Mapping the sirodesmin PL biosynthetic pathway — A remarkable intrinsic steric deuterium isotope effect on a ^{1}H NMR chemical shift determines β-proton exchange in tyrosine

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Abstract: Sirodesmin PL is both an antibiotic and a phytotoxin produced by a fungal plant pathogen (*Leptosphaeria maculans,* asexual stage *Phoma lingam*) that causes blackleg disease on crucifers. To determine potential biosynthetic precursors of sirodesmin PL, deuterated compounds were synthesized and incubated with cultures of *L. maculans*. Incorporations of deuterium into sirodesmin PL (**7**) and its precursor phomamide (**4**) were determined using ^{1}H and ^{13}C NMR spectroscopy, LC-HRMS-ESI and HRMS-EI spectrometry. Spectroscopic analyses established that [3,3-^{2}H$_2$]L-tyrosine (**1a**), [3,3-^{2}H$_2$]*O*-prenyl-L-tyrosine (**9a**), [3,3,5′,5′,5′-^{2}H$_5$]*O*-prenyl-L-tyrosine (**9b**), and [5,5-^{2}H$_2$]phomamide (**4a**) were incorporated efficiently into sirodesmin PL (**7**). Interestingly, an unexpected "twist" revealed that one of the β-deuteria (*pro-R*) of [3,3-^{2}H$_2$]L-tyrosine (**1a**) was exchanged stereospecifically before incorporation into sirodesmin PL (**7**). As well, our studies revealed that *O*-prenyl-L-tyrosine is likely to be the first committed precursor en route to sirodesmin PL (**7**).

Key words: biosynthesis, isotope effect, deuterium incorporation, *Leptosphaeria maculans*, phytotoxin, sirodesminbiosynthèse, effet isotopique, incorporation de deutérium, , *Leptosphaeria maculans*, phytotoxine, sirodesmine.

Résumé : La sirodesmine PL est à la fois un antibiotique et une phytotoxine produite par un champignon pathogène (*Leptosphaeria maculans*, stade asexuel du Phoma lingam) qui provoque la maladie de la jambe noire chez les crucifères. Dans le but de déterminer la nature des précurseurs biosynthétiques potentiels de la sirodesmine PL, on a effectué la synthèse de produits deutériés qui ont été incubés avec des cultures de *L. maculans*. L'incorporation du deutérium dans la sirodesmine PL (7) et dans son précurseur, la phomamide (**4**), a été déterminé en faisant appel à la RMN du ^{1}H et du ^{13}C ainsi que par chromatographie liquide liée à la spectrométrie de masse à haute résolution avec ionisation par impact électronébulisation (CL-SMHR-IEN) et par spectrométrie de masse à haute résolution avec ionisation par impact électronique (SMHR-IE). Les analyses spectroscopiques ont permis de déterminer que la [3,3-^{2}H$_2$]L-tyrosine (**1a**), la [3,3-^{2}H$_2$]*O*-prényl-l-tyrosine (**9a**), la [3,3,5′,5′,5′-^{2}H$_5$]*O*-prényl-l-tyrosine (**9b**) et la [5,5-^{2}H$_2$]phomamide (**4a**) s'incorporent d'une façon efficace dans la sirodesmine PL (**7**). Un fait intéressant à été noté, à savoir celui qu'un des atomes de deutérium en β, le *pro-R*, de la [3,3-^{2}H$_2$]L-tyrosine (**1a**) s'échange d'une façon stéréospécifique avant l'incorporation dans la sirodesmine PL (**7**). Des plus, nos études ont révélé que la *O*-prényl-l-tyrosine est probablement le premier précurseur dédié en route vers la sirodesmine PL (**7**).

[Traduit par la Rédaction]

Introduction

Mapping biosynthetic pathways used by living organisms to assemble complex libraries of natural products is generally accomplished using isotopically labeled precursors. The choice of isotope depends on the chemical structure of the natural product and hypothetical precursors. Currently, the use of stable isotopes is common due to the availability of highly sensitive analytical tools, while a few decades ago only radioactive isotopes were thinkable.[1] Nonetheless, great challenges remain, particularly for unusual and complex pathways assembled by nonribosomal peptide synthases, such as those producing sirodesmins.[2] Sirodesmins are epipolythio-2,5-dioxopiperazines (ETPs) produced by two unrelated fungal species, *Sirodesmium diversum* (sirodesmins A–G)[3] and *Leptosphaeria maculans* (asexual stage *Phoma lingam*, sirodesmin PL, deacetylsirodesmin PL,[4] and sirodesmins H,[5] J, and K[6]). Sirodesmin PL (**7**) is both an antibiotic and a phytotoxin, i.e., toxic to plant cells. Interest in ETPs has increased over the past years because of their biological activity[7] and potential ecological roles. The current focus on sirodesmin PL (**7**) is motivated by its potential involvement in the interaction of the plant pathogen *L. maculans* with its host plants.[8,9]

The primary biosynthetic precursors of sirodesmin PL (**7**) together with its biosynthetic pathway were proposed based on results of experiments using radiolabeled (^{3}H and ^{14}C) and stable (^{13}C) isotopes,[10] and later on confirmed by Bu'Lock and Clough.[11] The proposed pathway involved dioxopiperazine formation between L-tyrosine (**1**) and L-serine (**2**), isoprenylation with dimethylallylpyrophosphate (DMAPP) to form phomamide (**4**), followed by a number of oxidative rearrangements. As well, the isolation of a toxin structurally related to sirodesmin PL (**7**), phomalirazine (**6**), led us to propose a modification of the Curtis–Férézou pathway.[12]

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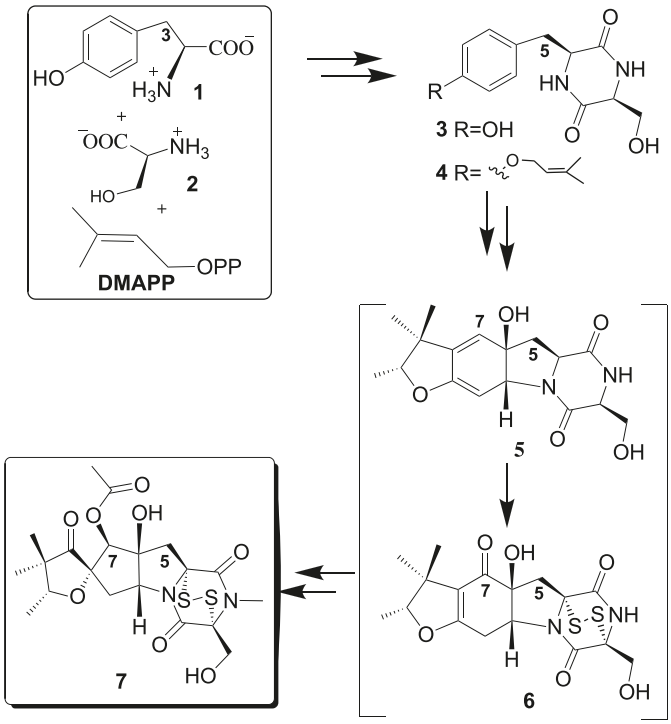
Scheme 1. Biosynthetic pathway of sirodesmin PL (**7**): precursors L-tyrosine (**1**), L-serine (**2**), and DMAPP (dimethylallylpyrophosphate) and potential intermediates **3**–**6**.



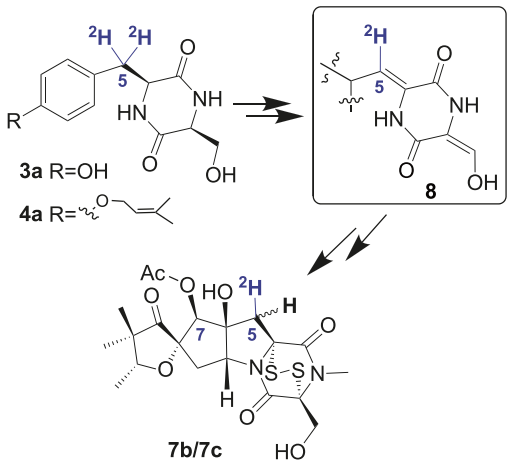
The recent cloning of a gene cluster involved in the biosynthesis of sirodesmin PL (**7**) suggested a more detailed pathway that took into account the ascribed functions of the cloned genes.[8] A simplified pathway showing relevant potential intermediates is depicted in Scheme 1.

These recent developments reawakened our interest in the sirodesmin PL (**7**) biosynthetic pathway. Specific goals to uncover the structure(s) of intermediate(s) immediately preceding insertion of the disulfide bridge and to determine the stage at which prenylation occurred directed our experimental design. Based on the structures of metabolites recently isolated,[13] we hypothesized that a compound containing a bis-ylidine 2,5-dioxopiperazine moiety (**8**) might be a plausible substrate for enzymatic introduction of the disulfide bridge into sirodesmin PL (**7**). If **8** was a precursor, then incorporation of dideuterated [3,3-$^2H_2$]L-tyrosine (**1a**) or [5,5-$^2H_2$]phomamide (**4a**) would lead to the incorporation of only one deuterium into sirodesmin PL (**7**, ^{2}H–C-5, Scheme 2). Alternatively, the presence of two deuteria in sirodesmin PL would eliminate **8** from this biosynthetic map (Scheme 2). Toward this end, we describe work to establish biosynthetic precursors of phomamide (**4**) and sirodesmin PL (**7**).

Results and discussion

The deuterated compounds [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**),[14] [3,3,5′,5′,5′-$^2H_5$]*O*-prenyl-L-tyrosine (**9b**), [5,5-$^2H_2$]cyclo-L-Tyr-L-Ser (**3a**), and [5,5-$^2H_2$]phomamide (**4a**)[15] were prepared from [3,3-$^2H_2$]L-tyrosine (**1a**) following modifications of previously reported methods, as described in the Supplementary data. HRMS-EI analyses indicated that the percentage of deuterium in the synthetic deuterated compounds was higher than 98%.

Scheme 2. Hypothetical intermediate **8** for introduction of sulfur into sirodesmin PL (**7**).

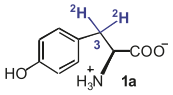
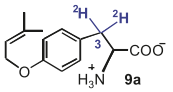
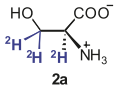
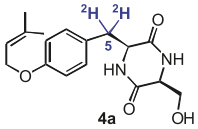
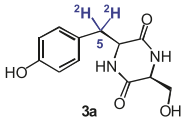
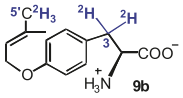


Incorporation experiments using deuterated compounds [3,3-$^2H_2$]L-tyrosine (**1a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**), [3,3,5′,5′,5′-$^2H_5$]*O*-prenyl-L-tyrosine (**9b**), [2,3,3-$^2H_3$]L-serine (**2a**), [5,5-$^2H_2$]phomamide (**4a**), and [5,5-$^2H_2$]cyclo-L-Tyr-L-Ser (**3a**) were carried out using a wild type isolate of *L. maculans*. After incubation of the fungal cultures with deuterated compounds, cultures were extracted and phomamide (**4**) and sirodesmin PL (**7**) were purified as described in the experimental. Similar conditions were used for control experiments in which the corresponding natural abundance compounds were administered to fungal cultures. Incorporation of deuterium, or lack of it, was determined using ^{1}H and ^{13}C NMR spectroscopy, LC-HRMS-ESI and HRMS-EI spectrometry.

First analysis of HRMS data established that (*i*) [3,3-$^2H_2$]L-tyrosine (**1a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**), [3,3,5′,5′,5′-$^2H_5$]*O*-prenyl-L-tyrosine (**9b**), and [5,5-$^2H_2$]phomamide (**4a**) were incorporated efficiently (>10%) into sirodesmin PL (**7**); (*ii*) [3,3-$^2H_2$]L-tyrosine (**1a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**), and [3,3,5′,5′,5′-$^2H_5$]*O*-prenyl-L-tyrosine (**9b**) were incorporated efficiently (>20%) into phomamide (**4**); (*iii*) [5,5-$^2H_2$]cyclo-L-Tyr-L-Ser (**3a**) and [2,3,3-$^2H_3$]L-serine (**2a**) were incorporated poorly (<5%) into either phomamide (**4**) or sirodesmin PL (**7**) (Table 1). Detailed analyses of spectroscopic data obtained for phomamide (**4**) and sirodesmin PL (**7**) isolated from fungal cultures incubated with deuterated compounds (summarized in Table 1 and Fig. 1) provided important additional information, as described below.

The HRMS-EI data of purified phomamide (**4**) isolated from cultures incubated with [3,3-$^2H_2$]L-tyrosine (**1a**) indicated that its $[M + 1]^+$ and $[M + 2]^+$ were about 20% and 12% higher, respectively, than those of control samples, suggesting that 20% of phomamide (**4**) was monodeuterated (equation for calculation is shown in Table 1) and 12% was dideuterated, i.e., 32% of phomamide (**4**) resulted from incorporation of [3,3-$^2H_2$]L-tyrosine (**1a**) (Table 1, entry 1). The ^{1}H NMR spectrum (provided in the Supplementary data) of phomamide (**4**) showed that the protons H-5a (δ_H 3.30, dd, J = 14.0, 3.5 Hz) and H-5b (δ_H 2.99, J = 14.0, 8.9 Hz) integrated for ca. 0.7 and 0.9, respectively, whereas control samples showed the expected integration of ca. 1 for H-5a and H-5b (H-5a (δ_H 3.30) / H-5b (δ_H 2.99) = 1.0). The

Table 1. Percentages of incorporation of deuterated compounds into phomamide (**4**) and sirodesmin PL (**7**) in cultures of *Leptosphaeria maculans.*

Entry	Deuterated compound	Phomamide (**4**) (% of ^{2}H, analytical method)	Sirodesmin PL (**7**) (% of ^{2}H, analytical method)
1	 **1a**	32 (HRMS-EI: monodeuterated = 20; dideuterated = 12)[a]; 30 (^{1}H NMR, H_2-5a,5b)	32 (HRMS-EI: monodeuterated = 19; dideuterated = 13)[a]; 30 (^{1}H NMR, H-7)[b]
2	 **9a**	41 (HRMS-EI: monodeuterated = 24; dideuterated = 17)[a]; 39 (^{1}H NMR, H_2-5a,5b)	35 (HRMS-EI: monodeuterated = 21; dideuterated = 15)[a]; 32 (^{1}H NMR, H-7)[b]
3	 **2a**	3 (HRMS-EI)[a]	4 (HRMS-EI)[a]
4	 **4a**	—	30 (HRMS-EI: monodeuterated = 4; dideuterated = 26)[a]; 27 (^{1}H NMR, H-7)[b]
5	 **3a**	3 (HRMS-EI: monodeuterated = 1; dideuterated = 2)[a]	2 (HRMS-EI: monodeuterated = 0; dideuterated = 2)[a]
6	 **9b**	23 (HRMS-ESI: tetradeuterated = 16; pentadeuterated = 7)[c]; 26 (^{1}H NMR, H_2-5a,5b)	13 (HRMS-ESI: tetradeuterated = 9; pentadeuterated = 4)[c]; 23 (^{1}H NMR, H-7)[b]

[a]The percentage of incorporation (*I*) was determined by HRMS-EI and was calculated using the formula $I = \{([M + n]^+ - [M + n]^+_{Ctl}) / ([M]^+ + [M + 1]^+ + [M + 2]^+)\} \times 100$; $n = 1, 2$; $[M + n]^+$ = intensity of deuterated molecular ion; $[M + n]^+_{Ctl}$ = intensity of molecular ion of control samples (natural abundance).

[b]Resolution of signals was enhanced through a Lorentz–Gaussian lineshape transformation (gb = 0.3, lb = –1).

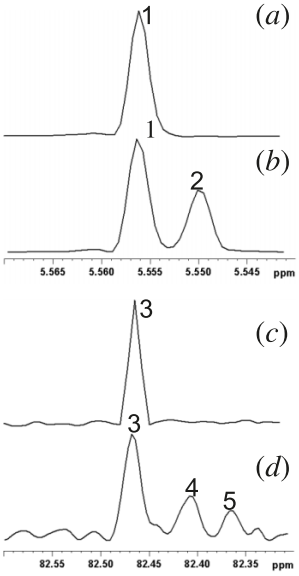
[c]The percentage of incorporation (*I*) was determined by HRMS-ESI (positive mode) and was calculated using the formula $I = \{([M + 1 + n]^+ - [M + 1 + n]^+_{Ctl}) / ([M + 1]^+ + [M + 2]^+ + [M + 5]^+ + [M + 6]^+)\} \times 100$; $n = 4, 5$; $[M + n]^+$ = intensity of deuterated molecular ion; $[M + n]^+_{Ctl}$ = intensity of molecular ion of control samples (natural abundance).

percentage of deuterium at the C-5 of phomamide resulting from feeding [3,3-$^2H_2$]L-tyrosine (**1a**) was calculated to be 30% for H-5a (δ_H 3.30) and 11% for H-5b (δ_H 2.99). These ^{1}H NMR data indicated that about 11% of phomamide (**4**) was dideuterated, whereas the remaining 19% of phomamide was monodeuterated (Table 1, entry 1), in complete agreement with the HRMS data. Similar results were obtained when cultures were incubated with [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**, Table 1, entry 2).

Similarly, the HRMS-EI spectroscopic data of purified sirodesmin PL (**7**) isolated from cultures fed with [3,3-$^2H_2$]L-tyrosine (**1a**) provided percentages of deuterium incorporation. Because the $[M]^+$ of sirodesmin PL (**7**) observed in the HRMS-EI spectrum was very weak, the base peak $[M - S_2]^+$ was used to calculate the percentage of deuterium incorporation, as follows. The intensity of the $[M - S_2 + 1]^+$ of sirodesmin PL was ca. 19% higher than that of control samples, whereas that of $[M - S_2 + 2]^+$ was ca. 13% higher than that of control samples. These results indicated that both monodeuterated (19%) and dideuterated (13%) sirodesmin PL (**7**) were biosynthesized from **1a** (Table 1, entry 1). Therefore, the total amount of deuterated **7** (mono plus di) calculated from the HRMS-EI data was ca. 32%.

The ^{1}H NMR spectrum of sirodesmin PL (**7**) was less informative (provided in the Supplementary data) than that of phomamide (**4**) because the signals due to protons H_2-5 (δ_H 3.27) appeared as a broad AB quartet; this signal integrated for ca. 2 in natural abundance samples of **7** and for <2 (1.4–1.6) in samples resulting from incorporation of [3,3-$^2H_2$]L-tyrosine (**1a**). Unexpectedly, a closer inspection of the signal due to H-7 (δ_H 5.56) in the ^{1}H NMR spectrum of sirodesmin PL (**7**) isolated from feeding experiments using [3,3-$^2H_2$]L-tyrosine (**1a**) showed two partially overlapping peaks attributable to H-7 (δ_H 5.56) that were not apparent in nondeuterated samples (i.e., natural abundance samples of **7**). Integration of these signals (δ_H 5.56) using resolution enhancement (Lorentz–Gaussian lineshape transformations and baseline corrections) showed resonances at δ_H 5.556 and δ_H 5.550 in a 2:1 ratio (Figs. 1*a* and 1*b*). The presence of both peaks suggested that replacement of H_2-5 of sirodesmin PL (**7**) with deuterium affected the chemical shift of H-7, i.e., the new peak at δ_H 5.550 was caused by the isotope shift effect of $^2H_2$-5 on H-7. The integrated area of this peak (δ_H 5.550) in conjunction with the HRMS-EI data (Table 1, entry 1) indicated that it accounted for the total amount of monodeuterated and dideuterated **7** (ca. 33%). Nonetheless, detection of a deuterium isotope shift effect over four covalent bonds was not expected.[1,16] To further understand this result, the proton decoupled ^{13}C NMR spectrum of this deuterated sample of sirodesmin PL (**7**) was obtained and analyzed. The signal of C-5 (single plus multiplet) had low intensity due to the presence of deuterium; as well, C-4 (δ_C 75.46, 75.40, 75.32) and C-6 (δ_C 82.44, 82.38, 82.34, Figs. 1*c* and 1*d*) displayed the expected two additional peaks

Fig. 1. Sections of NMR spectra of sirodesmin PL: (*a*) ^{1}H NMR, H-7 signal of natural abundance **7**; (*b*) ^{1}H NMR, H-7 signal (peaks 1 at δ 5.556 and 2 at δ 5.550) of **7** due to partial deuteration at C-5; (*c*) ^{13}C NMR, peak 3: C-6 signal of natural abundance **7**; (*d*) ^{13}C NMR, peak 3: C-6 signal of **7**, peak 4: C-6 signal of **7b**, and peak 5: C-6 signal of **7a** due to partial deuteration at C-5 (full ^{1}H NMR spectra of isolated deuterated phomamides and sirodesmins PL are provided in the Supplementary data).



due to the β-isotope shift.[1,16] Importantly, only one ^{13}C NMR peak was observed for C-7 (δ_C 79.2), indicating that the deuterium at C-5 caused no isotope shift effect on C-7, as expected due to the additional bond separation. Similar results were obtained when cultures were incubated with [3,3-$^{2}H_2$]*O*-prenyl-L-tyrosine (**9a**) (Table 1, entry 2). Furthermore, feeding [2,3,3-$^{2}H_3$]L-serine (**2a**) and [5,5-$^{2}H_2$]cyclo-L-Tyr-L-Ser (**3a**) led to very low incorporation of deuterium into sirodesmin PL (**7**) (Table 1, entries 3 and 5, respectively).

The results described above and summarized in entries 1 and 2 of Table 1 demonstrated that 30%–35% of both monodeuterated (**7b**) and dideuterated (**7a**) sirodesmin PL (**7**) was obtained from incorporation experiments with dideuterated precursors **1a** and **9a**, whereas mostly dideuterated **7a** was obtained from incorporation of **4a** (full ^{1}H NMR spectra of isolated deuterated phomamides and sirodesmins PL are provided in the Supplementary data). Consequently, these results indicated that deuterium exchange took place before the final step of phomamide (**4**) biosynthesis and thus the proposed bis-ylidine 2,5-dioxopiperazine (**8**) is not a likely intermediate of the biosynthetic pathway.

Further data analysis and additional calculations clarified the specific nature of this deuterium exchange, as follows. The HRMS-EI data (Table 1, entry 1) showed that the total amount of monodeuterated (19%) and dideuterated (13%) molecules of **7** was 32% (19% + 13%, relative to natural abundance compound), whereas the two ^{1}H NMR peaks due to H-7 at δ_H 5.556 and 5.550 (2:1, Figs. 1*a* and 1*b*) were due to the total amount of **7**, i.e., natural abundance, monodeuterated, and dideuterated. Since integration of both H-7 NMR peaks showed that the signal at δ_H 5.550 represented ca. 33% of H-7 and this amount corresponded to the total amount of deuterated **7** determined by HRMS-EI (19:13 ratio), the 33% accounted by ^{1}H NMR must necessarily represent a mixture of dideuterated (**7a**) and monodeuterated (**7b**) compound in a 19:13 ratio, respectively. This unexpected isotope shift effect (higher field signal of H-7, δ_H 5.550) could be explained by a through space intrinsic deuterium isotope effect on H-7.

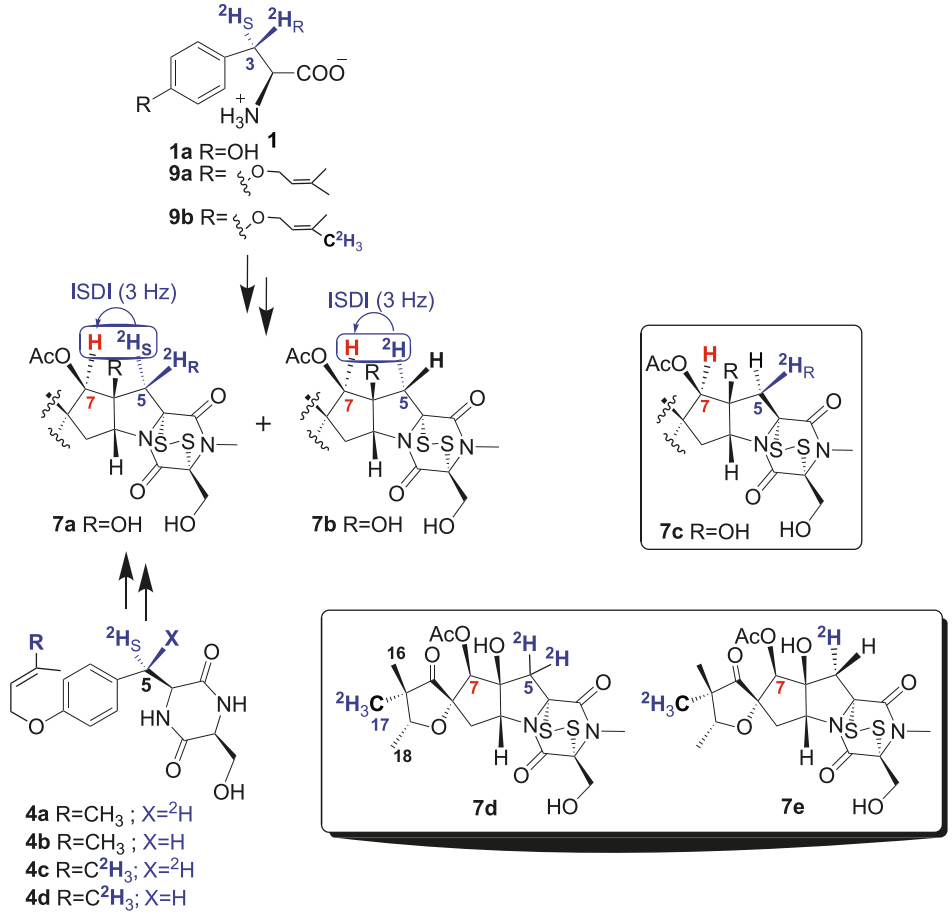
Isotope effects on proton and (or) carbon chemical shifts in compounds where deuterium and the observed proton(s)/carbon(s) were separated by four or more covalent bonds but where the nuclei were close together in space, termed "intrinsic steric isotope effects", have been reported and characterized.[17] The dideuterated sirodesmin molecules had both *pro-R* and *pro-S* deuteria at C-5 (13%), whereas the monodeuterated molecules (19%) in principle could have either the *pro-R* or the *pro-S* deuterium (corresponding to the *pro-R* or the *pro-S* hydrogens) or a mixture of *pro-R* and *pro-S* (e.g., a 1:1 ratio). However, considering the nature of intrinsic steric deuterium isotope effects, it would be expected that only the deuterated molecules having the deuterium close to H-7 (ca. <3 Å) would display this effect. Molecular mechanics calculations[18] showed that structure **7b** has the *pro-S* hydrogen at C-5 in closer proximity of H-7 (2.47 Å) than the *pro-R* hydrogen (3.59 Å). Therefore, these interatomic distances are in close agreement with previous reports[17] on intrinsic steric deuterium isotope effects on proton(s).

From the overall analysis it was concluded that only the monodeuterated structure **7b** was formed (but not **7c**), in addition to the dideuterated **7a**. Finally, extrapolation of this conclusion to the precursor [3,3-$^{2}H_2$]L-tyrosine (**1a**) indicated that its $^{2}H_R$ was partially and stereospecifically exchanged before incorporation into phomamide (**4**), which in turn was incorporated without further deuterium exchange into sirodesmin PL. Furthermore, considering that feeding either [3,3-$^{2}H_2$]L-tyrosine (**1a**) or [3,3-$^{2}H_2$]*O*-prenyl-L-tyrosine (**9a**) yielded monodeuterated products as well, it is surmised that deuterium exchange occurred before formation of phomamide, i.e., either in L-tyrosine or *O*-prenyl-L-tyrosine or both (Scheme 3).

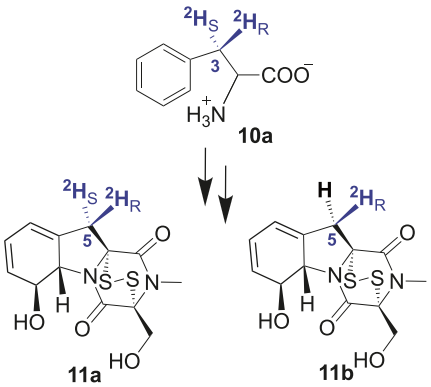
Comparable results in which dideuterated (**11a**) and monodeuterated (**11b**) gliotoxin were obtained from feeding experiments using [3,3-$^{2}H_2$]D,L-phenylalanine (**8a**) were reported previously;[19] however, stereospecific exchange of the *pro-S* deuterium of deuterated phenylalanine (Scheme 4) was established using stereospecifically monodeuterated phenylalanines **10b** and **10c**.

Finally, to clarify the stage at which prenylation occurred (i.e., amino acid or dioxopiperazine), incorporation of [3,3,5′,5′,5′-$^{2}H_5$]*O*-prenyl-L-tyrosine (**9b**) was investigated. The ^{1}H NMR spectra of both phomamide (**4**) and sirodesmin PL (**7**) isolated from cultures incubated with [3,3,5′,5′,5′-$^{2}H_5$]*O*-prenyl-L-tyrosine (**9b**) showed that integration of the peak area of protons H-5a, H-5b, and H_3-5′ of phomamide (**4**) and H_2-5 and H_3-17 of sirodesmin PL (**7**) were lower than those of control samples. That is, both pentadeuterated and tetradeuterated phomamide (**4c**, 7%; **4d**, 16%) and sirodesmin PL (**7d**, 4%; **7e**, 9%) were produced in cultures incubated with pentadeuterated prenyl tyrosine **9b** (Table 1, entry 6). These amounts of deuterium incorporation and the

Scheme 3. Incorporation of [3,3-$^2H_2$]L-tyrosine (**1a**), [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**), and [5,5-$^2H_2$]phomamide (**4a**) into sirodesmin PL showing partial structures of dideuterated (**7a**) and monodeuterated (**7b**) sirodesmin PL; **7c** is not formed; **7d** and **7e** result from incorporation of [3,3,5′,5′,5′-$^2H_5$]*O*-prenyl-L-tyrosine (**9b**). The curved arrow indicates an intrinsic steric deuterium isotope (ISDI) effect (ca. 3 Hz) of ^2H_S-5 on the ^1H NMR chemical shift of H-7.



Scheme 4. Incorporation of [3,3-$^2H_2$]D,L-phenylalanine (**10a**) into dideuterogliotoxin (**11a**) and monodeuterogliotoxin (**11b**).[19]



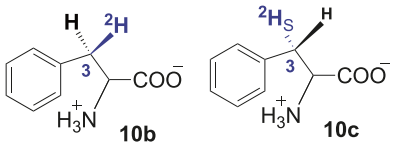
much lower incorporation of **3a** (≤2%) relative to compounds **1a**, **9a**, and **4a** indicated that **3** was not a likely intermediate in the sirodesmin PL (**7**) pathway. In addition, the incorporation of specifically deuterated prenyl derivative demonstrated clearly that CH_3-17 is derived from the deuterated methyl of substituted tyrosine **9b**, which agrees with previous data.[10] Substantially lower incorporations of serine than tyrosine may reflect a faster deployment of serine into other primary pathways (e.g., synthesis of proteins and tryptophan and (or) degradation to pyruvate and glycine). Previous results using radiolabeled serine and tyrosine reported a rather low incorporation of both amino acids into phomamide (**4**) and sirodesmin PL (**7**).[10]

Conclusion

In summary, this work demonstrated that both dideuterated and monodeuterated sirodesmin PL (**7a** and **7b**) and phomamide (**4a** and **4b**) were produced when the precursors [3,3-$^2H_2$]L-tyrosine (**1a**) and [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**) were incubated with cultures of *L. maculans*. Importantly, a remarkable intrinsic steric deuterium isotope effect detected on the NMR chemical shift of H-7 of monodeuterated sirodesmin PL (**7b**) revealed that the *pro-R* deuterium of sirodesmin PL exchanged stereospecifically. This stereospecific exchange occurred before incorporation of tyrosine (**1a**) into phomamide (**4**), thus the proposed bis-ylidine 2,5-dioxopiperazine (**8**) is not a likely intermediate of the sirodesmin pathway. That is, a percentage of [3,3-$^2H_2$]L-tyrosine (**1a**) is directly channelled into sirodesmin biosynthesis and another fraction rechannelled after diverging into a pathway(s) where a β-deuterium is exchanged stereospecifically. Such a pathway(s) might involve transamination via pyri-

doxal phosphate with concomitant stereospecific exchange of the *pro-R* β-deuterium with a proton.[20] A fraction of the resulting monodeuterated tyrosine ([3-^2H_S]L-tyrosine) could eventually be redirected into the sirodesmin pathway. In addition, our results strongly suggested that prenylation occurred before dioxopiperazine formation and thus *O*-prenyl-L-tyrosine is a likely intermediate of phomamide (**4**) en route to sirodesmin PL (**7**). Therefore, prenylation of tyrosine by the prenylase sirD appears to be the first committed step in the biosynthetic pathway to sirodesmin PL (**7**).

As a final point, it is of interest to highlight that, in general, demonstration of stereospecific isotope exchange observed in biological pathways[20,21] require feeding experiments with stereospecifically labeled substrates, e.g., phenylalanine (**10b** and **10c**) → gliotoxin (**11** and **11b**) (Scheme 4). By contrast, in this work, the detection and substantiation of an intrinsic steric deuterium isotope shift effect revealed clearly a stereospecific deuterium exchange (**7b** and **7e**) in the biosynthetic pathway to sirodesmin PL (**7**).



Materials and methods

General experimental procedures

All solvents were HPLC grade and used as such. Organic extracts were dried with Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator. Preparative thin layer chromatography was carried out on silica gel plates, Kieselgel 60 F_{254} (20 cm × 20 cm × 0.25 mm), compounds were visualized under UV light. NMR spectra were recorded on Bruker Avance 500 series spectrometers; for ^1H (500 MHz), δ values were referenced as follows for ^1H to $CHCl_3$ (7.27 ppm) and for ^{13}C to $CDCl_3$ (77.23 ppm). Mass spectra (MS) were obtained on a mass spectrometer using a solids probe.

Synthesis of labeled precursors

The deuterated materials [3,3-$^2H_2$]*O*-prenyl-L-tyrosine (**9a**), [3,3,5′,5′,5′-$^2H_5$]*O*-prenyl-L-tyrosine (**9b**), [5,5-$^2H_2$]cyclo-L-Tyr-L-Ser (**3a**), and [5,5-$^2H_2$]phomamide (**4a**) were prepared following modifications of previously reported methods, as described in the Supplementary data. All compounds gave satisfactory spectroscopic data; in each case the percentage of deuterated synthetic compound was ≥98% (HRMS-EI).

Incorporation of labeled precursors

Fungal cultures of *L. maculans* isolate BJ 125 were obtained from the IBCN collection, Agriculture and Agri-Food Canada Research Station, Saskatoon, SK. Cultures were handled as described previously.[22] Isolate BJ 125 was grown in 250 mL Erlenmeyer flasks containing 100 mL minimal media inoculated with fungal spores at 1×10^9 cells per flask. The cultures were incubated on a shaker at 130 rpm, at 24 ± 2 °C for 3 d, then deuterated compounds (dissolved in sterile distilled water, 5.0 mmol/L) were added to cultures. At the 5th day, the broth of each flask was extracted with EtOAc (100 mL × 3) and concentrated to dryness. The residue was separated by preparative TLC (MeOH–CH_2Cl_2, 10:90) to give sirodesmin PL (**7**) (R_f = 0.75, ca. 90 mg/L) and phomamide (**4**) (R_f = 0.25, ca. 10 mg/L).

Supplementary data

Supplementary data for this article are available on the journal Web site (canjchem.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Ottawa, ON K1A 0R6, Canada. DUD 3898. For more information on obtaining material refer to cisti-icist.nrc-cnrc.gc.ca/cms/unpub_e.shtml.

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References

(1) For a relevant review see: Schneider, B. *Prog. Nucl. Magn. Reson. Spectrosc.* **2007**, *51*, 155. doi:10.1016/j.pnmrs.2007.02.006.

(2) For a recent review on ETPs see: Gardiner, D. M.; Howlett, B. J. *Microbiology* **2005**, *151*, 1021. doi:10.1099/mic.0.27847-0. PMID:15817772.

(3) Curtis, P. J.; Greatbanks, D.; Hesp, B.; Forbes, C. A.; Freer, A. A. *J. Chem. Soc. Perkin Trans.* **1977**, *2*, 180. doi:10.1039/p19770000180.

(4) Quesneau-Thierry, A.; Boudart, G. *Nouv. J. Chim.* **1977**, *1*, 327.

(5) Pedras, M. S. C.; Abrams, S. R.; Séguin-Swartz, G. *Tetrahedron Lett.* **1988**, *29*, 3471. doi:10.1016/0040-4039(88)85192-X.

(6) Pedras, M. S. C.; Taylor, J. L.; Nakashima, T. T. *J. Org. Chem.* **1993**, *58*, 4778. doi:10.1021/jo00070a002.

(7) Kupfahl, C.; Heinekamp, T.; Geginat, G.; Ruppert, T.; Haertl, A.; Hof, H.; Brakhage, A. A. *Mol. Microbiol.* **2006**, *62*, 292. doi:10.1111/j.1365-2958.2006.05373.x. PMID:16956378.

(8) Gardiner, D. M.; Cozijnsen, A. J.; Wilson, L. M.; Pedras, M. S. C.; Howlett, B. J. *Mol. Microbiol.* **2004**, *53*, 1307. doi:10.1111/j.1365-2958.2004.04215.x. PMID:15387811.

(9) Elliot, C. E.; Gardiner, D. M.; Thomas, G.; Cozijnsen, A.; Van de Wouw, A.; Howlett, B. J. *Molecular Plant Pathology* **2007**, *8*, 791. doi:10.1111/j.1364-3703.2007.00433.x.

(10) Ferezou, J.-P.; Quesneau-Thierry, A.; Servy, C.; Zissmann , E.; Barbier, M. *J. Chem. Soc. Perkin Trans. 1* **1980**, 1739. doi:1.1039/P19800001739.

(11) Bu'Lock, J. D.; Clough, L. E. *Aust. J. Chem.* **1992**, *45*, 39. doi:10.1071/CH9920039.

(12) Pedras, M. S. C.; Abrams, S. R.; Séguin-Swartz, G.; Quail, J. W.; Jia, Z. *J. Am. Chem. Soc.* **1989**, *111*, 1904. doi:10.1021/ja00187a068.

(13) Pedras, M. S. C.; Yu, Y. *Bioorg. Med. Chem.* **2008**, *16*, 8063. doi:10.1016/j.bmc.2008.07.060. PMID:18701303.

(14) Fraile, J. M.; Garcia, J. I.; Mayoral, J. A.; Royo, A. J. *Tetrahedron Asymmetry* **1996**, *7*, 2263. doi:10.1016/0957-4166(96)00281-9.
(15) (a) Férézou, J. P.; Quesneau-Thierry, A.; Barbier, M.; Kollmann, A.; Bousquet, J. F. *J. Chem. Soc. Perkin Trans.* **1980**, *1*, 113. doi:10.1039/p19800000113.; (b) Mancilla, T.; Carrillo, L.; Zamudio-Rivera, L. S.; Beltran, H. I.; Farfan, N. *Org. Prep. Proced. Int.* **2001**, *33*, 34; (c) Nitecki, D. E.; Halpern, B.; Westley, J. W. *J. Org. Chem.* **1968**, *33*, 864. doi:10.1021/jo01266a091.
(16) Jurlina, J. L.; Stothers, J. B. *J. Am. Chem. Soc.* **1982**, *104*, 4677. doi:10.1021/ja00381a030.
(17) (a) Anet, F. A. L.; Dekmezian, H. J. *J. Am. Chem. Soc.* **1979**, *101*, 5449 doi:10.1021/ja00512a073.; (b) Ernst, L.; Eltamany, S.; Hopf, H. *J. Am. Chem. Soc.* **1982**, *104*, 299 doi:10.1021/ja00365a060.; (c) Saunders, M.; Wolfsberg, M.; Anet, F. A. L.; Kronja, O. J. *J. Am. Chem. Soc.* **2007**, *129*, 10276. doi:10.1021/ja072375r. PMID:17655301.
(18) Computational calculations of the interatomic distances in sirodesmin PL (**7**) were carried out using the Spartan 06 software package, method MMFF94.
(19) Bu'Lock, J. D.; Ryles, A. P.; Johns, N.; Kirby, G. W. *J. Chem. Soc. Chem. Comm.* **1972**, 100. doi:10.1039/c39720000100.
(20) Sawada, S.; Kumagai, H.; Yamada, H.; Hill, R. K. *J. Am. Chem. Soc.* **1975**, *97*, 4334. doi:10.1021/ja00848a033. PMID:1141596.
(21) Hanson, K. R. *Annu. Rev. Biochem.* **1976**, *45*, 307. doi:10.1146/annurev.bi.45.070176.001515. PMID:786152.
(22) Pedras, M. S. C.; Khan, A. Q. *J. Agr. Food Chem.* **1996**, *44*, 3403. doi:10.1021/jf960098u.