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Stress-driven discovery of metabolites from the phytopathogenic fungus *Leptosphaeria maculans*: Structure and activity of leptomaculins A–E

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Rutalexin
Sirodesmin
Spirobrassinin

ABSTRACT

A search for stress inducing metabolites produced by the plant pathogenic fungus *Leptosphaeria maculans* led to the isolation and structure elucidation of eight new metabolites, the leptomaculins and deacetylleptomaculins A–E. The chemical structures and absolute stereochemistry of the new metabolites were deduced by detailed analysis of 1D and 2D NMR spectroscopic data and chemical degradation of the toxin sirodesmin PL. Leptomaculins A and B are the first examples of naturally occurring 2,3-oxopiperazinethione and 2,3-dioxopiperazine, respectively. Stress inducing activity was found in the fungal phytotoxins sirodesmin PL and deacetylsirodesmin PL but not in any of the new leptomaculins, phomalide or phomamide. A metabolic pathway for biosynthesis of the first 2,3-(di)oxopiperazine(thione) from sirodesmin PL is proposed.

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1. Introduction

Plant defense responses to pathogen attack are mediated by specific molecules, elicitors, produced by pathogens (exogenous elicitors) or the plant itself (endogenous elicitors). Elicitors induce plant defense responses such as cell wall reinforcement by deposition of callose and lignin, and biosynthesis of phytoalexins and pathogenesis-related proteins, for example, chitinases and glucanases.[1] General elicitors are able to trigger defenses both in host and in non-host plants, whereas race specific elicitors induce defense responses leading to disease resistance only in specific hosts. Elicitors produced by plant pathogens display a wide range of chemical structures with no particular motifs, although a number of elicitors are oligosaccharides and peptides. Contrary to phytotoxins, which can affect detrimentally the plant without inducing plant defenses, elicitors act as signal compounds that trigger plant defenses and can be used in treatments to enhance plant disease resistance.[1–3] However, some phytotoxins can also induce defense responses, as, for example, the bacterial metabolite coronatine that displays both phytotoxin and elicitor activity.

The plant pathogenic fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] causes blackleg disease in rapeseed (*Brassica napus* L. and *B. rapa* L.) and canola (*B. napus* L. and *B. rapa* L.) in Canada and worldwide.[4–6] Recent canola field surveys showed the prevalence of *L. maculans* and an increase in more aggressive isolates in both Canada and USA, which suggests on-going evolution of blackleg causing isolates.[4] Consistent with these field surveys, metabolite profiles of isolates of *L. maculans* collected in different countries suggested that this species comprises distinct groups,[7] one of which is highly virulent on canola. These virulent isolates produce sirodesmin PL (**1**),[8] a non-host selective phytotoxin, in a chemically defined culture media. Other minor sirodesmins, deacetylsirodesmin PL (**2**),[8] and sirodesmins H (**3**),[9] J (**4**) and K (**5**)[10] and phomamide (**6**) are metabolites co-produced in liquid cultures.[11] As well, these virulent isolates produce a host-selective toxin, phomalide (**7**), *in planta* [12] and in culture. [13] Interestingly, we established that production of sirodesmin PL (**1**), but not phomalide (**7**), in fungal cultures was inhibited by the crucifer phytoalexin brassinin.[13] This observation provided an explanation for the absence of sirodesmin PL (**1**) in infected plant tissues,[12] although traces of **1** were recently reported in infected canola.[14]

Considering the importance of stress inducing metabolites on the plant-pathogen interaction and their potential application,[2] we initiated a search for elicitors produced by *L. maculans*, as no metabolites with such a bioactivity appear to have been reported to date. As a result of this search, eight new metabolites, leptomac-

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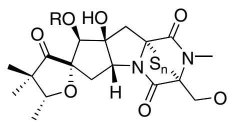
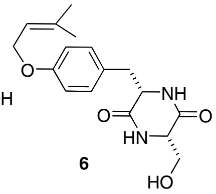


1 n=2, R = Ac
2 n=2, R=H
3 n=1, R = Ac
4 n=3, R = Ac
5 n=4, R = Ac



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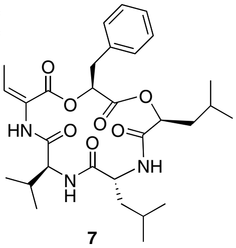

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ulins and deacetylleptomaculins A–E, were discovered, and elicitor activity was found in sirodesmins **1** and **2**. The structures of leptomaculins A (**8**) and B (**9**) are particularly remarkable as they are the first examples of a naturally occurring 2,3-oxopiperazinethione and a 2,3-dioxopiperazine, respectively. The chemistry involved in the structure determination of these metabolites is reported, and metabolic pathways for their biosyntheses are proposed.

2. Results and discussion

2.1. Metabolites from the culture broth

To search for specific elicitors, a bioassay using brown mustard (*B. juncea*, resistant to *L. maculans*) and canola (*B. napus*, susceptible to *L. maculans*) was developed as described in the experimental. Potential elicitor samples were applied to leaves of whole plants, and plants were incubated in a growth cabinet. A compound or fraction was considered to have elicitor activity when HPLC-DAD analyses of extracts of leaves incubated with solutions of the compound/fraction showed the presence of phytoalexins that were not detected in control plants.

Leptosphaeria maculans virulent isolate IBCN 57 (also called BJ 125) was grown in a chemically defined medium, as previously reported,[12] the cultures were filtered, the broth was extracted and the EtOAc broth extract was analyzed by HPLC-DAD. The EtOAc extracts were assayed on leaves of brown mustard and canola. Fractionation of extracts using various chromatographic techniques guided by the elicitor activity assay provided several fractions with elicitor activity. However, because the more polar sirodesmins **2**–**5** were components of several fractions (sirodesmins constitute ca. 90% of the broth extract), it was not clear whether the elicitor activity was due to the new but minor metabolites or to any of the more polar sirodesmins. Therefore, efforts were made to separate and characterize the minor extract components not available in our metabolite libraries and to determine their potential elicitor activity. Eight minor metabolites substantially more polar than sirodesmin PL (**1**) were isolated and their structures elucidated as described below.

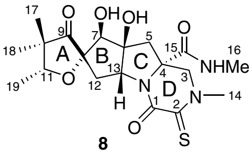
2.2. Chemical structures of new metabolites

The ^{1}H NMR spectral data of compound **8** showed several of the characteristic resonances of sirodesmin **1**, that is, two singlets (δ_H 1.03, 3H, and 0.98, 3H), a quartet (δ_H 3.87, 1H) and a doublet (δ_H 1.22, 3H) accounting for all protons of ring A, two doublets of doublets (δ_H 2.57, 1H and 1.93 1H) coupled to another doublet of doublets (δ_H 4.50, 1H) and a singlet (δ_H 3.80, 1H) accounting for ring B, and to two doublets coupled to each other (δ_H 2.83, 1H and 2.25, 1H) accounting for ring C (Table 1). In addition, the hydroxymethylene signals of the sirodesmin skeleton were replaced by two doublets at δ_H 4.03, 1H and 4.14, 1H. An additional singlet at δ_H 3.41 (H$_3$-14) was thought to be attached to the dioxopiperazine nitrogen, whereas a doublet at δ_H 2.76 (3H) was not assignable. The ^{13}C NMR spectrum displayed the ketone carbonyl at the expected chemical shift (δ_C 223.1) but the three remaining carbonyls (δ_C 158.2, 173.8 and 186.7) were rather different, and suggested a thiocarbonyl carbon (δ_C 186.7) not present in **1** (Table 1). The molecular formula of compound **8** ($C_{19}H_{27}N_3O_6S$) obtained by HRMS-EI confirmed the presence of a sulfur atom. The HMBC data showed a correlation of the carbonyl at δ_C 173.8 with proton doublets at δ_H 2.83 and 2.25, and δ_H 4.03 and 4.14 and the methyl group at δ_H 2.76. The absolute configurations of the stereogenic centres of compound **8** were assigned based on the stereochemistry of sirodesmin PL at C-6, C-7, C-8, C-11 and C-13, which is consistent with NOESY data. Finally, the structure of **8** was confirmed by X-ray diffraction analysis of a single crystal. Crystals of **8** suit-

Table 1
^{1}H NMR (CDCl$_3$, 500 MHz) and ^{13}C NMR (CDCl$_3$, 125 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of leptomaculins A (**8**) and B (**9**)

C/H #	Leptomaculin A (**8**) (CD$_3$OD)		Leptomaculin B (**9**) ((CD$_3$)$_2$SO)	
	δ_C	δ_H	δ_C	δ_H
1	158.2	–	155.5	–
2	186.7	–	157.4	–
3	60.4	4.03, 1H, d, (13.4) 4.14, 1H, d, (13.4)	56.1	3.75, 1H, d, (12.9) 3.94, 1H, d, (12.9)
4	69.8	–	67.8	–
5	46.0	2.83, 1H, d, (14.3) 2.25, 1H, d, (14.3)	45.2	2.62, 1H, d, (14.1) 2.09, 1H, d, (14.0)
6	85.8	–	83.2	–
7	80.4	3.80, 1H, s	78.4	3.61, 1H, d, (5.8)
8	92.7	–	91.3	–
9	223.1	–	221.7	–
10	48.6	–	46.9	–
11	80.9	3.87, 1H, q, (6.2)	79.2	3.83, 1H, q, (6.2)
12	37.5	1.93, 1H, dd, (14.9, 5.7) 2.57, 1H, dd, (14.9, 9.4)	35.0	1.71, 1H, dd, (14.4, 7.1) 2.44, 1H, dd, (14.4, 9.1)
13	69.3	4.50, 1H, dd, (9.3, 5.6)	66.0	4.15, 1H, dd, (8.9, 7.1)
14	43.9	3.41, 3H, s	34.3	2.82, 3H, s
15	173.8	–	171.5	–
16	27.3	2.76, 3H, s	26.5	2.65, 3H, d, (4.8)
17	20.4	1.03, 3H, s	19.6	0.97, 3H, s
18	17.3	0.98, 3H, s	16.3	0.87, 3H, s
19	14.7	1.22, 3H, d, (6.3)	14.1	1.14, 3H, d, (6.3)
Others				C-6 –OH, 4.60, s C-7 –OH, 5.57, d, (5.6) –NH, 8.32, q, (5.0)

able for X-ray diffraction were obtained by slow evaporation of a solution of **8** in MeOH/CH_2Cl_2/hexane (1:5:4). The ^1H and ^{13}C NMR spectral data of compound **9** suggested a chemical structure similar to compound **8**, which was consistent with the molecular formula of $C_{19}H_{27}N_3O_7$ (HRMS-EI). That is, compound **9** had a carbonyl instead of a thiocarbonyl group (Table 1). These compounds were named leptomaculin A (**8**) and leptomaculin B (**9**) by analogy to the name of the producing fungal species. 2,3-Oxopiperazinethiones and 2,3-dioxopiperazines do not appear to have been reported from natural sources.



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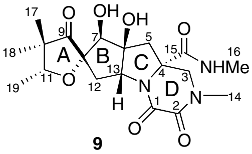

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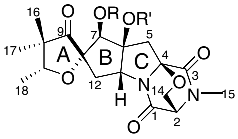
The molecular formula of compound **10** was established as $C_{20}H_{26}N_2O_8$ by HRMS-EI, that is, absence of two sulfur atoms relative to sirodesmin PL (**1**, $C_{20}H_{26}N_2O_8S_2$). The ^1H NMR spectral data of compound **10** showed most of the characteristic signals of sirodesmin **1** except for absence of an exchangeable proton due to the OH group at C-14 and the presence of an additional methine (δ_H 4.00, br s, H-2) coupled to a doublet of doublets at δ_H 4.07 (dd, H_a-14). Hence, two singlets at δ_H 1.06 (3H) and 1.01 (3H), a quartet at δ_H 3.88 (1H) and a doublet at δ_H 1.25 (3H) accounted for ring A, methylene protons at δ_H 2.54 (dd) and 1.59 (dd) together with a methine proton at δ_H 4.35 (dd) accounted for H_2-12 and H-13 and a singlet at δ_H 4.95 for H-7 (Table 2). Furthermore, two well-separated doublets (δ_H 3.50 and 2.54, H_2-5) indicated that one of the methylene protons at C-5 was substantially shielded, unlike sirodesmin **1** (3.27, ABq, H_2-5). The ^{13}C NMR spectral data of **10** was consistent with the conclusions drawn from the analysis of the ^1H NMR data, which were also supported by correlations observed in the HMBC spectrum. Acetylation of **10** (Ac_2O/py) provided only one product (**12**), confirming that the primary hydroxyl group at C-14 was not present. Altogether the spectroscopic data suggested an ether bridge formed between C-14 and C-4 replacing the disulfur bridge of **1**. The corresponding deacetyl derivative **11** was isolated from a more polar fraction, and confirmed the structural assignment of **10**. Assuming that the absolute configurations of compounds **10** and **11** at C-6, C-7, C-8, C-11 and C-13 are identical to those of sirodesmin PL (**1**), the stereochemistry at C-2 and C-4 was determined to be *cis* relative to H-13. This stereochemical assignment was based on an NOE effect observed between H-13 and H_2-14 of **12** (and vice versa). These compounds were named leptomaculin C (**10**) and deacetylleptomaculin C (**11**) by analogy to the name of the producing fungal species. No similar structures appear to have been described to date.



10 R=Ac; R'=H
11 R=R'=H
12 R=R'=Ac

The ^1H NMR spectral data of compound **13** showed most of the characteristic signals of sirodesmin **1**: two singlets at δ_H 1.11 (3H) and 1.03 (3H), a quartet at δ_H 3.95 (1H) and a doublet at δ_H 1.25 (3H) accounting for ring A, methylene protons at δ_H 2.92 (dd) and 1.63 (dd) together with a methine proton at δ_H 4.48 (dd) accounting for H_2-12 and H-13 of ring B. Furthermore, a methine proton at δ_H 4.03 (dd) coupled to two methylene protons at δ_H 4.00 (dd) and 4.08 (dd) indicated a difference at C-2 of ring D. Altogether the spectroscopic data suggested that the structural difference between the new compound and sirodesmin **1** was due to a double bond between C-4 and C-5, accounting for a singlet at δ_H 6.39 and two sp^2 carbons at 134.3 and 119.4 ppm. These assignments were confirmed using HMQC and HMBC spectral data. In addition, the HRMS-EI data indicated a molecular formula $C_{20}H_{26}N_2O_8$ and thus absence of sulfur. The C-2 epimer of compound **14** was also isolated from the same fraction, as revealed by analysis of spectroscopic data (Tables 3 and 4). To confirm the structure of new compounds **13** and **14**, sirodesmin **1** was converted to acetylated derivatives **19a** and **19b**, taking advantage of

Table 2

^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) chemical shifts (ppm) and multiplicities (*J* in Hz) of leptomaculin C (**10**) and ^1H NMR ($CDCl_3$, 500 MHz) of deacetylleptomaculin C (**11**) and acetylleptomaculin C (**12**)

C/H #	Leptomaculin C (**10**)		Deacetylleptomaculin C (**11**)	Acetylleptomaculin C (**12**)
	δ_C	δ_H	δ_H	δ_H
1	167.2	–	–	–
2	61.8	4.00, 1H, bs	4.00, 1H, d, (2.3)	4.01, 1H, bs
3	166.6	–	–	–
4	95.3	–	–	–
5	42.9	3.50, 1H, d, (16.2) 2.54, 1H, d, (16.3)	3.46, 1H, d, (15.4) 2.39, 1H, d, (15.4)	3.92, 1H, d, (16.7) 2.68, 1H, d, (16.7)
6	84.6	–	–	–
7	80.4	4.95, 1H, s	3.99, 1H, bs	5.64, 1H, s
8	89.4	–	–	–
9	221.2	–	–	–
10	47.6	–	–	–
11	80.3	3.88, 1H, q, (6.3)	3.85, 1H, q, (6.4)	3.70, 1H, q, (6.3)
12	35.9	2.54, 1H, dd, (13.9, 8.0) 1.59, 1H, dd, (14.0, 8.2)	2.45, 1H, dd, (14.5, 8.3) 1.85, 1H, dd, (14.9, 3.8)	2.32, 1H, dd, (14.8, 8.5) 1.88, 1H, bd, (14.6)
13	67.0	4.35, 1H, dd, (8.1, 8.1)	4.29, 1H, dd, (8.3, 3.7)	4.72, 1H, bd, (8.3)
14	66.1	4.07, 1H, dd, (9.1, 2.4) 4.01, 1H, bd, (8.4)	4.05, 1H, dd, (8.9, 2.2) 4.00, 1H, bd, (9.1)	4.04, 1H, bd, (9.7) 3.96, 1H, bd, (8.8)
15	31.7	3.10, 3H, s	3.10, 3H, s	3.09, 3H, s
16	20.5	1.06, 3H, s	1.04, 3H, s	1.02, 3H, s
17	17.6	1.01, 3H, s	1.01, 3H, s	0.93, 3H, s
18	14.4	1.25, 3H, d, (6.3)	1.24, 3H, d, (6.4)	1.24, 3H, d, (6.3)
19	169.6	–	–	–
20	20.7	2.10, 3H, s	–	2.09, 3H, s
22		–	–	2.04, 3H, s

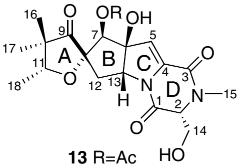
Table 3
^{1}H NMR ($CDCl_3$, 500 MHz) chemical shifts (ppm) and multiplicities (J in Hz) of leptomaculins D (**13**), E (**14**) and deacetylleptomaculins D (**15**) and E (**16**)

H #	Leptomaculin D (**13**) δ_H	Leptomaculin E (**14**) δ_H	Deacetylleptomaculin D (**15**) δ_H	Deacetylleptomaculin E (**16**) δ_H
2	4.03, 1H, dd, (3.1, 2.0)	4.01, 1H, dd, (3.6, 1.7)	4.02, 1H, s	4.00, 1H, s
5	6.39, 1H, s	6.39, 1H, s	6.08, 1H, s	6.15, 1H, s
7	4.86, 1H, s	4.85, 1H, s	3.94, 1H, s	3.93, 1H, s
11	3.95, 1H, q, (6.4)	3.94, 1H, q, (6.3)	3.88, 1H, q, (6.3)	3.95, 1H, q, (6.3)
12	1.63, 1H, dd, (14.0, 9.0) 2.92, 1H, dd, (14.0, 8.5)	1.55, 1H, dd, (13.9, 8.9) 2.85, 1H, dd, (13.9, 8.7)	1.60, 1H, dd, (14.3, 7.1) 2.72, 1H, dd, (14.3, 8.5)	1.72, 1H, dd, (14.1, 7.3) 2.80, 1H, dd, (14.1, 8.7)
13	4.48, 1H, dd, (8.8, 8.7)	4.50, 1H, dd, (8.7, 8.7)	4.55, 1H, dd, (8.3, 7.3)	4.48, 1H, dd, (7.9, 7.9)
14	4.00, 1H, dd, (11.2, 3.4) 4.08, 1H, dd, (11.2, 2.0)	3.99, 1H, dd, (11.2, 3.6) 4.09, 1H, dd, (11.2, 3.6)	4.05, 1H, dd, (11.9, 2.8) 4.07, 1H, dd, (11.8, 3.1)	4.01, 1H, dd, (11.8, 12.1) 4.05, 1H, dd, (11.8, 11.6)
15	3.07, 3H, s	3.08, 3H, s	3.09, 3H, s	3.08, 3H, s
16	1.11, 3H, s	1.11, 3H, s	1.04, 3H, s	1.09, 3H, s
17	1.03, 3H, s	1.03, 3H, s	1.00, 3H, s	1.01, 3H, s
18	1.25, 3H, d, (6.4)	1.25, 3H, d, (6.3)	1.22, 3H, d, (6.3)	1.24, 3H, d, (6.3)
20	2.10, 3H, s	2.09, 3H, s	—	—

the sulfur bridge lability under reductive conditions. First, the sulfur bridge of sirodesmin **1** was reduced using triphenylphosphine followed by acetylation to yield thiomethyl derivative **17**,[15] which upon treatment with Raney nickel yielded either **20** or **21** but not the desired derivative (Scheme 1). However, methanolysis of **17** followed by reduction with Raney nickel afforded a mixture of the desired compounds **18a** and **18b**. Chromatographic separation followed by acetylation established that these compounds were identical in all respects to the acetylated natural compounds **13** and **14**. The deacetyl derivatives of **13** and **14**, compounds **15** and **16**, were obtained from more polar fractions and their structures established similarly. Compounds **13** and **14** are likely to result from metabolism of sirodesmin **1** by fungal enzymes, whereas **15** and **16** are likely to result from metabolism of deacetylsirodesmin **2**. Thus, the absolute configurations of compounds **13**–**16** at C-6, C-7, C-8, C-11 and C-13 were expected to be identical to those of sirodesmins **1** and **2**, and were confirmed using X-ray diffraction data and NOESY correlations, as described below. These compounds were named leptomaculin D (**13**), leptomaculin E (**14**), deacetylleptomaculin D (**15**) and deacetylleptomaculin E (**16**) by analogy to the name of the producing fungal species. No similar structures appear to have been described to date.

Table 4
^{13}C NMR ($CDCl_3$, 125 MHz) chemical shifts (ppm) of leptomaculins D (**13**) and E (**14**) and deacetylleptomaculins D (**15**) and E (**16**)

C #	Leptomaculin D (**13**) δ_C	Leptomaculin E (**14**) δ_C	Deacetylleptomaculin D (**15**) δ_C	Deacetylleptomaculin E (**16**) δ_C
1	161.6	162.2	162.3	161.5
2	65.9	66.1	66.4	66.4
3	157.1	157.3	157.9	157.7
4	134.3	134.2	134.4	134.2
5	119.4	119.4	118.2	118.6
6	83.8	83.8	85.1	85.1
7	79.0	78.9	79.2	79.1
8	88.5	88.3	89.9	90.1
9	222.4	222.3	222.4	222.6
10	47.7	47.7	47.9	47.9
11	80.6	80.6	80.4	80.8
12	34.6	35.1	35.6	34.9
13	67.0	66.4	66.8	66.9
14	62.0	61.9	61.6	61.8
15	32.3	32.4	32.5	32.4
16	20.7	20.7	20.5	20.4
17	17.6	17.7	17.2	17.2
18	14.6	14.6	14.7	14.6
19	169.7	169.6	—	—
20	20.7	20.7	—	—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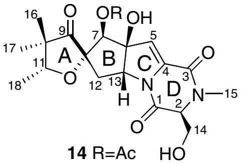
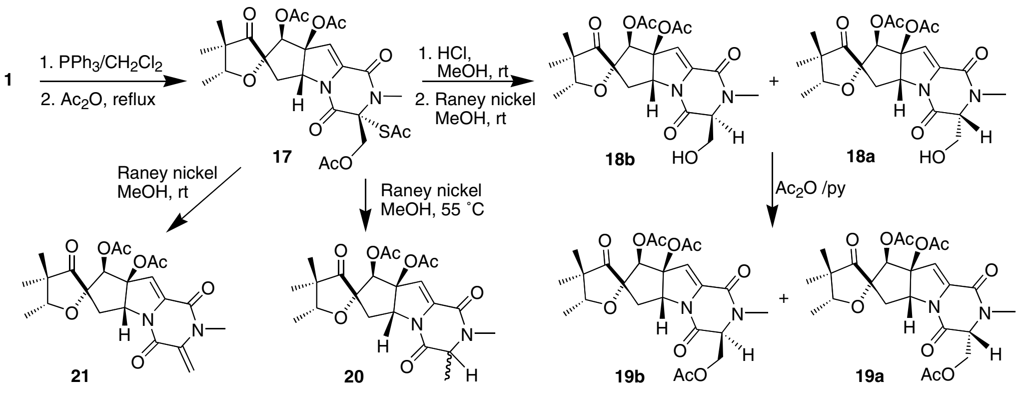
15 R=H


14 R=Ac
16 R=H

X-ray diffraction analysis of a single crystal of **17**, readily crystallized from CH_2Cl_2/hexane (1:9), confirmed the absolute configurations of compounds **13**–**16** at C-6, C-7, C-8, C-11 and C-13 (compounds **13**–**16** were refractory to crystallization). To establish



Scheme 1. Chemical conversion of sirodesmin PL (**1**) to diacetylleptomaculin D (**19a**) and diacetylleptomaculin E (**19b**).

the C-2 configurations of compounds **13**–**16**, NOESY spectral data were not useful due to signal overlap to signals of H-14 (δ_H 4.09) and H-2 (δ_H 4.01). However, because the acetylated derivatives of sirodesmin **1** showed well-separated ^{1}H NMR signals for H-14, H-13 and H-2,[10] the 6-monoacetyl derivatives **18b** and **18a** and diacetyl derivatives **19b** and **19a** of new compounds **13**–**16** were prepared. Both acetylated pairs showed the desired ^{1}H NMR spectra in which H-14, H-13 and H-2 signals were well separated. For example, **18b** (prepared from **14** and **16,** Scheme 1) showed a NOESY correlation between H-13 and H-14a (Fig. 1) whereas **18a** (prepared from **13** and **15**, Scheme 1) did not show a correlation between H-13 and H-14 (Fig. 1). A similar NOESY correlation between H-13 and H-14a was obtained for compound **17** whose configuration at C-2 was confirmed by X-ray crystallography. Since H-13 of compounds **13** to **16** is on the upper face of B/C rings, it is deduced that CH_2-14 is also on the upper face of D ring in acetylated compounds **18b** and **19b**, consequently new compounds **14** and **16** have *S* configuration at C-2 (similar to sirodesmin **1**). Likewise, following the same line of reasoning compounds **13** and **15** have *R* configuration at C-2.

Although a few trioxopiperazines and dioxopiperazinethiones have been isolated from fungi together with epipolythio-2,5-dioxopiperazines, including gliocladins,[16] silvathione[17] and variecolorins,[18] to the best of our knowledge no natural products containing a 2,3-oxopiperazinethione or a 2,3-dioxopiperazine have been reported to date. That is, the structures of leptomaculins A (**8**) and B (**9**) are the first naturally occurring examples of these unique chromophores. In this context, it is worthy to note that two important antibiotics (cefoperazone and piperacillin) that inhibit cell wall biosynthesis of bacteria contain a β-lactam attached to a 2,3-dioxopiperazine moiety.[19]

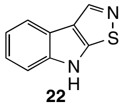
2.3. Elicitor activity of metabolites

Initially, to determine the scope of the bioassay, the potential elicitor activity of sirodesmin PL (**1**, non-selective toxin), phomamide (**6**, no toxicity) and phomalide (**7**, host-selective toxin) were determined. Of these three metabolites, only sirodesmin PL (**1**, 1.0 or 0.5 mM) showed elicitor activity, inducing the phytoalexins brassilexin (**22**), cyclobrassinin (**23**), rutalexin (**24**) and spirobrassinin (**25**) in brown mustard and spirobrassinin (**25**) in canola. Since there was selective and consistent phytoalexin elicitation (i.e., only sirodesmin elicited a response), the assay was used to search for elicitors produced by *L. maculans.* From elicitor-active fractions, sirodesmin PL (**1**), deacetylsirodesmin PL (**2**), sirodesmin H (**3**) and a mixture of sirodesmins J (**4**) and K (**5**) as well as new compounds **8**–**11** and **13**–**16** were isolated. Deacetylsirodesmin PL (**2**, 1.0 mM) elicited the production of spirobrassinin (**25**) in both mustard and canola. As well, the amounts of the plant stress metabolites indolyl-3-acetonitrile and 1-methoxyindolyl-3-acetonitrile increased up to eightfold after application and incubation of tissues with **1** and **2**. Sirodesmins **1** and **2** (ca. 0.5 mM), sirodesmin H (**3**, 0.5 mM) and a mixture of sirodesmins J (**4**, ca. 0.5 mM) and K (**5**, ca. 0.5 mM) caused necrosis on leaves of both mustard and canola; no elicitor activity was detected for sirodesmins **3**–**5**. Furthermore, the host-selective toxin phomalide (**7**) did not elicit the production of phytoalexins neither did phomamide (**6**).

Although sirodesmins **1** and **2** are well-characterized non-selective phytotoxins produced by *L. maculans*, their ability to elicit consistently the production of phytoalexins in blackleg susceptible and resistant crucifers does not appear to have been reported previously. Therefore, sirodesmins **1** and **2** are both phytotoxins and elicitors produced by *L. maculans*.

3. Conclusion

The bioassay guided isolation of elicitors produced by *L. maculans* in a chemically defined medium lead to the isolation of various known epipolythiodioxopiperazines, the sirodesmins, but not phomalirazine.[20] The large range of polarities of these sirodesmins together with the interconversion of sirodesmins J and K[10] rendered the purification of the new minor metabolites rather difficult and time consuming. Nonetheless, eight new metabolites, leptomaculins A–E (**8**–**11** and **13**–**14**), structurally related to sirodesmins were isolated from bioactive fractions; however, there was no elicitor activity detected nor phytotoxicity observed when these purified metabolites were tested on *B. juncea* cv. Cutlass (resistant) and *B. napus* cv. Westar (susceptible). Thus, the bioactivities of those fractions were most likely due to the presence of the polar sirodesmin **2**. Alternatively, a mixture of any of the leptomaculins could be required to elicit a plant defense response; however, this hypothesis could not be tested due to the small amounts isolated. Both sirodesmin PL (**1**) and deacetylsirodesmin PL (**2**) were shown to have substantial elicitor activity.



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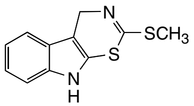

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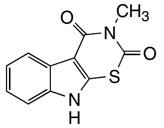

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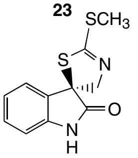

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Scheme 2 depicts a proposed pathway for metabolic conversion of sirodesmins **1** and **2** to leptomaculins A–E (**8**–**11** and **13**–**14**). The structures of leptomaculins A (**8**) and B (**9**) are unique and suggest interesting metabolic steps. By contrast, the formation of leptomaculins **10**, **11** and **13**–**16** from sirodesmin/deacetylsirodesmin PL appears straightforward. Specially considering that availability of sulfur after seven days of culture growth is likely to be low, the formation of **10**, **11** and **13**–**16** can provide a metabolic recovery of sulfur. However, the formation of leptomaculins **8** and **9** is intriguing, specially because these are the first naturally occurring

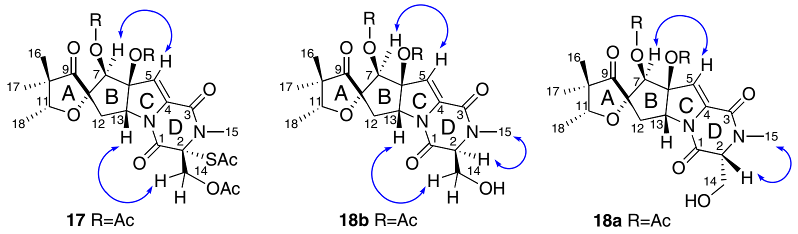
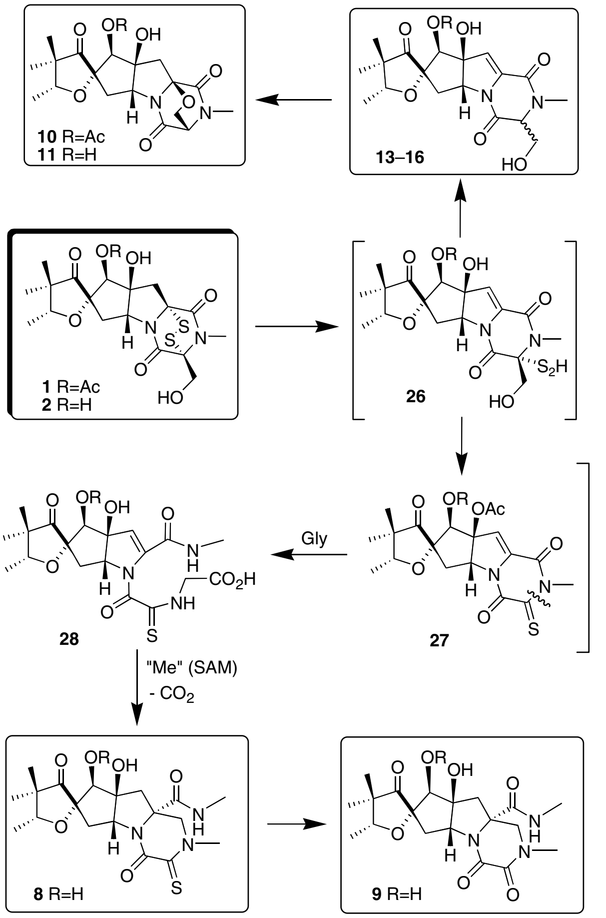


Figure 1. Partial NOESY correlations (double arrows) of compounds **17**, **18b** and **18a**.



Scheme 2. Proposed metabolic pathway for conversion of sirodesmins **1** and **2** to leptomaculins **8–11** and **13–16**; structures in brackets **26–28** are proposed intermediates.

examples of a 2,3-oxopiperazinethione/dioxopiperazine. It is suggested that intermediate **27** reacts with, for example, glycine to yield **28**, which upon decarboxylative cyclization and methylation can yield **8**. Nonetheless, at this stage it is not known whether sirodesmin PL (**1**) is indeed a precursor of **8** and **9**, which could be biosynthesized by an alternate route independent of sirodesmins. This proposition appears to be difficult to demonstrate since **8** and **9** are produced in rather small amounts. In any case, it is apparent that the biosynthetic pathway of **8** and **9** is rather complex, suggesting that these metabolites are likely to have a role in the pathogen's life cycle, albeit perhaps not plant stress inducing metabolites.

4. Experimental

4.1. Chemicals and instrumentation

All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON; solvents were of HPLC grade and used as such. Organic extracts were dried with Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230–400 Å. Preparative thin layer chromatography (prep. TLC) was carried out on silica gel plates, Kieselgel 60 F_{254} (20×20 cm $\times$ 0.25 mm), compounds were visualized under UV light. Specific rotations $[\alpha]_D$ were determined at ambient temperature on a polarimeter using a 1 mL, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations are reported in g/100 mL.

Nuclear magnetic resonance (NMR) spectra (^{1}H, ^{13}C, HMQC—heteronuclear multiple quantum coherence, HMBC—heteronuclear multiple bond coherence) were recorded on Bruker Avance 500 spectrometers. High resolution (HR) electron impact (EI) mass spectra (MS) were obtained on a VG 70 SE mass spectrometer, employing a solids probe.

HPLC analysis was carried out with Agilent high performance liquid chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), having an in-line filter. Mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and at a flow rate 1.0 mL/min.

X-ray diffraction data were collected at −100 °C on a Nonius Kappa CCD diffractometer, using the COLLECT program. Crystallographic data (excluding structure factors) for the structures **8** and **17** in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publications nos. CCDC 684017 and CCDC 684207, respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.2. Fungal cultures and elicitor activity assays

Minimal medium (MM) was prepared as previously reported.[12] Canadian virulent isolate of *L. maculans* IBCN 57 (or BJ 125) was grown in Erlenmeyer flasks (250 mL) containing MM (100 mL) inoculated with fungal spores (10^8) and incubated on a shaker at 120 rpm, at 23 ± 0.5 °C for 7 days.

Plants (*B. juncea* cv. Cutlass and *B. napus* cv. Westar) were grown in a growth chamber with 16 h light (fluorescent and incandescent, 450–530 μmol s^{-1} m^{-2}) and 8 h dark periods, at 24 ± 2° C. After the third leaf was half developed (about two weeks), mycelia extracts or fractions of extracts dissolved in MeOH/H_2O (1:1, v/v) solution were applied to leaves either by spraying or by pipetting droplets (10 μL) to puncture leaves. After two days, leaves (3 leaves per assay) excised at the base of the petiole were frozen in liquid N_2, crushed with a glass rod and extracted with EtOAc. EtOAc extracts were dried over Na_2SO_4 and concentrated in a rotary evaporator. The residue was dissolved in MeOH/CH_2Cl_2 (1:99), applied to a mini silica gel column (Pasteur pipette) and eluted with MeOH/CH_2Cl_2 (1:99, 5 mL), the solution was concentrated, the residue was dissolved in CH_3CN (80 μL) and analyzed by HPLC (20 μL injection volume) under the conditions reported above. Control leaves were treated similarly but employing a MeOH/H_2O (1:1) solution. Elicitor activity assays were carried out in triplicate, at least twice.

4.3. Fractionation and purification of metabolites

The broth and mycelium were separated by vacuum filtration, the broth (20 L) was concentrated to 5 L by freeze-drying and extracted with EtOAc. The EtOAc extract (7.2 g) was subjected to FCC on silica gel and eluted with a gradient of CH_2Cl_2–MeOH to yield 36 fractions (each 50 mL). HPLC-DAD and NMR analysis of each fraction indicated that fractions 14, 15, 23 and 26 contained new metabolites (Fig. 2).

The EtOAc extract of the broth was subjected to silica gel FCC (CH_2Cl_2/MeOH, 98:2, 600 mL, 12 fractions, CH_2Cl_2/MeOH, 94:6, 900 mL 18 fractions; CH_2Cl_2/MeOH, 80:20, 300 mL, 6 fractions). Fractions were concentrated to dryness, assayed for elicitor activity and analyzed by HPLC. Fractions 10–30 contained sirodesmins in various amounts. Active fractions were further separated as summarized in Figure 2. Fraction F_{14} was subjected to reversed phase chromatography, CH_3CN/H_2O, (25:75), then prep. TLC (toluene/EtOAc/HOAc, 50:40:10, multiple developments) of fraction 2 to yield **8**

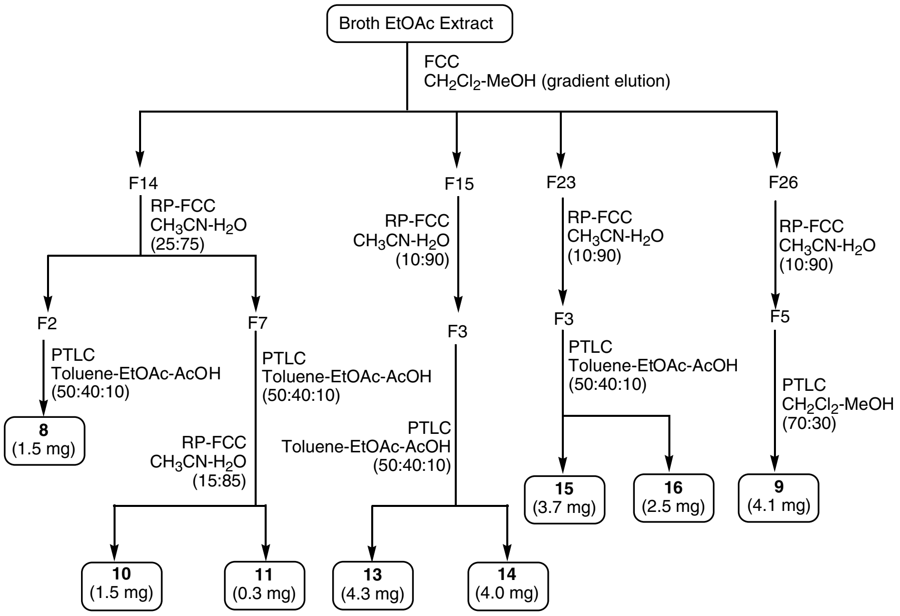


Figure 2. Flow chart of separation of leptomaculins **8**–**11** and **13**–**16** from the broth extract of cultures of *Leptosphaeria maculans*.

(1.5 mg) and prep. TLC of fraction 7 followed by reverse phase column to yield **10** (1.5 mg) and **11** (0.3 mg). Fraction F_{15} was subjected to reversed phase chromatography, CH_3CN/H_2O, 10:90 to 30:70, then prep. TLC (toluene/EtOAc/HOAc, 50:40:10, multiple developments) to yield **13** (4.3 mg) and **14** (4 mg). Fraction F_{23} was subjected to reversed phase chromatography, CH_3CN/H_2O, 10:90 to 30:70, then prep. TLC (toluene/EtOAc/HOAc, 50:40:10, multiple developments) to yield **15** (3.7 mg) and **16** (2.5 mg). Fraction F_{26} from FCC of EtOAc extracts was further subjected to reversed phase chromatography, CH_3CN/H_2O, 10:90 to 30:70, then prep. Si gel TLC (CH_2Cl_2/MeOH, 70:30) to yield **9** (4.1 mg).

4.4. Characterization and synthesis of metabolites and derivatives

4.4.1. Leptomaculin A (8)

HPLC: t_R = 4.7 min; mp 252–256 °C decomposed. HRMS-EI *m/z* 425.1614, $C_{19}H_{27}N_3O_6S$, calcd. 425.1621. MS-EI *m/z* 425 (61%), 367 (100%); FTIR (KBr): 3325, 3090, 2970, 2929, 2871, 1738, 1683, 1652 cm^{-1}; UV (MeOH): λ_{max} (logε) 202 (4.07), 274 (3.70), 312 (3.86) nm; $[\alpha]_D$ = +56 (c 0.10, MeOH).

4.4.2. Leptomaculin B (9)

HPLC: t_R = 3.3 min. HRMS-EI: *m/z* 409.1852, $C_{19}H_{27}N_3O_7$, calcd. 409.1849. MS-EI: *m/z* 409 (5%), 351(100%); FTIR (KBr): 3438, 3290, 2927, 2856, 1740, 1674 cm^{-1}; UV (MeOH): λ_{max} (logε) 224 (3.81) nm; $[\alpha]_D$ = −45 (c 0.10, MeOH).

4.4.3. Leptomaculin C (10)

HPLC: t_R = 7.4 min. HRMS-EI: *m/z* 423.1767, $C_{20}H_{27}N_2O_8$, calcd. 423.1767. MS-EI: *m/z* 423(10%), 422(8%), 365(100%), 323(57%). FTIR (KBr): 3379, 2974, 1751, 1710, 1395, 1236 cm^{-1}.

4.4.4. Deacetylleptomaculin C (11)

HPLC: t_R = 4.7 min. HRMS-ESI: *m/z* [M+1] 381.1660, $C_{18}H_{25}N_2O_7$, calcd. 381.1656. FTIR (KBr): 3368, 2927, 2841, 1743, 1674, 1017 cm^{-1}.

4.4.5. Acetylleptomaculin C (12)

HPLC: t_R = 12.4 min. Pyridine (100 μL) and acetic anhydride (100 μL) were added to compound **10** (1.0 mg) at room temperature and stirred overnight. The solvents were removed to yield compound **12** (1.1 mg). HRMS-EI: *m/z* 464.1799, $C_{22}H_{28}N_2O_9$, calcd. 464.1795. MS-EI: *m/z* 464 (22%), 407 (100%), 365 (54%); FTIR (KBr): 3401, 2959, 1755, 1712, 1232 cm^{-1}.

4.4.6. Leptomaculin D (13)

HPLC: t_R = 4.8 min. HRMS-EI: *m/z* 422.1678, $C_{20}H_{26}N_2O_8$, calcd. 422.1676. MS-EI: *m/z* 422 (4%), 380 (10%), 344 (18%), 223 (74%), 200 (86%), 140 (100%). FTIR (KBr): 3425, 2972, 2934, 2872, 1751, 1677, 1644, 1446, 1231, 1081, 738 cm^{-1}. UV (MeOH): λ_{max} (logε) 220 (3.92), 254 (3.87) nm; $[\alpha]_D$ = −47 (c 0.22, $CHCl_3$).

4.4.7. Leptomaculin E (14)

HPLC: t_R = 6.2 min. HRMS-EI: *m/z* 422.1675, $C_{20}H_{26}N_2O_8$, calcd. 422.1689. MS-EI: *m/z* 422 (21%), 223 (57%), 200 (67%), 140 (100%); FTIR (KBr): 3416, 2974, 2935, 1752, 1681, 1645 cm^{-1}. UV (MeOH): λ_{max} (log ε) 219 (4.01), 254 (3.97) nm; $[\alpha]_D$ = −66 (c 0.21, $CHCl_3$).

4.4.8. Deacetylleptomaculin D (15)

HPLC: t_R = 3.8 min. HRMS-EI: *m/z* 380.1591, $C_{18}H_{24}N_2O_7$, calcd. 380.1584. MS-EI: *m/z* 380(66%), 344(22%), 223(90%), 211(73%), 180(100%), 158(84%), 70 (46%); FTIR (KBr): 3375, 2927, 1750, 1675, 1640, 1464, 1405, 1085 cm^{-1}. UV (MeOH): λ_{max} (log ε) 221 (3.96), 255 (3.94) nm. $[\alpha]_D$ = −82 (c 0.14, $CHCl_3$).

4.4.9. Deacetylleptomaculin E (16)

HPLC: t_R = 3.1 min. HRMS-EI: *m/z* 380.1585, $C_{18}H_{24}N_2O_7$, calcd. 380.1584. MS-EI: *m/z* 380 (10%), 362 (12%), 223 (50%), 180 (43%), 158 (100%), 140 (43%), 70 (21%); FTIR (KBr): 3414, 2968, 2930, 1750, 1676, 1641, 1466, 1083 cm^{-1}; UV (MeOH): λ_{max} (log ε) 221 (4.04), 256 (3.99) nm; $[\alpha]_D$ = −93 (c 0.13, $CHCl_3$).

4.5. Preparation of derivatives of sirodesmin PL (1)

4.5.1. Compound 17

Acetic anhydride (5 mL) was added to sirodesmin H[9,15] (**3**, 589 mg, 1.30 mmol), and the reaction solution was heated at 110 °C, overnight. The reaction mixture was concentrated, and the residue was fractionated using FCC, EtOAc/CH_2Cl_2 (1:4), to give compound **17** (581 mg, yield 77%). HPLC: t_R = 20.1 min. ^1H NMR

(500 MHz, $CDCl_3$): δ6.49 (s, 1H), 5.40 (s, 1H), 5.03 (dd, *J* = 8.9, 4.9 Hz, 1H), 4.61 (d, *J* = 11.2 Hz, 1H), 4.37 (d, *J* = 11.2 Hz, 1H), 3.74 (q, *J* = 6.3 Hz, 1H), 3.07 (s, 3H), 2.54 (dd, *J* = 14.5, 8.9 Hz, 1H), 2.31 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H), 1.94 (dd, *J* = 14.5, 4.8 Hz, 1H), 1.21 (d, *J* = 6.3 Hz, 3H), 1.00 (s, 3H), 0.97 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 218.0, 191.8, 169.9, 169.3, 168.8, 159.3, 156.6, 135.6, 115.9, 90.8, 86.9, 79.9, 79.6, 77.0, 65.9, 63.4, 47.5, 34.8, 31.0, 30.1, 21.2, 20.7, 20.5, 20.1, 17.5, 14.2. HRMS-EI: *m/z* 580.1717, $C_{26}H_{32}N_2O_{11}S$, calcd. 580.1727. MS-EI: *m/z* 580 (6%), 505 (24%), 385 (49%), 287 (60%), 245 (100%), 205 (67%); FTIR (KBr): 2971, 2928, 1752, 1691, 1443, 1363, 1236, 1044 cm^{-1}; UV (MeOH): λ_{max} (log ε) 223 (4.09), 267 (4.15) nm; $[\alpha]_D$ = +43 (*c* 0.11, MeOH), $[\alpha]_D$ = +26 (c 0.20, $CHCl_3$).

4.5.2. Compounds 18a and 18b

Compound **17** (12.8 mg, 0.022 mmol) was dissolved in 1% HCl in MeOH (10 mL) at room temperature and kept overnight. After concentration, Raney nickel (1.5 mL) in MeOH (5 mL) was added to the residue, and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was filtered, and the solids were washed with MeOH (5 mL × 4), the combined methanolic phase was filtered through a Celite pad, and the filtrate was concentrated and applied to a RP-C_{18} FCC, CH_3CN/H_2O (20:80). Further chromatography of polar fraction (prep. TLC, MeOH/CH_2Cl_2, 5:95) yielded compound **18b** (0.9 mg, 9% yield). HPLC: t_R = 8.9 min. ^1H NMR (500 MHz, $CDCl_3$): δ 6.48 (s, 1H), 5.49 (d, *J* = 1.0 Hz, 1H), 4.92 (dd, *J* = 8.0, 1.0 Hz, 1H), 4.08 (d, *J* = 11.5 Hz, 1H), 4.02 (dd, *J* = 3.0, 1.5 Hz, 1H), 3.94 (dd, *J* = 11.5, 3.0 Hz, 1H), 3.77 (q, *J* = 6.0 Hz, 1H), 3.08 (s, 3H), 2.47 (dd, *J* = 15.0, 8.0 Hz, 1H), 2.35 (br s, 1H, OH), 2.24 (bd, *J* = 15.0 Hz, 1H), 2.05 (br s, 6H), 1.20 (d, *J* = 6.0 Hz, 3H), 0.98 (s, 3H), 0.97 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 216.9, 169.2, 168.8, 161.6, 156.7, 137.0, 114.6, 93.3, 87.5, 80.9, 79.8, 67.1, 66.2, 62.3, 47.5, 35.6, 32.3, 21.3, 20.8 (2C), 17.8, 15.3. HRMS-EI: *m/z* 464.1785, $C_{22}H_{28}N_2O_9$, calcd. 464.1795. MS-EI: *m/z* 464 (8%), 422 (38%), 404 (44%), 380 (54%), 362 (100%), 314 (52%), 223 (78%); FTIR (KBr): 3471, 2971, 2939, 1755, 1674, 1642, 1448, 1378, 1233, 1034 cm^{-1}. UV (MeOH): λ_{max} (log ε) 220 (4.05), 259 (4.06) nm; $[\alpha]_D$ = +77 (*c* 0.10, MeOH). Further chromatography of less polar fraction (prep. TLC (MeOH/CH_2Cl_2, 5:95) yielded compound **18a** (2.9 mg, 28% yield). HPLC: t_R = 9.5 min. ^1H NMR (500 MHz, $CDCl_3$): δ 6.29 (s, 1H), 5.18 (s, 1H), 5.00 (dd, *J* = 8.5, 6.5 Hz, 1H), 4.14 (d, *J* = 12.0 Hz, 1H), 4.03 (d, *J* = 3.5 Hz, 1H), 3.93 (dd, *J* = 12.0, 4.0 Hz, 1H), 3.80 (q, *J* = 6.0 Hz, 1H), 3.07 (s, 3H), 2.76 (br s, 1H, D_2O exchangeable), 2.62 (dd, *J* = 14.0, 8.5 Hz, 1H), 2.09 (s, 3H), 2.03 (s, 3H), 1.74 (dd, *J* = 14.0, 6.0 Hz, 1H), 1.22 (d, *J* = 6.0 Hz, 3H), 1.02 (s, 3H), 1.01 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.6, 169.9, 169.5, 162.9, 157.5, 137.4, 114.2, 90.5, 86.7, 80.4, 79.6, 66.4, 63.8, 61.9, 47.5, 36.1, 32.2, 21.5, 20.6 (2C), 17.4, 14.8. HRMS-EI: *m/z* 464.1807, $C_{22}H_{28}N_2O_9$, calcd. 464.1795. MS-EI: *m/z* 464 (5%), 422 (26%), 380 (29%), 362 (42%), 314 (100%), 223 (58%); FTIR (KBr): 3428, 2971, 2928, 1752, 1682, 1647, 1438, 1373, 1233, 1039 cm^{-1}; UV (MeOH): λ_{max} (log ε) 221 (3.91), 257 (3.94) nm; $[\alpha]_D$ = +4.6 (c 0.10, MeOH).

4.5.3. Compound 19b

Pyridine (100 μL) and acetic anhydride (200 μL) were added to compound **18b** (2.1 mg, 0.005 mmol) at room temperature. After standing overnight, the solution was concentrated to yield compound **19b** (2.3 mg, quantitative). HPLC: t_R = 14.6 min. ^1H NMR (500 MHz, $CDCl_3$): δ 6.48 (s, 1H), 5.46 (s, 1H), 4.97 (dd, *J* = 9.0, 3.5 Hz, 1H), 4.73 (dd, *J* = 11.5, 3.5 Hz, 1H), 4.33 (dd, *J* = 11.5, 3.0 Hz, 1H), 4.20 (dd, *J* = 3.5, 3.0 Hz, 1H), 3.79 (q, *J* = 6.4 Hz, 1H), 3.07 (s, 3H), 2.64 (dd, *J* = 14.9, 9.0 Hz, 1H), 2.06 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.92 (dd, *J* = 14.9, 3.4 Hz, 1H), 1.23 (d, *J* = 6.4 Hz, 3H), 1.01 (s, 3H), 1.00 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 170.1, 169.1 (2C), 160.1, 156.1, 136.1, 115.3, 92.2, 87.2, 80.8, 79.6, 66.2, 63.3, 62.7, 47.5, 36.7, 32.7, 21.5, 21.1, 20.7 (2C), 17.7, 15.6. HRMS-EI: *m/z* 506.1882, $C_{24}H_{30}N_2O_{10}$, calcd. 506.1900. MS-EI: *m/z* 506 (2%), 464 (10%), 422 (21%), 404 (100%), 344 (38%), 205 (45%); FTIR (KBr): 2970, 2928, 2874, 1750, 1688, 1647, 1375, 1236, 1044 cm^{-1}; UV (MeOH): λ_{max} (log ε) 220 (3.99), 260 (4.00) nm; $[\alpha]_D$ = +104 (*c* 0.10, MeOH); $[\alpha]_D$ = +136 (*c* 0.19, $CHCl_3$).

4.5.4. Compound 19a

Compound **19a** was obtained similarly to compound **19b** from acetylation of compound **18a**. HPLC: t_R = 14.2 min. ^1H NMR (500 MHz, $CDCl_3$): δ 6.44 (s, 1H), 5.44 (s, 1H), 4.95 (dd, *J* = 9.0, 2.5 Hz, 1H), 4.69 (dd, *J* = 11.5, 3.0 Hz, 1H), 4.37 (dd, *J* = 11.5, 2.0 Hz, 1H), 4.18 (dd, *J* = 3.0, 2.0 Hz, 1H), 3.75 (q, *J* = 6.5 Hz, 1H), 3.06 (s, 3H), 2.57 (dd, *J* = 14.5, 9.0 Hz, 1H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.92 (dd, *J* = 14.5, 2.5 Hz, 1H), 1.20 (d, *J* = 6.5 Hz, 3H), 1.00 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 170.2, 169.0, 168.9, 161.0, 156.6, 136.4, 115.0, 92.2, 87.1, 80.3, 79.6, 65.7, 63.7, 62.0, 47.5, 36.7, 32.4, 21.0, 20.8, 20.7, 20.6, 17.7, 15.0. HRMS-EI: *m/z* 506.1899, $C_{24}H_{30}N_2O_{10}$, calcd. 506.1900. MS-EI: *m/z* 506 (13%), 464 (70%), 422 (43%), 404 (100%), 344 (39%), 265 (47%); FTIR (KBr): 2960, 2925, 2854, 1746, 1690, 1649, 1377, 1242, 1048 cm^{-1}; UV (MeOH): λ_{max} (log ε) 221 (4.02), 257 (4.04) nm; $[\alpha]_D$ = +10 (c 0.a10, MeOH); $[\alpha]_D$ = +25 (c 0.15, $CHCl_3$).

4.5.5. Compound 20

Raney nickel (2 mL) in MeOH (5 mL) was added to compound **17** (34.2 mg, 0.059 mmol) in MeOH (5 mL) and heated at 55 °C for 6 h. After cooling to room temperature, the reaction mixture was filtered, and the solids were washed with MeOH (5 mL 4×), the combined methanolic phase was filtered through a Celite pad, the filtrate was concentrated and the residue was separated using prep. TLC (EtOAc/CH_2Cl_2, 2:3) to yield **20** (10.5 mg, 40% yield). HPLC: t_R = 13.7 min. ^1H NMR (500 MHz, $CDCl_3$): δ 6.46 (s, 1H), 5.52 (d, *J* = 1.0 Hz, 1H), 4.90 (d, *J* = 7.0 Hz, 1H), 4.02 (q, *J* = 7.0 Hz, 1H), 3.67 (q, *J* = 6.5 Hz, 1H), 3.05 (s, 3H), 2.40 (dd, *J* = 15.0, 8.0 Hz, 1H), 2.18 (d, *J* = 15.0, 1H), 2.05 (s, 6H), 1.53 (d, *J* = 7.0 Hz, 3H), 1.17 (d, *J* = 6.5 Hz, 3H), 0.97 (s, 3H), 0.95 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 169.2, 168.9, 154.0 (2C), 137.8, 136.2, 116.2, 103.7, 92.6, 87.2, 80.3, 79.6, 66.7, 47.5, 36.3, 30.0, 21.0, 20.7 (2C), 17.7, 15.0. HRMS-EI: *m/z* 448.1848, $C_{22}H_{28}N_2O_8$, calcd. 448.1846. MS-EI: *m/z* 448 (21%), 404 (64%), 344 (76%), 205 (100); FTIR (KBr): 2978, 1757, 1687, 1648, 1454, 1431, 1372, 1237 cm^{-1} UV (MeOH): λ_{max} (log ε) 258 (3.97), 220 (4.00) nm; $[\alpha]_D$ = +57 (c 0.11, MeOH).

4.5.6. Compound 21

Raney nickel (1 mL) in MeOH (5 mL) was added to compound **17** (28 mg, 0.048 mmol) in MeOH (5 mL) at room temperature and stirred for 1 h. The reaction mixture was filtered, and the solids were washed with MeOH (5 mL 4×), the combined methanolic layer was filtered through a Celite pad, the filtrate was concentrated and separated using prep. TLC (EtOAc/CH_2Cl_2, 2:3) to yield **21** (12 mg, 56% yield): HPLC: t_R = 17.5 min. ^1H NMR (500 MHz, $CDCl_3$): δ 6.54 (s, 1H), 5.88 (d, *J* = 1.5 Hz, 1H), 5.53 (d, *J* = 1.0 Hz, 1H), 5.02 (d, *J* = 1.5 Hz, 1H), 5.00 (dd, *J* = 9.0, 2.5 Hz, 1H), 3.72 (q, *J* = 6.5 Hz, 1H), 3.30 (s, 3H), 2.55 (dd, *J* = 15.0, 9.0 Hz, 1H), 2.08 (dd, *J* = 15.0, 2.5 Hz, 1H), 2.06 (s, 3H), 2.04 (s, 3H), 1.17 (d, *J* = 6.5 Hz, 3H), 0.99 (s, 3H), 0.96 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 217.3, 169.4, 168.9, 163.5, 155.7, 137.0, 114.5, 93.6, 87.8, 80.3, 80.0, 67.3, 60.3, 47.7, 35.3, 32.4, 21.1, 20.9, 20.5, 19.9, 17.9, 15.1. HRMS-EI: *m/z* 446.1694, $C_{22}H_{26}N_2O_8$, calcd. 446.1689. MS-EI: *m/z* 446 (21%), 404 (64%), 344 (76%), 205 (100); FTIR (KBr): 2970, 2928, 1755, 1689, 1609, 1432, 1233 cm^{-1} UV (MeOH): λ_{max} (log ε) 286 (4.27) nm; $[\alpha]_D$ = +48 (*c* 0.11, MeOH).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.07.060.

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