Steroids 150 (2019) 108424

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Three new C23 steroids from the leaves and stems of *Nicandra physaloides*

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ARTICLE INFO

Keywords:
Nicandra physaloides
Solanaceae
Withanolide
C23 steroids

ABSTRACT

Nicandra physaloides is a medicinal and edible plant and has been used as traditionally herbal medicine to treat various diseases in folk. Its characteristic withanolides, a kind of ergostane-type steroids, are reported to display plentiful biological activities that many explain the effect of *N. physaloides* to some extent. Thus, to further find bioactive steroids, the stems and leaves of *N. physaloides* were investigated and three new C23 steroids, nic-physatones I-J (**1**–**2**), and nic-physatone S (**3**), together with a known C25 steroid, nic 17 (**4**), were isolated. Their structures were elucidated by extensive 1D NMR and 2D NMR (HSQC, HMBC, ^{1}H-^{1}H COSY, and ROESY), UV and MS analyses. Compounds **1**–**3** possess a rare C23 steroid skeleton. Among them, compound **3** represented the first example of a C23 steroid featuring a benzene ring (D ring). The isolated compounds showed no cytotoxic activity.

1. Introduction

Nicandra physaloides (Solanaceae) is indigenous to Peruvian and widely cultured in Yunnan and Guangxi provinces, China. The seeds of *N. physaloides* are rich in edible pectin ingredients, which can be made into a cold drink or jelly in folk, while its fruits are a good source of antioxidants, such as lycopene, anthocyanin, chlorophyll and phenols [1]. Additionally, the whole plant of *N. physaloides* has been used as herbal medicine for the treatment of sedation, as an expectorant, for fever relieving and for detoxification due to flavonoids and withanloides [2]. Previous phytochemical investigation showed that it contains a series of compounds including steroids, flavonoids, alkaloids, aromatic glycosides and fatty acids [3]. Among them, withanolides [1,4–8] are the most peculiar constituent: they are C28 ergostane-type steroids with a 22 → 26 *δ*-lactone or 22,26-epoxy side chain [5]. Interestingly, nicandrenone was the first isolated withanolides characterized by a significant insect repellent activity [9], which attracted much attention from chemists [10,11]. Subsequently, many structurally diverse steroids, such as nicphysatone A with a 13,17-*seco*-17,26,22,26-diepoxy skeleton, nicphysatone B featuring a 13,17-*seco*-18,17-cyclo structure, and nic 17 belonging to a C25 steroid, were identified [12,13]. Moreover, pharmacological research found that withanolides displayed various biological activities, including antitumor [14–16], antibacterial [17–19], anti-inflammatory [20,21], and immunomodulatory [22,23] activities.

Thus, in this study, we carried out a detailed phytochemical investigation on the aerial parts (stems and leaves) of *N. physaloides* for gaining structurally diverse and biologically significant steroids. Our efforts led to the isolation of three new C23 steroids, nic-physatones I-J (**1**–**2**), nic-physatone S (**3**), as well as a known C25 steroid, nic 17 (**4**) [24] (Fig. 1). The isolation, structural elucidation and cytotoxicities of these steroids were reported as well.

2. Experimental

2.1. General

Optical rotations were obtained with a Jasco P-1020 polarimeter. UV spectra were run on a UV 210A spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured on Bruker AV-400 and DRX-500 instruments (Bruker, Zurich, Switzerland) with TMS (tetramethylsilane) as the internal standard. ESIMS and HRESIMS data were recorded on an API QSTAR Pulsar spectrometer and infrared spectra were recorded on a Bruker Tensor-27 instrument by using KBr pellets. Semi-preparative HPLC was performed on an Agilent 1100 liquid chromatography with an Agilent ZORBAX SB-C18 (5 μm, 9.4 × 250 mm) column. TLC was performed on precoated TLC plates (200–250 μM thickness, F254 Silical gel 60, Qingdao Marine Chemical, Inc.) with compounds visualized by

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https://doi.org/10.1016/j.steroids.2019.06.001
Received 17 December 2018; Received in revised form 14 May 2019; Accepted 3 June 2019
Available online 13 June 2019
0039-128X/

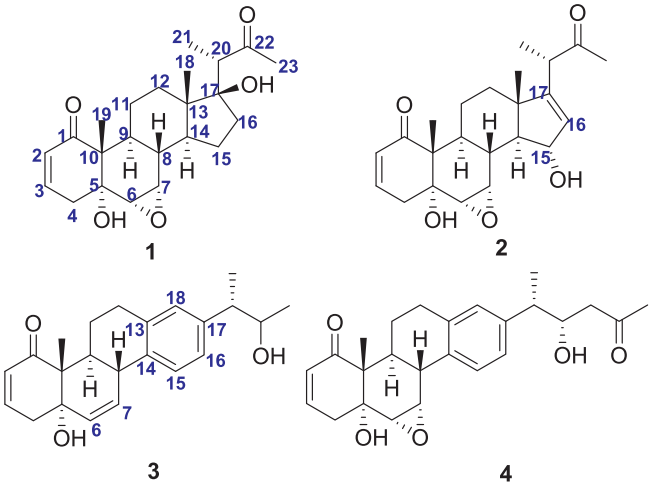

Fig. 1. Structures of compounds **1**–**4** from *N. physaloides*.

spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until dry. Silical gel (200–300) mesh, (Qingdao Marine Chemical, Inc.), Lichroprep RP-18 (40–63 μm, Merck) and Sephadex LH-20 (GE Healthcare, Piscataway, NJ, USA) were used for column chromatography.

2.2. Plant material

The dried aerial parts (stems and leaves) of *Nicandra physaloides* were collected in June 2013 from Baoshan of Yunnan province, People's Republic of China. The plant material was identified by Prof. Yang Chong-Ren. A voucher specimen (ZDSQ20130601) was stored at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy Sciences.

2.3. Extraction and isolation

The aerial parts (stems and leaves) (50 kg) of *N. physaloides* were air-dried and cut into small pieces, then extracted three times with methanol under reflux. The solvent was concentrated under vacuum to obtain the MeOH extract (3 kg), which was suspended in H_2O and extracted with petroleum ether (PE) and ethyl acetate (EA), respectively. On the basis of a sulfuric acid chromogenic reaction of PE and EA parts, steroids were mainly present in EA part. Thus, we continued to treat the EA part for steroids.

The EA extract (200 g) was chromatographed on silica gel chromatography ($CHCl_3$/MeOH, 150:1, 80:1, 50:1, 20:1, 10:1, 5:1) to afford six main fractions (Fr.1–Fr.6). Fr.2 (6.1 g) was subjected to silica gel column chromatography (CC) and eluted with a gradient system of petroleum ether/acetone (10:1, 8:1, 5:1, 3:1, 1:1) to yield seven subfractions (Fr.2–1–Fr.2–7) by TLC. Fr.2–1 (200.0 mg) was applied to Sephadex LH-20 using MeOH, silica gel CC using $CHCl_3$/MeOH (250:1, 100:1, 50:1) and P-TLC ($CHCl_3$/isopropanol 25:1) to produce compound **1** (3.0 mg). Fr.2–2 (279.2 mg) was successively isolated by Sephadex LH-20 ($CHCl_3$/MeOH 1:1), RP-18 column (MeOH/H_2O 40:60 → 80:20) and P-TLC ($CHCl_3$/MeOH) to yield compound **3** (3.0 mg). Fr.2–6 (215.5 mg) was isolated by silica gel ($CHCl_3$/MeOH 80:1, 50:1) to yield compound **4** (32.0 mg). Fr.3 (26.1 g) was fractionated by reversed-phase silica gel chromatography (MeOH/H_2O 50:50 → 100:0) to obtain seven subfractions (Fr.3–1–Fr.3–7). Fr.3–1 (2.9 g) was repeatedly isolated by Sephadex LH-20 (MeOH), silica gel CC ($CHCl_3$/isopropanol 80:1 and 50:1), P-TLC (PE/acetone), and semi-preparative HPLC (MeOH/H_2O, 50:50, flow rate 3.0 mL/min) to give compound **2** (3.0 mg, t_R = 18.3 min).

2.4. Nic-physatone I (1)

Yellow amorphous powder; $[\alpha]_D^{22}$ −16.2° (c 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 240 (3.52) nm; IR (KBr) ν_{max} 3452, 2928, 1684, 1630, 1461, 1377, 1290, 1177, 1080, 902 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 411 $[M+Na]^+$; HRESIMS, *m/z* at 411.2147 (calcd for $C_{23}H_{32}NaO_5$ $[M+Na]^+$, 411.2147).

Table 1
1D NMR spectroscopic data (600 MHz/150 MHz; $CDCl_3$) of compounds **1**–**3**.

position	**1**[a]		**2**		**3**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		206.1 s		202.7 s		203.5 s
2	5.73 dd (2.4, 10.2)	129.5 d	5.84 dd (1.9, 10.1)	129.0 d	5.94 dd (2.3, 10.1)	129.0 d
3	6.62 ddd (2.2, 5.2, 10.1)	142.2 d	6.59 dd (3.7, 9.3)	139.6 d	6.63 ddd (2.0, 5.1, 10.0)	140.7 d
4	2.43 dd (5.2, 19.1), 2.75 m	38.0 t	2.55 dd (3.6, 18.6), 2.69 d (18.6)	36.7 t	2.46 dd (5.1, 18.9), 2.68 m	36.4 t
5		74.9 s		73.4 s		74.4 s
6	3.01 d (3.9)	57.1 d	3.11 s	56.9 d	5.81 dd (2.8, 10.0)	130.2 d
7	3.25 dd (2.1, 3.6)	57.2 d	3.68 s	57.5 d	6.48 d (10.0)	130.7 d
8	1.79 m	37.4 d	2.09 m	33.9 d	3.23 d (10.9)	39.5 d
9	1.64 m	36.5 d	1.68 m	36.2 d	2.22 td (3.8, 10.8)	36.9 d
10		52.4 s		51.0 s		51.7 s
11	1.29 m, 2.70 m	22.7 t	1.37 m, 2.91 m	21.3 t	1.67 m, 2.89 m	24.3 t
12	1.56 m, 1.87 m	33.5 t	1.52 td (3.5, 12.5), 1.75 m	34.5 t	2.87 m, 3.03 m	29.7 t
13		49.5 s		47.7 s		137.4 s
14	2.15 m	47.0 d	1.59 m	61.5 d		136.9 s
15	1.38 m, 1.83 m	24.1 t	4.66 brs	76.8 d	7.23 d (8.4)	123.7 d
16	1.58 m, 1.91 m	38.4 t	5.50 s	130.1 d	7.06 dd (8.4, 2.9)	125.0 d
17		84.0 s		154.8 s		142.0 s
18	0.84 s	15.3 q	0.94 s	19.3 q	6.98 d (2.9)	128.5 d
19	1.20 s	15.3 q	1.22 s	14.9 q	1.27 s	14.3 q
20	2.91 q (7.1)	53.3 d	3.25 q (6.8)	46.5 d	2.70 d (6.9)	46.6 d
21	1.19 d (7.2)	12.5 q	1.26 d (6.9)	16.6 q	1.30 d (7.0)	15.9 q
22		217.2 s		208.8 s	3.86 m	72.3 d
23	2.21 s	29.9 q	2.14 s	27.5 q	1.09 d (6.3)	21.0 q

[a] Measured in CD_3OD.

2.5. Nic-physatone J (2)

White amorphous powder; $[\alpha]_D^{18}$ + 61.1° (c 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 240 (3.31) nm; IR (KBr) ν_{max} 3439, 2925, 2854, 1710, 1685, 1630, 1376, 1261, 1164, 1079, 908 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 409 $[M+Na]^+$; HRESIMS, *m/z* at 409.1995 (calcd for $C_{23}H_{30}NaO_5$ $[M+Na]^+$, 409.1991).

2.6. Nic-physatone s (3)

Yellow amorphous powder; $[\alpha]_D^{23}$ −10.9° (c 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 239 (3.68), 265 (3.61) nm; IR (KBr) ν_{max} 3433, 2926, 1680, 1454, 1379, 1268, 1206, 1091 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 375 $[M+Na]^+$; HRESIMS, *m/z* at 375.1929 (calcd for $C_{23}H_{28}NaO_3$ $[M+Na]^+$, 375.1936).

2.7. Cytotoxic activity assay

The cytotoxic activities of compounds **1**–**4** were evaluated against five human cancer cell lines: HL-60 (human leukemia cells), SMMC-7721 (human hepatoma cells), A-549 (human lung cancer cells), MCF-7 (human breast cancer cells), and SW480 (human colon cancer cells), which were acquired from ATCC (Manassas, VA, USA) and were cultured in RPMI-1640 or DMEM medium (Hy-clone, Logan, UT, USA) by means of 4-{5-[3-(carboxymethoxy)phenyl]-2-(4,5-dimethyl-1,3-thiazol-2-yl)-2H-tetrazol-3-ium-3-yl}benzene-1-sulfonate (MTS) assay, an analogues of MTT. Briefly, cells in RPMI-1640 or DMEM medium supplemented with 10% fetal bovine serum (FBS, Hyclone) were seeded into a 96-well cell culture plate in the presence of various concentrations of test compounds at 37 °C in a 5% CO_2 humidified incubator for 48 h. Reduced MTS crystals were dissolved in DMSO, and the optical density (OD) was measured at 490 nm in a 96-well microtiter plate reader (Bio-Rad 680) to determine cell growth inhibition. Cisplatin (MW 300) and paclitaxel were included as a positive control. The IC_{50} value of each compound was calculated by Reed and Muench's method [25].

3. Results and discussion

The methanol extracts of the aerial parts (stems and leaves) of *N. physaloides* were partitioned into petroleum ether and ethyl acetate fractions. The ethyl acetate fraction was repeatedly subjected to silica gel, Sephadex LH-20, RP-18 column, P-TLC and semi-preparative HPLC to yield three new C23 steroids (**1**–**3**), and a known C25 steroid (**4**).

Compound **1** was obtained as a yellow amorphous powder. It exhibited a $[M+Na]^+$ ion at *m/z* 411.2147 (calcd 411.2147) in the HRESIMS, compatible with the molecular formula of $C_{23}H_{32}NaO_5$. The IR spectrum showed characteristic absorption for an α,β-unsaturated carbonyl group (1684 cm^{-1}). The ^{13}C NMR and DEPT (Table 1) spectra showed 23 carbon signals, including four methyls, five methylenes, eight methines, and six quaternary carbons. Detailed comparison of 1D NMR data of **1** with those of nic-physatone B [13] showed that they have the same skeleton, except for the replacement of the oxymethine at C-12 and methine at C-17 in nicphysatone B by a methylene (δ_C 33.5) and an oxygenated quaternary carbon (δ_C 84.0) in **1**, respectively, which was confirmed by the HMBC correlations (Fig. 2) of H_3-21 (δ_H 1.19) with C-17 (δ_C 84.0), C-20 (δ_C 53.3), C-22 (δ_C 217.2), of H_3-23 (δ_H 2.21) with C-20 (δ_C 53.3), C-22 (δ_C 217.2), of H_3-18 with C-12 (δ_C 33.5), C-13 and C-14, together with ^1H-^1H COSY of H-9/H-11/H-12. In the ROESY spectrum, the correlation of H-20/H-14 (Fig. 2) indicated that 17-OH was β-orientated. Therefore, compound **1** was identified as nic-physatone I.

Compound **2** was isolated as a white amorphous powder. Its molecular formula, $C_{23}H_{30}NaO_5$, was determined by HRESIMS (*m/z* 409.1995, $[M+Na]^+$, calcd 409.1991). In the IR spectrum, the absorption band at 1685 cm^{-1} indicated the presence of an α,β-unsaturated carbonyl group. The ^{13}C NMR and DEPT (Table 1) spectra also showed 23 carbon resonances, which were similar to those of **1**. However, the signals at δ_C 130.1 (CH), δ_C 154.8 (C), and δ_C 76.8 suggested that a double bond and an oxygenated methine were present in **2**. Furthermore, the HMBC correlations (Fig. 3) of H_3-18 (δ_H 0.94), H_3-21 (δ_H 1.26) with C-17 (δ_C 154.8), and a proton signal at δ_H 4.66 attributing to H-15 with C-16 (δ_C 130.1), C-17(δ_C 154.8) illustrated that the double bond and the oxymethine were located at C-16, C-17 and C-15, respectively. The 15α-OH was established by the ROSEY correlation (Fig. 3) of H_3-18/H-15. Thus, Compound **2** was assigned as nic-physatone J.

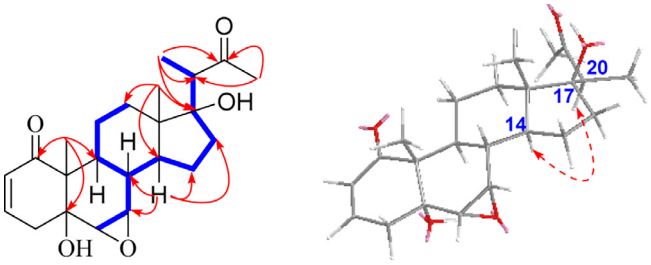

Fig. 2. Selected HMBC (H → C), ^1H-^1H COSY (▬) and ROESY (↔) correlations of **1**.

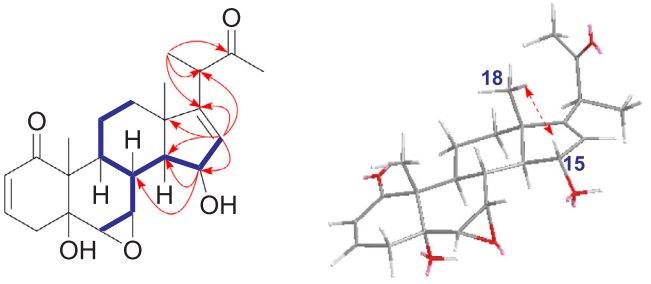

Fig. 3. Selected HMBC (H → C), ^1H-^1H COSY (▬) and ROESY (↔) correlations of **2**.

Compound **3** was isolated as a yellow amorphous powder. The molecular formula, $C_{23}H_{28}NaO_3$, was deduced from the HRESIMS (*m/z* 375.1929, $[M+Na]^+$, calcd 375.1936). The IR spectrum showed the presence of α,β-unsaturated carbonyl group (1680 cm^{-1}). The ^1H NMR spectrum of **1** showed one singlet methyl, two doublet methyls, seven aromatic or olefinic protons, and one oxygenated methine proton. Its ^{13}C NMR spectrum displayed 23 carbon resonances. Among them, the characteristic signals at δ_C 137.4, 136.9, 123.7 (δ_H 7.23, d, J = 8.4 Hz), 125.0 (δ_H 7.06, dd, J = 8.4 and 2.9 Hz), 142.0, and 128.5 (δ_H 6.98, d, J = 2.9 Hz) were representative of a trisubstituted benzene ring with the ABX spin system. Aforementioned data indicated that the structure of **3** resembles that of **4**, except for the presence of a double bond between C-6 and C-7, and the degradation of C-24 and C-25. Furthermore, a wide range of ^1H-^1H COSY correlations (Fig. 4) of H-6/H-7/H-8/H-9/H-11/H-12 and of H-21/H-20/H-22/H-23, along with the HMBC correlations (Fig. 4) of H_3-21 with C-20, C-17, C-22, of H-20 with C-22 and C-23, of H_3-23 with C-20 and C-22, and of H-6 and H-7 with C-5 and C-8 were observed in the 2D NMR spectra, which confirmed above deduction. Finally, compound **3** was identified as nic-physatone S.

All isolated compounds were evaluated for their cytotoxic activities

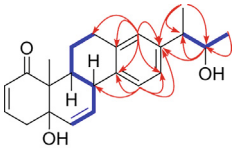

Fig. 4. Key HMBC (H → C), and ^1H-^1H COSY (▬) correlations of **3**.

against five human tumor cancer cell lines, HL-60, SMMC-7721, A-549, MCF-7, and S2480. The result showed that compounds **1–4** had no cytotoxicities with $IC_{50} > 40\,\mu M$.

4. Conclusion

In conclusion, three new C23 steroids (**1–3**) and a known C25 steroid (**4**) were isolated from the aerial parts of *N. physaloides*. Compound **3** represented the first example of a C23 steroid featuring a benzene ring (D ring). Many structurally diverse withanolides possessing an ether ring or a lactone ring in the side chain and steroids with a five-membered carbon ring or a benzene ring in the D ring have been identified [12,13]. Moreover, previous research showed that withanolides had cytotoxic activities and summarized that 2-en-1-one and 5*β*,6*β*-epoxy units in rings A and B played an key role in the cytotoxicity of withanolides [26]. However, in our study, compounds **1** and **2** with the previously mentioned key units displayed no cytotoxicity, suggesting that the significant bioactive functionalities could be target C28 skeleton. In view of the insecticidal activity of nicandrenone with an aromatic D-ring [9], compounds **3** and **4** will be tested for their insecticidal effects in the future study.

Declaration of Competing Interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This study was supported financially by the Foundational Project of Yunnan Key Laboratory of Tobacco Chemistry, R&D Center of China Tobacco Yunnan Industrial Co., Ltd. (KCFZ-2017-1096), Foundation of State Key Laboratory of Phytochemistry and Plant Resources in West China (P2015-ZZ09), and the Science and Technology Project of Yunnan Province (Preparation and application of plateau characteristic plant based functional materials and fragrance for simulated carbonation).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2019.06.001.

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