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TRITERPENOIDS FROM CALLUS TISSUE CULTURES OF *PAEONIA* SPECIES

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Key Word Index—*Paeonia japonica*; *P. lactiflora*; *P. suffruticosa*; Paeoniaceae; callus tissue; triterpenoid; chemotaxonomy; biogenesis.

Abstract—Six triterpenoids were isolated from the callus tissues of *Paeonia japonica*, *P. lactiflora* and *P. suffruticosa*. Four of the compounds are new from a natural source: 11α,12α-epoxy-3β,23-dihydroxyolean-28,13β-olide, 3β-hydroxy-11-oxo-olean-12-en-28-oic acid, 3β-hydroxy-oleana-11,13(18)-dien-28-oic acid and 3β,23-dihydroxy-oleana-11,13(18)-dien-28-oic acid. The postulated biosynthetic sequence of these compounds is discussed and the triterpenoids produced from the three callus tissues have been compared from a chemotaxonomical standpoint.

INTRODUCTION

Paeoniaceous plants have been used as an important source of crude drugs in traditional Chinese medicine and have been prescribed for women's diseases. In an earlier paper, we reported the accumulation of a large amount of triterpene and the characterization of six triterpenoids [oleanolic acid (**7**), hederagenin (**8**), betulinic acid (**9**), 23-hydroxybetulinic acid (**10**), 30-nor-hederagenin (**11**) and 24-methylenecycloartanol (**12**)] from the callus tissues of *Paeonia japonica* [1]. We now report the establishment of two further callus tissues from the stems of *P. lactiflora* and *P. suffruticosa* and the structural elucidation of the triterpenoids extracted from the callus tissues.

RESULTS AND DISCUSSION

The methanol extracts of the callus tissues derived from the stem of *P. japonica* were reinvestigated and chromatography yielded four triterpenoids (**1**–**4**) along with six other compounds reported previously [1]. The mass spectrum of **1** exhibited the $[M]^+$ at *m/z* 470. The IR spectrum of **1** showed an intense band at 1768 cm^{-1} indicating the presence of a γ-lactone and also at 868 cm^{-1} indicating the presence of an epoxide. The ^1H NMR spectrum (pyridine-d_5) of **1** showed the presence of seven characteristic tertiary methyl groups and two proton signals at δ3.14 (1H, *dd*, *J* = 4, 2 Hz) and 3.27 (1H, *d*, *J* = 4 Hz) ascribable to epoxide protons for C-11 and C-12. Furthermore, the ^{13}C NMR spectrum exhibited the characteristic peaks at δ52.8 (*d*) for C-11, 57.3 (*d*) for C-12, and 87.6 (*s*) for C-13, which agreed closely with those of the epoxy-γ-lactone moiety reported for compound **13** (3-epimer isolated from callus tissues of *Stauntonia hexaphylla*) (Table 1) [2]. Thus, **1** was identified as 3β-hydroxy-11α,12α-epoxy-olean-28,13β-olide, which was reported as the compound obtained by the photochemical oxidation of oleanolic acid (**7**) [3] and from the stem of *Lepechinia glomerata* [4].

The mass spectrum of **2** showed the peak $[M]^+$ at *m/z* 486 which was 16 mass units more than that of **1**, and the presence of a secondary hydroxyl group was suggested by the ^1H NMR chemical shifts (two double singlets at δ3.70 and 4.20, *J* = 10.5 Hz) and the ^{13}C NMR signals were similar to those of **1** except for the A/B rings having an additional secondary hydroxyl group (Table 1). Thus, **2** was identified as 11α,12α-epoxy-3β,23-dihydroxyolean-28,13β-olide, which was obtained from hederagenin (**8**) by anodic oxidation [3]. This is the first report of **2** from a natural source.

Compound **3** showed the peak $[M]^+$ at *m/z* 470 and the presence of an enone was revealed by the UV (λ_{max} at 250.9 nm) and the IR (at 1687 cm^{-1}) spectra. The ^1H NMR spectrum showed seven methyl signals and an olefinic proton at δ6.02.

Signals of an α,β-unsaturated carbonyl function at δ128.1, 169.8 and 200.1 were seen in the ^{13}C NMR spectrum. From the above spectral data, **3** was identified as 3β-hydroxy-11-oxo-oleañ-12-en-28-oic acid, which was reported as a bioconversion product of oleanolic acid (**7**) by the soil microbe *Cunninghamella blakesleeana* [5]. This is the first time that **3** has been reported from a natural source.

Compound **4** had a UV spectrum indicating the presence of a heteroannular conjugated diene system in the molecule (λ_{max} 244.1, 250.8 and 259.6 nm). The IR spectrum showed the presence of carbonyl (1700 cm^{-1}) and hydroxy (3448 cm^{-1}) functions. The mass spectrum of **4** showed the peak $[M]^+$ at *m/z* 454. The ^1H NMR spec-

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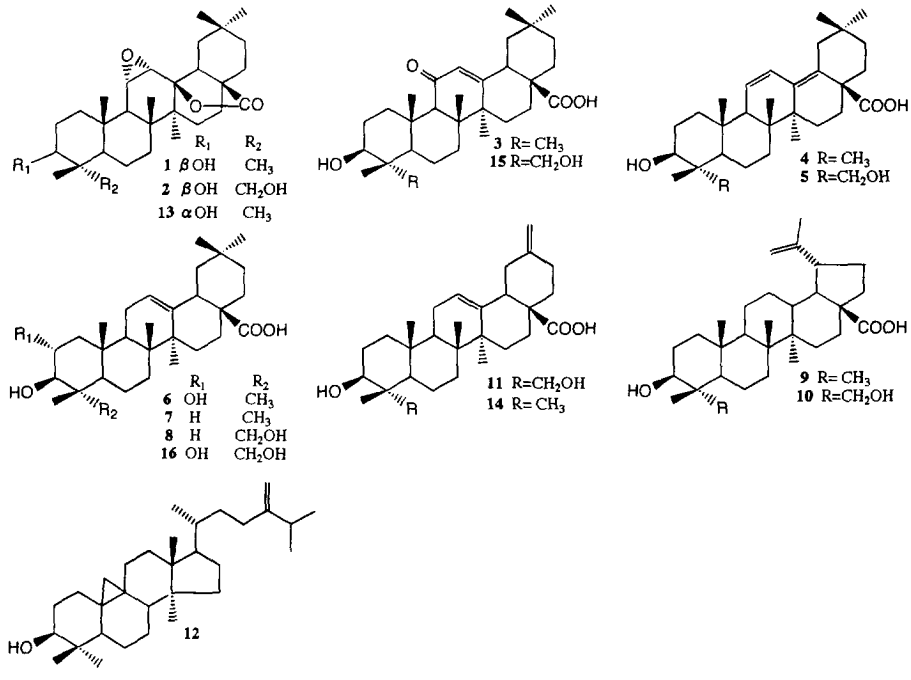
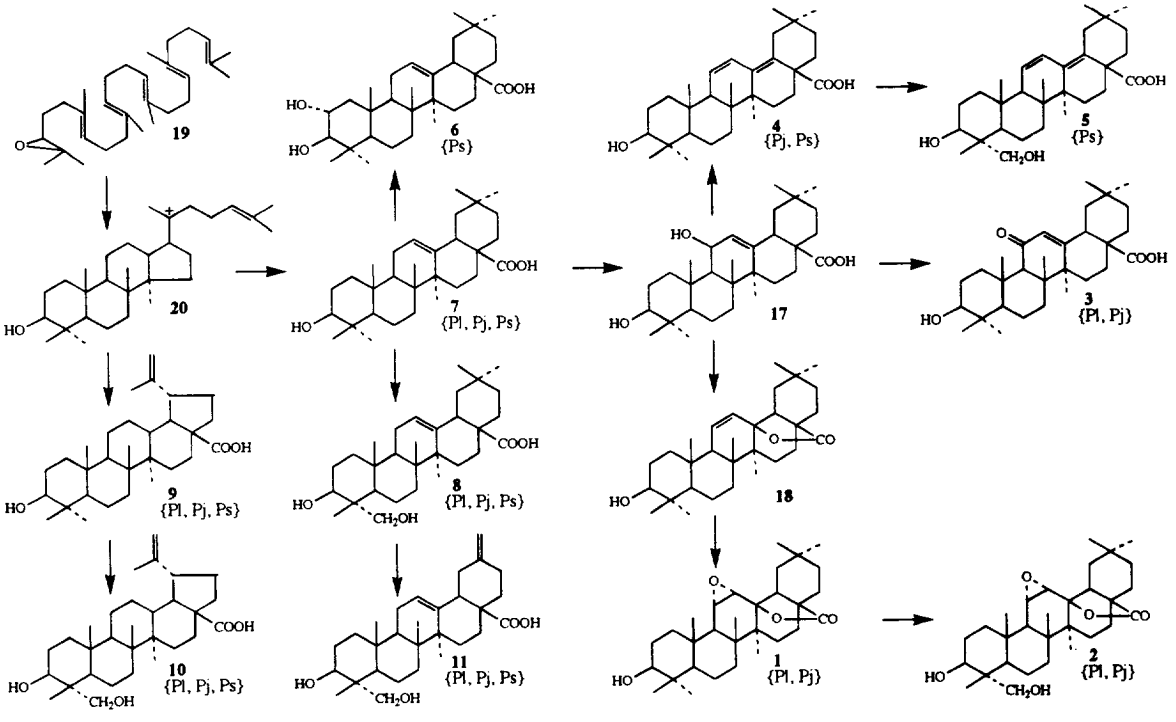


Table 1. ^{13}C NMR spectral data for **1**–**6** and **13*** in pyridine-d_5

C	1	2	3	4	5	6	13
1	38.7	38.6	39.8	38.5	38.3	47.8	33.0
2	27.6	27.3	28.2	28.0	27.6	68.6	27.5
3	77.9	72.8	77.9	78.1	73.1	83.8	75.1
4	39.4	43.0	37.9	39.5	43.1	39.8	37.0
5	55.0	47.8	55.4	55.3	48.2	55.9	48.6
6	18.8	17.8	17.9	18.8	18.5	18.9	17.3
7	31.4	31.1	33.3	32.9	32.6	33.2	31.5
8	40.9	40.9	44.0	41.1	41.1	48.2	40.9
9	49.8	49.8	62.2	54.8	54.9	48.2	49.8
10	36.8	36.7	37.9	37.1	36.9	38.5	37.0
11	52.8	52.8	200.1	126.0	126.0	23.9	52.9
12	57.3	57.3	128.1	127.0	127.1	122.5	57.4
13	87.6	87.6	169.8	136.6	136.6	144.9	88.0
14	41.7	41.6	45.3	42.5	42.5	42.2	41.9
15	27.0	27.0	28.3	33.3	33.3	28.3	27.0
16	21.6	21.6	23.4	25.6	25.6	23.6	21.7
17	44.1	44.0	46.2	48.7	48.7	46.7	44.1
18	51.2	51.3	42.4	133.8	133.2	46.4	51.1
19	38.0	38.0	44.6	41.0	41.0	42.0	38.0
20	31.5	31.5	30.8	32.8	32.8	30.9	31.6
21	34.4	34.4	32.2	37.5	37.4	34.2	34.4
22	27.7	27.6	34.0	36.3	36.2	33.2	27.7
23	28.4	67.0	28.8	28.5	67.4	29.3	29.1
24	16.0	12.6	16.6	16.0	12.7	16.9	22.2
25	17.3	17.6	16.7	18.4	18.7	17.5	17.3
26	18.9	18.8	19.4	17.1	17.1	17.7	18.8
27	20.4	20.4	23.6	20.1	20.1	26.2	20.5
28	178.9	178.9	179.7	178.9	178.9	180.2	180.0
29	33.0	33.0	32.9	32.4	32.4	33.2	33.6
30	23.4	23.4	23.4	24.4	24.3	23.7	23.5

*Assigned here from ref. [2].



Pl: *Paeonia lactiflora*, Pj: *Paeonia japonica*, Ps: *Paeonia suffruticosa*

Scheme 1. Postulated biosynthetic sequence for the production of triterpenoids in callus tissue of paeoniaceous plants.

trum exhibited two olefinic protons at δ5.79 (1H, *dd*, J = 1.5, 11 Hz) and 6.69 (1H, *dd*, J = 3, 11 Hz), respectively. The ^{13}C NMR spectrum showed four olefinic carbons at δ126.0, 127.0, 133.8 and 136.6, and further signals at δ78.1 and 178.9 attributable to C-3 and C-28, respectively. From these spectroscopic data, **4** was identified as 3β-hydroxy-oleana-11,13(18)-dien-28-oic acid. Compounds **3** and **4** have been reported as the bioconversion products of oleanolic acid (**7**) by the soil microbe *C. blakesleeana* [5]. Two other compounds (**5** and **6**) have been isolated from *P. suffruticosa* callus tissues. Compound **5** showed the peak $[M]^+$ at m/z 470 which was 16 mass units more than that of **4**. The ^{1}H NMR and ^{13}C NMR spectrum were similar to those of **4** except for a hydroxymethylene at C-23 (Table 1). Thus, compound **5** was recognized as 3β,23-dihydroxy-oleana-11,13(18)-dien-28-oic acid, which was reported as a product from the oxidation of hederagenin (**8**) [6], but this is the first report of **5** as a natural product. Compound **6** was identified by comparing the spectral data with 2α,3β-dihydroxy-olean-12-en-28-oic acid.

Six new triterpenoids, in addition to the six triterpenoids reported previously, were isolated from paeoniaceous plant callus tissues. The four hydroxymethylene derivatives at C-23 (**2**, **5**, **8** and **10**) can be derived from **1**, **4**, **7** and **9**, respectively. It is interesting from the biosynthetic point of view to identify a number of stepwise biogenetic triterpenoid intermediates from one plant tissue culture. The hypothetical biogenetic sequence for the four pairs of triterpenoids (**1**–**2**, **4**–**5**, **7**–**8** and **9**–**10**) and **11** shown in Scheme 1 can be theoretically proposed based on the co-occurrence of the compounds.

Lupeol, which is a precursor of betulinic acid (**9**), and oleanolic acid (**7**) may be biosynthesized at first by enlargement of ring D of a common intermediate, the protoeuphoid cation (**20**) biosynthesized from (3*S*)-2,3-oxidosqualene (**19**) (Scheme 1). Furthermore, **1** may be biosynthesized from **7** via the route **17**→**18** as the hypothetical intermediate obtained by hydroxylation, followed by the dehydration at C-11 and the formation of the lactone ring and with successive epoxydation at C-11 and C-12 as shown in Scheme 1. On the other hand, **17** may proceed to **4** by dehydration at C-11. The C-23 hydroxylated compounds (**2**, **5**, **8** and **10**) may be biosynthesized from **1**, **4**, **7** and **9**, respectively, although the biosynthetic route to **5** via **4** is present only in *P. suffruticosa* callus tissues. Compound **8** seems to proceed to **11** without going via **14**, since this compound has not been detected in the paeony callus tissues. However, **14** [7] and **11** [8] have been reported in lardizabalaceous callus tissues. The twelve triterpenoids (**1**–**12**) were isolated from the callus tissues of paeoniaceous plants. Oleanolic acid (**7**), hederagenin (**8**), betulinic acid (**9**), 23-hydroxybetulinic acid (**10**) and 30-norhederagenin (**11**) were produced as main constituents in all three callus tissues, *P. japonica*, *P. lactiflora* and *P. suffruticosa* (Table 2). 11α,12α-Epoxy-

Table 2. Comparison of triterpenes from paeoniaceous plant callus tissues

	7	8	9	10	14	11	1	2	3	15	4	5	6	16
P. lactiflora	+	+	+	+	—	+	+	+	+	—	—	—	—	—
	+	+	+				+	+						
	+	+	+											
P. japonica	+	+	+	+	—	+	+	+	+	—	+	—	—	—
		+	+				+	+						
		+	+											
P. suffruticosa	+	+	+	+	—	+	—	—	—	—	+	+	+	—
			+											

+ + + Large amount; + + middle amount; + small amount; —none detected.

3β-hydroxyolean-28,13β-olide(**1**), 11α,12α-epoxy-3β,23-dihydroxyolean-28,13β-olide(**2**) and 3β-hydroxy-11-oxo-olean-12-en-28-oic acid(**3**) are produced by *P. japonica* and *P. lactiflora* callus tissues, respectively. On the other hand, 2α,3β-dihydroxyolean-12-en-28-oic acid (**6**) and 3β-hydroxyolean-11,13(18)-dien-28-oic acid (**4**) were produced only by *P. suffruticosa* callus tissues (Scheme 1).

These paeoniaceous callus tissues produced almost the same triterpenoids as the main products, respectively (Table 1), but there are slightly differences between the two groups; Pl and Pj, and Ps for the production of the minor triterpenoid compounds (**1**, **2**, **3**, **4**, **5** and **6**) as shown in Scheme 1 and Table 2.

Many pairs of triterpenoids having a 4α-hydroxymethylene group are produced by paeoniaceous plant callus tissues, but **14** (30-noroleanolic acid) was not detected despite the presence in the callus tissues of **11** having a 4α-hydroxymethylene group. Compound **14** has been reported as the main triterpenoid from lardizabalaceous plant tissue cultures (*Akebia quinata*, *A. trifoliata* and *Staountonia hexaphylla*) [7–9].

The results are interesting from a chemotaxonomic and phylogenetic point of view. The above-mentioned triterpenoids (**1**–**12**) so far have not been reported from the paeoniaceous plants and the investigation of the triterpenes from the plants is now in progress. Furthermore, paeniflorine, the main component of three plants, was not detected in all three cultured cells.

EXPERIMENTAL

Mps are uncorr. ^{1}H and ^{13}C NMR: 500 and 125 MHz, respectively, room temp, pyridine-d_5. The chemical shifts are given in δ(ppm) with pyridine-d_5 (δ7.22 and 135.5 ppm respectively) as int. standard. Multiplicities for the ^{13}C NMR spectra were determined by DEPT experiment at 90 and 135°. MS: 70 eV, direct probe.

Derivation and culture of callus tissues. Callus tissues from the stems (*Paeonia japonica*, *P. lactiflora* and *P. suffruticosa*) were established in Murashige and Skoog medium (minus glycine) (M&S) containing 2,4-D(1 mg l^{-1}–3 mg l^{-1}) and kinetin(0.1 mg l^{-1}) as plant growth regulators which were used for induction of callus tissues. The callus tissues were subcultured every 5–6 weeks on to fresh M&S containing 2,4-D(1 mg l^{-1}) and kinetin (0.1 mg l^{-1}) at 25 ± 1° in the dark.

Extraction and isolation. The fr. callus tissues, *P. japonica* (813 g fr. wt, 27.5 g dry wt), *P. lactiflora* (683 g fr. wt., 24 g dry wt) and *P. suffruticosa* (1013 g fr. wt, 36.5 g dry wt) were extracted with cold MeOH and EtOAc in a Waring blender. The extracts were concd under red. pres. and the residue was partitioned between $CHCl_3$ and H_2O to obtain the organic solvent soluble fr. The $CHCl_3$ soln was evapd and the extracts chromatographed on a column of silica gel (Fuji gel BW 350) with gradient elution using $CHCl_3$ with increasing proportions of MeOH to afford the crude triterpenoid mixts. The mixts were purified repeatedly by CC on a silica gel column (CIG column system, Kusano) with hexane–EtOAc–MeCN, which afforded **1**–**6**.

11α,12α-*epoxy*-3β-*hydroxyolean*-28,13β-*olide* (**1**). Mp 258–263°; powder, $[\alpha]_D^{21}$ +72.1° ($CHCl_3$; *c* 0.73). IR ν_{max} cm^{-1}: 3368 (OH), 1768 (γ-lactone), 868 (epoxy). MS *m/z* (rel. int.): 470 $[M]^+$ (23), 263 (24), 204 (73), 189 (75). ^1H NMR (pyridine-d_5): δ0.88, 0.89, 0.98, 1.03, 1.17, 1.21, 1.22 (each 3H, each *s*), 2.55 (1H, *dd*, *J* = 3, 13.5 Hz), 3.14 (1H, *dd*, *J* = 4, 2 Hz), 3.27 (1H, *d*, *J* = 4 Hz), 3.44 (1H, *dd*, *J* = 4.5, 11 Hz).

11α,12α-*epoxy*-3β,23-*dihydroxyolean*-28,13β-*oxide* (**2**). Mp > 300°: powder, $[\alpha]_D^{21}$ + 63.6° ($CHCl_3$: *c* 0.68). IR ν_{max} cm^{-1}: 3404 (OH), 1766 (γ-lactone), 870 (epoxy). MS *m/z* (rel. int.): 486 $[M]^+$ (24), 263 (46), 251 (45), 204 (70), 189 (69). ^1H NMR (pyridine-d_5): δ0.78, 0.86, 1.04, 1.05, 1.14, 1.20 (each 3H, each *s*), 2.53 (1H, *dd*, *J* = 3, 13.7 Hz), 3.16 (1H, *dd*, *J* = 4, 2 Hz), 3.27 (1H, *d*, *J* = 4 Hz), 3.70 (1H, *d*, *J* = 10.5 Hz), 4.20 (1H, *d*, *J* = 10.5 Hz), 4.22 (1H, *dd*, *J* = 4.5, 11 Hz).

3β-*hydroxy-oleana*-11,13(18)-*dien*-28-*oic acid* (**4**). Mp 235–245°; powder, $[\alpha]_D^{21}$ − 50.9° ($CHCl_3$; *c* 0.44). IR ν_{max} cm^{-1}: 3448 (OH), 1700 (CO). UV λ_{max} nm (log ε): 201.2 (3.68), 244.1 (4.16), 259.6 (4.04). MS *m/z* (rel. int.): 454 $[M]^+$ (100), 409 (16), 301 (12), 255 (16), 234 (18), 203 (18), 189 (35). ^1H NMR (pyridine-d_5): δ0.91, 0.94, 0.99, 1.03, 1.10, 1.25 (each 3H, each *s*), 3.50 (1H, *dd*, *J* = 5, 11 Hz), 5.79 (1H, *dd*, *J* = 1.5, 11 Hz), 6.69 (1H, *dd*, *J* = 3, 11 Hz).

3β,23-*dihydroxy-oleana*-11,13(18)-*dien*-28-*oic acid* (**5**). Mp 273–276°; powder, $[\alpha]_D^{21}$ + 1.18° ($CHCl_3$: *c* 0.51). IR ν_{max} cm^{-1}: 3448 (OH), 1700 (CO). UV λ_{max} nm (log ε): 202.1 (3.97), 245.0 (4.23), 251.6 (4.20), 260.9 (4.09). MS *m/z* (rel. int.): 470 $[M]^+$ (41), 425 (10), 301 (10), 255 (18), 203 (22), 189 (38). ^1H NMR (pyridine-d_5): δ0.78, 0.86, 1.04, 1.05, 1.14, 1.20 (each 3H, each *s*), 2.18 (2H, *m*), 2.72 (1H, *dd*, *J* = 1.5, 14 Hz), 3.73 (1H, *d*, *J* = 10.5 Hz), 4.22 (1H, *d*, *J* = 10.5 Hz), 4.27 (1H, *dd*, *J* = 4.5, 11 Hz), 5.79 (1H, *dd*, *J* = 1.5, 11 Hz), 6.67 (1H, *dd*, *J* = 3, 11 Hz).

3β-*hydroxy*-11-*oxo-oleana*-12-*en*-28-*oic acid* (**3**). Mp 232–235°; powder, $[\alpha]_D^{21}$ + 56.3° ($CHCl_3$: *c* 0.96). IR ν_{max} cm^{-1}: 3450 (OH), 1687 (α,β-unsaturated carbonyl). UV λ_{max} nm (log ε): 200.7, 250.9 (3.94). MS *m/z* (rel. int.): 470 $[M]^+$ (37), 303 (55), 262 (100), 217 (32). ^1H NMR (pyridine-d_5): δ0.91, 0.92, 1.05, 1.19, 1.26, 1.42, (each 3H, each *s*), 2.56 (1H, *s*), 3.34 (1H, *dd*, *J* = 4, 14 Hz), 3.49 (1H, *dd*, *J* = 4.5, 12 Hz), 6.02 (1H, *s*).

2α,3β-*dihydroxy-oleana*-12-*en*-28-*oic acid* (**6**). Mp 236–238°; powder, $[\alpha]_D^{21}$ + 14.8° ($CHCl_3$; *c* 0.35). IR ν_{max} cm^{-1}: 3448 (OH), 1694 (CO). MS *m/z* (rel. int.): 472 $[M]^+$ (17), 248 (100), 203 (61), 189 (16). ^1H NMR (pyridine-d_5): δ0.92, 0.99, 1.00, 1.02, 1.08, 1.26, 1.28 (each 3H, each *s*), 3.30 (1H, *dd*, *J* = 4, 14 Hz), 3.40 (1H, *d*, *J* = 9.5 Hz), 4.10 (1H, *ddd*, *J* = 4.5, 9.5, 11.3 Hz), 5.47 (1H, *t*, *J* = 3.5 Hz).

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