# Isoaurostatin, a Novel Topoisomerase Inhibitor Produced by *Thermomonospora alba*

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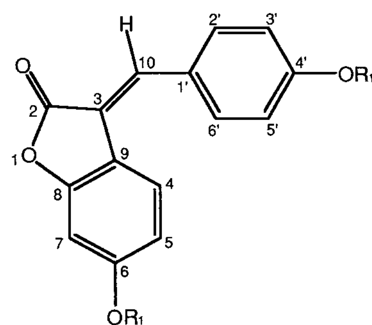
A novel inhibitor of topoisomerase I designated as isoaurostatin (**1**) was isolated from the culture filtrate of *Thermomonospora alba* strain No. 1520. The structure of **1** was determined to be 6,4′-dihydroxyisoaurone on the basis of spectroscopic (NMR and MS) methods. Compound **1** inhibited the relaxation activity of calf thymus topoisomerase I in a noncompetitive manner and did not inhibit the relaxation and decatenation of human placenta topoisomerase II. Compound **1** is an inhibitor belonging to cleavable complex-nonforming type without DNA intercalation.

Topoisomerases I and II [1,2] are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, and the enzymes are involved in producing the necessary topological and conformational changes in DNA that are critical to many cellular processes such as replication, recombination, and transcription.[3] In addition to their normal cellular functions, the enzymes are known as important cellular targets of some antitumor drugs.[4] In the search for topoisomerase inhibitors, we have screened various microorganisms and found four kinds of inhibitors, designated as 2280-DTI, 2890-DTI, macrostatin, and topostatin.[5−9] Topostatin isolated from *Thermomonospora alba* strain No. 1520 was found to be a novel inhibitor of cleavable complex-nonforming type that inhibits both topoisomerases I and II[7,8] containing both a novel 14-membered ring peptide and a terpenoid.[9] We have now found an additional inhibitor in the culture filtrate and named it isoaurostatin (**1**). Taxonomy of the producing organism was reported in the previous paper.[7] This report describes the isolation, structure elucidation, and the inhibitory properties of **1**.

## Results and Discussion

Cultivation of *Thermomonospora alba* strain No. 1520 was carried out under the cultural conditions described in the Experimental Section. Mycelial growth peaked on day four and thereafter gradually declined. Inhibitory activity of the culture filtrate against topoisomerase I increased rapidly on day four and was maximized on day five, followed by a rapid decline. For an optimum yield of the inhibitor, the culture broth was harvested on day five.

Compound **1** exhibited characteristic IR absorption bands at 3284 $cm^{-1}$ (hydroxyl) and 1630 $cm^{-1}$ ($\alpha,\beta$-unsaturated carbonyl). Compound **1** showed a $[M - H]^+$ peak at *m*/*z* 253 ($C_{15}H_9O_4$), and its acetate derivative (**1a**) showed a $M^+$ peak at *m*/*z* 338 and fragment peaks at *m*/*z* 296 and 254 in the negative FABMS and EIMS spectra, respectively. These results suggested the presence of two hydroxyl groups in **1**. In the $^1H$ NMR and $^1H-^1H$ COSY spectra of **1**, the proton signals at $\delta$ 6.83, 6.91, and 7.94 were assigned to ABX type (1,2,4-trisubstituted benzene ring), and the proton signals at $\delta$ 6.79 and 7.36 were assigned to $A_2B_2$ type (1,4-disubstituted benzene ring). The signal at $\delta$ 8.25 (1H, s) was assigned to a vinyl proton. The $^{13}C$ NMR spectrum of **1** exhibited 15-carbon signals, which were attributed to a 1,4-disubstituted benzene ring, a 1,2,4-trisubstituted benzene ring, a vinyl group ($\delta$ 123.6 and 152.9), and an ester carbonyl group ($\delta$ 174.8), suggesting that **1** is a stilbene derivative. Full assignments of the $^1H$ and $^{13}C$ signals were secured by the $^1H-^1H$ COSY, HMQC, and HMBC spectra. In the HMBC spectra (8 Hz) of **1**, the proton signal at $\delta$ 7.94 (H-4) correlated with the carbon signals at $\delta$ 157.6 (C-8), 162.8 (C-6), and 174.8 (C-2), and the proton signal at $\delta$ 8.25 (H-10) correlated with the carbon signals at $\delta$ 122.6 (C-1′), 123.6 (C-3), 157.6 (C-3), and 174.8 (C-2), as shown in Table 1. In the differential NOE spectra, the proton signal at $\delta$ 8.25 (H-10) correlated with $\delta$ 7.36 (C-2′ and H-6′), the signal at $\delta$ 8.32 (H-4) correlated with $\delta$ 7.18 (H-5) of **1a**, and the double bond was *E* configured. From the above evidence, the structure of **1** was elucidated as shown.




The effects of **1** on various topoisomerases and DNA-related enzymes were examined and are summarized in Table 2. For comparison, topostatin,[9] camptothecin,[10] and doxorubicin[11] were also examined as specific inhibitors against topoisomerases I and II. Compound **1** showed different inhibitory properties from those of other topoisomerase inhibitors; **1** inhibited only the relaxation activities of topoisomerases I from wheat germ and calf thymus gland. **1** (67 $\mu$M) gave 50% inhibition ($IC_{50}$) against wheat germ topoisomerase I.

The type of inhibition was determined by Lineweaver−Burk plots[12] of substrate concentrations against the rate of relaxation of supercoiled pBR322 DNA by topoisomerase I from calf thymus gland in the presence or absence of **1**.

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**Table 1.** $^{13}$C NMR and $^{1}$H NMR Assignments and $^{1}$H−$^{13}$C Long-Range Correlations of **1** by $^{1}$H−$^{1}$H COSY, HMQC, and HMBC in DMSO-$d_6$

| position | $\delta_C$ | $\delta_H$ | cross-peaks ($\delta_C$) in HMBC spectrum |
|---|---|---|---|
| 3 | 123.6 C | | |
| 4 | 127.3 CH | 7.94 (d, 8.5) | 174.8 (C-2), 157.6 (C-8), 162.8 (C-6) |
| 5 | 115.3 CH | 6.91 (dd, 2.4, 8.5) | 116.6 (C-9), 102.2 (C-7), 162.8 (C-6) |
| 6 | 162.8 C | | |
| 7 | 102.2 CH | 6.83 (d, 2.4) | 116.6 (C-9), 162.8 (C-6), 115.3 (C-5) |
| 8 | 157.6 C | | |
| 9 | 116.6 C | | |
| 10 | 152.9 CH | 8.25 (s) | 122.6 (C-1′), 123.6 (C-3), 174.8 (C-2), 157.6 (C-8) |
| 1′ | 122.6 C | | |
| 2′ | 130.1 CH | 7.36 (d, 8.5) | 157.2 (C-4′), 123.6 (C-3′) |
| 3′ | 115.0 CH | 6.79 (d, 8.5) | 122.6 (C-1′), 157.2 (C-4′) |
| 4′ | 157.2 C | | |
| 5′ | 115.0 CH | 6.79 (d, 8.5) | 157.2 (C-4′), 123.6 (C-3′) |
| 6′ | 130.1 CH | 7.36 (d, 8.5) | 122.6 (C-1′), 157.2 (C-4′) |

**Table 2.** Inhibitory Activity

| | inhibition (IC$_{50}$, $\mu$M) | | | |
|---|---|---|---|---|
| enzyme | **1** | topostatin | camptothecin | doxorubicin |
| topoisomerase I from wheat germ[a] | 67 | 1 | 3 | >200 |
| topoisomerase I from calf thymus gland[a] | 307 | 17 | 17 | >200 |
| topoisomerase I from NIH 3T3 cell[a] | >400 | 47 | 12 | >200 |
| topoisomerase I from Vero cell[a] | >400 | 17 | 27 | >200 |
| topoisomerase I from A549 cell[a] germ[a] | >400 | 55 | 4 | >200 |
| topoisomerase I from COLO 201 cell[a] | >400 | 50 | 17 | >200 |
| topoisomerase I from HeLa cell[a] | >400 | 29 | 9 | >200 |
| topoisomerase II from human placenta[a] | >400 | 4 | >300 | 1 |
| topoisomerase II from human placenta[b] | >400 | 4 | >300 | 1 |
| *Alu* I from *Arthrobacter luteus* | >400 | >250 | >300 | 24 |
| *Bam* HI from *Bacillus amyloliquefaciens* | >400 | 185 | >300 | >200 |
| *Eco* RI from *Escherichia coli* | >400 | >250 | >300 | >200 |
| *Hin* dIII from *Haemophilus influenzae* | >400 | 17 | >300 | 96 |
| *Pst* I from *Providencia stuartii* | >400 | 19 | >300 | >200 |
| *Sca* I from *Streptomyces caespitosus* | >400 | 7 | >300 | 25 |
| RNase A from bovine pancreas | >400 | >250 | >300 | >200 |
| DNase I from bovine pancrea | >400 | >250 | >300 | >200 |
| DNase II from porcine spleen | >400 | >250 | >300 | >200 |
| T4 ligase from *Escherichia coli* | >400 | >250 | >300 | 73 |
| telomerase from COLO201 cell | >400 | >250 | >300 | |

[a] Relaxation activity. [b] Decatenation activity.

As shown in Figure 1, **1** inhibited the enzyme activity noncompetitively with respect to pBR322 DNA, exhibiting a $K_i$ value of 0.5 mM, and the Michaelis constant ($K_m$ value) of the enzyme was 11.1 nM. From the results, **1** was considered to bind with a different site from the binding site of the substrate DNA in the enzyme molecule.

Topoisomerase I inhibitors of cleavable complex-forming type stabilize the cleavable complex (topoisomerase−DNA reaction intermediate) and inhibit DNA from rejoining the reaction of topoisomerase I, which is the inhibitory mechanism of the inhibitors.[10,13] Therefore, the inhibitors induce the nicked DNA in the cleavage assay.[11] The cleavage assay was carried out to determine whether or not **1** is an inhibitor of cleavable complex-forming type. Camptothecin was used as an inhibitor of cleavable complex-forming type against topoisomerase I. As shown in Figure 2, camptothecin induced the nicked DNA with increasing concentrations. Unlike camptothecin, **1** did not induce the nicked DNA. This result suggested that **1** is an inhibitor of cleavable complex-nonforming type which does not inhibit topoisomerase I by stabilizing the cleavable complex.

Some topoisomerase inhibitors are DNA intercalators.[14,15] To determine whether **1** has the ability to intercalate into DNA strands, the CD (circular dichroism) spectral change of DNA by the addition of **1** was measured. CD spectra are sensitive to the conformational changes of DNA by intercalators.[16] Camptothecin and doxorubicin,

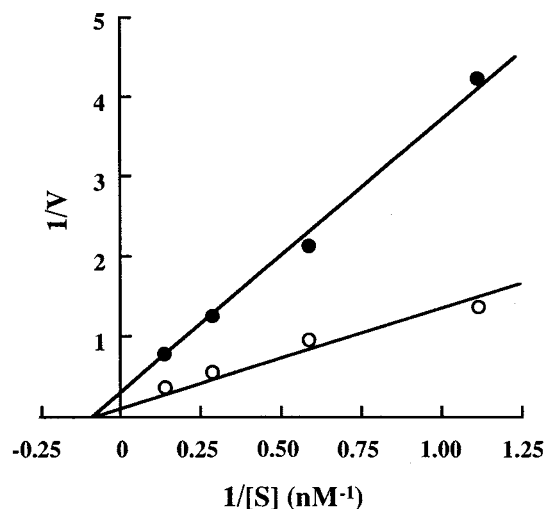


**Figure 1.** Lineweaver−Burk plots of substrate concentration against rate of relaxation by topoisomerase I with (●) and without (○) **1**. The reaction was performed in 20 $\mu$L of the mixture containing 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.6 $\mu$g of BSA, 1 unit of topoisomerase I from calf thymus gland, 785 $\mu$M **1**, and supercoiled pBR322 DNA. The concentrations of the supercoiled DNAs were 0.9, 1.7, 3.5, and 6.9 nM. After incubation for 40 min at 37 °C, the mixture was run into agarose gel at 50 V for 2 h, and relaxed pBR322 DNA on the gel was measured. The reciprocals of relaxed DNA concentrations were expressed as 1/V.

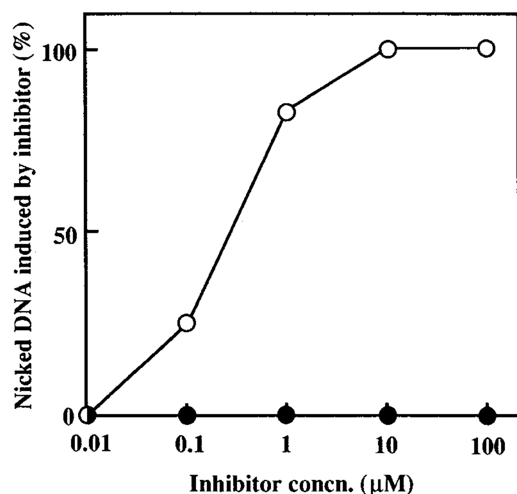


**Figure 2.** Stabilization of topoisomerase-cleavable complex by **1** (●) and camptothecin (○). The reaction was performed in 20 $\mu$L of the mixture containing 50 mM Tris-HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.6 $\mu$g of BSA, 20 units of topoisomerase I from calf thymus gland, 0.15 $\mu$g of supercoiled pBR322 DNA, and inhibitor. After incubation for 40 min at 37 °C, the reaction was terminated by adding 5 $\mu$L of the stop solution containing 5% SDS and 12.5 $\mu$g of proteinase K and thereafter incubated for an additional 30 min at 37 °C. The mixture was run into agarose gel containing 0.1% SDS at 50 V for 2 h. After agarose gel electrophoresis, the nicked pBR322 DNA on the gel was measured.

being a nonintercalator and an intercalator, respectively, were used as controls at the same concentration. As shown in Figure 3, the spectra of DNA changed greatly with increasing concentrations of doxorubicin and ethidium bromide, respectively. On the other hand, spectral changes by **1** and camptothecin did not occur. Therefore, it is clear that **1** has no ability to intercalate into DNA.

Thus, compound **1** is a specific inhibitor against topoisomerase I from wheat germ and calf thymus gland, and it is different from inhibitors causing DNA damage such as cleavable complex-forming inhibitors and DNA intercalators.

## Experimental Section

**General Experimental Procedures**. $^1$H NMR, $^{13}$C NMR, $^1$H−$^1$H COSY, HMQC, HMBC, and differential NOE spectra were measured using a JNM α-500 spectrometer. NMR spectra with tetramethylsilane (TMS) as an internal standard were taken in DMSO-$d_6$ solution at 500 MHz ($^1$H) and 125 MHz ($^{13}$C). FABMS and EIMS spectra were recorded with JMS-DX 303 HF and JMS GC-Mate spectrometers, respectively. UV and IR spectra were measured using Hitachi U-2000 and Hitachi 270-30 spectrophotometers, respectively. CD spectra were recorded in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 1 mM $MgCl_2$, and salmon sperm DNA (25 $\mu$g/mL) using a Jasco J-720 spectropolarimeter. CD spectral change was obtained by subtraction of the CD of the reagent from the CD of DNA with reagent. Extracts were chromatographed on silica gel 60 (100−210 mesh, Merck) and Florisil (100−200 mesh, Nacalai Tesque).

**Enzymes, Substrates, and Inhibitors**. Topoisomerase I (EC 5.99.1.2) from calf thymus gland, T4 DNA ligase (EC

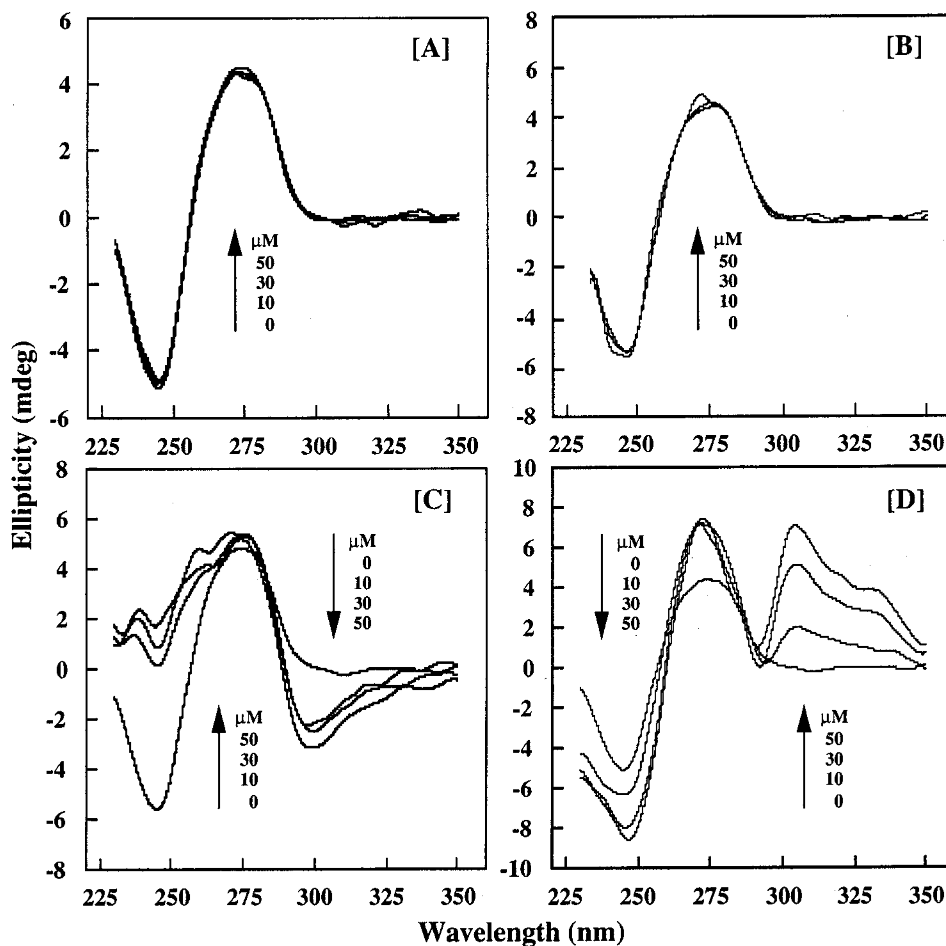


**Figure 3.** CD spectral changes of DNA by the addition of **1** [A], camptothecin [B], doxorubicin [C], and ethidium bromide [D]. CD spectra were recorded in 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM KCl, 1 mM $MgCl_2$, salmon sperm DNA (25 $\mu$g/mL), and inhibitor. The concentrations of inhibitors were 0, 10, 30, and 50 $\mu$M.

6.5.1.1), *Bam* HI (EC 3.1.23.6), *Eco* RI (EC 3.1.23.13), *Hin* dIII (EC 3.1.23.21), and supercoiled pBR322 DNA were purchased from MBI Fermentas. DNase I (EC 3.1.21.1) from bovine pancreas, DNase II (EC 3.1.22.1) from porcine spleen, RNase A (EC 3.1.27.5) from bovine pancreas, and RNA from yeast extract were obtained from Sigma. Salmon sperm DNA was purchased from Boehringer Mannheim GmbH. *Alu* I (EC 3.1.23.1), *Sca* I (EC 3.1.21.4), and *Pst* I (EC 3.1.23.31) were purchased from Gibco BRL. Topoisomerase II (EC 5.99.1.3) from human placenta and kinetoplast DNA were purchased from Topogen. Camptothecin and doxorubicin hydrochloride were obtained from Aldrich and Sigma, respectively. For preparation of topoisomerase I from COLO 201 (human colon carcinoma), HeLa (human cervix carcinoma), A549 (human lung carcinoma), Vero (African green monkey kidney), or NIH3T3 (mouse embryo) cells, each cell cultured for 5 days was washed with phosphate-buffered saline and harvested by centrifugation. The cell pellets ($1 \times 10^6$ cells) were resuspended in 200 $\mu$L of the cold lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 mM $\beta$-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 10% glycerin) and kept on ice for 30 min. The lysate was centrifuged and the supernatant was used as enzyme solution of the topoisomerase I obtained from each cell.

**Enzyme Reactions**. Relaxation activities of topoisomerases I and II were measured by detecting the conversion of supercoiled pBR322 DNA to its relaxed form.[2,17] Decatenation activity of topoisomerase II was measured by detecting the conversion of catenated kinetoplast DNA (kDNA) to minicircle monomers.[2,17] DNA cleavage activity of topoisomerase I (cleavage assay) was determined by measuring the increase of nicked pBR322 DNA induced by inhibitors.[11,18] Activities of restriction enzymes (*Alu* I, *Bam* HI, *Eco* RI, *Hin* dIII, *Pst* I, and *Sca* I) and nucleases (DNase I, DNase II, and RNase A) were determined by measuring the concentration of undigested supercoiled pBR322 DNA or RNA after enzyme reactions.[5,6] The assay of T4 DNA ligase was based on ligation of linearized pBR322 DNA which was cleaved by *Hin* dIII.[5,6] Telomerase activity was measured by TRAP-*eze* telomerase detection kit (Intergen Co.). After each enzyme reaction, the incubation mixture was subjected to gel electrophoresis, and DNA or RNA on the gel was measured by a densitometer with a transilluminator (Atto Co., AE-6900M). The assay conditions for inhibitory activity and electrophoresis have been described in the previous papers.[5–9] The inhibitory activity ($IC_{50}$) was defined as the amount of inhibitor that reduced each enzyme activity by 50%.

**Cultural Conditions for Production of 1**. A loopful of mature *Thermomonospora alba* strain No. 1520[7] from yeast malt extract agar slant was inoculated into sterilized S medium composed of 2.0% glucose, 3.0% starch, 1.0% corn steep liquor, 1.0% soybean flour, 0.5% peptone, 0.3% NaCl, and 0.5% $CaCO_3$ at pH 7.0. It was cultivated aerobically for 2 days at 28 °C and 180 rpm on a rotary shaker, termed seed culture. Main culture (S medium) was inoculated with 4.0% of seed culture and cultivated for 5 days at 28 °C for the production of **1**.

**Purification of 1**. After cultivation, mycelia and cellular residues of culture broth were removed by centrifugation at 4000*g* and 5 °C for 15 min. The supernatant was extracted with 2 volumes of ethyl acetate at pH 4.0 and dried in vacuo. The crude extract was applied to a column of silica gel 60 (1.5 × 15 cm) equilibrated with $CHCl_3$–MeOH (30:1) and eluted with $CHCl_3$–MeOH (20:1). The eluates were pooled and applied to a column of Florisil (1.9 × 17 cm) and eluted with $CHCl_3$–MeOH (20:1). The active fractions were pooled and dried *in vacuo* and termed purified isoaurostatin (**1**). Finally, 7.6 mg of **1** was obtained as an amorphous powder from 4 L of the culture filtrate.

A mixture of **1** (5 mg), acetic anhydride (0.3 mL), and pyridine (0.5 mL) was kept standing overnight at room temperature and then evaporated under reduced pressure to give a residue. The residue was chromatographied on silica gel 60 eluting with $CHCl_3$ to gave **1a** (2 mg).

**Isoaurostatin (1):** amorphous powder; UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 247.8 (3.46) nm; IR (KBr) $\nu_{max}$ 3284, 1629, 1585, 1581 $cm^{-1}$; $^1H$ and $^{13}C$ NMR in Table 1; negative HR FABMS *m*/*z* 253.0502 $[M - H]^-$ (calcd for $C_{15}H_9O_4$, 253.0501).

**Isoaurostatin diacetate (1a):** amorphous powder; $^1H$ NMR ($CDCl_3$) $\delta$ 2.32 (3H, s, C-4′-OAc), 2.36 (3H, s, C-6-OAc), 7.17 (2H, d, $J = 8.5$ Hz, H-3′ and H-5′), 7.18 (1H, dd, $J = 1.8$, 8.5 Hz, H-5), 7.32 (1H, d, $J = 1.8$ Hz, H-7), 8.01 (1H, s, H-10), 8.32 (1H, d, $J = 8.5$ Hz, H-4); EIMS *m*/*z* 338 $[M]^+$, 296 $[M - Ac]^+$, 254 $[M - 2Ac]^+$.

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