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Synthesis and antitumor activity of three novel ginsenoside M1 derivatives with 3′-ester modifications

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ABSTRACT

Ginsenoside M1 (M1) was considered to be the main antitumor component of ginsenoside metabolites in the body. In order to enhance its potency on antitumor effect, three novel M1 3′–ester derivatives (**1c**, **2c**, **3c**) were synthesized and evaluated. The yield of these derivatives was between 41% and 69%. Compared with M1, **2c** and **3c** can improve the efficacy of the inhibition on breast cancer MCF–7 and MDA–MB–231 cells, especially for MCF–7 (fold: 0.7–4.2, $p < 0.0001$). Further study suggested that **2c** and **3c** may cause cell autophagy and promote apoptosis in MCF–7 cells. The results indicated the 3′–ester modified M1 derivatives **2c** and **3c** possess higher abilities of inhibition growth towards triple–positive breast cancer and provided a new source for synthesis of potential anti-breast cancer drugs.

1. Introduction

Breast cancer is a heterogeneous disease with substantial genotypic and phenotypic diversity [1]. Worldwide, breast cancer is the second most common cancer and the fifth leading cause of cancer deaths among females [2]. In the United States, breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths among females [2]. In China, breast cancer has become the highest risk disease for women living in big cities. Most patients with the disease localized to the breast in clinical, approximately 20–85% of patients, will later develop recurrent and/or metastatic disease; whereas 10% of patients already present with locally advanced and/or metastatic breast cancer [3]. Since breast cancer is a very heterogeneous disease with distinct morphologies, molecular traits, prognoses, and treatment options, clinical decisions are mainly made on the basis of the tumor stage, lymph-node involvement, and molecular subtype, represented clinically by the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER2) [4]. According to the reported, intratumoral heterogeneity of HER2 expression and gene amplification has been in 16–36% of patients with HER2-positive breast cancer [5,6]. While, negative ER, PR and HER2 expression (triple negative breast cancer) accounts for 15–20% of all breast cancer cases [7,8]. Although breast cancer mortality has been decreasing since 2004 with the aid of early detection and more rational and scientific treatment, the five-year survival rate is still dissatisfactory [9]. Moreover, current clinical drugs such as tamoxifen citrate (tamoxifen), ethimetam (anoxine), trastuzumab (herceptin), doxorubicin and so on have various serious side effects. In the absence of approved targeted therapy, chemotherapy remains the mainstay of the limited treatment options for breast cancer at present, and there is an urgent need to find new and more effective agent for the treatment of breast cancer.

Panax ginseng (Ginseng) has been widely used as a natural medicine to treat a variety of ailments in China for thousands of years. As listed by the U.S. National Institutes of Health as a complementary and alternative medicine, the antitumor functions of ginseng are being increasingly recognized [10]. Ginseng played antitumor role in a variety of ways such as anti-inflammatory, anti-proliferation, apoptosis, free radical scavenging [11–14]. Ginsenoside, the active constituents of ginseng, was usually recognized as the main material basis to exert its anti-tumor effect [10]. Some ginsenosides such as Rg3 (Shenyi capsule) and Rh2 (Jinxing capsule) have been developed as antitumor ingredients for clinical use in China. However, since natural ginsenosides had many drawbacks in terms of drug development, synthesized or

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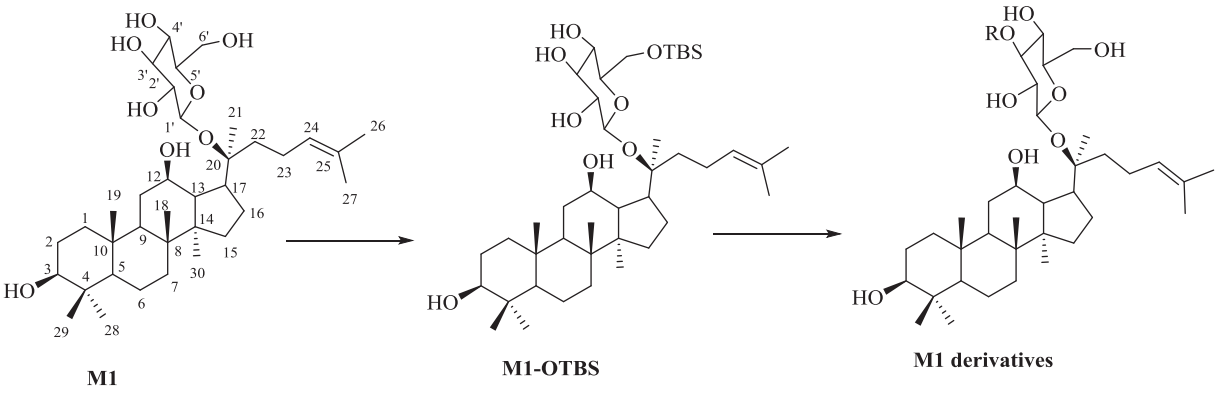


Fig. 1. M1, M1-OTBS and M1 3′-monoester derivatives.

modified natural ginsenosides become an effective method to make more efficacious, affordable and safe ginsenoside derivatives [15–17].

Ginsenoside M1 (M1) (Fig. 1) was a protopanaxadiol type ginsenoside, and was first obtained by bacterial hydrolysis of a mixture of ginsenosides Rb1, Rb2 and Rc [18]. Then, the following studies identified M1 as a metabolite from ginsenosides by intestinal flora [19], and proposed that the metabolized M1 was esterified with fatty acids in the liver, which resulted in longer permanence in the body and exhibited better antitumor activity [20]. M1 esterified derivatives may play a more important role for its efficacious on tumor cells. Although previous studies have shown that M1 exerted antitumor effects *in vitro* and *in vivo* through different mechanisms, including cytotoxicity, inhibition of tumor cell invasion and metastasis, inhibition of tumor growth [21], there were few related to M1 derivatives [22–24].

Among the diverse activities of ginsenoside, some specific ginsenoside compounds have estrogenic activity, such as ginsenoside Rg1 [25,26], Rb1 [27], Rh1 [28], Rh2 [29], Rb3 [30] and Re [31]. MDA-MB-231 and MCF-7 human cancer cell lines are estrogen receptor and estrogen receptor-positive cells, respectively. These cell lines are well-established *in vitro* models for evaluating estrogen-responsive or estrogen-independent antitumor drugs [32]. In this study, as an ongoing research of synthesized novel M1 derivatives with increased antitumor activities [33], three 3′–ester of M1 derivatives were synthesized for antitumor activities evaluation on MCF-7 and MDA-MB-231 cell lines.

2. Experimental section

2.1. Chemistry

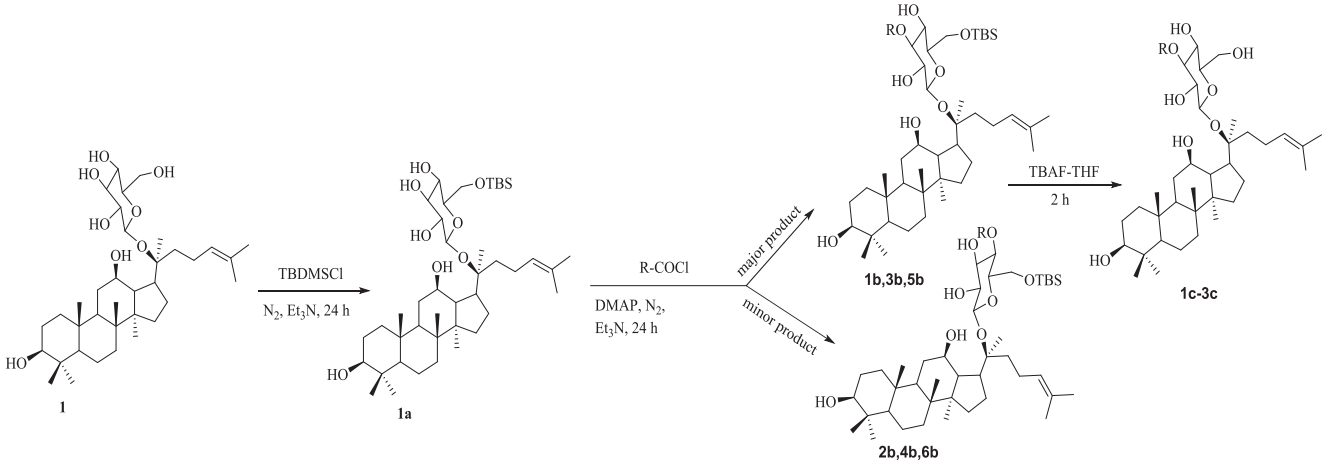
In order to obtain the 3′–monoester derivatives of M1, we adopted a strategy of protection–esterification–deprotection (Scheme 1, Table 1). Among the six hydroxyls in M1 (Fig. 1), 6′–hydroxyl group was most active [34], and esterification reaction was conducted after the optimum protective agent to 6′–hydroxyl. Then selective esterification was the reaction of the 6′–hydroxyl protected M1 with different acyl chloride in the presence of 4–dimethylaminopyridine (DMAP). The deprotection was achieved using tetrabutylammonium fluoride (TBAF) and monitored by HPLC. All the products were purified from the reaction mixture by silica gel column chromatography, the structures were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectra. Melting points were determined in open capillary tubes and are uncorrected. The MS data were obtained on an Agilent 1100–MDS SCIEX QSTAR instrument. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker DRX–500 spectrometer using tetramethylsilane as the internal standard. The major chemicals were purchased from Sigma–Aldrich.

Table 1
The yields of M1 3′-monoester derivatives through the whole procedure.

M1 derivatives	*R*	Total yields
1c	Decanoyl	69%
2c	Cyclohexaneformyl	58%
3c	Isobutyryl	41%

2.1.1. Procedure for the protection of hydroxyls in M1

Under nitrogen protection, M1 (1 equiv, 100 mg, 0.16 mmol) was reacted with *tert*–butyl dimethylchlorosilane (TBDMSCl) (5 equiv, 120 mg) in a solution of 5 mL triethylamine for 24 h at room temperature, and the reaction was terminated by adding a saturated ammonium chloride solution. After extracting the mixture product from



Scheme 1. Synthesis of M1 monoester derivatives and the description of protection–esterification–deprotection.

dichloromethane, washed (brine), dried (Na_2SO_4) and concentrated under reduced pressure. The crude products were chromatographed using silica gel and eluted with methanol/dichloromethane (1:4) to give the pure product **1a**.

2.1.2. Esterification of M1 silicon ether derivatives

1a (1equiv, 100 mg, 0.14 mmol) was added to DMAP (0.05 equiv, 0.85 mg) in a 3 mL solution of triethylamine under nitrogen and ice water to react 10 min, then different acyl chloride (0.05 equiv) was added to the mixture for 24 h at room temperature. After completion of the reaction (as monitored by TLC), it was terminated by adding a saturated ammonium chloride solution. The residue was extracted with dichloromethane, washed (brine) and dried (Na_2SO_4). The crude products were chromatographed using silica gel and eluted with ethyl acetate/ petroleum ether (3:1) to afford corresponding products **1b–6b**. The esterification mainly selected on the 3′–hydroxyl group with the yield between 72% and 80%, and a small amount of by–products on the 4′–hydroxyl group (15% to 18%) were also isolated.

2.1.3. Procedure for deprotection compounds 1c–3c

TBAF–THF was chosen as the desilyl reagent in this study. To a solution of the compounds **1b**, **3b** and **5b** (50 mg) in THF (20 mL), equivalent of TBAF was added under ice water bath, the mixture was allowed to stir for 2 h. After completion of the reaction (as monitored by HPLC) the solution was removed under reduced pressure. The residue was washed with brine and concentrated under reduced pressure to give the crude product. The crude products were chromatographed using silica gel and eluted with ethyl acetate/ petroleum ether (3:1) to afford monoester products **1c–3c**. The yield of this step was between 58% and 88%.

2.2. Antitumor bioassay

2.2.1. Cytotoxicity

MCF–7 and MDA–MB–231 cells were cultured in DMEM without phenol–red (Hyclone) and 10% charcoal-stripped fetal bovine serum (FBS, Gibco, USA) at 37 °C with 5% CO_2 in a humidified incubator. The cytotoxicity of the compounds **1c**, **2c**, **3c** was examined by Cell Counting Kit-8 (CCK-8, Byotime, C0042) method. Breast cancer cells MCF–7 and MDA–MB–231 in 100 μL culture medium with a density of 1.0×10^4 cells/well were added to 96-well plates and incubated for 6 h. Then cells were treated with different concentrations of the compounds M1, **1c**, **2c**, and **3c** (0, 1, 25, 50, 100 μM). The concentration of stock solution M1, **1c**, **2c**, **3c** is 10 mM in DMSO, so 0.1% DMSO as a vehicle control. After 48 h incubation, cell culture medium was discarded, and the cells were washed twice with PBS and further incubated with 100 μL fresh medium containing 10 μL of CCK–8. The absorbance at 450 nm was measured by Synergy™Mx Multi–Mode Microplate Reader. Each experiment was run in triplicate. Cell viability was calculated based on the equation:

$$\text{Cell viability (\%)} = (\text{Sample} - \text{blank})/(\text{Control} - \text{blank}) \times 100\%$$

2.2.2. Effect on the mitochondrial membrane potential

Rhodamine 123 was used to detect the mitochondrial membrane potential. Breast cancer cells MCF–7 and MDA–MB–231 (4×10^3 cells/well) were passed onto the 3 cm culture dishes and incubated for 6 h, then the culture medium was removed and added the fresh culture medium with a concentration of 50 μM compound M1, **1c**, **2c**, **3c** incubated for 24 h. After washing the culture dishes three times with PBS, Rhodamine 123 with flesh culture medium was added in cell culture dish and incubated for 15 min. Fluorescence imaging experiments were carried out on fluorescence microscope.

2.2.3. Effect on the autophagy

Autophagy was detected by Dansylcadaverine (MDC). Breast cancer cells MCF–7 and MDA–MB–231 (4×10^3 cells/well) were passed onto the 3 cm culture dishes and incubated for 6 h, then the culture medium was removed and added the fresh culture medium with a concentration of 50 μM compound M1, **1c**, **2c**, **3c** incubated for 24 h. After washing the culture dishes three times with PBS, dansylcadaverine (MDC) stain was added into dished and incubated for 15 min. Fluorescence imaging experiments were carried out on fluorescence microscope.

3. Results and discussion

3.1. Synthesis of M1 3′-ester

3.1.1. Characterization of M1-OTBS (1a)

Yield: 98%. mp: 142–143 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.12 (t, J = 5.5 Hz, 1H, H-24), 4.51 (d, J = 6.2 Hz, 1H, H-1′), 3.88 (dd, J = 8.5, 3.4 Hz, 1H, Ha-6′), 3.76 (dd, J = 8.5, 3.4 Hz, 1H, Hb-6′), 3.63 (m, 1H, H-4′), 3.58 (m, 1H, H-3′), 3.47 (t, J = 7.4 Hz, 1H, H-5′), 3.31 (m, 1H, H-2′), 3.21 (dd, J = 11.2, 4.8 Hz, 1H, H-3), 1.68 (s, 3H, H-26), 1.56 (s, 3H, H-27), 1.37 (s, 3H, H-21), 1.24 (s, 6H, H-19, H-28), 0.97 (s, 3H, H-30), 0.89 (s, 9H, H-2″-H-4″), 0.87 (s, 3H, H-18), 0.77 (s, 3H, H-29), 0.72 (d, J = 11.3 Hz, 1H, H-5), 0.07 (s, 6H, H-5″, H-6″); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 131.3 (C-25), 124.7 (C-24), 96.9 (C-1′), 83.9 (C-20), 78.8 (C-3), 75.1 (C-3′), 73.4 (C-5′), 71.7 (C-2′), 70.4 (C-12), 70.4 (C-4′), 64.1 (C-6′), 55.8 (C-5), 51.6 (C-17), 51.3 (C-14), 49.8 (C-9), 48.0 (C-13), 39.9 (C-8), 39.8 (C-1), 39.0 (C-4), 37.1 (C-10), 35.6 (C-22), 34.8 (C-7), 30.6 (C-11), 30.3 (C-15), 28.0 (C-28), 27.3 (C-2), 26.5 (C-16), 25.8 (C-2″, 3″, 4″), 25.7 (C-26), 22.3 (C-23), 21.5 (C-21), 18.3 (C-6), 18.2 (C-1″), 17.7 (C-27), 17.0 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19), −5.7 (C-5″, C-6″). ESI-MS: m/z 759.5 $[M+Na]^+$ (Calcd. for $C_{42}H_{76}NaO_8Si$, 759.5).

3.1.2. Characterization of esterification products of M1 silicon ether derivatives

3.1.2.1. (20S)-O-(3′-decanoyl-6′-tert-butyldimethylsilyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol (1b). Yield: 80%. mp: 132–133 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.12 (t, J = 5.6 Hz, 1H, H-24), 4.96 (t, J = 7.5 Hz, 1H, H-3′), 4.56 (d, J = 6.2 Hz, 1H, H-1′), 3.86 (dd, J = 8.5, 3.4 Hz, 1H, Ha-6′), 3.79 (dd, J = 8.5, 3.4 Hz, 1H, Hb-6′), 3.61 (m, 1H, H-4′), 3.46 (t, J = 7.4 Hz, 1H, H-5′), 3.34 (m, 1H, H-2′), 3.21 (dd, J = 11.2, 4.8 Hz, 1H, H-3), 2.40 (t, J = 6.0 Hz, 2H, H-2″), 1.69 (s, 3H, H-26), 1.60 (s, 3H, H-27), 1.38 (s, 3H, H-21), 1.23 (s, 6H, H-19, H-28), 0.97 (s, 3H, H-30), 0.88 (s, 9H, H-2‴-H-4‴), 0.87 (t, J = 7.0 Hz, 3H, H-10″), 0.86 (s, 3H, H-18), 0.77 (s, 3H, H-29), 0.72 (d, J = 11.3 Hz, 1H, H-5), 0.08 (s, 6H, H-5‴, H-6‴); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.1 (C-1″), 131.5 (C-25), 124.6 (C-24), 97.3 (C-1′), 84.4 (C-20), 78.9 (C-3), 78.0 (C-3′), 74.9 (C-5′), 71.9 (C-2′), 71.3 (C-4′), 70.4 (C-12), 64.4 (C-6′), 55.9 (C-5), 51.7 (C-17), 51.3 (C-14), 49.8 (C-9), 48.1 (C-13), 39.8 (C-8), 39.0 (C-1), 39.0 (C-4), 37.1 (C-10), 35.6 (C-22), 34.8 (C-7), 34.4 (C-2″-C-8″), 30.6 (C-11), 30.4 (C-15), 28.1 (C-28), 27.5 (C-2), 26.7 (C-16), 25.8 (C-2‴, 3‴, 4‴), 25.7 (C-26), 25.0 (C-3″), 22.7 (C-9″), 22.3 (C-23), 21.5 (C-21), 18.3 (C-6), 18.2 (C-1‴), 17.7 (C-27), 17.0 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19), 14.1 (C-10″), −5.7 (C-5‴, C-6‴). ESI-MS: m/z 913.6 $[M+Na]^+$ (Calcd. for $C_{52}H_{94}NaO_9Si$, 913.7).

3.1.2.2. (20S)-O-(4′-decanoyl-6′-tert-butyldimethylsilyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol (2b). Yield: 16%. mp: 118–119 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.16 (t, J = 7.5 Hz, 1H, H-24), 4.92 (t, J = 7.8 Hz, 1H, H-4′), 4.56 (d, J = 6.2 Hz, 1H, H-1′), 3.61 (m, 1H, Ha-6′), 3.56 (m, 1H, Hb-6′), 3.47 (m, 1H, H-3′), 3.43 (m, 1H, H-5′), 3.38 (t, J = 6.4 Hz, 1H, H-2′), 3.20 (dd, J = 9.0, 3.8 Hz, 1H, H-3), 1.69 (s, 3H, H-26), 1.60 (s, 3H, H-27), 1.38 (s, 3H, H-21), 1.26 (s, 6H, H-19, H-28), 0.97 (s, 6H, H-30, H-18), 0.88 (t, J = 7.0 Hz, 3H, H-10″), 0.86 (s, 9H, H-2‴-H-4‴), 0.77 (s, 3H, H-29), 0.71 (d, J = 11.3 Hz, 1H, H-5), 0.03 (s, 6H, H-5‴, H-6‴); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 173.4 (C-1″), 131.5 (C-25), 124.6 (C-24), 97.2 (C-1′), 84.4 (C-20), 78.9 (C-3), 74.9 (C-3′), 74.7 (C-5′), 72.1 (C-2′), 68.8 (C-4′), 70.4 (C-12), 62.7 (C-6′), 55.9 (C-5), 51.7 (C-17), 51.0 (C-14), 51.0 (C-9), 47.9 (C-13), 39.8 (C-8),

39.0 (C-1), 38.9 (C-4), 38.9 (C-10), 37.1 (C-22), 35.5 (C-7), 34.8 (C-2″), 34.3 (C-11), 34.2 (C-15), 34.8 (C-3″-C-8″), 30.6 (C-28), 28.1 (C-2), 27.4 (C-16), 26.6 (C-26), 25.8 (C-2‴, 3‴, 4‴), 25.7 (C-23), 22.7 (C-9″), 22.1 (C-21), 18.3 (C-1‴), 18.2 (C-6), 17.7 (C-27), 17.0 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19), 14.1 (C-10″), −5.5,-5.4 (C-5‴, C-6‴). ESI-MS: *m/z* 913.6 $[M+Na]^+$ (Calcd. for $C_{52}H_{94}NaO_9Si$, 913.7).

3.1.2.3. (20S)-O-(3′-cyclohexaneformyl-6′-tert-butyldimethylsilyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(3b)***. Yield: 76%. mp: 160–161 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 5.12 (t, *J* = 7.6 Hz, 1H, H-24), 4.92 (t, *J* = 7.4 Hz, 1H, H-3′), 4.56 (d, *J* = 6.2 Hz, 1H, H-1′), 3.86 (dd, *J* = 8.5, 3.7 Hz, 1H, Ha-6′), 3.80 (dd, *J* = 8.5, 4.6 Hz, 1H, Hb-6′), 3.58 (m, 1H, H-3′), 3.54 (m, 1H, H-5′), 3.46 (t, *J* = 6.9 Hz, 1H, H-4′), 3.21 (dd, *J* = 9.1, 3.7 Hz, 1H, H-3), 2.22 (m, 1H, H-2″), 1.69 (s, 3H, H-26), 1.60 (s, 3H, H-27), 1.36 (s, 3H, H-21), 1.26 (s, 6H, H-19, H-28), 0.97 (s, 6H, H-30, H-18), 0.88 (s, 9H, H-2‴-H-4‴), 0.77 (s, 3H, H-29), 0.72 (d, *J* = 11.3 Hz, 1H, H-5), 0.06 (s, 6H, H-5‴, H-6‴); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 177.4 (C-1″), 131.5 (C-25), 124.6 (C-24), 97.3 (C-1′), 84.3 (C-20), 78.9 (C-3), 78.0 (C-3′), 75.2 (C-5′), 72.4 (C-2′), 71.9 (C-4′), 70.4 (C-12), 64.2 (C-6′), 55.8 (C-5), 51.7 (C-17), 51.2 (C-14), 49.8 (C-9), 48.0 (C-13), 43.3 (C-2″), 39.8 (C-8), 39.0 (C-1), 38.9 (C-4), 38.9 (C-10), 37.1 (C-22), 35.5 (C-7), 34.8 (C-11), 34.8 (C-15), 30.6 (C-28), 29.1 (C-3″, C-7″), 29.0 (C-5″), 28.1 (C-2), 27.4 (C-16), 26.6 (C-26), 25.9 (C-2‴, 3‴, 4‴), 25.8 (C-23), 25.4 (C-4″, C-6″), 22.3 (C-21), 18.3 (C-1‴), 18.2 (C-6), 17.7 (C-27), 17.0 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19), −5.5 (C-5‴, C-6‴). ESI-MS: *m/z* 869.5 $[M+Na]^+$ (Calcd. for $C_{49}H_{86}NaO_9Si$, 869.6).

3.1.2.4. (20S)-O-(4′-cyclohexaneformyl-6′-tert-butyldimethylsilyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(4b)***. Yield: 18%. mp: 154–156 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 5.15 (t, *J* = 7.6 Hz, 1H, H-24), 4.91 (t, *J* = 7.8 Hz, 1H, H-4′), 4.56 (d, *J* = 6.2 Hz, 1H, H-1′), 3.61 (m, 1H, Ha-6′), 3.55 (m, 1H, Hb-6′), 3.51 (m, 1H, H-3′), 3.46 (m, 1H, H-5′), 3.38 (t, *J* = 5.6 Hz, 1H, H-2′), 3.20 (dd, *J* = 9.1, 3.7 Hz, 1H, H-3), 2.24 (m, 1H, H-2″), 1.70 (s, 3H, H-26), 1.60 (s, 3H, H-27), 1.38 (s, 3H, H-21), 1.27 (s, 6H, H-19, H-28), 0.97 (s, 6H, H-30, H-18), 0.86 (s, 9H, H-2‴-H-4‴), 0.77 (s, 3H, H-29), 0.72 (d, *J* = 11.3 Hz, 1H, H-5), 0.02 (s, 6H, H-5‴, H-6‴); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 175.7 (C-1″), 131.5 (C-25), 124.6 (C-24), 97.3 (C-1′), 84.5 (C-20), 78.8 (C-3), 75.0 (C-3′), 74.2 (C-5′), 72.4 (C-2′), 70.4 (C-12), 68.3 (C-4′), 62.5 (C-6′), 55.8 (C-5), 51.8 (C-17), 51.1 (C-14), 49.8 (C-9), 47.9 (C-13), 43.2 (C-2″), 39.8 (C-8), 38.9 (C-1), 38.9 (C-4), 38.0 (C-10), 37.1 (C-22), 35.6 (C-7), 34.8 (C-11), 34.8 (C-15), 30.4 (C-28), 28.8 (C-3″, C-5″, C-7″), 28.1 (C-2), 27.4 (C-16), 26.7 (C-26), 25.8 (C-2‴, 3‴, 4‴), 25.7 (C-23), 25.4 (C-4″, C-6″), 22.1 (C-21), 18.3 (C-1‴), 18.2 (C-6), 17.7 (C-27), 17.0 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19), −5.4,-5.6 (C-5‴, C-6‴). ESI-MS: *m/z* 869.5 $[M+Na]^+$ (Calcd. for $C_{49}H_{86}NaO_9Si$, 869.6).

3.1.2.5. (20S)-O-(3′-isobutyryl-6′-tert-butyldimethylsilyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(5b)***. Yield: 72%. mp: 166–168 °C. ^{1}H NMR (500 MHz, CD_3OD) *δ*: 5.13 (t, *J* = 5.6 Hz, 1H, H-24), 4.93 (t, *J* = 7.5 Hz, 1H, H-3′), 4.65 (d, *J* = 6.3 Hz, 1H, H-1′), 3.92 (dd, *J* = 8.9, 1.4 Hz, 1H, Ha-6′), 3.76 (dd, *J* = 8.9, 4.7 Hz, 1H, Hb-6′), 3.70 (m, 1H, H-4′), 3.46 (t, *J* = 7.4 Hz, 1H, H-5′), 3.40 (m, 1H, H-2′), 3.25 (dd, *J* = 11.2, 4.8 Hz, 1H, H-3), 2.64 (dt, *J* = 5.6 Hz, 1H, H-2″), 1.69 (s, 3H, H-26), 1.63 (s, 3H, H-27), 1.39 (s, 3H, H-21), 1.21 (d, *J* = 1.1 Hz, 3H, H-3″), 1.20 (d, *J* = 1.1 Hz, 3H, H-4″), 1.01 (s, 6H, H-19, H-28), 0.97 (s, 3H, H-30), 0.92 (s, 9H, H-2‴-H-4‴), 0.89 (s, 3H, H-18), 0.78 (s, 3H, H-29), 0.74 (d, *J* = 11.3 Hz, 1H, H-5), 0.09 (s, 6H, H-5‴, H-6‴); ^{13}C NMR (125 MHz, CD_3OD) *δ*: 178.7 (C-1″), 132.1 (C-25), 126.0 (C-24), 98.0 (C-1′), 85.1 (C-20), 79.6 (C-3), 79.5 (C-3′), 78.0 (C-5′), 73.6 (C-2′), 71.5 (C-12), 69.8 (C-4′), 64.1 (C-6′), 57.3 (C-5), 52.6 (C-17), 52.4 (C-14), 51.2 (C-9), 50.8 (C-13), 41.0 (C-8), 40.3 (C-1), 40.0 (C-4), 38.2 (C-10), 36.8 (C-22), 35.9 (C-7), 35.3 (C-2″), 31.7 (C-11), 31.6 (C-15), 29.8 (C-28), 28.7 (C-2), 28.0 (C-16), 27.3 (C-26), 26.5 (C-2‴, 3‴, 4‴), 26.0 (C-23), 23.8 (C-21), 19.5, 19.4 (C-3″, 4″), 19.2 (C-1‴), 18.6 (C-6), 18.0 (C-27), 17.5 (C-30), 16.7 (C-29), 16.4 (C-18), 16.1 (C-19), −5.0,-5.1 (C-5‴, C-6‴). ESI-MS: *m/z* 829.5 $[M+Na]^+$ (Calcd. for $C_{46}H_{82}NaO_9Si$, 829.6).

3.1.2.6. (20S)-O-(4′-isobutyryl-6′-tert-butyldimethylsilyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(6b)***. Yield: 15%. mp: 145–146 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 5.17 (t, *J* = 7.6 Hz, 1H, H-24), 4.93 (t, *J* = 7.9 Hz, 1H, H-4′), 4.57 (d, *J* = 6.2 Hz, 1H, H-1′), 3.62 (m, 1H, Ha-6′), 3.54 (m, 1H, Hb-6′), 3.51 (m, 1H, H-3′), 3.46 (m, 1H, H-5′), 3.39 (t, *J* = 6.4 Hz, 1H, H-2′), 3.20 (dd, *J* = 9.1, 3.7 Hz, 1H, H-3), 2.54 (dt, *J* = 5.6 Hz, 1H, H-2″), 1.69 (s, 3H, H-26), 1.61 (s, 3H, H-27), 1.39 (s, 3H, H-21), 1.27 (s, 6H, H-19, H-28), 1.15 (d, *J* = 1.6 Hz, 3H, H-3″), 1.11 (d, *J* = 1.6 Hz, 3H, H-4″), 0.97 (s, 6H, H-30, H-18), 0.86 (s, 9H, H-2‴-H-4‴), 0.77 (s, 3H, H-29), 0.72 (d, *J* = 11.3 Hz, 1H, H-5), 0.02 (s, 6H, H-5‴, H-6‴); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 176.7 (C-1″), 131.5 (C-25), 124.6 (C-24), 97.3 (C-1′), 84.5 (C-20), 78.8 (C-3), 75.0 (C-3′), 76.7 (C-5′), 74.9 (C-2′), 72.4 (C-4′), 70.4 (C-12), 62.6 (C-6′), 55.8 (C-5), 51.8 (C-17), 49.8 (C-14), 49.4 (C-9), 47.9 (C-13), 39.8 (C-8), 39.0 (C-1), 39.0 (C-4), 38.0 (C-10), 37.1 (C-22), 35.6 (C-7), 34.8 (C-2″), 34.0 (C-11), 33.9 (C-15), 30.4 (C-28), 28.1 (C-2), 27.4 (C-16), 26.7 (C-26), 25.8 (C-2‴, 3‴, 4‴), 25.7 (C-23), 22.1 (C-21), 18.9 (C-3″, 4″), 18.8 (C-1‴), 18.3 (C-6), 17.7 (C-27), 17.0 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19), −5.5,-5.6 (C-5‴, C-6‴). ESI-MS: *m/z* 829.5 $[M+Na]^+$ (Calcd. for $C_{46}H_{82}NaO_9Si$, 829.6).

3.1.3. Characterization of M1 esterification products

3.1.3.1. (20S)-O-(3′-decanoyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(1c)***. Yield: 88%. mp: 130–131 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 5.12 (t, *J* = 5.6 Hz, 1H, H-24), 4.50 (d, *J* = 7.4 Hz, 1H, H-1′), 4.36 (m, 1H, Ha-6′), 4.29 (dd, *J* = 11.8, 6.0 Hz, 1H, Hb-6′), 3.56 (m, 1H, H-4′), 3.43 (m, 1H, H-3′), 3.41 (m, 1H, H-2′), 3.33 (t, *J* = 6.3 Hz, 1H, H-5′), 3.20 (dd, *J* = 11.2, 4.8 Hz, 1H, H-3), 2.31 (t, *J* = 7.6 Hz, 2H, H-2″), 1.69 (s, 3H, H-26), 1.63 (s, 3H, H-27), 1.35 (s, 3H, H-21), 0.98, 0.97 (s, 6H, H-19, H-28), 0.90 (s, 3H, H-30), 0.88 (t, *J* = 7.0 Hz, 3H, H-10″), 0.87 (s, 3H, H-18), 0.78 (s, 3H, H-29), 0.73 (d, *J* = 11.3 Hz, 1H, H-5); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 174.1 (C-1″), 131.6 (C-25), 124.6 (C-24), 97.0 (C-1′), 84.4 (C-20), 78.9 (C-3), 76.6 (C-3′), 73.5 (C-5′), 73.4 (C-2′), 70.7 (C-12), 70.1 (C-4′), 63.3 (C-6′), 55.9 (C-5), 51.8 (C-17), 51.4 (C-14), 49.9 (C-9), 47.9 (C-13), 39.8 (C-8), 39.0 (C-1), 39.0 (C-4), 37.1 (C-10), 35.5 (C-22), 34.8 (C-7), 34.2 (C-2″), 31.9 (C-3″-C-8″), 30.7 (C-11), 30.6 (C-15), 28.1 (C-28), 27.4 (C-2), 26.8 (C-16), 25.7 (C-26), 24.9 (C-23), 22.7 (C-9″), 21.3 (C-21), 18.3 (C-6), 17.7 (C-27), 17.0 (C-30), 16.2 (C-29), 15.8 (C-18), 15.4 (C-19), 14.1 (C-10″). ESI-MS: *m/z* 799.5 $[M+Na]^+$ (Calcd. for $C_{46}H_{80}NaO_9$, 799.6).

3.1.3.2. (20S)-O-(3′-cyclohexaneformyl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(2c)***. Yield: 78%. mp: 140–141 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 5.13 (t, *J* = 7.4 Hz, 1H, H-24), 4.54 (d, *J* = 6.2 Hz, 1H, H-1′), 4.44 (m, 1H, H-3′), 4.15 (dd, *J* = 9.4, 4.8 Hz, 1H, Ha-6′), 3.67 (m, 1H, Hb-6′), 3.62 (m, 1H, H-4′), 3.43 (m, 1H, H-2′), 3.33 (m, 1H, H-5′), 3.21 (dd, *J* = 11.2, 4.8 Hz, 1H, H-3), 2.26 (m, 1H, H-2″), 1.68 (s, 3H, H-26), 1.59 (s, 3H, H-27), 1.36 (s, 3H, H-21), 1.27 (s, 6H, H-19, H-28), 1.03 (s, 6H, H-30, H-18), 0.77 (s, 3H, H-29), 0.73 (d, *J* = 11.3 Hz, 1H, H-5); ^{13}C NMR (125 MHz, $CDCl_3$) *δ*: 176.0 (C-1″), 131.1 (C-25), 125.0 (C-24), 96.8 (C-1′), 83.8 (C-20), 78.8 (C-3), 73.7 (C-2′), 73.5 (C-3′), 71.7 (C-5′), 70.4 (C-4′), 70.1 (C-12), 63.8 (C-6′), 55.8 (C-5), 51.5 (C-17), 51.3 (C-14), 49.8 (C-9), 48.3 (C-13), 43.2 (C-2″), 39.8 (C-8), 38.9 (C-1), 38.9 (C-4), 38.9 (C-10), 37.1 (C-22), 35.5 (C-7), 34.8 (C-11), 34.8 (C-15), 30.5 (C-28), 29.7 (C-3″, C-7″), 29.0 (C-5″), 28.0 (C-2), 27.4 (C-16), 26.5 (C-26), 25.8 (C-23), 25.4 (C-4″, C-6″), 22.2 (C-21), 18.3 (C-6), 17.6 (C-27), 17.1 (C-30), 16.1 (C-29), 15.8 (C-18), 15.4 (C-19). ESI-MS: *m/z* 755.4 $[M+Na]^+$ (Calcd. for $C_{43}H_{72}NaO_9$, 755.5).

3.1.3.3. (20S)-O-(3′-isobutyryl-β-D-glucopyranosyl)-dammarane-3β,12β-diol ***(3c)***. Yield: 58%. mp: 137–138 °C. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 5.11 (t, *J* = 6.5 Hz, 1H, H-24), 4.55 (d, *J* = 6.2 Hz, 1H, H-1′), 4.41 (m,

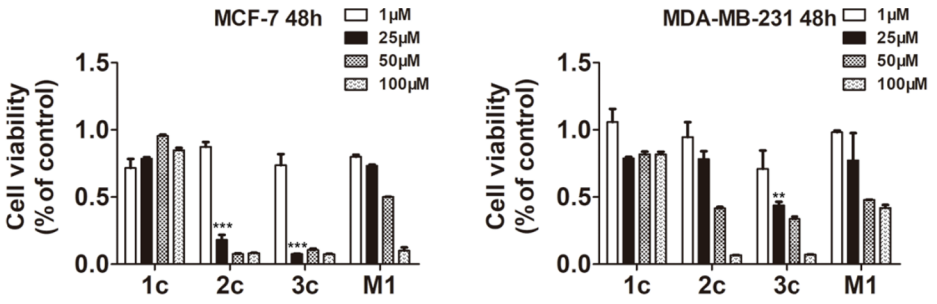


Fig. 2. The cytotoxicity assays of compound M1, **1c**, **2c**, **3c** deal with breast cancer cell MCF-7 and MDA-MB-231 for 48 h. Cell viability percentage were determined using 0.1%DMSO as a vehicle control. Values are means ± SD ($n = 3$). Bars with different superscripts are significantly different compared with the same concentration of M1 (**: $p < 0.001$; ***: $p < 0.0001$).

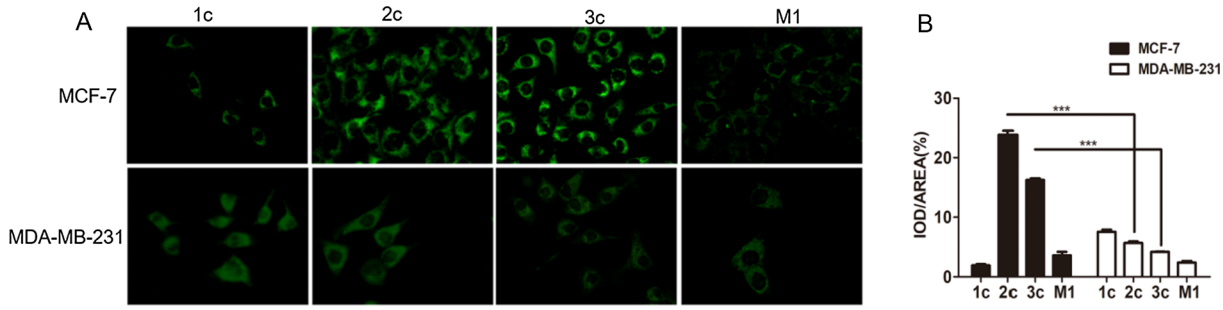


Fig. 3. The assays of mitochondrial membrane potential. A is the result that MCF-7 and MDA-MB-231 cells were treated with **1c**, **2c**, **3c** and M1 for 24 h and then stained with Rhodamine 123 for 30 min, the microscopic fields was set at 400 × magnification. B is statistical analysis of A (***: $p < 0.0001$).

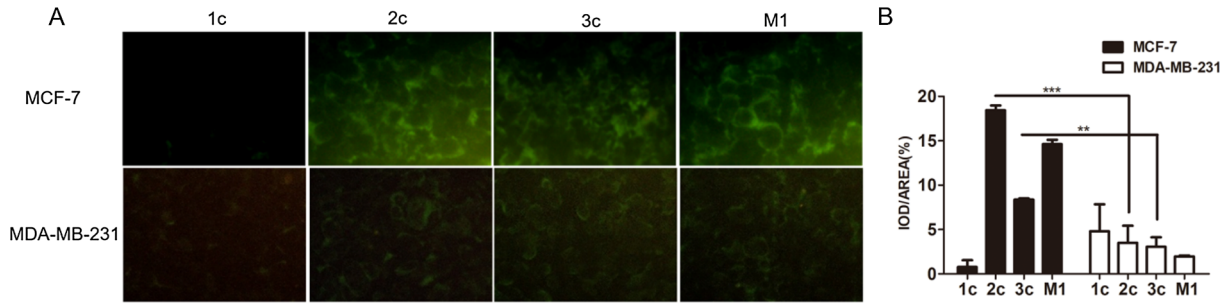


Fig. 4. The effect of autophagy of M1, **1c**, **2c** and **3c**. A is the result that MCF-7 and MDA-MB-231 cells were treated with **1c**, **2c**, **3c** and M1 for 24 h and then stained with MDC for 30 min, the microscopic fields was set at 400 × magnification. B is statistical analysis of A (**: $p < 0.001$; ***: $p < 0.0001$).

1H, H-3′), 4.21 (dd, $J = 9.4$, 4.8 Hz, 1H, Ha-6′), 3.82 (m, 1H, Hb-6′), 3.61 (m, 1H, H-4′), 3.44 (m, 1H, H-2′), 3.33 (t, $J = 6.4$ Hz, 1H, H-5′), 3.20 (dd, $J = 11.2$, 4.8 Hz, 1H, H-3), 2.56 (dt, $J = 5.6$ Hz, 1H, H-2″), 1.68 (s, 3H, H-26), 1.59 (s, 3H, H-27), 1.36 (s, 3H, H-21), 1.17 (d, $J = 1.5$ Hz, 3H, H-3″), 1.15 (d, $J = 5.6$ Hz, 3H, H-4″), 0.97 (s, 6H, H-30, H-18), 0.89 (s, 3H, H-28), 0.87 (s, 3H, H-19), 0.78 (s, 3H, H-29), 0.73 (d, $J = 11.3$ Hz, 1H, H-5); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 177.2 (C-1″), 131.5 (C-25), 124.7 (C-24), 96.6 (C-1′), 84.3 (C-20), 78.9 (C-3), 76.8 (C-3′), 73.6 (C-5′), 73.5 (C-2′), 70.7 (C-4′), 70.2 (C-12), 63.6 (C-6′), 55.9 (C-5), 51.8 (C-17), 51.4 (C-14), 49.9 (C-9), 48.0 (C-13), 39.8 (C-8), 39.0 (C-1), 38.9 (C-4), 38.0 (C-10), 37.1 (C-22), 35.6 (C-7), 34.8 (C-2″), 34.0 (C-11), 34.0 (C-15), 30.7 (C-28), 28.1 (C-2), 27.4 (C-16), 26.7 (C-26), 25.7 (C-23), 22.2 (C-21), 19.0 (C-3″, 4″), 18.3 (C-6), 17.7 (C-27), 17.1 (C-30), 16.2 (C-29), 15.8 (C-18), 15.4 (C-19). ESI-MS: m/z 715.4 $[M+Na]^+$ (Calcd. for $C_{40}H_{68}NaO_9$, 715.5).

3.2. Antitumor bioassay

3.2.1. Cytotoxicity

MCF–7 and MDA–MB–231 were two different types of breast cancer cells. MCF–7 were breast cancer cells with positive ER, PR and HER2, while MDA–MB–231 were breast cancer cells with negative ER, PR and HER2. We synthesized three new derivatives **1c**, **2c**, **3c** based on M1. Through the CCK–8 results (Fig. 2), it was shown that compared with M1, **1c** reduced the cytotoxicity and nearly didn't inhibit the growth of MCF–7 and MDA–MB–231 cells with concentration more than 50 μM. While, both **2c** and **3c** had a good inhibitory effect on the two types of breast cancer cells with relatively lower concentration and the inhibiton rate was more than 80% for MCF–7. However, for MDA–MB–231, higher concentration was needed to exhibt a better inhibitory effect. So, **2c** and **3c** had stronger inhibitory effects on the growth of triple negative breast cancer cells MDA–MB–231 at the higher concentrations tested, while just lower concentrations on triple positive breast cells MCF–7 was enough. Compared the results with the reported [24], 6′–ester M1 derivates also showed stronger cytotoxic activities at the concentration of 300 μg/mL on MCF–7 cells. So, monoesterification of M1 was an effective method to gain better anti-tumor effect on breast cancer cell.

3.2.2. Effect on the mitochondrial membrane potential and autophagy

Indicated by the result of Rhodamine 123 stain (Fig. 3), **2c** and **3c** can significantly change the mitochondrial membrane potential, and the changes in mitochondrial membrane potential can further cause the changes in membrane permeability, resulting in cell autophagy, etc. As shown in Fig. 4 of the results of dansylcadaverine (MDC) which indicate the degree of autophagy, **2c** and **3c** had a good ability to promote the MCF–7 to produce autophagy. **2c** and **3c** had inhibitory effect on the growth of breast cancer cells, while the effect of **1c** was not obvious. It showed that M1 had undergone significant changes in its biological activity through different modifications at the same site of the structure.

Through the above observation of the mechanism of M1 and its

derivatives on breast cancer cells, we found that **2c** and **3c** significantly changed the membrane potential of breast cancer MCF–7, and caused autophagy in breast cancer cells MCF–7, but they showed poor effect on triple–negative breast cancer cells MDA–MB–231, and its mechanism may be another way.

4. Conclusions

In summary, three novel M1 3′–ester derivatives were synthesized and their antitumor activity was evaluated on MCF–7 and MDA-MB–231 breast cancer cells. Through the strategy of protection–esterification–deprotection, the yield of M1 3′–ester derivatives was all above 41%. Different esterification groups at position C–3′ produced different antitumor effects. Compounds **2c** and **3c** showed effective inhibition of the growth of MCF–7, which was 0.7–4.2 times that of M1. Furthermore, compounds **2c** and **3c** caused the change of mitochondrial membrane potential, resulted in autophagy in MCF–7, and promoted the cells death. The specific mechanism needs further experimental, the treatment effects of **2c** and **3c** on animal models of breast cancer, and their safe dosage and side effects under effective treatment are also very important. The results could provide a new chemistry modification method for the drug development of M1 in triple–positive breast cancer.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103061.

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