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Original Research Article

Determination of Chemical Constituents of the Marine Pulmonate Slug, *Paraoncidium reevesii*

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Abstract

Purpose: *To isolate and identify the chemical components of Paraoncidium reevesii.*
Methods: *Silica gel column chromatography was used to isolate the components from petroleum ether and ethyl acetate fractions of the acetone extract, and the structures of the compounds were derived from ^{1}H-nuclear magnetic resonance (^{1}H-NMR), ^{13}C-nuclear magnetic resonance (^{13}C-NMR) and mass spectrometry (MS) analyses and also with the aid of literature data for authenticated samples.*
Results: *Cholesterol (1), baconipyrone D (2), chimyl alcohol (3), batyl alcohol (4), α-monpalmitin (5), stearic acid (6), 3-indolecarboxylic acid (7), uracil (8), thymine (9), uridine (10), thymidine (11) were isolated from the marine slug (Paraoncidium reevesii).*
Conclusion: *All the isolated compounds are being reported here for Paraoncidium reevesii for the first time. The results provide base data for further study of the Onchidiidae as a Chinese traditional remedy for asthma and athlete's foot.*

Keywords: *Mollusk, Paraoncidium reevesii, Baconipyrone D, Batyl alcohol, Chimyl alcohol Onchidiidae, Marine Pulmonate, Slug, Asthma, Athlete's foot*

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INTRODUCTION

Marine eupulmonates of the family Onchidiidae are shell-less, slug-like molluscs mainly living on sheltered intertidal shores, throughout the world, except the polar regions [1]. They are usually oval in shape with a dorsally arched notum bearing warts and papillae; and there are glands which apparently secrete noxious fluids at the sides of the body. Most species are marine and live in the middle and upper intertidal zone, either in rocky, muddy and sandy habitats. They are also known to be important members of mangrove communities. About 6 species in 5 genera are known from China [2].

Members of the genus *Paraoncidium reevesii* are widespread in littoral areas south of Zhejiang Province, China, and feed on organic detritus and unicellular algae in the surface mud. A series of compounds have been reported from the family Onchidiidae including onchidal, ilikonapyrones (For example, ilikonapyrone), onchitriols (e.g., onchitriol I), peroniatriols, onchidione and onchidins(example, onchidin) [3,4]. Some of these compounds are reported to display significant cytotoxic, antiviral activities. In

continuation of our research on the chemistry of the family Onchidiidae, we sought to investigate the chemical components of *Paraoncidium reevesii* from the intertidal zone along the coast of Haimen island, Xiamen Fujian Province, China during the summer of 2013.

EXPERIMENTAL

General

Melting points were determined using a Reichert hot-stage microscope and are uncorrected. Nuclear magnetic resonance (NMR) spectra, viz ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz), were recorded on Bruker Avance 600 NMR spectrometer, Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard, and coupling constants are given in Hz. Both ESIMS and HRESIMS spectra were recorded on a Micromass Q-TOF microinstrument. HRFABMS data were obtained on an Agilent 6538 UHD and Accurate-Mass Q-TOF/MS. Thin layer chromatography (TLC) was performed using Merck precoated plates (Silica gel 60 F254) of 0.25 mm thickness. Silica gel (200–300 mesh, Haiyang, Qingdao, China) was employed for column chromatography and Sephadex LH-20 (Pharmacia) was used for gel filtration chromatography. High performance liquid chromatography (HPLC) purification was carried out on a Shimadzu apparatus equipped with an LC-10ADVP pump and an UV SPD-10AVP detector by using reverse-phase semi-preparative column (250 × 10 mm, 5 μm, Phenomenex, Kromasil C18).

Animal material

A total of 1072 specimens of *Paraoncidium reevesii* were collected in the intertidal zone along the coast of Haimen Island, Fujian Province, China, during the summer of 2013. Species were identified by Professor Shen Heding. The specimens were frozen immediately after collection. A voucher specimen (XM2013-1) was deposited in the Aquatic Science and Technology Museum (ASTM) of Shanghai Ocean University.

Extraction and isolation

Specimens of *Paraoncidium reevesii* were dissected to remove the intestines and cut into small pieces, and subsequently, extracted repeatedly with acetone at room temperature (each 1 L × 3) by using ultrasound vibration. The acetone extracts were pooled and concentrated *in vacuo*, after three times of extraction, the total extracts were combined and concentrated again *in vacuo*, and the resulting residue was extracted with EtOAc (500 ml × 3). The EtOAc-soluble portion (30.7 g) was fractionated by silica gel column chromatography (Merck Kieselgel 60 powder) eluted with light petroleum ether (PE with boiling point of 60-90 ^{o}C) with increasing amounts of EtOAc (100:0 ~ 0:100) to afford 12 fractions (Fr.1-Fr.26) on the basis of TLC (Thin Layer Chromatography) analysis. Fr.2 was recrystallized in PE to yield compound 6 (8.1 mg). Fr.7 was recrystallized in EtOAc to yield compound 1 (19.2 mg), 5 (13.7 mg). Fr.12 eluted with PE/EtOAc (4:6) and was further separated with reversed-phase HPLC [Supelco-Discovery 5 um C18, 25 cm × 10 mm, 60 % H_2O / MeOH], yielding compound 2 (13.4 mg). Repeated chromatography of Fr.17 with silica gel (PE-EtOAc 7:3) gave compounds 3 (11.9 mg), 4 (21.6 mg). Fr. 19 was further purified by Sephadex LH-20 ($CHCl_3$-MeOH 1:1) to yield compound 7 (7.2 mg). Fr. 22 was subjected to Sephadex LH-20 ($CHCl_3$-MeOH 1:1) and RP-18 eluted with MeOH-H_2O (0-100) to give 8 (6.7 mg), 9 (4.1 mg), 10 (3.6 mg), and 11 (5.3 mg). The structures of each compound was obtained based on data from ^{1}H-NMR, ^{13}C-NMR and MS analysis as well as literature data for authenticated samples.

RESULTS

Spectroscopic data

Cholesterol **(1)**: $C_{27}H_{46}O$, White needle crystal, mp 144-146 ^{o}C. ESI-MS *m/z*: 387 $[M+1]^{+}$. ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 5.30 (1H, d, *J* = 4.3Hz, H-6), 3.48 (1H, m, H-3), 1.01 (3H, s, H-19), 0.91 (3H, d, *J* = 6. 6Hz, H-21), 0. 87 (3H, d, *J* = 5.5Hz, H-26, H-27), 0.86 (3H, d, *J* = 5.5Hz, H-26, H-27), 0.64 (3H, s, H-18); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 140.9 (C-5), 121.8 (C-6), 71.8 (C-3), 56.9 (C-14), 56.3 (C-17), 50.2 (C-9), 42.4 (C-4,13), 39.9 (C-12), 39.7 (C-24), 37.4 (C-1), 36.6 (C-10), 36.3 (C-22), 36.0 (C-20), 32.0 (C-7), 32.0 (C-8), 31.7 (C- 2), 28.4 (C-16), 28.2 (C-25), 24.5 (C-15), 24.0 (C-23), 23.0 (C-27), 22.8 (C-26), 21.2 (C-11), 19.6 (C-19), 18.9 (C-21), 12.0 (C-18). It was identified as thymine by comparison with physical and spectral data from the literature [5].

Baconipyrone D **(2)**: $C_{28}H_{43}O_8$, oil. UV (CH_3OH): 260nm. ^{1}H-NMR (600 MHz, $CDCl_3$) δ : 5.46 (1H, dd, *J* = 3.5 Hz, H-6), 4.13 (1H, *J* = 6.9 Hz, H-3), 3.46 (1H, br), 3.37 (1H, d, *J* = 10.5 Hz), 2.86 (3H, m), 2.72 (1H, dq, *J* = 7.2 Hz), 2.54 (2H, m), 2.34 (1H, dq, *J* = 7.2 Hz), 2.19 (3H, s), 2.05 (3H, s), 1.95 (3H, s), 1.38(1H, d, *J* = 6.9 Hz), 1.22 (3H, d, *J* = 7.2 Hz), 1.09 (3H, d, *J* = 7.2 Hz), 1.02 (3H, d, *J* = 7.2 Hz), 1.01 (3H, t, *J* = 7.2 Hz), 0.89 (3H, t, *J*

= 7.2 Hz), 0.86 (3H, d, *J* = 6.8 Hz) ; ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 211.8 (s), 210.9 (s), 210.4 (s), 210.4 (s), 179.4 (s), 173.9 (s), 160.8 (s), 160.3 (s), 120.4 (s), 119.1 (s), 77.6 (d), 73.8 (d), 51.1(d), 48.6 (d), 47.2 (d), 45.8 (d), 41.1 (d), 35.1 (2t), 17.5 (q), 15.0 (q), 14.2 (q), 13.4 (q), 13.1 (q), 10.0 (q), 9.8 (q), 9.6 (q), 7.7 (q), 7.3 (q). It was identified as baconipyrone D by comparison with physical and spectral data from the literature [6].

Chimyl alcohol **(3)**: $C_{19}H_{43}O_3$, mp 63~ 65 °C. ESI-MS *m /z*:317(M+H)$^+$, ^{1}H-NMR (600 MHz, $CDCl_3$) δ : 3.86 (1H, m, H-3), 3.71 (1H,dd,*J* =3.5,11.5Hz, H-1), 3.65 (1H, dd, *J* = 5.5,11.5Hz, H-20), 3.55 (1H, m, H-3), 3.52 (1H, m, H-1), 3.46 (2H, m, H-1′), 1.58 (2H, m, H-2′), 1.26 (3OH, brs), 0.88 (3H, t, *J*=7.0Hz, H-18′); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 71.9 (C-1), 72.5 (C-2), 64.3 (C-3), 70.4 (C-1′), 31.9 (C-2′), 26.6 (C-3′), 29.4~29.7 (C-5′~C-15′), 22.7 (C-16′), 31.7 (C-17′), 14.1 (C-18′). It was identified as chimyl alcohol by comparison with physical and spectral data from the literature [7].

Batyl alcohol **(4)**: $C_{21}H_{44}O_3$, white crystalline, mp. 62~64 °C. ESI-MS *m/z*: 343 [M-H]$^-$. ^{1}H-NMR (600 MHz, $CDCl_3$) δ : 3.71 (1H, dd, *J* = 3.5,11.5Hz, H-1a), 3.65 (1H, dd, *J* = 5.5, 11.5Hz, H-1b), 3.86 (1H, m, H-2), 3.49 (4H, m, H-3, H-1′), 1.58 (2H, m, H-2′), 1.26 (30H, brs, H-3′~ H-17′), 0.88 (3H, t, *J* = 7.0Hz, H-18′); ^{13}C-NMR (150 MHz, $CDCl_3$) δ : 71.9 (C-1), 72.5 (C-2), 64.3 (C-3), 70.4 (C-1′), 31.9 (C-2′), 26.6 (C-3′), 29.1~29.7 (C-5′~ C-15′), 22.6 (C-16′), 31.8 (C-17′), 14.1 (C-18′). It was identified as batyl alcohol by comparison with physical and spectral data from the literature [5].

α-monpalmitin **(5)**: white flaky crystals, mp 70~72 °C. ESI-MS *m/z*: 331 [M]$^+$. ^{1}H-NMR (600 MHz,$CDCl_3$) δ : 4.20 (1H, dd, *J* = 11.5, 4.5 Hz, H-1′a), 4.15 (1H, dd, *J* = 11.5, 6.5 Hz, H-1′b), 3.93 (1H, m, H-2′), 3.69 (1H, dd, *J* = 11.5, 4.0 Hz, H-3′b), 3.60 (1H, dd, *J* = 11.5, 5.5 Hz, H-3a), 2.35 (2H, t, *J* = 7.5 Hz, H-2), 1.62 (2H, m, H-3), 1.26~1.30 (24H, m), 0.88 (3H,t, *J* = 7.0 Hz, H-16) ; ^{13}C -NMR (150 MHz, CDCl3) δ : 70.3 (C-1′), 65.2 (C-2′), 63.3 (C-3′), 174.4 (C-1), 34.2 (C-2), 31.9 (C-3), 29.7~29.3 (C-4~13), 24.9 (C-14), 22.7 (C-15), 14.1 (C-16). It was identified as α-monpalmitin by comparison with physical and spectral data from the literature [8].

Stearic acid **(6)**: $C_{18}H_{36}O_2$, white waxy solid, mp 71~73 °C. ^{1}H-NMR (600 MHz, $CDCl_3$) δ : 2.34 (2H, t, *J* = 7.5 Hz, H-2), 1.61 (2H, m, H-3), 1.29 (28H, m, H-4 ~ H-17), 0.87 (3H, t, *J* = 6.5 Hz, H-18) ; ^{13}C-NMR (150 MHz, $CDCl_3$) δ : 179.9 (C-1), 34.2 (C-2), 24.8 (C-3), 29.9 ~ 29.3 (C-4 ~ C-15), 32.2 (C-16), 22.9 (C-17), 14.1 (C-18). It was identified as stearic acid by comparison with physical and spectral data from the literature [9].

3-indolecarboxylic acid **(7)**: $C_9H_7NO_2$, pale yellow powder. ^{1}H-NMR (600 MHz, CD_3OD) δ : 7.95 (1H, s, H-3), 7.46 (1H, s, H-5), 7.21 (2H, m, H-6,7), 8.09 (1H, d, *J* = 7.5 Hz, H-8); ^{13}C-NMR （150 MHz, CD_3OD） δ : 168.3 (C-1), 138.3 (C-9), 133.5 (C-4), 127.6 (C-2), 123.3 (C-7), 122.6 (C-5), 122.1 (C-6), 112.9 (C-8), 109.1(C-3). It was identified as 3-indolecarboxylic acid by comparison with the literature physical and spectral data [10].

Uracil **(8)**: $C_4H_4N_2O_2$, pale yellow needle crystal, mp 333~335 °C. ESI-MS *m/z*: 113(M+H)$^+$。 ^{1}H-NMR (600 MHz, DMSO-d_6) δ : 10.95 (1H, s, N-H), 10.81 (1H, s, N-H), 7.51(1H, t, *J* = 7.6 Hz, H-6), 5.49 (1H, d, *J* = 7.6 Hz, H-5); ^{13}C-NMR (150 MHz, DMSO-d_6) δ : 164.5 (C-1), 151.6 (C-2), 142.9 (C-6), 102.1 (C-5). It was identified as uracil by comparison with physical and spectral data from the literature [11].

Thymine **(9)**: $C_5H_6N_2O_2$, pale yellow needle crystal, mp 333~335 °C. ESI-MS *m /z*:126 (M)$^+$。 ^{1}H-NMR (600 MHz, DMSO-d_6) δ : 10.95 (1H, s, N-H), 10.51 (1H, s, N-H), 7.27 (1H, s, H-6), 1.72(3H, s, 5-CH3); ^{13}C-NMR (150 MHz, DMSO-d_6) δ : 166.5 (C-4), 151.6 (C-2), 142.9 (C-6), 108.1 (C-5), 11.9 (5-CH3). It was identified as thymine by comparison with physical and spectral data from the literature [11].

Uridine **(10)**: $C_9H_{12}N_2O_6$, white powder, mp 220~221 °C. ESI-MS *m /z*: 267 (M)$^+$. ^{1}H-NMR (600 MHz, DMSO-d_6) δ : 7.99 (1H, d, *J* = 8.4 Hz, H-6), 5.69 (1H, d, *J* = 8.4 Hz, H-5), 5.89 (1H, d, *J* =4.8 Hz, H-1′), 4.16 (1H, m, H-2′), 4.01 (1H, ddd, *J* =4.8, 3.4, 2.7Hz, H-4′), 4.14 (1H, t, *J* =4.8, 4.8 Hz, H-3′), 3.84 (1H, dd, *J* =12, 2.7 Hz, H-5′a), 3.72 (1H, dd, *J* =12, 3.4 Hz, H-5′b); ^{13}C-NMR (150 MHz, DMSO-d_6) δ : 166.2 (C-4), 152.6 (C-2), 142.9 (C-6), 102.6 (C-5), 86.4 (C-4′), 75.8 (C-2′), 71.3 (C-3′), 62.3 (C-5′). It was identified as uridine by comparison with physical and spectral data from the literature [11].

Thymidine **(11)**: $C_{10}H_{14}N_2O_5$, white powder, mp 184~185 °C. ESI-MS *m /z*: 259(M+H)$^+$. ^{1}H-NMR (600 MHz, DMSO-d_6) δ : 7.75 (1H, s, H-6), 5.77 (1H, d, *J* =5.1 Hz, H-1′), 4.10 (1H, m, H-2′), 4.00 (1H, m, H-3′), 3.89 (1H, dd, *J* =12.1, 2.8 Hz, H-4′), 3.54 (2H, m, H-5′); ^{13}C-NMR (150 MHz, DMSO-d_6) δ : 166.2 (C-4), 151.6 (C-2), 142.9 (C-6), 102.6 (C-5), 86.4 (C-4′), 75.8 (C-2′), 71.2 (C-3′), 62.3 (C-5′). It was identified as thymidine by comparison with physical and spectral data from the literature [11].

DISCUSSION

In the present study, various compounds have been isolated from the marine pulmonate slug *Paraoncidium reevesii*. To our knowledge, this is the first report of the presence of compounds 1–11 in this genus *Paraoncidium reevesii*, furthermore, all these compounds were reported for the first time from the family Onchidiidae.

Compounds (1, 3, 4 and 6) from *Paraoncidium reevesii* are very common secondary metabolites. Cholesterol (1) and stearic acid (6) were isolated from the pulmonate *Siphonaria japonica*. Baconipyrone D (2) was previously reported from the pulmonate *Siphonaria baconi* and *Siphonaria japonica*, which indicates a close relationship between Onchidiidae and *Siphonaria*. Recent molecular data also suggested that *Siphonaria* is closely related to Onchidiidae [2]. Baconipyrone D has been reported to display significant cytotoxic and antiviral activities. It is intensely bitter [12] which may be the reason Chinese people do not eat the onchidiid of *Paraoncidium reevesii*. Chimyl alcohol (3), batyl alcohol (4) Uracil (8), thymine (9) and thymidine (11) have been reported from the Opisthobranchia *Notarchus leachiifreeri*. Uridine (10) was previously reported from red alga *Gymnogongrus flabelliformis Harv*.

CONCLUSION

The eleven compounds isolated from the marine pulmonate slug *Paraoncidium reevesii*. Baconipyrone D (2) (polypropionate) is intensely bitter which is probably the reason Chinese people often do not eat *Paraoncidium reevesii*. These compounds from this source are reported here for the first time. The compounds have potential applications and therefore require further studies in view of the use of the slug as a traditional Chinese remedy for asthma and athlete's foot.

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