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New Dimeric Compounds of Avenanthramide Phytoalexin in Oats

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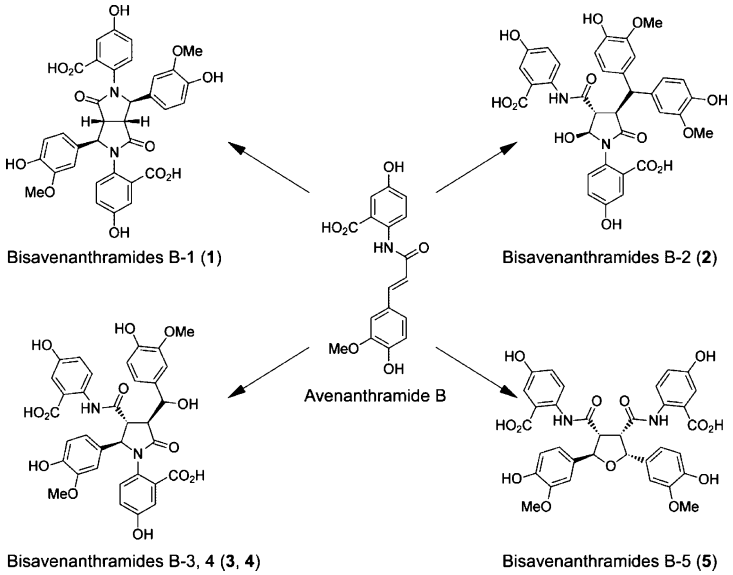

Avenanthramide B is an oat phytoalexin produced in response to pathogen attack and elicitation. We found the formation of new dimers (**1**−**5**) of avenanthramide B in elicited oat leaves. The dimers were synthesized by a reaction of peroxidase and avenanthramide B in the presence of hydrogen peroxide. The structures of **1**−**5** were determined by spectroscopic analyses, chemical derivatization, and ^{15}N labeling. Compound **1** was a dehydrodimer of avenanthramide B with a bisbutane lactam skeleton, while **2**−**4** were monohydrated dehydrodimers with butane lactam structures. Compound **5** was also a monohydrated dehydrodimer but with a tetrahydrofuran structure. All the compounds were classified into lignanamides that were formed by an 8′-8′ coupling reaction between two avenanthramide B units.

Introduction

Plants respond to pathogens by accumulating low-molecular-weight antimicrobial compounds called phytoalexins. Oat (*Avena sativa*) leaves produce phenolic phytoalexins, avenanthramides, upon infection with pathogenic fungi.[1] Avenanthramides are a series of hydroxycinnamic acid amides with hydroxyanthranilates,[2] and they are biosynthesized by a condensation reaction between hydroxycinnamoyl-CoA esters and hydroxyanthranilates[3] that are derived from the shikimate pathway.[4] The production of avenanthramides was evoked by the treatment of oat leaves with elicitors including oligo-*N*-

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acetylchitooligosaccharides,[5] the host-specific toxin victorin C,[6] and heavy metal ions.[7]

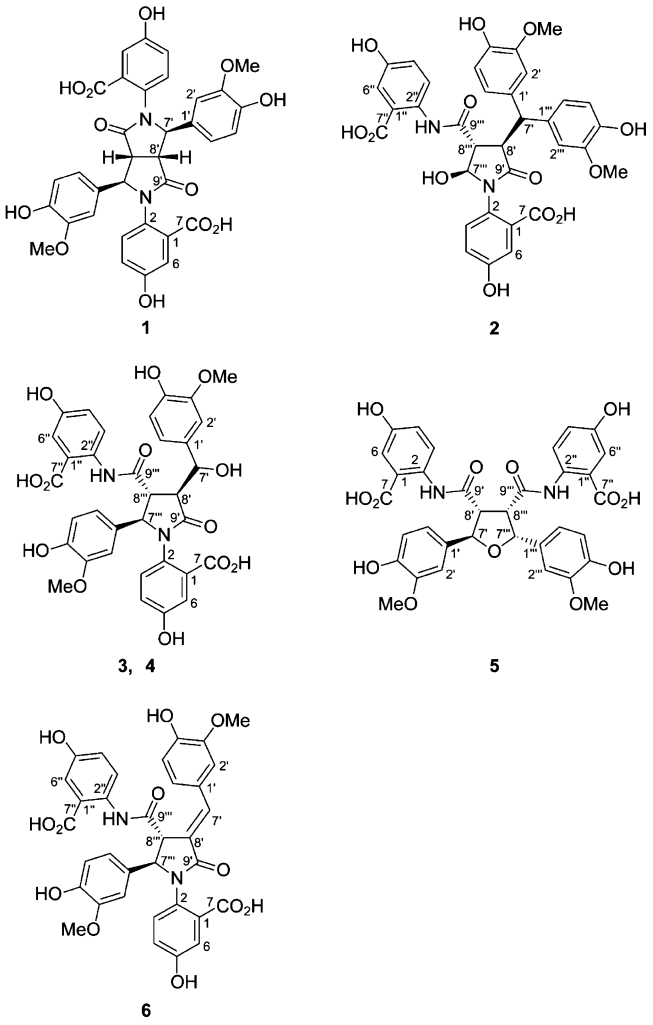
Oligomers of phenolic compounds occasionally have been present together with their monomer in plants. For instance, young barley seedlings accumulate hordatins, which are dimers of hydroxycinnamic acid amides with agmatine, together with their monomers.[8] In grapevine, the accumulation of phytoalexins, viniferins, accompanies the accumulation of their monomer, resveratrol, in tissues infected by phytopathogenic fungi.[9] In these cases, dimeric compounds have been shown to possess stronger antimicrobial activity than the original monomer. Some dimeric compounds play a role that is distinct from their original monomer. In opium poppy, bismorphine—the dimer of morphine—is formed in response to the mechanical wounding of tissues and is incorporated into cell walls, probably for the cross-linking of polysaccharide chains.[10] Thus, the formation of oligomers may not be a simple metabolic process leading to the removal of phenolic compounds.

During the course of the investigation of biosynthesis and metabolism of avenanthramides, we identified a dimeric compound of avenanthramide B in the elicited oat leaves.[11] We also discovered that radiolabeled avenanthramide B was converted into multiple metabolites in the elicited oat leaves.[12] On the basis of these findings, we extensively investigated the dimers of avenanthramides in the elicitor solution in this study and found five novel dimers of avenanthramide B (**1**−**5**). We report on the isolation and structures of **1**−**5** along with the preparation of the dimers, in which peroxidase extracted from the elicited oat leaves is used. The isolated compounds are classified into lignanamides that are defined as lignans bearing at least one amide bond, and we propose a mechanism for their formation in the peroxidase reaction.

Results

Detection of Avenanthramide Dimers. Oat leaves produce avenanthramides A, B, D, G, and L in response to elicitation. We attempted to detect dimeric compounds of the avenanthramide phytoalexins by LC-MS analysis with selected ion monitoring (SIM) of their protonated molecules because elicited oat leaves have been shown to accumulate the metabolites derived from avenanthramide B.[12] Primary leaves from 7-day-old oat seedlings were treated with penta-*N*-acetylchitopentaose (1 mM), which is an effective elicitor of phytoalexin production in oats. After an incubation for 72 h, the elicitor solution was subjected to LC-MS analysis. A new dehydrodimer of avenanthramide B (**1**: *m*/*z* 657, $[M + H]^+$) and four new monohydrated dehydrodimers of avenanthramide B (**2**−**5**: *m*/*z* 675, $[M + H]^+$) were detected in the elicitor solution along with a known dehydrodimer of avenanthramide B (**6**). These compounds (**1**−**6**) were referred to as bisavenanthramide B-1, bisavenanthramide B-2, and so on. The compounds were purified by ODS column chromatography and successive preparative HPLC using two different solvent systems. Dimerized compounds derived from other avenanthramides were not detected by the LC-MS analysis.

CHART 1. Structures of Bisavenanthramides B-1−B-6 (1−6)



It has been demonstrated that the dimers of hydroxycinnamic acids are formed by the peroxidase reaction.[13] We also had successfully synthesized **6** by employing this reaction in a previous study.[11] Thus, a similar reaction was employed for the large-scale preparation of **1**−**5**. Apoplastic peroxidase was extracted from the elicited oat leaves and used for the reaction. The HPLC analysis of the reaction mixture detected the compounds as major peaks that eluted at the same retention times with **1**−**5**. The enzyme reaction without peroxidase in the presence of hydrogen peroxide resulted in the formation of neglisible amounts of **1**−**5**. The comparison of the mass spectra obtained by a Q1 scan and product ion scan in the LC-MS analysis as well as the comparison of the UV spectra recorded on a photodiode array detector confirmed that the synthesized compounds were identical with **1**−**5**. Subsequently, the com-

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TABLE 1. ^{1}H NMR (400 MHz for 1 and 500 MHz for 1a) and ^{13}C NMR (125 MHz) Spectral Data for 1 in Acetone-d_6 and 1a in $CDCl_3$

	1		1a	
position	^{1}H multi, J (Hz)	^{13}C	^{1}H multi, J (Hz)	^{13}C
1		130.6[a]		128.7
2		129.9[a]		129.3
3	6.88 (1H, bd, 8.6)	129.9	7.06 (1H, d, 8.8)	128.3
4	6.76 (1H, dd, 8.6, 2.8)	119.7	6.91 (1H, dd, 8.8, 2.8)	118.4
5		156.7		158.1
6	7.25 (1H, d, 2.8)	118.2	7.41 (1H, d, 2.8)	115.9
7		167.5		166.0
MeO-C5			3.79 (3H, s)	55.6
MeO-C7			3.77 (3H, s)	52.5
1′		133.7		133.0
2′	7.06 (1H, bd, 1.8)	111.4	7.00 (1H, s)	110.0
3′		148.7		149.3
4′		147.2		148.8
5′	6.62 (1H, d, 8.0)	115.6	6.80 (1H, d, 8.6)	111.1
6′	6.74 (1H, dd, 8.0, 1.8)	121.2	6.99 (1H, d, 8.6)	119.4
MeO-C3′	3.65 (3H, s)	56.3	3.83 (3H, s)	56.0
MeO-C4′			3.85 (3H, s)	55.9
7′	5.20 (1H, s)	67.8	5.43 (1H, s)	67.0
8′	3.46 (1H, s)	49.4	3.59 (1H, s)	48.6
9′		174.0		173.3

[a] Assignments may be interchanged.

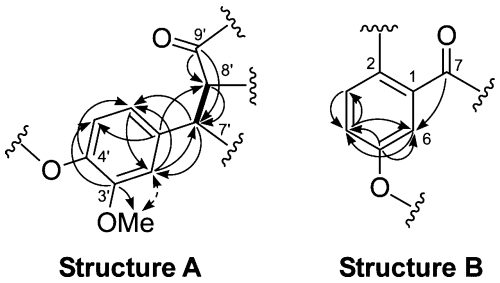

FIGURE 1. Key HMBC (indicated by arrows from ^{13}C to ^{1}H), COSY (indicated by bold lines), and NOESY correlations (indicated by a double headed arrows between two protons) for **1**.

pounds synthesized by the peroxidase reaction were purified and subjected to spectroscopic analysis and chemical derivatization.

Bisavenanthramide B-1 (1). Bisavenanthramide B-1 (**1**) had the molecular formula $C_{34}H_{28}N_2O_{12}$ as revealed by HRESIMS (*m*/*z* 657.1729, $[M + H]^+$), which was consistent with the molecular formula of the dehydrodimer of avenanthramide B. The ^{1}H and ^{13}C NMR spectra of **1** showed 9 and 16 signals, respectively, indicating that **1** had a symmetrical structure (see Tables 1 and S1 in Supporting Information). The multiplicities of carbons were determined based on DEPT and HMQC experiments. The ^{13}C NMR spectrum exhibited two methines (δ_C 49.4, 67.8), one methoxy (δ_C 56.3), and two carbonyl carbons (δ_C 167.5, 174.0). Besides, 11 signals were observed in the aromatic region of the ^{13}C NMR spectrum. The ^{1}H NMR spectrum showed two groups of typical ABX spin system signals corresponding to 1,2,4-trisubstituted benzene rings (Table S1). Therefore, in the aromatic region of the ^{13}C NMR spectrum, one of the 12 signals from two benzene rings was overlapping with another signal. All this information indicated that 17 nonequivalent carbons were present in **1**.

Since **1** had a symmetrical structure, one of the two identical structures was analyzed. The correlation of two methine protons at H-7′ and H-8′ in the COSY spectrum suggested a connection between C-7′ and C-8′ (Figure 1, structure A). Furthermore, in the HMBC spectrum, the signal of carbonyl carbon C-9′ showed cross-peaks between H-7′ and H-8′, indicating a connection between C-9′ and C-8′. These observations indicated a substituted 1-propanone structure (−CHCHC═O). One of the two trisubstituted benzene rings was determined to be a 3-methoxy-4-oxygenated phenyl group in the HMBC and NOESY experiments (Figure 1, structure A). The HMBC spectrum displayed correlations of H-7′, H-6′, and H-8′ with C-2′, C-7′, and C-1′, respectively, indicating a connection between C-1′ and C-7′. On the basis of the substitution pattern and HMBC correlation between C-3′ and the methoxy protons at δ_H 3.65, the benzene ring in structure A was derived from a ferulic acid of avenanthramide B. Therefore, the remaining trisubstituted benzene ring was derived from 5-hydroxyanthranilic acid. Supporting this, the HMBC spectrum displayed the correlation of carbonyl carbon C-7 with H-6 and that of oxygenated aromatic carbon C-5 (δ_C 156.7) with H-6 and H-4 (Figure 1, structure B).

Compound **1** was methylated with (trimethylsilyl)diazomethane to afford **1a**. The ion-spray MS of **1a** ($[M + H]^+$, *m*/*z* 741) indicated that six new methyl groups were introduced. NMR analyses revealed that the partial structures in Figure 1 were conserved in **1a**, and the HMBC and NOESY spectra of **1a** indicated that the newly formed methoxy groups were linked to C-5, C-7, and C-4′ (Tables 1 and S2). Therefore, these carbons were hydroxylated in **1**, and the atoms involved in the linkage of the partial structures in Figure 1 were C-2, C-7′, C-8′, C-9′, and the nitrogen atom.

The possibility that carbonyl carbon C-9′ formed a ketone was excluded based on its chemical shift (δ_C 173.3). Thus, C-9′ was expected to be connected to a nitrogen atom to form an amide because all the oxygen atoms in **1a** were found in methoxy, carbonyl, and methoxycarbonyl functions. A saturated methine carbon C-8′ (δ_C 48.6) was not expected to bind a nitrogen atom and therefore could bind a saturated carbon (C-7′ or C-8′ in the other structure B). The other saturated carbon C-7′ (δ_C 67.0) was expected to be connected to a heteroatom but not to a saturated carbon. All this information suggested that the two C-8′ atoms in **1a** were directly connected to the C-8′ atom in the other avenanthramide B unit and that C-7′ and C-9′ were linked via a nitrogen atom to form two amide

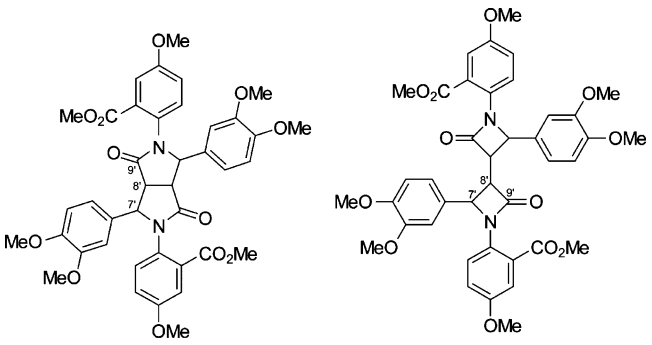

FIGURE 2. Two possible planar structures for **1a**.

linkages. Accordingly, two possible planar structures could be constructed (Figure 2): (i) a bicyclic structure composed of two five-membered lactams that share two C-8′ atoms as bridgehead atoms (structure C), and (ii) two four-membered lactams connected via two C-8′ atoms (structure D).

A small coupling constant of H-7′/H-8′ indicated the trans configuration of H-7′/H-8′. The chemical shifts of H-7′, C-7′, H-8′, and C-8′ of **1a** were compared with the corresponding signals in the compounds that have structures similar to the two candidate structures with the same configuration (Figure S1).[14] The similarity of the chemical shifts indicated that the structure of **1a** was structure C in Figure 2.

Two relative structures could be constructed for **1** depending on the relative configuration of two H-8′ at the bridgeheads: (i) if the configuration is cis, **1** is a chiral compound, and (ii) if the configuration is trans, **1** is a meso compound. Compound **1** prepared by the enzyme reaction was optically inactive, and chiral HPLC separated **1** into two peaks with almost identical areas. These two peaks showed contrasting CD spectra, indicating that **1** was a racemic mixture of around 1:1 ratio with two cis-configured bridgehead protons (H-8′).

Bisavenanthramide B-2 (2). The molecular formula of bisavenanthramide B-2 (**2**) was determined as $C_{34}H_{30}N_2O_{13}$ by HRESIMS (*m*/*z* 675.1740, $[M + H]^+$), which was consistent with the monohydrated dehydrodimer of avenanthramide B. The ^{13}C NMR spectra of **2** showed 33 signals (Table 2), including four methines (δ_C 50.3, 51.7, 58.0, 87.5), two methoxy (δ_C 56.2, 56.4), four carbonyl carbons (δ_C 168.5, 170.8, 171.1, 176.0), and 23 aromatic carbons. The ^1H NMR spectrum showed four groups of typical ABX spin system signals corresponding to 1,2,4-trisubstituted benzene rings (Tables 2 and S3), indicating that one of the 24 signals was overlapping with another aromatic carbon signal in the ^{13}C NMR spectrum. The index of hydrogen deficiency (= 21) suggested that **2** had one cyclic structure in addition to four benzene rings.

The HMBC and NOESY experiments revealed that the four benzene rings were two 3-methoxy-4-oxygenated benzenes (Figure 3 and Table S3) and two 2-carbonyl-4-oxygenated benzenes. COSY correlations between H-7′/H-8′, H-8′/H-8‴, and H-8‴/H-7‴ suggested that C-7′, C-8′, C-8‴, and C-7‴ were connected in this order. In the HMBC spectrum, correlations of C-7′ with H-2′, H-6′, H-2‴, and H-6‴ were observed, indicating that both C-1′ and C-1‴ were bound to C-7′. The

TABLE 2. ^1H (400 MHz) and ^{13}C (125 MHz) NMR Spectral Data for 2 in MeOH-d_4

	2	
position	^1H multi, *J* (Hz)	^{13}C
1		131.4
2		128.7
3	7.06 (1H, d, 8.5)	120.5
4	6.99 (1H, dd, 8.5, 2.9)	120.5
5		158.8
6	7.45 (1H, d, 2.9)	119.2
7		168.5
1′		135.9
2′	6.91 (1H, d, 1.9)	113.5
3′		148.69
4′		145.9
5′	6.65[a] (1H, d, 8.2)	115.9
6′	6.77 (1H, dd, 8.2, 1.9)	122.3
MeO-C3′	3.74 (3H, s)	56.4[b]
7′	4.47 (1H, d, 6.8)	51.7
8′	3.98 (1H, dd, 9.5, 6.8)	50.3
9′		176.0
1″		119.0
2″		134.5
3″	8.35 (1H, d, 9.0)	122.8
4″	6.96 (1H, dd, 9.0, 2.9)	121.9
5″		154.1
6″	7.45 (1H, d, 2.9)	118.2
7″		170.8
1‴		135.2
2‴	6.81 (1H, d, 1.9)	113.7
3‴		148.73
4‴		146.2
5‴	6.63[a] (1H, d, 8.2)	116.0
6‴	6.72 (1H, dd, 8.2, 1.9)	122.9
MeO-C3‴	3.73 (3H, s)	56.2[b]
7‴	5.39 (1H, d, 5.9)	87.5
8‴	2.89 (1H, dd, 9.5, 5.9)	58.0
9‴		171.1

[a,b] Assignments may be interchanged.

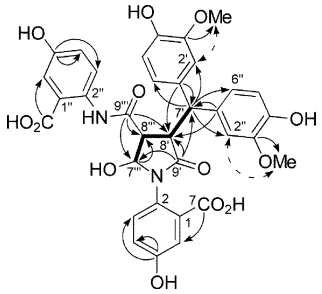

FIGURE 3. Key HMBC (indicated by arrows from ^{13}C to ^1H), COSY (indicated by bold lines), and NOESY correlations (indicated by a double headed arrows between two protons) for **2**.

HMBC correlations of C-9‴ with H-7‴, H-8‴, and H-8′ suggested the connection between C-9‴ and C-8‴, and the correlations of H-8′ and H-7′ with C-9′ suggested the connection between C-9′ and C-8′. The chemical shifts of C-9′ and C-9‴ (δ_C 176.0, 171.1) indicated the connection of a heteroatom. In the HMBC spectrum, H-7‴ showed a correlation with C-9′ but not with C-8′ and C-8‴, thus suggesting that C-7‴ was cross-linked to C-9′ via the heteroatom to form a five-membered lactam or lactone.

To determine the chemical shifts of two nitrogen atoms in **2**, **2*** enriched with ^{15}N was prepared from (^{15}N)avenanthramide B. In the ^{15}N NMR spectrum of **2***, two intense signals of enriched ^{15}N were observed at δ_N 129.9 and 149.5, indicating

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TABLE 3. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR Spectral Data for 3 in MeOH-d_4, and 4 in Pyridine-d_5

	3		4	
position	^{1}H multi, J (Hz)	^{13}C	^{1}H multi, J (Hz)	^{13}C
1		130.8		132.6[b]
2		129.7		129.9
3	6.71-6.82 (1H, m)	n.d.	7.11 (1H, bd, 8.4)	133.5
4	6.71-6.82 (1H, m)	120.1	7.03 (1H, dd, 8.4,2.5)	119.6
5		157.9		158.2
6	7.35 (1H, bd)	118.9	8.17 (1H, bd, 2.5)	119.3
7		168.8		169.0
1′		134.8		134.1[b]
2′	6.99 (1H, d, 1.6)	110.2	7.93 (1H, bs)	111.8
3′		148.6		148.6
4′		146.5		148.2
5′	6.59 (1H, d, 8.1)	115.8[a]	7.35-7.37 (1H)	116.2
6′	6.86 (1H, dd, 8.1, 1.6)	119.4	7.87 (1H, bs)	120.9
MeO-C3′	3.71 (3H, s)	56.1	3.82 (3H, s)	56.0
7′	5.47 (1H,s)	71.0	6.36 (1H, bm)	73.0
8′	3.56 (1H, m)	55.4	4.71 (1H, bm)	55.0
9′		176.4		175.2
1″		119.1		119.8
2″		133.9		134.6
3″	8.00 (1H, d, 9.0)	122.7	9.23 (1H, d, 9.0)	122.5
4″	6.85 (1H, dd, 9.0, 3.0)	121.6	7.38 (1H, dd, 9.0, 3.0)	121.5
5″		153.9		153.9
6″	7.33 (1H, d, 3.0)	117.8	8.14 (1H, d, 3.0)	118.2
7″		170.6		171.3
1‴		131.2		131.5
2‴	7.09 (1H, bs)	112.4	6.97-7.02 (1H)	111.8
3‴		149.4		149.0
4‴		147.9		147.6
5‴	6.62 (1H, d, 8.0)	115.9[a]	6.95 (1H, d, 8.0)	116.0
6‴	6.68 (1H, dd, 8.0, 1.2)	122.9	6.79 (1H, bs)	122.9
MeO-C3‴	3.80 (3H, s)	56.5	3.79 (3H, s)	55.9
7‴	4.97 (1H, bs)	68.9	6.06 (1H, d, 8.1)	68.3
8‴	3.56 (1H, m)	52.2	4.09 (1H, bm)	52.7
9‴		170.9		171.0

[a,b] Assignments may be interchanged.

the presence of two amide nitrogens.[15] In the ^{13}C NMR spectrum of **2***, the signals at δ_C 87.5 (d, $^1J_{CN}$ = 11.1 Hz, C-7‴), 128.6 (d, $^1J_{CN}$ = 15.5 Hz, C-2), 134.4 (d, $^1J_{CN}$ = 14.4 Hz, C-2″), 176.0 (d, $^1J_{CN}$ = 12.0 Hz, C-9′), and 171.0 (d, $^1J_{CN}$ = 15.0 Hz, C-9‴) were found to be split by heteronuclear spin couplings. The chemical shifts of two nitrogen atoms and their heteronuclear spin couplings indicated that C-7‴ and C-9′ were cross-linked via a nitrogen atom and that C-9‴ was connected to another nitrogen atom. In addition, because C-2 and C-2″ were doublets, two 2-carbonyl-4-oxygenated benzenes derived from 5-hydroxyanthranilic acids were connected to nitrogen atoms via C-2 and C-2″. The assignment of the two 2-carbonyl-4-oxygenated benzenes were based on the comparison of the chemical shifts of 5-hydroxyanthranilic acid moieties of **2** and **6**.

The saturated methine carbon C-7‴ did not connect with the hydroxyl and carboxyl groups on the benzene rings because it would have resulted in the formation of another cyclic structure that was not allowed by IHD. Thus, C-7‴ was connected with the remaining hydroxyl group that was expected from the molecular formula. On the basis of these observations, we concluded that **2** is a dimer of avenanthramide B formed with the translocation of a trisubstituted benzene ring derived from a ferulate unit (Figure 3). In the NOESY spectrum of **2**, H-7‴ showed a correlation with H-8′, while its vicinal proton H-8‴ did not show a correlation with H-7‴ and H-8′ (Table S3). Therefore, both H-7‴/H-8‴ and H-8‴/H-8′ had trans configurations.

Bisavenanthramides B-3 (3) and B-4 (4). Bisavenanthramides B-3 and B-4 (**3** and **4**) had the molecular formula $C_{34}H_{30}N_2O_{13}$ as revealed by HRESIMS (**3**: m/z 675.1835, $[M + H]^+$; **4**: m/z 675.1843, $[M + H]^+$), which was consistent with the monohydrated dehydrodimers of avenanthramide B. Compounds **3** and **4** showed similar ion-spray mass spectra to that of **6**, suggesting that **3** and **4** were simple hydrated compounds of **6**.

Compounds **3** and **4** displayed absorption maxima at 267 and 262 nm, respectively, while **6** exhibited an absorption maximum at 329 nm. These significant hypochromic shifts suggested that the conjugated double bond present in **6** was absent in **3** and **4** due to hydration. This was supported by the fact that the double bound observed in the ^{13}C NMR spectrum of **6** disappeared, whereas two methine carbons [δ 71.0 (C-7′) and 55.4 (C-8′) in **3**; δ 73.0 (C-7′), and δ 55.0 (C-8′) in **4**] were observed in the spectra of **3** and **4** (Tables 3, S4, and S5). The chemical shifts of C-7′ of **3** and **4** suggested that C-7′ was hydroxylated. The chemical shifts of the remaining signals of **3** and **4** in the ^{13}C NMR spectra corresponded to those of **6**. Therefore, **3** and **4** were monohydrated compounds of **6**, and they were diastereomers that had a different configuration at C-7′. The correlations in the HMBC and COSY spectra supported these structures (Tables S4 and S5). Compounds **3** and **4** showed similar CID spectra, which also supported the fact that they were simple

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TABLE 4. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR Spectral Data for 5 in MeOH-d_4[a]

	5	
position	^{1}H multi, *J* (Hz)	^{13}C
1		119.8
2		134.3
3	8.21 (1H, d, 9.1)	123.5
4	6.92 (1H, dd, 9.1, 3.0)	121.7
5		154.0
6	7.41 (1H, d, 3.0)	118.0
7		171.2
1′		135.6
2′	7.21 (1H, d, 1.8)	111.5
3′		149.0
4′		147.3
5′	6.82 (1H, d, 8.2)	116.3
6′	7.07 (1H, dd, 8.2, 1,8)	120.2
MeO-C3′	3.88 (3H, s)	56.5
7′	6.04 (1H, d, 8.3)	82.7
8′	3.8-3.86 (1H, m)	59.8
9′		169.9
1″		119.1
2″		134.3
3″	8.09 (1H, d, 9.0)	122.6
4″	6.81 (1H, dd, 9.0, 3.0)	121.4
5″		153.6
6″	7.30 (1H, d, 3.0)	117.9
7″		170.6
1‴		130.3
2‴	7.00 (1H, d, 1.7)	110.9
3‴		148.7
4‴		147.2
5‴	6.64 (1H, d, 8.1)	115.7
6‴	6.86 (1H, dd, 8.1, 1.7)	120.4
MeO-C3‴	3.67 (3H,s)	56.2
7‴	5.68 (1H, d, 4.6)	85.1
8‴	3.8--3.86 (1H, m)	59.6
9‴		169.7

[a] Assignments of two 5-hydroxyanthranilic acid units may be completely interchanged.

diastereomers. Compounds **3** and **4** were refluxed in formic acid to yield a dehydrated compound,[16] which was confirmed to be **6** by NMR and LC-MS analyses. In the NOESY spectra of **3** and **4**, H-7‴ showed a correlation with H-8′, but the correlations of H-8‴ with H-7‴ and H-8′ were not observed (Tables S4 and S5). Thus, the relative configuration of both H-7‴/H-8‴ and H-8‴/H-8′ in **3** and **4** were trans configurations.

Bisavenanthramide B-5 (5). Compound **5** had the molecular formula $C_{34}H_{30}N_2O_{13}$ as revealed by HRESIMS (*m*/*z* 675.1837, $[M + H]^+$), indicating that **5** was also a monohydrated dehydrodimer of avenanthramide B. The ^{13}C NMR spectra of **5** showed 33 signals including four methines (δ_C 59.6, 59.8, 82.7, 85.1), two methoxy (δ_C 56.2, 56.5), four carbonyl carbons (δ_C 169.7, 169.9, 170.6, 171.2), and 23 aromatic carbons (Table 4). Because the ^{1}H NMR spectrum showed four groups of typical ABX spin system signals corresponding to 1,2,4-trisubstituted benzene rings (Table S6), one of the 24 signals corresponding to the four benzene rings was overlapping with another aromatic carbon signal. The IHD (= 21) suggested that **5** had a cyclic structure apart from the four benzene rings.

The HMBC and NOESY experiments revealed that the four benzene rings comprised two 3-methoxy-4-oxygenated benzenes and two 2-carbonyl-4-oxygenated benzenes (Figure 4 and Table S6). The correlations between H-7′/H-8′, H-8′/H-8‴, and H-8‴/H-7‴ in the COSY spectrum and the respective correlations of C-9′ and C-9‴ with H-7′ and H-7‴ in the HMBC spectrum indicated a substituted 2,3-dicarbonylbutane structure (Figure 4). The HMBC spectrum also displayed correlations of C-7′ with H-2′ and H-6′ and correlations of H-7′ with C-2′ and C-6′, indicating a connection between C-1′ and C-7′. Similarly, H-2‴ and H-6‴ showed cross-peaks with C-7‴, indicating a connection between C-1‴ and C-7‴. The chemical shifts of C-7′ (δ_C 82.7) and C-7‴ (δ_C 85.1) indicated that these methines were connected to oxygen atoms. In the HMBC spectrum, the correlation of H-7′ with C-7‴ and not with C-8‴ was observed, suggesting that C-7‴ was linked to C-7′ via an oxygen atom to form a tetrahydrofuran structure (Figure 4).

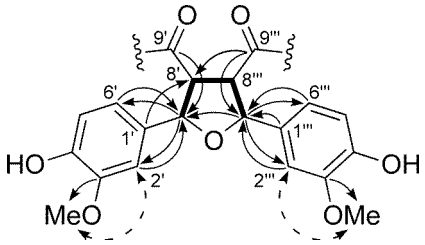


FIGURE 4. Key HMBC (indicated by arrows from ^{13}C to ^{1}H), and COSY (indicated by bold lines) and NOESY (indicated by a double headed arrow between two protons) correlations used to construct the planar structure of **5**.

The chemical shifts of C-9′ (δ_C 169.9) and C-9‴(δ_C 169.7) suggested that these carbonyl carbons were connected to a heteroatom. The chemical shifts of the ^{1}H and ^{13}C NMR signals of two 2-carbonyl-4-oxygenated benzenes in **5** were almost identical with that of the 5-hydroxyanthranilic acid moiety of avenanthramide B. This indicated that both C-9′ and C-9‴ were linked to 2-carbonyl-4-oxygenated phenyl groups via nitrogen atoms to form amides in a manner similar to avenanthramide B.

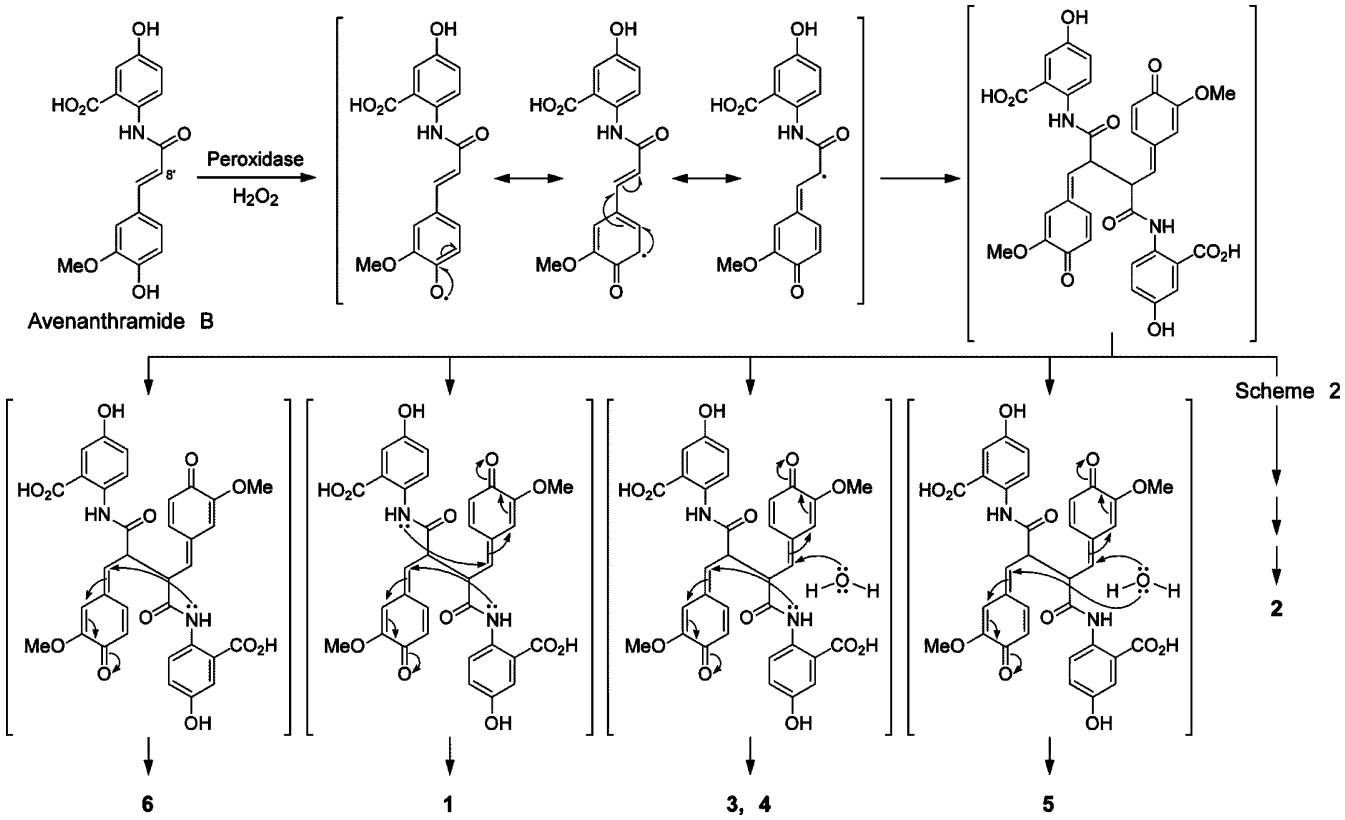
In the NOESY spectrum, H-7‴ correlated with H-8′ and H-8‴, whereas H-7′ did not correlate with other methine protons. Therefore, H-7′/H-8′, H-8′/H-8‴, and H-7′/H-7‴ had trans-, cis-, and cis-configurations, respectively.

Optical Resolution of Bisavenanthramides B-1 (1)−B-5 (5). In addition to bisavenanthramide B-1 (**1**), bisavenanthramide B-2 (**2**)−B-5 (**5**) synthesized by the peroxidase reaction were subjected to optical resolution by chiral HPLC with a CHIRALCEL OD-RH column. Compounds **2** and **4** were successfully separated into two peaks that have almost the same peak areas. Two peaks from a compound showed CD spectra that were almost mirror images of each other. Thus, **2** and **4** synthesized by a peroxidase reaction were racemic mixtures. Because chiral HPLC did not separate **3** into two peaks, we converted **3** to **6** by refluxing it in formic acid. The resulting compound **6** was separated into two peaks with the same areas using chiral HPLC under the conditions that were fixed for the separation of **6**,[11] indicating that **3** was also a racemic mixture. We could not find the HPLC conditions that separate **5** into two peaks on this column.

Bisavenanthramides B-1 (**1**)−B-4 (**4**) purified from the elicitor solution were analyzed by chiral HPLC under the same conditions as those for the synthesized compounds. The chiral HPLC separated **1**, **2**, and **4** into two peaks corresponding to their enantiomers with almost the same areas. Compound **3** that was purified from the elicitor solution was converted to **6** in the same way as in the case of the synthesized **3**, and the resulting compound **6** was separated into two peaks of enanti-

(16) Puterbaugh, W. H.; Hauser, C. R. *J. Am. Chem. Soc.* **1953**, *75*, 2415.

SCHEME 1. Possible Reaction Schemes for the Formation of 1, 3, 4, 5, and 6



omers with the same areas by chiral HPLC. Thus, **1**−**4** produced by oat leaves were racemic mixtures.

Discussion

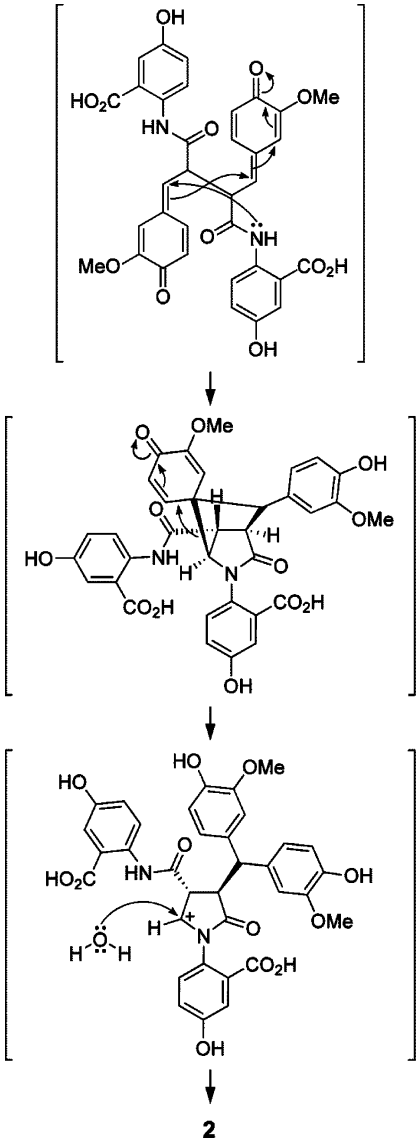
In this study, we identified five novel dimers of avenanthramide B. Together with the dehydrodimer of avenanthramide B (bisavenanthramide B) that had been identified in the previous study,[11] these dimers are collectively referred to as bisavenanthramides B-1−B-6. All the bisavenanthramides are hydroxycinnamic acid dimers that have two amide groups. Thus, they are classified into lignanamides that are defined as lignans bearing amide groups.[17] Bisavenanthramide B-1 (**1**) represents a new class of lignanamides because the bisbutane lactam skeleton has not been found in natural products. In addition, bisavenanthramide B-2 (**2**) has a trisubstituted benzene ring that is translocated from one avenanthramide B unit to the other. Thus, its carbon skeleton is also unique although it has a butane lactam structure, which was found in bisavenanthramides B-3 (**3**) and B-4 (**4**); this partial structure was common with that of the previously characterized bisavenanthramide B-6 (**6**).[11] Butane-lactam-type lignanamides have been found only in oats. Bisavenanthramide B-5 (**5**) is classified into tetrahydrofuran lignanamides that were found in the fruits of *Jacquemontia paniculata*.[18] The tetrahydrofuran skeleton is often found in lignans.

We prepared all the bisavenanthramides by using peroxidase extracted from elicited oat leaves. It has been demonstrated that peroxidase isoforms that accept avenanthramides as substrates are strongly induced by elicitation in apoplastic fractions.[12] The treatment of the elicited leaves with peroxidase inhibitors suppressed the metabolism of the labeled avenanthramide B in the elicitor solution. These findings indicate that elicitor-inducible peroxidases are responsible for the *in vivo* formation of bisavenanthramides. The involvement of peroxidases has been shown in the formation of dimers and oligomers of other phenolic compounds.[19] Compounds **1**−**4** prepared by the peroxidase reaction were confirmed to be racemic mixtures. Generally, the products of radial coupling reactions catalyzed by peroxidases are optically inactive. The involvement of dirigent proteins has been indicated in the enatiospecific formation of the radical coupling products in some plants.[20]

The possible formation mechanisms of bisavenanthramides by peroxidase are shown in Scheme 1. Peroxidase generates a phenoxy radical of avenanthramide B in the presence of hydrogen peroxide. A radical coupling of two avenanthramide B units at 8′-positions forms the quinone methide intermediate. Depending on the postcoupling rearomatization reactions, bisavenanthramides are (bio)synthesized. In the synthesis of **1**, two nitrogen atoms attack the double bonds of the other avenanthramide B units, resulting in the formation of a bicyclic structure consisting of two five-membered lactams. The translocation of a benzene ring derived from a ferulic acid is involved in the formation of **2** (Scheme 2). This process is thought to occur through a bicyclo[2.2.1]heptane-like intermediate. The

(17) McCredie, R. S.; Ritchie, E.; Taylor, W. C. *Aust. J. Chem.* **1969**, *22*, 1011.
(18) Henrici, A.; Kaloga, M.; Eckart, E. *Phytochemistry* **1994**, *37*, 1637.
(19) Ralph, J.; Bunzel, M.; Marita, J. M.; Hatfield, R. D.; Lu, F.; Kim, H.; Schatz, P. F.; Grabber, J. H.; Steinhart, H. *Phytochem. Rev.* **2004**, *3*, 79.
(20) (a) Davin, L. B.; Wang, H.-B.; Crowell, A. L.; Bedgar, D. L.; Martin, D. M.; Sarkanen, S.; Lewis, N. G. *Science* **1997**, *275*, 362. (b) Kim, M. K.; Jeon, J. H.; Fujita, M.; Davin, L. B.; Lewis, N. G. *Plant Mol. Biol.* **2002**, *49*, 199.

SCHEME 2. Possible Reaction Scheme for the Formation of 2



configuration of C-7‴ suggests that hydroxylation takes place after a carbocation is formed by the translocation of the benzene ring. In the formation of **3** and **4**, one of the nitrogen atoms of the avenanthramide B unit attacks a double bond in the other avenanthramide B unit, while H_2O attacks the remaining double bond (Scheme 1). The orientation of two avenanthramide B units in the synthesis of **5** is different from that in the rearomatization reactions of other bisavenanthramides. After a H_2O molecule is added to a double bond, the oxygen atom forms a tetrahydrofuran ring by attacking the other double bond. In the previous study,[12] we fed elicited oat leaves with radiolabeled avenanthramide B and found the accumulation of seven radioactive compounds in the elicitor solution. We have identified six compounds among them from this and previous studies although one hydrated dehydrodimer corresponding to a small radioactive peak could not be identified because of its instability. All the identified dimers had an 8′-8′ linkage (corresponding to the 8-8 linkage in the dimers of hydroxycinnamic acids) between the two avenanthramide B units. In contrast, coupling reactions between hydroxycinnamic acids or monolignols yield a mixture

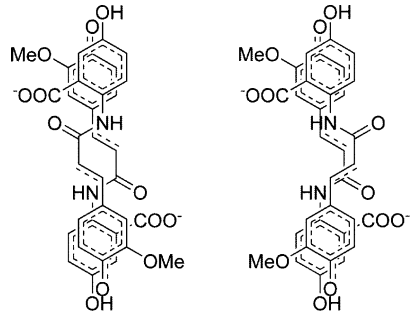


FIGURE 5. Possible intermediate π-complexes in the 8′-8′ coupling reaction between the two avenanthramide B units.

of products with 8-8, 8-O-4, and 8-5 linkages.[13,21] The preferred regiospecificity for the 8′-8′ coupling in bisavenanthramide formation may be attributed to the configuration of π-complexes as suggested to explain the difference in regiospecificity between the dimerization of coniferyl alcohol and cross-coupling of coniferyl alcohol with 2-methoxy-4-(1-hydroxyethyl)-phenol.[22] The presence of the benzene ring of anthranilate moiety in avenanthramide B may lead to π-complexes with a configuration favoring 8′-8′ coupling over 8′-O-4′and 8′-5′ couplings (Figure 5). The head-to-tail orientation found in bisavenanthramides other than **5** is likely preferred because of the negative charge of the carboxylic acid of the anthranilate moiety. These structural features of avenanthramides give rise to the formation of lignanamides with butane lactam structures, which have not been found in lignanamides synthesized from other hydroxycinnamic acid amides.

We have not detected dimers derived from other avenanthramides except avenanthramide B. Our previous study indicated that avenanthramide B is the best substrate for elicitor-inducible peroxidase.[12] The substrate specificity of inducible peroxidases probably affects the composition of bisavenanthramides. Another possible factor is radical transferring.[23] The major constituent of the avenanthramide mixture in the elicitor solution is avenanthramide A, which is composed of *p*-coumaric acid and 5-hydroxyanthranilate. It has been suggested that *p*-coumarate plays a role in radical transfer in the dehydrogenation of monolignols on the basis of the finding that the presence of *p*-coumarate enhanced the dehydrogenation of sinapyl alcohol.[19] Avenanthramide A, which has a *p*-coumaroyl moiety, may function in a similar manner in the radical transfer reaction.

Experimental Section

Analysis of Elicitor-Inducible Metabolites by Ion-Spray LC-MS. Oat leaves were treated with penta-*N*-acetylchitopentaose that acted as an elicitor, according to the previously reported procedure.[11] Dehydrodimers of avenanthramides were analyzed in the elicitor solution by LC-MS analyses with selected ion monitoring (SIM) in positive ion mode. HPLC conditions were as previously described.[11] The elution of compounds was monitored at *m*/*z* 597, 657, 565, and 649 to detect the homodimers of avenanthramides A, B, D, and L, respectively, and was monitored at *m*/*z* 627, 581, 623, 611, 653, and 607 to detect the heterodimers of avenanthra-

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(23) (a) Takahama, U.; Oniki, T. *Plant Cell Physiol.* **1994**, *35*, 593. (b) Takahama, U.; Oniki, T.; Shimokawa, H. *Plant Cell Physiol.* **1996**, *47*, 499.

mides. Hydrated dehydrodimers were also analyzed by SIM at an *m/z* value that was 18 mass units larger than those for dehydrodimers.

Enzymatic Preparation of 1−6 and 2*. Compounds **1**−**6** were synthesized from avenanthramide B (400 mg, 1.21 mmol) by an enzyme reaction using the peroxidase extracted from elicited oat leaves according to the methods previously reported.[11] The enzyme reaction was halted by adding 0.01 vol of AcOH, and the reaction mixture was applied to an ODS column (Cosmosil 75C18-OPN, Nacalai Tesque, Kyoto, 62 mm long, 32 mm i.d.) equilibrated with H_2O−AcOH (1000:5). The column was washed with 200 mL of H_2O and eluted with 150 mL of MeOH−H_2O (40:60). The eluate was concentrated and subjected to preparative HPLC [eluent, 0−42 min: B−A (30:70), 42−70 min: B−A (33:67); detection, 280 nm]. The fractions containing **1** (t_R 27.2 min), **2** (t_R 30.6 min), **3** (t_R 37.7 min), **4** (t_R 43.0 min), **5** (t_R 63.0 min), and **6** (t_R 67.5 min) were collected. Each fraction was diluted 4-fold with H_2O−AcOH (1000:1) and loaded on an ODS column (62 mm long, 32 mm i.d.) equilibrated with H_2O−AcOH (1000:1). After being washed with 500 mL of H_2O, the column was eluted with 150 mL of MeOH−H_2O (80:20). The eluates were concentrated *in vacuo* to yield **1** (9.3 mg, 28.4 μmol, yield 2.3%), **2** (12.2 mg, 18.1 μmol, yield 3.0%), **3** (16.3 mg, 24.2 μmol, yield 4.0%), **4** (50.1 mg, 74.3 μmol, yield 12.3%), **5** (5.9 mg, 8.75 μmol, yield 1.5%), and **6** (118.4 mg, 180 μmol, yield 29.7%). A similar reaction with (^{15}N)avenanthramide B (500 mg, 1.52 mmol) was carried out to obtain **2*** (21.8 mg, 32.2 μmol, yield 4.2%).

1: white solid; UV (MeOH) λ_{max} (log ϵ): 210 (4.83), 233 sh (4.44), 285 (4.04), 300 sh (3.84); ion-spray MS *m/z* (rel. int.): 679 $[M + Na]^+$ (6), 657 $[M + H]^+$ (100), 639 $[M + H - H_2O]^+$ (4); product ion scan (precursor ion: *m/z* 657) *m/z* (rel. int.): 639 (100), 621 (9), 515 (9), 458 (7), 336 (6); ^{1}H NMR and ^{13}C NMR, see Tables 1 and S1.

2: yellowish white solid; UV (MeOH) λ_{max} (log ϵ): 204 (5.04), 265 (4.23), 316 (3.07); ion-spray MS *m/z* (rel. int.): 697 $[M + Na]^+$ (11), 675 $[M + H]^+$ (52), 657 $[M + H - H_2O]^+$ (100), 533 $[M + H - H_2O - C_7H_8O_2]^+$ (29); product ion scan (precursor ion: *m/z* 675) *m/z* (rel.int.): 639 (100), 515 (42), 354 (67), 297 (63), 259 (63); ^{1}H NMR and ^{13}C NMR, see Tables 2 and S3

3: yellowish white solid; UV (MeOH) λ_{max} (log ϵ): 205 (4.76), 225 sh (4.42), 267 (3.98), 319 (3.49); ion-spray MS *m/z* (rel. int.): 697 $[M + Na]^+$ (8), 675 $[M + H]^+$ (6), 657 $[M + H - H_2O]^+$ (100), 639 $[M + H - 2(H_2O)]^+$ (3), 288 $[C_{15}H_{14}O_5N]^+$ (4); product ion scan (precursor ion: *m/z* 675) *m/z* (rel. int.): 639 (23), 486 (13), 458 (10), 323 (8), 288 (100); ^{1}H NMR and ^{13}C NMR, see Tables 3 and S4.

4: yellowish white solid; UV (MeOH) λ_{max} (log ϵ)): 204 (4.87), 225 sh (4.59), 262 (4.19), 319 (3.67); ion-spray MS *m/z* (rel. int.): 697 $[M + Na]^+$ (4), 675 $[M + H]^+$ (2), 657 $[M + H - H_2O]^+$ (100), 639 $[M + H - 2(H_2O)]^+$ (6), 288 $[C_{15}H_{14}O_5N]^+$ (9); product ion scan (precursor ion: *m/z* 675) *m/z* (rel. int.): 639 (8), 504 (6), 486 (7), 323 (5), 288 (100); ^{1}H NMR and ^{13}C NMR, see Tables 3 and S5.

5: yellow solid; UV (MeOH) λ_{max} (log ϵ)): 204 (4.79), 224 (4.51), 263 (4.19), 328 (3.75); ion-spray MS *m/z* (rel. int.): 697 $[M + Na]^+$ (17), 675 $[M + H]^+$ (100), 657 $[M + H - H_2O]^+$ (18), 522 $[M + H - C_7H_7O_3N]^+$ (19), 370 (4); product ion scan (precursor ion: *m/z* 675) *m/z* (rel. int.): 639 (19), 504 (12), 486 (19), 370 (54), 352 (40), 288 (100); ^{1}H NMR and ^{13}C NMR, see Tables 4 and S6.

2*: yellowish white solid; ion-spray MS *m/z* (rel. int.): 715 $[M + K]^+$ (6), 699 $[M + Na]^+$ (2), 677 $[M + H]^+$ (62), 659 $[M + H - H_2O]^+$ (100), 535 $[M + H - H_2O - C_7H_8O_2]^+$ (63); ^{1}H NMR (400 MHz, MeOH-d_4) δ 2.90 (1H, J = 9.4, 5.9 Hz, H-8‴), 3.735 (3H, MeO-C3′), 3.738 (3H, MeO-C3‴), 3.99 (1H, J = 9.4, 6.8 Hz, H-8′), 4.45 (1H, J = 6.8 Hz, H-7′), 5.40 (1H, J = 5.9 Hz, H-7‴), 6.64 (1H, J = 8.2 Hz, H-5‴), 6.66 (1H, J = 8.2 Hz, H-5′), 6.72 (1H, J = 8.2, 1.9 Hz, H-6‴), 6.78 (1H, J = 8.2, 1.9 Hz, H-6′), 6.82 (1H, J = 1.9 Hz, H-2‴), 6.92 (1H, J = 1.9 Hz, H-2′), 6.97 (1H, J = 9.1, 2.9 Hz, H-4″), 6.99 (1H, J = 8.5, 2.9 Hz, H-4), 7.06 (1H, $^3J_{HN}$ = 1.5 Hz, $^3J_{HH}$ = 8.5 Hz, H-3), 7.45 (2H, J = 2.9 Hz, H-6 and H-6″), 8.35 (1H, $^3J_{HN}$ = 1.2 Hz, $^3J_{HH}$ = 9.1 Hz, H-3″); ^{13}C NMR (125 MHz, MeOH-d_4) δ 50.3 ($^2J_{CN}$ = 7.6 Hz, C-8′), 51.7 (C-7′), 56.2 (MeO-C3‴), 56.3 (MeO-C3′), 58.0 ($^2J_{CN}$ = 0.48 Hz, C-8‴), 87.5 ($^1J_{CN}$ = 11.1 Hz, C-7‴), 113.4 (C-2′), 113.6 (C-2‴), 115.9 (C-5′), 116.0 (C-5‴), 118.1 (C-6″), 118.9 (C-1′), 119.2 (C-6), 120.4 (C-4), 121.8 (C-4″), 122.2 (C-6′), 122.77 (C-3″), 122.83 (C-6‴), 128.6 ($^1J_{CN}$ = 15.5 Hz, C-2), 131.4 (C-1), 134.4 ($^1J_{CN}$ = 14.4 Hz, C-2″), 135.1 (C-1‴), 135.9 (C-1′), 145.8 (C-4′), 146.1 (C-4‴), 148.6 (C-3′), 148.7 (C-3‴), 154.0 (C-5″), 158.8 (C-5), 168.4 (C-7), 170.7 (C-7″), 171.0 ($^1J_{CN}$ = 15.0 Hz, C-9‴), 176.0 ($^1J_{CN}$ = 12.0 Hz, C-9′); ^{15}N NMR (50 MHz, MeOH-d_4) δ 129.9, 149.5.

Optical Resolution of 1, 2, and 4. Compound **1** prepared by the enzyme reaction was subjected to optical resolution by chiral HPLC. The HPLC conditions were as follows: column, CHIRALCEL OD-RH (150 mm long, 4.6 mm i.d., Daicel); eluent, MeCN−100 mM KH_2PO_4 aq (13:87); flow rate, 0.5 mL min^{-1}; column temperature, 40 °C; detection, 280 nm. Compound **1** eluted as two peaks at t_R values of 12.5 and 21.2 min, with almost identical peak areas. The fractions corresponding to the two peaks were desalted by using a Sep-Pak Light C18 cartridge (Waters). The amounts of fast- and late-eluting enantiomers (**1**-1 and **1**-2) obtained were 0.752 mg (100% ee) and 0.896 mg (94.4% ee), respectively. CD (MeOH) **1**-1 λ_{max} ($\Delta\epsilon$): 209 (−22.4), 215 (−8.8), 221, (−8.0), 249 (5.9), 301 (−1.0); **1**-2 λ_{max} ($\Delta\epsilon$): 210 (30.4), 215 (15.5), 224 (10.6), 250 (−6.6), 299 (1.5).

The enantiomers of **2** and **4** were similarly separated by chiral HPLC. The HPLC conditions for **2** were as follows: eluent, MeCN−100 mM KH_2PO_4 aq (11:89); flow rate, 0.5 mL min^{-1}; column temperature, 27.5 °C; detection, 280 nm. The HPLC conditions for **4** were as follows: eluent, MeCN−100 mM KH_2PO_4 aq (12:88); flow rate, 0.5 mL min^{-1}; column temperature, 30 °C; detection, 265 nm. Both **2** and **4** eluted as two peaks (t_R values of 9.2 and 10.0 min for **2** and 9.3 and 10.2 min for **4**), with the areas of the two peaks from one compound being almost identical. The fractions corresponding to the peaks were desalted using a Sep-Pak Light C18 cartridge and the CD spectra were recorded. The amounts of fast- and late-eluting enantiomers of **2** (**2**-1 and **2**-2) were 0.459 mg (91.6% ee) and 0.516 mg (84.5% ee), respectively. The amounts of fast- and late-eluting enantiomers of **4** (**4**-1 and **4**-2) were 0.354 mg (100% ee) and 0.467 mg (95.1% ee), respectively. CD (MeOH) **2**-1 λ_{max} ($\Delta\epsilon$): 204 (−38.0), 217 (10.2), 236 (−4.2), 266 (6.3); **2**-2: 204 (31.8), 214 (−7.2), 234 (2.9), 266 (−5.4); **4**-1: 218 (−5.9), 223 (0.06), 241 (−6.2), 252 (1.9), 283 (2.0), 299 (−0.02); **4**-2: 220 (2.0), 225 (−2.5), 241 (5.3), 252 (−1.1), 268 (1.0), 284 (−1.7), 299 (1.4).

Isolation of 1−5 from Elicited Oat Leaves. Oat leaves (3.5 g) were floated in the elicitor solution (100 mL) for 72 h. After 1 mL of AcOH was added, the elicitor solution was applied onto an ODS column that had been equilibrated with H_2O−AcOH (100:1). The column was washed with H_2O−AcOH (100:1) and eluted with MeOH−H_2O (40:60). This MeOH−H_2O (40:60) fraction was concentrated, and the residue was dissolved in 50 μL of MeOH. The solution was subjected to preparative HPLC with a Mightysil RP-18 GP (150 mm long, 4.6 mm i.d.) column. A linear gradient with two solvents [A: H_2O−TFA (1000:1) and B: MeOH] was applied at a flow rate of 0.8 mL min^{-1}. The mobile phase at the initiation of each run was A:B = 80:20, and the ratio was increased to 50:50 within 40 min. The eluate from 18 to 33 min after injection was fractionated to 15 fractions (0.8 mL each). The fractions containing **1**−**5** were separately subjected to preparative HPLC with a Mightysil RP-18 GP (150 mm long, 4.6 mm i.d.) column. The mobile phase was generated by two solvents—H_2O−TFA (1000:1, A) and MeCN (B). A linear gradient of A:B from 90:10 to 30:70 within 30 min was applied with a flow rate of 0.8 mL min^{-1}. The fractions containing **1** (t_R 19.6 min, 51.5 μg), **2** (t_R 19.1 min, 19.7 μg), **3** (t_R 19.8 min, 41.0 μg), **4** (t_R 20.6 min, 124.8 μg), and

5 (t_R 27.1 min, 37.0 μg) were collected. UV spectra were recorded on photodiode array detector.

1: UV (MeCN−0.1% TFA in H_2O) λ_{max} (rel. abs.): 218 (100), 233 sh (67), 282 (13), 304 sh (21); ion-spray MS *m*/*z* (rel. int.): 679 $[M + Na]^+$ (9), 657 $[M + H]^+$ (100), 639 $[M + H - H_2O]^+$ (6); product ion scan (precursor ion: *m*/*z* 657) *m*/*z* (rel. int.): 639 (100), 621 (13), 515 (18), 458 (6), 336 (6);

2: UV (MeCN−0.1% TFA in H_2O) λ_{max} (rel. abs.): 216 (100), 228 sh (84), 267 sh (17), 324 (12); ion-spray MS *m*/*z* (rel. int.): 697 $[M + Na]^+$ (6), 675 $[M + H]^+$ (57), 657 $[M + H - H_2O]^+$ (100), 533 $[M + H - H_2O - C_7H_8O_2]^+$ (27); product ion scan (precursor ion: *m*/*z* 675) *m*/*z* (rel.int.): 639 (100), 515 (76), 354 (62), 297 (64), 259 (44);

3: UV (MeCN−0.1% TFA in H_2O) λ_{max} (rel. abs.): 230 (100), 260 (49), 319 (16); ion-spray MS *m*/*z* (rel. int.): 697 $[M + Na]^+$ (13), 675 $[M + H]^+$ (5), 657 $[M + H - H_2O]^+$ (100), 639 $[M + H - 2(H_2O)]^+$ (3), 288 $[C_{15}H_{14}O_5N]^+$ (3); product ion scan (precursor ion: *m*/*z* 675) *m*/*z* (rel. int.): 639 (20), 486 (12), 458 (8), 323 (8), 288 (100);

4: UV (MeCN−0.1% TFA in H_2O) λ_{max} (rel. abs.): 203 (100), 229 sh (53), 264 (21), 319 (7); ion-spray MS *m*/*z* (rel. int.): 697 $[M + Na]^+$ (11), 675 $[M + H]^+$ (1), 657 $[M + H - H_2O]^+$ (100), 639 $[M + H - 2(H_2O)]^+$ (4), 288 $[C_{15}H_{14}O_5N]^+$ (5); product ion scan (precursor ion: *m*/*z* 675) *m*/*z* (rel. int.): 639 (9), 504 (4), 486 (8), 323 (7), 288 (100);

5: UV (MeCN−0.1% TFA in H_2O) λ_{max} (rel. abs.): 224 (100), 260 (39), 328 (16); ion-spray MS *m*/*z* (rel. int.): 697 $[M + Na]^+$ (24), 675 $[M + H]^+$ (100), 657 $[M + H - H_2O]^+$ (17), 522 $[M + H - C_7H_7O_3N]^+$ (20), 370 (4); product ion scan (precursor ion: *m*/*z* 675) *m*/*z* (rel. int.): 639 (26), 504 (19), 486 (20), 370 (44), 352 (41), 288 (100).

The purified **1**, **2**, and **4** were subjected to chiral HPLC with a CHIRALCEL OD-RH column. The same HPLC conditions that were used for the separation of the enantiomers of synthesized compounds were applied. Compounds **1**, **2**, and **4** were eluted as two peaks corresponding to the enantiomers with the areas of the two peaks being almost identical. The purified compound **3** was dehydrated to **6** by refluxing in formic acid in the same way as described for the synthesized compound. The resulting compound **6** was analyzed by chiral HPLC and the two peaks corresponding to the enantiomers were observed with almost identical peak areas.

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Supporting Information Available: Experimental details; NMR spectral data of **1**−**5** (Tables S1−S6); the figure showing key NMR spectral data and structures of previously reported compounds that are similar to candidate compounds of **1a** (Figure S1); NMR spectra of **1**−**5** (Figure S2−S13), (^{15}N)5-hydroxy-2-nitrobenzoic acid (Figure S14−S16), (^{15}N)5-hydroxyanthranilic acid (Figure S17−S19), (^{15}N)avenanthramide B (Figure S20−S22), and **2*** (Figure S23−S25). This material is available free of charge via the Internet at http://pubs.acs.org.

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