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Dissecting metabolic puzzles through isotope feeding: a novel amino acid in the biosynthetic pathway of the cruciferous phytoalexins rapalexin A and isocyalexin A†

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Understanding defence pathways of plants is crucial to develop disease-resistant agronomic crops, an important element of sustainable agriculture. For this reason, natural plant defenses such as phytoalexins, involved in protecting plants against microbial pathogens, have enormous biotechnological appeal. Crucifers are economically important plants, with worldwide impact as oilseeds, vegetables of great dietetic value and even nutraceuticals. Notably, the intermediates involved in the biosynthetic pathways of unique cruciferous phytoalexins such as rapalexin A and isocyalexin A remain unknown. Toward this end, using numerous perdeuterated compounds, we have established the potential precursors of these unique phytoalexins and propose for the first time their detailed biosynthetic pathway. This pathway involves a variety of intermediates and a novel amino acid as the central piece of this complex puzzle. This work has set the stage for the discovery of enzymes and genes of the biosynthetic pathway of cruciferous phytoalexins of unique scaffolds.

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Introduction

Plants, microbes and animals produce an astonishing array of secondary metabolites, better known as natural products.[1,2] These products are biosynthesized from primary building blocks such as amino acids, the precursors of alkaloids, acetate/malonate, the precursors of polyketides, or shikimate, the precursor of phenylpropanoids, flavonoids and others. Such metabolic arrays are characteristic of individual groups of organisms and are crucial to their overall fitness. For example, plants produce vital defensive metabolites commonly known as phytoalexins and phytoanticipins that can be recruited into specific defence programs depending on the attacker. Phytoalexins are low molecular weight antimicrobial metabolites biosynthesized *de novo* by plants in response to pathogen attack or other types of stress such as UV radiation,[3] while phytoanticipins are constitutive metabolites.[4] Remarkably, most cruciferous phytoalexins,[5] and some phytoanticipins,[6] contain nitrogen and sulfur atoms in addition to the carbon scaffold, a rare feature in phytoalexins of other plant families. Indeed, cruciferous defence-related metabolites seem to combine structures concisely assembled, but containing a relatively high number of functional groups, which is possible due to the presence of sulfur, nitrogen and oxygen heteroatoms. Crucifers (plant family Brassicaceae) have worldwide impact due to all sorts of uses, from edible oils and vegetables (cauliflower, broccoli, cabbage) to condiments (mustard, wasabi), fodder, ornamentals, industrial oils and fuels.[7]

Since the discovery of the first cruciferous phytoalexins in 1986,[8] over 40 chemical structures have been solved.[5] Some of these metabolites have been found only in wild species, as for example wasalexins A (**5**) and B (**6**)[9] in *Thellungiella salsuginea* and camalexin (**8**)[10] in *Arabidopsis thaliana.* For decades, *A. thaliana*[11] and more recently *T. salsuginea*[12] have been developed and exploited as model-systems for dissecting cellular and metabolic processes. In fact, the availability of numerous *A. thaliana* mutants has facilitated the isolation and cloning of genes involved in the biosynthetic pathway of camalexin (**8**), but to date no genes or enzymes have been reported for any other cruciferous phytoalexin. The structural diversity of cruciferous phytoalexins points toward complex biosynthetic pathways and unique enzymes that have yet to be uncovered. (*S*)-Tryptophan (Trp) is the primary building block of cruciferous phytoalexins, including wasalexins and camalexins.[5] Comparison of the currently known phytoalexin structures (45) with that of Trp indicates only two carbon scaffold types, as summarized in Fig. 1: scaffold **A**, encompassing the majority of phytoalexins, which have indole plus a side-chain with one

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†Electronic supplementary information (ESI) available: General experimental details, Tables S1–S3 showing percentage of isotope incorporation, ^{1}H and ^{13}C NMR spectra of new compounds shown in Table 1. See DOI: 10.1039/c2ob27076e

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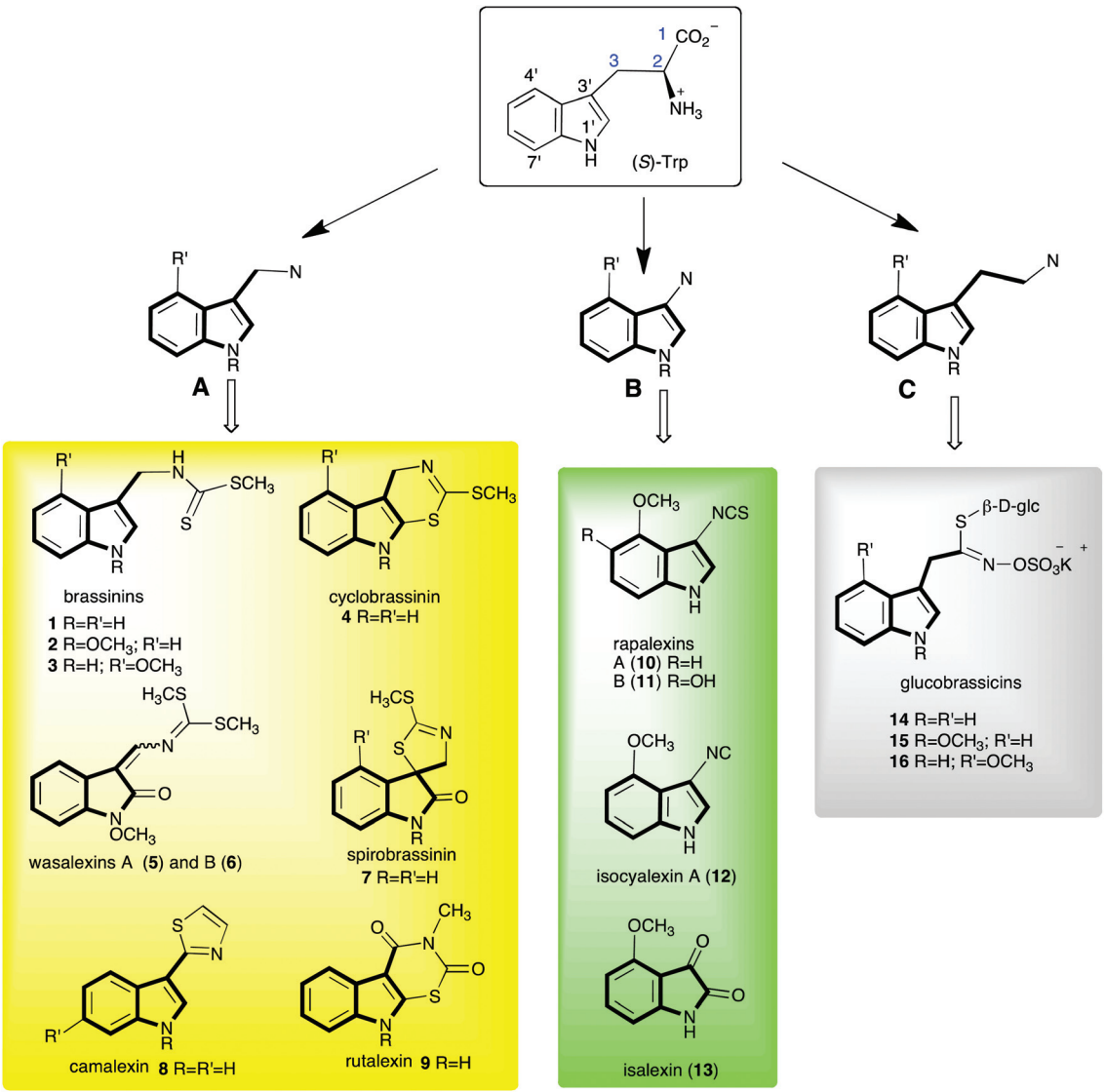

Fig. 1 Cruciferous metabolites grouped according to carbon scaffolds. Scaffold **A**, indole plus side-chain C–N; scaffold **B**, indole plus side-chain N or O; scaffold **C**, indole plus side-chain C–C–N.

carbon attached to a heteroatom (usually at C-3′; brassinin (**1**) and derivatives **2** and **3**, cyclobrassinin (**4**), camalexin (**8**), *etc.*, *ca.* 37 metabolites), and scaffold **B**, representing four phytoalexins, which have indole directly attached to a heteroatom (at C-3′; rapalexins A (**10**) and B (**11**), isocyalexin A (**12**) and isalexin (**13**)). Furthermore, the cruciferous phytoanticipins glucobrassicins **14–16** (indolyl-3′-methyl glucosinolates)[13] are constitutive metabolites also derived from (*S*)-Trp,[14,15] having scaffold **C**, which contains indole plus a side-chain with two carbons attached to nitrogen (at C-3′).

While most of the biosynthetic intermediates involved in the multistep biosynthesis of brassinins[16–18] and wasalexins[19] have been identified or deduced (Scheme 1), the biosynthetic steps of phytoalexins containing scaffold **B** remain undetermined. Although rapalexin A (**10**) has been shown to be a close precursor of isalexin (**13**)[20] and to incorporate perdeuterated Trp,[19] no other precursors have been revealed. In view of the recent discovery of isocyalexin A (**13**),[20] apparently the only plant isocyanide reported to date, and the exceptional opportunity to discover a novel route leading to scaffold **B**, we have undertaken to dissect this challenging biosynthetic pathway. This investigation has revealed that a novel amino acid derived from Trp is the central piece of this puzzle, setting the stage for biotechnological applications.

Results

Hypothetical pathway of rapalexin A (10) and isocyalexin A (12) and incorporation of (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp

Inspired by the presence of an isothiocyanate group in rapalexin A (**10**), we proposed that the hypothetical glucosinolate glucorapassicin (**21**, 4′-methoxyindolyl-3′-glucosinolate, trivial name derived from rapalexin and glucobrassicin) would be its

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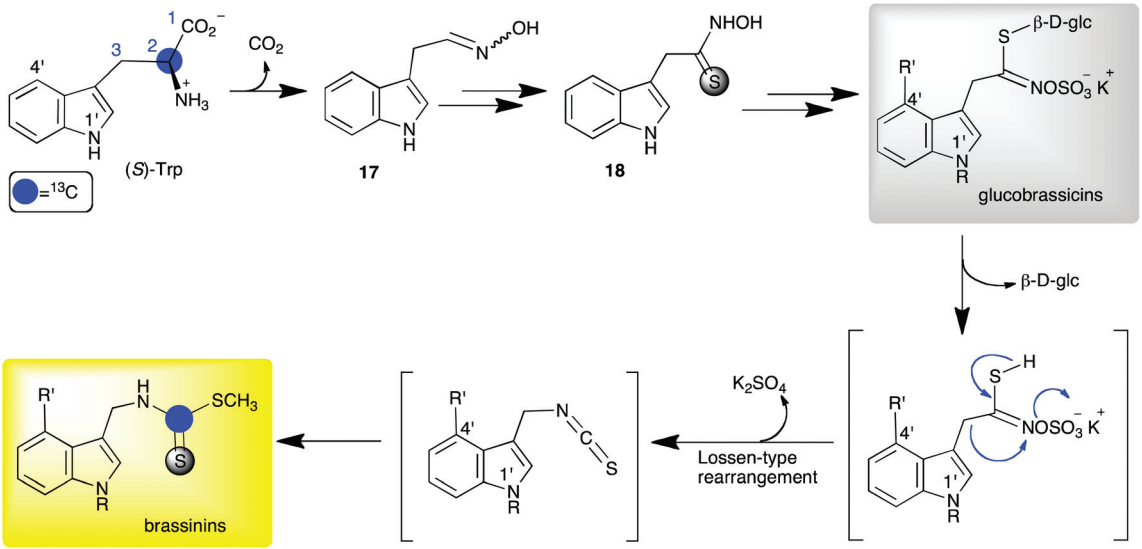
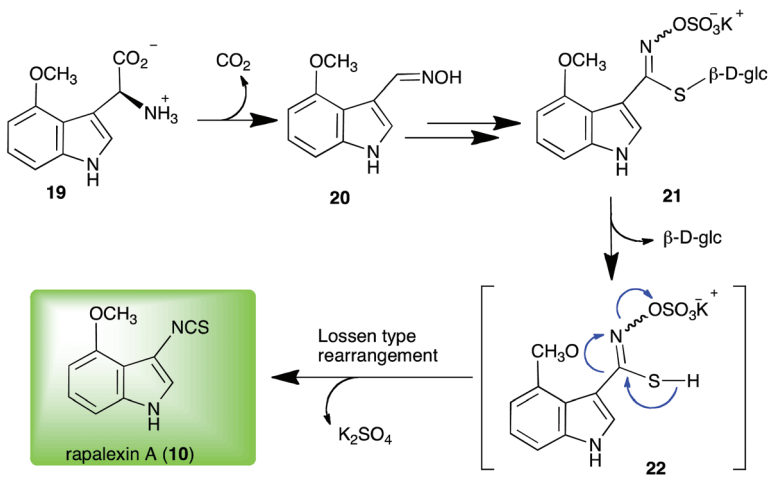


Scheme 1 Biosynthetic pathway from (*S*)-Trp to brassinins (two arrows indicate multiple steps; the compound in brackets is unstable, isolated only for quasi-natural products[18] with R = Ac or *t*-Boc).



Scheme 2 Proposed biosynthetic pathway of rapalexin A (**10**) (two arrows indicate multiple steps; the compound in brackets is expected to be unstable).

immediate precursor,[21] by analogy to the brassinins pathway (Scheme 1) and to reactions catalysed by plant myrosinases that use glucosinolates as substrates.[13] Further biosynthetic considerations suggest that the precursor of glucorapassicin (**21**) would be 4′-methoxyindole-3′-carboxaldehyde oxime (**20**), which in turn would derive from the corresponding amino acid 4′-methoxyindolyl-3′-glycine (**19**). Although this idea is a greatly appealing and exciting possibility, it has not been advanced or even proposed previously (Scheme 2), neither have glucorapassicin (**21**) or amino acid **19** been reported in crucifers or any other plants. Undoubtedly, an explanation for the conundrum of how amino acid **19** is formed *in planta* must be found.

Dissecting biosynthetic pathways involves retrobiosynthetic analysis of each structure to infer potential precursors of metabolite, followed by synthesis of isotopically labeled precursors and feeding of each labeled precursor to the particular organism. The choice of isotope(s) depends on the chemical structures of metabolites and hypothetical precursors.[22] The use of stable isotopes such as ^{2}H, ^{13}C, ^{15}N, ^{34}S instead of radioactive isotopes is possible and desirable due to the availability of highly sensitive analytical tools, namely HPLC-DAD-ESI-MS.[17,19] Rutabaga roots (*Brassica napus* L. ssp. *rapifera*) are a good choice to uncover the biosynthetic intermediates leading to rapalexin A (**10**) and isocyalexin A (**12**), due to the straightforward nature of elicitation and feeding experiments.[20]

To establish unambiguously the origin of the carbon and nitrogen atoms of both rapalexin A (**10**) and isocyalexin A (**12**), fully labeled (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp was used. Feeding experiments involved addition of aqueous solutions of labeled (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp to elicited slices of rutabaga roots, followed by

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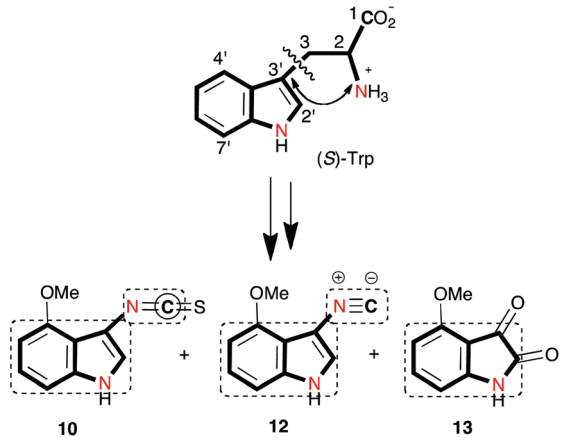
incubation and extraction of solutions and tissues. Control experiments were conducted similarly, but using either non-labeled, *i.e.* naturally occurring (*S*)-Trp, or the carrier solution only. HPLC-DAD-ESI-MS analysis of each extract was carried out; the identity of rutabaga metabolites corresponding to each peak detected in HPLC-DAD-ESI-MS chromatograms was established by direct comparison with authentic samples available in our libraries (UV spectra and ESI-MS data).

HPLC-ESI-MS analyses of extracts demonstrated that (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp was incorporated into various rutabaga metabolites (Table S1, ESI†). To our absolute delight, the mass spectral data (HPLC-ESI-MS) showed that both rapalexin A (**10**) and isocyalexin A (**12**) incorporated nine carbons (eight indole carbons plus one side-chain [$^{13}C_9$]) and both nitrogens ($^{15}N_2$) of Trp (determined from the presence of $[M - 1 + 11]^-$ ions in the mass spectrum of rapalexin A (**10**) and isocyalexin A (**12**), Fig. 2). These results clearly indicated that the carbons of the side-chain groups of both phytoalexins **10** and **12** resulted from (*S*)-Trp, suggesting that a rearrangement reaction occurred in the formation of both phytoalexins (Scheme 3; the nitrogen of the amino group of Trp became directly attached to C-3′ of indole). As well, isalexin (**13**) showed incorporation of all carbons and one nitrogen of the indole ring of (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp. Further examination of the chromatograms of extracts of fed tissues and analyses of mass spectra showed incorporation of carbons and nitrogens of (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp into the expected metabolites glucobrassicins **14–16**, cyclobrassinin (**4**), spirobrassinin (**7**) and rutalexin (**9**) (ESI Table S1†).



Scheme 3 Incorporation of (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp into rapalexin A (**10**), isocyalexin A (**12**) and isalexin (**13**) in rutabaga roots (determined by HPLC-ESI-MS).

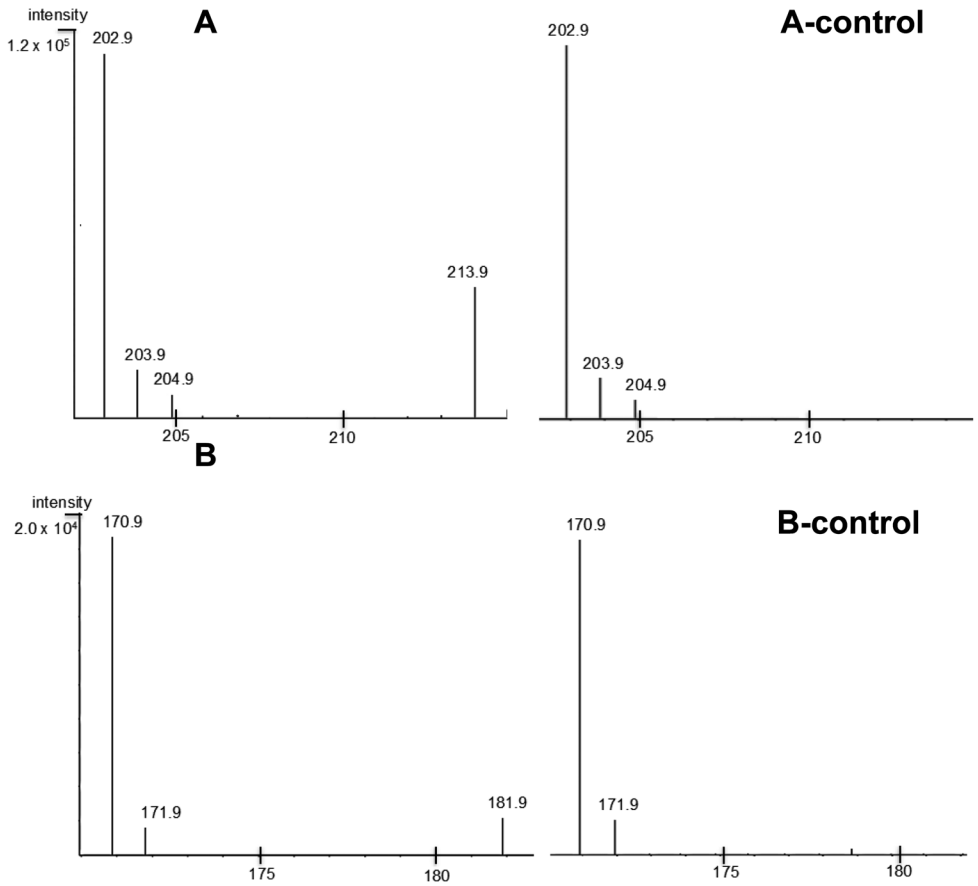


Fig. 2 ESI-MS spectra of: A, rapalexin A (**10**) $[M - 1]^-$ = 202.9, $[M - 1 + 1]^-$ = 203.9, $[M - 1 + 2]^-$ = 204.9, $[M - 1 + 11]^-$ = 213.9, and A-control; B, isocyalexin A (**12**) $[M - 1]^-$ = 170.9, $[M - 1 + 1]^-$ = 171.9, $[M - 1 + 11]^-$ = 181.9, B-control, resulting from incorporation of (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp showing ions corresponding to ^{13}C and ^{15}N enriched and natural abundance (control).

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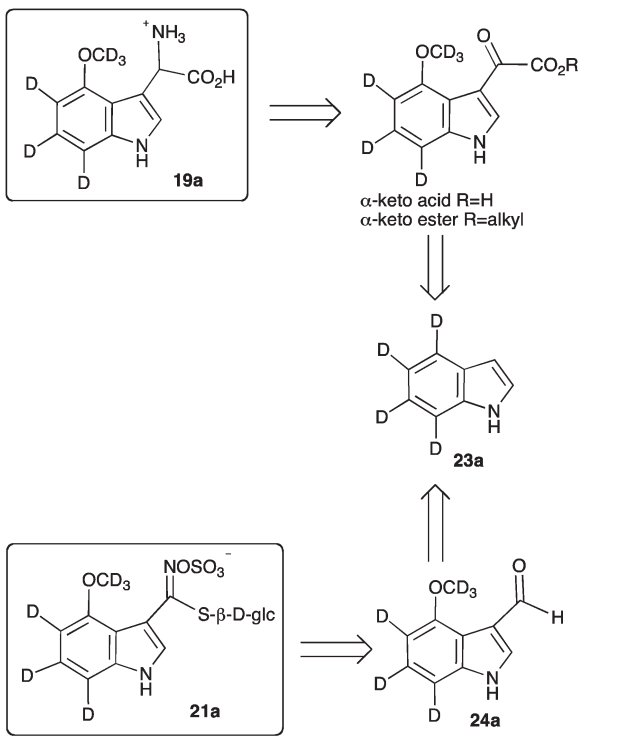
That is, this investigation confirmed previously established biosynthetic relationships among the various phytoalexins biosynthesized in rutabaga roots and other agronomic *Brassica* species.[5]

Synthetic planning and syntheses of perdeuterated intermediates

Excited with the promising results obtained from incorporation of (*S*)-[$^{13}C_{11}$,$^{15}N_2$]Trp, we set out to synthesize 4′-methoxyindolyl-3′-glycine (**19**), 4′-methoxyindole-3′-carboxaldehyde oxime (**20**) and glucorapassicin (**21**), and their perdeuterated counterparts [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindolyl-3′-glycine (**19a**), [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde oxime (**20a**), [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (**24a**) and [$^2H_3$CO,5′,6′,7′-$^2H_3$]glucorapassicin (**21a**). A fairly straightforward retrosynthetic analysis suggested tetradeuterated indole as the starting material for very concise syntheses of **19a** and **21a** *via* **24a** (Scheme 4). However, a concern remained regarding glucorapassicin (**21**), a yet unknown compound having somewhat unpredictable stability, but since the indole nitrogen could be protected, the synthetic challenge seemed solvable.

The synthesis of glucorapassicin (**21**) used *N*-*t*-Boc-4′-methoxyindole-3′-carboxaldehyde oxime (*N*-*t*-Boc-**20**), prepared from *N*-*t*-Boc-4′-methoxyindole-3′-carboxaldehyde, obtained from indole (**23**), following a standard 4-methoxylation procedure.[23] Chlorination of the resulting oxime *N*-*t*-Boc-**20** using *N*-chlorosuccinimide (NCS)[24] was followed by coupling with thioglucose tetraacetate and sulfonation using HSO_3Cl. Deprotection of tetraacetyl glucose with $KOCH_3$ yielded *N*-*t*-Boc-glucorapassicin (**28**), which on standing in either aqueous or organic solvents decomposed to various products over a period of 12–24 hours. Attempts to deprotect *N*-*t*-Boc-glucorapassicin (**28**) also led to extensive decomposition. For this reason, it was investigated whether desulfoglucorapassicin (**27**) could be prepared. Desulfoglucosinolates have been shown to be precursors of glucosinolates (introduction of sulfate is the last biosynthetic step), hence this alternative compound seemed a reasonable choice.[15,25] Deprotection of the indole nitrogen followed by methanolysis of *N*-*t*-Boc-desulfotetraacetyl glucorapassicin using $KOCH_3$ yielded desulfoglucorapassicin (**27**). Fortunately, desulfoglucorapassicin (**27**) was stable in aqueous solutions and in organic solvents, hence was judged satisfactory to use in feeding experiments. Synthesis of the corresponding hexadeuterated compound was carried out similarly, using *N*-*t*-Boc-**20a** (*N*-*t*-Boc-4′-methoxyindole-3′-carboxaldehyde oxime). The *t*-Boc deprotection step was carried out with deuterated TFA because the 4′-methoxy group caused exchange of indole deuterons ^{2}H-5′ and ^{2}H-7′ with protons of TFA (Scheme 5).



Scheme 4 Retrosynthetic analysis of [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindolyl-3′-glycine (**19a**) and [$^2H_3$CO,5′,6′,7′-$^2H_3$]glucorapassicin (**21a**).

The synthesis of indolyl-3′-glycine (**29**) was previously carried out by coupling indole (**23**) with oxalyl chloride followed by hydrolysis, oximation and reduction.[26,27] Following a similar route and starting with tetradeuteroindole (**23a**), [4′,5′,6′,7′-$^2H_4$]indolyl-3′-glycine (**29a**) was readily prepared. Nonetheless, synthesis of the corresponding [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindolyl-3′-glycine (**19a**) required a route modification because the starting material, hexadeuterated 4-methoxyindole, was not readily accessible. First, it was anticipated that the α-keto acid or α-keto ester **25a** might direct methoxylation of the indole nucleus to C-4′. Although the reaction did not work with the α-keto acid, the corresponding α-keto ester **25a** afforded the desired 4′-methoxyindolyl α-keto acid upon standard 4-methoxylation conditions applicable to indole derivatives.[23] Next, oximation of the α-keto acid yielded **26a**, which was reduced with $NaBH_4/NiCl_2$ to afford the desired amino acid **19a** (Scheme 5). In the first instance, synthesis of both enantiomers of **19a** was deemed unnecessary because the racemic form would provide 50% of the desired isomer. Additional perdeuterated compounds (Table 1, Fig. 3) used in feeding experiments described below were synthesized as reported in the Experimental section.

Feeding experiments and determination of deuterium incorporation

Feeding experiments were carried out with all deuterated compounds shown in Table 1 and the corresponding natural abundance compounds prepared using similar methods, to ensure that biotransformations were carried out with comparable materials. Rutabaga roots were elicited and incubated with aqueous solutions of perdeuterated compounds and solutions of the corresponding non-deuterated compounds, as described in the Experimental section. The percentages of deuterium incorporation into each metabolite were determined by

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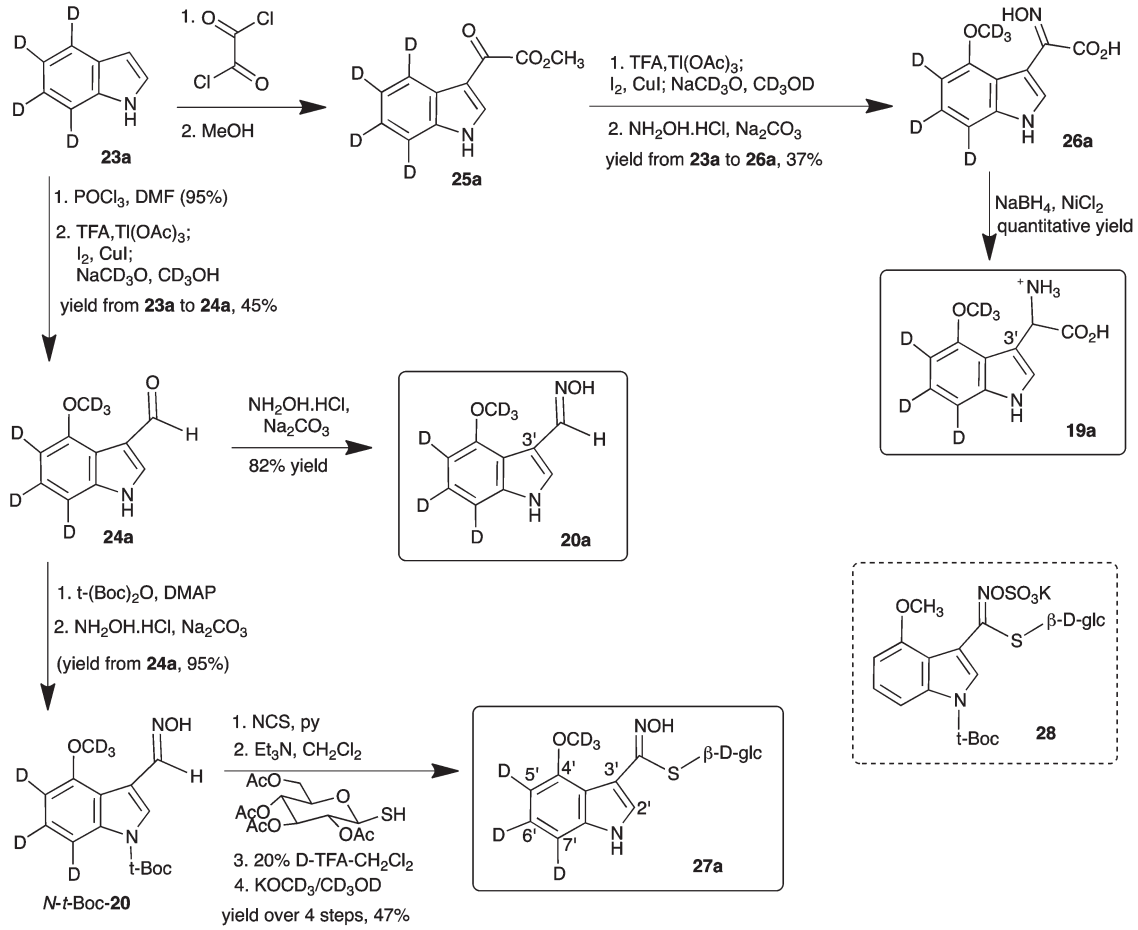

Scheme 5 Synthesis of perdeuterated precursors **19a**, **20a**, **24a** and **27a** (compound **28** decomposed upon standing in solution).

Table 1 Perdeuterated compounds used in precursor feeding experiments

Deuterated compound (origin)	Compound number or abbreviation
(*S*)-[U-$^{13}C_{11}$,U-$^{15}N_2$]Trp (commercial)	(*S*)-[$^{13}C_{11}$, $^{15}N_2$]Trp
[2,2,4′,5′,6′,7′-$^2H_6$]Glucobrassicin (synthetic)[19]	**14a**
(*R*,*S*)-[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-Methoxyindolyl-3′-glycine (this work)	**19a**
[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-Methoxyindole-3′-carboxaldehyde oxime (this work)	**20a**
[$^2H_3$CO]-4′-Methoxyindole-3′-carboxaldehyde (this work)	**24b**
[$^2H_3$CO,5′,6′,7′-$^2H_3$]Desulfoglucorapassicin (this work)	**27a**
(*R*,*S*)-[4′,5′,6′,7′-$^2H_4$]Indolyl-3′-glycine (this work)	**29a**
[4′,5′,6′,7′-$^2H_4$]Indole desulfoglucosinolate (this work)	**30a**
[4′,5′,6′,7′-$^2H_4$]Indole-3′-carboxaldehyde oxime (this work)	**31a**
[$^2H_3$CO]-4-Methoxyindole (this work)	**32a**
[$^2H_3$CO]-4′-Methoxyindolyl-3′-acetonitrile (this work)	**33a**
[4′,5′,6′,7′-$^2H_4$]Desulfoglucobrassicin (this work)	**34a**

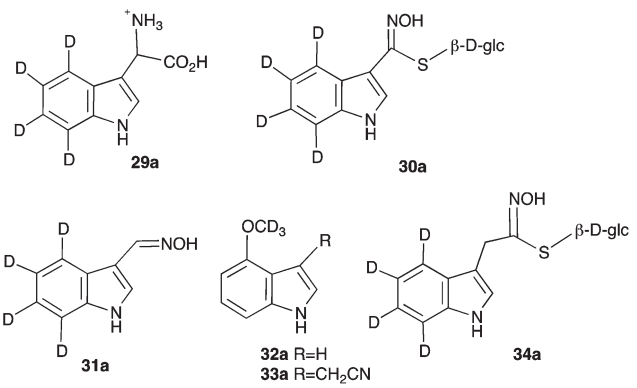

Fig. 3 Chemical structures of perdeuterated compounds used in feeding experiments.

analysis of data obtained by HPLC-ESI-MS (positive or negative ion modes), as described in the Experimental section and Tables S1–S3 (ESI†).

Incorporations of perdeuterated 4′-methoxyindolyl-3′-glycine (19a), 4′-methoxyindole-3′-carboxaldehyde oxime (20a) and desulfoglucorapassicin (27a)

Perdeuterated compounds 4′-methoxyindolyl-3′-glycine (**19a**), 4′-methoxyindole-3′-carboxaldehyde oxime (**20a**) and desulfoglucorapassicin (**27a**) were added to rutabaga root slices, roots were incubated, extracted and the extracts analyzed by

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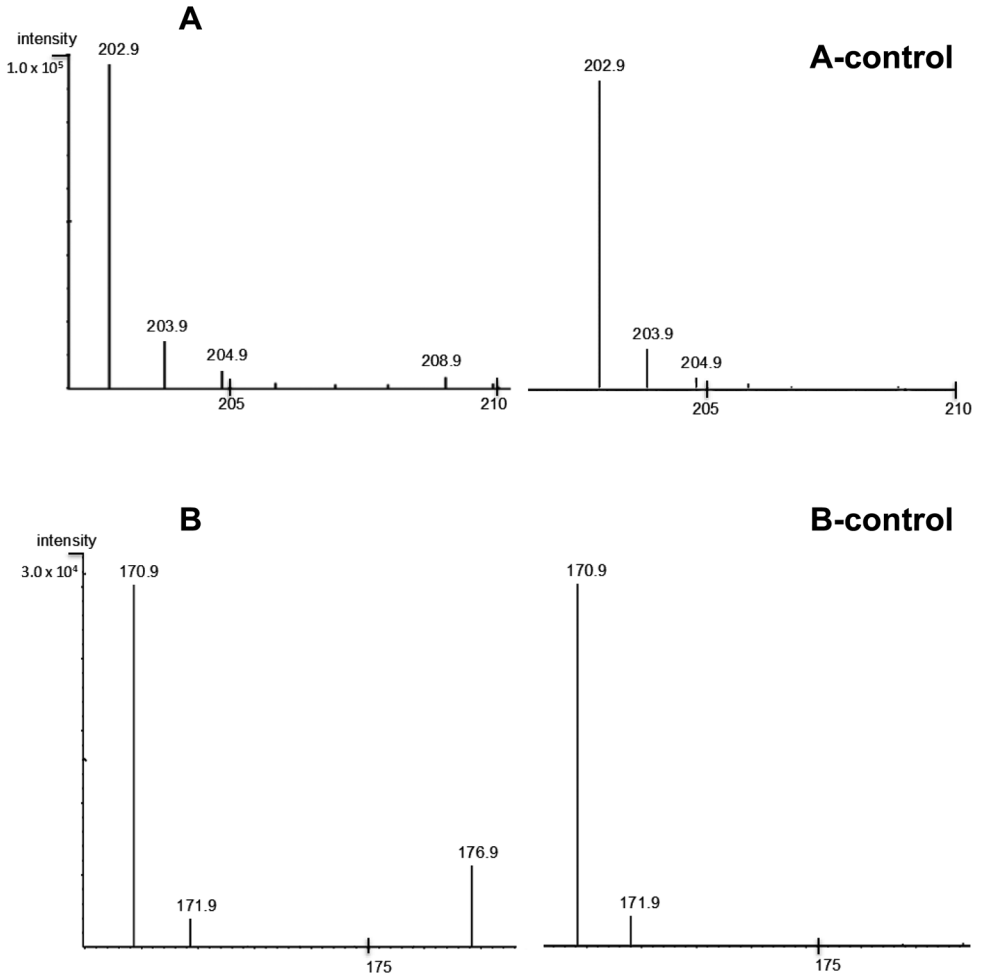

Fig. 4 ESI-MS spectra of: A, rapalexin A (**10**) $[M - 1]^-$ = 202.9, $[M - 1 + 1]^-$ = 203.9, $[M - 1 + 2]^-$ = 204.9, $[M - 1 + 6]^-$ = 208.9, A-control; B, isocyalexin A (**12**) $[M - 1]^-$ = 170.9, $[M - 1 + 1]^-$ = 171.9, $[M - 1 + 6]^-$ = 176.9, B-control, resulting from incorporation of (*R*,*S*)-[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindolyl-3′-glycine (**19a**) showing ions corresponding to deuterated and natural abundance (control).

HPLC-DAD-ESI-MS. From the amounts of deuterium present in each phytoalexin, it was concluded that the amino acid **19a** and the oxime **20a** were both incorporated into rapalexin A (**10a**), isocyalexin A (**12a**) and isalexin (**13a**), while desulfoglucorapassicin (**27a**) was incorporated into rapalexin A (**10a**) and isalexin (**13a**), but not into isocyalexin A (**12a**) (Fig. 4–6, Table S2, ESI†). Similarly conducted experiments using the perdeuterated and non-methoxylated counterparts, *i.e.* indolyl-3′-glycine (**29a**), indole-3′-carboxaldehyde oxime (**31a**) and indole desulfoglucosinolate (**30a**), followed by HPLC-DAD-ESI-MS analyses of extracts showed that these compounds were not incorporated into rapalexin A (**10a**), isocyalexin A (**12a**), isalexin (**13a**) or any of the metabolites shown in Table S2 (ESI†).

These results indicate that 4′-methoxyindolyl-3′-glycine (**19a**) is a precursor of phytoalexins with carbon scaffold type **B**: rapalexin A (**10a**), isocyalexin A (**12a**) and isalexin (**13a**). Furthermore, desulfoglucorapassicin (**27a**) is a precursor of rapalexin A (**10a**) and isalexin (**13a**), but is not a precursor of isocyalexin A (**12a**), that is **12a** is formed upstream of **27a** (Scheme 6). As expected, none of the metabolites with scaffold types **A** or **C** incorporated any of these perdeuterated metabolites (Table S2, ESI†). In addition, similarly conducted feeding experiments using [$^2H_3$CO]-4-methoxyindole (**32a**), [$^2H_3$CO]-4′-methoxyindole-3′-carboxaldehyde (**24b**) and [$^2H_3$CO]-4′-methoxyindolyl-3′-acetonitrile (**33a**) followed by HPLC-DAD-ESI-MS analyses of the tissues indicated that these compounds were not incorporated into any of the metabolites shown.

Incorporations of perdeuterated glucobrassicin (14a) and desulfoglucobrassicin (34a)

Previously, we have demonstrated that administration of hexadeuterated glucobrassicin (**14a**) led to incorporation of several deuterium atoms into both wasalexins A (**5a**) and B (**6a**) and into methoxyglucobrassicins **15a** and **16a**.[19] In this work, although only a maximum of three deuteria from hexadeuterated glucobrassicin (**14a**) could be incorporated into rapalexin A (**10a**), isocyalexin A (**12a**) and isalexin (**13a**), the availability of hexadeuterated glucobrassicin (**14a**) was crucial to

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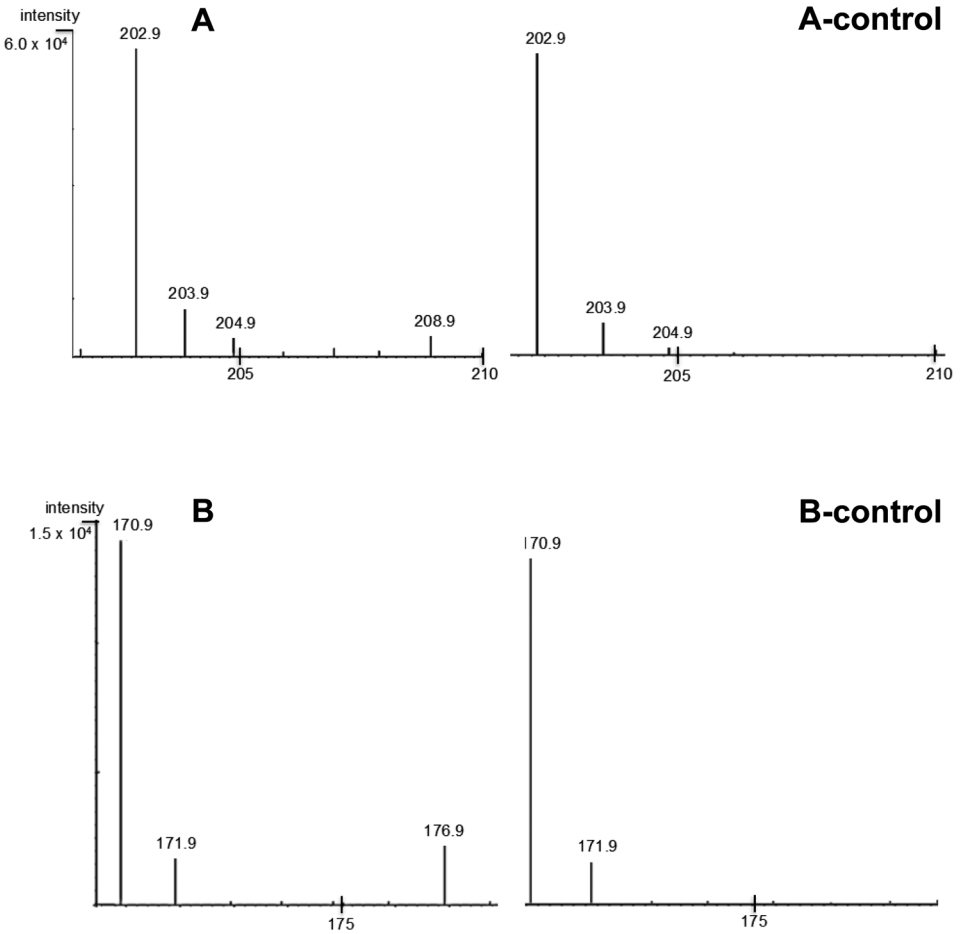

Fig. 5 ESI-MS spectra of: A, rapalexin A (**10**) $[M-1]^-$ = 202.9, $[M-1+1]^-$ = 203.9, $[M-1+2]^-$ = 204.9, $[M-1+6]^-$ = 208.9, A-control; B, isocyalexin A (**12**) $[M-1]^-$ = 170.9, $[M-1+1]^-$ = 171.9, $[M-1+6]^-$ = 176.9, B-control, resulting from incorporation of [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde oxime (**20a**) showing ions corresponding to deuterated and natural abundance (control).

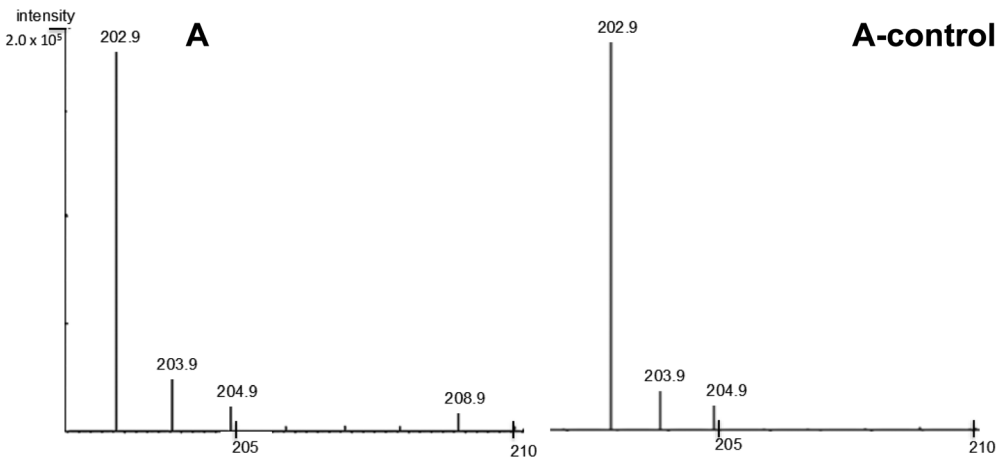

Fig. 6 ESI-MS spectra of: A, rapalexin A (**10**) $[M-1]^-$ = 202.9, $[M-1+1]^-$ = 203.9, $[M-1+2]^-$ = 204.9, $[M-1+6]^-$ = 208.9, A-control, resulting from incorporation of [$^2H_3$CO,5′,6′,7′-$^2H_3$]desulfoglucorapassicin (**27a**) showing ions corresponding to deuterated and natural abundance (control); ESI-MS peaks corresponding to isocyalexin A (**12**) indicated no incorporation of deuterium.

distinguish between two potential biosynthetic routes to phytoalexins of carbon scaffold **A** (brassinins, cyclobrassinins, *etc.*): degradation of the side chain of **14a** followed by metabolic recycling of [4,5,6,7-$^2H_4$]indole (**23a**) to (*S*)-[4′,5′,6′,7′-$^2H_4$]Trp to yield tetradeuterated metabolites, or direct incorporation of **14a** to yield hexadeuterated

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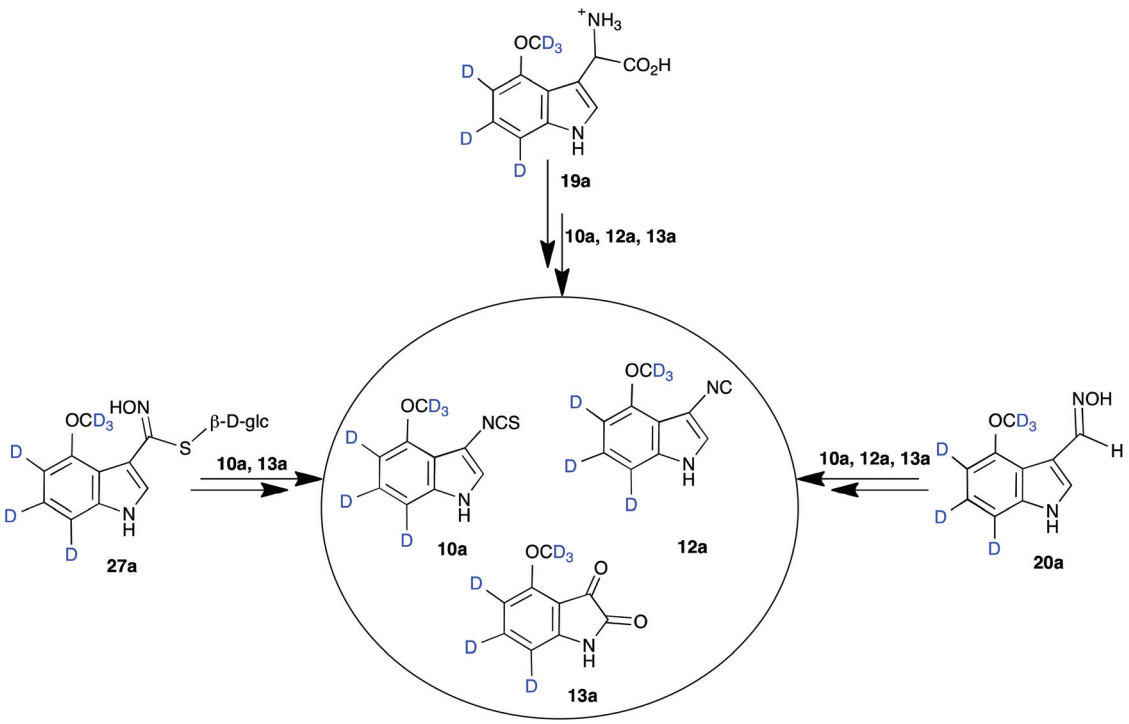
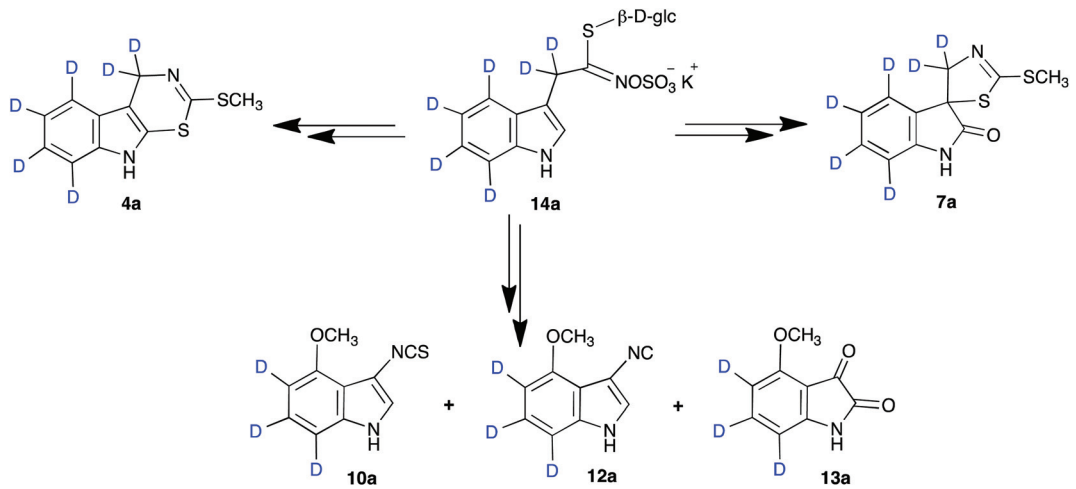


Scheme 6 Incorporation of perdeuterated compounds **19a**, **20a** and **27a** into rapalexin A (**10**), isocyalexin A (**12**) and isalexin (**13**) in rutabaga roots (determined by HPLC-ESI-MS).



Scheme 7 Incorporation of perdeuterated glucobrassicin (**14a**) into spirobrassinin (**7a**), cyclobrassinin (**4a**), rapalexin A (**10a**), isocyalexin A (**12a**) and isalexin (**13a**) in rutabaga roots (determined by HPLC-ESI-MS).

metabolites. To establish if any of these alternative routes was operating, [2,2,4′,5′,6′,7′-$^{2}H_6$]glucobrassicin (**14a**) was synthesized and added to prepared slices of rutabaga roots. After incubation and extractions, fractions were analyzed by HPLC-DAD-ESI-MS and the percentages of deuterium content were determined (Scheme 7, Table S3, ESI†). The presence of six deuteria in phytoalexins with carbon scaffold **A** (cyclobrassinin and spirobrassinin) demonstrated that **14a** was incorporated intact into these phytoalexins (Scheme 7 and Table S3, ESI†). Similarly, three deuteria of hexadeuterated glucobrassicin (**14a**) were present in rapalexin A (**10a**), isocyalexin A (**12a**) and isalexin (**13a**), demonstrating that **14a** was also incorporated into phytoalexins of scaffold type **B**. Furthermore, considering the substantial percentages of deuterium incorporation from glucobrassicin (**14a**) into both 1-methoxyglucobrassicin (**15a**) and 4-methoxyglucobrassicin (**16a**), the precursor relationship was once more confirmed.[19] Similarly, [4′,5′,6′,7′-$^{2}H_4$]desulfoglucobrassicin (**34a**) was synthesized and added to prepared slices of rutabaga roots, as reported above for **14a**. After incubation, extractions, and analyses using

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HPLC-DAD-ESI-MS, the percentages of deuterium content were determined (Table S3, ESI†). The presence of four deuteria in phytoalexins of carbon scaffold **A** (cyclobrassinin (**4a**) and spirobrassinin (**7a**)) and three deuteria in rapalexin A (**10a**) and isocyalexin A (**12a**) demonstrated that **34a** was incorporated into phytoalexins of scaffold types **A** and **B**. Furthermore, **34a** was incorporated into both 1-methoxyglucobrassicin (**15a**) and 4-methoxyglucobrassicin (**16a**) (Table S3, ESI†).

Discussion

The biosynthetic pathway to rapalexin A (**10**), isocyalexin A (**12**) and isalexin (**13**) starting from the primary building block (*S*)-Trp can be proposed as shown in Scheme 8. This pathway is based on incorporations of labeled Trp and perdeuterated compounds shown in Table 1 and Fig. 3. The key step in the biosynthetic puzzle of phytoalexins with carbon scaffold **B** is the transformation of 4-methoxyglucobrassicin (**16**) to 4′-methoxyindolyl-3′-glycine (**19**), for which we propose a Neber-type rearrangement *via* an azirine intermediate.[28] By analogy to the base-catalysed chemical hydrolysis of benzylglucosinolate to phenyl glycine,[28] it is proposed that glucosinolate **15** rearranges under enzymatic catalysis involving formation of the thioglucose azirine intermediate **35** followed by hydrolysis to the novel 4-methoxyindolyl containing amino acid **19**. Transformation of 4′-methoxyindolyl-3′-glycine (**19**), the central piece of this puzzle, to glucorapassicin (**21**) is proposed to follow a biosynthetic pathway similar to that of glucosinolates.[15] First, oxidative decarboxylation of **19** to oxime **20**, followed by oxidation to the nitrile oxide **36** and introduction of sulfur likely *via* glutathione (GST) is proposed. Next, hydrolysis of the glutamyl residue of GST followed by lyase mediated C–S cleavage would lead to thiohydroximic acid **37**. Glucosylation of **37** (likely the K salt) to yield desulfoglucorapassicin (**27**) followed by sulfation would yield glucorapassicin (**21**). Lossen-type rearrangement of **21** *via* **39** can lead to rapalexin A (**10**), similar to the rearrangement of **40** to isothiocyanate **41**, *en route* to brassinin (**1**). Finally, oxidation of rapalexin A (**10**) can lead to isalexin (**13**). The fact that perdeuterated compounds **14**, **19a**, **20a** and **27a** were incorporated into rapalexin A (**10**) and isalexin (**13**) strongly supports this hypothesis. Furthermore, since the non-methoxylated perdeuterated compounds indolyl-3′-glycine (**29a**), indole desulfoglucosinolate (**30a**) and indole-3′-carboxaldehyde oxime (**31a**) were not incorporated, it is likely that the enzymes downstream from 4′-methoxyindolyl-3′-glycine (**19**) are selective. The biosynthetic pathway to isocyalexin A (**12**) is interesting in its own right, rather different from the biosynthesis of the isocyanide functional group in other organisms.[29] Hence, the biosynthesis of isocyalexin A (**12**) is proposed to involve sulfation of oxime **20** followed by a Beckmann-type rearrangement, because it incorporated **19a** and **20a**, but did not incorporate desulfoglucorapassicin (**27a**) or the non-methoxylated counterparts. A pertinent question could be asked about the chemical stability of glucorapassicin (**21**) since its synthesis could not be completed (the indole deprotection step failed) possibly due to its low chemical stability. However, this reasoning may not be applicable to the chemical stability of glucorapassicin (**21**) in plant cells, since the conditions are quite different. Detailed steps of the transformation of Trp to glucobrassicins **14** and **16** are not shown in Scheme 8, as the glucosinolate pathway has been the subject of extensive studies reviewed recently.[14,15,30] Nonetheless, it is pertinent to stress that our data confirm that all carbon (except for the methyl group of **16**) and nitrogen atoms of glucobrassicins **14–16** derive from (*S*)-Trp.

Although our proposed metabolic map of rapalexin A (**10**), isocyalexin A (**12**) and isalexin (**13**) is complex (Scheme 8), considering that most of the enzymes of the glucosinolate pathway have been cloned,[15] this knowledge should facilitate the isolation of the corresponding genes involved in the biosynthesis of phytoalexins with scaffold **B**. Specially because rapalexin A is resistant to degradation by the blackleg fungus,[31] one of the major pathogens of oilseed *Brassica* species, this phytoalexin is likely to motivate great interest in breeding programs aiming to generate plants resistant to blackleg disease.

Overall, it appears that the important difference between the pathways of phytoalexins with scaffolds **A** or **B** lies in the stability of isothiocyanates **10** and **41**; while isothiocyanate **10** is chemically stable, **41** is not. As it stands, this difference could directly influence the number/variety of phytoalexins having scaffolds **A** and **B**. Furthermore, the discovery of this new pathway derived from a new glucosinolate suggests that other aromatic glucosinolates might be precursors of novel cruciferous phytoalexins. For example, benzyl glucosinolates might be precursors of yet unknown phytoalexins containing the benzyl scaffold and functional groups similar to those found in scaffolds **A** and **B**. Hence, to evaluate the diversity and evolution of phytoalexin structures, future work should include additional plant families of the order Brassicales[32] known to produce aromatic glucosinolates other than those containing indole. It would only be surprising if novel structures were not discovered, as metabolic pathways of plants are highly evolvable.[33,34]

Previously, it was shown that the callose defence response in *A. thaliana* triggered by a microbial associated molecular pattern (Flg22, a synthetic 22-amino acid peptide) involved GST, ascorbate, cadmium, and 4-methoxyglucobrassicin (**16**). That is, callose deposition in *A. thaliana*, an innate immune response of plants to pathogens, requires the simultaneous induction of three pathways, including 4-methoxylation (oxidation followed by methylation) of glucobrassicin (**14**) and hydrolysis of 4-methoxyglucobrassicin (**16**).[35] Importantly, independent work confirmed these findings and established that only metabolite X derived from 4-methoxyglucobrassicin (**16**) was important for the restriction of fungal growth.[36] Efforts to determine the structure of this metabolite X did not lead to a concrete structure, but a reference was made to the potential involvement of cruciferous phytoalexins. Considering that the steps required for callose deposition and formation of metabolite X are also required for the biosynthesis of

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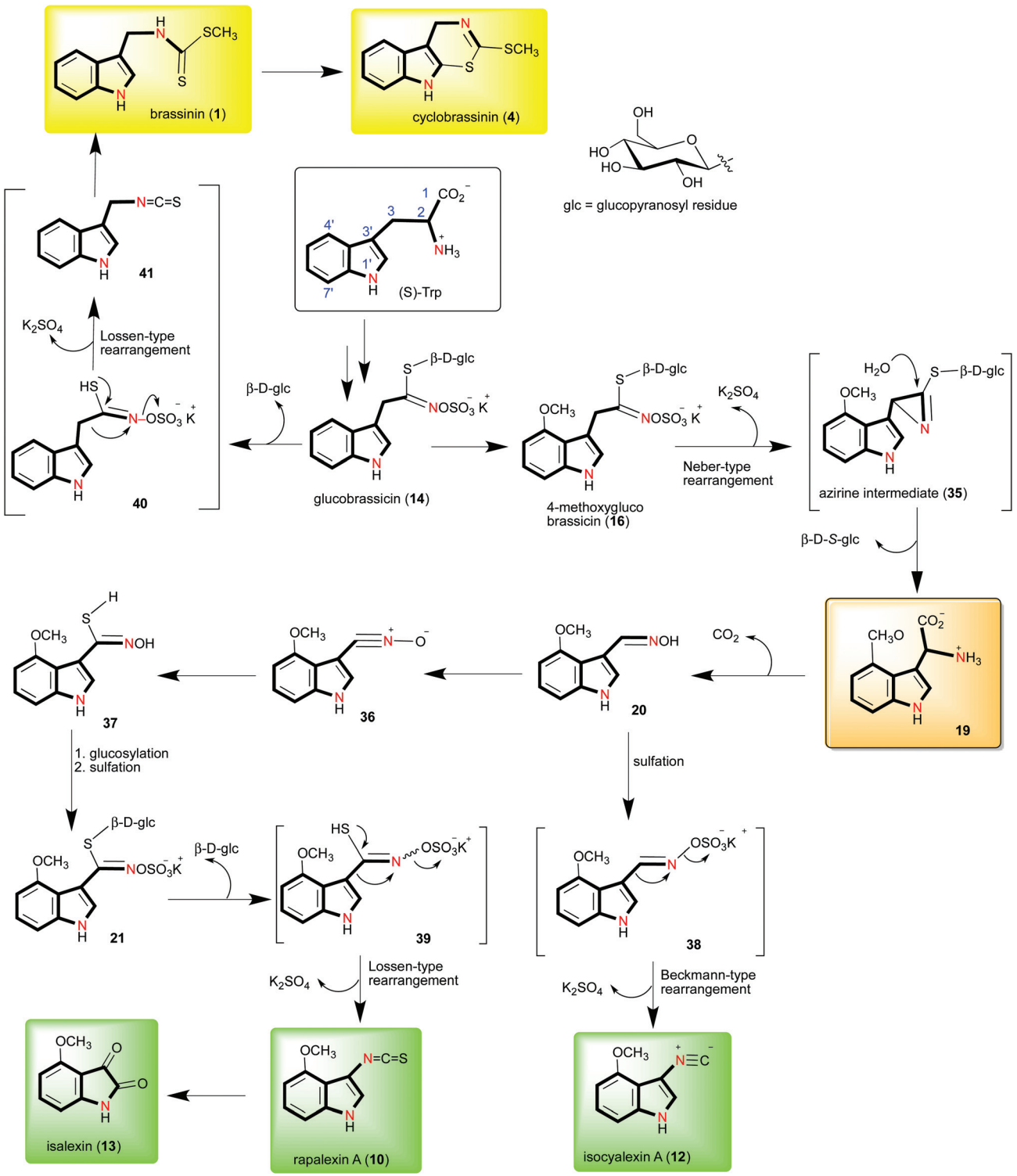

Scheme 8 Biosynthetic pathway of phytoalexins rapalexin A (**10**), isocyalexin A (**12**) and isalexin (**13**) and biosynthetic relationships of glucobrassicin (**14**) with brassinin (**1**) and cyclobrassinin (**4**).

4′-methoxyindolyl-3′-glycine (**19**), rapalexin A (**10**), isocyalexin A (**12**) and isalexin (**13**), it is possible that one of these compounds is either metabolite X or a precursor. Although we have previously detected rapalexin A in *A. thaliana*,[9] we cannot confirm this hypothesis as the phytoalexin profiles of rutabaga are more complex than those of *A. thaliana*.

As a final point, it is important to stress that breeding strategies to enhance natural resistance traits of plants to microbial

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pathogens can be improved if detailed metabolic maps of defence pathways are available. Yet, among the 45 cruciferous phytoalexins reported thus far, only genes of the camalexin (**8**) pathway in the model plant *A. thaliana* have been cloned.[5] To facilitate similar discoveries within economically important crops, we have investigated the potential precursors of the phytoalexins rapalexin A, isocyalexin A and isalexin using numerous perdeuterated compounds, and proposed for the first time their detailed biosynthetic pathway. We have shown that the novel amino acid 4′-methoxyindolyl-3′-glycine is the central piece of this puzzle, filling an important void in the cruciferous phytoalexin biosynthetic conundrum. The stage is set to facilitate the discovery of the corresponding enzymes and genes of the biosynthetic pathway of cruciferous phytoalexins of scaffolds **A** and **B**.

Experimental

General experimental

All solvents were HPLC grade and used as such, except for those used in chemical syntheses, as noted. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Labeled (*S*)-Trp containing U-$^{13}C_{11}$, 97–99%; U-$^{15}N_2$, 97–99% was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. Rutabagas were purchased from local markets throughout the year, no substantial seasonal changes were observed.

Flash column chromatography (FCC): silica gel, grade 60, 230–400 μm. Organic extracts were dried over Na_2SO_4 and the solvents were removed using a rotary evaporator.

NMR spectra were recorded on Bruker Avance 500 MHz spectrometers. For ^{1}H NMR (500 MHz) and ^{13}C NMR (125.8 MHz) spectra, the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS; spectra were calibrated using the solvent peak; spin coupling constants (J) are reported to the nearest 0.5 Hz. Fourier transform infrared (FT-IR) data were recorded on a spectrometer and spectra were measured by the diffuse reflectance method on samples dispersed in KBr. MS [high resolution (HR), electron impact (EI)] were obtained on a VG 70 SE mass spectrometer employing a solids probe.

HPLC analyses and calculation of deuterium incorporation

HPLC-DAD-ESI-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separations were carried out at room temperature using an Eclipse XDB-C-18 column (5 μm particle size silica, 150 mm × 4.6 mm I.D.). The mobile phase consisted of a linear gradient of: Method A (used with non-polar extracts), in H_2O (with 0.2% HCO_2H)–CH_3CN (with 0.2% HCO_2H) from 75 : 25 to 25 : 75 in 25 min, to 0 : 100 in 5 min and a flow rate of 1.0 mL min^{-1}; method B (used with polar extracts), H_2O (with 0.2% HCO_2H)–CH_3CN (with 0.2% HCO_2H) from 90 : 10 to 50 : 50 in 25 min and a flow rate of 1.0 mL min^{-1}. Data acquisition was carried out in positive and negative polarity modes in a single LC run, and data processing carried out with Agilent Chemstation Software. Samples were dissolved in CH_3CN or CH_3CN–H_2O.[19] Additional conditions are as reported in ESI.†

The percentage of deuterium incorporation into each metabolite was established from analysis of data obtained by HPLC-ESI-MS (positive or negative ion modes) using the expressions shown in footnotes to each table presented in ESI† (*e.g.*, % of ^{2}H incorporation = $\{[M \pm 1 + n]^{+/-}/([M \pm 1]^{+/-} + [M \pm 1 + n]^{+/-})\} \times 100$ (n = 3, 4, 6), where n is the number of deuterium atoms, and M is the peak intensity of the quasi-molecular ion peak $[M \pm 1]^{+/-}$); the normalized peak intensities of deuterated and corresponding non-deuterated compounds were identical; unless otherwise stated, the ions $[M \pm 1 + n]^{+/-}$ were not detected in natural abundance samples.

Compound synthesis and characterization

All compounds gave satisfactory spectroscopic data; in each case the percentage of deuterium in the synthetic compound was ≥97%. All deuterated compounds have the additional letter **a** (*e.g.* **19a**), except for [$^2H_3$CO]-4′-methoxyindole-3′-carboxaldehyde (**24b**).

(*R*,*S*)-[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-Methoxyindolyl-3′-glycine (19a)

Oxalyl chloride (52 μL, 0.60 mmol) was added to a solution of [4,5,6,7-$^2H_4$]indole (60 mg, 0.50 mmol) in anhydrous diethyl ether (2 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C, was filtered and the residue was washed with ice cold ether and concentrated under reduced pressure to yield crude [4′,5′,6′,7′-$^2H_4$]indole-3′-oxalylchloride quantitatively. [4′,5′,6′,7′-$^2H_4$]Indole-3′-oxalylchloride (100 mg, 0.48 mmol) was dissolved in MeOH (2 mL) and stirred at rt for 1 h. The solvent was evaporated under reduced pressure to yield methyl indolyl-3′-oxoacetate quantitatively. Thallium trifluoroacetate (TTFA, 209 mg, 0.38 mmol) was added to a solution of methyl indolyl-3′-oxoacetate (53 mg, 0.26 mmol) in TFA (2 mL) and stirred for 15 h at 60 °C. The solvent was evaporated under reduced pressure, DMF (2 mL), I_2 (195 mg, 0.77 mmol) and CuI (195 mg, 1.0 mmol) were added to the reaction mixture and stirred for 1 h at 25 °C. $NaOCD_3$ (prepared from Na, 400 mg, 17.4 mmol, in anhydrous CD_3OD, 3 mL) was added to the reaction mixture and refluxed at 110 °C for 1 h. The reaction mixture was acidified with HCl (1 M, pH 3), filtered through celite and extracted with EtOAc. The organic layer was dried and concentrated. The crude product was subjected to FCC (CH_2Cl_2–MeOH–AcOH, 80 : 20 : 1) to yield [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4-methoxyindolyl-3′-oxo acid (0.090 mmol, 22 mg, 37%). A solution of NH_2OH·HCl (13 mg, 0.19 mmol) and Na_2CO_3 (10 mg, 0.09 mmol) in water (1 mL) was added to a solution of [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4-methoxyindolyl-3′-oxo acid (21 mg, 0.090 mmol) in EtOH (2 mL). The reaction mixture was stirred for 3 h at 70 °C, EtOH was removed under reduced pressure and the residue was washed with CH_2Cl_2. The residue

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was dissolved in water (10 mL), acidified with HCl (1 M, pH 3) and extracted with EtOAc. The organic extract was dried and concentrated to afford crude [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4-methoxyindolyl-3′-oximino acid quantitatively. $NiCl_2{\cdot}6H_2O$ (11 mg, 0.05 mmol) in EtOH (0.5 mL) was added to a solution of [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4-methoxyindolyl-3′-oximino acid (11 mg, 0.05 mmol) in EtOH (1 mL) followed by addition of $NaBH_4$ (5.2 mg, 0.12 mmol) over a 15 min period. The reaction mixture was stirred at rt for 48 h, acidified (HCl, 1 M) and the solvent was removed. The residue was extracted with MeOH and the extract was concentrated to yield [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4-methoxyindolyl-3′-glycine (**19a**) quantitatively (21 mg, 0.090 mmol). The corresponding non-deuterated compound **19** was prepared similarly but using non-deuterated materials.

Compound 19. HPLC t_R = 6.8 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 265. FTIR (KBr, cm^{-1}) ν_{max} 3181, 2928, 1624, 1504, 1466, 1362, 1246, 1088, 731. ^{1}H NMR (500 MHz, CD_3OD) δ 7.11 (1H, s), 6.97 (1H, dd, J = 8, 8 Hz), 6.91 (1H, d, J = 8 Hz), 6.46 (1H, d, J = 7.5 Hz), 5.03 (1H, s), 3.83 (3H, s). ^{13}C NMR (125.8 MHz, CD_3OD) δ 173.9, 154.5, 139.9, 125.7, 124.2, 117.4, 109.9, 106.6, 100.9, 55.9, 53.9. HR-ESI-MS m/z: $[M + H - NH_2]^+$ 204.0662, calcd for $C_{11}H_{10}NO_3$ 204.0665. HPLC-ESI-MS m/z $[M - H]^-$ 219.2 (41%), 202.2 (24%), 176.2 (29%), 158.2 (100%).

Compound 19a. HPLC t_R = 6.8 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 265. ^{1}H NMR (500 MHz, CD_3OD) δ 7.43 (1H, s), 5.32 (1H, s). HR-ESI-MS m/z: 210.1033 $[M - NH_2]^+$, calcd for $C_{11}H_4{}^2H_6NO_3$ 210.1031. HPLC-ESI-MS m/z: $[M - H]^-$ 224.9 (34%), 207.9 (47%), 180.9 (38%), 162.9 (100).

[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde oxime (20a) and [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (24a)

Freshly distilled $POCl_3$ (96 μL, 1.02 mmol) was added dropwise to a solution of [4,5,6,7-$^2H_4$]indole (83 mg, 0.69 mmol) in anhydrous DMF (1 mL) at 0 °C. The reaction mixture was stirred for 1 h at rt, basified with aq. NH_3 (28%), diluted with water and extracted with EtOAc. The organic extract was dried and concentrated to yield [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldehyde (98 mg, 0.66 mmol, 95%). A solution of TTFA (260 mg, 0.49 mmol) in TFA (2 mL) was added to [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldehyde (49 mg, 0.33 mmol), the mixture was stirred overnight at 30 °C, and the solvent was removed under reduced pressure. DMF (2 mL), iodine (260 mg, 1.02 mmol) and CuI (260 mg, 1.36 mmol) were added to the residue and the reaction mixture was stirred for 2 h at 25 °C. $NaOCD_3$ (prepared from Na 400 mg, 17.4 mmol in CD_3OD 3 mL) was added to the reaction mixture and the mixture was further stirred at 110 °C for 1 h. The reaction mixture was cooled to room temperature, neutralized (HCl, 1 M) and filtered through celite. The filtrate was diluted with water and extracted with EtOAc. The organic layer was dried, concentrated and the crude product was subjected to FCC (EtOAc–Hex, 3 : 7) to afford [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (**24a**, 27 mg, 0.09 mmol, 45%).

A solution of $NH_2OH{\cdot}HCl$ (15 mg, 0.22 mmol) and Na_2CO_3 (13 mg, 0.12 mmol) in water (1 mL) was added to a solution of [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (**24a**, 20 mg, 0.11 mmol) in MeOH (2 mL) at 60 °C. After 3 h, the solvent was removed, the residue was dissolved in water, extracted with EtOAc, the organic phase was dried, concentrated and subjected to FCC (CH_2Cl_2–MeOH, 98 : 2) to yield [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde oxime (**20a**) (17.6 mg, 0.090 mmol, 82%). The corresponding non-deuterated compound **20** was prepared similarly but using non-deuterated materials.

Compound 20. HPLC t_R = 7.9 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 240, 295. FTIR (KBr, cm^{-1}) ν_{max}: 3260, 2932, 1620, 1379, 1267, 1100, 1026. ^{1}H NMR (500 MHz CD_3CN) δ 9.68 (1H, brs), 8.23 (1H, brs), 8.22 (1H, s), 8.21 (1H, d, J = 3 Hz), 7.13 (1H, dd, J = 8, 8 Hz), 7.08 (1H, d, J = 8 Hz), 6.64 (1H, d, J = 7.5 Hz), 3.94 (3H, s). ^{13}C NMR δ (125.8 MHz, CD_3CN) 155.5, 146.7, 142.2, 137.7, 131.1, 124.3, 108.0, 106.0, 102.0, 55.9. HR-EI-MS m/z 190.0741 (M^+), calcd for $C_{10}H_{10}N_2O_2$ 190.0742 (32%), 172.06 (100%), 157.04 (65%), 129.05 (72%). HPLC-ESI-MS m/z $[M + H]^+$ 191.0 (100%), 174.0 (11%).

Compound 20a. HPLC-DAD, t_R = 7.9 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 240, 295. ^{1}H NMR (500 MHz CD_3CN) δ 9.68 (1H, brs), 8.23 (1H, s), 8.21 (1H, s). HR-EI-MS m/z 196.1124 (M^+), calcd for $C_{10}H_4{}^2H_6N_2O_2$ 196.1119 (88%), 178.10 (100%), 160.05 (62%), 132.06 (44%). HPLC-ESI-MS m/z: $[M + H]^+$ 197.0 (100%), 180.0 (12%).

[$^2H_3$CO]-4′-Methoxyindole-3′-carboxaldehyde (24b)

A solution of TTFA (280 mg, 0.51 mmol) in TFA (2 mL) was added to indole-3′-carboxaldehyde (50 mg, 0.34 mmol).[23] The mixture was stirred for 3 h at 30 °C and the solvent was removed under reduced pressure. Iodine (260 mg, 1.02 mmol), CuI (260 mg, 1.36 mmol) and DMF (2 mL) were added to the reaction mixture and the mixture was stirred for 1 h at 25 °C. $NaOCD_3$ (prepared from Na 400 mg, 17.4 mmol in CD_3OD, 3 mL) was added and the reaction mixture was further stirred at 110 °C for 1 h. The reaction mixture was cooled, neutralized (HCl, 1 M), filtered through celite, the filtrate was diluted with water and extracted with EtOAc. The organic layer was dried, concentrated and the crude product was subjected to FCC (EtOAc–Hex, 3 : 7) to yield [$^2H_3$CO]-4′-methoxyindole-3′-carboxaldehyde (47 mg, 0.26 mmol, 78%). The corresponding non-deuterated compound **24** was prepared similarly but using non-deuterated materials.

Compound 24. HPLC t_R = 7.3 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 210, 250, 325. FTIR (KBr, cm^{-1}) ν_{max}: 3194, 1718, 1644, 1624, 1512, 1383, 1271, 1100, 781. ^{1}H NMR (500 MHz, CD_3CN) δ 10.41 (1H, s), 10.2 (1H, brs), 7.90 (1H, s), 7.19 (1H, dd, J = 8, 7.5 Hz), 7.14 (1H, d, J = 8 Hz), 6.75 (1H, d, J = 7.5 Hz), 3.95 (3H, s). ^{13}C NMR δ (125.8 MHz, CD_3CN) 188.2, 155.4, 139.1, 129.8, 125.0, 119.9, 116.9, 106.6, 103.4, 56.1. HR-EI-MS m/z 175.0629 (M^+), calcd for $C_{10}H_9NO_2$ 175.0633 (100%), 160.04 (28%), 144.04 (26%), 129.06 (43%). HPLC-ESI-MS m/z $[M - H]^-$ 173.9 (100%), 158.9 (98%).

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Compound 24b. HPLC t_R = 7.3 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 210, 250, 325. ^{1}H NMR (500 MHz, CD_3Cl) δ 10.51 (1H, s), 8.95 (1H, brs), 7.93 (1H, d, J = 3 Hz), 7.22 (1H, dd, J = 8, 8 Hz), 7.09 (1H, d, J = 8 Hz), 6.72 (1H, d, J = 8 Hz). HR-EI-MS m/z 178.0819 (M^+), calcd for $C_{10}H_6{}^2H_3NO_2$ 178.0821 (100%), 145.05 (21%), 131.07 (32%). HR-EI-MS m/z 175.0629 (M^+), calcd for $C_{10}H_9NO_2$ 175.0633 (100%), 160.04 (28%), 144.0. HPLC-ESI-MS m/z: $[M - H]^-$ 176.9 (92%), 158.9 (100%).

[$^2H_3$CO,5′,6′,7′-$^2H_3$]Desulfoglucorapassicin (27a)

(t-Boc)$_2$O (23 mg, 0.19 mmol) and DMAP (2 mg, 0.02 mmol) were added to a solution of [$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (40 mg, 0.22 mmol) in THF (3 mL) at 0 °C. The reaction mixture was stirred at rt for 2 h, was neutralized (HCl, 1 M), diluted with water and extracted with CH_2Cl_2. The organic phase was dried and concentrated to yield crude N-t-Boc[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (63 mg, 0.22 mmol). A solution of NH_2OH·HCl (31 mg, 0.44 mmol) and Na_2CO_3 (24 mg, 0.22 mmol) in water (1 mL) was added to a solution of t-Boc[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde (63 mg, 0.22 mmol) in MeOH (4 mL) at 60 °C. After 3 h the solvent was removed, the residue was dissolved in water and extracted with CH_2Cl_2. The organic phase was dried, and concentrated to yield crude N-t-Boc-[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde oxime (65 mg, 0.21 mmol, 95%). NCS (16 mg, 0.12 mmol) was added to a solution of N-t-Boc[$^2H_3$CO,5′,6′,7′-$^2H_3$]-4′-methoxyindole-3′-carboxaldehyde oxime (35 mg, 0.12 mmol) in CH_2Cl_2 (1 mL) and pyridine (100 μL) at 0 °C and the reaction mixture was stirred for 30 min at rt. 1-Thio-β-D-glucopyranose-2,3,4,6-tetraacetate (26 mg, 0.060 mmol) in CH_2Cl_2 (200 μL) and Et_3N (50 μL, 0.36 mmol) were added to the reaction mixture and the mixture was stirred for 3 h at rt. The reaction mixture was diluted with water, extracted with CH_2Cl_2, the organic layer was dried, concentrated and the residue was subjected to FCC (EtOAc–Hex, 1 : 1) to yield t-Boc[$^2H_3$CO,5′,6′,7′-$^2H_3$]desulfoglucorapassicin tetraacetate (41 mg, 0.060 mmol, 52%). t-Boc [$^2H_3$CO,5′,6′,7′-$^2H_3$]desulfoglucorapassicin tetraacetate (41 mg, 0.06 mmol) was dissolved in CH_2Cl_2 (3.7 mL) and D-TFA (750 μL) and stirred at rt for 3 h. The reaction mixture was diluted with water and extracted with CH_2Cl_2. The organic layer was dried, concentrated and subjected to FCC (EtOAc–Hex, 1 : 1) to yield [$^2H_3$CO,5′,6′,7′-$^2H_3$]desulfoglucorapassicin tetraacetate (31 mg, 0.05, 92%). Freshly prepared $KOCH_3$ (1 mM, 75 μL) was added to the solution of [$^2H_3$CO,5′,6′,7′-$^2H_3$] desulfoglucorapassicin tetraacetate (31 mg, 0.06 mmol) in anhydrous MeOH (1 mL) and stirred at rt for 30 min. The reaction mixture was neutralized with acetic acid and the solvent was evaporated under reduced pressure to yield [$^2H_3$CO,5′,6′,7′-$^2H_3$]-desulfoglucorapassicin (**27a**) (33 mg, 0.08 mmol) quantitatively. The corresponding non-deuterated compound **27** was prepared similarly but using non-deuterated materials.

Compound 27. HPLC t_R = 9.3 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 270. FTIR (KBr, cm^{-1}) ν_{max}: 3160, 1667, 1405. ^{1}H NMR (500 MHz, CD_3OD) δ 7.32 (1H, s), 7.08 (1H, dd, J = 8, 8 Hz), 7.02 (1H, d, J = 8 Hz), 6.56 (1H, d, J = 7.5 Hz), 4.19 (1H, d, J = 10 Hz), 3.87 (3H, s), 3.45–3.23 (4H, m), 2.99 (1H, dd, J = 9, 9 Hz), 2.24–2.21 (1H, m). ^{13}C NMR (125.8 MHz, CD_3OD) δ 155.3, 153.2, 139.0, 127.0, 124.3, 118.7, 107.7, 106.2, 102.1, 85.3, 81.6, 79.8, 73.7, 70.7, 61.9, 56.2. HR-EI-MS m/z 423.0632 $[M + K]^+$, calcd for $C_{16}H_{20}N_2O_7SK$ 423.0622 (100%). HPLC-ESI-MS m/z $[M + H]^+$ 385.0 (100%), 223.0 (38%).

Compound 27a. HPLC t_R = 9.3 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 270. ^{1}H NMR (500 MHz, CD_3OD) δ 7.28 (1H, s), 4.20 (1H, d, J = 10 Hz), 3.43–3.23 (4H, m), 2.98 (1H, dd, J = 9, 9 Hz), 2.24–2.21 (1H, m). HR-MS-ESI m/z 429.1009 $[M + K]^+$, calcd for $C_{16}H_{14}{}^2H_6N_2O_7SK$ 429.1004 (100%), 391.1440 $[M + 1]^+$ (82%), 229.0 (17%), 201 (36%). HPLC-ESI-MS m/z $[M + H]^+$ 391.0 (100%), 229.0 (38%).

(*R*,*S*)-[4′,5′,6′,7′-$^2H_4$]Indolyl-3′-glycine (29a)

Oxalyl chloride (52 μL, 0.6 mmol) was added to a solution of [4,5,6,7-$^2H_4$]indole (60 mg, 0.50 mmol) in anhydrous Et_2O (2 mL) at 0 °C, and the reaction mixture stirred for 1 h at 0 °C. The reaction mixture was filtered and the residue was washed with ice cold ether and concentrated under reduced pressure to yield crude [4′,5′,6′,7′-$^2H_4$]indolyl-3′-oxalylchloride in quantitative yield. NaOH (20%, w/v) was added to a suspension of [4′,5′,6′,7′-$^2H_4$]indolyl-3′-oxalylchloride (105 mg, 0.50 mmol) in THF to obtain pH 10. The reaction mixture was stirred at room temperature for 30 min, acidified (HCl, 2 M) and further stirred for 30 min. The reaction mixture was diluted with water, extracted with EtOAc, the organic layer was dried and concentrated to yield [4′,5′,6′,7′-$^2H_4$]indolyl-3′-oxo acid (72 mg, 0.37 mmol, 75% over two steps). A solution of NH_2OH·HCl (108 mg, 1.55 mmol) and NaOAc (211 mg, 1.55 mmol) in water (1 mL) was added to a solution of [4′,5′,6′,7′-$^2H_4$]indolyl-3′-oxo acid (50 mg, 0.26 mmol) in EtOH (2 mL). The reaction mixture was stirred for 3 hours at 70 °C, EtOH was removed under reduced pressure and the residue was washed with CH_2Cl_2. The residue was diluted with water (10 mL), acidified (HCl, 1 M) and extracted with EtOAc. The organic extract was dried and concentrated to yield crude [4′,5′,6′,7′-$^2H_4$]indolyl-3′-oximino acid, which was taken to the next step without further purification. A solution of $NiCl_2$·$6H_2O$ (61 mg, 0.26 mmol) in EtOH (1 mL) was added to a solution of [4′,5′,6′,7′-$^2H_4$]indole-3′-oximino acid (54 mg, 0.26 mmol) in EtOH (3 mL), followed by addition of $NaBH_4$ (29.6 mg, 0.78 mmol) over a 20 min period. The reaction mixture was stirred at rt for 48 h, was acidified with HCl (1 M, pH 3), the solvent was removed under reduced pressure, the residue was extracted with MeOH and the extract was concentrated to yield [4′,5′,6′,7′-$^2H_4$]indolyl-3′-glycine (**29a**) (50 mg, 0.26 mmol) quantitatively. The corresponding non-deuterated compound **29** was prepared similarly but using non-deuterated materials.

Compound 29. HPLC t_R = 6.9 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 275. FTIR (KBr, cm^{-1}) ν_{max}: 3389, 3000, 1737, 1488, 1454, 1426, 1193, 1105, 753. ^{1}H NMR (500 MHz, D_2O) δ 7.68 (1H, d, J = 8 Hz), 7.59 (1H, s), 7.57 (1H, d, J = 8.5 Hz), 7.31 (1H, dd, J = 8, 8 Hz), 7.23 (1H, dd, J = 8, 8 Hz), 5.51 (1H, s). ^{13}C NMR (125.8 MHz, D_2O) δ 171.7, 136.2, 127.0, 124.4, 122.7, 120.4, 117.9, 112.4, 105.0, 49.7. HR-ESI-MS m/z

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174.0556 $[M - NH_2]^+$, calcd for $C_{10}H_8NO_2$ 174.0555 (100%). HPLC-ESI-MS *m/z* $[M - H]^-$ 189.1 (100%), 146.2 (16%).

Compound 29a. HPLC t_R = 6.9 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 275. ^1H NMR (500 MHz, CD_3OD) δ 7.58 (1H, s), 5.50 (1H, s). HR-ESI-MS *m/z* 178.0796 $[M - NH_2]^+$, calcd for $C_{10}H_4{}^2H_4NO_2$ 178.0800 (100%). HPLC-ESI-MS *m/z*: $[M - H]^-$ 193.1 (100%).

[4′,5′,6′,7′-$^2H_4$]Indole desulfoglucosinolate (30a)

To a solution of [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldehyde (39 mg, 0.26 mmol) in EtOH (2 mL), a solution of Na_2CO_3 (28 mg, 0.26 mmol) and $NH_2OH{\cdot}HCl$ (36 mg, 0.52 mmol) in water (1 mL) was added and stirred at 70 °C for 3 h. EtOH was removed, the reaction mixture was diluted with water and extracted with EtOAc. The organic layer was dried and concentrated under reduced pressure to yield the corresponding [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldoxime quantitatively. NCS (27 mg, 0.20 mmol) was added to a solution of [4′,5′,6′,7′-$^2H_4$]-indole-3′-carboxaldoxime (28 mg, 0.17 mmol) in pyridine (100 μL) and CH_2Cl_2 (1 mL) at 0 °C. The reaction mixture was stirred for 30 min at rt, 1-thio-β-D-glucopyranose-2,3,4,6-tetra-acetate (37 mg, 0.10 mmol) in CH_2Cl_2 (0.2 mL) was added, and then Et_3N (71 μL, 0.51 mmol) and the reaction mixture was stirred for 3 h at rt. The reaction mixture was diluted with water (5 mL), extracted with CH_2Cl_2, the organic layer was dried and concentrated. The residue was subjected to FCC (EtOAc–Hex, 1 : 1) to yield the tetraacetyl derivative (20 mg, 0.04 mmol, 22% from oxime). $KOCH_3$ (75 μL, 1 mM) was added to the solution of the tetraacetyl derivative (20 mg, 0.04 mmol) in anhydrous MeOH (1 mL) to obtain pH 9. The reaction mixture was stirred for 30 min and neutralized with acetic acid. The solvent was evaporated, the residue was dissolved in water and freeze dried to yield [4′,5′,6′,7′-$^2H_4$]indole desulfoglucosinolate (**30a**) in quantitative yield (22 mg, 0.06 mmol). The corresponding non-deuterated compound **30** was prepared similarly but using non-deuterated materials. Compound **30** was previously synthesized to be used as internal standards in seed meals, but only the ^1H NMR data were reported.[37]

Compound 30. HPLC t_R = 6.1 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 270. FTIR (KBr, cm^{-1}) ν_{max}: 3270, 2932, 1565, 1413, 1105, 1048, 747. ^1H NMR (500 MHz, CD_3OD) δ 7.83 (1H, d, *J* = 8 Hz), 7.70 (1H, s), 7.39 (1H, d, *J* = 8 Hz), 7.15 (1H, dd, *J* = 7, 8 Hz), 7.08 (1H, dd, *J* = 7, 8 Hz), 4.52 (1H, d, *J* = 10 Hz), 3.66 (1H, dd, *J* = 12, 2 Hz), 3.54 (1H, dd, *J* = 12, 5.5 Hz), 3.29–3.25 (2H, m), 3.13 (1H, dd, 9, 9), 2.74 (1H, m). ^{13}C NMR δ (125.8 MHz, CD_3OD) 149.3, 137.9, 128.7, 127.6, 123.4, 121.6, 121.3, 112.6, 110.4, 86.1, 82.0, 79.6, 74.4, 71.1, 62.5. HR-ESI-MS *m/z* 393.0572 $[M + K]^+$, calcd for $C_{15}H_{19}N_2O_6SK$ 393.0517 (16%). HPLC-ESI-MS *m/z* $[M + 1]^+$ 355.1 (96%), 193.1 (100%).

Compound 30a. HPLC t_R = 6.1 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 270. ^1H NMR δ (500 MHz, CD_3OD) 7.71 (1H, s), 4.54 (1H, d, *J* = 10 Hz), 3.66 (1H, dd, *J* = 12, 2 Hz), 3.56 (1H, dd, *J* = 12, 5 Hz), 3.28–3.15 (2H, m), 3.15 (1H, dd, 9, 9), 2.77 (1H, m). HR-ESI-MS *m/z* 397.0769 $[M + K]^+$, calcd for $C_{15}H_{14}{}^2H_4N_2O_6SK$ 397.0768 (28%), 224.11 (100%). ESI-MS *m/z*: $[M + 1]^+$ 197.1 (100%), 359.1 (96%).

[4′,5′,6′,7′-$^2H_4$]Indole-3′-carboxaldehyde oxime (31a)

Freshly distilled $POCl_3$ (96 μL, 1.02 mmol) was added dropwise to a solution of [4,5,6,7-$^2H_4$]indole (83 mg, 0.69 mmol) in anhydrous DMF (1 mL) at 0 °C. The reaction mixture was stirred for 1 h at room temperature, basified with NH_3 (28%), diluted with water and extracted with EtOAc. The organic extract was dried over Na_2SO_4 and concentrated to yield [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldehyde (98 mg, 0.54 mmol, 95%). A solution of $NH_2OH{\cdot}HCl$ (69.5 mg, 1.0 mmol) and Na_2CO_3 (53 mg, 0.5 mmol) in water (1 mL) was added to a solution of [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldehyde (75 mg, 0.5 mmol) in EtOH (2 mL) at 60 °C. After 3 h the solvent was removed, the residue was dissolved in water and extracted with EtOAc. The organic phase was dried, concentrated and subjected to FCC (CH_2Cl_2–MeOH, 98 : 2) to yield [4′,5′,6′,7′-$^2H_4$]-indole-3′-carboxaldoxime (**31a**) (76 mg, 0.46 mmol, 92%).

Compound 31a. HPLC-DAD, t_R = 6.3 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 230, 260. ^1H NMR (500 MHz CD_3CN) δ 9.76 (1H, brs), 8.25 (1H, d, *J* = 2.5 Hz), 7.73 (1H, s). HR-EI-MS *m/z* 164.0885 (M^+), calcd for $C_9H_4{}^2H_4N_2O$ 164.0887 (20%), 147.08 (100%), 119.07 (35%). HPLC-ESI-MS *m/z* $[M + H]^+$ 165.1 (100%), 148.1 (48%).

[$^2H_3$CO]-4-Methoxyindole (32a)

A solution of 3-nitrophenol (100 mg, 0.70 mmol) in THF (1 mL) was added to a suspension of NaH (24 mg, 1 mmol) in THF (2 mL). The reaction mixture was stirred at room temperature for 10 min, ^2H_3CI (90 μL, 1.4 mmol) was added and the reaction mixture was stirred at 65 °C for 5 hours. The reaction mixture was diluted with water, extracted with CH_2Cl_2, the organic layer was dried and concentrated to yield crude 3-[$^2H_3$C-O]nitrobenzene (112 mg, 0.73 mmol) in quantitative yield. A solution of 3-[$^2H_3$C-O]nitrobenzene (110 mg, 0.70 mmol) and 4-chlorophenyl acetonitrile (127 mg, 0.75 mmol) in DMF (2 mL) was slowly added to a suspension of *t*-BuOK (300 mg, 2.67 mmol) in DMF (1.5 mL) at −20 °C. The reaction mixture was stirred at −20 °C for 2 h, quenched with ice cold HCl (1 M) and extracted with EtOAc. The organic extract was washed with NaOH (2 M), dried and concentrated. The crude reaction mixture was subjected to FCC (EtOAc–Hex 1 : 3) to yield (6-[$^2H_3$C-O]-2-nitrophenyl)acetonitrile (37 mg, 0.19 mmol, 26%). Palladium on carbon (10% Pd/C, 10 mg) was added to the solution of (6-[$^2H_3$CO]-2-nitrophenyl)acetonitrile (50 mg, 0.25 mmol) in MeOH (2.5 mL) and AcOH (244 μL) and the resulting reaction mixture was stirred under a H_2 atmosphere (balloon pressure) at rt. After 12 h, the catalyst was filtered off, and the filtrate was concentrated under reduced pressure. The residue was suspended in sat. $NaHCO_3$ (5 mL) and extracted with CH_2Cl_2. The organic layer was washed with water, dried and concentrated under reduced pressure to yield 4-[$^2H_3$C-O]indole (**32a**, 30.4 mg, 0.20 mmol, 81%). Compound **32** was synthesized similarly but using non-deuterated materials.

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Compound 32. HPLC t_R = 12.5 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 263. Other data as previously reported.[38]

Compound 32a. HPLC t_R = 12.5 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 263. ^{1}H NMR (500 MHz, CD_3Cl) δ 8.16 (1H, brs), 7.13 (1H, dd, J = 8, 7.5 Hz), 7.13 (1H, s), 7.03 (1H, d, J = 8 Hz), 6.67 (1H, broad s), 6.53 (1H, d, J = 7.5 Hz). HR-EI-MS m/z 150.0872 (M^+), calcd for $C_9H_6{}^2H_3NO$ 150.0872 (100%), 132.04 (73%), 104.04 (36%). HPLC-ESI-MS m/z: $[M + H]^+$ 151.1 (100%), 133.0 (44%).

[$^2H_3$CO]-4′-Methoxyindolyl-3′-acetonitrile (33a) (arvelexin)

A solution of TTFA (280 mg, 0.51 mmol) in TFA (2 mL) was added to indole-3′-carboxaldehyde (50 mg, 0.34 mmol). The mixture was stirred for 3 h at 30 °C. Solvent was removed under reduced pressure. Iodine (260 mg, 1.02 mmol), CuI (260 mg, 1.36 mmol) and DMF (2 mL) were added and stirred for 1 h at 25 °C. $NaOCD_3$ (prepared from Na 400 mg, 17.4 mg in CD_3OD 3 mL) was added and the reaction mixture was further stirred at 110 °C for 1 h. The reaction mixture was cooled, neutralized (HCl, 1 M), filtered through celite and the filtrate was diluted with water and extracted with EtOAc. The organic layer was dried, concentrated and the crude product was subjected to FCC (EtOAc–Hex, 3 : 7) to afford [$^2H_3$CO]-4′-methoxyindole-3′-carboxaldehyde (47 mg, 0.26 mmol, 79%). NH_4OAc (14 mg, 0.18 mmol) was added to a solution of [$^2H_3$CO]-4′-methoxyindole-3′-carboxaldehyde (65 mg, 0.36 mmol) in nitromethane (1 mL) and refluxed at 105 °C for 90 min. The reaction mixture was allowed to cool, diluted with water, extracted with CH_2Cl_2 and concentrated to yield crude 4-[$^2H_3$C-O]-3-nitrovinylindole, which was taken to the next step without further purification. $NaBH_4$ (42 mg, 1.1 mmol) was added to a solution of crude [$^2H_3$CO]-4-methoxy-3-nitrovinylindole (79 mg, 0.36 mmol) in THF (3 mL) and MeOH (300 μL). The reaction mixture was stirred for 3 h at rt, after which excess $NaBH_4$ was destroyed by adding ice-cold water to the reaction mixture. The reaction mixture was extracted with CH_2Cl_2, concentrated and subjected to FCC (CH_2Cl_2, 100%) to yield [$^2H_3$CO]-4-methoxy-3-(2′-nitroethyl)indole (27 mg, 0.12 mmol, 34%). Et_3N (324 μL, 2.3 mmol) and CS_2 (72 μL, 1.2 mmol) were added to a solution of [$^2H_3$CO]-4-methoxy-3-(2′-nitroethyl)indole (26 mg, 0.12 mmol) in acetonitrile (1.5 mL) and the reaction mixture was stirred at 40 °C for 20 h. The solvent was removed, the residue was diluted with water (10 mL) and extracted with CH_2Cl_2. The organic layer was dried, concentrated and the residue was subjected to FCC (CH_2Cl_2, 100% to CH_2Cl_2–MeOH, 98 : 2) to yield [$^2H_3$CO]-4′-methoxyindolyl-3′-acetonitrile (**33a**, 14 mg, 0.07 mmol, 62%). The corresponding non-deuterated compound **33** was prepared similarly but using non-deuterated materials.

Compound 33. HPLC t_R = 13.7 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 260. Other data as previously reported.[39]

Compound 33a. HPLC t_R = 13.7 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 220, 260. ^{1}H NMR (500 MHz, CD_3Cl) δ 8.16 (1H, brs), 7.13 (1H, dd, J = 8, 8 Hz), 7.10 (1H, s), 6.98 (1H, d, J = 8 Hz), 6.51 (1H, d, J = 8 Hz), 4.06 (2H, s). HR-EI-MS m/z 189.0977 $[M]^+$, calcd for $C_{11}H_7{}^2H_3N_2O$ 189.0981 (100%), 171.05 (92%). HPLC-ESI-MS m/z: $[M + H]^+$ 190.2 (100%), 163.2 (48%), 150.3 (89%).

[4′,5′,6′,7′-$^2H_4$]Desulfoglucobrassicin (34a)

NH_4OAc (12.9 mg, 0.16 mmol) was added to a solution of [4′,5′,6′,7′-$^2H_4$]indole-3′-carboxaldehyde in nitromethane (1 mL) and refluxed at 105 °C for 90 min. The reaction mixture was diluted with water, extracted with CH_2Cl_2 and concentrated to yield crude [4,5,6,7-$^2H_4$]-3-(2′-nitrovinyl)indole, which was taken to the next step without further purification. $NaBH_4$ (38 mg, 0.96 mmol) was added to a solution of crude [4,5,6,7-$^2H_4$]-3-(2′-nitrovinyl)indole (61.6 mg, 0.32 mmol) in THF (2 mL) and MeOH (200 μL). The reaction mixture was stirred for 3 h at rt and excess $NaBH_4$ was destroyed by adding water to the reaction mixture. The reaction mixture was extracted with CH_2Cl_2, the solvent was removed and the residue subjected to FCC (100% CH_2Cl_2) to yield [4,5,6,7-$^2H_4$]-3-(2′-nitroethyl)indole (23 mg, 0.12 mmol, 38%). $NaOCH_3$ (23 mg mL^{-1}, 1 mL) was added to a solution of [4,5,6,7-$^2H_4$]-3-(2′-nitroethyl)indole (23 mg, 0.12 mmol) in MeOH (1 mL), the reaction mixture was stirred for 30 min at rt and the solvent was removed. The residue was suspended in DME (1 mL) at −40 °C, thionyl chloride (35 μL, 0.29 mmol) in DME (0.5 mL) was added dropwise and the reaction mixture was stirred for 1 h at −40 °C. The solvent was removed, the residue was diluted with water, extracted with CH_2Cl_2 and concentrated to yield crude oximoyl chloride, which was taken to the next step without further purification. A solution of 1-thio-β-D-glucopyranose tetraacetate (22 mg, 0.09 mmol) in anhydrous CH_2Cl_2 (1 mL) and Et_3N (50 μL, 0.36 mmol) in Et_2O (1 mL) were successively added with stirring to a solution of crude oximoyl chloride in anhydrous Et_2O–CH_2Cl_2 (2 : 1, 3 mL) at rt. After 3 h, the reaction mixture was diluted with water, extracted with CH_2Cl_2 and concentrated. The residue was subjected to FCC (CH_2Cl_2–MeOH, 98 : 2) to yield [4′,5′,6′,7′-$^2H_4$]tetraacetyl desulfoglucobrassicin (22 mg, 0.040 mmol, 33%). Freshly prepared $KOCH_3$ (1 mM, 75 μL) was added to a solution of [4′,5′,6′,7′-$^2H_4$]tetraacetyl desulfoglucobrassicin (22 mg, 0.04 mmol) in anhydrous MeOH (1 mL) at rt under an inert atmosphere, and the mixture was stirred for 30 min. The reaction mixture was neutralized with acetic acid and concentrated. The residue was dissolved in water and freeze-dried to yield [4′,5′,6′,7′-$^2H_4$]desulfoglucobrassicin (23 mg, 0.08 mmol) quantitatively. The corresponding non-deuterated compound **34** was prepared similarly but using non-deuterated materials.

Compound 34. HPLC t_R = 11.9 min, method B. UV (CH_3CN–H_2O) λ_{max} (nm): 222, 280. FTIR (KBr, cm^{-1}) ν_{max}: 3389, 2889, 1456, 1422, 1340, 1048, 745. ^{1}H NMR δ (500 MHz, CD_3OD) 7.64 (1H, d, J = 8 Hz), 7.33 (1H, d, J = 8 Hz), 7.13 (1H, s), 7.09 (1H, dd, J = 8, 7.5 Hz), 7.01 (1H, dd, J = 7.5, 7.5 Hz), 4.68 (1H, d, J = 10 Hz), 4.25 (1H, d, J = 16 Hz), 3.94 (1H, d, J = 16 Hz), 3.80 (1H, d, J = 12 Hz), 3.59 (1H, m), 3.23 (1H, dd, J = 9.5, 9 Hz), 3.14 (1H, dd, J = 9, 9 Hz), 3.09–3.03 (2H, m). ^{13}C NMR δ (125.8 MHz, CD_3OD) 154.3, 138.2, 128.3, 124.1, 122.7, 120.0, 119.7, 112.4, 111.7, 83.0, 82.1, 79.5, 74.6, 71.3, 62.8, 30.4. HR-EI-MS m/z 156.0685 $[M - (S\text{-glc}) - OH]^+$, calcd for $C_{10}H_8N_2$

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156.0687 (72%), 130.06 (100%). HPLC-ESI-MS m/z $[M + 1]^+$ 368.9 (42%), 207.0 (100%), 174.0 (27%), 130.0 (40%).

Compound 34a. HPLC t_R = 11.9 min, method A. UV (CH_3CN–H_2O) λ_{max} (nm): 222, 280. ^1H NMR δ (500 MHz, CD_3OD) 7.11 (1H, s), 4.68 (1H, d, J = 10 Hz), 4.23 (1H, d, 16), 3.92 (1H, dd, 16, 1), 3.78 (1H, dd, 12, 2), 3.59 (1H, dd, 12, 6), 3.22 (1H, dd, 9.5, 9.5), 3.13 (1H, dd, 9.5, 9), 3.06–3.03 (2H, m). HR-EI-MS m/z 160.0931 [M – (*S*-glc) – OH]$^+$, calcd for $C_{10}H_4{}^2H_4N_2$ 160.0938 (74%), 134.09 (100%). HPLC-ESI-MS m/z $[M + 1]^+$ 373.1 (41%), 211.1 (100%).

Preparation of rutabaga roots, feeding experiments and extractions

Rutabaga (*B. napus* ssp. *rapifera*) root tubers were cut horizontally in 10–15 mm slices, cylindrical wells (*ca*. 16 mm in diameter) were made on one side of the slices with a cork-borer and slices were incubated for 24 h in the dark (Pedras *et al*., 2004). Compounds (5×10^{-4} M) dissolved in H_2O, H_2O–CH_3OH–Tween 80 (95 : 5 : 0.05, v/v), or H_2O–CH_3OH (90 : 10, v/v) were added to each well (500 μL per well) and the slices were further incubated. All feeding experiments with labeled compounds were conducted in triplicate. Following adsorption of the solution, the wells were filled with distilled water. After further incubation for 48 h, the aqueous solutions in wells of each slice were collected, extracted (EtOAc) and the solvent was removed under reduced pressure. The residue obtained was dissolved in CH_3CN and was analyzed by HPLC-DAD-ESI-MS. Control experiments were similarly carried out by incubating rutabaga root tubers with non-labeled precursors or with carrier solution only. The tissue around each well was cut, was ground and extracted with CH_3OH; the MeOH extracts were concentrated and analyzed by HPLC-DAD-ESI-MS.

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