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One unique steroidal sapogenin obtained through the microbial transformation of ruscogenin by *Phytophthora cactorum* ATCC 32134 and its potential inhibitory effect on tissue factor (TF) procoagulant activity

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ABSTRACT

With the aim to obtain more effective tissue factor (TF) inhibitors, the microbial transformation of three steroidal sapogenins, ruscogenin (**1**), diosgenin (**2**) and sarsasapogenin (**3**), was carried out and only ruscogenin was selectivity converted to 1-hydroxy-spirost-4-en-3-one (**4**) by *Phytophthora cactorum* ATCC 32134. The in vitro anti-TF procoagulant activity of this metabolite was enhanced almost 10 times to an IC_{50} value of 0.29 μM. The chemical assignments of compound **4** were made unambiguously using ESI-MS, IR and 2D NMR spectroscopy.

Tissue factor (TF) is the main initiator of the coagulation cascade and it plays a key role in the formation of thrombin and blood clots in vivo.[1,2] Inhibition of TF expression is an attractive target for the treatment of cardiovascular diseases.[3] Recent studies on the inhibition of TF procoagulant activities have shown that TF inhibitors may provide effective anticoagulation while lessening the risk of bleeding side effect.[4–8] The discovery of new TF inhibitors from nature is a promising approach for developing new anticoagulant drugs.[9]

Ruscogenin (**1**), diosgenin (**2**) and sarsasapogenin (**3**, Fig. 1) three important steroidal sapogenins, have strong anti-arrhythmia,[10] anti-ischemia,[11] anti-thrombosis activities[12,13] and anti-inflammatory.[14,15] In the preliminary screening of steroidal inhibitors of anti-TF procoagulant activities, we found that compounds **1**, **2** and **3** showed significant inhibitory effects on TF expression in vitro. In order to obtain more active and/or less toxic derivatives, the microbial transformation of these three steroidal sapogenins was investigated since microbial transformation is a very useful approach to expand the chemical diversity of natural products[16,17] and has long been valued for the production of almost every class of steroid hormone products.[18] After having screened about 50 microbes, we found that only **1** could be converted to one less polar product by *Phytophthora cactorum*, a kind of eumycetes and a main plant pathogen, obtained by the courtesy of Professor J. P. N. Rosazza of University of Iowa, USA.

The preparative scale biotransformation of **1** by *P. cactorum* was performed by a two-stage procedure in potato medium (PDA). A total of 120 mg of **1** was used and 30 mg of the corresponding metabolite (**4**) was obtained.[19]

Compound **4** was obtained as a white powder (methanol). It showed a positive Liebermann–Burchard reaction. The molecular formula of **4** was established as $C_{27}H_{40}O_4$ by HR-ESI-FTMS in which a pseudo-molecular ion was detected at *m/z*: 429.6106 $[M+H]^+$ {calcd for $C_{27}H_{41}O_4$: 429.6120}, revealing the two mass units less in comparison with **1**. In the IR spectrum, the carbonyl bond nature of the strong absorption band at 1670.74 cm^{-1} and the characteristic peaks of spirostanol-type steroids at 986.22, 924.27, 898.14, 866.29 cm^{-1} suggested a carbonyl group might exist in the spirostanol steroid. In the ^1H NMR spectrum, the four characteristic methyl signals δ_H 0.92 (s, H-18), δ_H 1.37 (s, H-19), δ_H 1.13 (d, J = 6.5 Hz, H-21), δ_H 1.08 (d, J = 7.0 Hz, H-27) further confirmed **4** was a spirostanol steroid. Comparing **4** with **1**, the multiplet of H-3 and diplet of H-6 were lost while a new singlet appeared at δ_H 5.97 (s), suggesting the possible microbial structural modification sites of **1** were at C-3 and C-6. In the ^{13}C NMR spectrum of **4**, by contrast with **1**, the new resonance signals of δ_C 73.6, 197.8 and 170.7 instead of C-1 (δ_C 78.2), C-3 (δ_C 68.2) and C-5 (δ_C 139.2) (Table 1) suggested the oxidation position was most probably at

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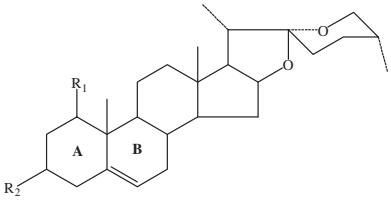
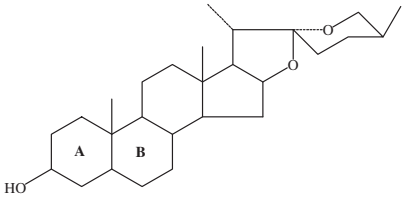
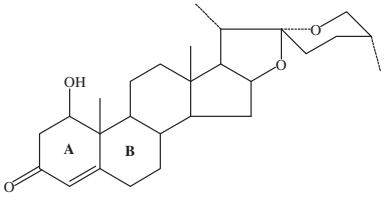


1 $R^1=R^2=OH$ ruscogenin
2 $R^1=H, R^2=OH$ diosgenin



3 sarsasapogenin



4 1-hydroxy-spirost-4-en-3-one

Figure 1. Chemical structures of compounds **1**–**4**.

C-1, 3 and 5. In the 2D NMR spectrum, the direct correlation of δ_H 4.21 (dd, J = 5.5 Hz, 16.9 Hz) and δ_C 73.6 and an indirect correlation between δ_H 4.21 and C-19, C-2 confirmed the oxidation position was not at C-1, and δ_C 73.6 was the resonance signal of C-1 of **4**. The direct correlation of δ_H 5.97 (s) to the alkene δ_C 124.5 and the indirect correlations between δ_H 5.97 (s) and δ_C 197.8 ppm, δ_C 170.7 ppm revealed an allylic ketone group in **4**.[20] Further analysis of HMBC spectrum revealed that there were correlations between H-2 and δ_C 197.8 (the carbonyl), suggesting that the C-3 of **1** was oxidized into carbonyl. Comparing the data of compound **4** with those reported in the literature,[21] the structure of **4** could be unambiguously identified as 1-β-hydroxy-spirost-4-en-3-one (Fig. 2), a unique 4-en-3-one steroidal sapogenin. Since there are no NMR data reported for **4**, a complete, unambiguous assignment was done through 2D NMR techniques (Table 1).

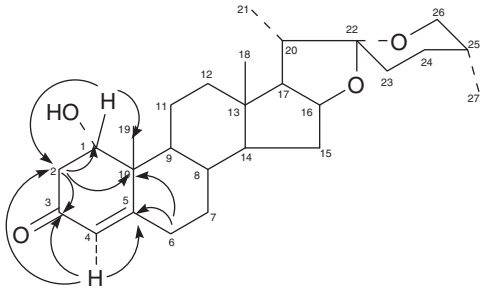


Figure 2. Key H–C HMBC spectra of compound **4**.

Table 2
Inhibitory effects of compound **1**–**4** on TF expression in monocytes (n = 5)

Compound	IC_{50} (μM)
1	2.27 ± 0.32
2	6.96 ± 0.53
3	3.30 ± 0.28
4	0.29 ± 0.08
Curcumin	1.21 ± 0.11

There are two hydroxyl groups on the A ring of ruscogenin, but only a C-3 dehydrogenation product was obtained, this reaction showed high regio-selectivity. In the experiment that diosgenin (**2**) and sarsasapogenin (**3**) were employed for parallel biotransformation by *P. cactorum* under the same conditions, it was interesting to note that no metabolite was detected and the main structural differences among the three sapogenins are the absence of a hydroxyl group at C-1 position. This suggested that the C-1 hydroxyl group on the substrate was an essential feature for the reaction of the reorganization with the enzyme(s) responsible for dehydrogenation of the hydroxyl group at C-3. Meanwhile there were at least two reactions in the biotransformation, dehydrogenation and the double bond migration, but there was no intermediates detected during the biotransformation procedure. The detailed reaction pathway and the enzyme system are now under further investigation.

It was reported that dehydrogenated derivatives of steroids may be more effective than their precursors in treating diseases[22] and the introduction of double bonds into the molecule of certain steroids is essential to increase their activities such as anti-inflammation.[23] We tested the inhibiting effects of compound **4** along with **1**, **2** and **3** on TF expression in human monocyte cell line THP-1 cells stimulated by tumor necrosis factor-alpha (TNF-α).[24] The results revealed that all the tested compounds showed strong inhibitory effects against TF expression in vitro (Table 2) and it is particularly that the IC_{50} value of compound **4** reached 0.29 μM,

Table 1
NMR data of compound **4**

Carbon numbers	δ_C	δ_H	Carbon numbers	δ_C	δ_H
C-1	73.6	4.21 (dd, J = 5.5, 16.9 Hz)	C-15	33.3	1.70 (m); 0.90 (m)
C-2	44.9	2.87 (m)	C-16	81.1	4.53 (m)
C-3	197.8	—	C-17	63.0	1.82 (m)
C-4	124.5	5.97 (s)	C-18	16.6	0.92 (s)
C-5	170.7	—	C-19	13.1	1.37 (s)
C-6	33.6	2.37 (m); 2.18 (m)	C-20	42.5	1.92 (m)
C-7	32.2	2.14 (m); 1.18 (m)	C-21	14.8	1.13 (d, J = 6.5 Hz)
C-8	36.0	1.75 (m)	C-22	109.7	—
C-9	54.8	1.12 (m)	C-23	27.6	1.59 (m)
C-10	45.4	—	C-24	26.2	2.15 (m); 1.36 (m)
C-11	24.0	2.39 (m); 1.69 (m)	C-25	26.4	1.91 (m); 1.46 (m)
C-12	40.3	1.76 (m); 1.17 (m)	C-26	65.1	4.06 (t, J = 10.5 Hz); 3.36 (d, J = 11.0 Hz)
C-13	40.5	—	C-27	16.3	1.08 (d, J = 7.0 Hz)
C-14	56.1	1.08 (m)			

almost 10 times greater than that for **1**. This confirmed that dehydrogenation and the formation of olefin–ketone moiety in the A ring of **1** remarkably increase the anti-TF activity of ruscogenin.

As the pharmaceutical uses of ruscogenin continue to be an attractive area of research, studies on the structure–activity relationship (SAR) of **1** and its derivatives are of great interest in the discovery of bioactive lead compounds for anticoagulant drugs. Our research findings should be helpful in exploring the therapeutic TF inhibitors from steroidal saponins, and they provide new insights into the SAR of ruscogenin and its analogues versus cardiovascular diseases.

Acknowledgments

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24. *The measurement of TF procoagulation activity*:[25] Materials and chemicals: RPMI 1640 medium was purchased from Invitrogen Corporation (USA). TNF-α was from HUMAN ZYME (Chicago, USA), chromogenic substrate Xa was obtained from Sigma (USA). Human prothrombin complex (300 IU, containing factor II, VII, etc.) was obtained from Hualan Bioengineering Company (Xinxiang, China). Cell culture: THP-1 cells were obtained from the cell bank of type culture collection of Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Chinese Academy of Sciences), and cultured in RPMI-1640 medium with 10% heat-inactivated newborn calf serum at 37 °C in 5% CO_2. Induction of TF and drug treatment: The human monocyte cell line THP-1 cells ($1–2 \times 10^6$ cells/mL) were initially incubated with control vehicle (DMSO, 0.1% vol/vol) or experimental vehicle for 1 h in 96-well plate, and then stimulated with 25 ng/mL TNF-α for 5 h to induce TF activity. At the end of incubation, cells were sedimented by centrifugation and resuspended in RPMI 1640. The cell suspension was frozen at −20 °C until TF activity measurement. The cell lysates were frozen and thawed three times before they were used in the assay. With several modifications, cell lysates (45 μL) were incubated with a reagent mixture (5 μL, pH 7.3) containing 10 g/L prothrombin complex and 100 mM $CaCl_2$ in a 96-well plate at 37 °C for 15 min. Then, 50 μL of factor Xa chromogenic substrate (0.5 mM) containing 100 mM EDTA (pH 8.4) was added, and the absorbance was measured at 405 nm. Purified reconstituted human TF was used to generate a standard curve.
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