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Relative and Absolute Stereochemistry of Quinadoline B, an Inhibitor of Lipid Droplet Synthesis in Macrophages

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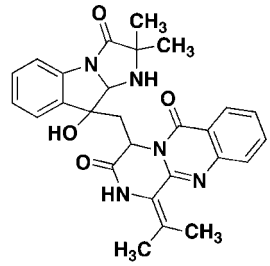
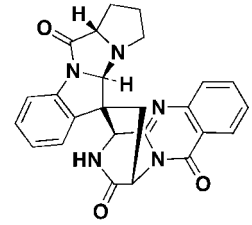
ABSTRACT



Quinadoline A (1)



Quinadoline B (2)

New fungal metabolites, designated quinadolines A (1) and B (2), were isolated from culture broth of *Aspergillus* sp. FKI-1746, and their structures were elucidated by NMR spectroscopy. The complete relative and absolute stereochemistry of 2 was determined by X-ray crystallography and amino acid analysis using a chiral column. Quinadolines moderately inhibited lipid droplet synthesis in mouse macrophages.

In the previous study, isobisvertinol was discovered as an inhibitor of lipid droplet synthesis in mouse macrophages from the culture broth of *Aspergillus* sp. FKI-1746.[1] Fungi are reported to produce secondary metabolites in response to culture condition.[2] Further precise analysis of metabolites obtained under various culture conditions led to the discovery of new inhibitors, designated quinadolines A (**1**) and B (**2**), in medium fermented under shaking and static conditons, respectively. In this study, their isolation, structure elucidation, and biological activity and the relative and absolute stereochemistry of **2** are described. Fungal strain *Aspergillus* sp. FKI-1746 was used to produce **1** and **2**. Compound **1** was isolated from culture broth fermented for 6 days under shaking conditions. The whole broth (20 L) was treated with ethanol, and the supernatant was concentrated under reduced pressure. The resulting aqueous layer was extracted twice with ethyl acetate. The organic layer was concentrated to give a dark brown oil (35.5 g). This oil was applied to a silica gel column (350 g) and eluted stepwise with a $CHCl_3-CH_3OH$ solvent system (500 mL each). The fraction (100% $CHCl_3$) containing **1** was concentrated, and the resulting oil (5.0 g) was subjected to a second silica gel column (50 g). After the column was washed with hexane,

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materials were eluted with a similar $CHCl_3-CH_3OH$ solvent system (300 mL each). Compound **1** was recovered from the 50:1 fraction, which was concentrated to give a yellow brown oil (432 mg). The oil was dissolved in a small amount of CH_3CN, subjected to an ODS column (4 g), and eluted stepwise with 30%, 50%, 70%, and 100% CH_3CN (15 mL each). Crude **1** was recovered from the 50% CH_3CN fraction, which was purified with preparative HPLC under the following conditions: column, PEGASIL ODS (Senshu Sci. i.d. 20 × 250 mm); solvent, 20% CH_3CN; flow rate, 6 mL/min; detection, UV at 210 nm. The fraction eluted as a peak with a retention time of 28 min was collected and concentrated to give a yellow powder (11.7 mg). Enriched **1** was repurified with preparative HPLC under the same conditions, except that 20% CH_3CN containing 0.05% trifluoroacetic acid was used as a solvent. The fraction eluted as a peak with a retention time of 22 min was collected and concentrated to give pure **1** (6.5 mg) as a white powder.

Compound **2** was isolated from culture broth fermented for 10 days under static conditions. The whole broth (3 L) was extracted with ethyl acetate to give a dark brown oil (5.8 g). This oil was applied to a silica gel column (30 g), and materials were eluted stepwise with 100:0, 100:1, 50:1, 10:1, and 1:1 (v/v) of $CHCl_3-CH_3OH$ solvents (500 mL each). Compound **2** was recovered from the 50:1 fraction, which was concentrated to yield a yellow powder (974.8 mg). The materials were finally purified with preparative HPLC (column, PEGASIL ODS (i.d. 20 × 250 mm); solvent, 35% CH_3CN; flow rate, 6 mL/min; detection, UV at 210 nm). The fraction eluted as a peak with 31 min was collected and concentrated to give pure **2** (5.3 mg) as a white powder.

Quinadoline B (**2**) showed a molecular ion peak at *m*/*z* 440 $(M + H)^+$ in FAB-MS, and the molecular formula $C_{25}H_{21}N_5O_3$ was assigned on the basis of HRFAB-MS [*m*/*z* 440.1771 $(M + H)^+$, Δ +0.5 mmu], requiring 18 degrees of unsaturation. IR absorptions at 3448, 1716, and 1623 cm^{-1} indicated the presence of an amide linkage. The ^1H and ^{13}C NMR spectra of **2** showed 21 proton and 25 carbon signals, and the multiplicity of carbon signals was classified into 4 sp^3 methylenes, 8 sp^2 methines, 4 sp^3 methines, 1 sp^3 quaternary carbon, 5 sp^2 quaternary carbons, and 3 amide carbonyl carbons by analysis of HSQC data. The connectivity of proton and carbon atoms was established by HSQC (Table 1). Analysis of $^1H-^1H$ COSY revealed the presence of the five partial structures, I−V, shown by the bold lines in Figure 1. Furthermore, $^1H-^{13}C$ long-range couplings of ^2J and ^3J were measured in the HMBC spectrum. First, it became clear that an indoline moiety is connected to a proline moiety and a δ-lactam moiety due to the following observations: (1) Cross-peaks from H21 (δ 7.42) and H23 (δ 7.10) to C25 (δ 136.8) and from H24 (δ 7.50) to C20 (δ 137.7) suggested the presence of 1,2-disubstituted benzene, which contained the partial structure I. (2) Cross-peaks from H15 (δ 1.76, 2.36) to C18 (δ 69.4) and from H17 (δ 1.80, 1.90) to C19 (δ 174.1) in HMBC experiments and chemical shifts of C15 (δ 56.5) and C18 (δ 69.4) indicated a proline moiety, which contained the partial structure II. (3) The cross-peak from H14 (δ 4.93) to C19 in HMBC experiments and chemical shifts of C14 (δ 91.8) and C19 (δ 174.1) showed that a heterocyclic five-membered ring should be formed with C14, N15, C18, C19, and N19 (Figure 1). (4) Cross-peaks from H14 and H24 to C13 (δ 53.5) suggested linkages between C13 and C25 and between C13 and C14. The chemical shift of C20 (δ 137.7) showed a connection between N19 and

Table 1. ^1H (400 MHz) and ^{13}C NMR (100 MHz) Chemical Shifts of **1** and **2** (in $CDCl_3$)

	quinadoline A (**1**)		quinadoline B (**2**)	
position	δ_c	δ_H	δ_c	δ_H
1	168.0		170.7	
2	120.5		60.6	4.62 d
3	145.2		151.1	
4	146.9		146.6	
5	127.6	7.70 dd	127.1	7.64 dd
6	134.9	7.79 ddd	135.0	7.74 ddd
7	127.6	7.50 ddd	127.8	7.50 ddd
8	127.0	8.29 dd	127.3	8.28 dd
9	119.8		120.9	
10	160.4		158.7	
11	51.3	5.87 dd	53.0	5.69 dd
12	40.2	2.35 dd, 2.65 dd	33.9	1.86 dd, 2.97 dd
13	74.4		53.5	
14	77.9	5.37 s	91.8	4.93 s
15	65.7		56.5	1.76 m, 2.36 dt
16	173.9		25.0	1.40 m
17	137.8		29.7	1.80 m, 1.90 m
18	115.9	7.54 dd	69.4	3.74 dd
19	130.4	7.32 ddd	174.1	
20	125.5	7.11 ddd	137.7	
21	124.2	7.33 dd	116.8	7.42 dd
22	136.7		129.6	7.28 ddd
23	132.7		126.7	7.10 ddd
24	21.9	2.02 s	123.9	7.50 dd
25	21.2	2.42 s	136.8	
26	25.6	1.50 s		
27	24.8	1.62 s		
CONH		7.98 brs		7.70 d

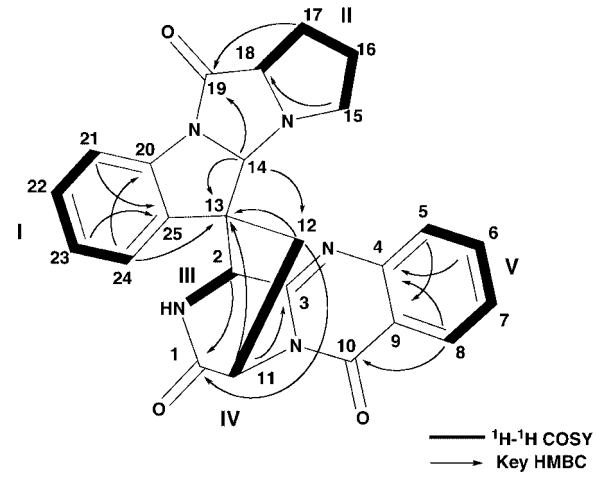

Figure 1. $^1H-^1H$ COSY and HMBC correlations of **2**.

C20, resulting in the formation of an prolinyl-indoline moiety. (5) Cross-peaks from H2 (δ 4.62) to C1 (δ 170.7), from H11 (δ 5.69) to C13, and from H12 (δ 1.86, 2.97) to C1 and C13 showed a connection between the partial structures III and IV, indicating that a δ-lactam ring is attached to the indoline moiety. Second, it became clear that a quinazolinone ring is fused with the δ-lactam moiety due to the following observations: (1) Cross-peaks from H5 (δ 7.64) to C9 (δ 120.9), from H6 (δ 7.74) to C4 (δ 146.6), and from H8 (δ 8.28) to C4 suggested the presence of 1,2-disubstituted benzene, which contained the partial structure V. (2) The cross-peak from H8 to C10 suggested that an amide carbonyl group was attached to C9. This was supported by the chemical shift of C10 (δ 158.7).

Taking the molecular formula into consideration, the presence of an imino group with a remaining sp^2 quaternary carbon is suggested as a residual unit. The chemical shifts of C3 and C4 showed that a nitrogen atom and C3 in the imino group are connected to C4 and N10[3], respectively, suggesting the presence of a quinazolinone ring, which contained 1,2-disubstituted benzene. Furthermore, the remaining unsaturation number indicated that a heterocyclic six-membered ring should be formed with C1, C2, C3, C11, N1, and N10 (Figure 1). The chemical shift of C11 (δ 53.0) and the cross-peak from H11 to C3 indicated linkages between C11 and N10. Moreover, the chemical shift of C2 (δ 60.6) showed that C2 should be linked to C3, resulting in the formation of a tetrayclic ring containing a δ-lactam ring. Taken together, the structure of **2** was deduced as shown in Figure 1.

To confirm the structure, **2** was crystallized for X-ray crystallographic analysis. Compound **2** yielded a good crystal from a methanol solvent. The ORTEP drawing of **2** is shown in Figure 2. The structure of quinadoline B, including the relative stereochemistry, was elucidated as shown in Figure 2.

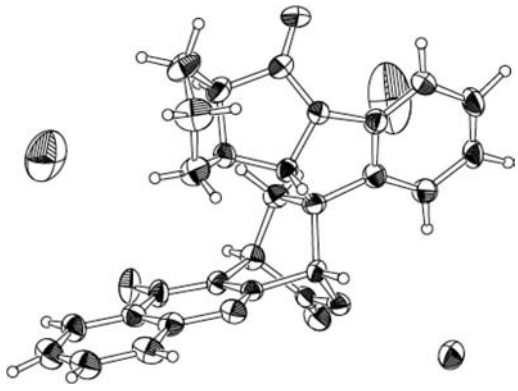

Figure 2. ORTEP drawing for **2**.

A fungal metabolite fiscalin A, originally isolated as a substance P antagonist,[4] is structurally related to **2**. Wong *et al*. reported that an alanine residue buried in the fiscalin molecule was released by acid hydrolysis, and they analyzed its stereochemistry to determine its absolute stereochemistry. Similarly, compound **2** had a proline in the molecule; therefore, we tried to elucidate the absolute stereochemistry of **2** by analyzing the proline residue presumably obtained by acid hydrolysis of **2** at 110 °C for 21 h using 6 N HCl. The hydrolysate was derivatized with NBD-F (4-fluoro-7-nitrobenzo-2-oxa,-1,3-diazole),[5] and the NBD-proline fraction (including *D*- and *L*-proline) was first isolated by reversed-phase HPLC with an ODS column as previously reported.[6] *D*- and *L*-proline in the fraction were subsequently enantioseparated by HPLC under the following conditions: column, Sumichiral OA-3100 (Sumika Chemical Analysis Service, i.d. 4.6 × 250 mm); solvent, methanol/acetonitrile (95/5) containing 1 mM citric acid; flow rate, 1 mL/min; detection, fluorescent intensity at 530 nm with excitation at 470 nm; temperature, 33 °C. Authentic *D*- and *L*-proline derivatives were eluted with retention times of 33.2 and 36.3 min, respectively. Thus, the proline residue obtained from **2** was identified as *L*-form. Taken together, the absolute stereochemistry of **2** was determined to be 2*S*, 11*S*, 13*S*, 14*R*, 18*S*.

Quinadoline A (**1**) showed a molecular ion peak at *m*/*z* 486 $(M + H)^+$ in FAB-MS, and the molecular formula $C_{27}H_{27}N_5O_4$ was assigned on the basis of HRFAB-MS [*m*/*z* 486.2126 $(M + H)^+$, Δ −0.2 mmu], requiring 17 degrees of unsaturation. Comparison of ^{1}H and ^{13}C NMR spectral data of **1** with those of **2** indicated that **1** shares the same core of indoline and quinazolinone moieties as **2**, but three methylenes (C15 to C17) and two methines (C2 and C18) in **2** are missing in **1**, indicating modification of the proline and δ-lactam moieties of **2**. Analyses of ^{1}H−^{1}H COSY and HMBC spectral data clarified their structural differences (Figure 3). First, an α-aminoisobutyric acid moiety is present in place of the proline moiety in **2** due to the following observations: (1) Cross-peaks from H26 (δ 1.50) and H27 (δ 1.62) to C15 (δ 65.7) and C16 (δ 173.9) indicated the presence of an α-aminoisobutyric acid moiety. In fact, the chemical shift of C15 is comparable to that of a carbon at α-position of amino acid. (2) Cross-peaks from H14 (δ 5.37) to C13 (δ 74.4) and C16 indicated that the indoline moiety extends to the α-aminoisobutyric acid moiety to form tricyclic structure A (Figure 3). Second, the δ-lactam moiety in **2** is replaced by a 2-methyl-2-propene due to the following observations: (1) Cross peaks from H24 (δ 2.02) and H25 (δ 2.42) to C2 (δ 120.5) and C23 (δ 132.7) showed the presence of 2-methyl-2-propene moiety. (2) Cross-peaks from H24 and H25 to C3 (δ 145.2) indicated that the 2-methyl-2-propene moiety is attached to the quinazolinone ring at C3. (3) The sequence of H11 (δ 5.87) and H12 (δ 2.35, 2.65) was determined by ^{1}H−^{1}H COSY. The cross-peak from H12 to C1 (δ 168.0) and the chemical shift of C11 (δ 51.3) showed that C11 is attached to N10. Furthermore, taking the degree of unsaturation and the molecular formula into consideration, a six-membered heterocyclic ring should be fused with the quinazolinone moiety to form the tricyclic

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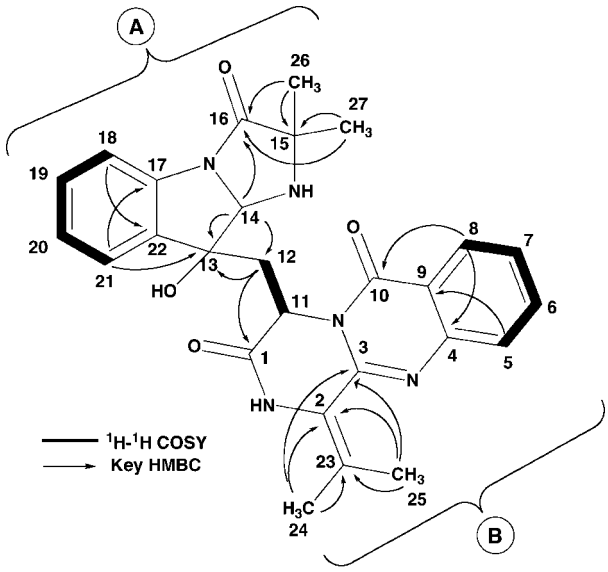


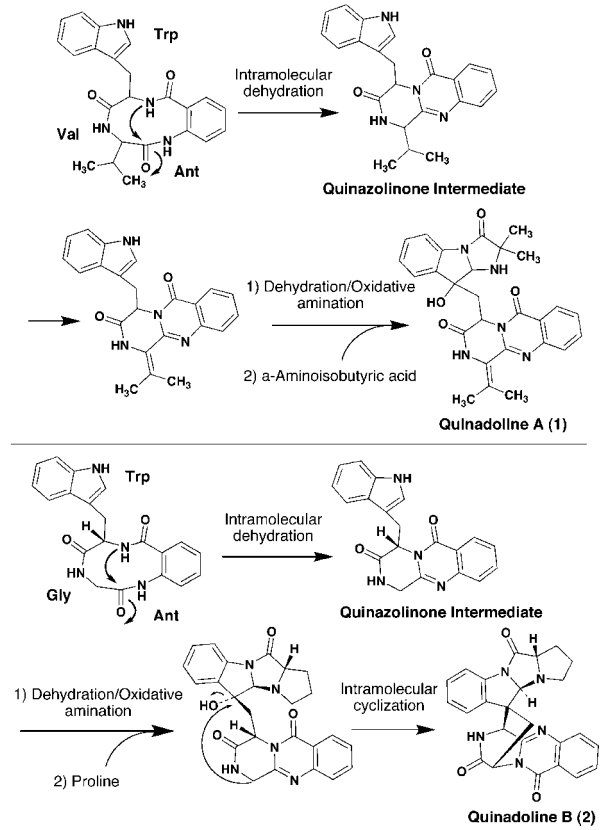
Figure 3. ^{1}H−^{1}H COSY and HMBC correlations of **1**.

structure B (Figure 3). Finally, cross-peaks from H14 to C12 (δ 40.2) and C13 and from H12 to C13 indicated that the two tricyclic structures A and B should be connected as shown in Figure 3. This conclusion was supported by the chemical shifts of **1** comparable to those of fiscalin C, except for C2 and C23.[4]

Fungal quinazolinone metabolites such as glyantrypine, fiscalins, and fumiquinazolines have been reported[7] into which anthranilic acid, tryptophan, and an amino acid are incorporated. Among them, only glyantrypin was demonstrated to be biosynthesized from anthranilic acid, tryptophan, and glycine.[7] Quinazolinones belong to this family, but one more amino acid appears to be fused with the indoline moiety. The plausible biosynthetic pathways of **1** and **2** are shown in Scheme 1. Fundamental quinazolinone intermediates for **1**/**2** are generated via intramolecular dehydration from anthranilic acid, tryptophan, and valine/glycine, respectively. After that, α-aminoisobutyric acid/proline is then bound to the indoline moiety via the dehydration and the oxidative amination to form **1** or precursor of **2**. Furthermore, in the case of **2**, subsequent intramolecular cyclization occurs between C2 and C13 to form **2** containing a δ-lactam ring.

Compounds **1** and **2** inhibited lipid droplet synthesis in mouse macrophages in a dose-dependent manner (50−200 μM, data not shown). No cytotoxic effects on macrophages were observed even at 300 μM. An assay for the synthesis of cholesteryl ester (CE) and triacylglycerol (TG), the main constituents of lipid droplets in macrophages, was then carried out by the method described previously.[8] Compound **1** inhibited CE synthesis with an IC_{50} value of 93 μM but had almost no effect on TG synthesis even at the highest dose (192 μM). On the other hand, **2** inhibited both CE and TG synthesis with IC_{50} values of 125 and 243 μM, respectively.

Scheme 1. Hypothetical Biosynthesis **1** and **2**



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Supporting Information Available: NMR and X-ray crystal data of quinadolines A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Quinadoline A (**1**): white amorphous solid; $[\alpha]^{22}_D$ −19.56 (*c* 0.01, MeOH); IR (KBr) ν_{max} 3432, 1685, 1596 cm^{-1}; UV (MeOH) λ_{max} 227 (ε 25682), 300 (ε 10180); ^{1}H and ^{13}C NMR data (Table 1); FAB-MS *m/z* 486 $(M + H)^+$; HRFAB-MS *m/z* 486.2126 $(M + H)^+$, calcd for $C_{27}H_{27}N_5O_4$ 486.2141.

(10) Quinadoline B (**2**): white amorphous solid; $[\alpha]^{22}_D$ −44.68 (*c* 0.01, MeOH); IR (KBr) ν_{max} 3448, 1716, 1623 cm^{-1}; UV (MeOH) λ_{max} 226 (e 30187), 267 (ε 9916), 278 (ε 9408), 303 (ε 3726), 315 (ε 2977); ^{1}H and ^{13}C NMR data (Table 1); FAB-MS *m/z* 440 $(M + H)^+$; HRFAB-MS *m/z* 440.1771 $(M + H)^+$, calcd for $C_{25}H_{21}N_5O_3$ 440.1723.