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REGULAR ARTICLE

Synthesis of diosgenin prodrugs: anti-inflammatory and antiproliferative activity evaluation

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Abstract. In this work, we evaluated the antiproliferative and anti-inflammatory activities of two diosgenin prodrugs. The prodrugs were obtained by esterification of diosgenin at position 3 with 4-oxo-4-(prop-2-yn-1-yloxy)butanoic acid followed by click reaction on terminal alkyne with 3-azidopropan-1-ol *N*-alkylated dendrons, resulting in a prodrug with methyl ester end-groups and a derivative with *tert*-butyl ester end-groups, hydrolysis of *tert*-butyl ester derivative afforded a prodrug with carboxylic acid terminals. All compounds were fully characterized by ^{1}H and ^{13}C NMR, ATR-FTIR and HR-ESI TOF. Studies of the anti-inflammatory effects on mouse ear edema of prodrugs methyl ester and carboxylic acid, ended, using diosgenin and dexamethasone as positive controls, showed the superiority of methyl ester ended prodrug with an ED_{50} four times lower than that of dexamethasone. Further, carboxylic acid ended prodrug was found to be more active than diosgenin as an antiproliferative agent, according to crystal violet assay.

Keywords. Antiproliferative activity; CV assay; click reaction; mechanosynthesis; 1,2,3-triazole, 4-oxo-4-(prop-2-yn-1-yloxy) butanoic acid.

1. Introduction

Diosgenin (25*R*-spirost-5-en-3β-ol), a steroidal sapogenin is widely distributed in plants, obtained mainly from some wild species of Mexican yam (*Dioscorea* sp.) or, as an alternative, from fenugreek (*Trigonella foenum-graecum* L.).[1] This metabolite has been the starting material of the corticosteroids (prednisolone, hydrocortisone and prednisone), sex hormones and oral contraceptives, as well as other steroidal drugs. Additionally, there are several studies on the potential of diosgenin for the treatment of various types of disorders such as leukemia, inflammation, hypercholesterolemia and cancer.[2,3]

Regarding inflammation, Kim *et al.* have suggested that diosgenin can be considered as a candidate for the treatment and prevention of inflammatory reactions in

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the skin. Their study showed that skin inflammation induced by phthalic anhydride is reduced by diosgenin by suppression of two important cytokines, IL-4 and IL-6 both involved in skin inflammation.[4] Also, it has been shown that diosgenin acts as anti-inflammatory and protects the heart, liver and brain, ameliorating atherosclerotic progression in the heart and suppressing inflammatory mediators in the liver and brain of Wistar rats by regulating inflammatory mediators COX-2 and TNF-α.[5] In this regard, Jung *et al.* studied the effect of diosgenin in macrophages, finding that this steroidal sapogenin inhibits macrophage-derived inflammatory mediators through downregulation of inflammatory meditators.[6]

Concerning cancer treatment, studies using F344 rats have shown that diosgenin suppresses the incidence of both invasive and non-invasive colon adenocarcinomas up to 60% via attenuation of tumor cell proliferation.[7] Additionally, *in vitro* activity of diosgenin against various human cancer cell lines has been studied extensively, showing inhibition of MCF-7 and MDA human breast carcinoma,[8] HT-29 and HCT-116 human colon adenocarcinoma,[3] PC-3[9] and DU145 human prostate cancer cells,[10] and pancreatic cancer[11] among others.

The versatile anticancer and anti-inflammatory activity exhibited by diosgenin indicates that this molecule could be a starting point for developing a new medicine, as an alternative drug of natural origin capable of diminishing the side-effects caused by allopathic drugs. However, diosgenin is practically insoluble in physiological media and has low absorption and a high percentage of the absorbed drug is metabolized rapidly.[12] Therefore, with the aim of improving the administration distribution metabolism excretion and toxicity (ADMET) properties[13] and taking into consideration that the triazole ring has been shown to increase anticancer[14] as well as antifungal activity of diosgenin derivatives.[15,16] In this study, diosgenin prodrugs were designed as hemisuccinate esters which were linked to 3-azidopropan-1-ol dendron via click reaction to improve their biological activity by incorporation of a triazole ring. In addition, the dendrons improve water solubility in the derivative **11** and show affinity to skin administration in derivative **9**.

To evaluate the performance of the new prodrugs, *in vivo* anti-inflammatory tests were performed on mouse ear edema as well as antiproliferative assays on breast cancer cell line MCF-7 and normal human fibroblast using the crystal violet (CV) test.

2. Experimental

2.1 *Materials and physical measurements*

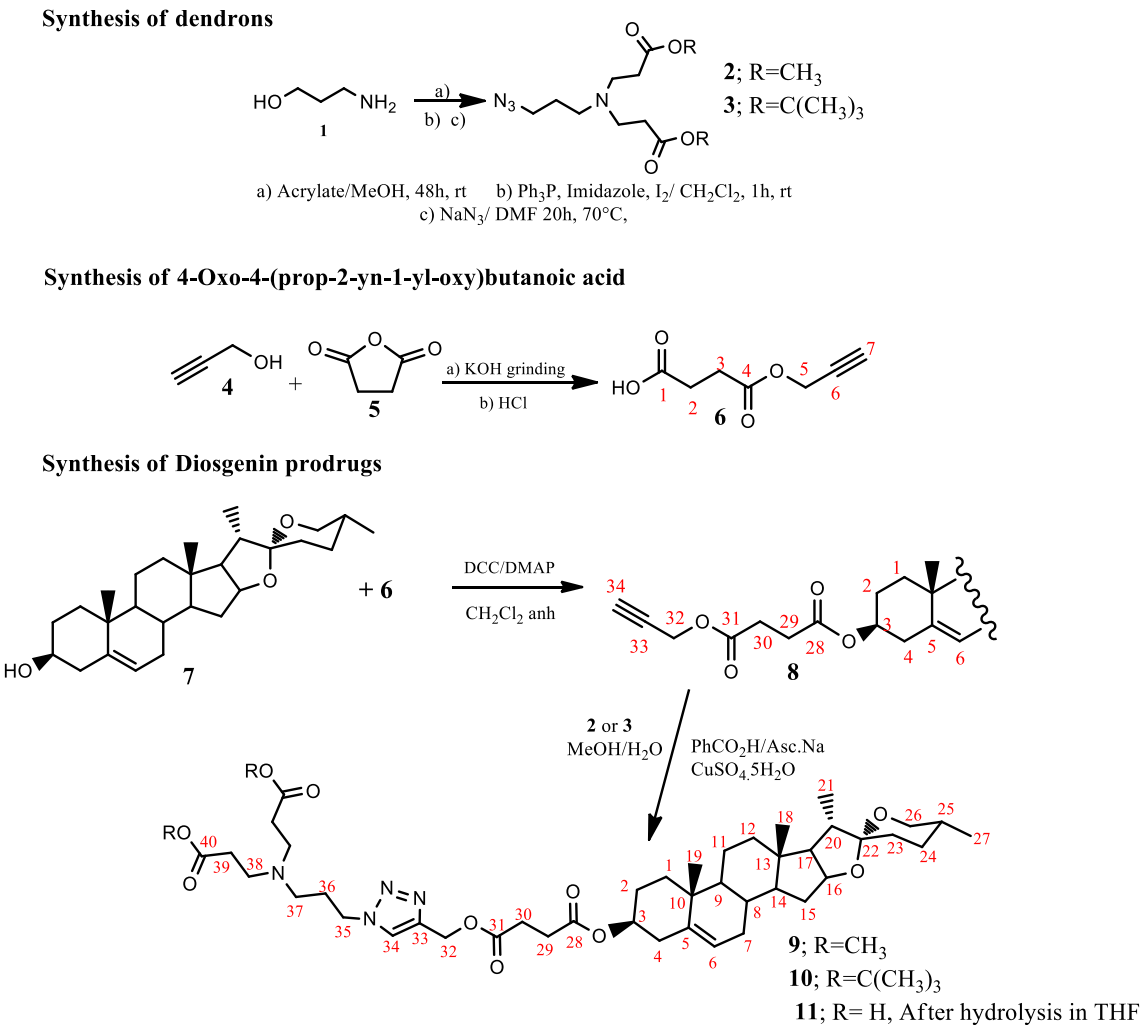
All reagents and solvents were purchased from Sigma Aldrich and used without purification, only DMF and CH_2Cl_2 were dried with CaH_2. Uncorrected melting points were determined on an electrothermal Fisher 9100 Melting Point Apparatus. Nuclear Magnetic Resonance (NMR) spectra were recorded on a JEOL ECA+500 or Bruker 400 instrument and attenuated total reflectance–fourier-transform infrared (ATR-FTIR) spectra on a Varian 600-IR series spectrometer. High resolution mass spectra (HRMS) were recorded on an agilent TOF mass spectrometer (MS TOF) using the ESI (+) technique. ^{13}C NMR assignment of diosgenin derivatives was made by comparison with the chemical shifts reported by Puri *et al.* for diosgenin.[17]

2.2 *Synthesis of diosgenin prodrugs*

The methodology for carrying out the structural modification of diosgenin is summarized in Scheme 1. Compounds **2** and **3** were synthesized as previously described.[18]

2.2a *Compound* ***6****, 4-oxo-4-(prop-2-yn-1-yloxy)butanoic acid*: KOH (1.5 g, 2.67 mmol) was ground in a mortar, followed by addition of 1 mL of propargyl alcohol **4** and further grinding until a paste was formed. To this paste, 1.960 g (1.96 mmol) of succinic anhydride **5** was added with further grinding until propargylic alcohol was not apparent by thin layer chromatography (TLC) between 15 and 30 min. The solid was treated with a 20 % HCl solution to pH 2; and extracted with CH_2Cl_2/H_2O (3 × 20 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated, and the product was recrystallized from hexane to give **6** as a bright white solid. Yield 48 % (0.812 g). ATR-FTIR (cm^{-1}): ν_{O-H} 3400–2500, $\nu_{C-H,\ alkyne}$ 3288, $\nu_{C-H\ aliph}$ 2917, 2849, $\nu_{C=O\ ester}$ 1729, $\nu_{C=O\ acid}$ 1691.[1] H-NMR (400 MHz, δ, ppm in $CDCl_3$): 11.41 (br s, 1H, COOH), 4.72 (s, 2H, H-5), 2.70 (m, 4H, H-2, H-3), 2.51 (d, 1H J = 1.8 Hz, H-7). ^{13} C-NMR (100 MHz, δ, ppm in $CDCl_3$): 178.5, 171.4, 77.3, 75.1, 52.3, 28.8 and 28.6.

2.2b *Compound* ***8****, Prop-2-yn-1-yl (3β, 25R)-spirost-5-en-3-yl-succinate*: Steglich esterification was used: to a 50 mL round-bottom flask equipped with a magnetic stirrer was immersed in a bath at 0°C, and 0.100 g of diosgenin **7** (0.24 mmol), 0.075 g of compound **6** (0.48 mmol) and 0.018 g of dimethylaminopyridine (DMAP, 0.15 mmol) dissolved in 5 mL of dry CH_2Cl_2 was added under N_2 atmosphere. Next, a solution of 0.118 g of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 0.55 mmol) in 2 mL of dry CH_2Cl_2 was added dropwise, and the reaction mixture was stirred for 1 h at 0°C and then for 12 h at room temperature (25–28°C). The precipitated urea was filtered off and the organic phase was extracted with CH_2Cl_2/



Scheme 1. Synthesis of prodrugs **9** and **11** from diosgenin through esterification and click reaction as anti-inflammatory and antiproliferative compounds.

H_2O, washed with 0.5 N HCl (3 × 5 mL), saturated solution of $NaHCO_3$ (3 × 5 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The product was purified by column chromatography using increasing solvent polarity, and compound **8** was obtained with hexane:AcOEt (95 Hex:5 AcOEt) as a white solid. Yield 90% (0.121 g). M.p.: 101–102°C. ATR-FTIR (cm^{-1}): $\nu_{C-H\ alkyne}$ 3287, $\nu_{C-H\ aliph}$ 2984, 2849, $\nu_{C\equiv C\ alkyne}$ 2128, $\nu_{C=O}$ 1731. ^{1}H-NMR (500 MHz, δ, ppm in $CDCl_3$): 5.36 (d, 1H, J = 4.6 Hz, H-6), 4.70 (br s, 2H, H-32), 4.61 (m, 1 H, H-3), 4.38 (dd, 1H, J= 15.0, 7.4 Hz, H16), 3.44 (d, 1H, J = 9.1 Hz, H-26α), 3.34 (t, 1H, J = 10.9 Hz, H-26β), 2.64 (d, 2H, J = 5.8 Hz, H-29*), 2.60 (d, 2H, J = 5.8Hz, H-30*), 2.47 (t, 1H J = 2.47 Hz, H-34), 1.00 (s, 3H, H-19), 0.96 (d, 3H, J = 6.7 Hz, H- 21), 0.78 (s, 6H, H-18, H-27). ^{13}C-NMR (125 MHz, δ, ppm in $CDCl_3$): 171.6, 171.4, 139.6, 122.4, 109.3, 80.8, 77.5, 75.1, 74.4, 66.8, 62.30, 56.4, 52.2, 49.9, 41.7, 40.3, 39.9, 38.1, 37.0, 36.8, 32.1, 31.9, 31.5, 30.4, 29.4, 29.0, 28.9, 27.8, 20.9, 19.4, 17.2, 16.4, 14.6. HR-ESI-TOF (m/z): calculated for $[C_{35}H_{45}N_4O_2 + H]^+$, 553.3537, found 553.3527.

2.2c *Methodology for click reaction*: In a round-bottom flask equipped with a magnetic stirrer, sodium ascorbate (AsNa), (0.41 equivalents), benzoic acid (2.0 equivalents) and $CuSO_4.5H_2O$ (0.20 equivalents) were dissolved in MeOH/H_2O (2:1) at 40°C (an orange coloration is indicative of the reduction of copper), followed by the addition of alkyne derivative **8** (0.9 equivalents) and the corresponding azide (1 equivalent). The reaction mixture was left stirring at 40°C and monitored by TLC until the reaction was completed. Once the reaction was completed, 5 mL of CH_2Cl_2 was added, and the organic layer was washed with a saturated solution of NH_4Cl (3 × 5 mL), followed by the addition of a saturated solution of $NaHCO_3$ (3 × 5mL), and finally with water (3 × 5 mL). The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum. The crude product was purified by column chromatography using increasing solvent polarity, and compounds **9** and **10** were eluted with hexane:AcOEt (5:5).

*2.2.2a. Compound **9**, (1-(3-(bis(3-methoxy-3-oxopropyl)amino)propyl)-1H-1,2,3-triazol-4-yl)methyl (3β, 25R)-spirost-5-en-3-yl-succinate:* According to the general procedure, 0.672 g (5.50 mmol) of benzoic acid, 0.227 g (1.15 mmol) of AsNa and 0.092 g (0.57 mmol) of $CuSO_4.5H_2O$ were dissolved in 20 mL of MeOH: H_2O (2:1). Compounds **8** (1.370 g, 2.48 mmol) and **2** (0.749 g, 2.75 mmol) were added together, previously dissolved in 5 mL of DMF and 7 mL of CH_2Cl_2. After 24 hours, 100 % conversion was observed (TLC). The raw material was purified by column chromatography to give product **9** as a white solid. Yield 50% (1.050 g). M.p.: 89–90°C. ATR-FTIR (cm^{-1}): $\nu_{C-H\ aliph}$ 2947, 2902, 2847, $\nu_{C=O}$ 1729. ^{1}H-NMR (500 MHz, δ, ppm in $CDCl_3$): 7.72 (s, 1H, H-34), 5.34 (d, 1H, *J* = 4.4 Hz, H-6), 5.22 (br s, 2H, H-32), 4.57 (ddd, 1H, *J* = 10.8, 8.9, 4.3 Hz, H-3), 4.39 (dd, 1H, *J* = 15.1, 7.3 Hz, H-16), 4.34 (t, 2H, *J* = 7.1 Hz, H-35), 3.66 (s, 6H, H-41), 3.45 (dd, 1H, *J* =10.1, 3.2 Hz, H-26α), 3.33 (m, 1H, H26β), 2.71 (m, 6H, H-37, H38), 2.62 (d, 2H, *J* = 5.5 Hz, H-29*), 2.59 (d, 2H, *J* = 5.5 Hz, H-30*), 2.40 (m, 4H, H-39), 1.00 (s, 3H, H-19), 0.96 (d, 3H, *J* = 6.7 Hz, H-21), 0.77 (s, 6H, H-18, H-27). ^{13}C-NMR (125.7 MHz, δ, ppm in $CDCl_3$): 173.0, 172.3, 171.6, 142.6, 139.7, 124.2, 122.5, 109.4, 80.9, 74.4, 66.9, 62.1, 58.1, 56.5, 51.7, 50.4, 49.9, 49.2, 48.0, 41.7, 40.3, 39.8, 38.1, 37.0, 36.8, 32.6, 32.5, 32.1, 31.9, 31.5, 30.4, 29.8, 29.4, 28.8, 28.2, 27.8, 20.9, 19.4, 17.2, 16.4, 14.6. HR-ESI-TOF (m / z): calculated for $[C_{45}H_{68}N_4O_{10} + H]^+$, 825.5021, found 825.5007. *These signals could be interchanged.

*2.2.2b. Compound **10**, (1-(3-(bis(3-tert-butoxy-3-oxopropyl)amino)propyl)-1H-1,2,3-triazol-4-yl)methyl (3β, 25R)-spirost-5-en-3-yl-succinate* According to the general procedure, 0.654 g (5.35 mmol) of benzoic acid, 0.221 g (1.11 mmol) of AsNa and 0.088 g (0.55 mmol) of $CuSO_4.5H_2O$ were dissolved in 20 mL of MeOH:H_2O (2:1). Compound **8** (1.318 g, 2.38 mmol) and compound **3** (0.944 g, 2.74 mmol) were added together dissolved in 5 mL of dry DMF. The reaction mixture was left stirring for 20 h (100% conversion by TLC). After workup, the crude product was purified by flash chromatography to obtain **10** as a slightly beige solid. Yield 52 % (0.750 g). M.p.: 80–81°C. ATR-FTIR (cm^{-1}): $\nu_{C-H\ aliph}$ 2927, 2902, 2849, $\nu_{C=O}$ 1725. ^{1}H-NMR (500 MHz, δ, ppm in $CDCl_3$): 7.71 (d, 1H, *J* = 8.3 Hz, H-34), 5.35 (d, 1H, *J* = 4.7 Hz, H-6), 5.23 (br s, 2H, H-32), 4.59 (ddd, 1H, *J* = 16.2, 10.8, 5.3, H-3), 4.39 (dd*[1], 1H, H-16), 4.37 (t, 2H, *J* = 7.2 Hz, H-35), 3.46 (dd, 1H, *J* = 10.2, 3.2, H-26α), 3.36 (t, 1H, *J* = 10.2, H-26β), 2.70 (t, 4H, *J* = 6.9 Hz, H-38), 2.62 (m, 4H, H-29*, H-30*), 2.41 (t, 2H, *J* = 6.1 Hz, H-37*), 2.32 (t, 4H, *J* = 6.9 Hz, H-39), 2.04 (dt, 2H, *J* =14.3, 7.2 Hz, H-36), 1.42 (s, 18H, H-42), 1.02 (s, 3H, H-19), 0.96 (d, 3H, *J* = 6.7Hz, H-21), 0.78 (d, 6H, H-18, H27). ^{13}C-NMR (125.7 MHz, δ, ppm in $CDCl_3$): 172.3, 172.1, 171.7, 142.6, 139.7, 124.1, 122.5, 109.4, 80.9, 80.6, 77.4, 66.9, 62.2, 58.1, 56.5, 50.4, 49.9, 49.2, 48.1, 41.7, 40.4, 39.8, 38.1, 37.0, 36.8, 33.6, 32.1, 31.9, 31.5, 30.4, 29.8, 29.5, 29.2, 28.9, 28.4, 28.3, 27.8, 20.9, 19.4, 17.3, 16.4, 14.7. HR-ESI-TOF (m/z): calculated for $[C_{51}H_{80}N_4O_{10} + H]^+$, 909.5974, found 909.5947. *[1] H-16 is overlapped with H-35 and it is not possible to determine the *J*.

2.2d *Compound **11**, 3,3′-((3-(4-(((4-oxo-4-(((3β,25R)-spirost-5-en-3-yl)oxy)butanoyl)oxy)methyl)-1H-1,2,3-triazol-1-yl)propyl)azanediyl)dipropannoic acid*: In a 50 mL round-bottom flask equipped with a magnetic stirrer, 0.200 g of compound **10** (2.06 mmol) was dissolved in 5 mL of trifluoroacetic acid (TFA), 2 drops of water were added and the mixture was stirred for 2 h. Once the reaction was completed according to TLC, approximately 3 hr, TFA was removed by air flux and the product was washed with acetone (5 × 3 mL) and CH_2Cl_2 (2 × 2 mL) to remove the remaining acid and dried in vacuum. Finally, **11** was obtained as a beige solid. Yield 90% (0.208 g). M.P.: 159–160°C. ATR-FTIR (cm^{-1}): ν_{O-H} 3400–2600, $\nu_{C=O}$ 1722, $\nu_{C=O}$ 1667. ^{1}H-NMR (400 MHz, δ, ppm in $CDCl_3$): 12.05 (br s, 1H, H-40 COOH), 8.15 (s, 1H, H-34), 5.35 (d, 1H, *J* = 3.9 Hz, H-6), 5.12 (br s, 2H, H-32), 4.38 (br s, 3H, H-3 and H-35), 4.29 (m, 1H, H-16), 3.39 (m, 6H, H-26, H38), 2.99 (m, 4H, H-29, H-30), 0.97 (s, 3H, H-19), 0.90 (d, 3H, *J* = 6.7 Hz, H-21), 0.74 (s, 6H, H-18, H-27). ^{13}C-NMR (100 MHz, δ, ppm in $CDCl_3$): 172.6, 172.2, 171.7, 142.3, 139.3, 125.2, 122.4, 108.9, 80.5, 74.0, 66.3, 62.3, 57.9, 56.3, 50.2, 49.9, 48.9, 47.4, 41.6, 38.5, 38.0, 36.9, 36.7, 31.9, 31.4, 30.3, 29.7, 29.8, 29.3, 29.0, 28.8, 27.8, 25.4, 20.8, 19.5, 17.5, 16.5, 15.1. HR-ESI-TOF (m/z): calculated for $[C_{43}H_{65}N_4O_{10} + H]^+$, 797.4695, found 797.4698.

2.3 *In vivo anti-inflammatory assay*

The tests were performed in the Bioterio of the University of Papaloapan, campus Tuxtepec. The experiments were developed with strict adherence to the requirements of the official Mexican standard of care of experimental animals (NOM-062-ZOO-1999) and international ethical guidelines for the use and care of experimental animals. The trial inflammation model induced by 12-ortho-tetradecanoylphorbol-13-acetate (TPA) was used.[19] Mice of strain ICR-CD1, weighing 28–30 g were used in groups of five, which were placed in transparent acrylic boxes at a constant temperature of 24°C, with a photoperiod of 12/12 h light/darkness with water and food. Under general anesthesia with sodium pentobarbital (35 mg/kg), each animal received 2.5 μg of dissolved TPA in 20 μL of acetone on the right ear and 20 μL of acetone on the left ear (10 μL to the internal surface and 10 μL to the external surface). After 15 min of the application of TPA, a test was performed with the target compounds (diosgenin **7**, **9** and **11**) which were dissolved in acetone and applied topically on both ears. Initially, all compounds were administered at a dose of 0.5

mg/ear to select the active compounds. Additionally, the active compounds (diosgenin **7** and **9**) were diluted to be administered at doses of 0.500, 0.250, 0.125 and 0.062 mg/ear to determine the effective dose 50 (ED_{50}), and dexamethasone as a positive control was administered at 1 mg/ear (2.55 μmol/ear) because it is the value at which an average of 50% of inflammation inhibition is obtained, according to the group experience. Four hours later, the mice were sacrificed by overexposure to anesthetic, and circular segments of 6 mm diameter of the auricle were obtained. The circular sections from left and the right ear were weighed immediately on an analytical balance, to determine the differential weight between both samples. The percentage of inhibition of edema was calculated using equation (1):

$$\%\,Inflammation\,inhibition = \frac{(C-E)}{C} \times 100, \quad (1)$$

where C = edema of the control group (treated with TPA), E = edema of the experimental group (TPA plus target compound). Statistical analysis was performed using Sigma Stat version 11.0 for Windows (Systat Software, San Jose, USA). Using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for anti-inflammatory activity, $P < 0.05$. ED_{50} values were obtained from linear regression with a coefficient factor of R^2 = 0.9618 and 0.9352.

2.4 *Antiproliferative assay*

To compare the potential anticancer activity of diosgenin **7**, and compounds **9** and **11**, antiproliferative tests on the cell line MCF-7 and a human fibroblast cell line (hFB) were performed by CV assay, according to the method described by Saotome *et al.*,[20] with minor modifications. In this regard, the concentrations of the compounds required for the reduction of cell viability by 50% (CC_{50}) were determined when it was possible. For this purpose, both cell lines were grown routinely in DMEM-F12 medium (cat. D2906, Sigma Aldrich) without phenol red and supplemented with 10% FBS (cat. 35-010-CV, Mediatech), at 37°C in 5% CO_2 and humidified atmosphere. For the toxicity test, the cells were seeded into 96-well microplates at a density of $2x10^4$ cells/well (100 μL/well). Cells were then incubated for 24 h under normal culture conditions to allow cell adhesion. After incubation, the medium was removed and a new medium containing increasing concentrations of the investigated compounds was added to the wells (final 500.000, 250.000, 125.000, 62.500, 31.250 and 15.625 μM). Control cells were exposed only to a medium containing 0.005% DMSO (the highest concentration of solvent in the samples), and pure DMSO as a control of the toxic effect. The plates were then incubated for 48 h under normal culture conditions. Further, the medium was removed and the wells were washed with water for four times. Next, the cells were dried by inversion on filter paper and incubated with 50 μL of 0.5% CV in methanol for 25 min at room temperature. The CV was then removed, and the cells were washed with water for four times and dried as mentioned. Finally, 200 μL of methanol was added to each well to dissolve the dye. Optical density (OD) was measured by a spectrophotometric plate reader at 541 nm. The percentage of relative cell viability was calculated as [OD treated/OD control] × 100%. Data were obtained from three independent experiments replicated thrice. Using the Statistical Program GraphPad Prism 7, the CC_{50} values were calculated from concentration-effect curves after fitting all corresponding data for each compound to a sigmoidal dose-response equation.

3. Results and Discussion

3.1 *Synthesis, characterization and structure activity relationship*

To preserve the structural integrity of diosgenin, since both molecular modeling and experimental studies have revealed that the presence of a hetero-sugar-like moiety fused at C-16 and C-17 as well as the presence of 5,6-double bond in its structure are the necessary structural parameters responsible for its activity,[21] and taking into consideration that ester prodrugs improve skin permeation and can activate esterases that promote hydrolysis,[22] we established a route to obtain diosgenin prodrugs as esters of an oxobutanoic acid 1,2,3-triazolyl derivative.

Compounds **2** and **3** were synthetized as previously described,[21] starting from 3-aminopropan-1-ol **1** (Scheme 1). 4-Oxo-4-(prop-2-yn-1-yloxy)butanoic acid (**6**) was obtained by mechanosynthesis,[23] avoiding pyridine or DMAP and anhydrous solvents. Compound **6** showed the characteristic four signals at 11.41, 4.72, 2.70 and 2.51 ppm in the ^{1}H NMR spectrum associated with the hydroxyl, methylene protons from the propargylic chain, methylene from the succinic residue, and alkyne protons, respectively. This reaction was carried out manually using a mortar, with yields from 36 to 48%, however, the yield could be improved by a more homogenous grinding in a ball mill.

Once **6** was obtained, Steglich esterification with diosgenin **7** was carried out to obtain compound **8**. Formation of compound **8** was corroborated by ^{1}H and ^{13}C NMR spectra, which evidenced the signals corresponding to the fragment derived from **6**, except for the acidic proton (Figure 1). Further evidence of esterification was the chemical shift observed for H-3 from 3.51 in diosgenin to 4.61 ppm in compound **8**.

Click reaction of compounds **2** and **3**, having azide as focal point with diosgenin derivative **8**, having a terminal alkyne, yielded new 1,2,3-triazolyl

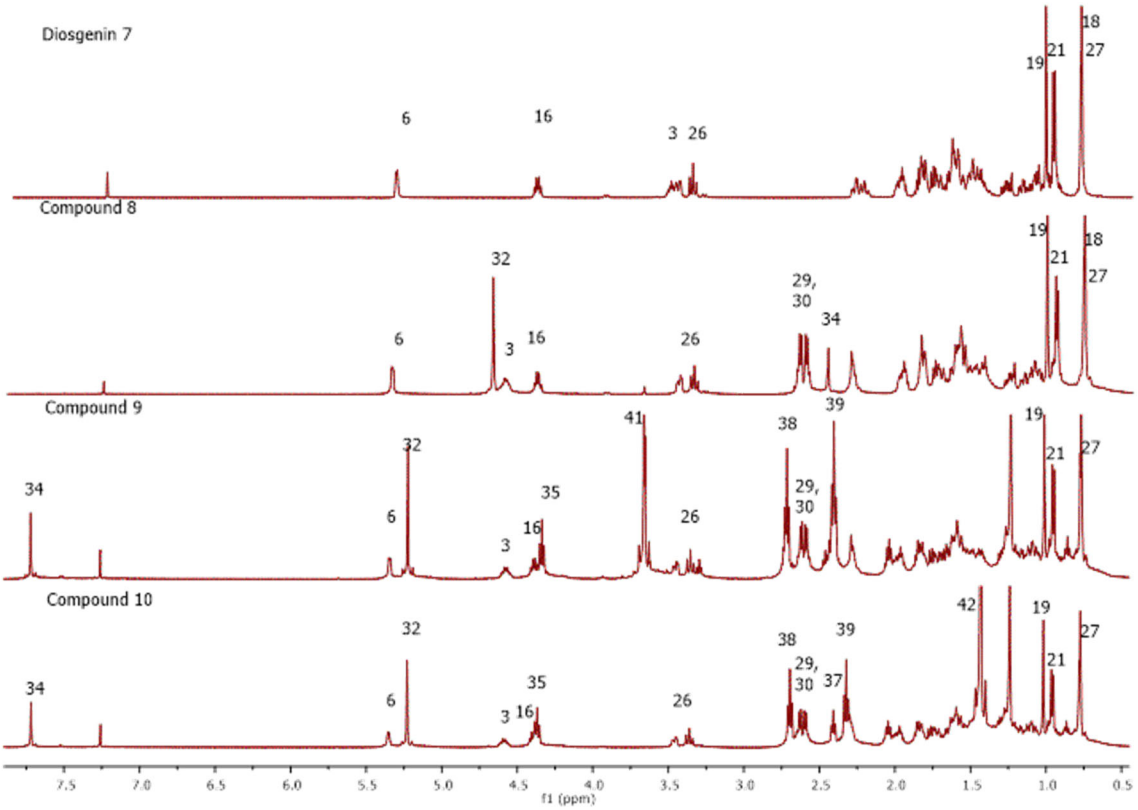


Figure 1. ^{1}H NMR spectra from diosgenin **7**, compounds **8**, **9** and **10** in $CDCl_3$.

derivatives **9** and **10**. Characteristic signals in the ^{1}H NMR spectra of diosgenin (**7**), compounds **8**, **9** and **10** are shown in Figure 1. A comparison of the spectra allows corroboration of the formation of the 1,2,3-triazolyl moiety in **9** and **10** from the signals at 7.74 and 7.72 ppm, respectively, as well as from the disappearance of the signal at 2.47 ppm, assigned to the alkyne (H-34) proton in compound **8**. Similarly, the signals associated with methyl ester and *tert*-butyl ester groups were assigned at 3.66 and 1.42 ppm for compounds **9** and **10**, respectively, confirming the formation of the products. The HR-MS TOF shows the molecular ion for **9** at 825.5007 corresponding to the formula $[C_{46}H_{65}N_8O_6 + H]^+$ 825.5021, while **10** shows the M^+ at 909.5947 in agreement with the theoretical value. Figure 1 depicts the most representative protons according to the structures.

Hydrolysis of compound **10** containing *tert*-butyl ester terminal groups using trifluoroacetic acid was confirmed by disappearance of the signal at 1.42 ppm (*t*-Bu group) in the ^{1}H NMR spectrum and the observation of a new signal at 11.9 ppm due to the proton of the acid; this induces a shift in the signal of the triazole ring to 8.13 ppm due to interactions between the nitrogen in the ring and the carboxylic acid terminals. Also, the ^{13}C spectrum evidences the disappearance of the quaternary carbon of the *t*-butyl group at 28.9 ppm, while the rest of the signals are not significantly modified. Finally, the formation of **11** was corroborated by HR-MS TOF which shows the molecular ion at 797.4698, in agreement with the formula $[C_{43}H_{65}N_4O_{10}+H]^+$.

As a consequence of the structural changes, the melting points of prodrugs **9** (89–90°C) and **11** (159–160°C) were below that of diosgenin (205-208°C), evidencing that formation of an ester linkage in the 3-O position of diosgenin decreases hydrogen bond interactions[24] and lowers intermolecular cohesion.

3.2 *In vivo evaluation of anti-inflammatory effect*

According to the established methodology, the percentages of inhibition of inflammation in mouse ear edema were determined in groups of five with inflammation induced by TPA. In the initial assay, all compounds were tested at 0.5 mg/ear, and only diosgenin and prodrug **9** showed an activity higher than 50%. To determine the ED_{50} of active compounds, serial dilutions were made and dexamethasone was evaluated at 2.55 µmol/ear, value at which an average

Table 1. Inhibition of inflammation in mouse as a function of concentration of prodrugs **9** and **11**.

Compound	Doses mg/ear (μmol/ear)	Edema ± ES	% Inhibition of inflammation
TPA	2.5 μg	8.40 ± 1.49	–
Dexamethasone	1.000 (2.55)	3.92 ± 0.85*	53.23
	0.500 (1.27)	6.26 ± 0.51*	25.39
Diosgenin **7**	0.500 (1.21)	3.80 ± 0.37*	54.76
	0.250 (0.60)	5.15 ± 0.18*	38.71
	0.125 (0.30)	6.43 ± 0.57*	23.42
	0.062 (0.15)	7.25 ± 0.39	13.73
Prodrug **9**	0.500 (0.61)	4.01 ± 0.32*	52.38
	0.250 (0.30)	7.19 ± 0.74	14.41
	0.125 (0.15)	7.28 ± 0.85	13.35
	0.062 (0.08)	8.19 ± 0.86	2.451
Prodrug **11**	0.500 (0.63)	6.20 ± 0.51*	21.48

The values are the average ± standard error (n = 5), *ANOVA post-test of Dunnett showed that there is statistically significant difference at $P < 0.001$ compared to control group TPA (12-*O*-tetradecanoylphorbol-13-acetate).

of 50% of inflammation inhibition was obtained according to the group experience. The percentage of inflammation inhibition as a function of concentration is shown in Table 1.

The data in Table 1 allows corroboration of the anti-inflammatory effect of diosgenin induced by TPA, which results almost twice as effective as dexamethasone, since the dose necessary to reach 54 and 53% of inhibition was 1.21 and 2.55 μmol/ear for diosgenin and dexamethasone, respectively. Interestingly, prodrug **9** turned out to be four times more active than dexamethasone and twice as active as diosgenin, since the dose required to cause 52% of inhibition was 0.61 μmol/ear. These results can be explained considering that diosgenin prodrug **9** with 4 methyl ester terminal groups promotes stratum corneum transport, and as this diosgenin prodrug reaches the dermis, soluble esterases cleave off the ester groups and release the active group into the systemic circulation[25] more efficiently than prodrug **11** with only two ester groups. As a result, the anti-inflammatory effect in compound **11** with carboxylic acid end-groups decreases. The ED_{50} for **7** and **9** were 1.02 and 0.61 μmol/ear, respectively. The potent anti-inflammatory action of diosgenin and its analogues could be attributed to the inhibition of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β.[26]

Taking into consideration that dexamethasone can cause side-effects,[27] the therapeutic use of diosgenin and prodrug **9** as anti-inflammatory topical agents could be beneficial, provided factors such as cytotoxicity, skin reactions and bioconversion as well as the mechanism of action are taken into account.

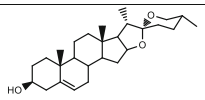
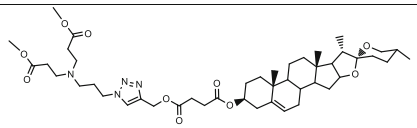
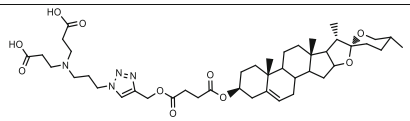
3.3 *Evaluation of the antiproliferative activity*

Diosgenin **7** and prodrugs **9** and **11** were screened for antiproliferative activity against a breast cancer cell line (MCF-7) and on human fibroblasts (hFB) as a healthy cell line. Preliminary screening of the compounds was carried out from 3.82 to 100 μM; however, the CC_{50} was not reached and a new set of concentrations up to 500 μM were tested. Starting from concentrations of 26.5 μM, diosgenin and compound **11** displayed antiproliferative effect in a dose-dependent manner on both MCF-7 and hFB. Compared to diosgenin, compound **11** showed improved antiproliferative activity over MCF-7, with a CC_{50} of 211.70 μM, while the CC_{50} of diosgenin was > 500 μM (Table 2). Additionally, the acid terminals in **11** changed the solubility, providing the possibility of administering it intravenously as a salt.

However, the same tendency was observed on fibroblasts. For compound **11**, a CC_{50} of 248.72 μM was determined, while for diosgenin, the value was over 500 μM. These results indicate that a change in solubility can modulate the activity, but this improvement affects both cell lines; therefore, further studies on cell death mechanism are required to establish the optimal chemical modification that leads to improvement of ADMET properties without affecting normal cells.

Concerning compound **9**, the response was unexpected and with MCF-7, the response starts at 250 μM with a $CC_{50} > 500$ μM. This reduced activity can be attributed to the lack of solubility. Nonetheless, hFB cells are affected starting at doses of 31.3 μM, and CC_{50} of 115.62 μM; i.e., compound **9** has a higher

Table 2. CC_{50} of diosgenin and prodrugs **9** and **11** on MCF-7 and hFB cell lines.

Compound	Structure	CC_{50} (μM) MCF-7	CC_{50} (μM) hFB
Diosgenin		> 500	> 500
Prodrug 9		> 500	115.62
Prodrug 11		211.70	248.72

antiproliferative effect than compound **11** on hFB cells. In addition, compound **9** had a greater effect on hFB than on MCF-7 cells. Overall, it is proposed that further research on the mechanism of cell death induced by these compounds is necessary.

4. Conclusions

Two diosgenin prodrugs were synthesized by esterification with 1,2,3-triazole dendrimeric fragments to preserve the chemical structure of diosgenin. The incorporation of the dendrimeric fragment with methyl ester **9** and carboxylic acid **11** end-groups allowed modulation of the solubility, and consequently, the anti-inflammatory and antiproliferative activity properties were improved. The results show that compound **9** with methyl ester end-groups may be more suitable than diosgenin and dexamethasone as anti-inflamatory compound for topical applications. For antiproliferative activity, prodrug **11** with carboxylic acid terminals was more effective than diosgenin or compound **9**; however, increase in activity towards the MCF-7 cancer cell line was not selective, and hFB were also affected. The results highlight the need to investigate cell death mechanisms induced by diosgenin derivatives, in order to improve the chemical design of diosgenin prodrugs.

Supplementary Information (SI)

Supplementary Figures 1–16 (^{1}H-NMR, ^{13}C NMR, FTIR and HRMS spectrums) are available as Supplementary Information at http://www.ias.ac.in/chemsci.

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