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# Designer phytoalexins: probing camalexin detoxification pathways in the phytopathogen *Rhizoctonia solani*†

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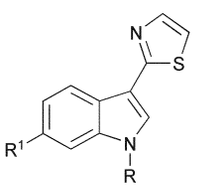
To probe the specificity of a camalexin detoxifying enzyme(s) produced by *Rhizoctonia solani*, the putative 5-camalexin hydroxylase (5-CAHY), the naturally occurring phytoalexin 1-methylcamalexin and designer phytoalexins in which the H-5 of camalexin was replaced with either a methyl group or a fluorine atom were synthesised. This investigation showed that biotransformation of 5-fluorocamalexin by *R. solani* was substantially slower than that of camalexin (12 days *vs*. six to eight hours), 5-methylcamalexin (5–6 days) or 1-methylcamalexin (5–6 days). Antifungal bioassays showed that 5-fluorocamalexin, 5-methylcamalexin and 1-methylcamalexin were more inhibitory to *R. solani* than camalexin, whereas their metabolic products displayed substantially lower inhibitory activity. It was concluded that detoxification *via* oxidation of the indole moiety of camalexins is predominant in the biotransformation of both camalexin and 5-methylcamalexin and likely catalysed by a specific 5-CAHY. By contrast, the pathways for detoxification of 1-methylcamalexin and 5-fluorocamalexin are likely catalysed by non-specific "house-keeping" enzymes. Most importantly, because 1-methylcamalexin showed stronger antifungal activity and was metabolised at substantially slower rate than camalexin this work suggested that, from a plant's perspective 1-methylcamalexin could be a more effective antifungal defence than camalexin.

## Introduction

Phytoalexins such as camalexins§ **1**–**3** are important secondary metabolites produced *de novo* by crucifer plants (Crucifer family) in response to pathogen attack and other forms of stress.[1,2] Recent studies show that crucifer phytoalexins can selectively inhibit phytopathogenic fungi and fungal pathogens can react differently to phytoalexins.[1] Some phytopathogenic fungi are able to metabolise these chemical defences utilising a variety of enzymatic reactions that lead to products devoid of antifungal activity.[1,3] These reactions facilitate pathogen invasion and make the plant more vulnerable to disease. Ongoing investigations of crucifer phytoalexins and their economically important pathogens showed that some of the strongest antifungal phytoalexins are detoxified by fungal pathogens.[1] For example, the root rot fungus (*Rhizoctonia solani* Kuhn) detoxified camalexin (**1**) to 5-hydroxycamalexin (**4**), which was slowly transformed into more polar 5-hydroxy metabolites **5** and **6** (Scheme 1).[4,5] In addition, both camalexins **1** and **2** were detoxified by the stem rot fungus (*Sclerotinia sclerotiorum* (Lib.) de Bary) to 6-*O*-β-D-glucopyranosyl-camalexin (**8**) *via* 6-hydroxycamalexin (**7**).[6] By contrast, "blackleg" (*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.) and "black-spot" (*Alternaria brassicae* (Berk.) Sacc.) fungi, as well as plant pathogenic bacteria did not transform camalexin (**1**).[7] Such results suggest that, in their continuous adaptation, both *R. solani* and *S. sclerotiorum* evolved enzymatic systems able to overcome camalexin (**1**), an otherwise effective plant defence.

In instances where rapid phytoalexin detoxification occurs, it was proposed that an environmentally attractive strategy to control such plant pathogens could make use of inhibitors of those fungal detoxifying enzymes to protect the plant against these pathogens.[1,2,8] Considering that the main detoxification step of camalexin (**1**) in *R. solani* leads to 5-hydroxy derivative **4**,[4,5] it is likely that inhibitors of this enzymatic reaction could prevent or slow down the fungal metabolism of camalexin (**1**). If the enzyme(s) catalysing camalexin detoxification is specific, the putative 5-camalexin hydroxylase (5-CAHY), it is anticipated that replacement of H-5 of camalexin with a different atom/group will slow down if not stop this oxidative step. Hence, 5-substituted camalexin derivatives are good lead structures for a rational design of potential detoxification inhibitors. Towards this end, the specificity of camalexin detoxifying enzyme(s) was probed with substrates in which the H-5 of camalexin was replaced with either a methyl group (**9**) or a fluorine atom (**10**). In addition, the naturally occurring 1-methyl derivative **3** was used to probe the effect of structural parameters such as hydrogen bond, hydrophobicity, and steric bulk of the substrate on the transformation process.




**1** R=$R_1$= H
**2** R=H; $R_1$= $OCH_3$
**3** R=$CH_3$; $R^1$=H

Here we report for the first time the metabolic pathways of the naturally occurring phytoalexin 1-methylcamalexin (**3**) and designer camalexins **9** and **10**, as well as the antifungal activity of camalexins **3**,**9**,**10** and their metabolites. *R. solani* transformed 1-methylcamalexin (**3**), 5-methylcamalexin (**9**) and 5-fluorocamalexin (**10**) at substantially slower rates than camalexin (**1**). Importantly, 1-methylcamalexin (**3**) was metabolised at a much slower rate than camalexin (**1**) (five to six days *vs*. six to eight hours), while the biotransformation of **10** was much slower (10–12 days). These findings suggest that, from a plant's perspective, 1-methylcamalexin (**3**) is a more effective

† Electronic supplementary information (ESI) available: $^1$H and $^{13}$C NMR spectral data for camalexin (**1**). See http://www.rsc.org/suppdata/ob/b4/b400031e/

‡ Work taken from thesis submitted in partial fulfilment of M.Sc. degree

§ For reviews on cruciferous phytoalexins see references 1 and 2; for plant species producing camalexins see ref. 1.

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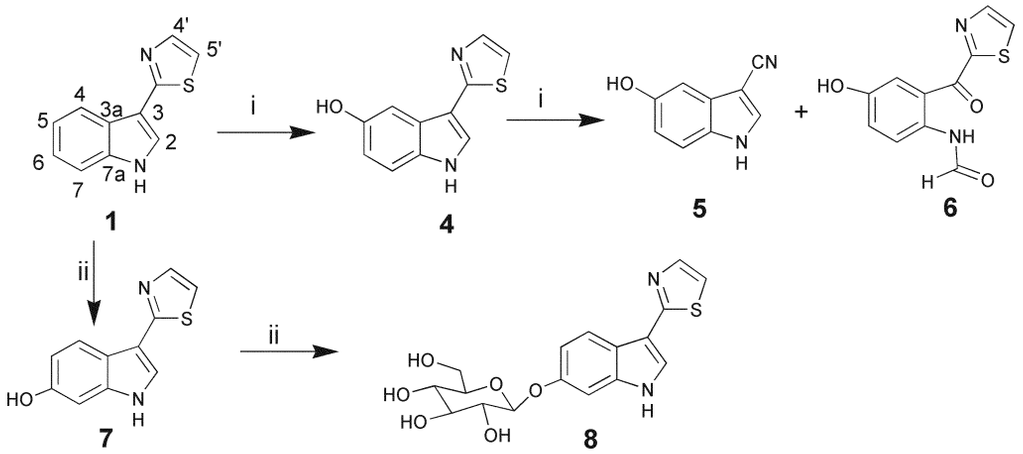
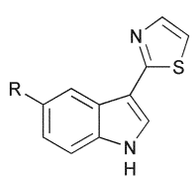


**Scheme 1** Detoxification pathway of camalexin (**1**) in phytopathogenic fungi: i) *Rhizoctonia solani*; ii) *Sclerotinia sclerotiorum*.

antifungal defence than camalexin (**1**) and that 5-fluorocamalexin (**10**) is the best designer phytoalexin against *R. solani*.

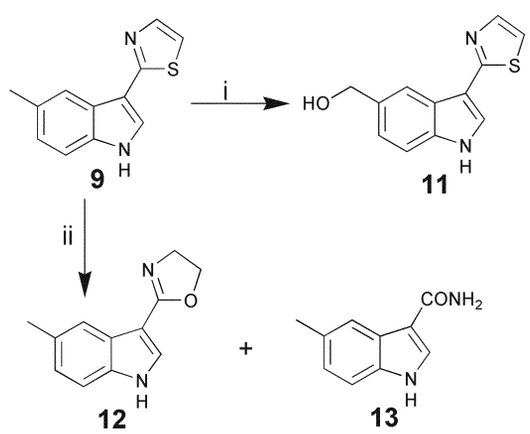
## Results

Camalexin (**1**) and designer camalexins **9** and **10** were synthesised from the respective indoles,[9] whereas **3** was obtained from camalexin following treatment of with NaH/MeI. To establish if compounds **3**, **9** and **10** were metabolised and the optimum time to isolate putative intermediates/products of each transformation, each compound (final concentration $1.0 \times 10^{-4}$ M) was incubated with *R. solani*. For comparison of biotransformation rates, camalexin (**1**, final concentration $1.0 \times 10^{-4}$ M) was incubated with *R. solani* under similar conditions. Samples were withdrawn from liquid cultures every 6 hours for 24 h, and every 24 h up to 12 days, were extracted with EtOAc and the extracts were analysed by HPLC. Comparison of the HPLC chromatograms showed that camalexin (**1**) was transformed almost completely in six to eight hours of incubation, while 5-methylcamalexin (**9**) and 1-methylcamalexin (**3**) were transformed only after five to six days of incubation. Importantly, *R. solani* transformed 5-fluorocamalexin (**10**) at much slower rate, achieving complete transformation only after 10–12 days of incubation. Next, larger scale cultures of *R. solani* incubated with **3**,**9** and **10** allowed the isolation of several metabolites. The chemical structures of major and minor metabolites were deduced from their spectroscopic data (NMR data in Tables 1 and 2) and comparison with those of camalexins and related compounds, is as follows.



**9** R=$CH_3$
**10** R=F

5-Methylcamalexin (**9**) was transformed by *R. solani* to yield mainly a single metabolite (HPLC $R_t$ = 10.4 min) after three to four days of incubation (Table 3). Relative to 5-methylcamalexin ($C_{12}H_{10}N_2S$), this new metabolite (**11**) contained an additional oxygen atom ($C_{12}H_{10}N_2OS$) as determined by HR-EIMS (*m*/*z* found 230.0512, calcd. 230.0514) in combination with $^1$H and $^{13}$C NMR spectral data. Comparison of the $^1$H and $^{13}$C NMR spectral data (Tables 1 and 2) of metabolite **11** with that of 5-methylcamalexin (**9**) indicated that both the chemical shifts and the coupling patterns of the protons on the indole and thiazole rings were similar, suggesting that both rings were intact. However, the proton and carbon signals due to the methyl group appeared at much lower field ($\delta_H$ 2.54, s, $\delta_C$ 22.1 $\delta_H$ in **9** *vs*. 4.70 ppm and $\delta_C$ 64.9 ppm in **11**), suggesting the presence of a hydroxymethylene instead of a methyl group. This hypothesis was supported by the FTIR spectrum, which showed a hydroxyl characteristic absorption at 3245 $cm^{-1}$ (OH).



**Scheme 2** Biotransformation of 5-methylcamalexin (**9**) by *Rhizoctonia solani*; i) major pathway, **11** (>80% after 4 days of incubation); ii) minor pathway, **12** and **13** (<10% after 4 days of incubation).

On the basis of these data, the structure of this new metabolite was assigned as 5-hydroxymethylcamalexin (**11**). Two additional but minor metabolites (corresponding to less than 10% of camalexin) were also isolated from biotransformation of 5-methylcamalexin (**9**) by *R. solani*. The structures of these minor metabolites were determined by analysis of spectroscopic data to be 2-(5-methyl-3-indolyl)-oxazoline (**12**) and 5-methylindole-3-carboxamide (**13**), as follows. Metabolite **12** contained oxygen and no sulfur ($C_{12}H_{12}N_2O$, *m*/*z* found 200.0945 [$M^+$], calc. 200.0950). The $^1$H NMR spectrum of metabolite **12** indicated the presence of an intact indole ring and the absence of thiazole protons; two new triplets due to four methylene protons indicated that the thiazole moiety of 5-methylcamalexin (**9**) had been reduced. This hypothesis was also supported by the $^{13}$C NMR spectrum, which displayed two signals indicative of $sp^3$ carbon atoms ($\delta_C$ 66.4 and 55.0), in addition to the nine carbon signals due to $sp^2$ carbon atoms. The structure of **12** was further corroborated by comparison of the $^1$H and $^{13}$C NMR data with that of 2-(3-indolyl)-oxazoline, a similar metabolite previously obtained from camalexin (**1**).[4] The identity of metabolite **13** was deduced from its spectroscopic data and further confirmed by synthesis from 5-methylindole through acylation with chlorosulfonyl isocyanate followed by hydrolysis in acetic acid, as described in the experimental.[10] To the best of our knowledge, 5-methyl-

**Table 1** $^{1}$H NMR chemical shifts (ppm) and multiplicities (*J* in Hz) of 1-methylcamalexin (**3**), 5-methylcamalexin (**9**), 5-fluorocamalexin (**10**) in $CDCl_3$ and metabolites **11**–**12** in $CD_3CN$, and **13**–**19** in $CD_3OD$

| H | **3** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 7.78 s | 8.08 s | 7.98 s | 7.90 s | 7.71 s | 7.89 s | 7.91 s | 7.80 s | 7.87 s | 8.02 s | 7.89 s | 8.00 s |
| 4 | 8.26 m | 7.81 d (2.5) | 7.81 dd (9, 2.5) | 8.22 d (1.5) | 7.97 d (1.5) | 7.92 s | 7.72 d (8) | 8.06 d (8) | 8.12 d (8) | 7.34 dd (9, 2.5) | 7.71 dd (10, 2.5) | 7.81 dd (10, 2.5) |
| 5 | 7.35 m | — | — | — | — | — | 7.29 dd (8, 7.5) | 7.21 dd (8, 7.5) | 7.22 dd (7.5, 7) | — | — | — |
| 6 | 7.32 m | 7.13 d (8) | 7.03 ddd (9, 9, 2.5) | 7.25 dd (8.5, 1.5) | 7.09 dd (8.5, 1.5) | 7.05 d (8.5) | 7.37 dd (8, 7.5) | 7.28 dd (8, 7.5) | 7.28 dd (8, 7.5) | 7.09 ddd (9, 9, 2.5) | 6.99 ddd (9, 9, 2.5) | 6.98 ddd (9, 9, 2.5) |
| 7 | 7.40 m | 7.32 d (8) | 7.46 dd (9.0, 4.5) | 7.48 d (8.5) | 7.39 d (8.5) | 7.33 d (8.5) | 7.55 d (8) | 7.46 d (8) | 7.46 d (8) | 7.52 dd (9, 4.5) | 7.42 dd (9, 4.5) | 7.42 dd (9, 4.5) |
| 4′ | 7.83 d (3) | 7.86 d (3.5) | 7.80 d (3.5) | 7.80 d (3.5) | 4.00 t (9.5) | — | — | 4.02 t (9.5) | — | — | 4.02 t (9.5) | — |
| 5′ | 7.23 d (3) | 7.25 d (3.5) | 7.45 d (3.5) | 7.35 d (3.5) | 4.33 t (9.5) | — | — | 4.11 t (9.5) | — | — | 4.46 t (9.5) | — |
| Other | N$CH_3$ 3.87 s | C$H_3$ 2.54 s NH, 8.66, br s | — | C$H_2$OH 4.70 s NH, 9.70, br s | C$H_3$ 2.47 s NH, 9.64, br s | C$H_3$ 2.47 s | N$CH_3$ 3.89 s | N$CH_3$ 3.87 s | N$CH_3$ 3.88 s | — | — | — |

**Table 2** $^{13}$C-NMR chemical shifts (ppm) of 1-methylcamalexin (**3**), 5-methylcamalexin (**9**), 5-fluorocamalexin (**10**) in $CDCl_3$ and metabolites **11**–**12** in $CD_3CN$, and **13**–**19** in $CD_3OD$

| C | **3** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 129.4 | 125.0 | 127.4 | 126.3 | 128.7 | 129.2 | 135.5 | 133.2 | 133.0 | 135.1 | 130.8 | 130.4 |
| 3 | 111.3 | 112.4 | 111.3, d $^{4}J_{C-F}$ = 5 | 111.7 | 104.8 | 109.7 | 82.7 | 103.0 | 109.2 | 85.6 | 104.2 | 110.3, d $^{4}J_{C-F}$ = 5 |
| 3a | 125.6 | 125.3 | 125.2, d $^{3}J_{C-F}$ = 10 | 124.9 | 126.5 | 126.4 | 126.5 | 126.3 | 126.8 | 128.1, d $^{3}J_{C-F}$ = 10 | 126.3, d $^{3}J_{C-F}$ = 10 | 127.0, d $^{3}J_{C-F}$ = 10 |
| 4 | 121.1 | 120.6 | 104.9, d $^{2}J_{C-F}$ = 25 | 119.2 | 121.0 | 120.4 | 114.5 | 121.1 | 121.0 | 103.6, d $^{2}J_{C-F}$ = 25 | 105.7, d $^{2}J_{C-F}$ = 25 | 105.8, d $^{2}J_{C-F}$ = 25 |
| 5 | 123.1 | 131.4 | 159.1, d $^{1}J_{C-F}$ = 235 | 135.3 | 130.6 | 130.5 | 120.6 | 121.2 | 121.3 | 159.5, d $^{1}J_{C-F}$ = 234 | 159.1, d $^{1}J_{C-F}$ = 234 | 159.2, d $^{1}J_{C-F}$ = 234 |
| 6 | 121.5 | 125.2 | 110.9, d $^{2}J_{C-F}$ = 26 | 122.9 | 124.5 | 124.0 | 122.3 | 122.7 | 122.6 | 112.1, d $^{2}J_{C-F}$ = 26 | 110.8, d $^{2}J_{C-F}$ = 26 | 110.7, d $^{2}J_{C-F}$ = 26 |
| 7 | 110.1 | 111.6 | 113.0, d $^{3}J_{C-F}$ = 10 | 112.2 | 111.8 | 111.5 | 109.4 | 109.9 | 109.9 | 113.9, d $^{3}J_{C-F}$ = 10 | 112.8, d $^{3}J_{C-F}$ = 10 | 112.7, d $^{3}J_{C-F}$ = 10 |
| 7a | 137.7 | 135.1 | 133.9 | 136.4 | 135.0 | 135.5 | 135.2 | 137.8 | 137.8 | 132.5 | 133.6 | 133.6 |
| 2′ | 163.6 | 163.9 | 164.4 | 163.7 | 161.4 | 170.1 | 114.5 | 163.7 | 169.5 | 115.7 | 163.7 | 169.5 |
| 4′ | 142.8 | 142.9 | 142.0 | 143.0 | 55.0 | — | — | 53.7 | — | — | 53.7 | — |
| 5′ | 115.9 | 116.1 | 116.1 | 116.5 | 66.4 | — | — | 66.9 | — | — | 67.0 | — |
| Other | N$CH_3$ 33.6 | C$H_3$ 22.1 | — | C$H_2$OH 64.9 | C$H_3$ 21.0 | C$H_3$ 20.7 | N$CH_3$ 31.2 | N$CH_3$ 32.3 | N$CH_3$ 32.4 | — | — | — |

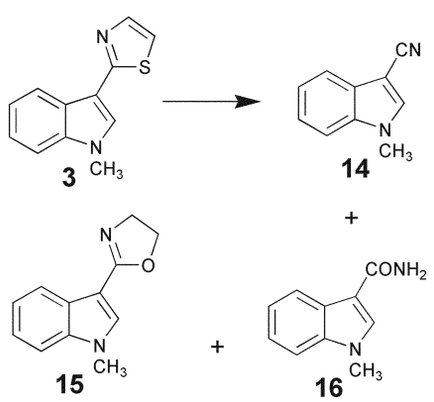
**Table 3** Products of metabolism of camalexins **1**, **3**, **9** and **10** by *Rhizoctonia solani*[a]

| Compound added to fungal cultures[a] | Products (%)[b] of metabolism after incubation for 24 h up to 10 days; no camalexin recovered after 6 days |
|---|---|
| Camalexin (**1**) | Biotransformation to **4** (90%) in 6–8 h |
| 1-Methylcamalexin (**3**) | Biotransformation to **14** (30%), **15** (<5%) and **16** (<5%) in 4 days; no 1-methylcamalexin recovered after 6 days |
| 5-Methylcamalexin (**9**) | Biotransformation to **11** (80%), **12** (<5%) and **13** (<5%) in 4 days; no 5-methylcamalexin recovered after 6 days |
| 5-Fluorocamalexin (**10**) | Biotransformation to **17** (20%), **18** (<5%) and **19** (<5%) in 8 days; *ca*. 10% 5-fluorocamalexin recovered after 10 days |

[a] Compounds were dissolved in DMSO ($1 \times 10^{-4}$M), added to 48-h-old cultures, and incubated at 24 ± 2 °C. [b] Percentage yields were calculated by HPLC analysis using calibration curves constructed for each compound.

camalexin (**9**) and metabolites **11**–**13** are new compounds. Metabolites **11**–**13** were further metabolised to undetermined products.

1-Methylcamalexin (**3**) was transformed by *R. solani* to yield mainly three metabolites (HPLC $R_t$=14.6, 8.7, 5.8 min) after 5–6 days of incubation (Table 3). These metabolites were identified as 1-methylindole-3-carbonitrile (**14**), 1-methylindole-3-carboxamide (**16**) and 2-(1-methyl-3-indolyl)-oxazoline (**15**), as shown in Scheme 3. Metabolite **14** was the major compound, accounting for *ca*. 20–30% of the total amount of 1-methylcamalexin, whereas both **15** and **16** amounted to less than 10%. The molecular formula of compound **14** was determined to be $C_{10}H_8N_2$ by HR-EIMS (*m*/*z* found 156.0687 [M$^+$], calcd. 156.0687) in combination with $^1$H and $^{13}$C NMR spectral data. Analysis of the $^1$H and $^{13}$C NMR spectra indicated an intact indole ring with two substituents, a 1-methyl signal at $\delta_C$ 31.2 and a signal at $\delta_C$ 114.5, which was assigned to a CN substituent at C-3. The $^{13}$C-NMR signal at $\delta_C$ 82.7 was assigned to C-3, which relative to the parent compound was shifted to higher field due to attachment to the CN group. An FTIR absorption band at 2223 cm$^{-1}$ also supported the presence of a CN group, hence the major metabolite from 1-methylcamalexin was carboxynitrile **14**. Metabolite **16** had a molecular formula $C_{10}H_{10}N_2O$ as determined by HR-EIMS (*m*/*z* found 174.0793 [M$^+$], calcd. 174.0793). Relative to 1-methylcamalexin (**3**, $C_{12}H_{10}N_2S$) this metabolite had two carbons less than **3**, corroborated by $^{13}$C NMR data showing a carbon signal at $\delta_C$ 169.5 but no thiazole ring. FTIR showed characteristic absorption bands at 3385 and 3191 cm$^{-1}$ (NH) and 1641 cm$^{-1}$ (C=O). According to these data, the structure was determined to be 1-methylindole-3-carboxamide (**16**). Metabolite **15** also contained oxygen and no sulfur (*m*/*z* found 200.0951 [M$^+$], calc. 200.0950, determined molecular formula: $C_{12}H_{12}N_2O$). Comparison of the $^1$H NMR spectrum of this metabolite with that of 1-methylcamalexin (**3**), indicated the presence of an intact indole ring and the absence of thiazole protons; two new triplets due to four methylene protons indicated that the thiazole moiety of 1-methylcamalexin (**3**) had been reduced. This hypothesis was also supported by the $^{13}$C NMR spectrum, which displayed two signals indicative of sp$^3$ carbon atoms ($\delta_C$ 66.9 and 53.7), in addition to the nine carbon signals due to sp$^2$ carbon atoms. The structure was further confirmed to be 2-(1-methyl-3-indolyl)-oxazoline (**15**) by comparison of the $^1$H and $^{13}$C NMR data with that of a similar metabolite of camalexin.[4] Metabolites **14**–**16**, which were further metabolised to undetermined products, are known compounds but the detailed spectroscopic data has not been described. Both **14** and **16** were recently obtained in one-electron oxidation of 1-methylindole-3-carbaldehyde oximes,[11] whereas metabolite **15** was described in the synthesis of azoles and diazoles containing 1-methylindole fragments.[12]




**Scheme 3** Biotransformation of 1-methylcamalexin (**3**) by *Rhizoctonia solani;* **14** (*ca*. 20% after 5–6 days of incubation); **15** and **16** (<10% after 5–6 days of incubation).

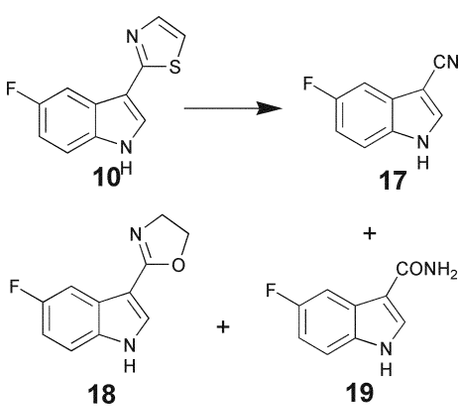
5-Fluorocamalexin (**10**) was transformed by *R. solani* to yield mainly three metabolites (HPLC $R_t$ = 12.2, 7.3, 4.4 min) after 10–12 days of incubation (Table 3). These metabolites were identified as 5-fluoroindole-3-carbonitrile (**17**) (15%), 5-fluoro-indole-3-carboxamide (**19**) (4%) and 2-(5-fluoro-3-indolyl)-oxazoline (**18**) (3%), based on analysis of the spectroscopic data. The molecular formula of metabolite **17** was determined to be $C_9H_5N_2F$ on the basis of HR-EIMS (*m*/*z* found 160.0432 [M$^+$], calcd. 160.0437) in combination with $^1$H and $^{13}$C NMR spectral data. The FTIR displayed characteristic absorption bands for –CN at 2228 cm$^{-1}$ and –NH 3257 cm$^{-1}$. Analyses of both the $^1$H NMR and $^{13}$C NMR data showed an indole ring similar to the parent compound **10**. On the basis of the spectroscopic data, metabolite **17** was identified as 5-fluoroindole-3-carbonitrile. The HR-EIMS (*m*/*z* found 178.0539 [M$^+$], calcd. 178.0542) of metabolite **19** indicated a molecular formula of $C_9H_7N_2OF$. The FTIR spectrum exhibited characteristic bands at 3191 cm$^{-1}$ (NH) and 1636 cm$^{-1}$ (C=O). The $^1$H NMR spectrum showed a 3,5-disubstituted indole, which was corroborated by $^{13}$C NMR data. Thus, to accommodate two nitrogen atoms and an oxygen atom in the molecular formula, the side chain of compound **19** was assigned as an amide group. This structure was confirmed to be 5-fluoroindole-3-carboxamide (**19**) by synthesis *via* acylation of 5-fluoroindole with chlorosulfonyl isocyanate, followed by hydrolysis in acetic acid,[10] as described in the Experimental section. The next metabolite (**18**) was determined to have a molecular formula of $C_{11}H_9N_2OF$ (*m*/*z* found 204.0693 [M$^+$], calcd. 204.0699) by HR-EIMS. Comparison of the $^1$H NMR spectrum of this compound with that of 5-fluorocamalexin (**10**) indicated the presence of an intact indole ring and the absence of thiazole protons; two new triplets due to four protons ($\delta_H$ 4.02 and 4.46) indicated that the thiazole moiety of 5-fluorocamalexin (**10**) had been reduced. This hypothesis was also supported by the $^{13}$C NMR spectrum, which displayed two signals indicative of sp$^3$ C atoms ($\delta_C$ 53.7 and 67.0), in addition to nine carbon signals due to sp$^2$ C atoms. These spectroscopic features suggested that this metabolite contained an oxazoline instead of a thiazole moiety, hence, it was assigned as 2-(5-fluoro-3-indolyl)-oxazoline (**18**). Metabolites **17**–**19**, which were further metabolised to undetermined products, have not been reported previously.

The antifungal activity of naturally occurring camalexins **1** and **3**, analogues **9** and **10**, and metabolites **11**–**19** against *R. solani* were tested using the radial mycelial growth inhibition assay described in the experimental. Solutions of each compound in potato dextrose broth were inoculated with *R. solani* and incubated under constant light for 96 hours (Table 4). Camalexin (**1**) and the structural analogues **3**,**9** and **10** showed strong inhibitory effect against *R. solani*; however, camalexin (**1**)

**Table 4** Bioassay results of camalexins **1**, **3**, **9**, **10** and metabolites **11**–**19** against *Rhizoctonia solani* after 4 days of incubation

| Compound | Concentration/M | % Inhibition[a] |
|---|---|---|
| Camalexin (**1**) | $2.5 \times 10^{-4}$ | C. I.[b] |
| | $1.3 \times 10^{-4}$ | N. I.[c] |
| 1-methylcamalexin (**3**) | $2.5 \times 10^{-4}$ | C. I. |
| | $1.3 \times 10^{-4}$ | 90 ± 1 |
| 5-Methylcamalexin (**9**) | $2.5 \times 10^{-4}$ | C. I. |
| | $1.3 \times 10^{-4}$ | 60 ± 1 |
| 5-Fluorocamalexin (**10**) | $1.3 \times 10^{-4}$ | C. I. |
| | $0.8 \times 10^{-4}$ | C. I. |
| 5-Hydroxymethylcamalexin (**11**) | $5.0 \times 10^{-4}$ | N. I. |
| 2-(5-Methyl-3-indolyl)-2-oxazoline (**12**) | $5.0 \times 10^{-4}$ | N. I. |
| 1-Methylindole-3-carboxamide (**13**) | $5.0 \times 10^{-4}$ | N. I. |
| 1-Methylindole-3-carbonitrile (**14**) | $5.0 \times 10^{-4}$ | 88 ± 1 |
| | $2.5 \times 10^{-4}$ | 60 ± 1 |
| | $1.3 \times 10^{-4}$ | N. I. |
| 2-(1-Methyl-3-indolyl)-2-oxazoline (**15**) | $5.0 \times 10^{-4}$ | N. I. |
| 1-Methylindole-3-carboxamide (**16**) | $5.0 \times 10^{-4}$ | N. I. |
| 5-Fluoroindole-3-carbonitrile (**17**) | $5.0 \times 10^{-4}$ | 93 ± 1 |
| | $2.5 \times 10^{-4}$ | 50 ± 1 |
| | $1.3 \times 10^{-4}$ | 44 ± 1 |
| 2-(5-Fluoro-3-indolyl)-2-oxazoline (**18**) | $5.0 \times 10^{-4}$ | N. I. |
| 5-Fluoroindole-3-carboxamide (**19**) | $5.0 \times 10^{-4}$ | N. I. |

[a] % inhibition = 100 – [(growth on treated/growth in control) × 100] ± SD; results are the means of at least three separate experiments. [b] C. I. = complete inhibition. [c] N. I. = no inhibition.




**Scheme 4** Biotransformation of 5-fluorocamalexin (**10**) by *Rhizoctonia solani;* **17** (*ca*. 15% after 9–10 days of incubation); **12** and **13** (<8% after 9–10 days of incubation).

inhibited the fungal growth less than compounds **3**, **9** and **10** did. As shown in Table 4, at a concentration of $1.3 \times 10^{-4}$ M, camalexin (**1**) had no inhibitory effect after incubation, whereas 5-fluorocamalexin (**10**) showed the strongest fungal growth inhibition of all compounds. The antifungal activities of metabolites isolated from large-scale biotransformation experiments of 5-methylcamalexin (**9**), 5-fluorocamalexin (**10**), and 1-methylcamalexin (**3**) were also determined and were compared with that of their parent compound to determine whether the biotransformations are detoxification processes. It was determined that 5-hydroxymethylcamalexin (**11**), the major metabolite from 5-methylcamalexin (**9**), did not inhibit mycelial growth, even at the highest concentration ($5.0 \times 10^{-4}$ M). In addition, 5-fluoroindole-3-carbonitrile (**17**) and 1-methylindole-3-carbonitrile (**14**), major metabolites from 5-fluorocamalexin (**10**) and 1-methylcamalexin (**3**), respectively, showed partial mycelial growth inhibition, but both were substantially less inhibitory than their starting materials. Thus, it can be concluded that the transformations of 1-methylcamalexin (**3**), 5-methylcamalexin (**9**), and 5-fluorocamalexin (**10**) by *R. solani* are detoxification processes.

## Discussion

Previous results[4,5] on the metabolism of camalexin (**1**) suggested that the most important step in camalexin detoxification involves enzymatic oxidation of C-5 by a putative 5-CAHY; this step is followed by further transformations of minor consequence to the overall detoxification process (Scheme 1) carried out by *R. solani.* To further understand the parameters affecting these *in vivo* transformations, in particular, the substrate specificity of the putative 5-CAHY, three metabolic probes, 5-methylcamalexin (**9**), 5-fluorocamalexin (**10**), and 1-methylcamalexin (**3**) were synthesised. We established that these three compounds were transformed by *R. solani* but at rather different rates; while 5-fluorocamalexin (**10**) remained in culture up to 12 days, both 5-methylcamalexin (**9**) and 1-methylcamalexin (**1**) were metabolised in five to six days. Under similar conditions, camalexin was transformed in six to eight hours. Considering that hydroxylation of 5-methylcamalexin (**9**) occurred at the methyl group at the 5-position of the indole moiety, similar to camalexin (**1**), it is likely that the putative 5-CAHY also catalyses this step; however, its affinity for substrate **9** appears to be much lower (substantially slower rate). It appears that 5-CAHY is rather specific since neither 5-fluorocamalexin (**10**) nor 1-methylcamalexin (**3**) were transformed through hydroxylation at C-5 of indole. Interestingly, although no substituent blocked the C-5 position of the indole moiety of 1-methylcamalexin (**3**), we did not isolate or detect compounds resulting from C-5 oxidation. Perhaps the methyl group at *N*-1 of **3** makes the molecular fit unsuitable or a higher hydrophobicity prevents **3** from reaching the target site. These different biopathways suggest that *R. solani* employs less selective enzymes to detoxify **3** and **10**. Hence, it is concluded that the pathway leading to oxidation of the indole moiety is predominant in the biotransformation of both camalexin (**1**) and 5-methylcamalexin (**9**) and likely catalysed by the specific 5-CAHY. By contrast, the pathways for detoxification of 1-methylcamalexin (**3**) and 5-fluorocamalexin (**10**) are likely catalysed by non-specific "house-keeping" enzymes which transform the thiazole moiety, similar to a minor pathway occurring in weakly virulent isolates of *R. solani*.[4] Further understanding of the enzymatic mechanisms involved in these fungal transformations will require kinetic studies with the putative 5-CAHY and/or other detoxifying enzyme(s). These enzymes are of primary importance to design and screen potential detoxification inhibitors of camalexin metabolism by *R. solani*.

Bioassays with camalexins **1**, **3**, **9** and **10** against *R. solani* showed that these compounds were stronger mycelial growth inhibitors than camalexin (**1**) itself. Of these analogues, 5-fluoro-

camalexin (**10**) exhibited the strongest inhibitory activity. The metabolites resulting from transformation of **3**, **9** and **10** were much less toxic against *R. solani* than their starting materials. 5-Fluoroindole-3-carbonitrile (**17**) and 1-methylindole-3-carbonitrile (**14**) showed an inhibitory effect, whereas the other metabolites had little effect on fungal growth (Table 4).

In conclusion, *R. solani* transformed 5-methylcamalexin (**9**), 1-methylcamalexin (**3**) and 5-fluorocamalexin (**10**) at substantially slower rates than that of camalexin (**1**). Importantly, transformation of 5-fluorocamalexin (**10**) was substantially slower (up to 12 days) than camalexins **3** and **9**, suggesting that 5-fluorocamalexin (**10**) is the best designer phytoalexin against *R. solani*. Overall, from a plant's perspective, our results indicate that 1-methylcamalexin (**3**) is a more effective antifungal defence than camalexin (**1**). Considering that *R. solani* is an important pathogen of oilseed crops and that 1-methylcamalexin (**3**) is a naturally occurring phytoalexin, crops producing 1-methylcamalexin (**3**) might be more suitable to overcome this economically important pathogen. Because camalexin (**1**) is produced by a crucifer whose genome has been sequenced, *Arabidopsis thaliana*,[13] it would be possible and relevant to engineer a 1-methylcamalexin (**3**) producing plant and compare the reactions of such a transformant with those of wild type lines of *A. thaliana* to *R. solani.*

## Experimental

### General experimental procedures

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for $CH_2Cl_2$ and $CHCl_3$ that were redistilled. Solvents utilized in syntheses were dried over the following drying agents prior to use: benzene, THF, and $Et_2O$: Na/benzophenone; $CH_2Cl_2$: $CaH_2$. Organic extracts were dried over anhydrous $Na_2SO_4$ and solvents removed under reduced pressure in a rotary evaporator.

HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. Mobile phase: 75% $H_2O$–25% $CH_3CN$ to 100% $CH_3CN$, for 35 min, linear gradient, and a flow rate 1.0 mL $min^{-1}$. NMR spectra were recorded on Bruker AMX 500 spectrometers; for $^1H$ (500 MHz), $\delta$ values were referenced as follows: $CDCl_3$ ($CHCl_3$ 7.27 ppm), $CD_3OD$ ($CD_2HOD$ 3.31 ppm), $CD_3CN$ ($CD_2HCN$ 1.94 ppm); for $^{13}C$ (125.8 MHz) referenced to $CDCl_3$(77.73 ppm), $CD_3OD$ (49.15 ppm), or $CD_3CN$ (1.39, 118.69 ppm), $CD_3S(O)CD_3$ (39.51 ppm). Fourier transform infrared (FTIR) spectra were obtained on a Bio-Rad FTS-40 spectrometer using a diffuse reflectance cell. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer.

### Fungal cultures

*R. solani* isolate AG 2-1 was obtained from Agriculture and Agri-Food Canada Research Station, Saskatoon, Saskatchewan. The fungus was grown on potato dextrose agar (PDA) plates at 24 ± 2 °C, under constant light. Solid cultures were started by placing a plug of mycelium (6 mm diameter) at the centre of the plate containing the agar. Liquid cultures were initiated by cutting plugs of mycelium from the edges of 5-day-old cultures and placing them in PDB medium (5 plugs per 100 mL of medium).

### Fungal growth assays

The antifungal activity of compounds **1**, **3** and **9**–**16** was investigated using the following mycelial radial growth bioassay. Solutions of each compound in DMSO ($5.0 \times 10^{-2}$ M) were used to prepare assay solutions in potato dextrose broth (PDB) at concentrations shown in Table 4; control solutions contained 1% DMSO in PDB. Sterile tissue culture plates (12 wells) containing test solutions and control (1 mL per well) were inoculated with mycelium plugs placed upside down on the centre of each plate (5 mm cut from 5-day-old potato dextrose agar plates of *R. solani* isolate AG 2-1) and incubated under constant light at 23 ± 2 °C for 7 days; measurement of the mycelium radial growth was carried out every 24 h up to 7 days. Control plates containing only DMSO and PDB were prepared and incubated with plugs of *R. solani*, similarly.

### Fungal metabolism

**Time-course study and metabolism of camalexins 1, 3, 9, 10.** Erlenmeyer flasks (250 mL) each containing 100 mL of PDB media were employed. Flasks were inoculated with mycelium plugs of *R. solani* and the flasks were incubated at 25 × 2 °C on a shaker at 120 rpm under constant light. After 48 h a solution of each compound in DMSO was added to fungal cultures (final concentration $1.0 \times 10^{-4}$ M or $2.0 \times 10^{-4}$ M) and to medium (compound stability control); DMSO (200 μl) was added to control cultures. Samples (10 mL each) were withdrawn from the flasks immediately after adding the compounds. Subsequently 10 mL samples were taken every 6 h for 24 h and then every 24–48 h for 10–12 days. The samples were either immediately extracted or frozen for later extraction. Each sample was first extracted with EtOAc; the resulting aqueous layer was acidified (to pH 2 with HCl) and extracted with EtOAc. Finally, the acidic aqueous layer was made alkaline (to pH 10 with 28% aqueous ammonia) and extracted with chloroform. After concentration of the solvent, the extracts were analyzed by HPLC.

**Large scale metabolism experiments.** To obtain larger amounts of each extract to isolate the products of metabolism of each camalexin analogue, experiments were carried out with 2 L batches, as described above for time-course studies.

**Analysis and isolation of metabolites 11–16.** The analyses of the organic extracts and biotransformation products were performed with the HPLC system described above. Only the chromatograms of the EtOAc extracts of the neutral broth showed peaks not present in chromatograms of extracts of control cultures; *n*-butanol extracts, as well as EtOAc extracts of acidic and basic broths were similar to those of control cultures.

The extracts were fractionated by FCC on silica gel with gradient elution ($CH_2Cl_2$–MeOH, 96 : 4 to 90 : 10). Each fraction was analyzed by HPLC. The metabolites were isolated by preparative TLC (silica gel, $CH_2Cl_2$–MeOH, 96 : 4, or benzene–EtOAc–acetic acid, 5 : 4 : 1, or toluene–EtOAc–acetic acid, 6 : 3 : 1).

### Synthesis

**Syntheses of camalexins 1, 3, 9 and 10.** Camalexin (**1**) was synthesized as previously reported.[9] 5-Methylcamalexin (**9**, 421 mg, 79% based on 2-bromothiazole) was synthesised similarly but replacing indole with 5-methylindole (663 mg, 5.06 mmol). 5-Fluorocamalexin (**10**, 129 mg, 60% yield based on recovered 5-fluoroindole) was synthesised similarly but replacing indole with 5-fluoroindole (150 mg, 1.10 mmol). 1-Methylcamalexin (**3**) (in dry THF, 4 mL) was synthesised from camalexin (**1**, 46.0 mg, 0.23 mmol) by treatment with NaH (19.2 mg, 60%, 0.480 mmol) and $ICH_3$ (20 μl, 0.32 mmol). The crude product (58.6 mg) was purified by FCC (hexane–EtOAc, 75 : 25) to afford 1-methylcamalexin (**3**) (39.0 mg, 80%).

**Synthesis of 5-methylindole-3-carboxamide (13).** To a solution of 5-methylindole (50 mg, 0.38 mmol) in 2.5 mL of

acetonitrile, 40 µl (70.8 mg, 0.50 mmol) of chlorosulfonyl isocyanate was added dropwise at 50 °C while stirring. After stirring at 50 °C for 3.5 h, the reaction mixture was concentrated on a rotary evaporator, acetic acid (2 mL) was added to the residue and stirred for 15 min at 60 °C. Removal of the solvent gave crude product (148.4 mg), which was separated by FCC with gradient elution $CH_2Cl_2$–MeOH (93 : 7 to 90 : 10) to yield 5-methylindole-3-carboxamide (**13**) as yellow crystals (23 mg, 40% based on recovered 5-methylindole).

**Synthesis 5-fluoroindole-3-carboxamide (19).** To a solution of 5-fluoroindole (50 mg, 0.37 mmol) in 2.5 mL of acetonitrile, 50 µl (81.3 mg, 0.57 mmol) chlorosulfonyl isocyanate was added dropwise at 45 °C with stirring. After 30 min, the reaction mixture was concentrated on a rotary evaporator, acetic acid (2 mL) was added to the residue which was stirred for 40 min at 60 °C. Removal of the solvent gave a crude product (138.6 mg), which was separated by FCC with $CH_2Cl_2$–MeOH (90 : 10) to yield 5-fluoroindole-3-carboxamide (**19**) as light yellow crystals (49 mg, 74% based on recovered 5-fluoroindole).

**Camalexin (1).** HPLC $R_t$ = 26.1 min, remaining data in supporting information.

**1-Methylcamalexin (3).** HPLC $R_t$ = 23.0 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI: *m*/*z* (% relative abundance): measured 214.0561 $[M]^+$ (100), (calculated for $C_{12}H_{10}N_2S$, 214.0565), 156 (31); FTIR: 3107, 3072, 1928, 1553, 1463, 1355, 1247, 1211, 1164 $cm^{-1}$.

**5-Methylcamalexin (9).** HPLC $R_t$ = 21.4 min. $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 214.0562 $[M]^+$ (100), (calculated for $C_{12}H_{10}N_2S$, 214.0565), 213 (34), 155 (18); FTIR: 3387, 3112, 2916, 1544, 1473, 1437, 1240, 1114, 912, 864 $cm^{-1}$.

**5-Fluorocamalexin (10).** HPLC $R_t$ = 24.0 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 218.0311 $[M]^+$ (100), (calculated for $C_{11}H_7N_2FS$, 218.0314); FTIR: 3174, 2922, 1553, 1463, 1194, 1176, 865 $cm^{-1}$.

**5-Hydroxymethylcamalexin (11).** HPLC $R_t$ = 6.8 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 230.0512 $[M]^+$ (100), (calculated for $C_{12}H_{10}N_2OS$, 230.0514), 229 (22), 213 (24), 201 (49); FTIR: 3245, 2922, 2862, 1654, 1547, 1439, 1242 $cm^{-1}$.

**2-(5-Methyl-3-indolyl)-oxazoline (12).** HPLC $R_t$ = 8.3 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 200.0945 $[M]^+$ (100), (calculated for $C_{12}H_{12}N_2O$, 200.0950), 199 (20), 171 (15), 170 (61), 158 (18); FTIR: 3191, 2916, 2862, 1636, 1535, 1439, 1242, 1134 $cm^{-1}$.

**5-Methylindole-3-carboxamide (13).** HPLC $R_t$ = 5.2 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 174.0790 $[M]^+$ (74), (calculated for $C_{10}$ $H_{10}N_2O$, 174.0793), 158 (100); FTIR: 3383, 3305, 3197, 2922, 1666, 1445 $cm^{-1}$.

**1-Methylindole-3-carbonitrile (14).** HPLC $R_t$ = 14.6 min: $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2: HRMS-EI *m*/*z* (% relative abundance): measured 156.0687 $[M]^+$ (100), (calculated for $C_{10}H_8N_2$, 156.0687), 155 (38); FTIR: 3331, 3116, 2932, 2223, 1528, 1459, 1383, 1335, 1254, 1195, 1136 $cm^{-1}$.

**2-(1-Methyl-3-indolyl)-oxazoline (15).** HPLC $R_t$ = 8.7 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 200.0951 $[M]^+$ (100), (calculated for $C_{12}H_{12}N_2O$, 200.0950), 199 (23), 170 (54), 158 (23); FTIR: 2928, 2863, 1657, 1469, 1098, 1001 $cm^{-1}$.

**1-Methylindole-3-carboxamide (16).** HPLC $R_t$ = 5.8 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 174.0798 $[M]^+$ (69), (calculated for $C_{10}H_{10}N_2O$, 174.0793), 158 (100); FTIR: 3385, 3191, 3110, 1641, 1604, 1528, 1469, 1378, 1249 $cm^{-1}$.

**5-Fluoroindole-3-carbonitrile (17).** HPLC $R_t$ = 12.2 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 160.0432 $[M]^+$ (100), (calculated for $C_9H_5N_2F$, 160.0437), 133 (35); FTIR: 3257, 2916, 2228, 1499, 1170, 931, 847 $cm^{-1}$.

**2-(5-Fluoro-3-indolyl)-oxazoline (18).** HPLC $R_t$ = 7.3 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 204.0693 $[M]^+$ (100), (calculated for $C_{11}H_9N_2OF$, 204.0699), 203 (20), 174 (69); FTIR: 3700, 1642, 1463, 1259, 1188, 1134, 1002, 943 $cm^{-1}$.

**5-Fluoroindole-3-carboxamide (19).** HPLC $R_t$ = 4.4 min; $^1$H NMR and $^{13}$C NMR spectra, see Tables 1 and 2; HRMS-EI *m*/*z* (% relative abundance): measured 178.0539 $[M]^+$ (71), (calculated for $C_9H_7N_2OF$, 178.0542), 162 (100); FTIR: 3191, 2922, 1636, 1589, 1445, 1218, 936 $cm^{-1}$.

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