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Semisynthetic modifications of antitubercular lanostane triterpenoids from *Ganoderma*

Panida Chinthanom[1] · Vanicha Vichai[1] · Kanchana Dokladda[1] · Malipan Sappan[1] · Chawanee Thongpanchang[1] ·
Masahiko Isaka [1]

Abstract

Antitubercular lanostane triterpenoids isolated from mycelial cultures of the basidiomycete *Ganoderma australe* were structurally modified by semisynthesis. One of the synthetic compounds, named GA003 (**9**), showed more potent activity against *Mycobacterium tuberculosis* H37Ra than the lead natural lanostane (**1**). GA003 was also significantly active against the virulent strain (H37Rv) as well as extensively drug-resistant tuberculosis strains.

Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2019, an estimated 10 million people fell in TB worldwide, and 1.4 million died from the disease [1]. Problematic issues in TB treatment include the increased incidence of multidrug-resistant TB (MDR-TB), which is a form of TB caused by bacteria that do not respond to isoniazid and rifampicin. Extensively drug-resistant TB (XDR-TB) is a more serious form of MDR-TB caused by bacteria that do not respond to the most effective second-line anti-TB drugs, often leaving patients without any further treatment options. The increasing emergence of drug resistance highlights the need to develop novel chemical entity of TB drugs.

Ganoderma is a genus of bracket fungi including several medicinal mushroom species such as lingzhi (*Ganoderma lucidum*). Lanostane triterpenoids have been considered as one of the key ingredients of lingzhi, and a wide range of their biological activities related to human health promoting effects have been reported [2, 3]. A number of lanostanes

and modified lanostanes have been isolated from natural or cultivated fruiting bodies and/or mycelial cultures of this genus [3, 4]. We previously reported that several lanostanes from mycelial cultures of *Ganoderma* species exhibit significant growth inhibitory activities against *Mycobacterium tuberculosis* H37Ra [5–7]. In the conclusive full paper [5], the structure–activity relationships (SARs) of the natural lanostanes were proposed, wherein (24*E*)−3β,15α-diacetoxylanosta-7,9(11),24-trien-26-oic acid (ganodermic acid S, **1**) was the optimal anti-TB *Ganoderma* lanostanoid (Fig. 1). In particular, 3β- and 15α-acetoxy groups were shown to be critical for the antimycobacterial activity. In a later study (unpublished), a methyl ester of **1** was inactive in the assay, which indicated the importance of the C-26 carboxyl group. On the basis of these results, we had planned semisynthetic modifications of compound **1** to discover more potent anti-TB agents.

Results and discussion

We had planned synthesis of derivatives of ganodermic acid S (**1**) [8] with various acyl groups at C-15 and C-3 (R^1 and R^2 in Fig. 1). As substrates for the semisynthesis, compounds **2** (ganodermic acid T-O) [9], **3** (ganodermic acid T-N) [9], and **4** (ganodermic acid Jb) [10] were prepared (Fig. 2). In our previous chemical investigations of mycelial cultures of several *Ganoderma* species, *Ganoderma australe* BCC 22314 was selected as the most suitable sources of the desired substrates, since the strain produces 3β,15α-diacetoxy- and 3β-acetoxy-15α-hydroxy-lanostanes **1**, **2**,

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Fig. 1 Structure–activity relationships (SARs) for antitubercular activity of natural lanostanes from *Ganoderma*
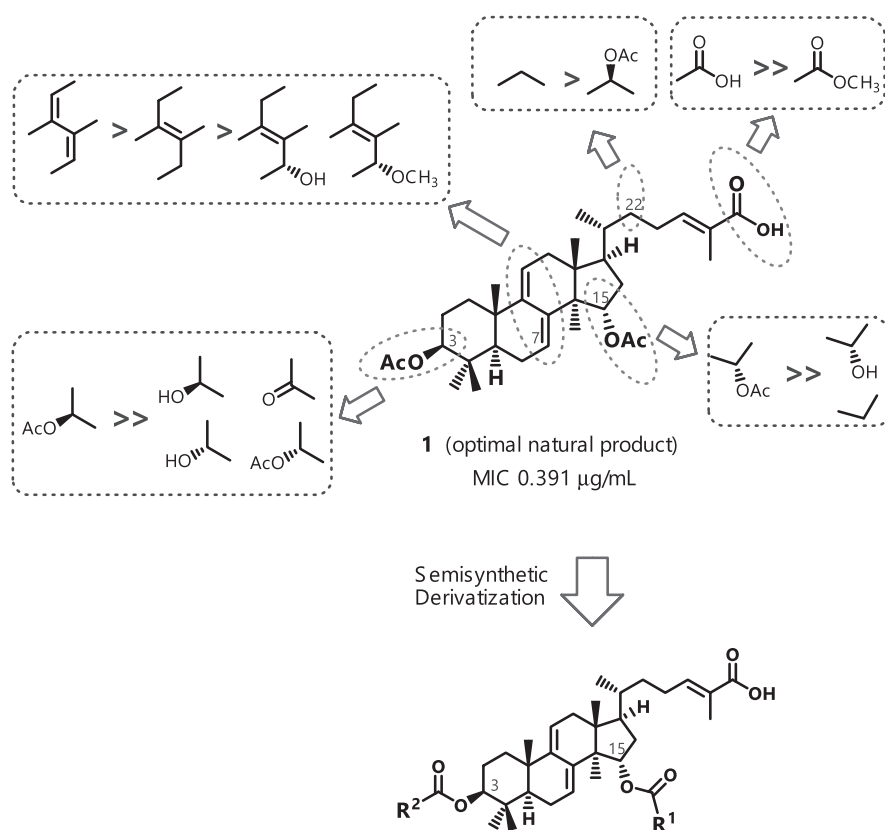
and **5–7** as major secondary metabolites. Compounds **5–7** are 7α-hydroxy-8-ene or 7α-acetoxy-8-ene derivatives that are acid sensitive and can be easily converted to 7,9(11)-diene derivatives **1** and **2** under acidic conditions [7, 11]. For simplification of the lanostane compositions, the mycelial extract was treated with *p*-TsOH·H$_2$O in CH$_2$Cl$_2$ then the extract was subjected to chromatographic separations to furnish **1** and **2** as major lanostanes.

Compound **3** was prepared by partial alkaline hydrolysis of **1**. Fortunately, 3-*O*-deacetylation was faster than 15-*O*-deacetylation; however, the regioselectivity of the hydrolysis was not so high. Therefore, the reaction was terminated at an appropriate stage to obtain a mixture mainly composed of **1**, **3**, and **4**. Compound **4** was synthesized by complete hydrolysis of **1**.

Derivatives **8–18** (Fig. 3) were synthesized using compounds **2–4**. The formylated derivatives **8** and **13** were successfully prepared from **2** and **3**, respectively, by using formic acid–2,2,2-trifluoroethyl formate [12]. All other acylated derivatives were synthesized by acylation of **2–4** with excess acyl chloride in pyridine–CH$_2$Cl$_2$.

Semisynthetic derivatives **8–18** were tested for activity against *M. tuberculosis* H37Ra (Table 1). Most notably, 15-*O*-propionyl derivative **9**, named GA003, showed higher antimycobacterial activity (MIC 0.0977 µg/ml) than **1**. Comparison of the activities of **1** and **8–12**, possessing different 15-*O*-acyl (R^1), demonstrated that propionyl (R^1 = Et) derivative (**9**)

has the most suitable size of the R^1 alkyl group. Similarly, the activities of **1** and **13–16** with different 3-*O*-acyl (R^2) suggested that acetyl (**1**, R^2 = Me) and propionyl (**14**, R^2 = Et) have suitable size of R^2 alkyl group. The activities of dipropionyl and dibutyryl derivatives **17** and **18** were reasonably consistent with these partial SARs.

Cytotoxicity of these compounds to noncancerous Vero cells (African green monkey kidney fibroblasts) was also evaluated. Compound **9** and many other compounds showed moderate or weak cytotoxicity; however, there were reasonable selectivity with the antimycobacterial activities. The SAR of cytotoxicity to Vero cells was different from that for the anti-TB activity, which indicated that the positive assay results for growth inhibition of TB mycobacteria are not related to the cytotoxic properties of the lanostanes.

The most potent antimycobacterial compound **9** (GA003) also showed significant activity against the virulent strain *M. tuberculosis* H37Rv (ATCC 27294) with an MIC value of 0.313 µg ml^{-1}. Then, it was further tested for activities against drug-resistant strains of *M. tuberculosis*, pre-XDR strain (THX-0001), and XDR strains (THX-0002, THX-0003). GA003 was active against these strains exhibiting MIC values of 0.313, 0.625, and 1.25 µg ml^{-1}, respectively.

In the present study, derivatives of the natural anti-TB lanostane **1** were prepared by semisynthesis. Among them, GA003 (**9**) exhibited superior activity than **1** against *M. tuberculosis* H37Ra. This compound was active against the

Fig. 2 Preparation of **2**, **3**, and **4** as substrates for semisynthesis of derivatives of **1**. Reagents and conditions: (a) the mycelial extract was treated with *p*-TsOH·H$_2$O in CH$_2$Cl$_2$, rt, 1 h; (b) 2 M NaOH–MeOH (1:6), rt, 5 h; (c) 3 M NaOH–MeOH (1:10), rt, 23 h
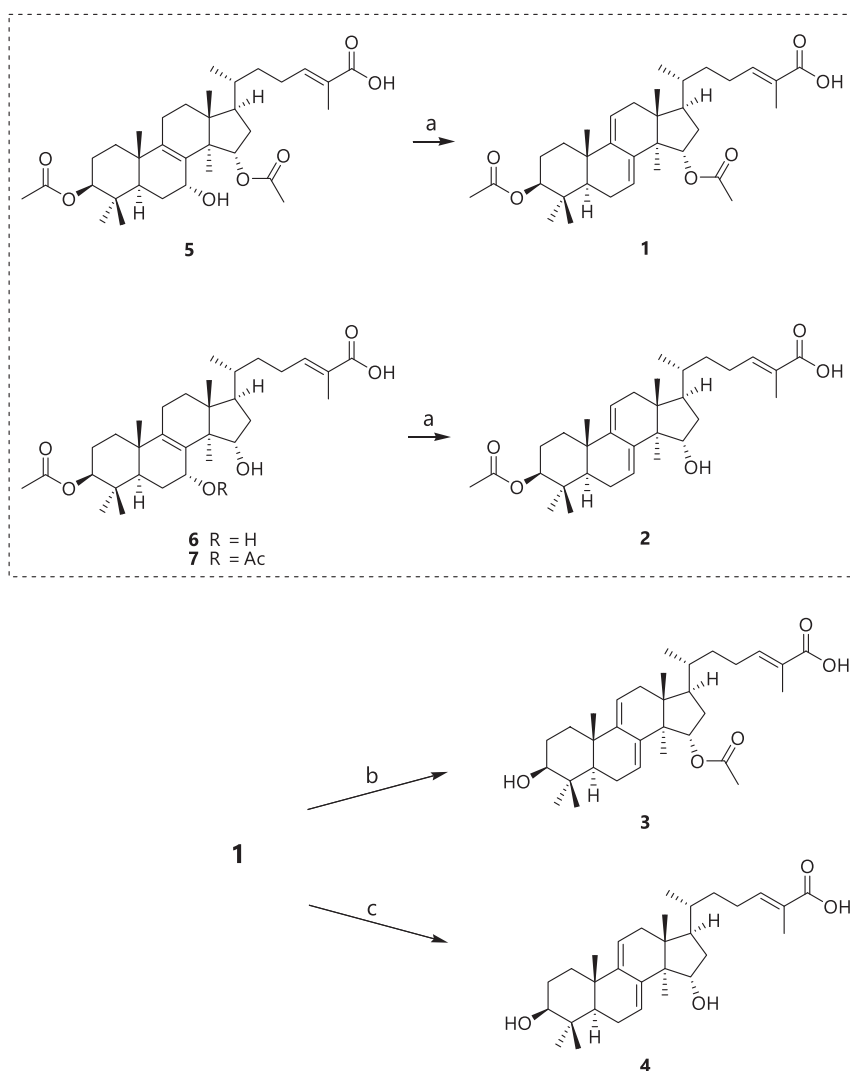
virulent strain *M. tuberculosis* H37Rv, moreover, also against the XDR strains. The MIC values against XDR strains were relatively larger than those against drug-sensitive strains. However, considering the urgent need for the discovery of novel chemotypes against drug-resistant TB, these activities will be reasonably good enough to consider further biochemical and pharmacological evaluations as a lead compound.

Materials and methods

General experimental procedures

Optical rotations were determined using a JASCO P-2000 digital polarimeter. FTIR spectra were acquired using a Bruker ALPHA spectrometer. NMR spectra were recorded on a Bruker DRX400 spectrometer. ESI-TOF mass spectra were measured using a Bruker micrOTOF mass spectrometer.

Fungal material

Ganoderma australe was isolated from a dead oil palm (*Elaeis guineensis*) trunk in the plantation area, Klong Thom, Krabi Province, Thailand, on May 4, 2006. The mushroom specimen was deposited in the BIOTEC Bangkok Herbarium as BBH 19074, and the living culture was deposited in the BIOTEC Culture Collection as BCC 22314. Identification of this fungus is based on the morphology and ITS rDNA sequence data (GenBank accession number: KX421866) [5].

Fermentation of *G. australe* BCC 22314, extraction, and isolation of 1 and 2

The fungus BCC 22314 was maintained on potato dextrose agar at 25 °C. The agar was cut into small plugs and inoculated into 12 × 250-ml Erlenmeyer flasks containing 25 ml of malt extract broth (MEB; malt extract 6.0 g l^{-1}, yeast

Fig. 3 Synthesis of the derivatives of **1**. Reagents and conditions: (a) formic acid–2,2,2-trifluoroethyl formate, 60 °C, 3 h; (b) RCOCl (excess), pyridine–CH_2Cl_2 (2:1), rt, 3 h
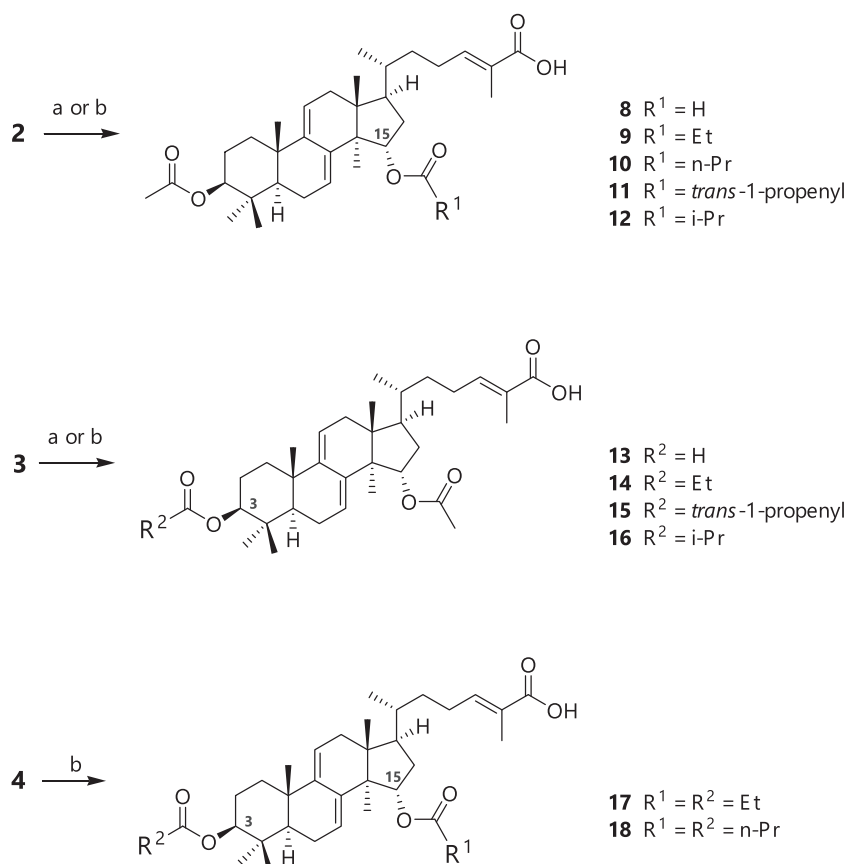

8	R^1 = H
9	R^1 = Et
10	R^1 = n-Pr
11	R^1 = *trans*-1-propenyl
12	R^1 = i-Pr

13	R^2 = H
14	R^2 = Et
15	R^2 = *trans*-1-propenyl
16	R^2 = i-Pr

| **17** | R^1 = R^2 = Et |
| **18** | R^1 = R^2 = n-Pr |

Table 1 Antimycobacterial and cytotoxic activities of the lanostanes

	Antituberculosis	Cytotoxicity
Compound	*M. tuberculosis* H37Ra H37Ra MIC, μg ml^{-1}	Vero cells IC_{50}, μg ml^{-1}
1	0.391	18
8	3.13	15
9 (GA003)	0.0977	9.2
10	1.56	12
11	0.781	12
12	0.781	9.5
13	1.56	16
14	0.391	16
15	>50	18
16	0.781	15
17	0.781	8.7
18	>50	13
Rifampicin[a]	0.025	–
Isoniazid[a]	0.0469	–
Ellipticine[b]	–	1.3

[a]Standard anti-TB drugs

[b]Standard compound for the cytotoxicity assay

extract 1.2 g l^{-1}, maltose 1.8 g l^{-1}, dextrose 6.0 g l^{-1}). After incubation at 25 °C for 7 days on a rotary shaker (200 rpm), each primary culture was transferred into a 1000-ml Erlenmeyer flask containing 250 ml of the same liquid medium (MEB), and incubated at 25 °C for 7 days on a rotary shaker (200 rpm). The secondary cultures were pooled and each 25 ml portion was transferred into 120 × 1000-ml Erlenmeyer flasks containing 250 ml of MEB, and the final fermentation was carried out at 25 °C for 90 days under static conditions. The cultures were filtered, and the residual wet mycelia were macerated in acetone (6 l, 25 °C, 7 days) and filtered. The filtrate was concentrated under reduced pressure, and the residue was diluted with H_2O (600 ml) and extracted with EtOAc (2.0 l). The EtOAc solution was concentrated under reduced pressure to obtain a brown gum (mycelial extract, 6.30 g). This extract was dissolved in CH_2Cl_2 (50 ml) and p-TsOH·H_2O (74 mg) was added. After stirring for 1 h, H_2O (20 ml) and 28% aqueous ammonia solution (0.2 ml) were added, and then partially concentrated by evaporation. The residual aqueous solution was diluted with EtOAc (100 ml) and washed with H_2O (50 ml). The H_2O layer was extracted with EtOAc (2 × 100 ml). The combined organic layer was concentrated under reduced pressure to obtain a brown gum

(acid-treated mycelial extract; 6.14 g). This extract was subjected to column chromatography on silica gel (EtOAc–hexane, step gradient elution from 0:100 to 100:0), then the fractions containing the target compounds were further purified by preparative HPLC (Waters SunFire Grom-Sil 120 ODS-4 HE, 20×150 mm, 5 μm; mobile phase MeCN–H_2O, gradient from 70:30 to 100:0 over 30 min; flow rate 12 ml min^{-1}) to furnish **1** (792 mg) and **2** (511 mg).

Preparation of 3 by partial alkaline hydrolysis of 1

Compound **1** (103 mg, 0.19 mmol) was partially hydrolyzed in 2 M NaOH (1 ml) – MeOH (6 ml) at room temperature for 5 h. The mixture was acidified to pH 2 by addition of 1 M HCl and partially concentrated by evaporation. The residue was diluted with H_2O and extracted with EtOAc. The organic layer was concentrated under reduced pressure to obtain a pale yellow gum, which was subjected to column chromatography on silica gel (EtOAc–hexane) to furnish **3** (27 mg, 28%).

Preparation of 4 by complete alkaline hydrolysis of 1

Compound **1** (56 mg, 0.10 mmol) was completely hydrolyzed in 3 M NaOH (0.4 ml) – MeOH (4 ml) at room temperature for 23 h. The mixture was acidified by addition of 1 M HCl and partially concentrated by evaporation. The residual aqueous solution was diluted with H_2O and extracted with EtOAc. The organic layer was concentrated in vacuo to obtain **4** (46 mg, 97%).

Synthesis of 8

A mixture of **2** (8.6 mg, 17 μmol) and formic acid (20 μl) in 2,2,2-trifluoroethyl formate (0.2 ml) was stirred on a 60 °C heating bath for 3 h. After cooling to room temperature, the mixture was concentrated in vacuo. The residue (10 mg) was purified by preparative HPLC (MeCN–H_2O) to furnish **8** (3.0 mg, 33%).

(24*E*)−3β-Acetoxy-15α-formyloxylanosta-7,9(11),24-trien-26-oic acid (**8**): colorless amorphous solid; IR (ATR) ν_{max} 2929, 1724, 1686, 1375, 1247, 1177 cm^{-1}; ^1H NMR (400 MHz, CDCl$_3$), selected resonances, δ 8.11 (1H, s), 6.84 (1H, t, $J = 7.0$ Hz), 5.50 (1H, d, $J = 6.0$ Hz), 5.33 (1H, d, $J = 5.7$ Hz), 5.19 (1H, dd, $J = 9.9$, 5.4 Hz), 4.51 (1H, dd, $J = 11.2$, 4.5 Hz), 2.33 (1H, br d, $J = 16.7$ Hz), 2.32 (2H, t, $J = 7.5$ Hz), 2.06 (3H, s), 1.83 (3H, s), 1.17 (1H, dd, $J = 11.3$, 4.2 Hz), 1.03 (3H, s), 0.99 (3H, s), 0.94 (3H, s), 0.92 (3H, d, $J = 6.4$ Hz), 0.89 (3H, s), 0.66 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 172.1, 170.9, 161.1, 145.8, 144.8, 140.2, 126.9, 121.3, 116.2, 80.7, 77.2, 51.6, 49.2, 49.0, 44.2, 38.1, 37.7, 37.4, 37.0, 36.0, 35.5, 34.7, 28.1, 25.9, 24.3, 22.9, 22.8, 21.2, 18.4, 18.2, 16.9, 16.0, 12.1; HRMS

(ESI-TOF) m/z 563.3330 $[M + Na]^+$ (calcd. for C$_{33}$H$_{48}$O$_6$Na, 563.3343).

Synthesis of 9 (GA003)

Compound **2** (64 mg, 0.125 mmol) was treated with propionyl chloride (0.20 ml) in pyridine (0.8 ml)/CH$_2$Cl$_2$ (0.80 ml) at room temperature for 3 h. The mixture was diluted with EtOAc (50 ml) and washed with H_2O (3 ml). The organic layer was concentrated under reduced pressure to obtain a pale yellow solid, which was purified by preparative HPLC (Waters SunFire Prep C18 OBD, 19×250 mm, 10 μm; gradient of MeCN–H_2O from 80:20 to 100:0 over 30 min; flow rate 12 ml/min) to furnish **9** (32 mg, 45%; t_R 26 min).

(24*E*)−3β-Acetoxy-15α-propionyloxylanosta-7,9(11),24-trien-26-oic acid (**9**, GA003): colorless solid; $[α]^{22}_D$ +99 (*c* 0.17, CHCl$_3$); IR (ATR) ν_{max} 2936, 1731, 1686, 1375, 1247, 1029 cm^{-1}; ^1H NMR (CDCl$_3$, 400 MHz) δ 6.85 (1H, t, $J = 7.0$ Hz, H-24), 5.46 (1H, d, $J = 4.8$ Hz, H-7), 5.31 (1H, d, $J = 5.9$ Hz, H-11), 5.07 (1H, dd, $J = 9.8$, 5.0 Hz, H-15), 4.50 (1H, dd, $J = 11.3$, 4.5 Hz, H-3), 2.36 (2H, q, $J = 7.5$ Hz, propionyl H-2″), 2.32 (1H, br d, $J = 16.5$ Hz, H$_α$−12), 2.23 (1H, m, H$_a$−23), 2.11 (1H, m, H$_β$−16), 2.10 (1H, m, H$_b$−23), 2.08 (1H, m, H$_β$−6), 2.06 (3H, s, acetyl H-2′), 2.05 (1H, m, H$_α$−6), 1.99 (1H, m, H$_β$−1), 1.83 (3H, s), 1.72 (1H, m, H$_α$−2), 1.70-1.67 (3H, m, H$_β$−2, H$_α$−16, and H-17), 1.53-1.50 (2H, m, H$_α$−1 and H$_a$−22), 1.40 (1H, m, H-20), 1.17 (1H, m, H-5), 1.17 (3H, t, $J = 7.5$ Hz, propionyl H-3″), 1.13 (1H, m, H$_b$−22), 1.00 (3H, s, H-30), 0.98 (3H, s, H-19), 0.94 (3H, s, H-29), 0.91 (3H, d, $J = 6.4$ Hz, H-21), 0.89 (3H, s, H-28), 0.65 (3H, s, H-18); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 174.5 (C, propionyl C-1″), 172.5 (C, C-26), 171.0 (C, acetyl C-1′), 145.6 (C, C-9), 145.1 (CH, C-24), 140.2 (C, C-8), 126.7 (C, C-25), 121.1 (CH, C-7), 116.1 (CH, C-11), 80.7 (CH, C-3), 77.1 (CH, C-15), 51.4 (C, C-14), 48.9 (CH, C-17), 48.8 (CH, C-5), 44.1 (C, C-13), 37.9 (CH$_2$, C-12), 37.6 (C, C-4), 37.3 (C, C-10), 37.1 (CH$_2$, C-16), 35.9 (CH, C-20), 35.3 (CH$_2$, C-1), 34.6 (CH$_2$, C-22), 28.1 (CH$_3$, C-28), 28.0 (CH$_2$, propionyl C-2″), 25.9 (CH$_2$, C-23), 24.2 (CH$_2$, C-2), 22.8 (CH$_3$, C-19), 22.8 (CH$_2$, C-6), 21.3 (CH$_3$, acetyl C-2′), 18.4 (CH$_3$, C-30), 18.1 (CH$_3$, C-21), 16.9 (CH$_3$, C-29), 16.0 (CH$_3$, C-18), 12.0 (CH$_3$, C-27), 9.3 (CH$_3$, propionyl C-3″); HRMS (ESI-TOF) m/z 591.3659 $[M + Na]^+$ (calcd. for C$_{35}$H$_{52}$O$_6$Na, 591.3656).

General procedure for the 15-*O*-acylation of 2: synthesis of 10–12

Compound **2** (20–45 μmol) was treated with corresponding acyl chloride (excess; 50–70 μl) in pyridine–CH$_2$Cl$_2$ (2:1) at room temperature for 3 h. The mixture was diluted with EtOAc and washed with H_2O, and the aqueous layer was

extracted with EtOAc. The combined EtOAc solution was concentrated under reduced pressure, and the residue was purified by preparative HPLC (MeCN–H$_2$O) to furnish the 15-O-acyl derivative.

(24E)−3β-Acetoxy-15α-butyryloxylanosta-7,9(11),24-trien-26-oic acid (**10**): colorless amorphous solid, yield 32% (3.8 mg); IR (ATR) ν_{max} 2929, 1731, 1686, 1375, 1247, 1030 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.85 (1H, t, J = 7.1 Hz), 5.47 (1H, m), 5.32 (1H, d, J = 5.6 Hz), 5.07 (1H, dd, J = 9.7, 4.9 Hz), 4.50 (1H, dd, J = 11.2, 4.4 Hz), 2.32 (2H, t, J = 7.5 Hz), 2.06 (3H, s), 1.83 (3H, s), 1.17 (1H, dd, J = 10.7, 5.0 Hz), 1.00 (3H, s), 0.99 (3H, s), 0.97 (3H, t, J = 7.5 Hz), 0.94 (3H, s), 0.91 (3H, d, J = 6.4 Hz), 0.89 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 173.5, 170.7, 170.2, 146.1, 144.8, 140.6, 126.7, 121.3, 116.3, 80.9, 77.0, 51.8, 49.4, 49.2, 44.4, 38.4, 37.8, 37.6, 37.2, 36.9, 36.1, 35.7, 34.9, 28.2, 26.0, 24.4, 23.0, 22.9, 21.1, 18.6, 18.5, 18.3, 16.9, 16.2, 13.7, 12.1; HRMS (ESI-TOF) m/z 605.3810 [M + Na]$^+$ (calcd. for C$_{36}$H$_{54}$O$_6$Na, 605.3813).

(24E)−3β-Acetoxy-15α-crotonyloxylanosta-7,9(11),24-trien-26-oic acid (**11**): colorless amorphous solid, yield 22% (5.1 mg); IR (ATR) ν_{max} 2940, 1717, 1685, 1375, 1247, 1029, 756 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.99 (1H, dq, J = 15.6, 6.8 Hz), 6.84 (1H, t, J = 7.2 Hz), 5.90 (1H, dd, J = 15.6, 1.4 Hz), 5.43 (1H, d, J = 5.5 Hz), 5.32 (1H, d, J = 5.8 Hz), 5.12 (1H, dd, J = 10.2, 5.5 Hz), 4.51 (1H, dd, J = 11.2, 4.5 Hz), 2.32 (1H, br d, J = 17.7 Hz), 2.05 (3H, s), 1.89 (3H, d, J = 6.8 Hz), 1.82 (3H, s), 1.17 (1H, dd, J = 10.9, 4.4 Hz), 1.03 (3H, s), 0.99 (3H, s), 0.94 (3H, s), 0.92 (3H, d, J = 6.4 Hz), 0.88 (3H, s), 0.66 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 172.0, 171.0, 166.6, 145.6, 145.1, 144.5, 140.2, 126.6, 123.2, 121.2, 116.1, 80.7, 77.2, 51.5, 48.9, 48.9, 44.1, 38.0, 37.6, 37.3, 35.9, 36.1, 35.3, 34.6, 28.1, 25.9, 24.2, 22.8, 22.8, 21.3, 18.4, 18.2, 18.0, 16.9, 16.0, 12.0; HRMS (ESI-TOF) m/z 603.3649 [M + Na]$^+$ (calcd. for C$_{36}$H$_{52}$O$_6$Na, 603.3656).

(24E)−3β-Acetoxy-15α-isobutyryloxylanosta-7,9(11),24-trien-26-oic acid (**12**): colorless amorphous solid, yield 26% (6.8 mg); IR (ATR) ν_{max} 2972, 2932, 1731, 1686, 1375, 1247 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.85 (1H, t, J = 7.0 Hz), 5.46 (1H, m), 5.32 (1H, d, J = 6.0 Hz), 5.05 (1H, dd, J = 10.0, 5.2 Hz), 4.51 (1H, dd, J = 11.3, 4.5 Hz), 2.58 (1H, m), 2.32 (1H, br d, J = 17.7 Hz), 2.06 (3H, s), 1.83 (3H, s), 1.19 (6H, d, J = 7.0 Hz), 1.01 (3H, s), 0.98 (3H, s), 0.94 (3H, s), 0.92 (3H, d, J = 6.4 Hz), 0.89 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 177.1, 172.4, 171.0, 145.6, 145.1, 140.3, 126.7, 121.0, 116.1, 80.7, 77.2, 51.5, 48.9, 48.9, 44.1, 37.9, 37.6, 37.3, 37.1, 36.0, 35.3, 34.6, 34.3, 28.1, 26.0, 24.2, 22.8, 22.8, 21.3, 19.0, 19.0, 18.4, 18.2, 16.9, 16.0, 12.0; HRMS (ESI-TOF) m/z 605.3814 [M + Na]$^+$ (calcd. for C$_{36}$H$_{54}$O$_6$Na, 605.3813).

Synthesis of 13

A mixture of **3** (5.7 mg, 11 µmol) and formic acid (20 µl) in 2,2,2-trifluoroethyl formate (0.2 ml) was stirred on a 60 °C heating bath for 3 h. After cooling to room temperature, the mixture was concentrated in vacuo. The residue was purified by preparative HPLC (MeCN–H$_2$O) to furnish **13** (3.6 mg, 60%).

(24E)−15α-Acetoxy-3β-formyloxylanosta-7,9(11),24-trien-26-oic acid (**13**): colorless amorphous solid; IR (ATR) ν_{max} 2938, 1721, 1686, 1376, 1249, 1176 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 8.12 (1H, s), 6.84 (1H, t, J = 7.0 Hz), 5.48 (1H, d, J = 5.6 Hz), 5.33 (1H, d, J = 6.0 Hz), 5.07 (1H, dd, J = 10.3, 5.3 Hz), 4.64 (1H, dd, J = 10.2, 5.6 Hz), 2.32 (1H, br d, J = 17.6 Hz), 2.32 (2H, t, J = 7.5 Hz), 2.09 (3H, s), 1.83 (3H, s), 1.19 (1H, dd, J = 11.2, 4.5 Hz), 1.01 (3H, s), 1.00 (3H, s), 0.97 (3H, s), 0.92 (3H, s), 0.91 (3H, d, J = 6.4 Hz), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 171.1, 170.2, 161.0, 145.7, 144.8, 140.4, 126.8, 121.1, 116.3, 80.9, 77.0, 51.4, 49.1, 48.9, 44.2, 38.1, 37.6, 37.4, 37.0, 36.0, 35.5, 34.7, 28.1, 25.9, 24.4, 22.8, 22.8, 21.3, 18.4, 18.2, 16.9, 16.0, 12.1; HRMS (ESI-TOF) m/z 563.3340 [M + Na]$^+$ (calcd. for C$_{33}$H$_{48}$O$_6$Na, 563.3343).

General procedure for 3-O-acylation of 3: synthesis of 14–16

Compound **3** (10–11 µmol) was treated with corresponding acyl chloride (excess, 30 µl) in pyridine (100 µl) – CH$_2$Cl$_2$ (50 µl) at room temperature for 3 h. The mixture was diluted with EtOAc and washed with H$_2$O, and the aqueous layer was extracted with EtOAc. The combined EtOAc solution was concentrated under reduced pressure, and the residue was purified by preparative HPLC (MeCN–H$_2$O) to furnish the 3-O-acyl derivative.

(24E)−15α-Acetoxy-3β-propionyloxylanosta-7,9(11),24-trien-26-oic acid (**14**): colorless amorphous solid, yield 42% (2.4 mg); IR (ATR) ν_{max} 2923, 1734, 1686, 1376, 1248 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.85 (1H, t, J = 7.0 Hz), 5.47 (1H, d, J = 5.5 Hz), 5.32 (1H, d, J = 6.1 Hz), 5.07 (1H, dd, J = 10.2, 5.4 Hz), 4.52 (1H, dd, J = 11.2, 4.6 Hz), 2.33 (2H, q, J = 7.5 Hz), 2.09 (3H, s), 1.83 (3H, s), 1.17 (1H, dd, J = 10.7, 4.5 Hz), 1.15 (3H, t, J = 7.5 Hz), 1.00 (3H, s), 0.98 (3H, s), 0.95 (3H, s), 0.91 (3H, d, J = 6.4 Hz), 0.89 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 173.9, 171.4, 170.8, 146.0, 144.6, 140.5, 126.5, 121.2, 116.3, 80.5, 76.9, 51.6, 49.3, 49.0, 44.3, 38.2, 37.8, 37.5, 37.0, 36.0, 35.6, 34.8, 28.1, 28.0, 25.9, 24.3, 22.9, 22.8, 21.3, 18.3, 18.2, 16.9, 16.1, 12.0, 9.2; HRMS (ESI-TOF) m/z 591.3657 [M + Na]$^+$ (calcd. for C$_{35}$H$_{52}$O$_6$Na, 591.3656).

(24*E*)−15α-Acetoxy-3β-crotonyloxylanosta-7,9(11),24-trien-26-oic acid (**15**): colorless amorphous solid, yield 22% (1.3 mg); IR (ATR) ν_{max} 2930, 1717, 1686, 1376, 1249, 1181 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.96 (1H, dq, $J = 15.5, 6.7$ Hz), 6.84 (1H, t, $J = 7.2$ Hz), 5.86 (1H, dd, $J = 15.6, 1.4$ Hz), 5.47 (1H, d, $J = 5.9$ Hz), 5.33 (1H, d, $J = 5.7$ Hz), 5.06 (1H, dd, $J = 10.3, 5.4$ Hz), 4.57 (1H, dd, $J = 11.5, 4.2$ Hz), 2.32 (1H, br d, $J = 17.7$ Hz), 2.09 (3H, s), 1.88 (3H, d, $J = 6.8$ Hz), 1.83 (3H, s), 1.19 (1H, dd, $J = 11.4, 3.9$ Hz), 1.00 (3H, s), 0.99 (3H, s), 0.97 (3H, s), 0.91 (3H, d, $J = 6.4$ Hz), 0.90 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 172.0, 171.0, 166.6, 145.6, 145.1, 144.5, 140.2, 126.6, 123.2, 121.2, 116.1, 80.7, 77.2, 51.5, 48.9, 48.9, 44.1, 38.0, 37.6, 37.3, 35.9, 36.1, 35.3, 34.6, 28.1, 25.9, 24.2, 22.8, 22.8, 21.3, 18.4, 18.2, 18.0, 16.9, 16.0, 12.0; HRMS (ESI-TOF) *m/z* 603.3658 [M + Na]$^+$ (calcd. for C$_{36}$H$_{52}$O$_6$Na, 603.3656).

(24*E*)−15α-Acetoxy-3β-isobutyryloxylanosta-7,9(11),24-trien-26-oic acid (**16**): colorless amorphous solid, yield 53% (3.4 mg); IR (ATR) ν_{max} 2935, 1731, 1686, 1376, 1248 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.86 (1H, t, $J = 7.1$ Hz), 5.48 (1H, d, $J = 4.8$ Hz), 5.33 (1H, d, $J = 5.4$ Hz), 5.07 (1H, dd, $J = 10.3, 5.4$ Hz), 4.50 (1H, dd, $J = 11.2, 4.8$ Hz), 2.55 (1H, m), 2.32 (1H, br d, $J = 17.7$ Hz), 2.09 (3H, s), 1.83 (3H, s), 1.19 (3H, d, $J = 6.4$ Hz), 1.17 (3H, d, $J = 6.4$ Hz), 1.00 (3H, s), 0.99 (3H, s), 0.96 (3H, s), 0.92 (3H, d, $J = 6.3$ Hz), 0.89 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 176.6, 170.9, 170.1, 146.1, 144.7, 140.6, 126.7, 121.3, 116.3, 80.4, 77.1, 51.7, 49.4, 49.1, 44.4, 38.3, 37.9, 37.6, 37.1, 36.1, 35.7, 34.9, 34.6, 28.2, 26.0, 24.4, 23.0, 22.9, 21.2, 19.1, 19.0, 18.4, 18.3, 17.0, 16.1, 12.1; HRMS (ESI-TOF) *m/z* 605.3814 [M + Na]$^+$ (calcd. for C$_{36}$H$_{54}$O$_6$Na, 605.3813).

General procedure for diacylation of 4: synthesis of 17 and 18

Compound **4** (10–23 μmol) was treated with corresponding acyl chloride (excess, 50 μl) in pyridine (0.2 ml) – CH$_2$Cl$_2$ (0.1 ml) at room temperature for 3 h. The mixture was diluted with EtOAc and washed with H$_2$O, and the aqueous layer was extracted with EtOAc. The combined EtOAc solution was concentrated under reduced pressure, and the residue was purified by preparative HPLC (MeCN–H$_2$O) to furnish the diacylated derivative.

(24*E*)−3β,15α-Dipropionyloxylanosta-7,9(11),24-trien-26-oic acid (**17**): colorless amorphous solid, yield 23% (3.1 mg); IR (ATR) ν_{max} 2930, 1733, 1686, 1376, 1277, 1195 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.85 (1H, t, $J = 7.1$ Hz), 5.46 (1H, d, $J = 4.9$ Hz), 5.32 (1H, d, $J = 6.0$ Hz), 5.07 (1H, dd, $J = 9.8, 5.1$ Hz), 4.52 (1H, dd, $J = 11.2, 4.6$ Hz), 2.36 (2H, q, $J = 7.5$ Hz), 2.33 (2H, q, $J = 7.5$ Hz), 2.09 (3H, s), 1.83 (3H, s), 1.17 (3H, t, $J = 7.5$ Hz), 1.15 (3H, t, $J = 7.5$ Hz), 1.01 (3H, s), 0.99 (3H, s), 0.95 (3H, s), 0.91 (3H, d, $J = 6.4$ Hz), 0.89 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 174.5, 174.3, 171.6, 145.7, 145.1, 140.3, 126.6, 121.1, 116.1, 80.4, 77.1, 51.4, 48.9, 48.9, 44.1, 38.0, 37.7, 37.3, 37.1, 36.0, 35.4, 34.6, 28.1, 28.1, 28.1, 26.0, 24.2, 22.8, 22.8, 18.4, 18.2, 16.9, 16.0, 12.0, 9.3, 9.3; HRMS (ESI-TOF) *m/z* 605.3819 [M + Na]$^+$ (calcd. for C$_{36}$H$_{54}$O$_6$Na, 605.3813).

(24*E*)−3β,15α-Dibutyryloxylanosta-7,9(11),24-trien-26-oic acid (**18**): colorless amorphous solid, yield 48% (3.0 mg); IR (ATR) ν_{max} 2963, 2933, 1731, 1686, 1376, 1257, 1179, 985 cm^{-1}; ^{1}H NMR (400 MHz, CDCl$_3$), selected resonances, δ 6.86 (1H, t, $J = 7.1$ Hz), 5.47 (1H, d, $J = 4.3$ Hz), 5.32 (1H, d, $J = 5.4$ Hz), 5.07 (1H, dd, $J = 9.8, 4.8$ Hz), 4.52 (1H, dd, $J = 11.4, 4.0$ Hz), 2.32 (2H, t, $J = 7.2$ Hz), 2.29 (2H, t, $J = 7.5$ Hz), 1.83 (3H, s), 1.18 (1H, dd, $J = 11.0, 4.3$ Hz), 1.00 (3H, s), 0.98 (3H, s), 0.97 (3H, t, $J = 7.5$ Hz), 0.96 (3H, t, $J = 7.5$ Hz), 0.95 (3H, s), 0.91 (3H, d, $J = 6.3$ Hz), 0.89 (3H, s), 0.65 (3H, s); ^{13}C NMR (100 MHz, CDCl$_3$) δ 173.8, 173.5, 171.7, 145.6, 145.1, 140.2, 126.6, 121.7, 116.1, 80.4, 77.0, 51.4, 48.9, 48.9, 44.0, 37.9, 37.6, 37.3, 37.1, 36.7, 36,7, 35.9, 35.3, 34.6, 28.1, 28.1, 25.9, 24.2, 22.8, 22.8, 18.6, 18.6, 18.4, 18.1, 17.0, 13.7, 13.7, 12.0; HRMS (ESI-TOF) *m/z* 633.4124 [M + Na]$^+$ (calcd. for C$_{38}$H$_{58}$O$_6$Na, 633.4126).

Antitubercular activity against *M. tuberculosis* H37Ra and cytotoxicity of the lanostane triterpenoids

Growth inhibitory activity of the lanostane triterpenoids against *M. tuberculosis* H37Ra was evaluated using the green fluorescent protein (GFP)-based microplate assay [13]. Cytotoxicity of the lanostane triterpenoids against Vero cells (African green monkey kidney fibroblasts) was also evaluated using the GFP-based microplate assay [14]. Details of the bioassay procedures are described in the Supplementary material.

Antitubercular activities of compound 9 (GA003) against *M. tuberculosis* H37Rv (virulent strain) and XDR strains

Activities of compound **9** against *M. tuberculosis* H37Rv (ATCC 27294, virulent strain) and drug-resistant strains, pre-XDR (THX-0001) and XDR strains (THX-0002, THX-0003), were evaluated by the Microplate Alamar Blue Assays [15]. Rifampicin (MIC 0.32 μg ml^{-1}) and isoniazid (MIC 0.08 μg ml^{-1}) were used as positive controls for the activity against *M. tuberculosis* H37Rv. Drug-resistant strains of *M. tuberculosis* used in these assays are clinical isolates from patients in Thailand and preserved at the laboratory of Dr. Angkana Chaiprasert, Drug-Resistant Tuberculosis Research Fund, Siriraj Foundation, Siriraj

Hospital, Bangkok, Thailand. Details of the bioassay procedures are described in the Supplementary material.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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