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Synthesis and evaluation of cytotoxic effects of novel α-methylenelactone tetracyclic diterpenoids

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ABSTRACT

A series of tetracyclic diterpenoids bearing the α-methylenelactone group have been synthesized and screened for their in vitro anti-tumor activities against six human cancer cell lines. The results showed that compounds **1c**, **2a** and **2b** exhibited significant cytotoxicity superior to the positive control doxorubicin hydrochloride against MDA-MB-231, K562 and HepG2 cell lines. In particular, compound **2b** was identified as the most promising anticancer agent against HepG2 cells with IC_{50} value of 0.09 μM.

Cancer is one of the leading causes of death worldwide and causes serious problems in human life. Therefore, various categories of anti-tumor agents have been developed. However, some side effects could happen simultaneously and the resistance to available chemo-therapeutic agents was rising. Hence, it is urgent to develop novel compounds as anticancer agents with higher bioactivities and lower toxicities.[1,2]

Natural products have always been interesting sources for developing novel leading compounds. Stevioside (Fig. 1) is the primary sweet component in the leaves of *Stevia rebaudiana* Bertoni which is a plant native to South America.[3,4] Stevioside consists of three molecules of glucose and steviol as its aglycone. A large number of researches have suggested that stevioside tastes 300 times sweeter than sucrose and can be used as a non-caloric sweetener in South America, Japan, and China. Moreover, stevioside along with its metabolic components steviol[5] and isosteviol[6] possesses multiple pharmacological activities including anti-hyperglycemic, anti-inflammatory, anti-tumor and anti-diarrheal. It has been shown that these three compounds strongly inhibited the cancer formation induced by TPA (12-*o*-tetra-decanoylphorbol-13-acetate) and DMBA (7,12-dimethylbenz[α]anthracene) in a two-stage carcinogenesis test in mouse. In addition, isosteviol inhibited both mammalian DNA polymerases and human DNA topoisomerase II. Taken together, these compounds could be served as promising chemopreventive agents against chemical carcinogenesis.[7,8] In order to develop potential anticancer agents of higher cytotoxicity, some structural modifications have been done. In our previous work, we built up a crucial fragment of *exo*-methylene cyclopentanone in the ring D of steviol and isosteviol and obtained some compounds with significantly improved cytotoxicity.[9] Tao and coworkers. synthesized various 15- and 16-substituted isosteviol derivatives by means of functional interconversions, then obtained some compounds with promising activities against B16-F10 melanoma cells.[10]

Plenty of investigations have reported that α-methylenelactone is a crucial building block of many natural products and exhibits wide-ranging biological activities such as anti-tumor, anti-inflammatory, antimicrobial and so on.[11] Therefore, the synthesis of this structural moiety has received much attention,[12,13] and the relationship between its activities and structure has also been studied. It appears that α-methylenelactone may be regarded as alkylating agents by virtue of Michael addition with biological nucleophiles such as L-cysteine or thiol-containing enzymes (Enz-SH).[14] Many sesquiterpene lactones isolated from different kinds of natural plants have been reported to display interesting biological activities. For instance, costunolide (Fig. 1) isolated from the root of *Saussurea lappa* exhibited potent cytotoxicity against HepG2, OVCAR-3 and HeLa cell lines with CD_{50} values of 1.6, 2.0, 2.0 μg/mL, respectively.[15] Kupchan et al. discovered that vernolepin (Fig. 1) bearing two α-methylenelactone groups showed significant cytotoxicity activity against Walker intramuscular carcinosarcoma in vitro

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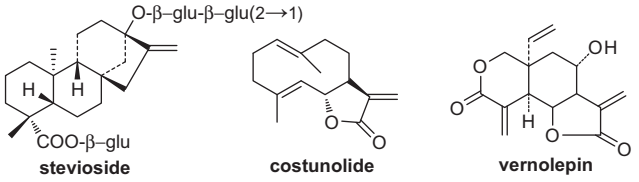
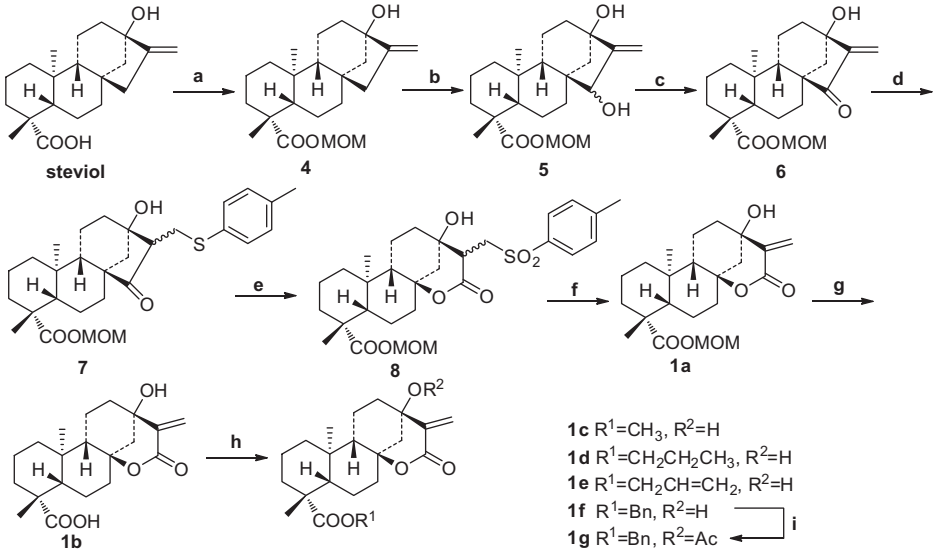


Figure 1. Chemical structures of stevioside, costunolide and vernolepin.

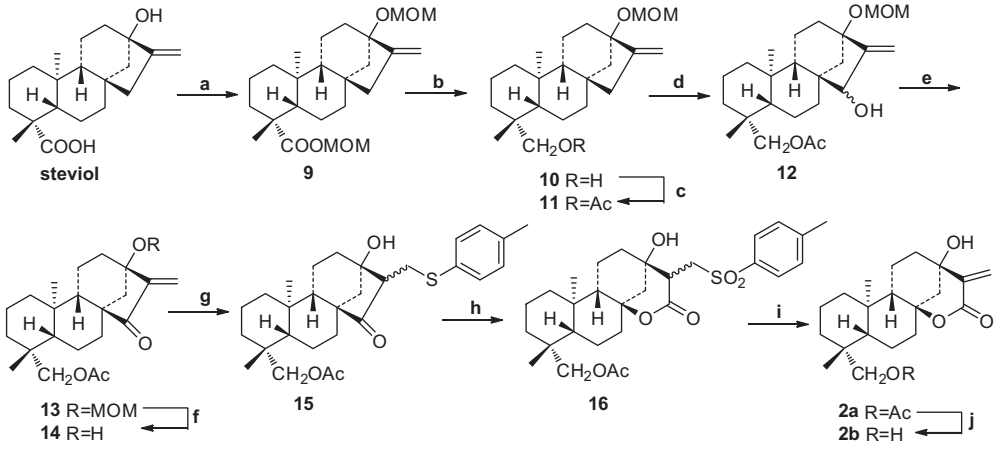
and in vivo in rats.[16] However, *ent*-kaurane diterpenoids possessing α-methylenelactone group are rare in the natural products discovered recently. Therefore, we tried to introduce this critical moiety into steviol and isosteviol and obtained three scaffolds of *ent*-kaurene diterpenoids. Some derivatives were also synthesized and screened for their anticancer activities against six cancer cell lines in vitro by MTT method.

The synthetic route towards the target compounds was described as follows: first, treatment of steviol with chloromethyl methyl ether and *N*,*N*-diisopropylethylamine afforded **4** in 1 h,[17] then reaction of **4** with selenium oxide and *tert*-butyl hydroperoxide led to **5** (Scheme 1).[18] Oxidation of **5** with PDC provided compound **6**.[19] We next tried a phenylthio group as a stable protecting group of the α-methylene unit. Conjugate addition of *p*-thiocresol to enone **6** produced β-thioketone **7**[20] which was successfully transformed into sulfone lactone **8** by Baeyer–Villiger oxidation with excessive *m*CPBA.[21,22] Finally, desulfonylation of **8** with DBU in THF under mild condition gave the desired compound **1a**.[23] Compound **1b** was prepared from **1a** by deprotection of methoxymethyl group with 10% HCl in THF.[24] Esterification of **1b** with different kinds of halohyrocarbons afforded **1c–f**. Compound **1g** could be obtained by acylation of **1f** with acetic anhydride in the presence of DMAP.[25]

The synthetic approach employed to prepare **2a** was outlined in Scheme 2. First, we attempted to reduce **4** with $LiAlH_4$ in anhydrous THF under refluxing condition. Although the reaction could proceed smoothly, the yield was very low due to the poor liposolubility of the product. Therefore, we had to protect the 13-hydroxy of steviol with excessive MOM ether as well. By doing this, compound **10** could be obtained in a good yield (86%). Acylation of



Scheme 1. Reagents and conditions: (a) MOMCl, DIPEA, DMF (90.0%); (b) SeO_2, *t*-BuOOH, THF (85.0%); (c) PDC, DMF (75.0%); (d) *p*-thiocresol, Et_3N, THF (65.6%); (e) 85% *m*CPBA, $NaHCO_3$, CH_2Cl_2 (53.8%); (f) DBU, THF (69.9%); (g) 10% HCl, THF, H_2O (84.0%); (h) R^1R (for **1c**, R = I; for **1d** and **1f**, R = Br; for **1e**, R = Cl), K_2CO_3, DMF, KI (64.0–81.4%); (i) Ac_2O, Et_3N, THF, DMAP (65.2%).



Scheme 2. Reagents and conditions: (a) MOMCl, DIPEA, DMF (85.3%); (b) $LiAlH_4$, THF, reflux (86.0%); (c) Ac_2O, Et_3N, THF, DMAP (77.6%); (d) SeO_2, *t*-BuOOH, THF (75.6%); (e) PDC, DMF (72.0%); (f) 10% HCl, THF, H_2O (82.3%); (g) *p*-thiocresol, Et_3N, THF (87.6%); (h) 85% *m*CPBA, $NaHCO_3$, CH_2Cl_2 (55.2%); (i) DBU, THF (70.6%); (j) 10% $KHCO_3$, CH_3OH, reflux (71.0%).

10 with acetic anhydride afforded **11**. The following procedures for preparing **2a** were exactly the same as for preparing **1a**. Finally, **2a** was converted to **2b** with 10% $KHCO_3$ in refluxing CH_3OH. It was noteworthy that conjugate addition of *p*-thiocresol to **13** could not get the β-thioketone, so we had to deprotect the MOM ether to reduce the steric hindrance.

Scheme 3 illustrated the synthesis of compounds **3a–d** starting from isosteviol. First of all, we attempted to construct a hydroxymethyl in the α-position of 16-ketone with aqueous formaldehyde under base condition. Surprisingly, the 16-ketone was reduced at the same time. The mechanism of the reaction had been reported and was proposed as a one-spot 'Aldol–Cannizzaro reaction' process.[26] After the diol **18** had been successfully achieved, we chose acetyl, a cleanly and conveniently deprotected group, to protect the primary alcohol selectively. Oxidation of **19** with PDC provided **20**. However, the key intermediate lactone **22** couldn't be prepared from **20** with excessive *m*CPBA until the deprotection of acetyl was completed. Treatment of **22** with *p*-toluenesulfonyl chloride in pyridine produced tosylate **23** which was heated under reflux in pyridine for 6 h to give the target compound **3a**.[27] Compound **3b** was obtained via elimination of **24** which was accessed by removing the benzyl of **23** with 10% Pd-C. Eventually, two analogues (**3c, d**) of **3b** have been synthesized.

In order to investigate whether the α-methylenelactone group is essential for the bioactivity of the compound; we changed the α-methylene group into the epoxy. Oxidation of **1f** and **3a** with excessive *m*CPBA afforded compounds **1h**[28] and **3e**, respectively (Scheme 4).

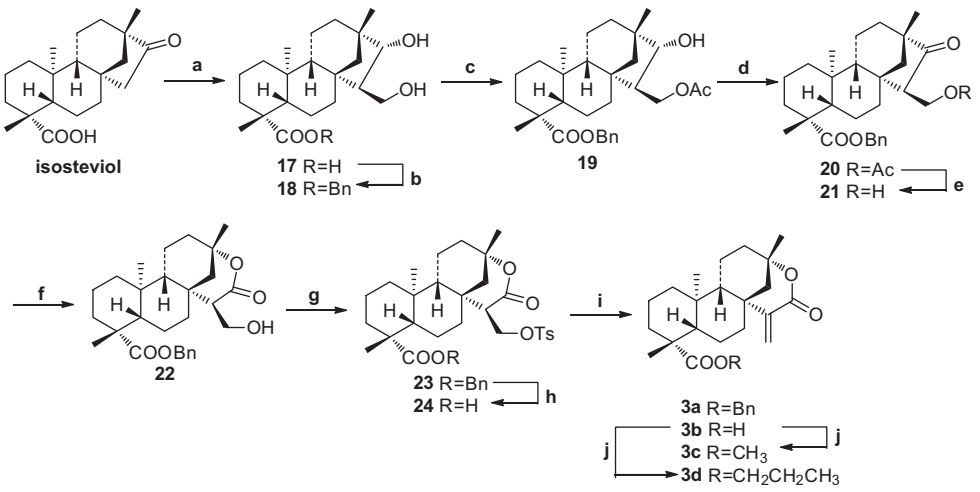
The structures of the target compounds were elucidated using spectroscopic techniques (IR, ^1H and ^{13}C NMR, ESI/MS, HRMS).

The cytotoxic activities[29] of compounds **1a-3e**[30] were determined in vitro against six cell lines: prostatic carcinoma (PC-3), colotectal carcinoma (HCT-116), breast carcinoma (MDA-MB-231), human erythroleukemic cell line (K562), hepatocellular carcinoma (HepG2) and gastric carcinoma (MGC-803). Doxorubicin hydrochloride was selected as a positive control. The IC_{50} values were used to determine the growth inhibition in the presence of tetracyclic diterpenoids **1a–3e** against PC-3, HCT-116, MDA-MB-231, K562, HepG2 and MGC-803 cancer cell lines. From the IC_{50} values summarized in Table 1, the compounds **1c**, **2a** and **2b** have

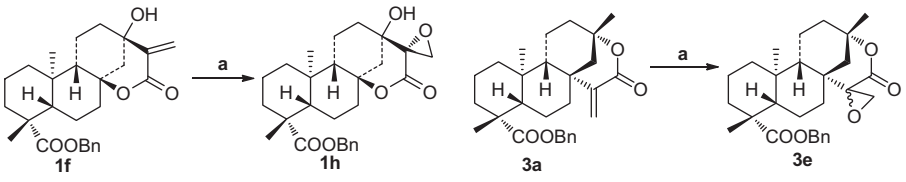
Table 1
Cytotoxicities of compounds **1a–3e** in vitro[a]

Compound	Anti-tumor activity in 48 h[b] (IC_{50}, μM)					
	PC-3	HCT-116	MDA-MB-231	K562	HepG2	MGC803
1a	0.12	2.56	0.78	24.21	0.38	2.23
1b	8.32	2.31	21.10	18.4	5.68	3.23
1c	1.56	0.22	0.56	0.13	0.16	1.21
1d	88.67	67.56	36.43	24.43	67.21	35.14
1e	56.32	24.12	37.32	22.12	67.21	34.18
1f	15.52	45.24	28.23	6.23	78.15	2.12
1g	10.11	35.23	18.10	0.34	25.23	1.56
1h	56.24	45.46	38.69	102.10	48.34	29.97
2a	5.71	0.34	1.10	0.89	0.12	2.56
2b	2.10	3.83	0.22	0.16	0.09	1.26
3a	88.76	78.90	102.50	19.53	28.65	19.92
3b	56.21	35.23	78.43	56.24	46.54	98.78
3c	34.21	56.45	22.87	25.45	35.24	11.23
3d	65.35	23.45	56.76	35.23	12.39	19.76
3e	189.92	222.62	127.35	87.81	231.26	123.54
Dox[c]	1.18	3.03	1.45	2.35	1.09	2.56

[a] Inhibition of cell growth by the listed compounds was determined using MTT assay.
[b] Data represent the mean value of three independent determinations.
[c] Dox = Doxorubicin hydrochloride.



Scheme 3. Reagents and conditions: (a) HCHO (aq), NaOH, C_2H_5OH–H_2O, 75 °C, 3 h (56.1%); (b) BnBr, K_2CO_3, DMF, KI (70.4%); (c) Ac_2O, Et_3N, THF, DMAP, 45 min (85.2%); (d) PDC, DMF (83.1%); (e) 10% KOH, CH_3OH, rt (89.4%); (f) 85% *m*CPBA, $NaHCO_3$, CH_2Cl_2 (56.1%); (g) TsCl, pyridine, DMAP (53.7%); (h) 10% Pd-C, H_2, C_2H_5OH (92.3%); (i) pyridine, DMAP, reflux (66.2%); (j) RR^1(for **3c**, R^1 = I; for **3d**, R^1 = Br), K_2CO_3, DMF, KI (75.2%, 80.1%).



Scheme 4. Reagents and conditions: (a) 85% *m*CPBA, $NaHCO_3$, CH_2Cl_2 (51.3%, 55.7%).

shown significant cytotoxities against all the six cell lines with the IC_{50} values ranging from 0.09 to 5.71 μM. Compounds **1a** and **1c** were found to be more effective than doxorubicin hydrochloride in HCT-116, MDA-MB-231, HepG2 and MGC803 cell lines. Esterification of 19-acid with MOM ether or methyl improved the cytotoxicity (**1a** vs **1b**, **1b** vs **1c**), while esterification of 19-acid with propyl, allyl and benzyl group decreased the activity (**1b** vs **1d**, **1b** vs **1e**, **1b** vs **1f**). Compound **1f** showed selective inhibition against K562 (IC_{50} = 6.23 μM) and MGC803 (IC_{50} = 2.12 μM) cell lines. Compound **1g** with the 13-hydrogen acylated exhibited slightly higher activity compared to compound **1f**. On the contrary, removing the 19-acetyl of **2a** afforded **2b** with better cytotoxity against all the cell lines except HCT-116. Compared with **1h**, compound **1f** bearing the α-methylenelactone group displayed better activity, which indicated that α-methylenelactone group played an important role in their anticancer activities. However, the compounds (**3a–e**) with the isosteviol scaffold exhibited weak activities.

In summary, we have successfully synthesized three scaffolds of tetracyclic diterpenoids bearing the α-methylenelactone moiety and evaluated their anticancer activities against six cell lines. We also proved that α-methylenelactone group was essential for the bioactivity of the compound, which was consistent with the previous literature.[11] Compound **2b** was found to be the most potent compound in HepG2 with IC_{50} value of 0.09 μM. Further researches on identifying their cellular targets are ongoing in our laboratory and the results will be reported in due course.

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29. Cytotoxicity assay in vitro: The human cancer cell lines were provided by Mr. Yangchang Wu research group (China Medical University) and maintained in a humidified atmosphere at 37 °C in 5% CO_2. The cells were cultured in RPMI-1640 media containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Cell cytotoxity was determined by MTT assay. Briefly, cells were seeded in 96-well-plate (1×10^4 cells/well) and incubated for 48 h. Then, the tested compounds with various concentrations were added to the wells and 48 h later the MTT solution (0.5 mg/mL) was added and incubated for 4 h. Two hundred microliters of DMSO was added to each well to dissolve the reduced MTT crystals. The absorbance of each well was measured at 570 nm with a microplate reader.
30. Selected spectral data for compounds **1a–2b**: Compound **1a**: white solid, mp 172–174 °C; IR (KBr, cm^{-1}) 3415, 2952, 2928, 2879, 1731, 1717, 1696, 1629, 1466, 1384, 1370, 1311, 1170, 1136, 1073, 1035, 999, 942; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.56(s, 1H), 6.07(s, 1H), 5.29(d, *J*=6 Hz, 1H), 5.16(d, *J* = 6 Hz, 1H), 3.49(s, 3H), 2.35(d, *J* = 13.2 Hz, 1H), 2.26(d, *J* = 13.41 Hz, 1H), 1.98–2.04(m, 3H), 1.86–1.94(m, 4H), 1.43–1.83(m, 6H), 1.26(s, 3H), 0.91–1.21(m, 3H), 0.88(s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 176.6, 165.4, 143.3, 125.8, 90.5, 84.9, 70.3, 57.9, 56.1, 50.2, 43.9, 42.6, 41.7, 40.9, 39.7, 38.1, 37.4, 28.8, 20.6, 19.1, 18.8, 16.4; ESI/MS: 393 $[M+H]^+$; HRMS: calcd for $C_{22}H_{32}NaO_6$ $[M+Na]^+$ 415.2091, found 415.2102. Compound **1b**: white solid, mp 218–220 °C; IR (KBr, cm^{-1}) 3539, 3423, 3221, 2957, 2868, 1704, 1630, 1467, 1385, 1370, 1353, 1292, 1264, 1234, 1202, 1164, 1095, 999, 970, 956, 812, 499; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.1(s, 1H), 6.26(d, *J* = 1.77 Hz, 1H), 5.94(d, *J* = 1.1 Hz, 1H), 2.20(d, *J* = 13.38 Hz, 1H), 2.04(d, *J* = 12.93 Hz, 1H), 1.77–1.90(m, 5H), 1.61–1.75(m, 4H), 1.41–1.45(m, 1H), 1.33(m, 1H), 1.18–1.27(m, 2H), 1.14(s, 3H), 0.93–1.05(m, 2H), 0.88(s, 3H), 0.74–0.85(m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 178.5, 164.7, 144.1, 124.6, 84.4, 68.6, 54.9, 50.1, 42.6, 41.6, 41.2, 40.4, 39.2, 38.3, 37.1, 28.4, 20.4, 18.9, 18.5, 15.9; ESI/MS: 331 $[M+1-H_2O]^+$. Compound **1c**: white solid, mp 200~202 °C; IR (KBr, cm^{-1}) 3437, 2954, 2928, 2868, 1721, 1697, 1625, 1456, 1437, 1371, 1307, 1291, 1237, 1202, 1167, 1149, 1118, 1083, 1039, 995, 971; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.55(s, 1H), 6.07(s, 1H), 3.65(s, 3H), 2.35(d, *J* = 13.2 Hz, 1H), 2.23(d, *J* = 13.32 Hz, 1H), 1.96–2.0(m, 2H), 1.85–1.91(m, 4H), 1.76–1.82(m, 2H), 1.62–1.74(m, 2H), 1.25–1.58(m, 4H), 1.21(s, 3H), 1.03–1.18(m, 1H), 0.93–1.00(m, 1H), 0.85(s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 177.6, 165.4, 143.3, 125.8, 85.0, 70.3, 56.0, 51.3, 50.2, 43.6, 42.5, 41.7, 40.9, 39.6, 38.1, 37.5, 28.7, 20.6, 19.1, 18.8, 16.2; ESI/MS: 380 $[M+NH_4]^+$; HRMS: calcd for $C_{21}H_{30}NaO_5$ $[M+Na]^+$ 385.1985, found 385.1996. Compound **1f**: colorless oil, IR (KBr, cm^{-1}) 3427, 2956, 2932, 2871, 1718, 1627, 1455, 1370, 1351, 1275, 1259, 1231, 1207, 1163, 1141, 1092, 999, 996, 806, 755, 745, 699; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.34(m, 5H), 6.54(s, 1H), 6.05(s, 1H), 5.15(d, *J* = 12.34 Hz, 1H), 5.04(d, *J* = 12.33 Hz, 1H), 2.25(d, *J* = 13.17 Hz, 1H), 1.68–2.00(m, 9H), 1.57–1.63(m, 2H), 1.45–1.55(m, 3H), 1.24(s, 3H), 0.92–1.22(m, 3H), 0.76(s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 176.8, 165.3, 143.3, 135.8, 128.5, 128.3(2), 128.2(2), 125.8, 84.9, 70.4, 66.1, 56.2, 50.1, 43.76, 42.5, 41.7, 40.9, 39.6, 38.1, 37.5, 28.8, 20.6, 19.1, 18.8, 16.3; ESI/MS: 461 $[M+Na]^+$. Compound **2a**: white solid, mp 152–154 °C; IR (KBr, cm^{-1}) 3433, 2935, 2866, 1717, 1654, 1629, 1458, 1384, 1374, 1307, 1275, 1260, 1241, 1159, 1090, 1036, 1000; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.48(s, 1H), 6.00(s, 1H), 4.11(d, *J* = 11.1 Hz, 1H), 3.83(d, *J* = 11 Hz, 1H), 2.26(d, *J* = 13.2 Hz, 1H), 1.98(s, 3H), 1.82–1.94(m, 5H), 1.71–1.81(m, 2H), 1.58–1.67(m, 2H), 1.36–1.53(m, 4H), 1.26–1.32(m, 1H), 1.15–1.23(m, 1H), 1.03–1.09(m, 1H), 0.99(s, 3H), 0.93(s, 3H), 0.78–0.90(m, 1H); ESI/MS: 375 $[M-H]^-$. **2b**: white solid, 92–94 °C; IR (KBr, cm^{-1}) 3424, 2961, 2930, 2870, 1701, 1626, 1475, 1446, 1384, 1369, 1348, 1307, 1267, 1174, 1164, 1029, 1000; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 6.26(s, 1H), 5.94(s, 1H), 3.45–3.51(m, 1H), 3.16–3.22(m, 1H), 2.22(d, *J* = 13.4 Hz, 1H), 1.80–1.98(m, 4H), 1.67–1.76(m, 4H), 1.42–1.54(m, 3H), 1.23–1.36(m, 3H), 1.18(m, 1H), 1.03–1.15(m, 1H), 0.99(s, 3H), 0.89(s, 3H), 0.83(m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 164.7, 144.3, 124.5, 84.6, 68.6, 63.0, 55.5, 51.1, 43.5, 41.8, 41.7, 40.1, 39.2, 38.3, 35.1, 27.7, 18.8, 18.4, 18.0, 17.7; ESI/MS: 333 $[M-H]^-$; HRMS: calcd for $C_{40}H_{60}NaO_8$ $[2M+Na]^+$ 691.4180, found 691.4207.