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Synthesis of Pironetin and Related Analogs:

Studies on Structure-Activity Relationships as Tubulin Assembly Inhibitors

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Pironetin (**1**) and demethylpironetin (**2**) are potent inhibitors of tubulin assembly. They arrested the mammalian cell cycle in M-phase and showed antitumor activity against a murine tumor cell line, P388 leukemia, transplanted in mice. To investigate the chemical and biological properties of **1**, we synthesized several derivatives and investigated the structure-activity relationships. All synthesized derivatives decreased biological activities, such as inhibition of cell cycle progression, and disruption of the microtubule network *in situ*. The most drastic decrease was observed in **6**, **8** and **10**. These results suggested that α,β-unsaturated lactone, chirality at the 7-position bearing a hydroxyl group and the terminal portion of the alkyl chain are important for microtubule inhibitory activity of pironetins.

Tubulin is the main structural component of microtubules, which play an important roles in mitosis, cell signaling and motility in eucaryotes and interacts with a wide variety of small ligands, including divalent cations, guanine nucleotides and antimitotic drugs. These small ligands regulate microtubule dynamics. Paclitaxel and vinblastine bind to different sites of tubulin and show opposite effects *in vitro*; paclitaxel induces microtubule bundling and vinblastine induces microtubule disassembly.[1] We recently reported that tryprostatin A inhibited the microtubule-associated protein-dependent tubulin assembly by binding tubulin on different binding sites of colchicine and vinblastine.[2] The drugs bind to their own sites on the tubulin or microtubule-associated protein, suggesting that novel tubulin binding drugs may be discovered.

Pironetin (**1**)[3,4] and demethylpironetin (**2**)[5] possess plant growth regulatory and immunosuppressive activities. We recently reported that these compounds exhibited antitumor activity in a murine model.[6] In the course of our investigation of the mechanism, we found that **1** and **2** are potent inhibitors of tubulin assembly.[7] Compound **1** directly binds to tubulin and inhibits the tubulin assembly in a vinblastine-like manner. Interestingly, the effective dose of **1** is slightly higher than that of vinblastine with respect to the cytotoxicity; the K_d value determined by the surface plasmon resonance method is 10-fold lower than that of vinblastine. These data indicate that the affinity of pironetin to tubulin is stronger than that of vinblastine but the membrane permeability of **1** may be less than that of vinblastine. Compound **1** and **2** have unique structures containing only one pyran residue and an alkyl chain. The structures are simple compared to those of other M-phase inhibitors. These distinctive features of pironetins from known tubulin binding agents suggest that it is possible to create a new drug useful for cancer therapy from **1** as a lead compound. In this paper, we synthesized several pironetin derivatives to investigate the structurally essential parts for its function.

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Scheme 1. Pironetin derivatives.

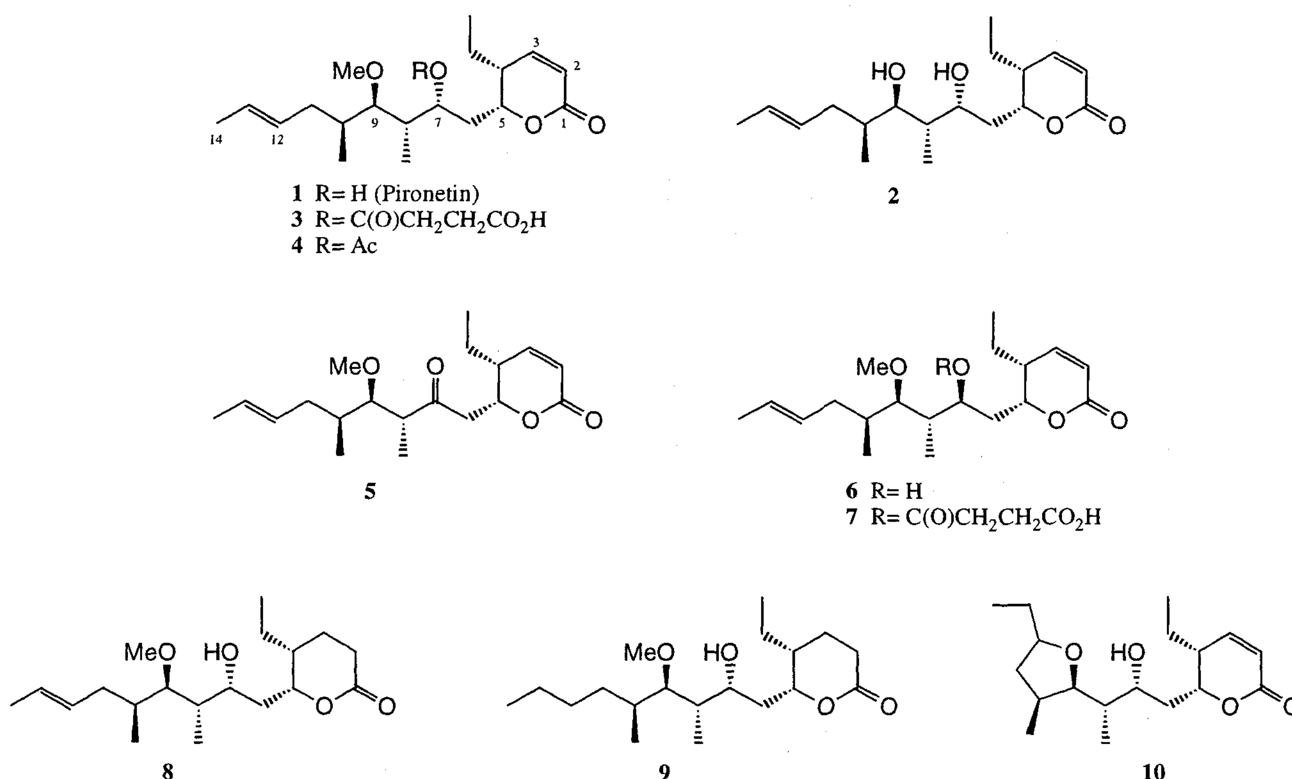

Table 1. Synthesis of the pironetin derivatives.

Compound	Starting material	Reagents and conditions	Yield (%)
1	See reference 8	—	—
2	See reference 8	—	—
3	**1**	succinic anhydride, TsOH, C_6H_6, reflux	76
4	**1**	Ac_2O, pyridine, rt	quant.
5	**1**	Dess-Martin periodinane, CH_2Cl_2, 0°C	94
6	See reference 8	—	—
7	**6**	succinic anhydride, pyridine, DMAP, 90°C	80
8	Naturall-occurring	—	—
9	**1** or **8**	H_2 (1 atm), Lindlar cat., EtOAc, rt	quant.
10	**1**	TMSCl, NaI, MeCN, rt	84

Results and Discussion

Chemistry

Naturally-occurring pironetin (**1**) and demethylpironetin (**2**) were efficiently prepared as shown in our previous paper, and 7-epipironetin (**6**) could also be obtained by the same route.[8] Using these compounds, several analogs were synthesized (Scheme 1 and Table 1). At first, to investigate the need of a C(7)-hydroxyl group, 7-oxopironetin (**5**) was synthesized by Dess-Martin oxidation.[9] Although the acetylation of the 7-hydroxyl group of **1** was achieved in the usual manner (Ac_2O, pyridine) to give **4** in quantitative yield, the esterification with succinic anhydride in pyridine resulted in no reaction even in the presence of an equivalent of DMAP. The C(7) position of **1** was sterically hindered

Table 2. Effects on the cell cycle progression and microtubule network *in situ* of pironetin derivatives.

Compound	Effective concentrations (μg/mL)		Fold [c]
	M-phase arrest [a]	Microtubule disassembly *in situ* [b]	
1	0.005	0.005	—
2	0.005	0.005	1
3	0.2	0.2	40
4	0.5	0.5	100
5	0.5	0.5	100
6	5.0	5.0	1000
7	>10.0	>10.0	>2000
8	5.0	5.0	1000
9	10.0	10.0	2000
10	5.0	5.0	1000

a. Concentration of pironetin derivatives, at which M-phase arrest was induced in 3Y1 cells. The distributions of DNA content in the cell populations were determined by flow cytometry. M-phase arrest was judged by the accumulation of cells containing 4C DNA (20% above against total cells).

b. Concentrations of pironetin derivatives, at which microtubule network was disassembled in 3Y1 cells. The concentrations, which induced microtubule disassembly completely, were determined.

c. Relative concentration folds, that were required for both M-phase arrest and microtubule disassembly *in situ*. The concentration of **1**, required for M-phase arrest and microtubule disassembly, was defined as **1**.

and the reaction with 1,1′-thiocarbonyldiimidazole[10] or phenyl chlorothionoformate[11] intended to prepare a deoxygenation product gave the same results. The acylation, however, was achieved under acidic conditions (*p*-TsOH, benzene, reflux) to give succinate (**3**) in 76% yield. On the other hand, the reaction of 7-epimer (**6**) with succinic anhydride in pyridine in the presence of a catalytic amount of DMAP afforded **7** in 80% yield. The low reactivity of the hydroxyl group of **1** might be caused by the strong hydrogen bonding most probably to the pyron oxygen suggested by the ^{1}H NMR spectrum; –OH=δ 3.48 (1H, d, J=2.6 Hz).

The importance of a highly reactive α,β-unsaturated lactone moiety could be expected, in comparison with the other tubulin binders, but we fortunately got a suitable compound, $\Delta^{2,3}$-dihydro derivative (**8**), as a very minor natural product component. The absolute structure of **8** was determined by converting it to **9**, whose specific rotation was identical with that of a hydrogenation product of **1**. In order to get demethylation product **2**, treatment of **1** with TMSI, prepared from TMSCl and NaI *in situ*,[12] afforded **10**, which would be a cyclization product *via* **2**.

Biological Activities

Inhibition of cell cycle progression and disruption of the microtubule network *in situ* were examined, and the biological activities of the synthetic derivatives were found to be decreased (Table 2). Esterification of the 7-hydroxyl residues with succinate (**3**) and acetate (**4**) resulted in 40- and 100-times decrease of activity *in situ*. Drastic decrease of activity was observed in 7-epipironetin (**6**). Interestingly, 7-ketopironetin (**5**) showed only 100-times decrease of activity, whereas 7-epipironetin (**6**) lost almost all inhibitory activity *in vitro* and *in situ*, respectively. These results suggest that the chirality of the 7-hydroxyl group is important for interaction with tubulin and its interaction is thought to be hydrogen bonding.

Another derivative showing drastic decrease of activity was $\Delta^{2,3}$-dihydro derivative (**8**) with a saturated lactone moiety. Recently, it has been reported that lactone-containing drugs bind to their target biomolecules covalently. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region.[13] A leptomycin B derivative, which contained a $\Delta^{2,3}$-dihydrolactone structure, is also inactive and does not inhibit CRM1-mediated protein nuclear export. As the same result was obtained in the case of pironetin, an α,β-unsaturated lactone might be necessary for tubulin assembly inhibition through covalent binding.

A drastic decrease in activity was also observed in **10**. We previously reported that epoxypironetin, a naturally-occurring pironetin derivative, which contains an epoxy ring instead of the double bond in the branched alkyl side

chain, had decreased inhibitory activity. These results suggest that the structure of the terminal portion of the alkyl chain is important for microtubule inhibitory activity of pironetins.

In conclusion, α,β-unsaturated lactone, chirality of the hydroxyl group at the 7-position, and the terminal portion of the alkyl chain are important for microtubule inhibitory activity of pironetins, and it is suggested that pironetin has a minimum structure as a tubulin assembly inhibitor.

Experimental

Cell Culture, Cell Cycle Analyses and Immunofluorescence Procedures

Rat normal fibroblast 3Y1 cells were grown in DULBECCO's modified EAGLE's medium supplemented with 10% (v/v) fetal calf serum in a humidified atmosphere containing 5% CO_2.

For cell cycle analysis, the exponentially growing 3Y1 cells were treated with various concentrations of compounds. After 24 hours treatment, DNA contents were quantified by flow cytometry. Immunofluorescence observation of tubulin was performed as described previously.[2] The cytoskeletons were photographed using a cooled CCD camera (Olympus PROVIS AX70, Tokyo, Japan).

Compounds

All melting pounts (mps) were uncorrected. Infrared spectra (IR) were measured on a Jasco FT/IR-230 spectrometer. Proton magnetic resonance spectra (^{1}H-NMR) were recorded on a BRUKER AC300 spectrometer. Chemical shifts are reported in parts per million (δ) relative to internal chloroform (δ 7.26). Optical rotations were measured on a Jasco DIP 1000 polarimeter. Analytical thin-layer chromatography was carried out using 0.25 mm Merck silica gel 60 F_{254} precoated glass -backed plates. Compounds were visualized by ultraviolet light (254 mm), iodine vapor, or phosphomolybdic acid spray reagent. Preparative TLC was carried out using 0.5 mm Merck silica gel 60. F_{254} precoated glass -backed plates. All column chromatography was performed on Merck silica gel 60. All solvents were reagent grade. Dichloromethane and benzene were distilled from calcium hydride and stored over 4A-molecular sieves.

(2*Z*,4*R*,5*R*,7*R*,8*S*,9*R*,10*S*,12*E*)-7-(3-Carboxypropanoyloxy)-4-ethyl-9-methoxy-8,10-dimethyl-2,12-tetradecadien-5-olide (3)

A mixture of **1** (10.0 mg, 0.031 mmol), succinic anhydride (30 mg, 0.30 mmol), *p*-toluenesulfonic acid monohydrate (1 mg), and benzene (3 ml) was heated under reflux for 12 hours. After cooling, brine (10 ml) was added and the mixture was extracted with diethyl ether (3×10 ml). The combined organic phase was washed with 2% aqueous sodium acetate (10 ml), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by preparative TLC developed with *n*-hexane/ethyl acetate/acetic acid (50 : 50 : 1) to give **3** (10.0 mg, 76%) as a colorless oil.

(2*Z*,4*R*,5*R*,7*R*,8*S*,9*R*,10*S*,12*E*)-7-Acetoxy-4-ethyl-9-methoxy-8,10-dimethyl-2,12-tetradecadien-5-olide (4)

A solution of **1** (10.0 mg, 0.031 mmol) and acetic anhydride (30 μl, 0.32 mmol) in pyridine (1 ml) was stirred at room temperature for 12 hrous. The mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel (5 g) eluted with *n*-hexane/ethyl acetate (3 : 1) to give **4** (11.3 mg, quantitative) as a white solid. Recrystallization from *n*-hexane gave colorless needles; mp 71~72°C; $[\alpha]_D^{28}$ $-134°$ (*c* 0.08, $CHCl_3$); IR (KBr) ν_{max} 2966, 1726, 1703, 1380, 1261, 1240, 1135, 1097, 1063, 1025, 962, 833 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.81 (3H, d, *J*=6.8 Hz), 0.88 (3H, d, *J*=7.0 Hz), 0.97 (3H, t, *J*=7.5 Hz), 1.43~1.82 (4H, m), 1.66 (3H, d, *J*=5.5 Hz), 1.89~2.17 (4H, m), 2.06 (3H, s), 2.22~2.30 (1H, m), 2.88 (1H, dd, *J*=2.0, 9.4 Hz), 3.39 (3H, s), 4.46 (1H, ddd, *J*=3.6, 6.0, 7.7 Hz), 5.26 (1H, dt, *J*=1.8, 6.8 Hz), 5.38 (1H, dt, *J*=15.2, 5.9 Hz), 5.46 (1H, dq, *J*=15.2, 5.5 Hz), 6.02 (1H, d, *J*=9.8 Hz), 7.00 (1H, dd, *J*=6.2, 9.8 Hz).

(2*Z*,4*R*,5*R*,8*R*,9*R*,10*S*,12*E*)-4-Ethyl-9-methoxy-8,10-dimethyl-7-oxo-2,12-tetradecadien-5-olide (5)

To a solution of **1** (10.0 mg, 0.031 mmol) in dichloromethane (3 ml) at 0°C was added Dess-Martin periodinane (16 mg, 0.038 mmol). After being stirred for 5 hours, 10% aqueous sodium thiosulfate (5 ml) and saturated aqueous sodium bicarbonate (5 ml) was added. The mixture was stirred at room temperature for 1 hour and then extracted with dichloromethane (2×20 ml). The combined organic phase was washed with brine (20 ml), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was chromatographed on silica gel (5 g) eluted with *n*-hexane/ethyl acetate (3 : 1) to give **5** (9.3 mg, 94%) as a colorless oil; $[\alpha]_D^{28}$ $-215°$ (*c* 0.11, $CHCl_3$); IR (film) ν_{max} 2966, 1723, 1457, 1383, 1246, 1093, 1025, 968, 825 cm^{-1};

^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.86 (3H, d, J=6.9 Hz), 0.96 (3H, t, J=7.5 Hz), 0.97 (3H, d, J=7.3 Hz), 1.42~1.68 (3H, m), 1.67 (3H, d, J=5.6 Hz), 1.97 (1H, ddd, J=6.4, 6.8, 13.6 Hz), 2.13 (1H, dt, J=13.6, 6.4 Hz), 2.46~2.53 (1H, m), 2.83 (1H, m), 2.84 (1H, dd, J=7.3, 17.7 Hz), 3.16 (1H, dd, J=6.2, 17.7 Hz), 3.27 (1H, dd, J=2.4, 8.3 Hz), 4.98 (1H, ddd, J=3.8, 6.2, 7.3 Hz), 5.38 (1H, dt, J=15.5, 6.4 Hz), 5.47 (1H, dq, J=15.5, 5.6 Hz), 6.04 (1H, d, J=9.8 Hz), 7.01 (1H, dd, J=6.1, 9.8 Hz); HR-MS *m/z* (M^+): Calcd for $C_{19}H_{30}O_4$: 322.2144, Found: 322.2160.

(2*Z*,4*R*,5*R*,7*S*,8*S*,9*R*,10*S*,12*E*)-7-(3-Carboxypropanoyloxy)-4-ethyl-9-methoxy-8,10-dimethyl-2,12-tetradecadien-5-olide (**7**)

A mixture of **6** (10.0 mg, 0.031 mmol), succinic anhydride (30 mg, 0.30 mmol), 4-dimethylaminopyridine (1 mg), and pyridine (3 ml) was stirred at 90°C for 12 hours. After cooling, the mixture was concentrated *in vacuo* and brine (10 ml) was added to the residue. The mixture was extracted with diethyl ether (3×10 ml). The combined organic phase was washed with 2% aqueous sodium acetate (10 ml), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by preparative TLC developed with *n*-hexane/ethyl acetate/acetic acid (50 : 50 : 1) to give **7** (10.5 mg, 80%) as a colorless oil.

(4*R*,5*R*,7*R*,8*S*,9*R*,10*S*,12*E*)-4-Ethyl-7-hydroxy-9-methoxy-8,10-dimethyl-12-tetradecen-5-olide (**8**)

This compound was isolated from *Streptomyces* NK10958. Recrystallization from *n*-hexane gave colorless needles; mp 81°C; $[\alpha]_D^{27}$ +65.7° (*c* 0.10, $CHCl_3$); IR (KBr) ν_{max} 3528, 2963, 1725, 1467, 1383, 1318, 1261, 1134, 1083, 1035, 970 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.95 (3H, t, J=7.1 Hz), 0.95 (3H, d, J=6.3 Hz), 0.98 (3H, d, J=7.0 Hz), 1.20~1.32 (2H, m), 1.47~1.99 (8H, m), 1.66 (3H, d, J=5.4 Hz), 2.03~2.13 (1H, m), 2.49 (1H, dt, J=20.0, 6.9 Hz), 2.57 (1H, dt, J=20.0, 7.6 Hz), 2.98 (1H, dd, J=4.5, 5.8 Hz), 3.44 (1H, d, J=3.0 Hz), 3.47 (3H, s), 4.18 (1H, br d, J=9.4 Hz), 4.67 (1H, dt, J=9.2, 3.0 Hz), 5.37 (1H, dt, J=15.2, 5.1 Hz), 5.44 (1H, dq, J=15.2, 5.4 Hz); *Anal.* Calcd. for $C_{19}H_{34}O_4$: C, 69.90; H, 10.50. Found: C, 69.68; H, 10.44.

(4*R*,5*R*,7*R*,8*S*,9*R*,10*S*)-4-Ethyl-7-hydroxy-9-methoxy-8,10-dimethyltetradecan-5-olide (**9**)

From **1**: To a solution of **1** (10.0 mg, 0.031 mmol) in ethyl acetate (3 ml) was added Lindlar catalyst (2 mg, Aldrich Chemical Co.). After being stirred vigorously under hydrogen atmosphere (1 atm) at room temperature for 5 hours, the mixture was filtered through a celite pad and then concentrated *in vacuo*. The residue was chromatographed on silica gel (5 g) eluted with *n*-hexane/ethyl acetate (3 : 1) to give **9** (10.1 mg, quantitative) as a white solid. Recrystallization from *n*-hexane gave colorless needles; mp 84°C; $[\alpha]_D^{26}$ +34.9° (*c* 0.07, $CHCl_3$); IR (KBr) ν_{max} 3531, 2964, 2927, 1737, 1463, 1382, 1352, 1316, 1276, 1152, 1097, 1076, 1034, 985, 957 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.90 (3H, t, J=7.2 Hz), 0.95 (3H, d, J=7.5 Hz), 0.95 (3H, d, J=6.6 Hz), 0.99 (3H, d, J=7.1 Hz), 1.10~1.42 (6H, m), 1.46~1.68 (4H, m), 1.70~1.86 (4H, m), 1.90~2.00 (1H, m), 2.49 (1H, dt, J=20.0, 7.5 Hz), 2.57 (1H, dt, J=20.0, 6.8 Hz), 2.97 (1H, dd, J=4.5, 6.5 Hz), 3.47 (3H, s), 3.48 (1H, d, J=3.0 Hz), 4.16 (1H, br d, J=9.2 Hz), 4.66 (1H, dt, J=9.0, 3.3 Hz); *Anal.* Calcd. for $C_{19}H_{36}O_4$: C, 69.47; H, 11.05. Found: C, 69.53; H, 11.04.

From **8**: To a solution of **8** (10.0 mg, 0.031 mmol) in ethyl acetate (3 ml) was added Lindlar catalyst (2 mg, Aldrich Chemical Co.). After being stirred vigorously under hydrogen atmosphere (1 atm) at room temperature for 5 hours, the mixture was filtered through a celite pad and then concentrated *in vacuo*. The residue was chromatographed on silica gel (5 g) eluted with *n*-hexane/ethyl acetate (3 : 1) to give **9** (10.1 mg, quantitative) as a white solid. Recrystallization from *n*-hexane gave colorless needles; $[\alpha]_D^{26}$ +34.0° (*c* 0.10, $CHCl_3$); The ^{1}H-NMR and IR spectral data, melting point were identical with above data.

(2*Z*,2′*R*,3′*S*,4*R*,5*R*,5′*R* or *S*,7*R*,8*S*)-4-Ethyl-8-(5′-ethyl-3′-methyloxolan-2′-yl)-7-hydroxy-2-nonen-5-olide (**10**)

To a solution of **1** (10.0 mg, 0.031 mmol) and sodium iodide (19 mg, 0.13 mmol) in acetonitrile (3 ml) at room temperature was added chlorotrimethylsilane (15 μl, 0.12 mmol). After being stirred for 12 hours, 10% aqueous sodium thiosulfate (3 ml) and saturated aqueous sodium bicarbonate (3 ml) was added. The mixture was extracted with ethyl acetate (3×10 ml). The combined organic phase was washed with brine (10 ml), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was chromatographed on silica gel (5 g) eluted with *n*-hexane/ethyl acetate (3 : 1) to give **10** (8.0 mg, 84%) as a colorless oil; $[\alpha]_D^{28}$ −122° (*c* 0.11, $CHCl_3$); IR (film) ν_{max} 3479, 2963, 2931, 2876, 1725, 1463, 1383, 1256, 1098, 1068, 1028, 1004, 822 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.82 (3H, d, J=7.1 Hz), 0.89 (3H, t, J=7.4 Hz), 0.93 (3H, d, J=7.1 Hz), 0.97 (3H, t, J=7.6 Hz), 1.39~1.77 (7H, m), 1.89 (1H, ddd, J=1.4, 9.4, 14.2 Hz), 1.96~2.04 (1H, m), 2.21~2.30 (1H, m), 2.32~2.39 (1H, m), 3.69 (1H, d,

J=9.5 Hz), 3.73 (1H, dd, J=4.2, 10.8 Hz), 3.85 (1H, br t, J=9.5 Hz), 4.04 (1H, dq, J=9.3, 6.3 Hz), 4.83 (1H, dt, J=9.4, 3.5 Hz), 6.02 (1H, dd, J=1.0, 9.8 Hz), 7.02 (1H, dd, J=5.8, 9.8 Hz). The stereochemistry at C5′ position (ethyl substituent on tetrahydrofuran ring) was not determined, but the ^{1}H-NMR spectra suggested it is a single isomer.

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