Unveiling the phytoalexin biosynthetic puzzle in salt cress: unprecedented incorporation of glucobrassicin into wasalexins A and B†

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Salt cress (*Thellungiella salsuginea* also known as *T. halophila*) is a wild cruciferous extremophile highly resistant to salt, drought, and cold. The recent discovery that salt cress produces the phytoalexins wasalexins A and B, and the phytoanticipins 1-methoxyglucobrassicin and 4-methoxyglucobrassicin in relatively higher amounts than other cruciferous species, prompted investigation of their biosynthetic relationships. Toward this end, perdeuterated 1-methoxybrassinin, L-Trp, glucobrassicin, 1-methoxyindolyl-3-acetaldoxime, brassinin, and methionine, as well as the corresponding natural abundance compounds, were administered to salt cress plants previously irradiated with UV-light (λ_{max} 254 nm). Remarkably, administration of hexadeuterated glucobrassicin led to incorporation of several deuterium atoms into wasalexins A and B, 1-methoxyglucobrassicin and 4-methoxyglucobrassicin. This unprecedented discovery suggests that glucobrassicin is a biosynthetic precursor of wasalexins and methoxylated glucosinolates in salt cress.

Introduction

Plants are able to protect themselves from microbial pathogens using complex metabolic pathways that synthesize a great variety of natural products/secondary metabolites, some of which are only produced under stress conditions. Phytoalexins are antimicrobial metabolites biosynthesized *de novo* by stressed plants from precursors that are common to most, if not all plant families.[1,2] However, interestingly, to synthesize phytoalexins, each plant is able to recruit common precursors into unique metabolic pathways that are generally characteristic of a given family (or related families). For example, the phytoalexins produced by plants of the family Brassicaceae, commonly known as crucifers, are unique in that they contain sulfur and are biosynthesized from the amino acid L-tryptophan (L-Trp), which is produced in plants and microbes but not in animals.[3] Wasalexins A (**1**) and B (**2**) are the main phytoalexins produced by phylogenetically diverse cruciferous species: wasabi (*Wasabia japonica*, first isolated from $CuCl_2$ stressed plants),[4] water cress (*Thlaspi arvense*),[5] and salt cress (*Thellungiella salsuginea/halophila*).[6] Though unrelated, these species show resistance to economically important fungal pathogens.

While to date no biosynthetic relationships have been established between wasalexins A (**1**) and B (**2**) and other cruciferous phytoalexins, a retro-biosynthetic analysis suggests that both could derive from 1-methoxybrassinin (**6**), after enzymatic oxidation and methylation (Scheme 1). Hence, the potential biosynthetic pathway of wasalexins A (**1**) and B (**2**) and biswasalexins A1 (**3**) and A2 (**4**) can be probed using 1-methoxybrassinin (**6**) deuterated at key positions (*e.g.* indole ring and SMe group). 1-Methoxybrassinin (**6**) is known to derive from L-Trp *via* 1-methoxyindolyl-3-acetaldoxime (**7**), in rutabaga roots, as summarized in Scheme 1.[7]

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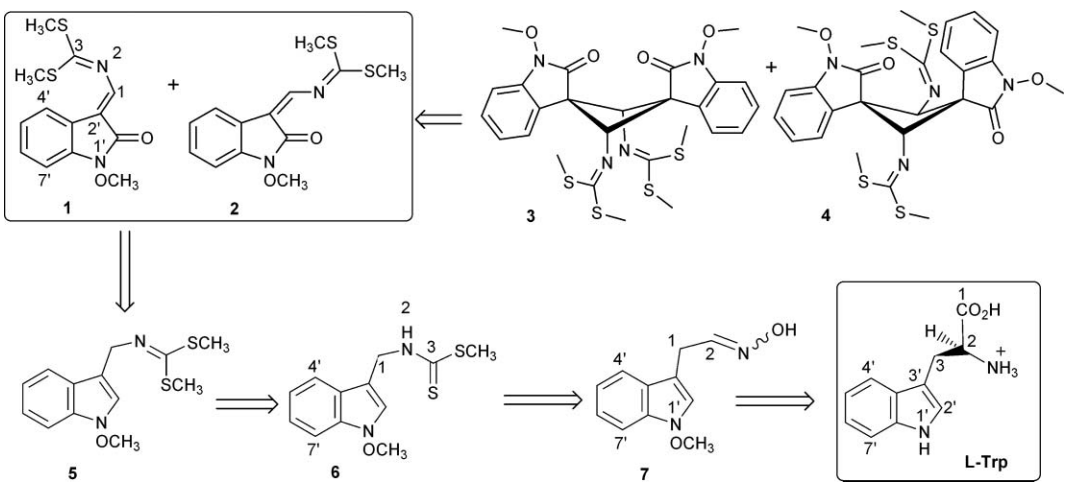
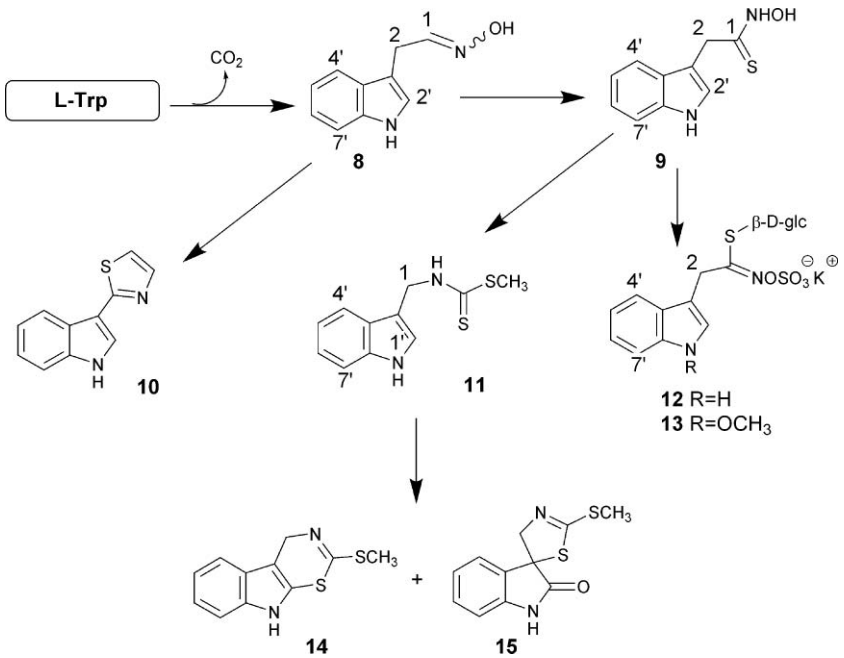


Scheme 1 Retro-biosynthetic analysis of wasalexins A (**1**) and B (**2**) and biswasalexins A1 (**3**) and A2 (**4**).



Scheme 2 Biosynthetic relationship between L-Trp, indolyl-3-acetaldoxime (**8**), indolyl-3-acetothiohydroxamic acid (**9**), glucobrassicin (**12**), and phytoalexins camalexin (**10**), brassinin (**11**), cyclobrassinin (**14**) and spirobrassinin (**15**).

Furthermore, the elusive relationship between the phytoanticipin glucobrassicin (**12**) and wasalexins A (**1**) and B (**2**) can be probed similarly. Glucobrassicin (**12**) is a constitutive metabolite also derived from L-Trp, whose metabolic products have protective and defensive roles in a very large number of cruciferous species (*i.e.* **12** is a phytoanticipin).[8,9] Indeed, due to the similarity between the chemical structures of brassinin (**11**) and glucobrassicin (**12**) and 1-methoxybrassinin (**6**) and 1-methoxyglucobrassicin (**13**), it was suspected that glucobrassicin (**12**) was a precursor of brassinin (**11**). However, administration of tetradeuterated glucobrassicin (**12**) to different *Brassica* species did not reveal deuterium incorporation into brassinin (**11**), whereas deuterium incorporation from tetradeuterated indolyl-3-acetaldoxime (**8**) was significant.[10,11] Thus, it was inferred that indolyl-3-acetaldoxime (**8**) was a precursor of brassinin (**11**), but glucobrassicin (**12**) was not. Currently, indolyl-3-acetaldoxime (**8**) is the last known intermediate common to camalexin (**10**),[12,13] brassinin (**11**), and glucobrassicin (**12**), whereas [4′,5′,6′,7′-$^{2}H_4$]indolyl-3-[^{34}S]acetothiohydroxamic acid (**9a**) is the last known intermediate common to glucobrassicin (**12**) and brassinin derived phytoalexins (cyclobrassinin (**14**) and spirobrassinin (**15**), Scheme 2).[14]

Although the importance of phytoalexins in the resistance of plants to particular pathogens is well known, fundamental questions regarding the cruciferous phytoalexins remain to be deciphered. Namely, the biosynthetic intermediates, enzymes and genes of cruciferous phytoalexins are of primary importance, but this biosynthetic puzzle is rather incomplete. A biosynthetic study of cruciferous phytoalexins is particularly relevant in salt cress (*T. salsuginea*) for two main reasons: it is a cruciferous extremophile highly resistant to salt, drought, and cold,[15] which are very important agronomical traits, and its sequenced genome is in the final assembly status to be released to public database.[16] Furthermore, the recent discovery that salt cress (*T. salsuginea*) produces wasalexins A (**1**) and B (**2**), biswasalexins A1 (**3**) and A2 (**4**), and methoxyglucobrassicins **13** and **26** in relatively higher amounts than other cruciferous species,[17,18] urged this investigation of their potential biosynthetic relationships. Toward this end, perdeuterated 1-methoxybrassinin (**6**), L-Trp and other compounds were administered to UV-irradiated salt cress plants. Remarkably, administration of hexadeuterated glucobrassicin (**12a**) led to incorporation of several deuterium atoms into both wasalexins A (**1**) and B (**2**) and into methoxyglucobrassicins **13** and **26**. This unprecedented discovery suggests that in salt cress glucobrassicin (**12**) is a precursor of wasalexins and methoxylated indolyl glucosinolates. In addition, administration of perdeuterated brassinins **6** and **11** and trideuterated methionine provided unique information that allows us to propose a fairly complete map of the biosynthetic pathway to wasalexins.

Results and discussion

The biosynthetic pathway of wasalexins A (**1**) and B (**2**) (and related metabolites) in salt cress was probed with several deuterated compounds (Table 1). The incorporation of deuterium atoms from potential metabolic precursors into wasalexins was established by HPLC-MS. First, [$^{2}H_3$C-S; 4′,5′,6′,7′-$^{2}H_4$]-1-methoxybrassinin (**6a**) was synthesized from [4,5,6,7-$^{2}H_4$]indole as previously reported.[19] Petiolated leaves of salt cress previously elicited by exposure to UV light (λ_{max} 254 nm) were immersed in aqueous solutions of [$^{2}H_3$C-S; 4′,5′,6′,7′-$^{2}H_4$]-1-methoxybrassinin (**6a**) as described in the Experimental. After 48 h, leaves were frozen in liquid nitrogen, extracted, and the extracts were fractionated to give a non-polar fraction containing the expected phytoalexins, and a polar fraction containing indolyl glucosinolates, as described in the Experimental. HPLC-DAD-MS analysis of the fractions and comparison of the UV and MS-ESI spectra of the components with those available in our metabolite spectral database showed the presence of wasalexins A (**1**) and B (**2**) and

Table 1 Perdeuterated compounds used in precursor administration experiments

Deuterated compound (#)[a]	Origin[reference]
[$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]-1-methoxybrassinin (**6a**)	synthetic[19]
[4′,5′,6′,7′-$^2H_4$]brassinin (**11a**)	synthetic[19]
[2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**)	synthetic, this work
[$^2H_3$C-O]-1-methoxyindolyl-3-acetaldoxime (**7a**)	synthetic[19]
L-[2′,4′,5′,6′,7′-$^2H_5$]tryptophan (L-Trp)	commercial
L-[$^2H_3$C-S]methionine (L-Met)	commercial

[a] Deuterated compounds are referred to by a number followed by the letter **a**.

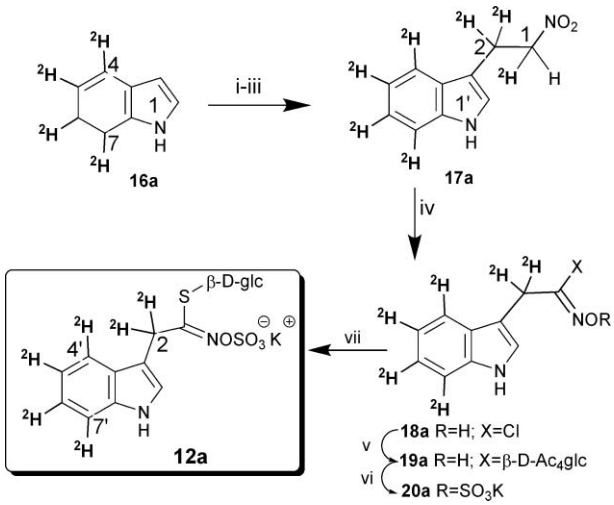
Table 2 Metabolism of [$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]-1-methoxybrassinin (**6a**) in UV-irradiated leaves (UV-elicited) of *Thellungiella salsuginea*

Metabolites isolated (nmoles ± Std/g of fresh tissue)[a]	% Incorporation of deuterium ± Std
[$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]Wasalexin A (**1a**) (794 ± 54)	19.2 ± 3.9[b]
[$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]Wasalexin B (**2a**) (103 ± 16)	16.7 ± 4.3[b]
[$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]Biswasalexin A1 (**3a**) (129 ± 38)	10.7 ± 1.6[b]
[$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]Biswasalexin A2 (**4a**) (<0.5)	8.1 ± 0.4[b]
Glucobrassicin (**12**) (n.d.)	no deuterium detected (≤0.1%)[c]
1′-Methoxyglucobrassicin (**13**)	no deuterium detected (≤0.1%)[c]
4-Methoxyglucobrassicin (**26**)	no deuterium detected (≤0.1%)[c]

[a] Deuterated compounds are referred to by a number followed by the letter **a**; nmoles = total amount of deuterated plus non-deuterated compound; n.d. = not determined. [b] Positive ion mode. Incorporations calculated from HPLC-MS-ESI (peak intensities); % of ^2H incorporation = {[M + 1+ n]$^+$/([M + 1]$^+$ + [M +1 + n]$^+$)} × 100 (n = 7) ± Std (standard deviation), where n = number of deuterium atoms. [c] Negative ion mode.

biswasalexins A1 (**3**) and A2 (**4**) in the non-polar fraction, and the glucosinolates glucobrassicin (**12**), 1-methoxyglucobrassicin (**13**), and 4-methoxyglucobrassicin (**26**) in the polar fraction. HPLC-MS analysis of each fraction indicated the levels of deuterium incorporation into each metabolite, as shown in Table 2. As predicted, [$^2H_3$C-S; 4′,5′,6′,7′-$^2H_4$]-1-methoxybrassinin (**6a**) was incorporated in high percentage into wasalexins A (**1a**) (19.2%) and B (**2a**) (16.7%), suggesting that only a few enzymatic steps separate phytoalexins **1** and **2** from **6**. In addition, biswasalexins A1 (**3a**) and A2 (**4a**) also showed a substantial amount of deuterium incorporation (10.7% and 8.1%, respectively). Similar experiments were carried out in parallel using natural abundance 1-methoxybrassinin (**6**); as expected, no [M + n]$^±$ ion peaks due to deuterium atoms were detected in non-polar or polar fractions.

The availability of hexadeuterated glucobrassicin (**12**) instead of tetradeuterated was crucial to distinguish between two possible metabolic routes to wasalexins: (i) degradation of the side chain of [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**) followed by metabolic recycling of [4,5,6,7-$^2H_4$]indole (**16a**) to L-[4′,5′,6′,7′-$^2H_4$]Trp to yield tetradeuterated wasalexins A/B, or (ii) incorporation of [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**) to yield pentadeuterated wasalexins A/B. To establish if any of these alternative routes was operating, [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**) was synthesized. A synthetic route modified from that previously reported by Rollin's group,[20] starting with [4,5,6,7-$^2H_4$]indole[19] and deuterated NaB^2H_4 was used, as summarized in Scheme 3. The reductive step



Scheme 3 Synthesis of [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**); reagents and reaction conditions: i. $POCl_3$–DMF-d_7, 94%; ii. CH_3NO_2, ammonium acetate, 120–130 °C; iii. NaB^2H_4, THF–MeOH-d_4, 39%; iv. MeON-a–MeOH, $SOCl_2$, DME; v. thioglucose tetraacetate, Et_3N, Et_2O–CH_2Cl_2, 38%; vi. HSO_3Cl, pyridine, $KHCO_3$, 66%; vii. MeOK–MeOH, quantitative.

using NaB^2H_4 was carried out in THF–MeOH-d_4 to ensure complete deuteration of the C-2 methylene of 3′-(1-nitroethyl)indole (**17a**). Unfortunately, despite several attempts, the yields obtained in this step were *ca.* 20% lower than those obtained with the non-deuterated reagent; these lower yields might reflect a slower reaction rate, thus lower conversion (the reaction time was not increased due to low product stability).

[2,2,4′,5′,6′,7′-$^2H_6$]Glucobrassicin (**12a**) in aqueous solutions was administered to elicited leaves of salt cress, as reported in the Experimental. After incubation and extractions, fractions were analyzed by HPLC-MS and the percentages of deuterium content were determined and calculated as reported in Table 3. Unexpectedly, pentadeuterated wasalexins A (**1a**) (3.7%) and B

Table 3 Metabolism of [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**) in UV-irradiated leaves (UV-elicited) of *Thellungiella salsuginea*

Metabolites isolated (nmoles/g of fresh tissue)[a]	% Incorporation of deuterium ± Std
[1,4′,5′,6′,7′-$^2H_5$]Wasalexin A (**1a**) (550 ± 36)	3.7 ± 1.0[b]
[1,4′,5′,6′,7′-$^2H_5$]Wasalexin B (**2a**) (54 ± 6)	2.4 ± 0.6[b]
[1,4′,5′,6′,7′-$^2H_5$]Biswasalexin A1 (**3a**) (128 ± 18)	2.0 ± 0.6[b]
[1,4′,5′,6′,7′-$^2H_5$]Biswasalexin A2 (**4a**)	Not detected[b]
[2,2,4′,5′,6′,7′-$^2H_6$]Glucobrassicin (**12a**) (≤1)	77.5 ± 13.8[c]
[2,2,4′,5′,6′,7′-$^2H_6$]-1′-Methoxyglucobrassicin (**13a**) (154 ± 10)	4.7 ± 1.6[c]
[2,2,5′,6′,7′-$^2H_5$]-4-Methoxyglucobrassicin (**26a**) (366 ± 21)	13.9 ± 2.6[c]

[a] All deuterated compounds are referred to by a number followed by the letter **a**; conc. = total concentration of deuterated plus non-deuterated compound. [b] Positive ion mode. Incorporations calculated from HPLC-MS-ESI (peak intensities); % of ^2H incorporation = {[M + 1 + n]$^+$/([M + 1]$^+$ + [M + 1 + n]$^+$)} × 100 (n = 5) ± Std (standard deviation), where n = number of deuterium atoms. [c] Negative ion mode. Incorporations calculated from HPLC-MS-ESI; % of ^2H incorporation = {[M−1 + n]$^-$/([M−1]$^-$ + [M−1 + n]$^-$)} × 100 (n = 5 or 6) ± Std (standard deviation), where n = number of deuterium atoms.

Table 4 Metabolism of [$^2H_3$C-O]-1-methoxyindolyl-3-acetaldoxime (**7a**) in UV-irradiated leaves (UV-elicited) of *Thellungiella salsuginea*

Metabolites isolated (nmoles/g of fresh tissue)[a]	% Incorporation of deuterium ±Std
Wasalexin A (**1**) (501 ± 32)	no deuterium detected (≤0.1%)[b]
Wasalexin B (**2**) (56 ± 2)	no deuterium detected (≤0.1%)[b]
Biswasalexin A1 (**3**) (152 ± 39)	no deuterium detected (≤0.1%)[b]
Biswasalexin A2 (**4**) (<0.5)	no deuterium detected (≤0.1%)[b]
Glucobrassicin (**12**) (≤1 nmoles)	no deuterium detected (≤0.1%)[b]
[$^2H_3$C-O]-1′-Methoxyglucobrassicin (**13a**) (49 ± 6)	3.4 ± 0.9[c]
4-Methoxyglucobrassicin (**26**) (423 ± 52)	no deuterium detected (≤0.1%)[c]

[a] Deuterated compounds are referred to by a number followed by the letter **a**; nmoles = total amount of deuterated plus non-deuterated compound. [b] Positive ion mode. [c] Negative ion mode. Incorporations calculated from HPLC-MS-ESI (peak intensities); % of ^2H incorporation = $\{[M-1+n]^-/([M-1]^- + [M-1+n]^-)\} \times 100$ ($n = 3$) ± Std (standard deviation), where n = number of deuterium atoms.

Table 5 Metabolism of L-[2′,4′,5′,6′,7′-$^2H_5$]tryptophan (L-Trp) in UV-irradiated leaves (UV-elicited) of *Thellungiella salsuginea*

Metabolites isolated (nmoles/g of fresh tissue)[a]	% Incorporation of deuterium ±Std
[4′,5′,6′,7′-$^2H_4$]Wasalexin A (**1a**) (280 ± 14)	4.6 ± 0.8[b,c]
[4′,5′,6′,7′-$^2H_4$]Wasalexin B (**2a**) (16 ± 3)	2.8 ± 0.3[b,c]
[4′,5′,6′,7′-$^2H_4$]Biswasalexin A1 (**3a**) (60 ± 4)	2.5 ± 1.9[b,c]
[4′,5′,6′,7′-$^2H_4$]Biswasalexin A2 (**4a**) (<0.5)	no deuterium detected (≤0.1%)[b]
[2′,4′,5′,6′,7′-$^2H_5$]Glucobrassicin (**12a**) (≤1)	11.1 ± 2.7[d]
[2′,4′,5′,6′,7′-$^2H_5$]-1′-Methoxyglucobrassicin (**13a**) (118 ± 6)	22.8 ± 2.6[d]
[2′,5′,6′,7′-$^2H_4$]-4-Methoxyglucobrassicin (**26a**) (412 ± 24)	11.9 ± 2.5[d]

[a] Deuterated compounds are referred to by a number followed by the letter **a**; nmoles = total amount of deuterated plus non-deuterated compound. [b] Positive ion mode. [c] Incorporations calculated from HPLC-MS-ESI (peak intensities); % of ^2H incorporation = $\{[M+1+n]^+/([M+1]^+ + [M+1+n]^+)\} \times 100$ ($n = 4$) ± Std (standard deviation), where n = number of deuterium atoms. [d] Negative ion mode. % of ^2H incorporation = $\{[M-1+n]^-/([M-1]^- + [M-1+n]^-)\} \times 100$ ($n = 4$ or 5) ± Std (standard deviation), where n = number of deuterium atoms.

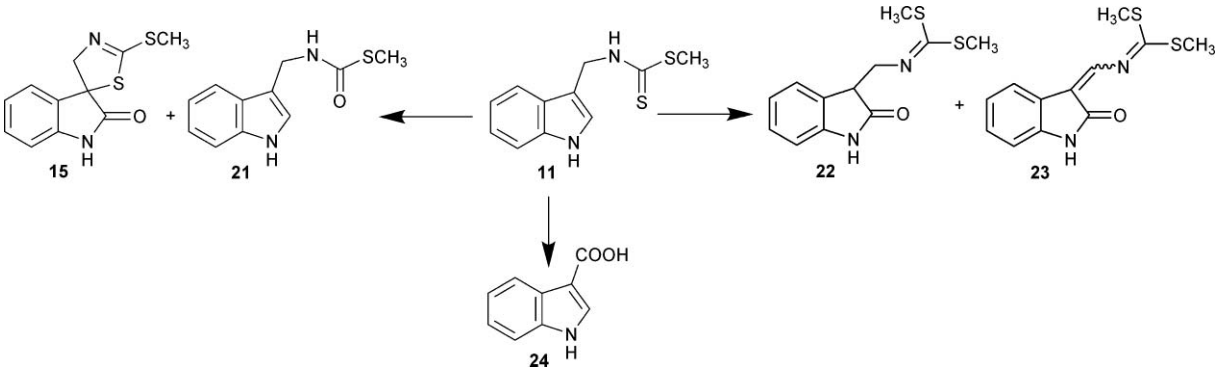
(**2a**) (2.4%), and biswasalexin A1 (**3a**) (2.0%) were detected in reasonable amounts. Previously, despite numerous experiments using various plant tissues and different species (*B. juncea* and *B. rapa*), incorporation of [4′,5′,6′,7′-$^2H_4$]glucobrassicin (**12b**) into brassinin derived phytoalexins was not detected.[10,11] Hence, the deuterium incorporation results described here suggest for the first time that wasalexins and biswasalexins are likely derived from glucobrassicin (**12**). Alternatively, the direct participation of either 3-acetothiohydroxamic acid (**9**), or desulfoglucobrassicin (**25**), or the corresponding 1-methoxy derivatives cannot be ruled out. By inference, brassinin (**11**), an important phytoalexin from various *Brassica* species, is also likely to derive from glucobrassicin (**12**), although this hypothesis cannot be probed because salt cress does not produce detectable amounts of brassinin (**11**). Furthermore, considering the substantial percentages of deuterium incorporation from glucobrassicin (**12**) into both 1-methoxyglucobrassicin (**13**) and 4-methoxyglucobrassicin (**26**), a precursor relationship is likely to exist also in this pathway.

[$^2H_3$C-O]-1-Methoxyindolyl-3-acetaldoxime (**7a**) in aqueous solutions was also administered to elicited leaves of salt cress, as reported in the Experimental. HPLC-MS analyses of extracts showed that [$^2H_3$C-O]-1-methoxyindolyl-3-acetaldoxime (**7a**) was incorporated into 1′-methoxyglucobrassicin (**13a**) (3.4%), but no deuterium incorporation was detected in any of the wasalexins (Table 4). Altogether these results suggest that 1′-methoxyglucobrassicin (**13**) is either formed *via* two parallel pathways (*i.e.* from 1-methoxyindolyl-3-acetaldoxime (**7**) and from glucobrassicin (**12**)) or that the enzymes that convert **8** to **12** are not substrate specific. Previously, we have found that [$^2H_3$C-O]-1-methoxyindolyl-3-acetaldoxime (**7**) was incorporated into 1-methoxybrassinin (**6**).7

Deuterium incorporations into wasalexins A (**1a**) (4.6%) and B (**2a**) (2.8%) from L-[2′,4′,5′,6′,7′-$^2H_5$]Trp confirmed their *de novo* biosynthesis from a primary precursor (Table 5); similar results were previously observed for brassinin (**11**)[21] and other phytoalexins.[22] As well, HPLC-MS analysis of the polar fraction showed high level of incorporations of L-[2′,4′,5′,6′,7′-$^2H_5$]Trp into 1-methoxyglucobrassicin (**13a**) (22.8%) and 4-methoxyglucobrassicin (**26a**) (11.9%), also confirming their biosynthetic origin from L-Trp (Table 5). The substantially higher incorporation percentages observed in **13a** are not understood at present.

Additional experiments were designed to determine the origin of the methyl groups of wasalexins, using [$^2H_3$C-S]methionine (Met), a common methyl donor *via* *S*-adenosylmethionine (SAM). Thus, administration of solutions of trideuterated Met to elicited leaves of salt cress led to the incorporation of nine (1.6/1.3%), six (3.7/2.7%), and three (7.6/5.6%) deuterium atoms into wasalexins A (**1**) and B (**2**), respectively, and three deuteria into glucobrassicins **13a** (8.1%) and **26a** (12.5%), as determined by HPLC-MS (Table 6). These results indicate that SAM is a methyl donor in the formation of these metabolites.

To probe *in vivo* the specificity of the enzymes involved in the biosynthesis of wasalexins, [4′,5′,6′,7′-$^2H_4$]brassinin (**11a**) was administered to UV-elicited leaves of salt cress. Interestingly, HPLC-MS analysis of the non-polar extracts of leaves showed the presence of the tetradeuterated phytoalexins spirobrassinin (**15a**) and brassitin (**21a**), as well as [4′,5′,6′,7′-$^2H_4$]indole-3-carboxylic acid (**24a**) (Table 7). Because only the deuterated compounds were detected in the extracts (*i.e.* >98% deuterium incorporation), they must necessarily result from metabolism of tetradeuterobrassinin (**11a**). In addition, two other peaks were detected and assigned to the new compounds identified as [4′,5′,6′,7′-$^2H_4$]demethoxydihydrowasalexin (**22a**) and [4′,5′,6′,7′-$^2H_4$]demethoxywasalexin (**23a**), as summarized in Scheme 4 (Table 7). The chemical structures of compounds **22** and **23** were confirmed by synthesis, as described in the Experimental. None of the corresponding non-deuterated compounds were detected in the extracts. Analysis of polar extracts did not show the presence of deuterated compounds. Similar feeding experiments using natural abundance brassinin (**11**) followed by extraction and HPLC-MS analysis of non-polar extracts showed the presence of non-deuterated spirobrassinin (**15**), brassitin (**21**),



Scheme 4 Metabolism of [4′,5′,6′,7′-$^2H_4$] brassinin (**11a**) in leaves of *Thellungiella salsuginea*.

Table 6 Metabolism of L-[$^2H_3$C-S]methionine Met) in leaves UV-irradiated leaves (UV-elicited) of *Thellungiella salsuginea*

Metabolites isolated (nmoles/g of fresh tissue)[a]	% Incorporation of deuterium ±Std
[x^2H_3C]Wasalexin A (**1a**) (612 ± 38)	$x = 1 \Rightarrow {}^2H_3$ 7.6 ± 2.3[b] $x = 2 \Rightarrow {}^2H_6$ 3.7 ± 1.2[b] $x = 3 \Rightarrow {}^2H_9$ 1.6 ± 0.4[b]
[x^2H_3C]Wasalexin B (**2a**) (91 ± 4)	$x = 1 \Rightarrow {}^2H_3$ 5.6 ± 1.8[b] $x = 2 \Rightarrow {}^2H_6$ 2.7 ± 0.9[b] $x = 3 \Rightarrow {}^2H_9$ 1.3 ± 0.3[b]
[x^2H_3C]Biswasalexin A1 (**3a**) (223 ± 10)	$x = 1 \Rightarrow {}^2H_3$ 4.6 ± 1.8[b] $x = 2 \Rightarrow {}^2H_6$ 1.6 ± 1.6[b]
[x^2H_3C]Biswasalexin A2 (**4a**) (<0.5)	$x = 1 \Rightarrow {}^2H_3$ 2.3 ± 0.6[b]
Glucobrassicin (**12**) (≤1)	no deuterium detected (≤0.1%)[c]
[$^2H_3$C]-1′-Methoxyglucobrassicin (**13a**) (129 ± 30)	8.1 ± 1.6[c]
[$^2H_3$C]-4-Methoxyglucobrassicin (**26a**) (321 ± 53)	12.5 ± 1.6[c]

[a] Deuterated compounds are referred to by a number followed by the letter **a**; nmoles = total amount of deuterated plus non-deuterated compound. [b] Positive ion mode. Incorporations calculated from HPLC-MS-ESI (peak intensities); % of ^2H incorporation = $\{[M + 1 + n]^+/([M + 1]^+ + [M + 1 + n]^+)\} \times 100$ (n = 3, 6 or 9) ± Std (standard deviation), where n = number of deuterium atoms. [c] Negative ion mode. Incorporations calculated from HPLC-MS-ESI; % of ^2H incorporation = $\{[M-1 + n]^-/([M-1]^- + [M-1 + n]^-)\} \times 100$ (n = 3) ± Std (standard deviation), where n = number of deuterium atoms.

Table 7 Metabolism of [4′,5′,6′,7′-$^2H_4$]brassinin (**11a**) in UV-irradiated leaves (UV-elicited) of *Thellungiella salsuginea*

Metabolites isolated[a]	% Incorporation of deuterium[b]
Wasalexin A (**1**)	no deuterium detected (≤0.1%)[b]
Wasalexin B (**2**)	no deuterium detected (≤0.1%)[b]
[4′,5′,6′,7′-$^2H_4$]spirobrassinin (**15a**)	≥98%[b]
[4′,5′,6′,7′-$^2H_4$]brassitin (**21a**)	≥98%[b]
[4′,5′,6′,7′-$^2H_4$]demethoxydihydrowasalexin (**22a**)	≥98%[b]
[4′,5′,6′,7′-$^2H_4$]demethoxywasalexin (**23a**)	≥98%[b]
[4′,5′,6′,7′-$^2H_4$]indole-3-carboxylic acid (**24a**)	≥98%[b]

[a] Deuterated compounds are referred to by a number followed by the letter **a**; only deuterated compounds detected by HPLC-MS-ESI (confirmed by HRMS). [b] Positive ion mode.

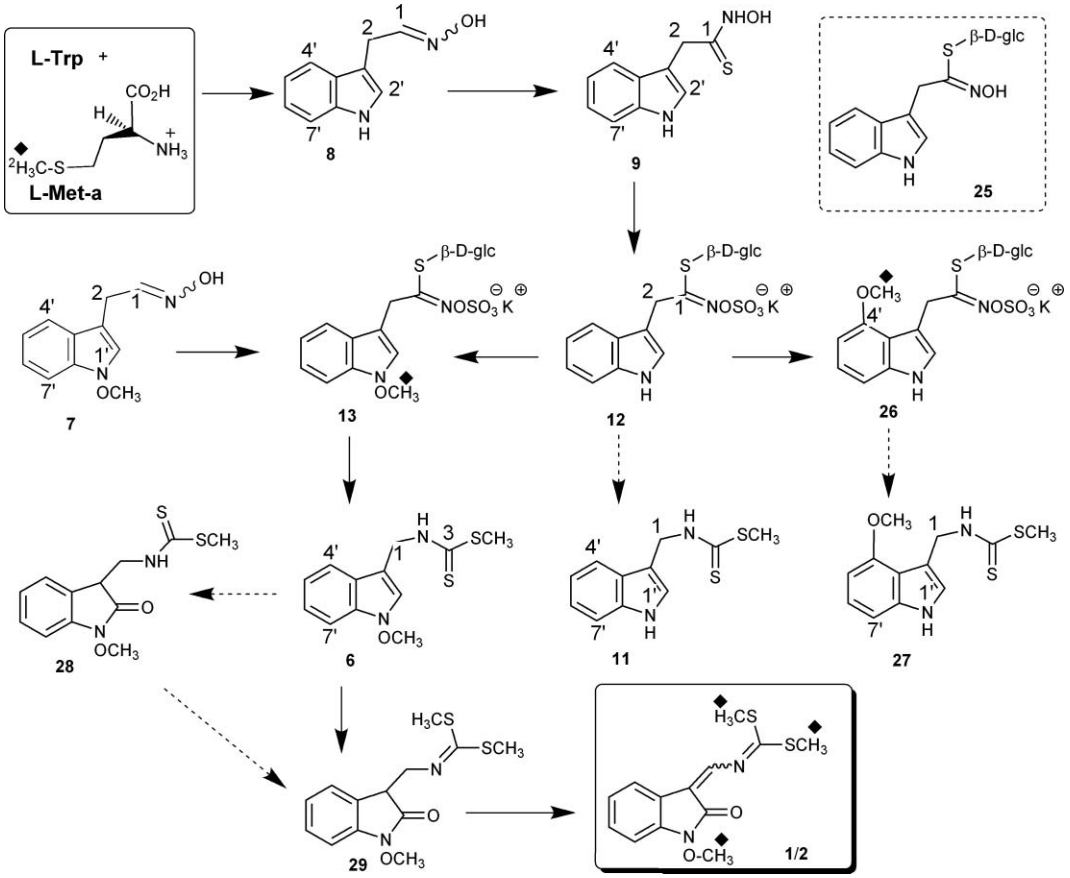
demethoxydihydrowasalexin (**22**), demethoxywasalexin (**23**) and indole-3-carboxylic acid (**24**).

Altogether these results suggest that some enzymes involved in the biosynthesis of wasalexins are also able to accept non-methoxylated compounds, *e.g.* brassinin (**11**), as substrate. In this context, the formation of spirobrassinin (**15**) is particularly interesting because the corresponding 1-methoxylated metabolite was not detected in extracts of salt cress. Nonetheless, one must conclude that enzymes able to convert **11** to **15** are produced in salt cress. These findings direct us to hypothesize that the methoxylated counterpart of compound **22**, that is metabolite **29**,[23] is a putative intermediate between 1-methoxybrassinin (**6**) and wasalexins **1** and **2**. Indeed, **29** was detected in all extracts of elicited leaves.

Based on the results obtained from deuterium incorporation experiments described above (Tables 2–7), and our most recent results,[14,22] the putative intermediates of the biosynthetic pathway to brassinins **6**, **11** and **27** and wasalexins A (**1**) and B (**2**) can be proposed starting from the primary building block L-Trp, as shown in Scheme 5. Because five deuteria from [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**) were incorporated into wasalexins, it is likely that **12** is their precursor in salt cress; however, we cannot exclude the possibility that **9** or **25** (potentially formed from enzymatic degradation of **12**) or one of the corresponding methoxylated metabolites is an immediate precursor. To differentiate among these possibilities, the availability of mutants of salt cress deficient in *e.g.*, desulfoglucobrassicin (**25**) and thiohydroxamic acid **9** would substantiate the current proposal. As a final point, it is important to stress that, although both biswasalexins A1 (**3**) and A2 (**4**) are derived from wasalexins, it remains to be determined whether their formation involves enzymatic catalysis or is a spontaneous transformation (under normal light conditions).

Conclusion

Breeding strategies to enhance natural resistance traits of plants to microbial pathogens can be improved if a detailed understanding of defense pathways is available. Yet, among the 44 cruciferous phytoalexins reported thus far, only genes of the camalexin (**10**) pathway have been cloned.[24] This enterprise was accomplished in the cruciferous species *Arabidopsis thaliana*, a model plant having a large array of well-characterized mutants.[12,13] Nonetheless, because *A. thaliana* produces mainly the phytoalexin camalexin (**10**), this species is not useful to investigate the



Scheme 5 Proposed biosynthetic intermediates between L-Trp and the phytoalexins brassinins **6**, **11**, **27** and wasalexins A (**1**) and B (**2**); L-[$^2H_3$-S]Met is the methyl donor; black ◆ indicates that methyl groups are derived from methionine (determined by HPLC-MS-ESI); dashed arrows indicate proposed transformation.

biosynthesis of other cruciferous phytoalexins.[6] On the other hand, because the species *T. salsuginea* has a relatively small genome, it is a valuable model to investigate the intermediates, enzymes and genes involved in the biosynthesis of wasalexins. Toward this end, using various perdeuterated compounds, we have investigated the potential precursors of the wasalexins A (**1**) and B (**2**), and proposed for the first time their detailed biosynthetic pathway. This pathway fills an important void in the cruciferous phytoalexin biosynthetic puzzle. Notwithstanding, considering that previously other *Brassica* species did not incorporate glucobrassicin (**12**) into phytoalexins, further work needs to be carried out before a general conclusion can be drawn. These results will facilitate the discovery of the corresponding enzymes and genes of the biosynthetic pathway to wasalexins and, by inference, those of most phytoalexins derived from brassinins **6**, **11** and **27**.

Experimental

General experimental

All solvents were HPLC grade and used as such, except for THF (dried over sodium). Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. 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. Fourier transform infrared (FT-IR) data were acquired on a spectrometer and spectra were measured by the diffuse reflectance method on samples dispersed in KBr.

HPLC analysis was carried out with either Agilent 1100 series or Hewlett Packard HPLC systems equipped with quaternary pump, autosampler, diode array detector (DAD, wavelength range 190–600 nm), and degasser. Method A (phytoalexins and non-polar metabolites) used a Zorbax Eclipse XDB-C18 column (5 μm particle size silica, 150 × 4.6 mm I.D.), equipped with an in-line filter, with the mobile phase H_2O–CH_3OH from 50 : 50 to 0 : 100, linear gradient for 25 min, and a flow rate of 0.75 ml min^{-1}; Method B (indolyl glucosinolates and other polar metabolites) used a Zorbax SB-C18 column (3.5 μm particle size silica, 100 × 3.0 mm I.D.), equipped with an in-line filter, with the mobile phase H_2O (with 0.1% TFA)–CH_3OH (with 0.1% TFA) from 85 : 15 to 70 : 30 in 25 min, to 50 : 50 in 5 min, to 40 : 60 in 5 min and a flow rate of 0.40 ml min^{-1}. Samples were dissolved in CH_3OH for Method A, and in H_2O–CH_3OH (1 : 1) for Method B. HPLC-DAD-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 × 4.6 mm I.D.). The mobile phase consisted of a linear gradient of: Method A, 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, 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 for Method A, and in methanol–water (50 : 50) for Method B. HPLC-HRMS-ESI was performed on an Agilent HPLC 1100 series directly connected to a QSTAR XL Systems Mass Spectrometer (Hybrid Quadrupole-TOF LC/MS/MS) with turbo spray ESI source. Chromatographic separation was carried out at room temperature using a Hypersil ODS C-18 column (5 µm particle size silica, 200 × 2.1 mm I.D.) or a Hypersil ODS C-18 column (5 µm particle size silica, 100 × 2.1 mm I.D.). The mobile phase consisted of a linear gradient of: Method A, 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 0.25 ml min^{-1}. Data acquisition was carried out in either positive or negative polarity mode per LC runs. Data processing was carried out by Analyst QS Software. Samples were dissolved in CH_3CN or CH_3OH–H_2O (1 : 1).

Plant material

Thellungiella salsuginea ecotype Shandong was obtained from TAIR (envelope labeled as *Thellungiella halophila*). Seeds were sown in a perlite and nutrient free LG-3 soil (Sun Gro Horticulture Canada) in a Petri dish and incubated at 4 °C. After 10 days, the Petri dishes containing seeds were transferred to a growth chamber at 16 h of light/8 h of dark, 23 °C day/16 °C night, light intensity of 150 µE m^{-2} s^{-1}, and with ambient humidity. After seven days, the seedlings were transferred into soil in small pots (50 pots/tray), and kept under the same conditions. For elicitation of phytoalexins, four-week-old plants were used.

Synthesis and characterization of new compounds

All compounds gave satisfactory spectroscopic data; in each case the percentage of deuterated synthetic compound was about 98%. Deuterated compounds are numbered and have the additional letter **a** (*e.g.* **1a**). Deuterated compounds [$^2H_3$C-S 4′,5′,6′,7′-$^2H_4$]-1-methoxybrassinin (**6a**),[19] [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**), and [$^2H_3$C-O]-1-methoxyindolyl-3-acetaldoxime (**7a**)[19] were prepared from [4,5,6,7-$^2H_4$]indole (**16a**), which was prepared from the readily available NMR solvent [2,3,4,5,6-$^2H_5$]nitrobenzene (99%, $^2H_5$) and 2-chloroacetonitrile, as previously reported.[22]

Synthesis of [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (12a)

A solution of [4,5,6,7-$^2H_4$]indole (**16a**, 84 mg, 0.69 mmol) in DMF-d_7 (120 µl, 2.1 mmol) was added to freshly distilled $POCl_3$ (70 µl 0.76 mmol) and stirred at r.t. for 60 min. The reaction mixture was poured into water and the resultant yellow solution was basified (pH 9) with aq. NaOH solution (2 M). The suspension was extracted with EtOAc (3 × 20 ml) and the combined organic extract was dried and concentrated to dryness to yield [1,4′,5′,6′,7′-$^2H_5$]indole-3-carboxaldehyde (98 mg, 94% yield). Ammonium acetate (25 mg, 0.33 mmol) was added to a suspension of [1,4′,5′,6′,7′-$^2H_5$]indole-3-carboxaldehyde (98 mg; 0.65 mmol) in nitromethane (700 µl). The mixture was vigorously stirred under reflux at 120–130° C for 2 h, the reaction mixture was cooled to room temperature, diluted with water (20 ml), and extracted with CH_2Cl_2 (3 × 40 ml). The combined organic layer was dried and concentrated under reduced pressure to yield 120 mg of crude product, which was directly used in the next step without purification. To a stirred mixture of crude [1,4′,5′,6′,7′-$^2H_5$]-3′-(1-nitrovinyl)indole (89 mg; 0.46 mmol) in THF (2.2 ml) and MeOH-d_4 (280 µl), NaB^2H_4 (58 mg, 1.4 mmol) was added in portions over a period of 15 min. After 6 h, the remaining NaB^2H_4 was quenched with aq HCl (1 M), the reaction mixture was filtered, the filtrate was dried and concentrated under reduced pressure to yield the crude reaction mixture. Separation over silica gel using CH_2Cl_2–hexane (4 : 1) as the eluent yielded a mixture of heptadeuterated (3′-(1-nitroethyl)indole (**17a**, 50 mg, 39%). Deuteration at C-1 was not necessary because C-1 is completely oxidized in later steps.

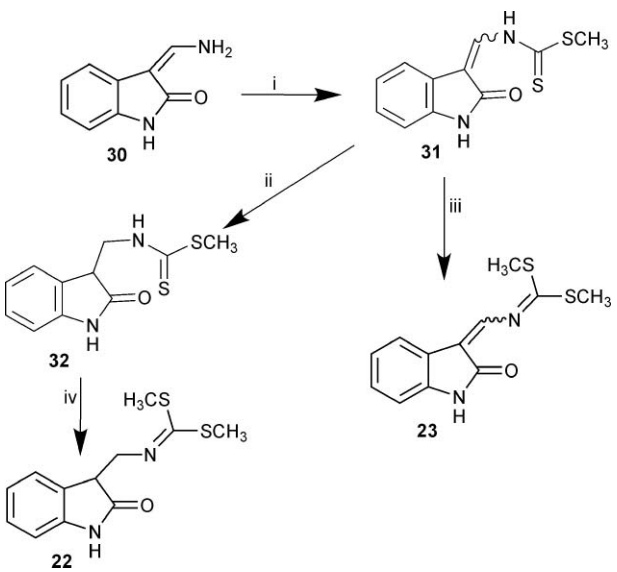
Next, a solution of perdeuterated 3′-(1-nitroethyl)indole (**17a**, 91 mg, 0.46 mmol) in MeOH (0.9 ml) was added to a stirred solution of MeONa (21 mg Na in anhydrous MeOH, 0.9 ml). After stirring for 30 min, the solvent was evaporated at room temperature, the reaction mixture was suspended in cooled DME (1 ml, –40 °C), and a solution of $SOCl_2$ (90 µl, 1.20 mmol) in DME (1 ml) was added dropwise *via* syringe under argon. After stirring for 1 h at –40 °C, the mixture was diluted with H_2O (5 ml), was concentrated (evaporation of most of the DME) under vacuum, and extracted with CH_2Cl_2 (3 × 25 ml). The combined organic layer was dried and concentrated under reduced pressure at room temperature to yield crude [2,2,4′,5′,6′,7′-$^2H_6$]indolyl-3-acetohydroximoylchloride (**18a**, 85 mg), which was directly used for the next step without further purification. 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranose (146 mg; 0.40 mmol) in dry CH_2Cl_2 (2 ml) and triethylamine (170 µl, 1.20 mmol) diluted with dry Et_2O (1 ml) were successively added to the crude [2,2,4′,5′,6′,7′-$^2H_6$]indolyl-3-acetohydroximoylchloride (**18a**, 85 mg, 0.40 mmol) in Et_2O–CH_2Cl_2 (6 ml, 2 : 1 v/v) under argon. After stirring for 3 h at room temperature, the reaction mixture was acidified with H_2SO_4 (0.5 M), extracted with CH_2Cl_2 (3 × 40 ml), and the combined organic layer was dried and concentrated under reduced pressure to yield 105 mg of crude product **19a**. After column chromatography (silica gel, CH_2Cl_2–MeOH, 98 : 2), [2,2,4′,5′,6′,7′-$^2H_6$]indolyl-3-tetraacetylglucoacetothiohydroximate (**19a**) was obtained in 38% yield (82 mg). A solution of chlorosulfonic acid (90 µl, 1.3 mmol) in dry diethyl ether (600 µl) was added over a period of 30 min to a cooled and stirred solution of **19a** (70 mg; 0.13 mmol) in dry pyridine (800 µl) and dry CH_2Cl_2 (600 µl) under argon. After stirring for 24 h at room temperature under argon, the reaction mixture was diluted with an aq. $KHCO_3$ solution (0.1 g in 2.0 ml), stirred for another hour, and extracted with $CHCl_3$ (3 × 50 ml). The combined organic layer was dried and concentrated under reduced pressure to yield 100 mg of crude reaction product. Chromatography over silica gel (CH_2Cl_2–MeOH, 95 : 5, v/v) afforded d_6-tetraacetylated glucobrassicin (**20a**, 54 mg) in 66% yield. Hydrolysis of d_6-tetraacetylated glucobrassicin (**20a**, 56 mg;

0.085 mmol) in anhydrous MeOH (2 ml) plus KOMe (1 M) to obtain pH 8, under argon at room temperature for 24 h, followed by neutralization with AcOH and concentration to dryness yielded 26.7 mg of [2,2,4′,5′,6′,7′-$^2H_6$]glucobrassicin (**12a**) containing KOAc.

HPLC t_R = 4.6 min. $[\alpha]_D$ = –22 (*c* 0.17, H_2O). UV (H_2O) λ_{max} nm (log ε): 218 (5.3), 277 (4.6). FTIR (KBr, cm^{-1}) ν_{max}: 1570, 1406, 1260, 1061, 786 cm^{-1}. ^1H NMR δ (500 MHz, D_2O) 7.32 (1 H, s), 3.54 (2H, d, *J* = 2.6 Hz), 3.32 (1H, dd, *J* = 9.6, 9.4 Hz), 3.26 (1H, dd, *J* = 9.6, 9.4 Hz), 3.17 (1H, dd, *J* = 9.0, 9.0 Hz), 2.90–2.92 (1H, m). ^{13}C NMR δ (125.8 MHz, D_2O) 163.6, 136.7, 126.5, 124.6, 108.4, 81.8, 80.3, 77.3, 72.2, 69.0, 62.9, 60.6. HR-ESI-MS *m/z* calc. for $C_{16}H_{13}{}^2H_6N_2O_9S_2$: 453.0906; found 453.0914 $[M-1]^-$.

Syntheses of demethoxydihydrowasalexin (22) and demethoxywasalexins A and B (23)

NaH (60% suspension in mineral oil, 10 mg, 0.25 mmol) and CS_2 (30 µl, 0.5 mmol), were added to a stirred solution of 3-aminomethylene-2-oxindole[25] (80 mg, 0.50 mmol) in dry THF (1.0 ml, cooled to 0 °C), and after 10 min CH_3I (15 µl, 0.23 mmol). The mixture was stirred for 30 min at 0 °C, was diluted with brine (10 ml), was extracted (EtOAc), and the combined organic extract was dried and concentrated to yield crude **31**. Pyridine (30 µl, 0.37 mmol) and Et_3N (50 µl, 0.36 mmol) were added to a solution of **31** (10 mg, 0.040 mmol) in CH_2Cl_2 (3.0 ml) and the reaction mixture was stirred at 30 °C for 10 min. MeI (8.0 µl, 0.13 mmol) was added to the reaction mixture and the solution was stirred for 14 h at 30 °C. The reaction mixture was diluted with brine, extracted with EtOAc, and the organic extract was concentrated. After separation of the extract by prep TLC, demethoxywasalexin (**23**, yellow solid, mixture of *E* and *Z* isomers 4 : 1) was obtained in 53% yield (5 mg) (Scheme 6).



Scheme 6 Synthesis of demethoxydihydrowasalexin (**22**) and demethoxywasalexins (**23**); reagents and reaction conditions: i. NaH–THF, CS_2, MeI, 0 °C; ii. $NaBH_3CN$, AcOH, 55%; iii. Py–Et_3N, MeI, 53%; iv. $(CH_3O)_2SO_2$, K_2CO_3, acetone, 58%.

$NaBH_3CN$ (90 mg, 1.4 mmol) was added to a solution of compound **31** (18 mg, 0.072 mmol) in AcOH (1.0 ml). The mixture was stirred at 60 °C for 18 h, was diluted with water (3 ml) and the pH of the solution adjusted to 6 with Na_2CO_3 (sat. solution) and the solution was extracted (EtOAc). The combined organic extract was concentrated and the residue was subjected to chromatography over silica gel (EtOAc–hexane, 3 : 7) to yield **32** (10 mg, 55%). Dimethylsulfate (4 µl, 0.04 mmol) and K_2CO_3 (6 mg, 0.04 mmol) were added to a solution of **32** (8 mg, 0.03 mmol) in acetone (200 µl) and the mixture was stirred at 30 °C for 20 h. The reaction mixture was concentrated, then diluted with water (3 ml) and extracted with EtOAc. The combined organic extract was dried, was concentrated and the residue was subjected to column chromatography (EtO_2) to afford demethoxydihydrowasalexin (**22**, yellow solid, 5 mg, 58%).

Demethoxywasalexins A and B (23). HPLC t_R = 16.0 min. UV (CH_3CN) λ_{max} (nm): 254, 286, 362. FTIR (KBr, cm^{-1}) ν_{max}: 3167, 2918, 1695, 1624, 1613, 1466, 1176, 935, 781. ^1H NMR δ (500 MHz, CD_2Cl_2) 8.00 (1 H, s), 7.88 (1H, s, D_2O exchangeable), 7.86 (1H, d, *J* = 7.6 Hz), 7.18 (1H, dd, *J* = 7.7, 7.7 Hz), 6.99 (1H, dd, *J* = 7.6, 7.6 Hz), 6.86 (1H, d, *J* = 7.7), 2.69 (3H, s). ^{13}C NMR δ (125.8 MHz, CD_2Cl_2) 176.6, 170.8, 140.4, 138.7, 128.7, 125.1, 123.6, 122.3, 119.5, 109.7, 16.2 (2C). HREI-MS *m/z* calc. for $C_{12}H_{12}N_2OS_2$: 264.0391; found 264.0396 $[M]^+$ (57), 271.0 (100), 202.0 (43), 144.0 (24). MS-ESI *m/z* $[M+1]^+$ 265.0 (100), 217.0 (64).

Demethoxydihydrodwasalexin (22). HPLC t_R = 14.8 min. UV (CH_3CN) λ_{max} (nm): 242. FTIR (KBr, cm^{-1}) ν_{max}: 3180, 2922, 2851, 1703, 1621, 1574, 1468, 747. ^1H NMR δ (500 MHz, CD_2Cl_2) 8.22 (1 H, s), 7.29 (1H, d, *J* = 7.4), 7.20 (1H, dd, *J* = 7.8, 7.8 Hz), 6.99 (1H, dd, *J* = 7.4, 7.4 Hz), 6.86 (1H, d, *J* = 7.8 Hz), 3.98 (1H, dd, *J* = 13.5, 3.3), 3.81–3.74 (2H, m), 2.51 (3H, s), 2.19 (3H, s). ^{13}C NMR δ (125.8 MHz, CD_2Cl_2) 178.7, 142.6, 129.9, 128.4, 125.2, 122.6, 118.5, 109.6, 53.6, 47.9, 15.2, 14.9. HREI-MS *m/z* calc. for $C_{12}H_{14}N_2OS_2$: 266.0548; found 266.0542 (6), 146.0 (100). MS-ESI *m/z* $[M+1]^+$ 267 (100), 219 (47), 146 (50).

Biosynthesis: precursor administration experiments

Four-week-old potted plants were irradiated with a 30 W (UV λ_{max} 254 nm) lamp for 60 min in a laminar flow cabinet. After UV-irradiation, plants were allowed to stand in a flow cabinet under fluorescent light for 3 h, and then were uprooted, the leaves were cut at the base of the petiole and were immediately immersed in tubes containing an aqueous solution of the precursor (4 ml, 5×10^{-4} M dissolved in H_2O or H_2O–CH_3OH–Tween 80, 90 : 10 : 0.1, v/v). On average, leaves of two plants were used per precursor, experiments were conducted in triplicate and repeated at least twice for each precursor. Following the uptake of each precursor solution (*ca.* 12 h), the tubes were filled with H_2O and leaves were further incubated for 48 h under continuous fluorescent light. The leaves were frozen in liq. nitrogen, extracted with methanol (20 ml), the methanol extract was filtered off, and was concentrated to dryness. The methanolic residue was extracted with CH_2Cl_2, the CH_2Cl_2 fraction was concentrated under reduced pressure, and the residue was dissolved in acetonitrile to yield the non-polar fraction containing mostly phytoalexins for HPLC-MS analysis. The insoluble residue was dissolved in MeOH–H_2O (1 : 1) and was filtered to yield the polar fraction for HPLC-MS analysis.

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