First Isolation and Confirmation of Sterol Based on *β*-sitosterol Skeleton from the Leaves of *Podocarpusnagi* Planted in Fujian, Preliminary *in vitro* Anticancer Activity and the Crystal Structure①

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ABSTRACT The crystal of (24R)-3*β*,5*α*-dihydroxy-24-ethyl-5*α*-cholestan-6-one (**1**) was isolated from the leaves of *P. nagi* planted in Fujian for the first time with the same skeleton as 26,27-dinorcholest-5-en-3-*β*-ol (**2**) and *β*-sitosterol (**3**) that have been reported before. Compound **1** crystallizes in monoclinic, space group $P2_1$ with a = 10.8482(4), b = 7.3671(3), c = 33.7860(15) Å, β = 93.103(4)°, V = 2696.21(19) Å^3, Z = 75, M_r = 446.70, ρ_{calc} = 1.145 g/cm^3, F(000) = 1032, μ = 0.572 mm^{-1}, $GOOF$ = 1.034, the final R = 0.0467 and wR = 0.1129 for 6989 observed reflections with $I > 2\sigma(I)$. Compound **2** was selected to evaluate for their preliminary *in vitro* anticancer activity against four cancer cell lines for the first time. The results showed that compound **2** exhibited great inhibition against gastric cancer, breast cancer MCF-7, lung cancer A549 and Helacell lines with the inhibitions of 89.16% ±1.17, 97.02%±0.53, 47.20% ±2.58 and 36.89%±1.22 respectively at the concentration of 1.4×10^{-2} M, which means that we found the new anticancer compound in this plant medicine.

Keywords: *Podocarpusnagi*, crystal structure, sterols, isolation, anticancer evaluation;
DOI: 10.14102/j.cnki.0254–5861.2011–2981

1 INTRODUCTION

Podocarpusnagi (*P. nagi*, named Zhubai in Chinese) is widely distributed in south districts of Yangtze River, such as Fujian, Hunan, Guangxi, Guangdong, etc. This plant contains different kinds of biological compounds (such as volatile oil, flavonoids, steroids, sugar and glycosides, lactones and so on) and exhibits a wide spectrum of biological activities like hemostasis, bone setting, anti-bacterial, anti-tumor, antiviral, antioxidant and detume-scence activities[1]. According to the folk records of the Yao Nationality, *P. nagi* has ever been used to treat trauma, stop-bleeding, fractures, knife wounds, gunshot wounds, body odor, eye diseases, colds, and so forth. The fresh bark or root of *P. nagi* was also used to treat the rheumatoid arthritis[2-4]. Some work about the chemical components and biological activities of *P.*nagi has been reported: Ye Yang and XuYaming`s groups isolated *Podocarpus* nagilactones from *P. nagi*planted in Guangdong province and evaluated their biological activity. The results showed that most of them exhibited higher antitumor activity[5,6]. Chen Yegao`s group isolated several bioflavonoids and few steroids from the leaves of *P. nagi* grown in Yunnan[7]. However, *P. nagi* was also distributed in Nan-

Received 14 September 2020; accepted 23 November 2020 (**CCDC 1985853**)
① This work was financially supported by the National Natural Science Foundation of China (21875252) and Self-created Area Project of Major Science Technology Innovation Platform of Xiamen (3502ZCQ20171002)
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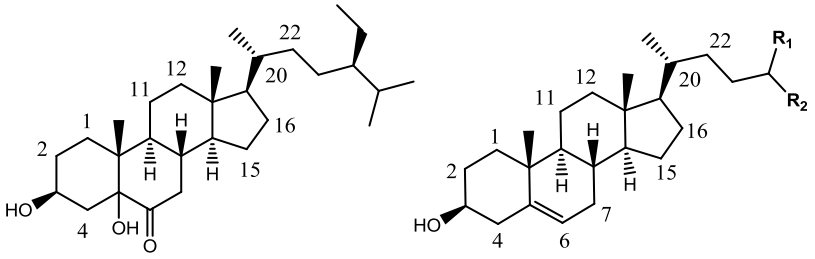
ping of Fujian province. In recent years, a large scale of *P. nagi* was planted in Yangli town of Fujian province. Our research group has extracted the essential oil from its fruits, confirmed its chemical components, and evaluated its biological activities. The results showed this oil contains many active components: such as abundant unsaturated fatty acids, flavonoids, *β*-vanillin, vitamin E and essential microelements for human body; the biological evaluation results showed that it exhibited higher anti-oxidant[8]. We checked the published papers and some old records and found that there are some differences about the *P. nagi* grown in Guangdong, Yunnan and Fujian. To the best of our knowledge, there are no reports about the chemical components of *P. nagi* planted in Fujian, so our research group took the lead to isolate the chemical components from the leaves of *P. nagi* planted in Fujian.

In this work, three sterols, (24R)-3*β*,5*α*-dihydroxy-24-ethyl-5*α*-cholestan-6-one (**1**), 26,27-dinorcholest-5-en-3-*β*-ol (**2**), and *β*-sitosterol (**3**), were isolated using the silica gel column chromatography. The preparative thin layer chromatography (PTLC) together with the recrystallization from the leaves of *P.nagi* and their structures was confirmed by NMR and XRD methods. Compound **1** was isolated from nature source for the first time and its crystal structure has not been reported. The crystal structure of **1** clearly explained its absolute configuration, and provided the reference for the assign of this kind of compounds. The crystal structures of **2** and **3** have been reported[9-17]. The chemical structures of **1**～**3** are listed in Fig. 1. Compounds **1** and **2** are rare compounds with the same skeleton as the *β*-sitosterol, and were isolated for the first time from the leaves of *P.nagi* grown universally in Fujian. Compound **2** showed good to moderate *in vitro* anticancer activity against gastric cancer (NCI-N87), breast cancer MCF-7 (HTB-22), lung cancer A549 (CCL-185) and Hela (CCL-2)cell lines using the cell counting kit-8 (CCK-8) method[18].



1

2 R_1=H, R_2=CH_3
3 R_1=CH_2CH_3, R_2=$CH(CH_3)_2$

Fig. 1. Chemical structures of compounds 1～3

2 EXPERIMENTAL

2. 1 Materials and instruments

Plant materials The leaves of *Podocarpusnagi* were collected in September of 2018 from the Yangli town of Fujian province, China and identified by one of the authors (J.P. Yong).

Instruments NMR spectra were recorded on a Bruker AV-400 spectrometer. Column chromatography (CC) was carried out onsilica gel (100～200 mesh, Qingdao Marine Chemical Inc., Qingdao, China). Melting points were determined on a XT-4 apparatus equipped with a microscope and uncorrected. Crystallography data were obtained from Rigaku SuperNova, with CCD detector and X-ray source of Cu*Kα* radiation (λ = 1.54184 Å). The structure was solved by direct methods with Olex2 Crystallographic Software.

2. 2 Extraction and isolation

The detailed isolation processes are listed below: 10 kilograms of the air-dried and powdered leaves were added into a 25 L container and the material was dipped in 20 L70 % ethanol-water solution for one month and then filtered. The solution was concentrated under the reduced pressure, and the residue was dispersed in 5 L water and extracted with 1 L ethyl acetate for three times. The ethyl acetate layers were combined and concentrated under the reduced pressure to obtain another residue, which was rechromatographed over a column of silica gel with petroleum ether, petroleum ether-ethyl acetate ($V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 10:1 to 0:1) as eluents to obtain some fractions: 10 fractions using petroleum ether as eluent; 10 fractions using $V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 10:1 as eluent; 18 fractions using $V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 5:1 as eluent; 20 fractions using $V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 2:1 as eluent; 48 fractions using $V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 1:1 as eluent; and 21 fractions using ethyl acetate as eluent. After

the simple TLC analysis, we selected some fractions and combined to obtain another 6 fractions for further isolation:fraction 1 (petroleum ether as eluent); fraction 2 ($V_{\text{petroleum ether}}$:$V_{\text{ethyl acetate}}$, 10:1 as eluent); fraction 3 ($V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 5:1 as eluent); fraction 4 ($V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 2:1 as eluent); fraction 5 ($V_{\text{petroleumether}}$:$V_{\text{ethyl acetate}}$, 1:1 as eluent) and fraction 6 (ethyl acetate as eluent).

Compounds **1**, **2** and **3** were isolated from the fraction 3 using silica gel column separation, preparative thin layer chromatrgraphy (PTLC) together with recrystallization. We checked the crystals under microscope and found that the appearance of the crystals was very different. We selected different crystals and analyzed their structures by XRD method, obtaining three different structural compounds.

2. 3 Structure characterization

The isolated compounds were characterized using NMR and XRD methods. NMR was recorded on a 400 MHz Bruker AVANCE III spectrometer in $CDCl_3$. The chemical shifts were expressed in ppm relative to tetramethylsilane (TMS) as the internal standard. XRD were recorded on a SuperNova, Dual, Cu at zero, Atlas diffractometer equipped with graphite-monochromated Cu$K\alpha$ radiation (λ = 1.54184 Å).

Compound **1**: white lamellar single crystal, m.p.: 253～256 °C; HR-MS for $C_{29}H_{50}O_3Na$, $[M+Na]^+$: Calcd. 469.3652, found: 469.3652. This compound was confirmed by XRD analysis. A white lamellar single crystal of compound **1** with dimensions of 0.18mm × 0.18mm × 0.06mm was used for X-ray diffraction analysis. A total of 10599 reflections were collected at 100.01(16) K in the range of $5.24 \leqslant 2\theta \leqslant 149.60^{\circ}$ by using an ω-scan mode, of which 6989 were unique with R_{int} = 0.0419 and R_{sigma} = 0.0595 and 6989 were observed with $I > 2\sigma(I)$. The final R = 0.0561 and wR = 0.1209. The structure was solved by direct methods with SHELXS-2014 and refined by full-matrix least-squares methods with SHELXL-2014 program package[19]. All of the non-hydrogen atoms were located with successive difference Fourier synthesis. Hydrogen atoms were added in idealized positions. The non-hydrogen atoms were refined anisotropically. Selected bond lengths and bond angles from XRD data are listed in Table 1. The XRD data are ideal and physical data agree well with (24R)-3β,5α-dihydroxy-24-ethyl-5α-cholestan-6-one[9]. The HR-MS result was also consistent well with its molecular weight.

Table 1. Selected Bond Lengths (Å) and Bond Angles (°) of Compound 1

Bond	Dist.	Angle	(°)
C(3)–O(1)	1.433(5)	O(1)–C(3)–C(2)	110.1(3)
C(5)–O(2)	1.442(4)	O(1)–C(3)–C(4)	108.8(3)
C(6)=O(3)	1.217(5)	O(2)–C(5)–C(4)	107.5(3)
C(5)–C(6)	1.540(4)	O(2)–C(5)–C(6)	105.2(3)
		O(2)–C(5)–C(10)	109.6(3)
		O(3)=C(6)–C(5)	123.3(3)
		O(3)=C(6)–C(7)	122.1(4)

Compound **2** is a white thin lamellar single crystal, m.p.: 127～128 °C, and compound **3** is a white needle crystal, m.p.: 140～141 °C. The NMR data, XRD analysis and relevant biological evaluation have been reported earlier[9-18].

2. 4 Preliminary *in vitro* anticancer evaluation

Compound **2** was selected to evaluate for their preliminary *in vitro* anticancer activity against gastric cancer (NCI-N87), breast cancer MCF-7 (HTB-22), lung cancer A549 (CCL-185) and Hela (CCL-2) cell lines using the CCK-8 method. Briefly, the cancer cell lines were seeded in 96-well plates (5000 cells/well) with 100 μL DMEM supplemented with 10% fetal bovine serum, and cultured at 37 °C in a humidified CO_2 incubator (95% air, 5% CO_2) for 24 h. While the cell lines grew to 90% in logarithmic growth, the culture medium was removed from each well, and 100 μL fresh DEME was added to each well. Then, 10 μL solution of compound **2** was added into each well (The experiment was repeated for 5 times) and the plates were incubated for another 48 h at 37 °C. Subsequently, 10 μL CCK8 was added to each well, and the plates were cultured at 37 °C for another 4 hours. The optical density was measured at a wave-length of 450 nm on an ELISA microplate reader. DMEM and DMSO solution (V/V: 10/1) was used as a negative control. The results were expressed as the inhibition calculated at the ratio [(1-(OD_{450} treated/OD_{450} negative control)) × 100].

3 RESULTS AND DISCUSSION

During the isolation of fraction 3, after the silica gel column isolation, the preparative thin layer chromatography (PTLC) was used to isolate one compound. However, it was confirmed to be the mixture of compounds **1**, **2** and **3** based on ^{1}H-NMR analysis. Because the R_f values of these compounds (**1**, **2** and **3**) were almost the same, it is so difficult to differentiate them using TLC analysis and also very difficult to isolate them through PTLC. However, this mixture is very easy to form crystals in solution ($V_{\text{petroleum ether}}$:$V_{\text{ethyl acetate}}$, 1:1). We checked the crystals under microscope and found that their appearances are very different. We selected different crystals and analyzed their structures by XRD method, obtaining three different structural compounds: compound **1** (9 mg) as a white lamellar single crystal, compound **2** as a white thin lamellar single crystal (11 mg), and compound **3** as a colorless acicular single crystal (186 mg). It exhibited that compound **1** is in the very lower content in fraction 3 and the amount is not enough for NMR analysis, so we only finished the XRD and HR-MS analyses.

The molecular structure and ORTEP diagram of compound **1** are shown in Fig. 2. The skeleton of steroid is the same as that of compounds **2** and **3**, while the hydroxyl at C(5) and carbonyl at C(6) of compound **1** are unique in comparison with compounds **2** (two hydroxyl groups at C(5) and C(6)) and **3** (double bonds between C(5) and C(6)). Besides, there are many differences of the dihedral angles of C(4)–C(10)–C(5)–C(6) between rings A and B of compounds **1** and **3**. C(4)–C(5)–C(6) and C(10)–C(5)–C(6) are different planes of rings A and B, respectively. The value for **1** is 126.694º, while that for **3** is 177.734º, with the deviation to be 51.04º. The difference of dihedral angles might be caused by the type of bonds between C(5) and C(6): single bond (1.540 Å) in **1** but double bond (1.337 Å) in **3**. The big groups at C(5) and C(6) of compound **1** increase the spatial effect and make rings A and B turn round accordingly. The schematic diagrams of the crystal cells and intermolecular hydrogen bonds of compound **1** are shown in Fig. 3 and Table 2. It is a supramolecular laminated structure, in which the branched alkanes of ten carbons can easily rotate and interact with the adjacent crystal cell layer. Intermolecular hydrogen bonds are formed by hydroxyl groups at C(5) between compound molecules (d2). Hydroxyl groups at C(3), C(5) and carbonyl groups at C(6) can also form hydrogen bonds with water (d1, d3, d5, d6). Effective hydrogen bonds can be formed in three-dimensional space, and strong intermolecular forces are found by calculating effective distances, the range of hydrogen bonds: 1.929～2.077 Å. The melting point (253～256 ℃) of compound **1** is much higher than that of compounds **3** (140～141 ℃) aroused by the intermolecular hydrogen bond.

Table 2. Hydrogen Bonds for Compound 1

D–H···A	d(D–H)/Å	d(H···A)/Å	d(D···A)/Å	∠D–H···A/°
O(2)–H(2)···O(2)^1	0.82	2.08	2.899(3)	177
O(2)–H(2)···O(H_2O)	0.82	2.02	2.840(3)	173
O(1)–H(1)···O(H_2O)	0.82	2.00	2.757(3)	154
O(2)–H(2)···O(2)	0.82	2.08	2.872(3)	163
O(H_2O)–H(H_2O)···O(H_2O)^2	0.95	1.93	2.869(3)	170

^{1}2 – x, –1/2 + y, 1 – z; ^{2}2 – x, 1/2 + y, 1 – z

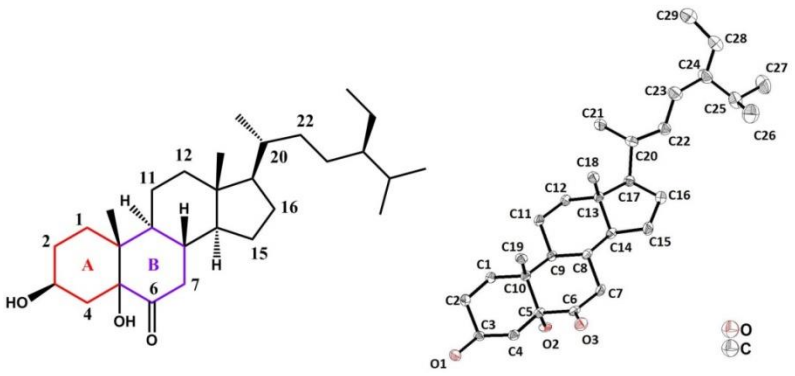


Fig. 2. Molecular structure and ORTEP diagram of compound 1

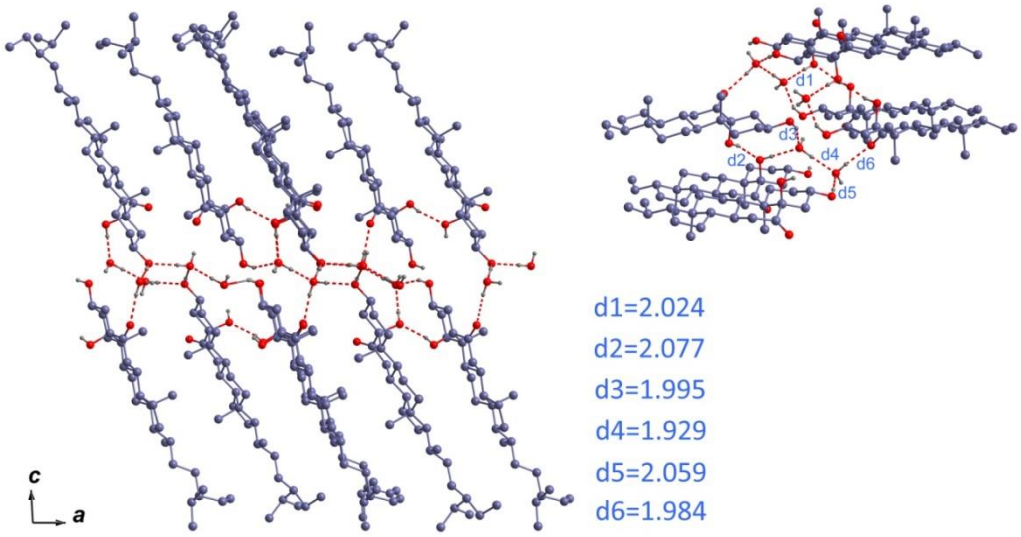


Fig. 3. Crystal cell and hydrogen bonds of compound 1

In this work, three compounds were isolated and confirmed from the leaves of *P. nagi*. Compound **1** is very rare compounds with the same skeleton as *β*-sitosterol and was isolated for the first time from the leaves of *P. nagi*. Its absolute configuration was confirmed using XRD. During isolation of compounds **1**, **2** and **3**, we used PTLC together with recrystallization methods. In addition, we checked the appearances of the crystals under microscope to distinguish one from another, and selected out one by one from the mixture. According to the reported results[5, 7], only *β*-sitosterol was isolated from the leaves of *P. nagi* grown in Yunan and Guangdong provinces. Maybe there are compounds **1** and **2** in the leaves of *P. nagi* grown in Yunan and Guangdong provinces (This assumption needs to be determined by comparing the chemical components planted in different provinces through studying the finger print of this plant later), but the authors did not isolate them, because these two compounds are so difficult to discover and isolate. But we obtained compounds **1** and **2** through selecting the crystals under microscope. This work provides an effective and worthy separation method for some compounds with the smaller differences of polarity.

It was reported that compound **3** could inhibit the proliferation of cancer cells and induce apoptosis[16, 17], indicating that this series of compounds are promising for *in vitro* anticancer. In current work, we tested compound **2** for its preliminary *in vitro* anticancer against gastric cancer, breast cancer (MCF-7), lung cancer (A549) and Hela cell lines. The results showed that compound **2** exhibited good to moderate inhibition against the four cancer cell lines with the inhibition of 89.16% ± 1.17, 97.02% ± 0.53, 47.20% ± 2.58 and 36.89% ± 1.22, respectively at the concentration of 1.4×10^{-2} M. We have improved the cell viability experiment *in vitro* and enriched the tested cancer cell lines. These results indicated that these series of sterols maybe have good anticancer activity. It means that we found the new anticancer agent in this plant medicine. Inspired by this wok, more compounds will be isolated and their anticancer activity will be evaluated for the development of anticancer drugs.

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