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New Cytotoxic Steroid from *Stachyurus himalaicus* var. *himalaicus*

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Abstract: A phytochemical study of the ethanolic extract of *Stachyurus himalaicus* var. *himalaicus* was undertaken and as a result a new polyoxygenated steroid, named stachsterol ((20*S*)-20, 25-dihydroxy-4-cholesten-3-one, **1**) and three known ecdysteroids, 20-hydroxyecdysone (**2**), 20-hydroxyecdysone-20, 22-monoacetonide (**3**) and polypodine B-20,22-monoacetonide (**4**), were isolated. Their structures were elucidated by spectroscopic methods, including UV, NMR, MS and HR-MS. The purified product **1** was found to have *in vitro* cytotoxic activity against human Hela cell lines with an IC_{50} value of 2.5 μg/mL. This is the first time that phytoecdysteroids have been found in the genus *Stachyurus*.

Keywords: Steroids, *Stachyurus himalaicus* var. *himalaicus*, Stachyuraceae, Cytotoxity

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Introduction

Since ancient times, medicinal plants have been used in Asian cultures as a source of remedies and for healthcare preparations. In fact, the word medicine was used for herbal remedies for centuries.

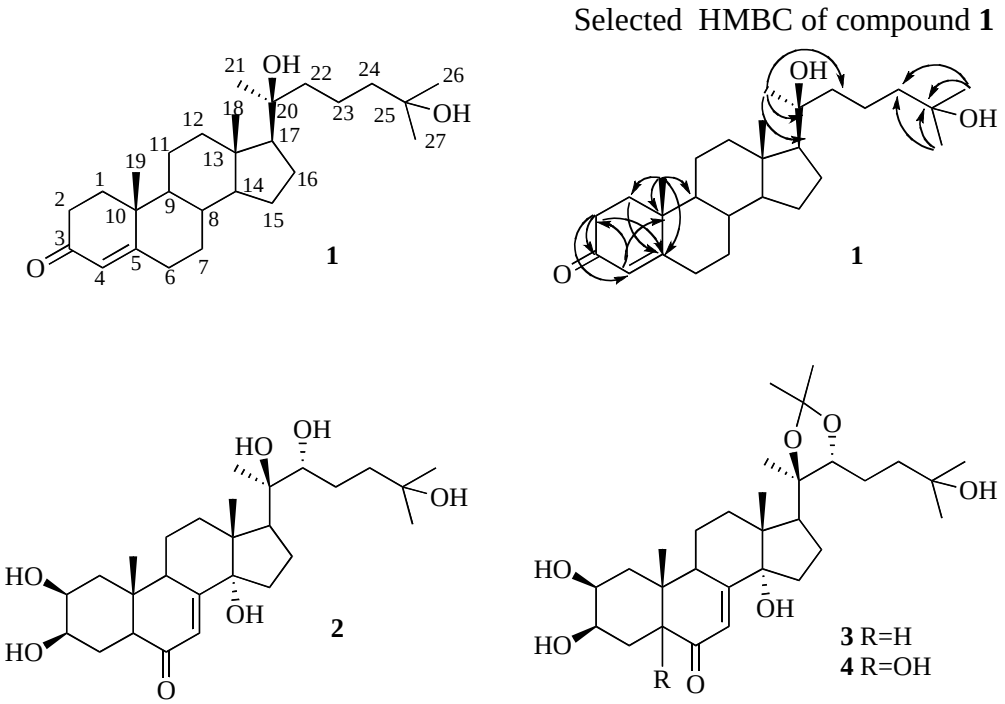
Only after the 1950s has the use of synthetic drugs became so dominant as to totally eclipse herbal cures. Recently, however, renewed interest in medicinal plants for restoring and maintaining health has emerged. Members of the genus *Stachyurus* have been used in Chinese folk medicines for a long time and earlier work reported the isolation of tannins from the genus [1, 2]. *Stachyurus himalaicus var. himalaicus,* known as 'Tong-Cao" in Chinese folklore, is used for the treatment of dropsy and gonorrhea [3]. However, to our knowledge no work has been reported on the biologically active constituents of this species. A preliminary pharmacological study on this plant showed that the 95% EtOH extract had cytotoxic activity against human Hela cell lines at a concentration of 10 μg/mL. A bio-assay guided study revealed that an EtOAc fraction of the extract also displayed strong cytotoxic activity. In the previous phytochemical study, we isolated two new polyoxygenated triterpenoids, one of which was found to have mild cytotoxic activity against human Hela cell lines *in vitro* [4].

In this study, we report the bioactivity-guided isolation and structural determination of four steroids (**1**-**4**; Figure 1) from *Stachyurus himalaicus* var. *himalaicus*. One of these steroids, **1**, is a new 3-oxo-4-ene steroid and the structure was elucidated in detail. The purified product **1** was found to have cytotoxic activity against human Hela cell lines *in vitro* with an IC_{50} value, 2.5 μg/mL. Phytoecdysteroids **2**, **3**, and **4** were isolated for the first time from the genus *Stachyurus*.

Results and Discussion

In order to obtain more information about the bioactive compositions of *Stachyurus himalaicus* var. *himalaicus*, the EtOAc-soluble fraction of a crude ethanolic extract was analyzed by HPLC-UV. The early-running compounds possessed the UV spectra of flavonoids, whilst the later-running compounds had simple UV spectra and could not be correlated with a specific chemical class. The present study focused on the purification and identification of the latter minor components.

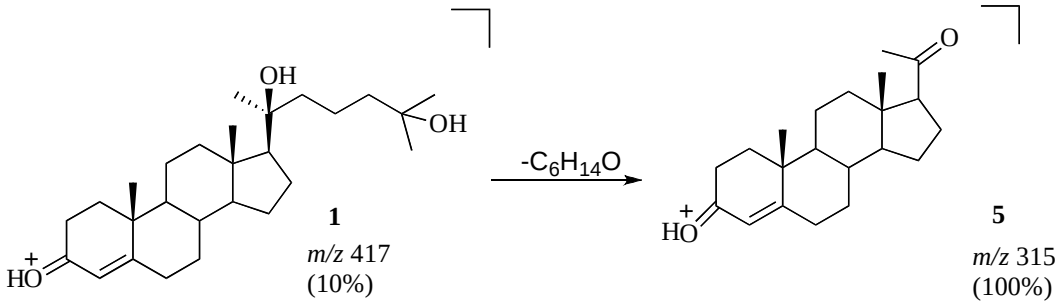
Figure 1. Structures of compounds **1,2,3** and **4.**



The twigs and leaves of *Stachyurus himalaicus* var. *himalaicus*, collected from Wenshan County, Yunnan province, were extracted with 95% ethanol and then the concentrated extract suspended in water was partitioned successively with petroleum ether, EtOAc and n-BuOH. The EtOAc extract was subjected to chromatography on silica gel, Sephadex LH-20, and RP-18 to yield compounds **1**, **2**, **3** and **4** (Figure 1).

Compound **1** was isolated as white powder. The color test indicated **1** to be a steroid. The HR-ESI/MS determined the molecular formula to be $C_{27}H_{44}O_3$ (*m/z* 417.3377 $[M+H]^+$), indicating six degrees of instauration, ascribed to α, β-unsaturated ketone and four steroid rings. The UV absorption at 243 nm suggested the presence of an α, β-unsaturated ketone, which was indicative of a conjugated 4-ene-3-one [5]. The IR spectrum (KBr) displayed absorption bands for hydroxyl (3443 cm^{-1}) and α,β-unsaturated ketone (1667, 1652 cm^{-1}) groups. The ^{1}H-NMR and ^{13}C-NMR (DEPT) of **1** revealed signals due to five methyls, eleven methylenes, five methines and six quaternary carbons. Its ^{1}H-NMR indicated the presence of five methyl groups at δ_H 0.86 (3H, s, CH_3-18), 1.16 (3H, s, CH_3-19), 1.20 (6H, s, CH_3-26, 27), 1.27 (3H, s, CH_3-21). Twenty-seven carbons including five methyls suggested that **1** was an analogue of cholesterol. A series of proton signals at δ 0.9-2.5 were attributed to resonances of overlapping of methylenes and methines of framwork of steroid. All of the protonated carbons were assigned by HMQC experimental. The proton NMR spectrum of **1** displayed a vinyl signal at δ 5.70 (1H, s), which is fully consistent with those reported for 3-keto-4-ene steroids [5, 6]. In the HMBC spectrum, the correlations between δ_H 5.70 (1H, s, H-4) and δ_C 39.0 (C-10), 172.0 (C-5), 200.1(C-3), 33.3 (C-2) supported the presence of 3-keto-4-ene unit. The ^{1}H-NMR signal at δ_H 1.20 (6H, s) indicated the side chain C-26/C-27 was intact. Its ^{13}C NMR spectrum showed two oxygen-bearing quaternary carbon signals at δ_C 75.5 and 71.4. The presence in the ^{1}H-NMR spectrum of a methyl singlet at δ 1.27 suggested the location of one hydroxyl function at C-20, and this was supported by a quaternary carbon signal at δ 75.5 ppm in the ^{13}C-NMR spectrum. Furthermore, in the HMBC experiments, the methyl proton signals at δ_H 1.27 (CH_3-21) correlated with the signals at δ_C 58.1 (C-17), 75.5 (C-20) and 44.7 (C-22), and that at δ_H 1.20 (6H, s, C-26, C-27-CH_3) correlated with the signals at δ_C 44.7 (C-24) and 71.4 (C-25). Thus, two hydroxyl groups were assigned to C-20 and C-25, respectively. The ESI$^+$MS of **1** gave a significant base peak at *m/z* 315 ($[M^+-C_6H_{14}O]$, derived from the cleavage at the C-20-C-22 bond from $[M+H]^+$ (as shown in Figure 2), which also confirmed that the hydroxyl groups were located on the side chain. The value of the chemical shift of CH_3-21 at δ 1.27 was indicative for the common 20*S*-configuration hydroxyl group ((20*S*)-and (20*R*)-20-hydroxycholestane: δ 1.28 and 1.13, respectively) [7]. The observation of NOESY correlations between H_3-21 and H-12β and between H-17 and H-14 was in agreement with the α-orientation of H-17 and the *R* stereochemistry of C-20, which features normally exhibited by a steroid skeleton [8]. Following detailed consideration of molecular models, a (20*S*)-20,25-dihydroxy-4-cholesten-3-one structure was established for the new steroid **1**, which was named stachsterol (Figure 1). The new steroid **1,** a polyoxygenated 3-oxo-4-ene sterol, was found to have cytotoxic activity against human Hela cell lines *in vitro* with an IC_{50} value 2.5 μg/mL. Literature reportes indicate that 3-oxo-4-ene sterols (OH-substituted at $C_{1\sim15}$ hydrocarbon chain) have been utilized in the treatment of conditions associated with rapidly growing cells and cancers such as melanoma [9] and except for the estrogens, many steroid hormones have the 3-oxo-4-ene (Δ^4)-structure [10].

Figure 2. Partial MS analysis of **1.**



In addition to **1**, the three known ecdysteroids 20-hydroxyecdysone (**2**) [11], 20-hydroxyecdysone-20,22-monoacetonide (**3**) [12] and polypodine B-20,22-monoacetonide (**4**) [13], were isolated from *Stachyurus himalaicus* var. *himalaicus*. Their structures were identified by comparison of their spectroscopic data (FABMS, ^{1}H- and ^{13}C-NMR) with those reported in the literature. Ecdysteroids were discovered decades ago as hormones of arthropods; they have effects on moulting and development. There are several plants containing ecdysteroids, the so-called phytoecdysteroids. These substances have proved to be extremely interesting because of their capability for enhancing the biosynthesis of protein in the organism of experimental animals in a manner similar to the steranabols, but without at the same time exhibiting androgenic, thymolytic and other side effects. Ecdysteroids are even used for doping purposes. Since there is a great demand for compounds with this type of action at present, much research has and is being carried out to identify ecdysteroid-containing specimens [14]. This is the first time that ecdysteroids were found from the genus *Stachyurus* and we hope to find more ecdysteroids from the members of *Stachyurus*.

Conclusions

A new polyoxygenated steroid, named stachsterol ((20*S*)-20, 25-dihydroxy-4-cholesten-3-one**, 1**) and three known ecdysteroids 20-hydroxyecdysone (**2**), 20-hydroxyecdysone-20,22-monoacetonide (**3**) and polypodine B-20,22-monoacetonide (**4**), were isolated from *Stachyurus himalaicus* var. *himalaicus*. The purified product **1** was found to have cytotoxic activity against human Hela cell lines *in vitro* with an IC_{50} value, 2.5 μg/mL. This is the first time that phytoecdysteroids have been found in the genus *Stachyurus*.

Experimental

General

Optical rotations $[\alpha]_D$ were measured on a Jasco-20 MC Polarimeter. IR spectra were taken on a Nicolet AVATAR-360 spectrophotometer, ν_{max} in cm^{-1}. UV was determined on a Shimadzu UV-2401PC spectrophotometer, λ_{max} in nm. Commercial Silica gel plates (QingDao Marine Chemical Group Co.) were used for TLC. The chromatograms were sprayed with 10% H_2SO_4 and heated at 80°C to detect the spots. ^{1}H-, ^{13}C- and 2D NMR were recorded on *a* Bruker AV 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) and AV-500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), respectively;

chemical shifts are given in ppm as δ values relative to TMS as internal standard. ESI /MS were obtained on a Finnigan-MAT (San José, CA, USA) TSQ-700 triple-stage quadrupole instrument.

Plant Material

Twigs and leaves of *Stachyurus himalaicus* var. *himalaicus* were collected in May 2002 from Wenshan County of Yunnan Province, P. R. China and identified by Prof. Hu Zhi-Hao, Yunnan University. A voucher specimen (YN 0205) has been deposited at Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, Yunnan University.

Extraction and Isolation

The powdered plant material of *Stachyurus himalaicus* var. *himalaicus* (33.0 kg) was repeatedly extracted with 95% EtOH at room temperature. The extract was then concentrated under reduced pressure to give a brown syrup, which was partitioned into petroleum ether-soluble (340.0 g), EtOAc-soluble (540 g), and *n*-BuOH-soluble (1.0 kg) fractions. The EtOAc-soluble portion was subjected to silica gel column chromatography eluting with $CHCl_3$-MeOH (99:1-1:1), whereby twenty fractions (I-XX) were obtained. Fraction VIII (8.7 g) was resubmitted to silica gel column chromatography with a step $CHCl_3$-MeOH gradient to obtain 10 fractions. The Fraction VIII-3 was subjected to repeated silica gel column and Sephadex LH-20 chromatography to yield (20*S*)-20,25-dihydroxy-4-cholesten-3-one (stachsterol, **1**, 15 mg). Fraction 16 (8.0 g) was subjected to chromatography over silica gel eluting with $CHCl_3$-MeOH (9:1, 8:2, 7:3), Sephadex LH-20 (MeOH) and RP-18 (MeOH: H_2O 7:3) to yield 20-hydroxyecdysone (50 mg, **2**) as a white amorphous powder. Fraction 11 (15.0 g) was subjected to repeated silica gel column and Sephadex LH-20 chromatography (MeOH) to yield 20-hydroxyecdysone-20,22-monoacetonide (10 mg, **3**) as a white amorphous powder. Fraction 14 (12.0 g) was subjected to repeated silica gel column, Sephadex LH-20 (MeOH) and RP-18 chromatography to yield polypodine B-20,22-monoacetonide (8 mg, **4**) as a white amorphous powder.

Stachsterol ((*20S)-20, 25-dihydroxy-4-cholesten-3-one*, **1**) white amorphous powder, $[\alpha]_{D.}^{25.4}$: +31.48 (c 0.667, chloroform); IR ν_{max}^{KBr} cm^{-1}: 3443 (-OH), 1667, 1652 (α,β-unsaturated ketone); UV (MeOH) λ_{max} nm: 243; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm): 0.86 (3H, s, CH_3-18), 1.16 (3H, s, CH_3-19), 1.20 (6H, s, CH_3-26, 27), 1.27 (3H, s, CH_3-21), 5.70 (1H, s, H-4), 2.22, 1.92 (each 1H, m, H-1), 2.42, 2.31 (each 1H, m, H-2), 2.11, 2.08 (each 1H, m, H-6), 1.40 (1H, m, H-8), 1.17, 2.04 (each 1H, m, H-12α, H-12β), 1.20 (1H, m, H-14), 1.42 (1H, m, H-17), 1.38 (2H, m, H-22), 1.24 (2H, m, H-23), 1.43 (2H, m, H-24); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 36.0t (C-1), 33.3t (C-2), 200.1s (C-3), 124.2d (C-4), 172.0s (C-5), 34.3t (C-6), 32.3t (C-7), 35.3d (C-8), 54.1d (C-9), 39.0s (C-10), 21.2t (C-11), 40.3t (C-12), 43.1s(C-13), 56.4d (C-14), 24.0t (C-15), 22.7t (C-16), 58.1d (C-17), 14.1q (C-18), 17.7q (C-19), 75.5s (C-20), 26.7q (C-21), 44.7t (C-22), 19.3t (C-23), 44.7t (C-24), 71.4s (C-25), 29.6q (C-26), 29.8q (C-27); FAB^+/MS *m/z* (relative intensity): 417 $[M+1]^+$; ESIMS *m/z*: 417 $[M+1]^+$ (10), 315 (100), 297 (45), 272 (40), 257 (35), 245 (15), 229 (10), 187 (8), 149 (23), 124 (30), 109 (85), 79 (15), 71 (20), 67 (12); HR-ESI^+MS *m/z*: 417.3377 $[M+1]^+$, (calcd for $C_{27}H_{44}O_3$ 417.3368).

Cytotoxic activity

Hela (human carcinoma of the cervix) cell lines were grown as a monolayer in Dulbecco's modified eagle's medium, DMEM (Gibco), supplemented with 10% newborn calf serum (Gibco) and 1% of penicillin-streptomycin mixture (10,000 UI/mL). The cells were maintained at 37 °C in 5% CO_2 and 90% humidity. The cytotoxic activitity was assessed using colorimetric MTT reduction assay [15]. The percentage viability was plotted against the compound concentrations, and the 50% cell viability (IC_{50}) was calculated from the curve. All the experiments were repeated three times. The assay showed the IC_{50} of **1** was 2.5 μg/mL.

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References

1. Li, H.; Hatano, T.; Okuda T.; Yoshida T. Tannins of *Stachyurus* species. III. Stachyuranins A, B, and C, three new complex tannins from *stachyurus praecox* leaves. *Chem. Pharm. Bull.* **1995**, *43*, 2109-2114.
2. Hatano T.; Yazaki K.; Okonogi A.; Okuda T. Tannins of *Stachyurus* species. II. Praecoxins A, B, C and D, four new hydrolysable tannins from S*tachyurus praecox* leaves. *Chem. Pharm. Bull.* **1991**, *39*, 1689-1693.
3. Institutum Botanicum Kunmingense Academiae Sinicae Edita. *Flora Yunnanica.* Science Press: Beijing, **1983**; Tomus 3, pp.339 (in Chinese).
4. Yang, J.H.; Wang, Y.S.; Huang, R.; Luo, S.D.; Zhang H.B.; Li, L. New polyoxygenated triterpenoids from *Stachyurus himalaicus* var. *himalaicus*. *Helv. Chimi. Acta.* **2006**, *89*, 2830-2835.
5. Wozny, M. E.E.; Smith, T.R.; Angus, R.L.; Kaiser, E.T. Transformation pf 25-hydroxycholesterol by *Rhizoctonia muneratii*. *Appl. Environ. Microbiol.* **1982**, *3*, 572-577.
6. Blunt, J.W.; Stothers, J.B. ^{13}C NMR spectra of steroids-a survey and commentary. *Org. Magn. Reson.* **1977**, *9*, 439-464.
7. Nes, W. R.; Varkey, T. E.; Crump, D. R.; Gut, M. (Z)-17(20)-Dehydrocholesterol. A New Sterol with C-21 and C-22 Spatially Fixed. *J. Org. Chem.***1976**, *41*, 3429-3433.
8. Duh, C.Y.; El-Gamal, A. A. H.; Song, P.Y.; Wang, S.K.; Dai, C.F. Steroids and Sesquiterpenoids from the Soft Corals *Dendronephthya gigantean* and *Lemnalia cervicorni*. *J. Nat. Prod.* **2004**, *67*, 1650-1653.
9. Axelon, M.; Nus, L.O. Cytostatic sterols for treatment of cancer and other conditions associated with rapidly growing cells. PCT Int. Appl. WO 97 45, 440 (Cl. C07J9/00), 4 Dec 1997, SE Appl. 96/2, 100, 30 May 1996; pp. 59. CA, 128:57450.

10. Higashi, T.; Takayama, N.; Shimada, K. Enzymic conversion of 3β-hydroxy-5-ene-steroids and their sulfates to 3-oxo-4-ene-steroids for increasing sensitivity in LC-APCI-MS. *J. Pharm. & Biomed. Anal.* **2005**, *39*, 718-723.
11. Singh, SB, Thakur RS. Structure and stereochemistry of paristerone, a novel phytoecdysone from the tubers of *Paris polyphylla*. *Tetahedron* **1982**, *38*, 2189-2194.
12. Zhang, M.N.; Michael, J.S.; Isao, K. Isolation of ecdysterids from *Vitex strickeri* using RLCC and recycling HPLC. *Phytochemistry* **1992**, *31*, 247-250.
13. Jaroslav, P.M.B.; Karel, V.V.L.; Juraj, H. Ecdysteroids from the roots of *Leuzea carthamoides*. *Phytochemistry* **1994**, *37*, 707-711.
14. Saatov, Z.; Agzamkhodzhaeva, D.A.; Syrov, V.N. Distribution of phytoecdysteroids in plants of Uzbekstan and the possibility of using drugs based on them in neurological practice. *Chem. Natur. Compd.* **1999**, *35*, 186-191.
15. González, A.G..; Alvarenga, N.L.; Bazzocchi, I.L.; Ravelo, A.G..; Moujir, L. A new bioactive norquinone-methide triterpene from *maytenus scutioides*. *Planta Med.* **1998**, *64*, 769-771.

Sample Availability: Available from the authors.