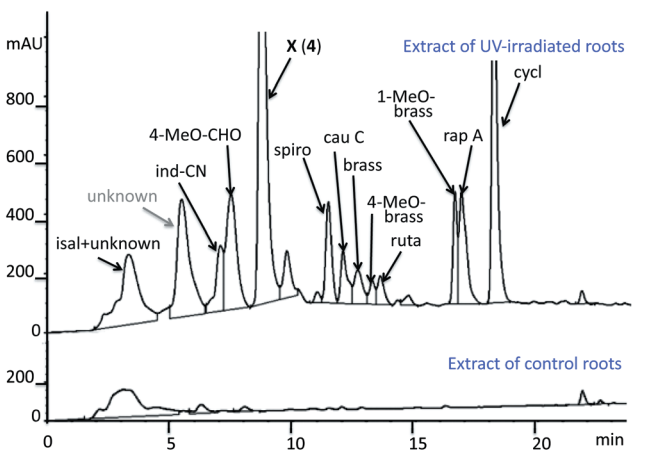
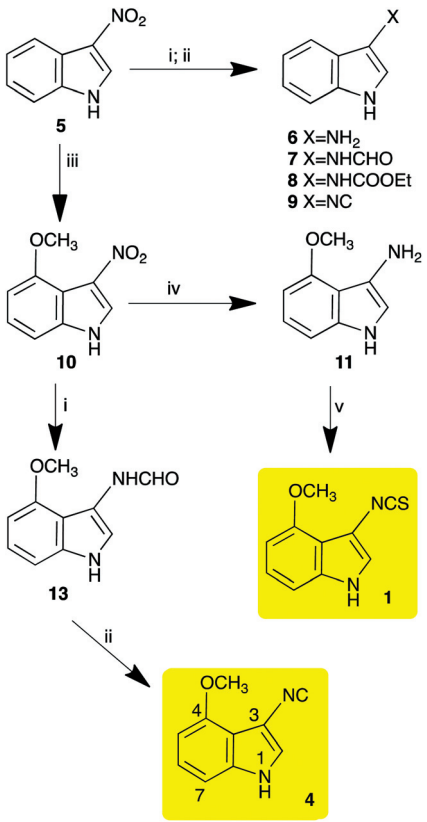


Fig. 2 HPLC-DAD chromatograms (C-18 reverse phase column, H_2O–MeOH elution, 50 : 50 to 0 : 100 linear gradient for 25 min, detection at 220 nm) of rutabaga root extracts: UV-irradiated for 90 min (isa = isalexin (**14**), ind-CN = indolyl-3-acetonitrile, 4-MeO–CHO = 4-methoxyindolecarboxaldehyde, spiro = spirobrassinin, caul C = caulilexin C, brass = brassinin, 4-MeO–brass = 4-methoxybrassinin, ruta = rutalexin, 1-MeO–brass = 1-methoxybrassinin, rap A = rapalexin A (**1**), cycl = cyclobrassinin§) and control (no irradiation).



Scheme 1 Synthesis of isocyalexin A (**4**) and rapalexin A (**1**). Reagents and conditions: (i) H_2–Pd/C, MeOH, HCO_2COCH_3, HCO_2H; **7**, 64%, **13**, 66%; (ii) $POCl_3$, THF, Et_3N; **4**, 41%, **9**, 54%; (iii) TFA, $Tl(OAc)_3$; I_2, CuI, DMF, NaOMe, MeOH; **10**, 64%; (iv) H_2–Pd/C, EtOH; (v) Cl_2CS; **12**, 51%.

carbonylindoles is known to yield substitution only at C-4.[20,21] Thus, it was surmised that a nitro group at C-3 of indole had the potential to afford similar regiocontrol, by directing substitution to C-4. To test this hypothesis, 4-methoxylation of 3-nitroindole (**5**) was attempted using $Tl(OAc)_3$ and $CuI–I_2$. We were delighted to find that this reaction worked well, and that the nitro group could direct thallation–iodination to the C-4 of indole. This is the first time that a nitro group at C-3 of indole has been reported to direct 4-substitution. Next, addition of MeONa to 4-iodo-3-nitroindole afforded the desired 4-methoxy-3-nitroindole (**10**) in reasonable yield. One-pot hydrogenation and formylation of **10**, followed by dehydration, as reported for isocyanide **9**, led to metabolite **4** in 27% overall yield. This route from 3-nitroindole (**5**) provided a convenient synthesis of **4** involving 4-steps, 3-pots and two chromatographic separations (Scheme 1).

Having sufficient amounts of ICs **4** and **9** in hand facilitated spectroscopic characterization by NMR (^1H and ^{13}C NMR, HMQC and HMBC); interestingly, spin–spin coupling between ^{13}C-3 (δ_C 103.2 in **4** and 104.3 ppm in **9**) and ^{14}N (t, $J_{C–N}$ = *ca.* 16 Hz) of the IC group was observed. To the best of our knowledge, a similar coupling has only been reported for brasilidene A, an indolyl-conjugated alkenyl isocyanide.[22]

To establish the potential ecological role of metabolite **4** in rutabaga roots and correlate functional group with antifungal activity, mycelial growth inhibition assays[23] were performed testing compounds **4**, **7**, **9** and **13** against four fungal species of economically important cruciferous pathogens (Table 1).

Among the four compounds tested, metabolites **4** and **9** displayed the highest antifungal activity against the root pathogen *Rhizoctonia solani* and the lowest activity against *Leptosphaeria maculans*. Because **9** displayed almost no inhibitory activity against *L. maculans*, it is concluded that the presence of the 4-MeO substituent has a stronger effect on this pathogen. These results indicated that **4** is a novel phytoalexin produced by stressed rutabaga tissues, which may have a significant role in protecting rutabaga against root pathogens. Importantly, this phytoalexin was also induced by *R. solani* incubation with rutabaga roots. Hence, this metabolite was named isocyalexin A (**4**).

Table 1 Inhibitory activity of isocyalexin A (**4**) and compounds **7**, **9** and **13** against mycelia[a] of plant pathogenic fungi *Alternaria brassicicola*, *Leptosphaeria maculans*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*[23]

Compounds[b]	*A. brassicicola*	*L. maculans*	*R. solani*	*S. sclerotiorum*
Isocyalexin A (**4**)				
5.0×10^{-4} M	47 ± 0	29 ± 2	100	65 ± 2
2.0×10^{-4} M	14 ± 2	9 ± 0	51 ± 4	40 ± 2
1.0×10^{-4} M	0	8 ± 2	32 ± 2	31 ± 5
Indole-3-isonitrile (**9**)				
5.0×10^{-4} M	42 ± 4	5 ± 0	100	60 ± 2
2.0×10^{-4} M	27 ± 4	0	51 ± 4	31 ± 2
1.0×10^{-4} M	17 ± 4	0	31 ± 4	17 ± 0
4-Methoxyindole-3-formamide (**13**)				
5.0×10^{-4} M	21 ± 2	27 ± 0	29 ± 4	32 ± 2
2.0×10^{-4} M	9 ± 4	7 ± 0	12 ± 2	25 ± 4
1.0×10^{-4} M	0	2 ± 2	6 ± 2	17 ± 0
Indole-3-formamide (**7**)				
5.0×10^{-4} M	42 ± 4	31 ± 3	15 ± 4	25 ± 0
2.0×10^{-4} M	27 ± 4	16 ± 2	0	19 ± 2
1.0×10^{-4} M	17 ± 4	5 ± 2	0	15 ± 2

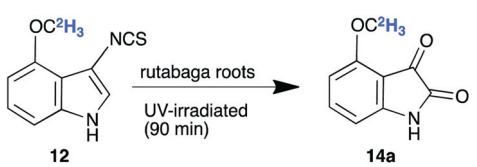
[a] Percentage of inhibition = 100 – [(growth on medium containing compound/growth on control medium) × 100)] ± standard deviation. Results are the averages ± standard deviations of independent experiments conducted in triplicate. [b] Compounds were dissolved in DMSO and added to potato dextrose agar medium (see ESI†).

A puzzling question regarding the biosynthetic origin of isocyalexin A (**4**) emerged. Since this is the first isocyanide of plant origin, there is no precedence for analogy reasoning.[24] Nonetheless, the close structural similarity between isocyalexin A (**4**) and rapalexin A (**1**) hinted that **4** might derive from **1** by enzymatic desulfurization. To verify this possibility, [$^2H_3$]rapalexin A (**12**) was synthesised and administered to UV-irradiated rutabaga roots, similar to experiments described previously.[14] After incubation and extraction, analysis of the extracts by HPLC-MS-ESI indicated a substantial amount of deuterium in isalexin (**14a**, 60% ± 8) relative to the total amount (*i.e.* deuterated **14a** plus natural abundance **14**), while no deuterium incorporation into isocyalexin A (**4**) was detected (Scheme 2). The high deuterium incorporation suggests that rapalexin A (**1**) is a close precursor of isalexin (**14**), while the lack of deuterium incorporation into isocyalexin A (**4**) suggests that **1** is not a precursor of **4**. Nonetheless, this hypothesis cannot be completely ruled out. It is worth stressing that rapalexin A (**1**) is the first precursor of isalexin (**14**) reported to date. Transformation of **1** to **14** suggests that this biosynthetic step is likely to involve a single oxidase, placing isalexin as a potential piece at the border of this biosynthetic puzzle.

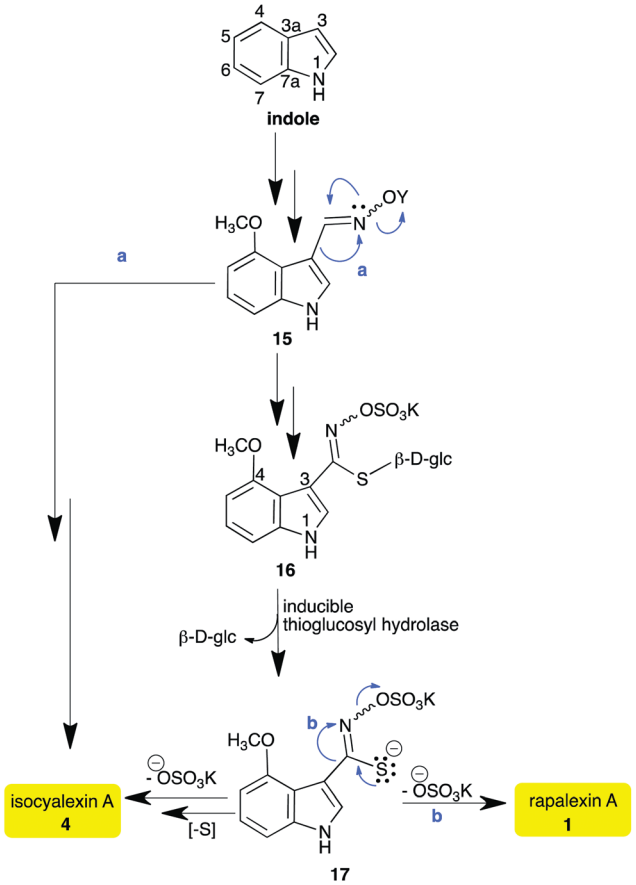
Alternatively, a common precursor transformed by different enzymes to isocyalexin A (**4**) and rapalexin A (**1**) could be proposed (Scheme 3). Hence, by analogy with the biosynthesis of other ITCs,[9] the as yet unknown 4-methoxyindole glucosinolate (**16**) could be the precursor common to both isocyalexin A (**4**) and rapalexin A (**1**). The formation of ITCs is mediated by thioglucosidases** and is followed by a spontaneous Lossen-type rearrangement. That is, rapalexin A (**1**) and isocyalexin A (**4**) could result from different enzymatic transformations of glucosinolate **16**. In this context, it is of interest to point out that the thermal rearrangement of isocyanides to cyanides is well known,[25] but equivalent enzymatic reactions do not appear to have been reported. Furthermore, a Beckman-type rearrangement[26] of the aldoxime derivative **15** to the corresponding isocyanide **4** could be considered also a possibility.

These hypotheses, as depicted in Scheme 3, need substantial experimental work before any sort of confirmation can be obtained. Nonetheless, such work is essential to design crops producing higher levels of phytoalexins. Increased concentrations of these metabolites in infected plants are expected to provide superior protection against plant pathogens.

Finally, the discovery of the first IC of plant origin attracts speculation concerning the absence of such metabolites from the



Scheme 2 Biotransformation of [$^2H_3$]rapalexin A (**12**) to [$^2H_3$]isalexin (**14a**) in UV-irradiated rutabaga roots.



Scheme 3 Proposed biosynthetic intermediates of isocyalexin A (**4**) and rapalexin A (**1**).

plant kingdom. To a greater extent, this is a more pertinent question in crucifers, since the corresponding ITCs and CNs are abundant. Toward this end, future work should include syntheses of IC libraries, the counterparts of naturally occurring ITCs, to establish their occurrence and function in crucifers. In addition, it will be of interest to determine if isocyalexin A (**4**) is produced in other *Brassica* species and its potential synergistic activity in naturally occurring blends of cruciferous phytoalexins.

Considering the novel chemical structure and its selective antifungal activity, phytoalexin **4** is a new but prominent jigsaw piece yet to find a position in the biosynthetic puzzle of cruciferous phytoalexins. Unquestionably, the importance of cruciferous phytoalexins would warrant further chemical and biochemical work to solve the puzzle.

Acknowledgements

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Notes and references

‡A report of constituents of floral scents of *Saponaria officinalis* suggested that benzylisocyanide was detected by GC-MS analysis; however, it was not established whether this volatile is an air pollutant or a plant metabolite.[27]

§Chemical structures of compounds are provided in the ESI.†

¶Isocyalexin A (**4**): HPLC: t_R = 11.0 min. UV (CH_3CN–H_2O, HPLC) λ_{max} (nm): 220, 269. FTIR (KBr, cm^{-1}) ν_{max}: 3305, 3129, 2134, 1592, 1514, 1447, 1366, 1336, 1272, 1079. ^{1}H NMR (500 MHz, CD_3OD) δ 7.45 (1H, s), 7.10 (1H, dd, J = 8, 8 Hz), 6.97 (1H, d, J = 8 Hz), 6.58 (1H, d, 8 Hz), 3.91 (3H, s). ^{13}C NMR δ (125.8 MHz, CD_3OD) δ 164.1, 154.8, 137.3, 125.5, 124.3, 113.9, 106.4, 103.2, ($J_{C\text{-}3\text{-}N}$ = 15 Hz), 102.1, 56.0. HREI-MS m/z 172.0633 (M^+), calcd for $C_{10}H_8N_2O$ 172.0632 (100%), 157.04 (69%), 129.04 (69%). HPLC-MS-ESI m/z [M − H]$^-$, 171.1 (11%), 156.0 (100%).

‖4-Methoxyindole-3-carbonitrile does not appear to have been described previously, synthesis and spectroscopic data are provided in ESI.†

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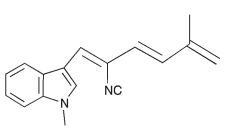

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