Isolation of Labeled 9-Dihydrobaccatin III and Related Taxoids from Cell Cultures of *Taxus canadensis* Elicited with Methyl Jasmonate

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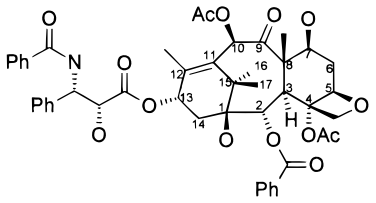
Cell suspension cultures of *Taxus canadensis* rapidly produced paclitaxel (**1**) and other taxoids in response to elicitation with methyl jasmonate. Three of these taxoids, of potential value in the synthesis of taxoid analogues, have been isolated from cell cultures of *Taxus canadensis* and identified as 13-acetyl-9-dihydrobaccatin III (**2**), baccatin VI (**3**), and 9-dihydrobaccatin III (**4**). Of these metabolites, 9-dihydrobaccatin III (**4**) has not been isolated from any *Taxus* species, whereas 13-acetyl-9-dihydrobaccatin III (**2**) and baccatin VI (**3**) have been isolated from a number of natural sources. 2D NMR techniques, mass spectrometry, and partial synthesis were used to rigorously elucidate the structure and stereochemistry of these natural products.

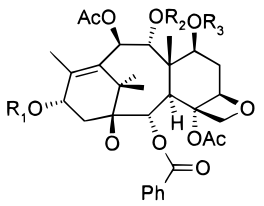
Paclitaxel (**1**), a diterpenoid extracted from the bark of the pacific yew (*Taxus brevifolia*) and other yew species, is one of the most recent and effective drugs in the treatment of cancer (Figure 1). Its activity against ovarian and breast cancer, its biology, and its biochemistry have been thoroughly reviewed.[1] It may also prove to be a useful treatment against many other types of cancer, including nonsmall-cell lung cancer and[2] gastric carcinoma,[3] and of noncancerous conditions, such as polycystic kidney disease.[4]

The greatest concentrations of paclitaxel (**1**) are about 0.02% to 0.1% of the dry weight of *T. brevifolia* bark.[5] The low concentration of paclitaxel (**1**) in the bark, the lethal nature of bark harvest, and the slow growth of the tree have led to the development of alternative means of paclitaxel (**1**) production. Semisynthetic methods have been developed for the production of paclitaxel (**1**) (and its analogue docetaxel or Taxotere) using advanced taxane diterpenoid (taxoid) metabolites isolated from readily available natural sources, such as 10-deacetylbaccatin III extracted from leaves of yew.[6] However, with increasing applications in medicine, especially in the treatment of additional cancer types, and its use much earlier in the course of intervention, the supply and cost of these drugs will remain important issues. Total synthesis of paclitaxel (**1**) has been achieved by several elegant routes, but the yields are too low to be commercially feasible.[7,8]

The supply of paclitaxel (**1**) and semisynthetic precursors must rely on biological methods of production, either from plants in the genus *Taxus* or in cell cultures derived from them.[9] Inducible *Taxus* cell cultures, capable of producing significant amounts of paclitaxel (**1**) and related taxoids, provide an excellent tool for the study of this complex biosynthetic pathway.[10−12] We have previously reported the production of up to 117 mg·L^{-1} of paclitaxel (**1**) within 5 days of elicitation.[10] However, paclitaxel (**1**) never accounted for more than 20% of the total taxoids in the culture, based on detector response. Here we report on the isolation and identification of several other taxoids present in elicited cultures and accounting for as much as 60% of the total taxoids in an elicited cell culture of *Taxus canadensis* Marsh (Taxaceae).



1 Paclitaxel (Taxol)



2 13-Acetyl-9-dihydrobaccatin III
R_1=Ac; R_2=R_3=H

3 Baccatin VI
R_1= R_2=R_3=Ac

4 9-Dihydrobaccatin III
R_1= R_2=R_3=H

Figure 1. Structure of paclitaxel (taxol) (**1**), 13-acetyl-9-dihydrobaccatin III (**2**), baccatin VI (**3**), and 9-dihydrobaccatin III (**4**).

Results and Discussion

Measurable differences in paclitaxel (**1**) concentration between elicited cultures and the control are detectable within 8 h of elicitation and are apparent under normal analytical conditions within 24 h.[10] Paclitaxel (**1**) concentration increases in the culture medium until 12 to 14 days after elicitation and then begins to decline as the cells senesce.[10] However, paclitaxel (**1**) is only one of several taxoids that accumulate in the medium and accounts for only 13−20% of the total taxoids present in the elicited culture.[10] Two other taxoids, eluting at approximately 7 and 19 min, together account for 39−62% of the total taxoids present in an elicited culture, depending on the culture age (Figure 2). These compounds were isolated by

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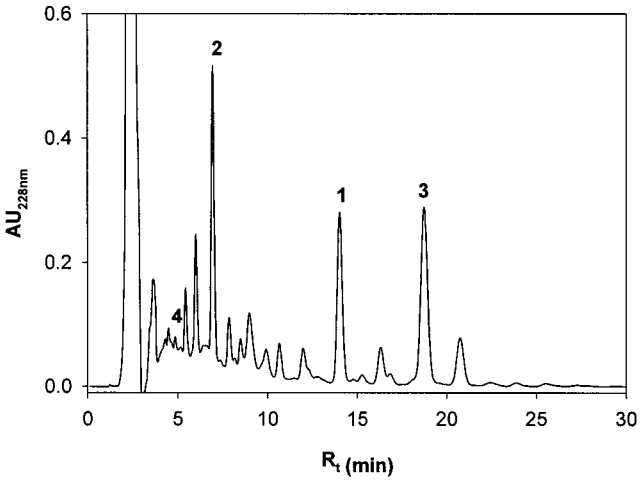


Figure 2. HPLC chromatogram of taxoids produced in suspension cultures of a 19 day-old suspension culture of *T. canadensis*. Culture was elicited with 200 μM methyl jasmonate on day 7 of the culture cycle and taxoids recovered from the medium 12 days later. Paclitaxel (**1**) concentration in the culture medium is 50 mg·L^{-1}. Peak identity: paclitaxel (**1**), 13-acetyl-9-dihydrobaccatin III (**2**), baccatin VI (**3**), 9-dihydrobaccatin III (**4**).

multiple injections of a partially purified and concentrated extract of the suspension medium on an analytical column with subsequent fractionation. Although the method was more time-consuming than separation using semipreparative HPLC, the use of an autosampler and fraction collector allowed for unattended collection of 1- to 8-mg quantities of as many as 25 peaks of interest in 48 h. The fractions collected were often sufficiently pure for subsequent NMR analysis, eliminating the need for additional purification.

To identify the structure of taxoid **2** (Figure 1), 2D NMR techniques including DQ−COSY, HMQC, and HMBC were used to establish unambiguously the chemical shifts of each of the protons and carbons as well as their points of attachment. The relative stereochemistry of the substituents on the carbon framework of the molecule was established from a 2D ROESY experiment. The spectroscopic data of taxoid **2** were found to be identical to those reported in the literature for 13-acetyl-9-dihydrobaccatin III isolated from various natural sources.[13] Thus, it was established unambiguously that taxoid **2** was 13-acetyl-9-dihydrobaccatin III.

From the ^{1}H and ^{13}C NMR spectra of taxoid **3**, it was deduced that it was very similar to that of 13-acetyl-9-dihydrobaccatin III (**2**) and that it also possessed two extra acetyl groups compared to **2**. Thus, taxoid **3** was tentatively identified as baccatin VI. After a careful comparison of the spectroscopic data of taxoid **3** with literature values for baccatin VI, it was observed that they were identical.[14−16] However, to confirm unambiguously the structure of taxoid **3**, its partial synthesis was carried out by acetylating the free alcoholic group at C-7 and C-9 of 13-acetyl-9-dihydrobaccatin III (**2**) with acetic anhydride and (dimethylamino)-pyridine (Scheme 1). The acetylated compound obtained from the reaction and the naturally isolated **3** coeluted on the HPLC and had identical ^{1}H NMR spectra[14−16] (Figure 3). Thus taxoid **3** was identified as baccatin VI.

Similarly, based on the ^{1}H and ^{13}C NMR spectra and MS data, taxoid **4** (Figure 1) was tentatively assigned as 9-dihydrobaccatin III. To assign the structure unambiguously, its partial synthesis was carried out from 13-acetyl-9-dihydrobaccatin III (**2**) as reported in the literature.[17,18] It was synthesized by reacting taxoid **2** with methyllithium, resulting in the deacetylation of the acetyl group at C-13 (Scheme 1). The compound obtained from the reaction and

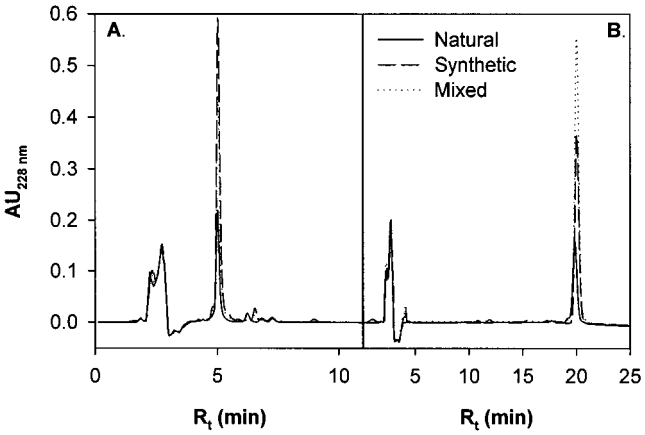
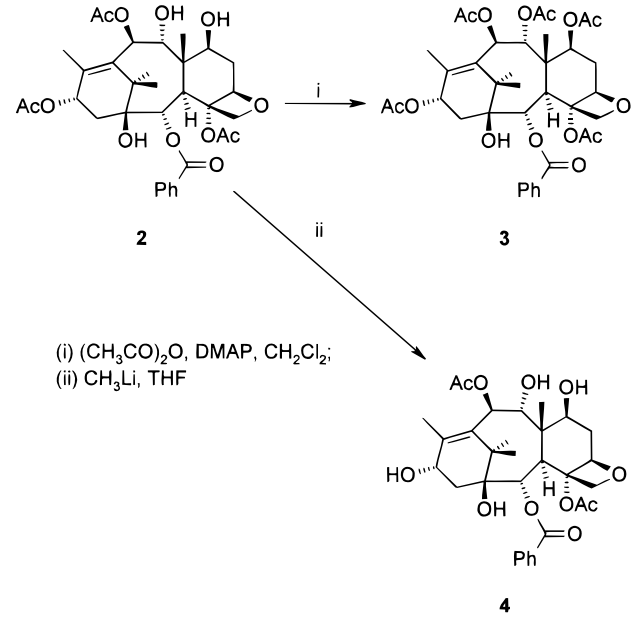


Figure 3. HPLC chromatograms of natural, synthetic, and natural + synthetic mixtures of (**A**) 9-dihydrobaccatin III (**4**) and (**B**) baccatin VI (**3**).

Scheme 1. Semisynthesis of Baccatin VI (**3**) and 9-Dihydrobaccatin III (**4**) from 13-Acetyl-9-dihydrobaccatin III (**2**)



the naturally isolated taxoid coeluted on the HPLC column (Figure 3). The ^{1}H NMR spectrum of 9-dihydrobaccatin III (**4**) reported in the literature was identical to that of the synthetic compound and that of the taxoid isolated from *Taxus canadensis*.[17,18] Thus, taxoid **4** was identified as 9-dihydrobaccatin III. This is the first report of the isolation of this compound from a natural source, and of any of these three 9-dihydrobaccatin III derivatives from a *Taxus* cell suspension culture.

Compounds **2**−**4** share the same basic structure, with the only differences being the degree to which the hydroxyl groups have been acylated. The difference between baccatin III and 9-dihydrobaccatin III (**4**) is the presence of the hydroxyl group at C-9, instead of the carbonyl found in paclitaxel and the more common taxoids (Figure 1).

Although the role of the 9-dihydrobaccatin III (**4**) taxoid derivatives is not clear, we have previously suggested that the immediate precursor to paclitaxel (**1**) is 9-dihydrobaccatin III (**4**), rather than baccatin III.[10] Experiments are currently planned to feed *Taxus* suspension cultures with radiolabeled 9-dihydrobaccatin III to see if the label is incorporated into more highly acylated taxoids.

Due to our interest in the biosynthesis of taxol, feeding studies with labeled acetate were performed on these suspension cell cultures. Biosynthetic studies on plants or needles of *Taxus* species suffer from a number of disadvantages, namely: (a) very low levels of incorporation of the label in the natural product occur, (b) low levels of production of the natural product are evident, and (c) there are difficulties in the isolation of the natural product itself. Carrying out biosynthetic studies in cell cultures is one possible method to circumvent some of these problems. Feeding studies were conducted by adding [1,2-$^{13}C_2$]acetate simultaneously with methyl jasmonate to *Taxus canadensis* suspension cultures. Paclitaxel (**1**) and 13-acetyl-9-dihydrobaccatin III (**2**) were isolated, purified, and analyzed by ^{13}C NMR spectroscopy. It was observed that the labeled acetate was incorporated intact into the acetyl groups at C-4, C-10, and C-13 (J = 30 Hz). No incorporation was observed in the benzoate moiety or in the terpene skeleton, thus supporting the recently proposed hypothesis that geranylgeranyl diphosphate required in the biosynthesis of paclitaxel (**1**) in *Taxus canadensis* is not derived from the mevalonate pathway requiring two acetates.[19] This observation is also consistent with an earlier report that [^{3}H]sodium acetate labeled similar positions of taxoids in stems and needles of *T. canadensis*.[20]

Because paclitaxel (**1**) is almost insoluble in water, a combination of polyethoxylated castor oil and ethanol (Cremophor EL−EtOH) is used to dilute the drug before administering it to patients. Cremophor is toxic, and the use of this delivery vehicle led to some extreme hypersensitivity reactions in some patients in early clinical trials. Now, patients are usually treated with a variety of drugs to block histaminic responses prior to treatment with paclitaxel (**1**).[21]

Consequently, there has been a great deal of interest in the synthesis of promising new taxoid analogues with greater anticancer activity or increased aqueous solubility. One such analogue, 9-dihydrotaxol, synthesized from 13-acetyl-9-dihydrobaccatin III (**2**) isolated from leaves of *T. canadensis*, has recently shown promise as an anti-cancer drug.[17,22] Relative to paclitaxel (**1**), 9-dihydrotaxol has greater stability, is 20 times more soluble in water; demonstrates less toxicity to mice, which allows for a four-fold increase in the daily dosage; and yields a six-fold greater cure rate against human MX-1 solid tumor xenografts in mice.[17,22] Should 9-dihydrotaxol become an important chemotherapeutic agent, *Taxus* cell suspension cultures developed in our laboratory provide a convenient, reliable, renewable, and rapidly produced source of the semisynthetic starting material, 13-acetyl-9-dihydrobaccatin III (**2**).

Experimental Section

General Experimental Procedures. All reactions requiring nonaqueous conditions were performed in oven-dried glassware under a positive pressure of N_2. All solvents were distilled. The term in vacuo refers to the removal of solvent to constant sample weight (<0.05 mmHg). Commercial TLC plates were Merck 60F_{254}. Flash column chromatography was performed according to the method of Still[23] on Merck type 60 Si gel, 230−240 mesh.

Growth Conditions. Cell line CO93P, initiated from *T. canadensis* embryos and maintained as previously described,[9,24] was used for these experiments. A 40-mL culture of 14-day-old cells was used to inoculate 160 mL of fresh B5NB medium, in a 500-mL Erlenmeyer flask with Bellco silicone/foam closures. Cultures were elicited as previously described.[10] Briefly, 7 days after inoculation, 200 μM (final concentration) of filter-sterilized methyl jasmonate was added to the flasks. Cells and medium were harvested at day 14, 7 days after elicitation.

Extraction. The extracellular medium (1200 mL), containing the majority of the taxoids in the culture, was frozen in a −58 °C shell freezer, and reduced to dryness in vacuo on a Labconco lyophilizer. The dried residue was extracted four times with Me_2CO and reduced to dryness in vacuo on a Büchi Rotovapor and yielded 412 mg of dried residue.

Solid-Phase Extraction Purification. The dried residue from the acetone extract was dissolved in 25 mL MeOH, diluted with 25 mL H_2O, and loaded onto a 10-g C_{18} (Varian Mega Bond Elut) solid-phase extraction (SPE) column. The column was washed with 50 mL of MeOH−H_2O (40:60), and taxoids eluted with 50 mL of MeOH−H_2O (80:20). The taxoid extract was evaporated to dryness in vacuo, first on a Büchi Rotovapor followed by freeze-drying on a Labconco lyophilizer. A solution containing 40 mg·mL^{-1} of this dried extract in MeOH was used for purification by HPLC.

Analysis and Purification of Taxoids. Paclitaxel (**1**) and other taxoids were extracted, analyzed, and verified using HPLC methods developed in this laboratory and previously described,[25,26] with minor modifications. The column used was a 250 × 4.6 mm Metachem Taxsil column, 5 μm particle size, with an Upchurch ODS guard column. For purification of individual taxoids, multiple 30-μL injections of the 40 mg·mL^{-1} methanolic SPE-purified suspension extract were made on the analytical column. Fractions were collected and evaporated to dryness for subsequent analysis by HPLC, NMR, and mass spectrometry.

^{13}C Labeling. Six flasks containing 200 mL of a 7-day-old culture were fed a filter-sterilized aqueous solution of [1,2-$^{13}C_2$]sodium acetate to give a final concentration of 2 mM. Cultures were elicited with 200 μM of methyl jasmonate at the same time that the labeled sodium acetate was added. Cultures were harvested at day 16 (9 days after elicitation), and the taxoids were extracted, purified, and analyzed by ^{13}C NMR.

NMR Methods. Nuclear magnetic resonance (NMR) spectra (^{1}H and ^{13}C) were recorded on a Bruker AF-300, a Varian VXR-400S, or a Varian Unity 500 instrument in the specified deuterated solvent with tetramethylsilane (TMS) as an internal standard for ^{1}H spectra and $CDCl_3$ as an internal standard for the ^{13}C spectra. 2D homonuclear ^{1}H NMR, DQCOSY, gradient-enhanced ^{1}H−^{13}C HMQC, and HMBC spectroscopy were performed on a Varian Unity 500 spectrometer operating at 499.93 MHz for proton and 125.72 MHz for carbon.[27,28] ROESY was performed on a Varian Unity 500 MHz spectrometer.

13-Acetyl-9-dihydrobaccatin III (2): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.10 (2H, d, J = 7.1 Hz, C_6H_5COO), 7.62 (1H, t, J = 7.4 Hz, C_6H_5COO), 7.48 (2H, t, J = 8.2 Hz, C_6H_5COO), 6.18 (1H, d, J = 10.9 Hz, H-10), 6.17 (1H, t, J = 9.2 Hz, H-13), 5.76 (1H, d, J = 6.0 Hz, H-2), 4.96 (1H, d, J = 8.7 Hz, H-5), 4.44 (2H, m, H-7, H-9), 4.32 (A of AB, 1H, d, J = 8.2 Hz, H-20A), 4.17 (B of AB, 1H, d, J = 8.2 Hz, H-20B), 3.06 (1H, d, J = 5.5 Hz, H-3), 2.55 (1H, m, H-6A), 2.29 (3H, s, C*H*$_3$COO), 2.22−1.90 (2H, m, H-14), 2.20 (3H, s, C*H*$_3$COO), 2.15 (3H, s, C*H*$_3$COO), 1.95 (3H, d, J = 1.1 Hz, H-18), 1.97−1.82 (1H, m, H-6B), 1.82 (3H, s, H-19), 1.78−1.66 (2H, m, C−O*H*), 1.68 (3H, s, H-17), 1.25 (3H, s, H-16); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.45 (s, CH_3*C*OO), 170.23 (s, CH_3*C*OO), 169.3 (s, CH_3*C*OO), 167.01 (s, C_6H_5*C*OO), 139.63 (s, C-12), 134.84 (s, C-11), 133.71 (s, *C*$_6$H$_5$COO), 130.07 (s, *C*$_6$H$_5$COO), 129.18 (s, *C*$_6$H$_5$COO), 128.63 (s, *C*$_6$H$_5$COO), 84.0 (s, C-5), 82.05 (s, C-4), 78.47 (s, C-1), 76.79 (s, C-9), 76.56 (s, C-20), 73.98 (s, C-7), 73.47 (s, C-2), 73.20 (s, C-10), 69.74 (s, C-13), 47.04 (s, C-3), 44.89 (s, C-8), 43.0 (s, C-15), 37.94 (s, C-6), 35.30 (s, C-14), 28.27 (s, C-17), 22.86 (s, *C*H$_3$COO), 22.56 (s, C-16), 21.34 (s, *C*H$_3$COO), 21.25 (s, *C*H$_3$COO), 14.87 (s, C-18), 12.48 (s, C-19); FABMS m/z 631 [(M + H)$^+$].

Baccatin VI (3). Acetic anhydride (0.048 g, 0.48 mmol) was added to a solution of 13-acetyl-9-dihydrobaccatin III (**2**) (0.0038 g, 4.8 μmol) and (dimethylamino)pyridine (0.058 g, 0.48 mmol) in dichloromethane (3 mL). The reaction was allowed

to stir for 4 h at room temperature. The reaction was quenched by the addition of 5% HCl (1 mL) and H_2O (5 mL). The organic layer was separated, and the aqueous layer once again was extracted with dichloromethane. The organic extracts were combined, dried over Na_2SO_4, and concentrated. Flash column chromatography (Si gel, MeOH−CH_2Cl_2, 2:98) afforded 3 mg (70%) of pure synthetic baccatin VI (**3**) as a white solid: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.10 (2H, d, J = 6.8 Hz, C_6H_5COO), 7.6 (1H, t, J = 7.3 Hz, C_6H_5COO), 7.49 (2H, d, J = 7.8 Hz, C_6H_5COO), 6.22 (1H, d, J = 11.2 Hz, H-10), 6.18 (1H, t, J = 11.2 Hz, H-13), 6.0 (1H, d, J = 11.2 Hz, H-9), 5.88 (1H, d, J = 6.4 Hz, H-2), 5.58 (1H, m, H-7), 4.98 (1H, d, J = 8.9 Hz, H-5), 4.34 (A of AB, 1H, d, J = 8.3 Hz, H-20A), 4.13 (B of AB, 1H, d, J = 8.3 Hz, H-20B), 3.19 (1H, d, J = 5.9 Hz, H-3), 2.49 (1H, m, H-6A), 2.30 (3H, s, CH_3COO), 2.28−2.10 (2H, m, H-14), 2.2 (3H, s, CH_3COO), 2.12 (3H, s, CH_3COO), 2.11 (3H, s, CH_3COO), 2.04 (3H, s, CH_3COO), 2.00 (3H, s, H-18), 1.9−1.82 (2H, m, H-6B, C−O*H*), 1.80 (3H, s, H-16), 1.58 (3H, s, H-19), 1.23 (3H, s, H-17); FABMS m/z 715 [(M + H)$^+$]; HRMS m/z 715.2970 (calcd for $C_{37}H_{47}O_{14}$, 715.2966).

9-Dihydrobaccatin III (4). Methyllithium (60 μL, 1.4 M solution in ethyl ether, 87 μmol) was added in a dropwise manner to a solution of 13-acetyl-9-dihydrobaccatin III (**2**) (0.0084 g, 13.3 μmol) in THF (1.5 mL), which had been cooled to −78 °C. The reaction was then stirred for 2 h at −78 °C. The reaction was quenched by addition of H_2O (2 mL) followed by EtOAc (3 mL). The organic layer was separated and the aqueous layer once again extracted with EtOAc. The organic extracts were combined, dried over Na_2SO_4, and concentrated. Flash column chromatography (Si gel, MeOH−CH_2Cl_2, 4:96) afforded 3.4 mg (43%) of pure synthetic 9-dihydrobaccatin III **(4)** as a white solid: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.10 (2H, m, C_6H_5COO), 7.6 (1H, m, C_6H_5COO), 7.48 (2H, m, C_6H_5COO), 6.13 (1H, d, J = 10.74 Hz, H-10), 5.72 (1H, d, J = 5.91 Hz, H-2), 4.92 (1H, d, J = 9.1 Hz, H-5), 4.80 (1H, br t, H-13), 4.44 (2H, m, H-7, H-9), 4.36 (A of AB, 1H, d, J = 8.1 Hz, H-20A), 4.16 (B of AB, 1H, d, J = 8.1 Hz, H-20B), 3.10 (1H, d, J = 5.91 Hz, H-3), 2.53 (1H, m, H-6A), 2.31−2.17 (2H, m, H-14), 2.28 (3H, s, CH_3COO), 2.17−2.03 (2H, m, C−O*H*), 2.13 (3H, s, CH_3-COO), 2.11 (3H, d, J = 1.1 Hz, H-18), 2.00−1.79 (2H, m, H-6, C−O*H*), 1.82 (3H, s, H-19), 1.65 (3H, s, H-17), 1.40−1.15 (1H, m, C−O*H*), 1.10 (3H, s, H-16); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.6, 170.4, 167.0, 142.8, 134.3, 133.7, 130.1, 129.3, 128.6, 84.2, 82.2, 78.6, 77.2, 73.9, 73.8, 73.4, 68.5, 46.8, 45.1, 42.6, 40.8, 38.7, 37.9, 28.3, 23.1, 22.1, 21.4, 15.2, 12.5; FABMS m/z 589 [(M + H)$^+$]; HRMS m/z 589.2638 (calcd for $C_{31}H_{41}O_{11}$, 589.2649).

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