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Novel antifungal polyene amides from the myxobacterium *Cystobacter fuscus*: isolation, antifungal activity and absolute structure determination

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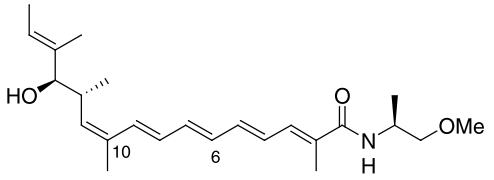
Abstract—Three new unstable metabolites, (6*E*,10*Z*)-2′-*O*-methylmyxalamide D (**1**), 2′-*O*-methylmyxalamide D (**2**) and (6*E*)-2′-*O*-methylmyxalamide D (**3**) were isolated from the myxobacterium *Cystobacter fuscus*. The planar structures were elucidated by spectroscopic analyses to be geometrical isomers of a polyene amide related to a myxobacterial metabolite, myxalamide D (**4**). Their absolute stereochemistry was determined by synthesis of degradation products. Antifungal activities of **1**–**3** as well as their acetates were evaluated against the phythopathogenic fungus *Phythopthora capsici*.

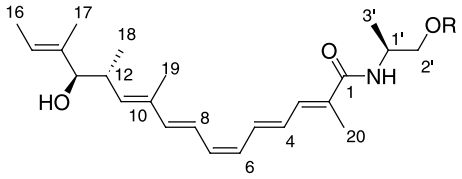
1. Introduction

Myxobacteria are unique gram-negative bacteria characterized by the gliding and fruiting body forming nature. They have recently been well established as a new and potent source for natural products with biological activities because of their potential to produce a considerable variety of metabolites.[1–3] Polyene antibiotics are a group of metabolites characteristic of the myxobacteria. Myxalamides, examples of such antibiotics from myxobacteria, have been discovered from *Myxococcus xanthus* Mx X12.[4,5]

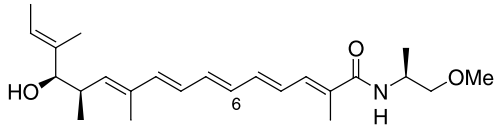
In the course of our screening for bioactive metabolites from myxobacteria, we previously discovered a series of antifungal metabolites named cystothiazoles from a myxobacterium species, *Cystobacter fuscus* AJ-13278.[6–8] On the other hand, a further search for additional antifungal agents from *C. fuscus* has resulted in the isolation of three new polyene amides that are structurally related to myxalamide D (**4**),[5] namely, (6*E*,10*Z*)-2′-*O*-methylmyxalamide D (**1**), 2′-*O*-methylmyxalamide D (**2**) and (6*E*)-2′-*O*-methylmyxalamide D (**3**) (Fig. 1). This paper reports the isolation,



(6*E*,10*Z*)-2′-*O*-methylmyxalamide D (**1**)



R = Me: 2′-*O*-methylmyxalamide D (**2**)
R = H: myxalamide D (**4**)



(6*E*)-2′-*O*-methylmyxalamide D (**3**)

Figure 1. Structures of myxalamide D (**4**) and its new derivatives **1**–**3**.

Keywords: Myxobacteria; *Cystobacter fuscus*; Antifungal; Polyene antibiotics.
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structure elucidation, antifungal activity and absolute stereochemistry for these new metabolites.

2. Results and discussion

Production of the antibiotics was performed as reported previously for the isolation of the cystothiazoles.[6,7] The bacterial cells and adsorbent resin obtained from a 150-L fermentation broth of *C. fuscus* were extracted with acetone and the extract was subjected to solvent partition. The non-polar fraction was chromatographed twice on silica gel to afford less polar fractions containing cystothiazoles[6–8] and a relatively polar fraction containing polyene metabolites. The latter fraction was further subjected to reversed-phase HPLC to give the myxalamide D derivatives **1** (0.6 mg), **2** (0.6 mg) and **3** (2.4 mg). The content of these metabolites should be potentially much higher (more than 10 times) than the isolated amounts, because the more they were purified, the more they decomposed due to their instability.

All three compounds **1–3** showed similar spectroscopic data to each other. The molecular formula of $C_{24}H_{37}NO_3$ for all three compounds were determined by HRMS measurement. The resonances at 168.3 ppm in the ^{13}C NMR spectra indicated the presence of an amide carbonyl carbon in all compounds, which was confirmed by the characteristic IR absorption band at 1653 cm^{-1}. Based on these findings, 2D NMR analysis was then performed to determine the planar structures of the compounds as discussed below.

Many olefinic signals observed between 5 and 7 ppm in the ^1H NMR spectrum of **1** (Table 1) are characteristic of polyenes like the myxalamides.[5,9] The proton–carbon direct connectivities in **1** were first determined by the HMQC spectrum. The chemical shifts are summarized in Tables 1 and 2. The partial structures of H3–H9, H11–H12(H18)–H13, H15–H16 and NH–H1′(H3′)–H2′ were revealed by the DQF-COSY data (Fig. 2). These were then successfully connected to each other by the HMBC spectrum to give the planar structure of **1** (Fig. 2). The singlet signal observed at δ 3.38 (3H) in the ^1H NMR spectrum was easily determined to be the 2′-*O*-methyl group from the HMBC correlation to C2′. The geometry of the double bonds at C4, C6, and C8 were determined from the proton coupling constants of $J_{4,5}=14.6$ Hz, $J_{6,7}=14.5$ Hz, $J_{8,9}=15.0$ Hz as 4*E*, 6*E*, 8*E*, respectively. The geometry of the trisubstituted olefins at C2, C10, and C14 was determined from the NOESY correlations of H4/H20, H9/H12, H11/H19, and H13/H15 as 2*E*, 10*Z*, 14*E*, respectively (Fig. 2). Although the molecular formula of **1** is the same as myxalamide C,[5] which is the C16-methylated homologue of myxalamide D (**4**), these were unambiguously distinguished by the NMR analysis, especially the HMBC correlation of OCH_3/C2′, Thus, the

Table 1. ^1H NMR spectral data for **1–3** in $CDCl_3$ (600 MHz)

Position	1	2	3
	δ_H (m, *J* in Hz)	δ_H (m, *J* in Hz)	δ_H (m, *J* in Hz)
3	6.96 (d, 11.0)	7.00 (d, 11.0)	6.95 (d, 11.3)
4	6.47 (dd, 14.6, 11.0)	6.48 (dd, 14.5, 11.0)	6.45 (dd, 14.6, 11.3)
5	6.54 (dd, 14.6, 10.6)	6.99 (dd, 14.5, 11.0)	6.53 (dd, 14.6, 10.0)
6	6.39 (dd, 14.5, 10.6)	6.10 (dd, 11.0, 11.0)	6.35 (dd, 15.0, 10.0)
7	6.48 (dd, 14.5, 10.8)	6.16 (dd, 11.3, 11.0)	6.40 (dd, 15.0, 10.0)
8	6.36 (dd, 15.0, 10.8)	6.69 (dd, 15.1, 11.3)	6.26 (dd, 15.0, 10.0)
9	6.73 (d, 15.0)	6.36 (d, 15.1)	6.36 (d, 15.0)
11	5.28 (d, 10.0)	5.46 (d, 9.8)	5.43 (d, 9.7)
12	2.83 (m)	2.75 (m)	2.73 (m)
13	3.64 (d, 8.9)	3.69 (d, 8.7)	3.68 (d, 8.8)
15	5.49 (q, 6.5)	5.49 (q, 6.3)	5.49 (q, 6.0)
16	1.64 (d, 6.5)	1.64 (d, 6.3)	1.63 (d, 6.0)
17	1.66 (s)	1.64 (s)	1.64 (s)
18	0.83 (d, 6.7)	0.84 (d, 6.7)	0.83 (d, 6.7)
19	1.91 (s)	1.91 (s)	1.86 (s)
20	1.98 (s)	1.99 (s)	1.97 (s)
1′	4.25 (m)	4.25 (m)	4.25 (m)
2′	3.43 (dd, 9.5, 4.0)	3.43 (dd, 9.5, 4.0)	3.42 (dd, 9.5, 4.0)
	3.39 (dd, 9.5, 4.0)	3.39 (dd, 9.5, 4.0)	3.39 (dd, 9.5, 4.0)
3′	1.23 (d, 6.8)	1.23 (d, 6.8)	1.22 (d, 6.2)
2′-OCH_3	3.38 (s)	3.38 (s)	3.37 (s)
NH	5.96 (d, 7.6)	5.98 (d, 7.6)	5.96 (d, 7.8)

Table 2. ^{13}C NMR chemical shifts for **1–3** in $CDCl_3$ (150 MHz)

Position	1[a]	2[a]	3
1	168.3	168.3	168.3
2	129.8[b]	130.5	129.6
3	133.6	133.5	133.7
4	127.7	128.0	127.3[b]
5	138.1	133.2	138.3
6	132.8	128.4	132.0
7	136.1	132.5	136.0
8	130.0[b]	122.3	127.2[b]
9	131.4	139.8	139.2
10	134.0	136.0	136.1
11	135.2	137.6	137.1
12	36.3	37.3	37.2
13	82.7	82.7	82.8
14	135.6	135.7	135.6
15	123.6	123.4	123.5
16	13.1	13.1	13.1
17	10.7	10.7	10.6
18	17.8	17.3	17.3
19	20.7	13.1	12.9
20	13.1	13.1	13.1
1′	45.0	45.0	45.0
2′	75.6	75.6	75.6
3′	17.8	17.8	17.8
2′-OCH_3	59.1	59.1	59.1

[a] Chemical shifts were partially determined by HMQC and HMBC data.
[b] Interchangeable signals.

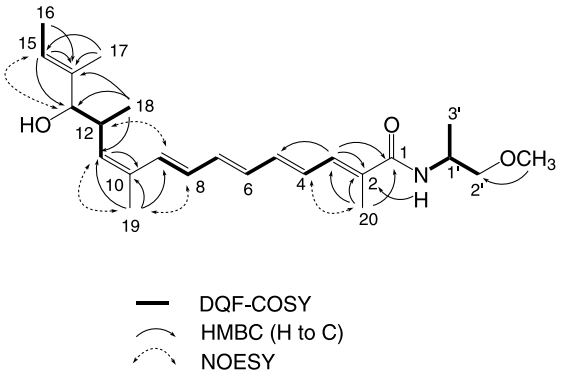


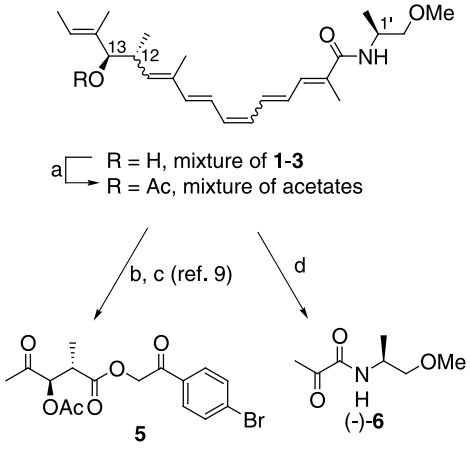
Figure 2. Gross structure of **1** determined by the 2D NMR correlations shown.

compound **1** was determined to be (6*E*,10*Z*)-2′-*O*-methylmyxalamide D.

The NMR data of **2** (Tables 1 and 2) were quite similar to those for **1**, suggesting that it was a stereoisomer of **1**. The planar structure of **2** was determined by 2D NMR in a similar manner to that for **1**. The 4*E*, 6*Z*, 8*E* geometry was determined from the coupling constants of $J_{4,5}=14.5$ Hz, $J_{6,7}=11.0$ Hz, $J_{8,9}=15.1$ Hz, respectively. The 2*E*, 10*E*, 14*E* geometry of the trisubstituted double bonds was determined from the NOESY correlations of H4/H20, H9/H11, H12/H19, and H13/H15. These findings revealed that the compound **2** was 2′-*O*-methylmyxalamide D.

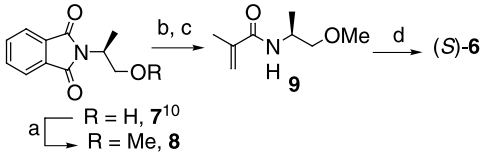
Similarly, the NMR data of **3** (Tables 1 and 2) suggested that this compound was a stereoisomer of **1** and **2**. The same planar structure as those of **1** and **2** was then determined by 2D NMR analysis. The 4*E*, 6*E*, 8*E* geometry were determined from the coupling constants as shown in Table 1, and the 2*E*, 10*E*, 14*E* geometry of the trisubstituted double bonds were determined from the NOESY correlations of H4/H20, H12/H19, and H13/H15. Thus, the compound **3** was determined to be (6*E*)-2′-*O*-methylmyxalamide D. The steric repulsion effect in the ^{13}C NMR data, that is, high-field shifts of the carbons adjacent to a *cis* olefin, also supported the geometry of these polyene metabolites **1**–**3**. Thus, high-field shifts compared to the all *E* compound **3** were observed for the following carbons of **1** and **2**: C9 ($\Delta\delta$ −7.8) in **1**, C5 ($\Delta\delta$ −5.1) and C8 ($\Delta\delta$ −4.9) in **2**.

To determine the absolute stereochemistry of the myxalamide D derivatives **1**–**3**, we followed the methodology described by Jansen et al.[9] with a slight modification. A mixture of **1**–**3** was used for degradation to obtain the fragments that contained asymmetric centers because **1**–**3** easily isomerized to each other under light and decomposed during purification. Acetylation of a mixture of **1**–**3** gave a mixture of the corresponding acetates, which was subjected to oxidative cleavage of the double bonds followed by esterification to yield *p*-bromophenacyl ester **5** (Scheme 1). The spectral data including specific rotation for **5** was identical to those reported,[9] revealing the 12*R*, 13*R* configuration of **1**–**3**. To determine the stereochemistry at C1′ a mixture of the acetates of **1**–**3** was treated with ozone followed by Me_2S to obtain keto amide (−)-**6** (Scheme 1). The configuration of (−)-**6** was determined by the synthesis of (*S*)-**6** from phthalimide **7**[10] in four steps as shown in Scheme 2. Methylation of the hydroxyl group in **7** afforded methyl ether **8**. Deprotection of the phthalimide group of **8** with hydrazine gave the corresponding free amine, which was treated with methacryloyl chloride and triethylamine to give acrylamide **9**. Finally, ozonolysis of **9** gave (*S*)-ketoamide **6**: $[\alpha]_D^{23}$ −13° (*c* 0.3, $CHCl_3$) [natural: −17° (*c* 0.03, $CHCl_3$)]. The NMR spectra and other spectral data including specific rotations of both synthetic and natural **6** were identical, indicating the 1′*S* configuration of **1**–**3**. The stereochemistry of **1**–**3** is therefore the same as that of the known myxalamides.



Reagents and conditions: (a) Ac_2O, pyridine, DMAP, rt; (b) O_3, MeOH, -78 °C, then H_2O_2, 100 °C; (c) *p*-Bromophenacyl bromide, Et_3N, acetone; (d) O_3, MeOH, -78 °C, then Me_2S, -78 °C to rt.

Scheme 1. Degradation routes to determine the absolute stereochemistry of **1**–**3**.



Reagents and conditions: (a) MeI, Ag_2O, CH_3CN, rt; (b) $H_2NNH_2.H_2O$, EtOH, 75 °C; (c) Methacryloyl chloride, Et_3N, -10 to 0 °C; (d) O_3, MeOH, -78 °C.

Scheme 2. Synthetic route to (*S*)-**6**.

The antifungal activities of the compounds **1**–**3** as well as their acetates was evaluated by a paper disc assay method against the phytopathogenic fungus *Phytophthora capsici*. The minimum dose to form a recognizable inhibition zone in the neighborhood of the paper disc was determined to be 2 μg/disc for all three compounds. The acetates of **1**–**3** as a mixture showed antifungal activity at a minimum dose of 5 μg/disc.

Discovery of the myxalamide D derivatives **1**–**3** from the myxobacterium *C. fuscus* suggests that polyene amides such as the myxalamides seem to be fairly common secondary metabolites in the myxobacteria. The myxalamides were also discovered from *Stigmatella aurantiaca*[11] and others[2] after the original discovery from *M. xanthus*,[4,5] although methyl ether-type derivatives such as **1**–**3** were the first examples in the myxalamides. *C. fuscus* seems to possess a metabolic system that is similar to that of the other myxalamide producers but is unique, because myxalamide-related metabolites other than **1**–**3** were not detected in photodiode array HPLC analysis of the extracts. Since all three isomers **1**–**3** showed the same antifungal activity against the fungus *P. capsici*, the olefin geometry plays no crucial role for their antifungal activity. The activity of the acetates (mixture) suggests that the free 13-OH group is not essential. In addition, the free 2′-hydroxyl group of the known myxalamides seems not to be very important, though a direct comparison of the activity was not performed

between the myxalamides and our compounds. Based on a structural similarity to the myxalamides the mode of action of our new myxalamide derivatives could be blocking the mitochondrial respiratory chain by inhibition of NADH oxidation at complex I.[4,12] It should be noted that more than one species of the myxobacteria can produce similar polyene amides with stereochemical homogeneity.

3. Experimental

3.1. General

Thin-layer chromatography (TLC) was performed by using pre-coated silica gel 60 F_{254} plates (Art. 5715, Merck) or RP-18 F_{254} plates (Art. 15389, Merck). Open column chromatography was performed using silica gel BW-300 (Fuji Silysia) or Cosmosil 75C_{18}-OPN (Nacalai Tesque). HPLC was performed on a high-pressure gradient system equipped with PU-980 pumps and UV-970 detector or an MD-915 photodiode array UV detector (JASCO). Specific rotations were obtained by using a DIP-370 digital polarimeter (JASCO). FT-IR spectra were recorded on a FT-IR-7000S spectrometer (JASCO). UV spectra were recorded on a Ubest-50 UV/VIS spectrophotometer (JASCO). Mass spectra (MS) were recorded on a Mariner Biospectrometry Workstation (Applied Biosystems) in the positive ESI mode. Residual phthalic acid anhydride (*m/z* 149.0233) and a peptide mixture (angiotensin I, bradykinin and neurotensin) were used as internal standards for high-resolution MS analysis. NMR spectra were recorded on an ARX 400 (400 MHz) or an AMX2 600 (600 MHz) spectrometer (Bruker). The NMR chemical shifts (ppm) were referenced to the solvent peaks of δ_H 7.26 (residual $CHCl_3$) and δ_C 77.0 for $CDCl_3$ solutions or δ_H 3.30 (residual CHD_2OD) and δ_C 49.0 for CD_3OD solutions. The assay method was the same as the previously reported one for the cystothiazoles.[6,7]

3.2. Isolation of 1–3

A large scale (150 L) fermentation of *C. fuscus* followed by extraction and silica gel chromatography was described previously.[6,7] The first chromatography of the crude hexane/EtOAc (3:1)–soluble material (14.2 g) on silica gel afforded five fractions. The third fraction yielded cystothiazole A as described in our reference (see Ref. 6). A portion (770 mg) of the fourth fraction (2.5 g) eluted with EtOAc was chromatographed on silica gel [Develosil LOP60 (∅ 20×300 mm), Nomura Chemical; 160 min linear gradient from 2 to 42% acetone in benzene, 5 mL/min]. The sixth fraction (42.8 mg) eluted from 8.25 to 8.5% acetone in benzene was applied to HPLC [TSK gel ODS-120T (∅ 20×250 mm), TOSOH, 72% aq MeOH, 5 mL/min, detected at 380 nm] to obtain (6*E*,10*Z*)-2′-*O*-methylmyxalamide D (**1**) (0.6 mg, t_R=101.0 min), 2′-*O*-methylmyxalamide D (**2**) (0.6 mg, t_R=105.4 min), and (6*E*)-2′-*O*-methylmyxalamide D (**3**) (2.4 mg, t_R=114.6 min) as pale yellow oils. The rest (1.73 g) of the fourth fraction eluted with EtOAc was used for obtaining a crude mixture of **1**–**3** (287 mg), which was used for degradation without further purification (see Section 3.3.1).

3.2.1. (6*E*,10*Z*)-2′-*O*-Methylmyxalamide D (1). Pale yellow oil; $[\alpha]_D^{21}$ −12° (*c* 0.03, MeOH); IR (film) ν_{max} 3370 (br), 1653, 1522, 1108, 1000 cm^{-1}; UV (MeOH) λ_{max} 260 (ε 6000), 340 (sh), 355 (30,000), 370 (sh) nm; MS (ESI^+) *m/z* 388 $[M+H]^+$, 410 $[M+Na]^+$. HRMS found 388.2794, calcd for $C_{24}H_{38}NO_3$ $[M+H]^+$ 388.2846. For NMR data refer to Tables 1 and 2.

3.2.2. 2′-*O*-Methylmyxalamide D (2). Pale yellow oil; $[\alpha]_D^{21}$ −27° (*c* 0.04, MeOH); IR (film) ν_{max} 3367 (br), 1653, 1522, 1107, 1000 cm^{-1}; UV (MeOH) λ_{max} 260 (ε 6000), 340 (sh), 355 (30,000), 370 (sh) nm; MS (ESI^+) *m/z* 388 $[M+H]^+$, 410 $[M+Na]^+$. HRMS found 388.2805, calcd for $C_{24}H_{38}NO_3$ $[M+H]^+$ 388.2846. For NMR data refer to Tables 1 and 2.

3.2.3. (6*E*)-2′-*O*-Methylmyxalamide D (3). Pale yellow oil; $[\alpha]_D^{21}$ −31° (*c* 0.017, MeOH); IR (film) ν_{max} 3365 (br), 1653, 1522, 1106, 998 cm^{-1}; UV (MeOH) λ_{max} 257 (ε 3000), 340 (sh), 356 (30,000), 370 (sh) nm; MS (ESI^+) *m/z* 388 $[M+H]^+$, 410 $[M+Na]^+$. HRMS found 388.2807, calcd for $C_{24}H_{38}NO_3$ $[M+H]^+$ 388.2846. For NMR data refer to Tables 1 and 2.

3.3. Degradation

3.3.1. 13-*O*-Acetyl derivatives of 1–3. A mixture of **1**–**3** (46.1 mg, 0.12 mmol) was treated with a mixture of pyridine (4 mL), DMAP (22 mg, 0.18 mmol) and Ac_2O (2 mL) at room temperature for 3 h. The mixture was concentrated and the residue was chromatographed on silica gel (hexane/EtOAc 2:1) to obtain a mixture of acetates as a pale yellow oil (20.7 mg, 40%): ^1H NMR (400 MHz, $CDCl_3$) δ 6.90–7.05 (1H, m, H-3), 6.05–6.68 (6H, m, H-4–H-9), 5.90–6.00 (1H, m, NH), 5.50–5.60 (1H, m, H-15), 5.37 (1H, m, H-11), 4.98 (1H, m, H-13), 4.25 (1H, m, H-1′), 3.35–3.45 (2H, m, H-2′), 3.33, 3.37 and 3.38 (total 3H, s each, 2′-OCH_3), 2.83–2.93 (1H, m, H-12), 1.97 and 1.98 (total 3H, s each, H-20), 1.94 and 1.95 (total 3H, s each, acetate), 1.86, 1.81 and 1.72 (total 3H, s each, H-19), 1.65 and 1.58 (total 6H, m, H-16, 17), 1.21, 1.22 and 1.23 (total 3H, d each, *J*=6.8 Hz, H-3′), 0.87 and 0.88 (total 3H, d each, *J*=6.8 Hz, H-18).

3.3.2. *N*-(2-Methoxy-1-methylethyl)-2-oxopropanamide (6). The above acetate mixture (20.7 mg, 0.048 mmol) was dissolved in MeOH (1.5 mL) and cooled to −78 °C. A stream of ozone (8%) in oxygen was passed through this solution for 1 h. The solution was flushed with oxygen, treated with Me_2S (0.5 mL) and then allowed to warm to room temperature with stirring for 3 h. Solvent evaporation gave an oily residue (4.9 mg), which was chromatographed on silica gel (hexane/acetone 10:1) to obtain pure **6** (0.5 mg, 7%) as a colorless oil: $[\alpha]_D^{23}$ −17° (*c* 0.03, $CHCl_3$). The NMR and other spectroscopic data were identical to synthetic (*S*)-**6** (refer to Section 3.4).

3.3.3. 3-Acetoxy-2-methyl-4-oxopentanoic acid *p*-bromophenacyl ester (5). The reaction was carried out in a manner similar to that described in Ref. 6. Briefly, a mixture of the above acetates (24.7 mg, 0.057 mmol) was subjected to ozonolysis followed by oxidative treatment with H_2O_2 and subsequent esterification of the resulting carboxylic

acid with *p*-bromophenacyl bromide (26 mg, 0.094 mmol) to give **5** (0.7 mg, 3%): $[\alpha]_D^{23}$ +10° (*c* 0.06, $CHCl_3$) (lit.:[9] $[\alpha]_D^{20}$ +11° (*c* 0.3, $CHCl_3$)). ^1H NMR spectrum was identical to that reported in the literature.[9]

3.4. Synthesis of ketoamide (*S*)-6

3.4.1. (*S*)-2-(2-Methoxy-1-methylethyl)-1*H*-isoindole-1,3(2*H*)-dione (8). To a solution of phthalimide **7**[10] (592 mg, 2.9 mmol) in CH_3CN (7.5 mL) was added Ag_2O (2 g, 8.7 mmol) and the mixture was heated at reflux temperature for 5 h in the dark. Ag_2O was filtered off by using a pad of celite and the filtrate was concentrated to afford **8** (602 mg, 95%) as chromatographically pure material: colorless oil, $[\alpha]_D^{23}$ +20° (*c* 0.83, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.81 (2H, m), 7.69 (2H, m), 4.61 (1H, m), 3.97 (1H, t, J=9.8 Hz), 3.53 (1H, dd, J=5.4, 9.8 Hz), 3.32 (3H, s), 1.44 (3H, d, J=7.1 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.5, 133.8, 132.1, 123.1, 72.9, 58.7, 46.3, 15.0; UV (MeOH) λ_{max} 219 (ε 31,000), 240 (7600, sh), 295 (1600) nm; IR (film) ν_{max} 2984, 2939, 2894, 1775, 1714, 1705, 1468, 1394, 1373, 1337, 1111, 1042, 878, 720, 532 cm^{-1}; HRMS *m*/*z* 220.0967 $(M+H)^+$, calcd for $C_{12}H_{14}NO_3$ 220.0968.

3.4.2. (*S*)-*N*-(2-Methoxy-1-methylethyl)-2-methyl-2-propenamide (9). Phthalimide **8** (96.4 mg, 0.44 mmol) was dissolved in EtOH (0.1 mL) in a sealed tube. To this solution was added hydrazine monohydrate (25 μL, 0.48 mmol), and the tube was sealed and heated at 75 °C for 5 h. After cooling to room temperature, Et_3N (0.61 mL, 4.4 mmol) was added and stirred for 5 min before cooling to −10 °C. Then methacryloyl chloride (0.43 mL, 4.4 mmol) was added dropwise and the mixture was stirred at 0 °C for 5 h. The reaction mixture was diluted with water and extracted with EtOAc three times. The combined organic layers were dried (anhyd Na_2SO_4) and concentrated to give a crude oil, which was chromatographed on silica gel (hexane/acetone 4:1) to give acrylamide **9** (21.6 mg, 31%): colorless oil, $[\alpha]_D^{23}$ −10° (*c* 0.23, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 6.02 (NH, br s), 5.65 (1H, s), 5.29 (1H, s), 4.19 (1H, m), 3.39 (1H, dd, J=4.2, 9.4 Hz), 3.36 (1H, dd, J=4.1, 9.4 Hz), 3.35 (3H, s), 1.94 (3H, s), 1.19 (3H, d, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.8, 140.2, 119.2, 75.4, 59.0, 44.8, 18.6, 17.6; UV (MeOH) λ_{max} 203 (ε 8700) and 296 (350, sh) nm; IR (film) ν_{max} 3312, 2981, 2930, 1721, 1678, 1659, 1624, 1530, 1455, 1154, 1111, 1038, 935 cm^{-1}; HRMS *m*/*z* 180.0991 $(M+Na)^+$, calcd for $C_8H_{15}NO_2Na$ 180.0995.

3.4.3. (*S*)-*N*-(2-Methoxy-1-methylethyl)-2-oxopropanamide [(*S*)-6]. A stream of ozone (8% in O_2) was passed through a solution of **9** (26.3 mg, 0.17 mmol) in MeOH (1.5 mL) at −78 °C for 1 h. After removal of ozone, the reaction mixture was concentrated to obtain (*S*)-**6** (6.6 mg, 25%): colorless oil, $[\alpha]_D^{23}$ −13° (*c* 0.3, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.06 (NH, br s), 4.10 (1H, m), 3.37 (2H, dd, J=1.0, 4.4 Hz), 3.35 (3H, s), 2.46 (3H, s), 1.21 (3H, d, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.2, 159.6, 75.1, 59.1, 45.1, 24.4, 17.2; UV (MeOH) λ_{max} 230 (ε 1440, sh) nm; IR (film) ν_{max} 3400, 1718, 1684, 1522, 1169, 1110, 669 cm^{-1}; HRMS *m*/*z* 182.0778 $(M+Na)^+$, calcd for $C_7H_{13}NO_3Na$ 182.0788. It must be noted that product (*S*)-**6** (both natural and synthetic) is a very volatile compound and most product was lost mainly during concentration between spectroscopic measurements.

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