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# Microbial Biotransformations of a Synthetic Immunomodulating Agent, HR325

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**Abstract**—The microbial biotransformation of HR325 [2-cyano-3-cyclopropyl-3-hydroxy-*N*-(4′-trifluoromethyl-3′-methylphenyl)-propenamide], a synthetic immunomodulating agent, has been investigated in order to be compared with animal metabolism and to prepare some metabolites which are difficult to obtain by chemical methods. Several fungal strains are able to completely metabolize this drug. *Mortierella isabellina* NRRL 1757 only achieves a benzylic hydroxylation on the aromatic methyl group, affording in high yield the corresponding hydroxymethyl derivative. In addition, other strains, such as *Cunninghamella elegans* ATCC 26269 or *Beauveria bassiana* ATCC 7159 can cleave both cyclopropyl and cyano groups in a new unknown oxidative biochemical reaction, which can be mimicked by *m*-chloroperbenzoate oxidation. The resulting cyanohydrin is hydrolyzed and reduced to a primary alcohol. In *B. bassiana*, the final incubation product is a β-4-*O*-methylglucoside derivative of this alcohol, and has been fully characterized by independent synthesis. The different metabolic patterns of HR325 in the three fungal strains are discussed, and a mechanistic hypothesis about the oxidative cleavage of the right part of the molecule is proposed. The production of microbial metabolites is compared to animal metabolism in terms of structure and efficiency. © 1997 Elsevier Science Ltd.

## Introduction

Microbial biotransformations are now accepted as useful complements of mammalian drug metabolic studies.[1–19] They can be used to produce significant amounts of known (or new) metabolites, in order to use them as standard specimens for qualitative and quantitative characterization of identified (or yet unidentified) mammalian metabolites, as well as for further pharmacological and toxicological studies. In some propitious cases, microbial transformations may allow us to rapidly obtain a sufficient amount of a selected metabolite without any time-consuming or uncertain chemical synthesis.

As part of a program aimed to validate and apply such a concept in drug metabolism studies, we have been interested in obtaining a simple microbial metabolization method to produce, in reasonable yield, the benzylic alcohol derivative RU34941 (**2**). The chemical synthesis of this material, which is one of the main animal and human metabolites of the synthetic immunomodulating agent, HR325 (**1**) [2-cyano-3-cyclopropyl-3-hydroxy-*N*-(4′-trifluoromethyl-3′-methyl-phenyl)-propen-amide], has proved to be relatively difficult, especially when needing to be applied to a radiolabeled compound.[20] In addition, other metabolites eventually formed were carefully examined, in order to compare them to the already known main products[21] of animal metabolism (Fig. 1), and to trace the fungal metabolic transformation pathways of this molecule.

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## Results

We have thus incubated the commercial drug **1** at 0.2 g/L concentration with a number of grown fungal cultures and the metabolites formed in the liquid medium were subsequently detected by reverse-phase HPLC with detection at 280 nm. Five groups presenting different metabolic patterns were characterized among a total of 16 strains tested (Table 1).

*Mortierella isabellina* NRRL 1757 was the best strain selected to obtain the desired derivative **2**. In small-scale preparative conditions, in 1 L of a 65-h culture, incubated with 200 mg of HR325, all the substrate disappeared from the incubation filtrate in three days (Figs 2 and 3), and after extraction of the incubation medium, flash chromatography, and crystallization, a 45–50% yield of a nearly unique pure metabolite **2** was reproducibly obtained. Mass spectral examination, accounting for the loss of the cyclopropane unit ($C_3H_5$) in the mass spectrometer, revealed a 16-mass units increase ($[M+NH_4]^+$, *m/z* 303), indicating the addition of one oxygen atom to HR325, while the UV absorption spectrum was unchanged, demonstrating that metabolite **2** had retained the conjugated enol chromophore. At last, beside the characteristic features of the starting product, $^1H$ and $^{13}C$ NMR data of metabolite **2** (see Tables 2 and 3) clearly indicated the replacement of the aromatic methyl group (2.47 and 19.4 ppm, respectively) by a hydroxymethyl group with a

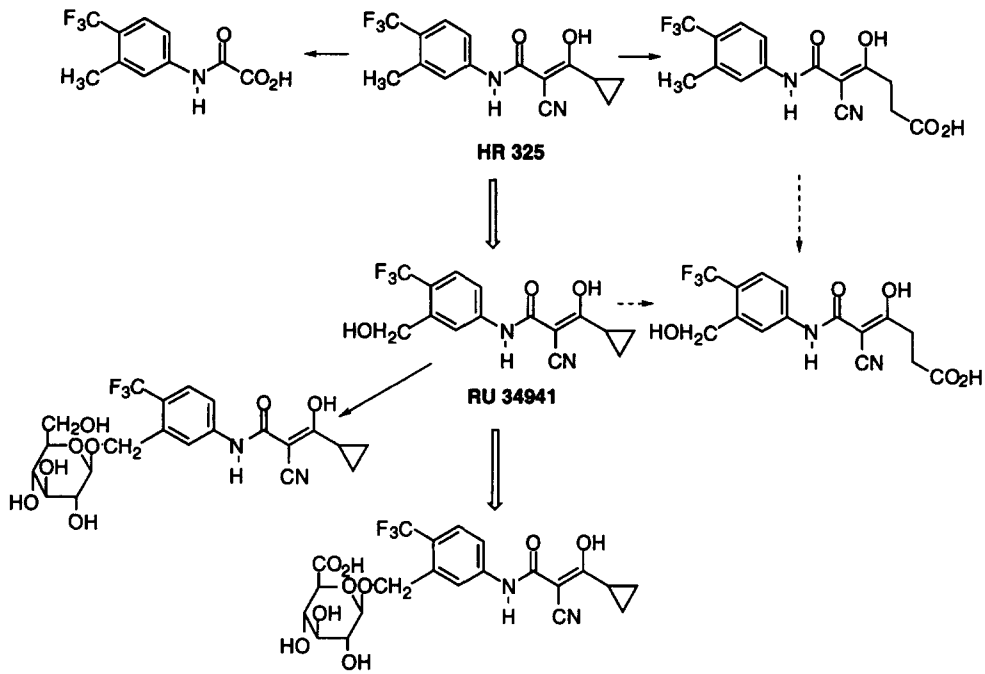


**Figure 1.** Urinary metabolites of **HR 325** in rat after a single oral administration: ⟹, main metabolic pathway; → and - - →, secondary metabolic pathways.[21]

2-proton singlet at 4.65 ppm and a $^{13}C$-methylene signal at 62.9 ppm. This biotransformation reaction was scaled-up to a 10-L fermenter culture with very similar yield and result.

*Cunninghamella elegans* ATCC 26269 formed in a first step the same benzylic hydroxy-derivative RU34941 (**2**) (Figs 4 and 5). However, after a few days of incubation, metabolite **2** disappeared from the incubation medium, while a more recent polar product, exhibiting a UV maximum absorption shifted to 240 nm, slowly appeared. This new product **3**, isolated in a 30% yield by extraction and flash chromatography from a four-day incubation supernatant, had obviously lost the conjugated right part of metabolite **2**, as confirmed by mass spectrometry ($M^+$ at *m/z* 249) and by the absence of the

**Table 1.** Metabolization of HR325 by some fungal strains

| | Microorganisms[a] | Biotransformations observed[b] |
|---|---|---|
| Group I | *Mortierella isabellina* NRRL 1757 | Immediate formation of RU34941 (**2**) the amount of which increases during the first two days, then remains constant (~50%). |
| Group II | *Cunninghamella elegans* ATCC 26269<br>*Cunninghamella elegans* ATCC 36112 | Immediate formation of RU34941 (**2**) (~30%). After two days the amount of **2** decreases while a second more polar product (**4**) appears. |
| Group III | *Cunninghamella echinulata* MMP 2203<br>*Mortierella isabellina* MMP 108 | Immediate formation of low amount of RU34941 (**2**, <5%) with no further increase. |
| Group IV | *Beauveria bassiana* ATCC 7159 | Several products (including RU34941) are formed then disappear during the first two to three days. After seven days, only two main products (**3** and **5**) are present. |
| Group V | *Acremonium alternatum* MMP 3010<br>*Actinomucor elegans* MMP 2092<br>*Aspergillus ochraceus* ATCC 10009<br>*Aspergillus terreus* MMP 2296<br>*Curvularia lunata* NRRL 2380<br>*Cunninghamella baineri* ATCC 9244<br>*Cunninghamella echinulata* NRRL 3655<br>*Mucor plumbeus* CBS 11107<br>*Rhizopus arrhizus* ATCC 11145<br>*Thamnostylum piriforme* ATCC 8992 | No product formed after six-day incubation. |

[a]Origin of strains and incubation conditions: see Experimental.
[b]Detected by reverse-phase HPLC of the incubation supernatant (Nucleosil C18); detection at 280 nm.

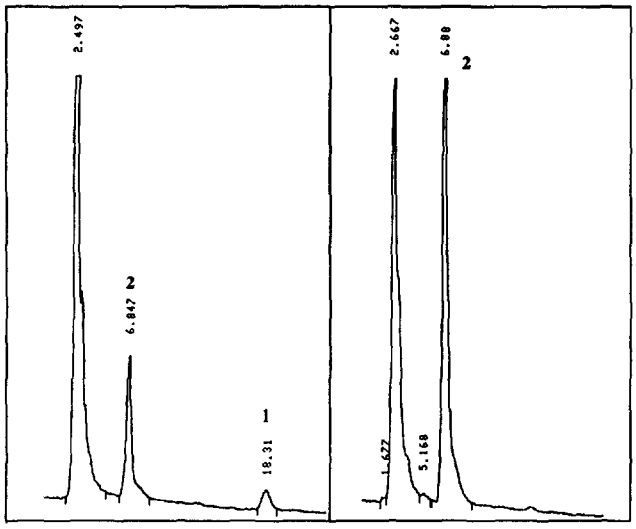


**Figure 2.** HPLC profiles of 24-h (left) and 48-h (right) incubations of **HR 325** (**1**, 0.2 g $L^{-1}$) with *M. isabellina* NRRL 1757, showing the formation of **RU 34941** (**2**); detection at 280 nm.

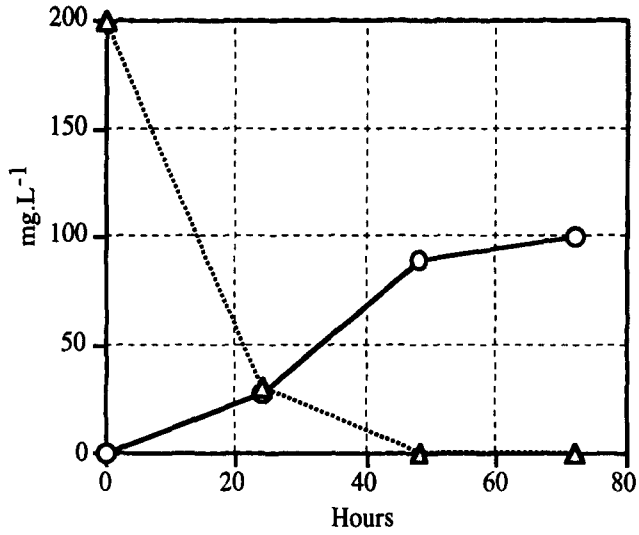


**Figure 3.** Time course of the formation of **RU 34941** (**2**, —○—) from **HR 325** (**1**, 0.2 g $L^{-1}$,···△···) by *M. isabellina* NRRL 1757.

characteristic cyclopropane signals in $^{1}H$ and $^{13}C$ NMR (Tables 2 and 3), replaced by a new $-CH_2OH$ signal at 4.14 (s) and 60.6 ppm, respectively, in addition to the 7-$CH_2OH$ signals preexisting in metabolite **2**, and assigned at 4.81 and 63.1 ppm, respectively.

Extraction of the incubated mycelium with organic solvent revealed the presence, in a very small amount (about 1%), of another new metabolite **4**, absorbing at 240 nm. This metabolite was identical in all respects with a synthetic compound prepared by the treatment of 4-trifluoromethyl-3-toluidine with acetoxyacetyl chloride, followed by alkaline hydrolysis of the resulting acetyl ester (Fig. 6 ).

*Beauveria bassiana* ATCC 7159 exhibited a more complex metabolic pattern: a small amount of RU34941 (**2**) was formed during the first three days, together with several other products (Figs 7 and 8), among which small amounts of metabolites **3** and **4** were detected. Most of them disappeared in the following days, except for a major metabolite **5**, absorbing at 240 nm, which was isolated by flash chromatography as a crystalline compound (mp 215–217 °C), in a 35% yield. $^{1}H$ NMR revealed the loss of the cyclopropane ring and the occurrence of the characteristic signals of a glucopyranoside-conjugated compound, with an anomeric 1′-H as a doublet ($J$ = 7.5 Hz) consistent with a β-configuration, and several –*CH*OH signals in the 3–3.8 ppm range; moreover, a sharp singlet integrating for three hydrogens at 3.53 ppm suggested the presence of an *O*-methyl substituted

**Table 2.** $^{1}H$ NMR data (250.13 MHz) for HR325 and its metabolites

| Position no. | 1 ($CDCl_3$) | 2 ($CDCl_3$+$CD_3OD$) | 3 | 4 ($CDCl_3$) | 5 (Acetone-$d_6$) |
|---|---|---|---|---|---|
| 2 | 7.41 (s) | — | 8.13 (s) | 7.46 (s) | 7.68 (s) |
| 5 | 7.58 (d, 8.0) | 7.28 (m) | 7.91 (d, 8.5) | 7.53 (d, 8.0) | 7.77 (d, 8.8) |
| 6 | 7.41 (d, 8.0) | — | 7.60 (d, 8.5) | 7.46 (d, 8.0) | 7.58 (d, 8.8) |
| 7 | 2.47(d, 1.5) | 4.65 (s) | 4.81 (s) | 2.43 (d, 1.5) | 2.43 (s) |
| NH-9 | 7.71 (s) | — | 9.43 (s) | 8.5 (br.s) | 9.82 (br.s) |
| OH-13 | — | — | — | 3.24 (s) | — |
| 11 | — | — | 4.14 (s) | 4.22 (s) | 4.25–4.34 (2d, 16.3) |
| 14 | 2.27 (m) | — | — | — | — |
| 15 | 1.32 (m) | 0.67–1.14 (m) | — | — | — |
| 16 | 1.14 (m) | — | — | — | — |
| 1′ | — | — | — | — | 4.48 (d, 8.0) |
| 2′ | — | — | — | — | 3.38 (dd, 8.0, 9.2) |
| 3′ | — | — | — | — | 3.57 (t, 9.2) |
| 4′ | — | — | — | — | 3.15 (dd, 8.8, 9.6) |
| 5′ | — | — | — | — | 3.35 (m) |
| 6′ | — | — | — | — | 3.68 (dd, 4.8, 11)<br>3.81 (dd, 2.4, 11) |
| 4′-$OCH_3$ | — | — | — | — | 3.53 (s) |

Chemical shifts in ppm, referenced to $CHCl_3$ (7.24 ppm). Coupling constants in Hz (±0.1 Hz).

**Table 3.** $^{13}C$ NMR data (62.9 MHz) for HR325 and its metabolites

| Position no. | 1 ($CDCl_3$) | 2 ($CD_3OD$) | 3 (Acetone-$d_6$) | 4 ($CDCl_3$) | 5 ($CD_3OD$) |
|---|---|---|---|---|---|
| 1,3,4 (ArC) | 126.4 | 125.6 | 123.7 | 127.0 | 129.4 |
| | 138.1 | 130.2 | 128.0 | 138.1 | 140.0 |
| | 138.9 | 144.1 | 143.1 | 139.6 | 144.0 |
| 2,5,6 (ArCH) | 117.6 | 120.2 | 118.0 | 116.4 | 119.8 |
| | 123.6 | 121.7 | 119.6 | 122.4 | 125.7 |
| | 126.9 | 129.3 | 127.4 | 126.9 | 129.4 |
| 7 | 19.4 | 62.9 | 63.1 | 19.4 | 21.3 |
| 10 | 192.4 | 196.2 | 171.9 | 170.0 | 172.7 |
| 11 | 127.0 | 146.4 | 60.6 | 62.5 | 71.6 |
| 12 | 168.0 | 171.4 | — | — | — |
| 13 | 116.9 | 144.4 | — | — | — |
| 14 | 15.9 | 20.2 | — | — | — |
| 15 | 11.1 | 10.4 | — | — | — |
| 16 | 11.1 | 11.5 | — | — | — |
| 1′ | — | — | — | — | 106.6 |
| 2′ | — | — | — | — | 76.8 |
| 3′ | — | — | — | — | 79.1 |
| 4′ | — | — | — | — | 79.7 |
| 5′ | — | — | — | — | 82.4 |
| 6′ | — | — | — | — | 63.8 |
| 4′-$OCH_3$ | — | — | — | — | 62.7 |

glucopyranose residue. It has previously been shown that *B. bassiana* can specifically produce 4-*O*-methyl-D-glucose-conjugated derivatives of phenols,[22–26] and, when given, the spectrochemical features of their glycosidic residue grossly corresponded to those found in metabolite **5**. In addition, $^{13}C$ NMR data were in full agreement with a β-*O*-methylglucopyranoside-conjugated simplified structure, involving again the loss of the right part of the starting molecule, and leaving an amidified hydroxyacetate residue glucosylated on its primary alcohol group. This was confirmed by high-resolution mass spectrometric analysis (FAB with $Li^+$ or $Na^+$ salts) which indicated a $C_{17}H_{23}F_3NO_7$ composition.

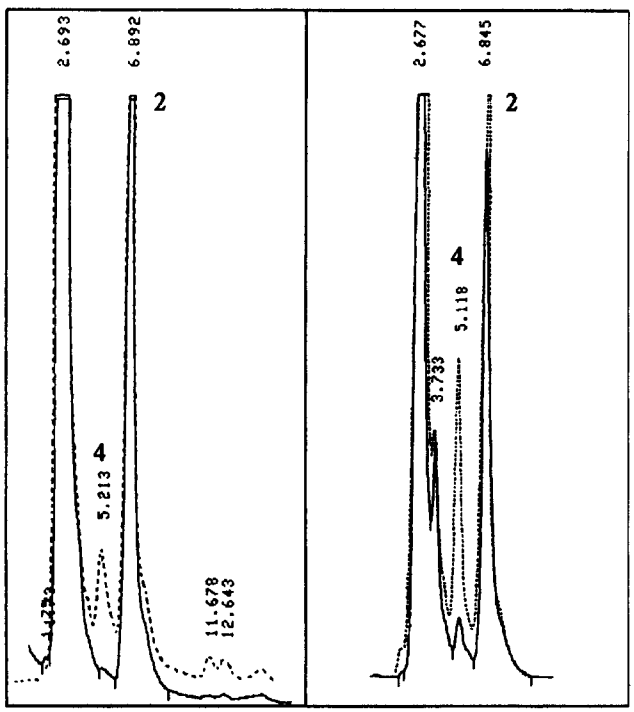


**Figure 4.** HPLC profiles of 24-h (left) and 48-h (right) incubations of **HR 325** (0.2 g $L^{-1}$) with *C. elegans* ATCC 26269 showing the formation of RU34941 (**2**) and metabolite **3**; detection at 280 (——) and 240 nm (– – –).

In order to establish with certainty the structure of the glycosyl moiety, as 4-*O*-methylglucose was not available, a synthesis (Fig. 9) of the corresponding 4-*O*-methyl-β-D-glucopyranoside conjugate was undertaken for a comparative study. We used a method[27] recently introduced for obtaining β-4-*O*-methyl-D-glucopyranoside derivatives through the condensation of 1,2-

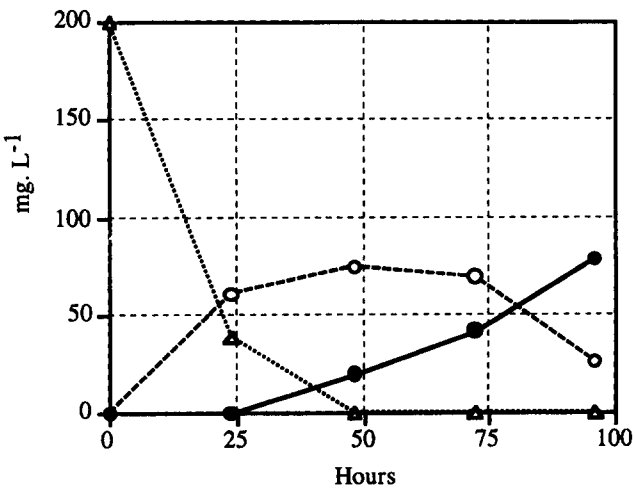


**Figure 5.** Time course of the formation of RU34941(**2**, - -○- -) and metabolite **3** (—●—) from **HR 325** (0.2 g $L^{-1}$, ···△···) by *C. elegans* ATCC 26269.

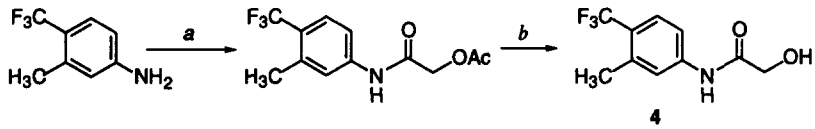


**Figure 6.** Chemical synthesis of metabolite **4**: (*a*) $ClCOCH_2OAc$–$Et_3N/CH_2Cl_2$ (90% yield); (*b*) 2 N NaOH–acetone (90% yield).

anhydro-3,6-di-*O*-benzyl-4-*O*-methyl-2-deoxy-α-D-glucopyranose (prepared in four steps from tri-*O*-acetyl-D-glucal) with a nucleophilic aglycone. Beside the expected protected 4-*O*-methyl-β-D-glucopyranoside, a smaller amount (ratio 1:2) of the corresponding protected 4-*O*-methyl-α-D-mannopyranoside, arising from the isomeric β-1,2-anhydrosugar,[27] was also obtained and characterized after chromatographic separation. Catalytic hydrogenation of the major isomer afforded the desired 4-*O*-methyl-β-D-glucopyranoside which was identical in all respects (mp, TLC, HPLC, NMR, and mass spectra) with metabolite **5**.

In order to obtain more detailed information about the involvement of the fragmentation alcohol metabolite **4** in the biotransformation pathways of HR325, synthetic **4** was incubated with *C. elegans* and *B. bassiana* cultures and the rate of formation of metabolites **3** or **5** was compared to that found using HR325 or its hydroxylated metabolite **2** as substrates (Table 4). With *C. elegans*, in similar conditions, **2** and **4** behaved as equally efficient precursors for the formation of metabolite **3**, allowing its formation at a rate comparable to that found from HR325. With *B. bassiana*, where the 4-*O*-methylglucoside **5** was the major HR325 metabolite found after 96 h, **4** was only a poor precursor. However, when Tween 80, a neutral detergent, was added to the incubation mixture (0.2% final concentration) in order to facilitate the dissolution and absorption of the added synthetic alcohol **4**, this one behaved as an excellent precursor of metabolite **5**. Similarly, the cyanohydrin **6**, obtained by the chemical oxidation of HR325, a hypothetic intermediate in the cleavage of the right part of the drug, and a possible precursor of the alcohol **4**, was incubated with a *B. bassiana* culture and the formation of products was compared to a control incubation without the microorganism. In the control experiment, **6** was rapidly converted into the corresponding aldehyde, which slowly disappeared in the medium, probably by slow hydrolysis at the amide bond (data not shown). On the contrary, the cyanohydrin **6** was rapidly converted by *B. bassiana* first to the alcohol metabolite **4**, then to the glucoside **5** in a comparable extent to experiments with metabolite **4** as substrate (Table 4).

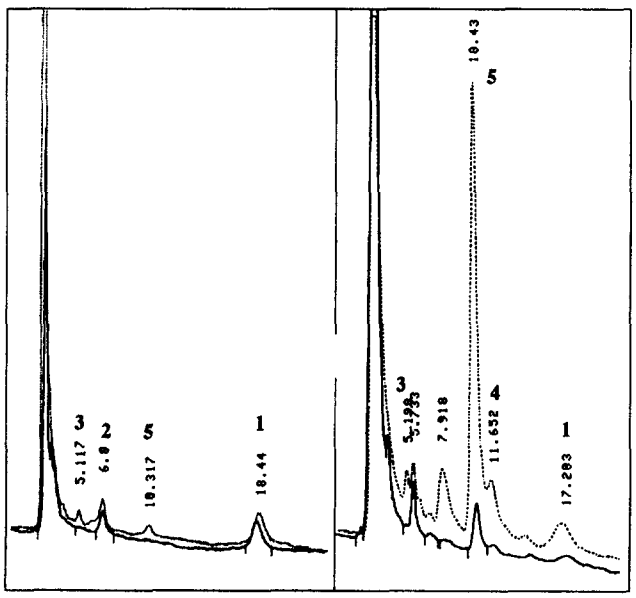


**Figure 7.** HPLC profiles of 48-h (left) and 96-h (right) incubations of HR325 (**1**, 0.3 g $L^{-1}$) with *B. bassiana* ATCC 7159 showing the formation of RU34941 (**2**) and metabolite **5**; detection at 280 (——) and 240 nm (– – –).

## Discussion

From the examination of the different products obtained, it is possible to devise several possible different metabolic pathways for HR325, depending on the strain used (Fig. 10).

It is clear that the detoxification metabolism of HR325 by *M. isabellina* is mainly limited to the hydroxylation of the aromatic methyl group, the product RU34941 (**2**) being excreted and accumulated in the incubation medium. The easy preparation of this metabolite in significant amounts obviated the need to achieve a complex and difficult chemical synthesis. However, as only 50% of HR325 could be isolated as metabolite **2**, it

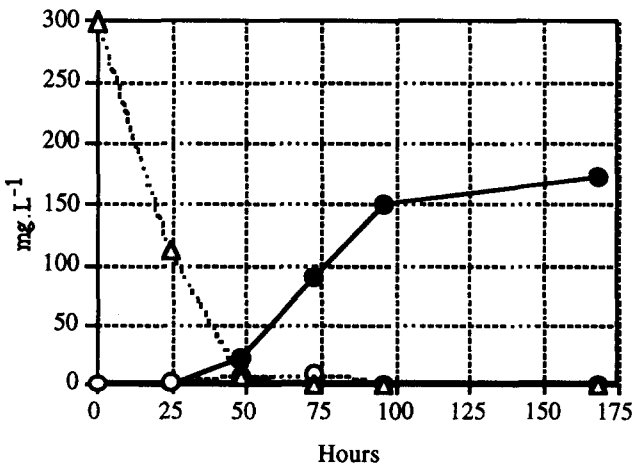


**Figure 8.** Time course of the formation of RU34941(**2**, - - ○ - -) and metabolite **5** (—●—) from HR325 (**1**, 0.3 g.$L^{-1}$, ···△···) by *B. bassiana* ATCC 7159.

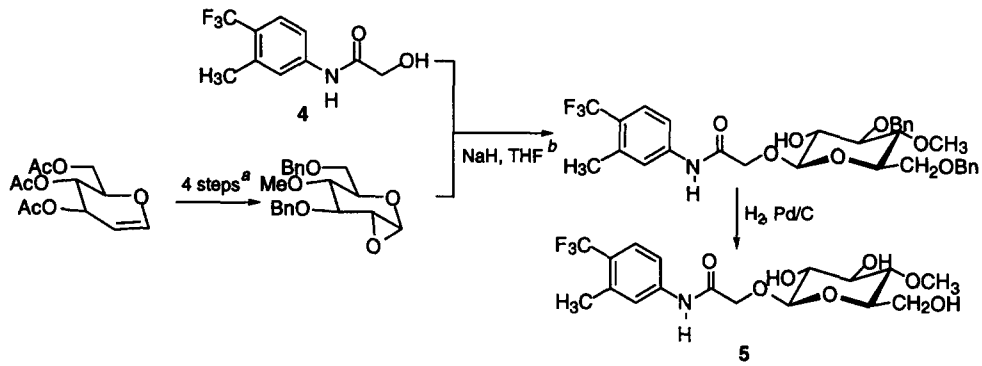


**Figure 9.** Synthesis of the 4-*O*-methyl-β-D-glucopyranoside **5**. (*a*) $Et_3N$:$H_2O$:MeOH (1:1:8); $Bu_2SnO$, MeOH; BnBr, $Bu_4N^+Br^-$, refluxing in cyclohexane; NaH, MeI, DMF; dimethyldioxirane, $CH_2Cl_2$. (*b*) The 3,6-di-*O*-benzyl derivative of **5** was separated from the minor accompanying dibenzyl-4-*O*-methyl-α-D-mannopyranoside by flash chromatography.

is possible that further degradation reactions to undetected products are occurring. *Mortierella isabellina* was already known as a good benzylic hydroxylation reagent,[28,29] but, to our knowledge, no example of the hydroxylation of a trifluoromethyl-containing aromatic has been previously described.

*Cunninghamella elegans* is apparently able to proceed further in the same metabolic pathway, by abstracting from metabolite **2**, in an unknown way, the cyclopropane and cyano groups, leaving a free additional hydroxymethyl group (metabolite **3**). At first glance, from the kinetic data obtained from the incubation medium (Fig. 5), metabolite **3** seems to originate from the further degradation of RU34941 (**2**). However, **3** may be formed by an alternate route, through an initial fragmentation to **4**, followed by hydroxylation of the aromatic methyl group. The fragmentation product **4** was not detected in the incubation medium, but was present in a small amount in the mycelium, from which it could be extracted by treatment with an organic solvent, indicating that such a product is still sufficiently hydrophobic to remain in the cellular compartment. Hydroxylation of the aromatic methyl group may thus occur on compound **4**, and it is highly probable that metabolite **3** can be formed in both ways. This is entirely confirmed by the formation of **3**, when the synthetic product **4** was incubated in the usual conditions with *C. elegans* (Table 4), at a slightly higher rate than that found when **1** was used as a substrate.

*Beauveria bassiana* presents a much more complex detoxification pattern. A minor part of HR325 is metabolized to **3** through a pathway probably identical to that found in *C. elegans*. However, HPLC records showed a number of products formed in a first step (see Fig. 7), then disappearing to lead to an accumulation of the new glucoside-conjugated metabolite **5**. As previously noted, the formation of 4-*O*-methylglucopyranosides by *B. bassiana* is not an unprecedented observation.[22–26] Again the fragmentation metabolite **4**, previously detected in *C. elegans* mycelium extracts, was thought to be a mandatory intermediate. However, incubation of *B. bassiana* with **4** did not allow the formation of metabolite **5** at a rate comparable to that found when using **1** as substrate (Table 4), unless small amounts of a detergent (Tween 80) were added in the *B. bassiana* incubation mixture. This promoted a fast formation of **5**, at a rate identical to that observed with **1**, thus demonstrating the actual involvement of **4** in the pathway conducting to **5**, and the need for a permeabilization reagent to incorporate **4** into the *Beauveria* mycelium.

While only metabolite **2** is common to animals and microorganisms, the prevalent occurrence of a micro-

**Table 4.** Compared formation of metabolites from HR325 (**1**), RU34941 (**2**), **4**, or **6** by *C. elegans* and *B. bassiana* cultures

| Microorganism | Substrate incubated[a] | Metabolite(s) observed | Amount of metabolites formed(mg $L^{-1}$)[b] after 24 h | 48 h | 72 h | 96 h |
|---|---|---|---|---|---|---|
| *C. elegans* | HR325 (**1**) | RU34941 (**2**) | 62 | 75 | 70 | 27 |
| | | Metabolite **3** | — | 20 | 43 | 80 |
| | RU34941 (**2**) | Metabolite **3** | 10 | 37 | 71 | 76 |
| | Metabolite **4** | Metabolite **3** | 20 | 45 | 92 | 100 |
| *B. bassiana* | HR325 (**1**) | Metabolite **5** | — | 13 | 41 | 140 |
| | Metabolite **4** | Metabolite **5** | 7 | 16 | 29 | 56 |
| | **4** + Tween 80[c] | Metabolite **5** | 8 | 27 | 102 | 156 |
| | **6** + Tween 80[c] | Metabolite **4** | 42 | 33 | 18 | 16 |
| | | Metabolite **5** | — | 21 | 47 | 62 |

[a]250 mg $L^{-1}$, dissolved in acetone (25 mg $mL^{-1}$).
[b]Determined by HPLC at 240 or 280 nm.
[c]0.2% final concentration.

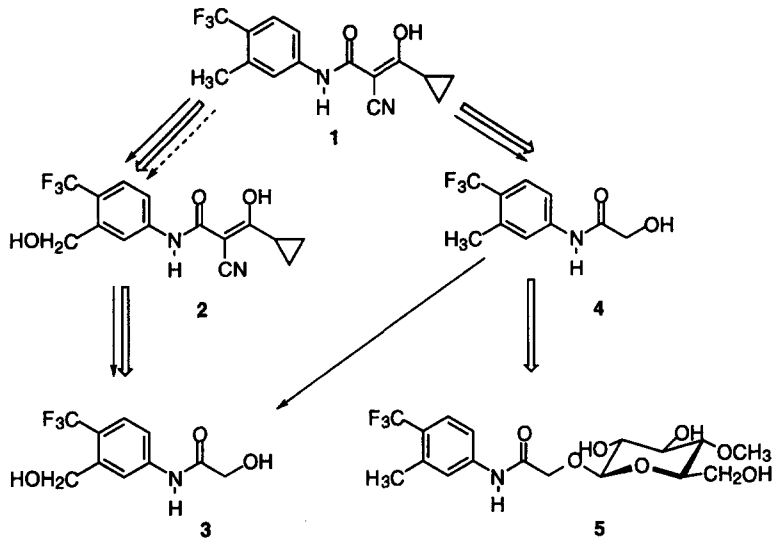

**Figure 10.** Metabolic pathways for the detoxification of HR325 by *M. isabellina* (- - →), *C. elegans*,(→) *and B. bassiana* (⟹).

bial fragmentation pattern eliminating the right part of the molecule (metabolites **3** and **4**) is reminiscent of the formation of the corresponding acid as one of the animal metabolites (see Fig. 1), and suggests that such alcohol or carboxylic acid fragmentation products could be formed from a common aldehyde intermediate. This raises the question of the nature of the reaction(s) involved in the elimination of the cyano- and cyclopropyl groups. While currently detected in the animal (see Fig. 1), no product arising from a simple opening of the cyclopropane ring could be detected in our microorganisms. On the contrary, the cyclopropane ring seems to be removed as a whole, and this removal is always accompanied by the elimination of the cyano group. An easily hydrolyzed cyanohydrin derivative **6** would constitute a very likely intermediate (Fig. 11), accounting for the intermediate formation of an aldehyde, which would be then either oxidized to the corresponding carboxylic acid in animals, or reduced to the corresponding alcohol in microorganisms. This hypothesis is clearly confirmed by incubations of cyanohydrin **6**, which, in the incubation medium, but in the absence of microorganisms, is rapidly converted to the corresponding aldehyde, which does not survive the incubation conditions. Conversely, in the presence of *B. bassiana*, this intermediate aldehyde, formed either from a nonenzymic reaction in the medium, or a cyanohydrolase-mediated reaction in the mycelium, is not detected because it is immediately reduced to the alcohol derivative **4**, which is then completely converted to the 4-*O*-methyl glucoside **5**.

However, the formation of such a cyanohydrin by known biochemical pathways is not easy to devise.

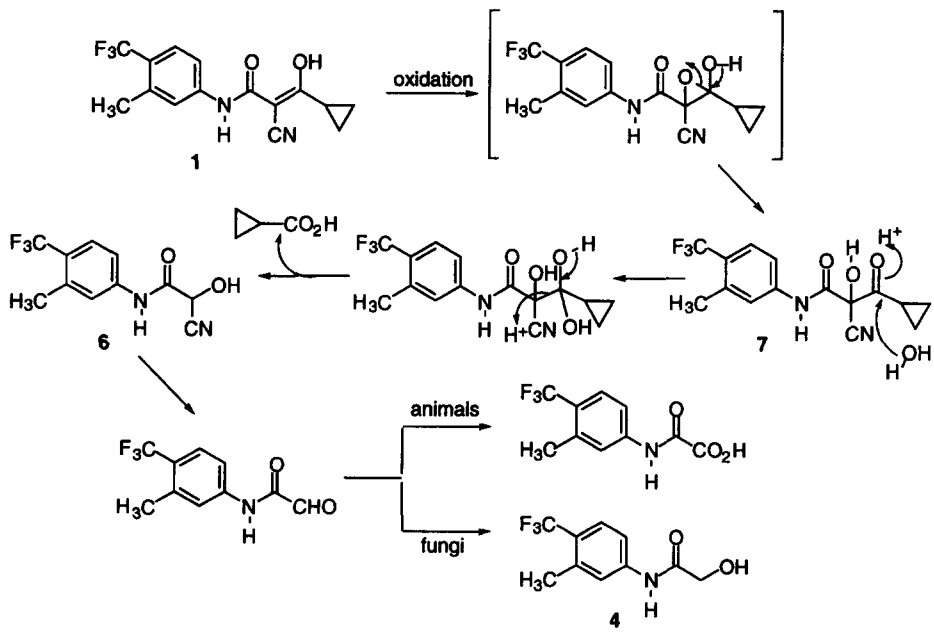

**Figure 11.** Putative reaction mechanism and intermediates involved in the oxidative elimination of the cyclopropane and cyano groups of HR325.

A Baeyer–Villiger-type reaction,[30] acting on a hypothetical keto form of HR325, is highly unlikely: such a reaction, though currently observed in microorganisms,[31–33] has never been observed[30] in highly enolized 1,3-dicarbonyl-2-substituted derivatives, such as HR325. However, using *m*-chloroperbenzoate (MCPBA), a usual Baeyer–Villiger reagent, as oxidant, the biological cleavage of the right part of the drug can be easily mimicked, affording rapidly and in good yield the desired cyanohydrin **6**, and this reaction was used to prepare it. Such a fast and high-yield oxidation with MCPBA, $H_2O_2$, or even $O_2$, for *O*-silylated enolates,[34,35] β-diketones,[36] or β-ketoesters[37–39] has been known for a long time and has been interpreted as a direct attack of the double bond of the enol (or silyl enol ether) to give an intermediate α-hydroxy (or α-silyloxy) epoxide derivative, easily opened to give an α-ketol (or its silyl ether).[40] Another oxidizing reagent, dimethyldioxirane, considered as a very convenient and versatile epoxidizing reagent,[41] has been extensively used for the epoxidation of electron-rich double bonds of enol derivatives.[42–44] This reagent was further shown to directly afford the α-hydroxy ketone rearrangement product of the epoxide from enol salts,[45–47] or, especially, from spontaneously enolized 1,3-dicarbonyl compounds.[46] From HR325, using excess dimethyldioxirane in the described conditions, it was possible to obtain as a unique product the crude hydroxyketone **7** (Fig. 11), easily characterized by $^1H$ and $^{13}C$ NMR spectroscopy, but which did not survive isolation by silica gel chromatography, affording again the cyanohydrin **6**. Reinvestigating the MCPBA oxidation product of HR325 showed that the same hydroxyketone **7** has been formed as the crude product, which was then quantitatively converted into the cyanohydrin during the chromatographic purification step. Both results[48] confirm the assumption of a primary epoxide formation in the reaction mechanism leading to the cyanohydrin **6** through MCPBA or dimethyldioxirane oxidation of HR325. Concerning the final outcome of the reaction (i.e. the splitting of α-ketol **7** to cyanohydrin **6**) one can speculate that, in the absence of an α-hydrogen, no stabilizing enolization of the α-ketol can now take place, and in the presence of silicic acid, a general acid-catalyzed elimination of the cyclopropyl-substituted keto group as a carboxylic acid is occurring, certainly favored by the additional electron-withdrawing effect of the cyano group. Cyclopropanecarboxylic acid was effectively detected by GC-MS as one of the silica gel-conversion products of the α-ketol **7**.

These reactions may account for a sequential elimination of the cyclopropane and cyano groups and suggest a very similar oxidative mechanism for the microbiological reaction (Fig. 11); however, it is possible that only a part of this mechanism (for example the first oxidation step) may be the result of an enzymic reaction in *C. elegans* or *B. bassiana*, the following rearrangement and elimination reactions being purely chemical ones. It is not known which enzymic activity is responsible for the oxidation step: a $P_{450}$-dependent monooxygenase activity acting on the electron rich-double bond of enol ethers has been already demonstrated,[49,50] but a flavine monooxygenase, acting through a hydroperoxy-flavine intermediate, or a peroxydase-type activity cannot be excluded. Work is in progress to determine, in our fungal strains, the exact nature of the enzymic reaction effecting the initial oxidation step.

## Conclusion

The variety of metabolites obtained from HR325 using a small number of fungal strains may exemplify some of the potential advantages of the so-called microbial models of animal metabolism: (a) similarity with animals in several detoxification reactions; (b) easy preparation in good yields and large quantities of known hydroxylated animal metabolites, without the use of chemical synthesis; (c) formation of glycosyl-conjugated (*phase II*) metabolites; and (d) preparation of new derivatives for pharmacological assays. In addition, a possibly new, unknown metabolic reaction of general use for the simultaneous elimination of cyano- and cyclopropyl groups has been recognized.

## Experimental

### General

$^1H$ and $^{13}C$ NMR (1-D and 2-D) spectra were performed at 250.13 and 62.9 MHz, respectively, on a Bruker WM250-FT instrument, using standard pulse sequences. Electron impact and chemical ionization ($NH_3$) mass spectrometric analyses were performed on a R10-10 Nermag instrument at the Laboratoire de Chimie, Ecole Normale Supérieure (Paris). UV spectra were recorded with a Uvikon 810 spectrophotometer (Kontron). Melting points were determined in capillary tubes with a Büchi instrument and are uncorrected. Optical rotations were measured using a Perkin–Elmer 241C spectropolarimeter, in a 1 dm cell.

### Microorganisms

All cultures were maintained on agar slants containing (per litre), yeast extract (Difco) 5 g, malt extract (Difco) 5 g, glucose 20 g, and Bacto-agar (Difco) 20 g, stored at 4 °C and subcultured before use. Fungi were purchased from the American Type Culture Collection (ATCC strains), Rockville, MD, U.S.A., the Northern Regional Research Laboratories (NRRL strains), Peoria, IL, U.S.A., the Centralbureau voor Schimmelcultures (CBS strains), Baarn, the Netherlands, or the Mycothèque of the Museum d'Histoire Naturelle (MMP strains), Paris, France. Some strains (no strain number) are of local origin.

### Analytical chromatographic procedures

Reverse-phase HPLC was carried out using the following conditions: column, Lichrospher 100 RP18 Merck

(125 × 4 mm); solvent, MeOH-0.1 M NaOAc (6:4), 0.8 mL/min, using a Beckmann 110A pump, a Gilson 231 sample injector equipped with a 20 μL loop, a Pye-Unicam LC-UV detector set at 280 or 240 nm and a Shimadzu C-R6A integrator recorder. TLC was carried out with the ascending method using silica gel $60F_{254}$ precoated aluminum sheets (Merck, Germany). Spots were detected under UV light and by spraying with a phosphomolybdic acid solution in 10% aq $H_2SO_4$ or a 5% solution of anisaldehyde in EtOH:concd $H_2SO_4$: AcOH (90:5:5).

### Substrates and reference compounds

HR325 and 4-trifluoromethyl-3-toluidine were kindly donated by Roussel Uclaf Co. 2,2-Dimethyldioxirane was prepared according to Halcomb and Danishefski[51] and Gordon et al.[52] All other chemicals were obtained from commercial sources and used without further purification.

### Microbial transformations

**Culture and screening procedures.** All microorganisms were grown at 27 °C in a liquid medium containing (per litre), corn steep liquor (Roquette, France) 10 g, glucose 30 g, $KH_2PO_4$ 1 g, $K_2HPO_4$ 2 g, $NaNO_3$ 2 g, KCl 0.5 g, $MgSO_4$, $7H_2O$ 0.5 g, $FeSO_4$, $7H_2O$ 0.02 g. For screening experiments, 250-mL conical flasks containing 100 ml of sterile liquid medium were inoculated with a few drops of spore suspensions obtained from freshly grown agar slants, and orbitally shaken (200 rpm) at 27 °C for 66–72 h in a Gallenkamp incubator. The substrates were then added as an acetone solution to yield a final concentration of 0.2 g/L. Samples (1–2 mL) were aseptically withdrawn every day, centrifuged, and the supernatants were microfiltered (0.45 μm). Aliquots of the filtrates were analyzed by reverse-phase HPLC, and the remaining fraction was saturated with sodium chloride and extracted with ethyl acetate for TLC analysis. Most transformations were continued until no further increase of metabolite(s) was observed (usually seven days). Control experiments performed by incubating fungi in the absence of substrate were simultaneously run to eliminate mycelium products possibly detected by HPLC.

**Microbial transformation of 1 by *M. isabellina*.** HR325 (200 mg) was added to a 1-L, 66-h-grown culture of *M. isabellina* NRRL 1757 and incubation was continued at 27 °C with orbital shaking. After three days' incubation, monitoring of the incubation supernatant by HPLC (detection at 280 nm) showed complete disappearance of the substrate, and stationary amounts of products. The mycelium was separated from the broth by filtration. The filtrate was saturated with sodium chloride, then extracted three times with 150 mL of ethyl acetate. The pooled organic extracts were washed with brine, dried over $MgSO_4$, then evaporated under reduced pressure to yield 280 mg of crude extract. This extract was deposited on a silica gel H60 (Merck, 230–400 mesh) column (2 × 15 cm) which was eluted with EtOAc:MeOH (95:5). Collected fractions were pooled to give metabolite **2**, which was crystallized in $CHCl_3$ (89.5 mg). Mp 165 °C dec, $^1H$ and $^{13}C$ NMR (see Tables 2 and 3). MS (CI, $NH_3$): 303 ($MNH_4^+ - C_3H_5$), 127.

**Microbial transformation of 1 by *C. elegans*.** HR325 (2 × 30 mg) was added to 2 × 100 mL, 66-h-grown cultures of *C. elegans* ATCC 36112, and incubation was continued at 27 °C with orbital shaking. After four days' incubation, monitoring of the incubation supernatant by HPLC (detection at 280 and 240 nm) showed complete disappearance of the substrate, and stationary amounts of products. The mycelium was separated from the broth by filtration. The filtrate was saturated with sodium chloride, then extracted three times with 150 mL of ethyl acetate. The pooled organic extracts were washed with brine, dried on sodium sulfate, then evaporated under reduced pressure to yield 50 mg of crude extract. The mycelium was repeatedly extracted with methanol. The extract was evaporated to dryness, suspended in ethyl acetate, and washed with brine. The organic phase was dried over $MgSO_4$ and pooled with the previous extract to give 170 mg of total extract which was deposited on two preparative 20-cm thick-layer silica gel plates. After migration of the solvent ($CH_2Cl_2$:MeOH, 95:5) and elution of the relevant portion at $R_f$ = 0.2 with EtOAc:MeOH (1:1), 18 mg of metabolite **3** were recovered. Mp 134–136 °C. $^1H$ and $^{13}C$ NMR (see Tables 2 and 3). MS (CI, $NH_3$): 267 [$MNH_4^+$], 250 [$MH^+$].

A small amount of metabolite **4** (about 1 mg) was also recovered at $R_f$ = 0.4 and identified by comparison with an authentic synthetic specimen.

**Microbial transformation of 1 by *B. bassiana*.** HR325 (240 mg) was added to a 800 mL, 66-h-grown culture of *B. bassiana* ATCC 7159 and incubation was continued at 27 °C with orbital shaking. After seven days' incubation, monitoring of the incubation supernatant by HPLC (detection at 280 nm) showed complete disappearance of the substrate, and stationary amounts of products. The mycelium was separated from the broth by filtration. The filtrate was saturated with sodium chloride, then extracted three times with ethyl acetate. The pooled organic extracts were washed with brine, dried over $MgSO_4$, then evaporated under reduced pressure to yield 330 mg of crude extract as a yellow oil. Chromatography on a silica gel H60 (Merck, 230–400 mesh) column (2 × 15 cm) eluted with EtOAc:MeOH (95:5) afforded metabolite **5** (110 mg), which was crystallized in ethanol. Mp 215–217 °C. $[\alpha]_D^{20}$ −33.4 (*c* 0.6, acetone). MS (FAB): 410 $(M+H)^+$, 432 $(M+Na)^+$, or 416 $(M+Li)^+$. HRMS, calcd for $C_{17}H_{23}NO_7F_3$, 410.141216; found, 410.142662. $^1H$ and $^{13}C$ NMR (see Tables 2 and 3).

Metabolite **5** (15 mg) in anhydrous pyridine (1 mL) and acetic anhydride (0.021 mL) was left overnight at room temperature After extraction with ether, the usual work up, and purification by TLC ($CH_2Cl_2$:MeOH, 96:4), 18

mg of the *2,3,6-triacetylglucoside* were obtained. $^1$H NMR ($CDCl_3$) δ 2.02, 2.06, and 2.10 (9H, 3s, $COCH_3$), 2.45 (3H, br.d., $J$ = 1.25 Hz, $ArCH_3$), 3.36 (1H, t, $J$ = 9.2 Hz, H-4′), 3.43 (3H, s, $OCH_3$), 3.59 (1H, ddd, $J$ = 2.1, 4.9, and 7.4 Hz, H-5′), 4.24 (1H, dd, $J$ = 4.9 and 12 Hz, H-6′), 4.36 (1H, dd, $J$ = 2.1 and 12 Hz, H-6′), 4.15 and 4.39 (2H, 2d, $J$ = 15 Hz, $COCH_2$), 4.54 (1H, d, $J$ = 8 Hz, H-1′), 4.98 (1H, dd, $J$ = 7.7 and 9.8 Hz, H-3′), 5.23 (1H, dd, $J$ = 8.1 and 9.8 Hz, H-2′), 7.52 (3H, m, ArH), 8.36 (1H, s, NH).

### Synthesis of 4-trifluoromethyl-3-toluidine hydroxyacetate 4

**4-Trifluoromethyl-3-toluidine acetoxyacetate**. To an ice-cold solution of 4-trifluoromethyl-3-toluidine chlorohydrate (1 g, 4.74 mmol) in $CH_2Cl_2$ (15 mL), and triethylamine (1.32 mL, 9.5 mmol), acetoxyacetyl chloride (0.508 mL, 4.75 mmol) was added dropwise with stirring. After additional stirring at room temperature for 5 h, the reaction mixture was washed with 1 N HCl, and the organic phase was dried on $MgSO_4$ and evaporated under reduced pressure to give a solid, which is used without further purification. A sample is crystallized in ether:cyclohexane. Mp 98–99 °C. $^1$H NMR ($CDCl_3$) δ 2.21 (3H, s, $COCH_3$), 2.43 (3H, br d, $J$ = 1.2 Hz, $ArCH_3$), 4.68 (2H, s, $CH_2$), 7.43 (1H, d, $J$ = 8 Hz, ArH), 7.49 (1H, s, ArH), 7.53 (1H, d, $J$ = 8 Hz, ArH), 8.0 (1H, s, NH). $^{13}$C NMR ($CDCl_3$) δ 19.37 ($ArCH_3$), 20.74 ($CO\underline{C}H_3$), 63.28 ($CH_2$), 116.74, 122.72, and 126.92 (arom. $\overline{C}H$), 126.84, 138.11 and 139.56 (arom. quat. C), 165.38 ($\underline{C}OCH_3$), 169.44 (CONH).

**4-Trifluoromethyl-3-toluidine hydroxyacetate 4**. Acetoxyacetamide (1.246 g, 4.7 mmol) was dissolved in acetone (10 mL), added with 2 N NaOH (4.7 mL) and stirred at room temperature for 4 h. After addition of water and neutralization with 2 N HCl, the mixture was extracted repeatedly with EtOAc which was dried ($MgSO_4$) and evaporated under reduced pressure. The residual oil was purified by flash chromatography (solvent cyclohexane:EtOAc, 1:1) to give **3** (1.165 g) as a colorless solid, which was crystallized in $CH_2Cl_2$–cyclohexane (0.879 g). Mp 84–85 °C. Anal. for $C_{10}F_3H_{10}NO_2$ (233.192): calcd C 51.51, F 24.44, H 4.32, N 6.01; found C 51.51, F 24.94, H 4.32, N 5.92. $^1$H and $^{13}$C NMR (see Tables 2 and 3).

### Synthesis of metabolite 5

1,5-Anhydro-3,6-di-*O*-benzyl-2-deoxy-D-arabino-hex-1-enopyranose was obtained[27] as a pale-yellow oil (42% yield) from 3,4,6-trihydroxy-D-glucal. $[\alpha]_D$ −32,5 (*c* 2.5, $CHCl_3$) (ref[27] −22.5). $^1$H NMR ($CDCl_3$) δ 2.87 (1H, br s, OH), 3.80 (2H, m, H-6), 3.98 (2H, m, H-4 and H-3), 4.09 (1H, m, H-5), 4.57 (1H, d, $J$ = 11.9 Hz, $OCH_2$), 4.60 (1H, d, $J$ = 11.9 Hz, $OCH_2$), 4.63 (1H, d, $J$ = 11.9 Hz, $OCH_2$), 4.69 (1H, d, $J$ = 11.9 Hz, $OCH_2$), 4.85 (1H, dd, $J$ = 2.4 Hz, 6.4, H-2), 6.40 (1H, dd, $J$ = 1.6 Hz, 6.4, H-1), 7.28–7.37 (1H, m, ArH). $^{13}$C NMR ($CDCl_3$) δ 68.7, 76.3, and 77.1 (CH-3, -4, and -5), 69.1, 70.7, and 73.6 ($CH_2$), 100.1 (CH-2), 127.8, and 128.5 (ArCH), 137.9 and 138.4 (quat. C), 144.7 (CH-1). 1,5-Anhydro-3,6-di-*O*-benzyl-4-*O*-methyl-2-deoxy-D-arabino-hex-1-enopyranose was obtained[27] as a colorless oil (72% yield) by methylation of the dibenzyl derivative. $[\alpha]_D$ −9.4 (*c* 1.4, $CHCl_3$) (ref[27] −9.8). $^1$H NMR ($CDCl_3$) δ 3.50 (3H, s, $OCH_3$), 3.63 (1H, dd, $J$ = 5.5 and 7.9 Hz, H-4), 3.76 (1H, dd, $J$ = 3.2 and 10.7 Hz, H-6), 3.82 (1H, dd, $J$ = 5.2 and 10.7 Hz, H-6), 4.06 (1H, ddd, $J$ = 8.3, 5.2 and 3.2 Hz, H-5), 4.12 (1H, dd, $J$ = 3 and 5.5 Hz, H-2), 4.57 (1H, d, $J$ = 13.9 Hz, $OCH_2$), 4.58 (1H, d, $J$ = 13.9 Hz, $OCH_2$), 4.59 (1H, d, $J$ = 11.2 Hz, $OCH_2$), 4.68 (1H, d, $J$ = 11.2 Hz, $OCH_2$), 4.87 (1H, dd, $J$ = 2.8 and 5.9 Hz, H-2), 6.42 (1H, dd, $J$ = 0.8 and 5.9 Hz, H-1), 7.25–7.37 (10H, m, ArH). $^{13}$C NMR ($CDCl_3$) δ 59.3 ($OCH_3$), 68.5, 70.4, and 73.5 ($CH_2$), 74.8, 76.3, and 76.5 (CH-3, -4, and C-5), 99.8 (CH-2), 127.6, 127.7, and 128.3 (ArCH), 138.1 and 138.4 (quat. C), 144.6 (CH-1). 1,2-Anhydro-3,6-di-*O*-benzyl-4-*O*-methyl-2-deoxy-D-glucopyranose was prepared[27] from the protected 4-*O*-methyl glucal by oxidation with dimethyldioxirane (quantitative yield) $[\alpha]_D$ +36 (*c* 0.76, $CHCl_3$) (ref[27] +36.4). $^1$H NMR ($CDCl_3$) δ 3.02 (1H, d, $J$ = 2 Hz, H-2), 3.36 (1H, dd, $J$ = 7.9 and 9.6 Hz, H-4), 3.49 (3H, s, $CH_3$), 3.63 (2H, m, H-5 and H-6), 3.72 (1H, dd, $J$ = 3.9 and 11.1 Hz, H-6), 3.85 (1H, dd, $J$ = 0.8 and 7.9 Hz, H-3), 4.54 (1H, d, $J$ = 11.6 Hz, $OCH_2$), 4.64 (1H, d, $J$ = 11.6 Hz, $OCH_2$), 4.70 (1H, d, $J$ = 11.6 Hz, $OCH_2$), 4.79 (1H, d, $J$ = 11.6 Hz, $OCH_2$), 4.95 (1H, d, $J$ = 1.6 Hz, H-1), 7.25–7.39 (10H, m, ArH). $^{13}$C NMR ($CDCl_3$) δ 52.6 ($CH_3$), 68.1, 72.3, and 73.5 ($CH_2$), 60.2, 69.4, 75.8, 77.4, and 78.8 (CH), 127.6, 127.7, 127.9, 128.3, and 128.5 (ArCH), 137.6 and 138 0 (quat. C).

**2-(3,6-di-*O*-benzyl-4-*O*-methyl-β-D-glucopyranosyl)-(3-methyl-4-trifluoromethyl-amidophenyl)-oxyacetate**. NaH (50% dispersion in oil, 8.4 mg, 0.17 mmol) and then the protected glucopyranosyl epoxide (125 mg, 0.35 mmol) dissolved in THF (1 mL) were added to 122 mg (0.52 mmol) of the amidoalcohol **3** dissolved in THF (1 mL) at 0 °C. After stirring at room temperature for 3 h, EtOAc was added and the mixture was washed with water, dried with $MgSO_4$ and evaporated under reduced pressure. The resulting colorless oil was flash chromatographed (cyclohexane:EtOAc, 7:3) to give 61 mg (30%) of the pure 3,6-di-*O*-benzyl-4-*O*-methyl-β-D-glucopyranoside. Mp 132 °C (after crystallization in $CH_2Cl_2$–cyclohexane). $[\alpha]_D$ +14.4 (*c* 1, $CHCl_3$). $^1$H NMR($CDCl_3$) δ 2.41 (3H, d, $J$ = 1.6 Hz, H-7), 2.86 (1H, s, OH), 3.34–3:47 (4H, m, H-2, -3, -4, and -5), 3.49 (3H, s, $OCH_3$), 3.71 (2H, d, $J$ = 2.4 Hz, H-6), 4.29 (1H, d, $J$ = 7.6 Hz, H-1), 4.27 (1H, d, $J$ = 16.7 Hz, H-11), 4.36 (1H, d, $J$ = 16.7 Hz, H-11), 4.50 (1H, d, $J$ = 11.9 Hz, $CH_2Ph$), 4.61 (1H, d, $J$ = 11.9 Hz, $CH_2Ph$), 4.73 (1H, d, $J$ = 11.6 Hz, $CH_2Ph$), 4,95 (1H, d, $J$ = 11.6 Hz, $CH_2Ph$), 7.28–7.49 (13H, m, ArH), 9.04 (1H, s, NH). $^{13}$C NMR ($CDCl_3$) δ 19.43 ($CH_3$, C-7), 60.57 ($CH_3$, $OCH_3$), 68.34, 69.45, 73.57, and 75.04 ($CH_2$, C-11, C-6′, and $CH_2Ph$), 73.30, 75.41, 79.39, and 84.21 (CH-2′, -3′, -4′, and -5′), 103.43 (CH-1′), 116.34, 122.27, 127.69, 127.77, 127.99, 129.13, 128.40, and 128.75 (ArCH), 140.23, 138.36, and 137.80 (quat. C-2, -5, and -6), 168.23 (CO).

Pure 3,6-di-*O*-benzyl-4-*O*-methyl-α-D-mannopyranoside (30 mg, 15%) was obtained in another fraction. Mp 90–95 °C dec (after crystallization in $CH_2Cl_2$–cyclohexane). $[\alpha]_D$ +76.4 (*c* 0.5, $CHCl_3$). $^1H$ NMR ($CDCl_3$) δ 2.44 (3H, d, *J* = 1.6 Hz, H-7), 2.85 (1H, s, OH), 3.50 (3H, s, $OCH_3$), 3.64–3.81 (6H, m, H-2′, -3′, -4′, -5′, and -6′), 4.14 (1H, d, *J* = 16.7 Hz, H-11), 4.27 (1H, d, *J* = 16.7 Hz, H-11), 4.54 (1H, d, *J* = 11.9 Hz, $CH_2Ph$), 4.66 (1H, d, *J* = 11.9 Hz, $CH_2Ph$), 4.72 (1H, d, *J* = 11.6 Hz, $CH_2Ph$), 4.86 (1H, d, *J* = 3.2 Hz, H-1′), 5.02 (1H, d, *J* = 11.6 Hz, $CH_2Ph$), 7.27–7.52 (13H, m, ArH), 9.33 (1H, s, NH).

**2-(4-*O*-methyl-β-D-glucopyranosyl)-(3-methyl-4-trifluoromethyl-amidophenyl)-oxyacetate (5).** A suspension of the 3,6-di-*O*-benzyl-4-*O*-methyl-β-D-glucopyranoside (60 mg) and Pd (10% on charcoal, 30 mg) in ethanol (5 mL) was stirred for 3 h at room temperature under a hydrogen atmosphere. The catalyst was filtered off with celite, rinsed with EtOAc, then evaporated under reduced pressure to give 30 mg (73%) of 4-*O*-methyl-β-D-glucopyranoside. Mp 215–217 °C (after recrystallization in ethanol). $[\alpha]_D$ −28.4 (*c* 0.52, acetone). $^1H$ and $^{13}C$ NMR, see Tables 2 and 3.

The 4-*O*-methyl-α-D-mannopyranoside (87% yield) was similarly obtained from the corresponding 3,6-di-*O*-benzyl derivative. Mp 128–130 °C. $[\alpha]_D$ +63.7 (*c* 0.76, MeOH). $^1H$ NMR (acetone-$d_6$) δ 10.04 (1H, br s, NH), 7.78 and 7.57 (2H, 2d, *J* = 8.7 Hz, H-5 and H-6), 7.65 (1H, s, H-2), 4.96 (1H, d, *J* = 3.9 Hz, H-1′), 4.28 and 4.14 (2H, 2d, *J* = 16.7 Hz, H-11), 3.87 (1H, t, *J* = 9.6 Hz, H-4′), 3.75–3.57 (3H, m, H-2′ and H-6′), 3.54 (3H, s, $OCH_3$), 3.40 (1H, m, H-5′), 3.16 (1H, t, *J* = 9.6 Hz, H-3′), 2.43 (3H, s, $ArCH_3$). $^{13}C$ NMR ($CD_3OD$) δ 21.32 ($CH_3$, C-7), 62.7 ($OCH_3$), 63.8 and 70.37 ($CH_2$-11 and -6′), 75.05, 75.42, 76.81, and 82.61 (CH-2′, -3′, -4′, and -5′), 103.44 (CH-1′), 119.74, 125.61, and 129.39 (CH-2, -5, and -6), 129.48, 140.67, and 144.13 (quat. C-1, -3, and -4), 172.75 (quat. C-10).

## Chemical oxidation of HR325

(A) A solution of **1** (200 mg, 0.64 mmol) and *m*-chloroperbenzoic acid (223 mg, about 1.1 mmol) in $CH_2Cl_2$ (5 mL) was shaken at ambient temperature for 2 h. After washing with a satd $NaHCO_3$ solution, then brine, and drying with $MgSO_4$, the dichloromethane solution was evaporated under vacuum to give a yellow oil (191 mg) identified as the α-ketol **7** by comparison (TLC, $^1H$ NMR) with the dimethyldioxirane reaction product (see below).

Flash chromatography on a silica gel column (cyclohexane:EtOAc, 7:3) afforded 117 mg (70%) of pure cyanohydrin **6**. Mp 75–77 °C. $^1H$ NMR (Acetone-$d_6$) δ 9.63 (1H, s, NH), 7.74–7.70 (2H, m, H-2 and H-5 or H-6), 7.61 (1H, d, *J* = 8.9 Hz, H-5 or H-6), 6.76 (1H, br s, OH), 5.43 (1H, s, H-11), 2.44 (3H, s, H-7). $^{13}C$ NMR (Acetone-$d_6$) δ 19.6 ($CH_3$-7), 63.3 (CH-11), 123.6 (quat. C-12), 118.0, 123.6, and 127.6 (CH-2, -5, and -6), 127.9, 138.6, and 141.7 (quat. C-1, -3, and -4), 206.9 (quat. C-10). HRMS (EI): calcd for $C_{11}H_9F_3O_2N_2$, 258.0616; found 258.0615.

(B) A freshly prepared dimethyldioxirane solution (0.45 mmol) in acetone (5 mL) was added, under nitrogen, to a solution of **1** (100 mg, 0.32 mmol) in $CH_2Cl_2$ (2 mL). The solution was stirred at ambient temperature for 4 h, then an identical amount of dimethyldioxirane solution was added, followed by a second addition (3 mL, 0.27 mmol) after 22 h. When HR325 had completely disappeared from the reaction medium (24 h), the solvents were evaporated under vacuum to give 85 mg (80%) of crystalline colorless **7**. Mp 92–95 °C. $^1H$ NMR (acetone-$d_6$) δ 9.82 (1H, s, NH), 7.77 and 7.65 (2H, 2d, *J* = 8.7 Hz, H-5 and H-6), 7.36 (1H, s, H-2), 2.68–2.78 (1H, m, H-13), 2.46 (3H, d, *J* = 1.6 Hz, H-7), 1.02–1.33 (4H, m, H-14 and H-15). $^{13}C$ NMR (acetone-$d_6$) δ 14.0 and 14.9 ($CH_2$-14 and -15), 17.2 (CH-13), 19.6 ($CH_3$-7), 80.3 (quat. C-11), 123.8 (quat. C-16), 127.5, 138.7, and 141.4 (quat. C-1, -3, and -4), (CH-2, -5, and -6), 162.6 and 198.9 (quat. C-10 and C-12). Flash chromatography on a silica gel column (cyclohexane:EtOAc, 7:3) afforded pure cyanohydrin **6**.

(C) A 5-mg sample of hydroxyketone **7** in 1 mL of cyclohexane:EtOAc (1:1) was stirred overnight with 5 mg of silica gel. Cyanohydrin **6** was quantitatively recovered by filtration and washing with EtOAc. Further elution of silica gel with 10% AcOH in EtOAc afforded a fraction containing cyclopropanecarboxylic acid, identified by GC-MS (retention time and EI-mass spectrum identical to an authentic specimen).

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