Isolation and characterization of spirocaesalmin, a novel rearranged vouacapane diterpenoid from *Caesalpinia minax* Hance

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Spirocaesalmin, a novel rearranged vouacapane diterpenoid that exhibits significant activity against respiratory syncytial virus, possesses a new carbon skeleton with a spiro-CD ring system.

Introduction

Respiratory syncytial virus (RSV) is a major pathogen of some respiratory infections.[1] The nucleoside analog ribavirin and immune serum globulin have been used for treating RSV infections.[2] Some strains of RSV have developed resistance to currently administered therapeutic agents and these agents have potential side effects, therefore a sustained effort for the development of new and more effective antiviral agents has been undertaken. Searching for natural products as antiviral agents against RSV has thus attracted considerable attention in recent years.[3]

Caesalpinia minax Hance, a member of the *Caesalpinia* genus, is a prickly shrub growing in the tropics and subtropics. The seeds of this plant, which is called 'ku-shi-lian', have long been used as Chinese folk medicine for the treatment of common cold, fever and dysentery.[4] In the course of our ongoing search for antiviral agents from natural sources, the chloroform fraction of the ethanol (95%) extract of the seeds was found to show *in vitro* anti-RSV activity, and a subsequent bioassay-guided study led to the isolation of a novel rearranged vouacapane diterpenoid possessing a new carbon skeleton, now designated spirocaesalmin (Fig. 1). This paper describes its isolation, structure elucidation and anti-RSV activity.

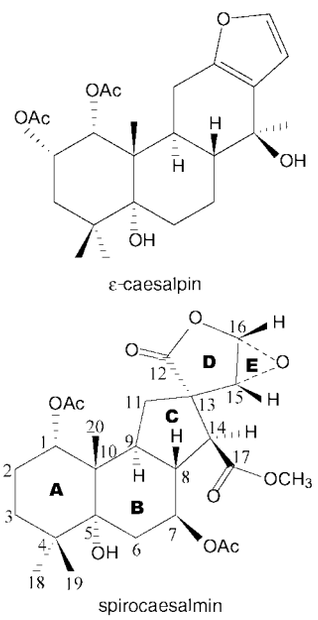

Fig. 1 Chemical formulae of ε-caesalpin and spirocaesalmin.

Results and discussion

Spirocaesalmin was isolated from the chloroform fraction of the 95% ethanol extract of the seeds followed by column chromatography and preparative TLC. The molecular formula of spirocaesalmin was assigned as $C_{25}H_{34}O_{10}$ based on high resolution liquid secondary ion mass spectrometry (HRLSIMS experimental $[MH]^+$ 495.2216, calculated 495.2220).

A close inspection of the ^{1}H NMR spectrum (Table 1) revealed the presence of six methyl groups including three tertiary methyl groups at δ_H 1.06 (3H, s, H-18), 1.12 (3H, s, H-19) and 1.22 (3H, s, H-20), two acetoxy groups at δ_H 1.98 (3H, s, OAc-1) and 2.08 (3H, s, OAc-7) and one methoxy group at δ_H 3.72 (3H, s, OCH_3-25), two low-field protons attached to carbon atoms bearing an oxygen function at δ_H 5.22 (1H, dt, J = 5.2, 15.9 Hz, H-7) and δ_H 4.82 (1H, m, H-1), and two adjacent epoxide protons at δ_H 5.81 (1H, d, J = 4.0 Hz, H-16) and δ_H 3.84 (1H, d, J = 4.0 Hz, H-15). The nature of all the 25 carbon atoms was revealed from ^{13}C-NMR and DEPT measurements, and this showed that spirocaesalmin has 4 carbonyls, 6 methyls, 4 methylenes, 7 methines and 4 quaternary carbon atoms. The low-field region of the ^{13}C-NMR spectrum contains signals for four carbonyls including one lactonic carbonyl δ_C 178.78 (s, C-12), one methoxycarbonyl δ_C 174.24 (s, C-17) and two acetate carbonyls δ_C 171.72 (s, C-23), 170.56 (s, C-21), three carbon atoms bearing oxygen functions δ_C 74.54 (d, C-1), 80.27 (s, C-5), 75.63 (d, C-7) and two epoxide carbon atoms δ_C 72.68 (d, C-15) and δ_C 106.49 (d, C-16). The more downfield nature of H-16 and C-16 relative to H-15 and C-15 indicates that C-16 is connected to two oxygen atoms to form a hemiketal structure. The full assignments and connectivities were determined by ^{1}H–^{1}H COSY, HMQC and HMBC spectra. The ^{1}H–^{1}H COSY spectrum establishes spin systems involving H-6, H-7, H-8, H-9 and H_2-11, and H-15 and H-16. The HMBC spectrum shows the following key correlations: H-1 ⟶ C-3, C-5, C-10, C-20 and C-21; H7 ⟶ C-6, C-8 and C-23; H14 ⟶ C8, C-9, C-11, C-12, C-13, C-15 and C-17; H15 ⟶ C-11, C-12, C-13, C-14 and C-16, and H16 ⟶ C-12, C-13 and C-15.

The molecular skeleton and relative stereochemistry of spirocaesalmin (Fig. 2) were established by single crystal X-ray analysis. The cyclohexane rings A and B adopt the normal chair conformation with mean torsion angles of 52.7 and 57.1°, respectively, and these values are comparable with the value of 56° found in cyclohexane. Ring C exists as a distorted envelope

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Table 1 ^{1}H-NMR (300 MHz, CD_3OD) and ^{13}C-NMR (75 MHz, CD_3OD) data

Position	δ_H (multiplicity, *J*/Hz)	δ_C DEPT
1	4.82 (1H, m)	74.54 d
2	1.61 (1H, m, H-2$^{\alpha}$) 1.90 (1H, m, H-2$^{\beta}$)	23.52 t
3	1.19 (1H, m, H-3$^{\alpha}$) 1.66 (1H, m, H-3$^{\beta}$)	34.68 t
4		38.79 s
5		80.27 s
6	1.74 (2H, m)	33.56 t
7	5.22 (1H, dt 5.2, 15.9)	75.63 d
8	2.13 (1H, m)	43.10 d
9	2.44 (1H, m)	34.92 d
10		46.81 s
11	2.93 (1H, dd 6.0, 13.8, H-11$^{\alpha}$) 3.63 (1H, dd 6.3, 13.8, H-11$^{\beta}$)	30.28 t
12		178.78 s
13		48.90 s
14	2.85 (1H, d 10.8)	48.52 d
15	3.84 (1H, d 4.0)	72.68 d
16	5.81 (1H, d 4.0)	106.49 d
17		174.24 s
18	1.06 (3H, s)	28.61 q
19	1.12 (3H, s)	24.98 q
20	1.22 (3H, s)	17.34 q
1-OAc	1.98 (3H, s)	170.56 s, 21.77 q
7-OAc	2.08 (3H, s)	171.72 s, 22.01 q
14-$COOCH_3$	3.72 (3H s)	57.35 q

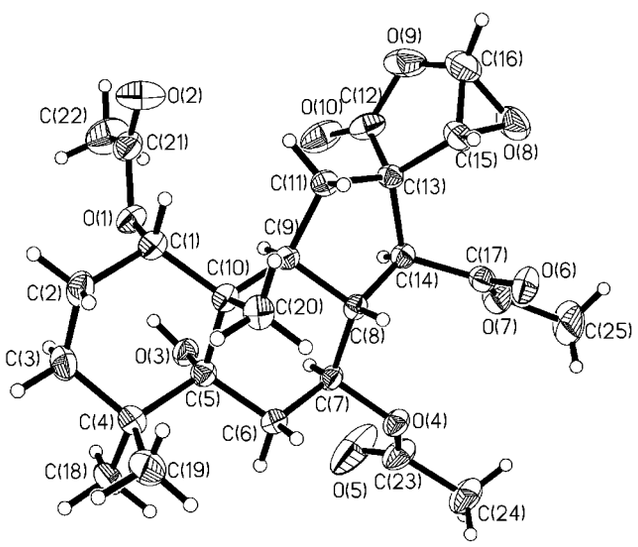

Fig. 2 Molecular structure of spirocaesalmin with atom labeling scheme. The C and O atoms are drawn as 30% thermal ellipsoids. Selected bond lengths (Å) and angles (°): C(15)–C(16) 1.440(5), O(8)–C(15) 1.456(4), O(8)–C(16) 1.399(5), C(16)–O(8)–C(15) 60.6(2), C(16)–C(15)–O(8) 57.8(2), O(8)–C(16)–C(15) 61.7(2).

with C-9 displayed 0.680 Å out of the best plane formed by C-8, C-11, C-13 and C-14. Lactone ring D is nearly planar with a mean deviation of 0.029 Å. Ring C and ring D are orthogonal as the dihedral angle between them is 90.0°. The epoxide ring E, having a short C–C bond length of 1.440 Å, makes a dihedral angle of 104.2° with ring D. The only hydrogen bond in the crystal structure is intramolecular, which involves O-3, H-3a and O-1, with an O-3 · · · O-1 distance of 2.761 Å.

Normally, vouacapane diterpenoids are characterized by a molecular skeleton constructed from the fusion of three cyclohexane rings and a furan ring. Diterpenoids of this kind are mostly distributed in the genera *Caesalpinia*[5–14] and *Pterodon*,[15–16] and differentiated by the presence of a C-5 hydroxy group in the former genus. All vouacapane diterpenoids isolated so far from *Caesalpinia* related species share the same carbon skeleton, and the β-oriented methyl group at C-10 and an α-oriented hydroxy group at C-5 are both well established; for example, the absolute configurations of ε-caesalpin[17] and caesaldekarin A[18] were established by X-ray analysis using the anomalous dispersion method (Fig. 1) and comparison of the NMR data of MTPA derivatives, respectively. Spirocaesalmin has an optical rotation of $[\alpha]_D^{20}$ + 15.8 and crystallizes in a non-centrosymmetric space group ($P2_12_12_1$), which indicates that it exists as a single enantiomer. Considering the biogenetic relationship in the vouacapane diterpenoids, spirocaesalmin can be inferred to have a similar absolute configuration to ε-caesalpin[17] as shown in Fig. 1. The A/B *trans* and B/C *trans* ring junctions are also the same as in other vouacapane diterpenoids. The present compound is a novel rearranged vouacapane diterpenoid with a spiro-heterocyclic skeleton which is considered to be reconstructed from ordinary vouacapane diterpenoid through oxidation of the two double bonds in the furan ring and migration of the C-11–C-12 bond to the C-13 position.

Spirocaesalmin has been found to exhibit significant activity against respiratory syncytial virus (IC_{50} = 19.5 ± 1.5 μg mL^{-1}, TC_{50} = 126.9 ± 2.0 μg mL^{-1} and SI = 6.5) in cell culture, and the corresponding values for the positive control (ribavirin) are 3.6 ± 0.2 μg mL^{-1}, 62.5 ± 1.9 μg mL^{-1} and 17.4, respectively. The *in vitro* antiviral activity was evaluated with the cytopathogenic effect (CPE) reduction assay as described in the literature.[19,20] The TC_{50} and IC_{50} values are estimated from the graphic plots as shown in Fig. 3 and the SI value is characterized by the ratio of TC_{50}/IC_{50}. Normally, the selectivity index SI > 4 is considered significant.[20] Thus isolation of spirocaesalmin with a novel spiro-heterocyclic ring skeleton and the first bioassay against RSV in the family of vouacapane diterpenoids provide a potentially useful lead to the search for antiviral drugs. Further investigations regarding the antiviral properties of other vouacapane diterpenoids and structural modification of spirocaesalmin are in progress.

Experimental

General

The optical rotation was recorded on a Perkin-Elmer 341 polarimeter in MeOH solution and is given in 10^{-1} deg cm^2 g^{-1}. The melting point was determined using a Fisher Scientific instrument and is uncorrected. The IR spectrum was recorded on a Nicolet Impact 420 FT-IR spectrometer with KBr pellets. All NMR spectra were obtained on a Bruker-300 MHz spectrometer in CD_3OD solution with TMS as internal standard, and the chemical shift values are in ppm. ESI-MS/MS was

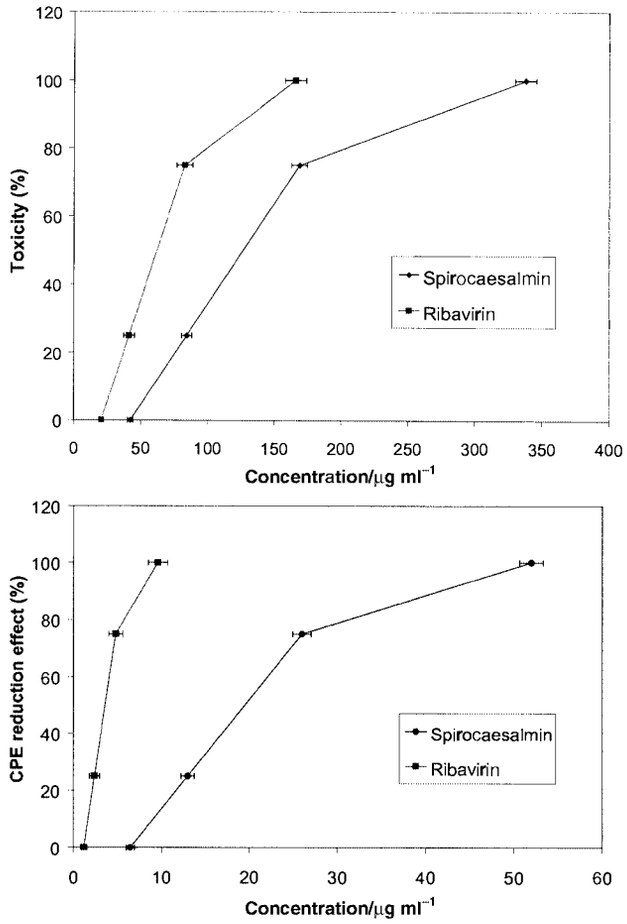

Fig. 3 a. Cell toxicity of spirocaesalmin and ribavirin on HEp-2 cell line; b. CPE reduction effects of spirocaesalmin and ribavirin on RSV. The TC_{50} and IC_{50} values are estimated from the graphic plots. The data are expressed as mean ± standard deviation ($n = 3$).

recorded on a Finnigan MAT TSQ 7000 instrument. The HRLSIMS measurement was made on an APEX 47e FTMS spectrometer.

Plant material

The seeds of *Caesalpinia minax* Hance (Ku-shi-lian in Chinese) were collected in Guangxi Province, P. R. China in September, 1999, and air-dried. Identification was provided by the Institute of Chinese Medicine, The Chinese University of Hong Kong, where a voucher specimen (No. cm-99) was deposited.

Extraction and isolation

The dry ground seeds of *Caesalpinia minax* Hance (5 kg) were refluxed with 95% ethanol. The solution was evaporated *in vacuo* to yield an ethanol extract (450 g) which was then suspended in distilled water (1500 mL) and extracted successively with hexane, chloroform, ethyl acetate and *n*-butanol. The chloroform extract (25 g) was subjected to silica gel column chromatography and eluted with hexane–acetone (20 : 1). Fractions of 150 ml each were taken and fractions 12 and 13 were further purified by preparative TLC (*n*-hexane–acetone 2 : 1, R_f 0.47) to afford 5.4 mg of spirocaesalmin (0.00011%).

Spirocaesalmin. $C_{25}H_{34}O_{10}$: prism, mp 289–291 °C, $[\alpha]_D^{20}$ + 15.8 (*c* 0.1 in CH_3OH); IR (KBr) ν_{max}/cm^{-1} 3583 (OH), 1736 (C=O); positive ESI-MS/MS 40 eV, *m*/*z* (relative intensity, %) 512 $[M + NH_4]^+$ (100), 494 $[M]^+$ (8), 463 $[M - OCH_3]^+$ (87), 403 $[M - OCH_3 - CH_3COOH]^+$ (17), 357 $[M - OCH_3 - CH_3COOH - CO - H_2O]^+$ (40), 325 $[M - OCH_3 - 2CH_3COOH - H_2O]^+$ (84); HRLSIMS *m*/*z* $[MH]^+$ 495.2216, calculated 495.2220.

X-Ray crystal structure analysis of spirocaesalmin

$C_{25}H_{34}O_{10}$, colorless prisms were re-crystallized from a hexane and acetone mixture, mp 289–291 °C, M = 494.52, orthorhombic, $P2_12_12_1$, a = 11.8436(6), b = 12.9482(6), c = 16.3048(8) Å, V = 2500.4 (2) Å^3, Z = 4, D_c = 1.314 g cm^{-3}, $F(000)$ = 1056, μ(Mo-Kα) = 0.101 mm^{-1}. Data collection was performed on a SMART 1000 CCD using graphite monochromatized radiation (λ = 0.71073 Å); 3594 unique reflections (R_{int} = 0.049) were collected to θ_{max} = 23.27°, in which 2598 reflections were observed [$F^2 > 4\sigma(F^2)$]. The crystal structure was resolved by direct method using SHELXS-97 and refined by full-matrix least-square method on F^2 using SHELXS-97. Non-hydrogen atoms were refined with anisotropic temperature factors. Hydrogen atoms bonded to carbons were placed at their geometrically ideal positions. Hydrogen atoms bonded to oxygen were located on difference Fourier map and included in the calculation of structure factors with isotropic temperature factors. In the final stage, R = 0.0391, Rw = 0.0645 and S = 0.958. CCDC 153781. See http://www.rsc.org/suppdata/p1/b1/b107473n/ for crystallographic files in .cif or other electronic format.

Anti-RSV assay

Bioassay materials. Respiratory syncytial virus (Long strain) and HEp-2 cells were obtained from American Type Culture Collection. Dulbecco's modified eagle's medium was purchased from Sigma Co., USA. Fetal bovine serum (FBS) was obtained from Biofluids Inc., USA.

Cytotoxicity assay. Cell toxicity was monitored by observing the effects of the natural products on cell morphology and cell viability. Confluent HEp-2 cell monolayers were incubated with serial twofold dilutions of spirocaesalmin in growth medium with 5% serum and were observed microscopically for detectable alterations, *i.e.* loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolisation in the cytoplasm. The cytopathogenic effect (CPE) was assessed using the established literature method.[19,20] The concentration causing visible changes in 50% cells was estimated from graphic plots and defined as TC_{50}.

CPE reduction assay. Serial diluted portions of spirocaesalmin were seeded into Confluent monolayers of HEp-2 cells cultivated in 96-well culture plates, using the maximal non-cytotoxic concentration as the limit. Ribavirin was used as a positive control, and an infection control was made in the absence of spirocaesalmin. An equal volume of respiratory syncytial virus suspension was added to the monolayers of cells. The plates were incubated at 37 °C in a humidified CO_2 atmosphere (5% CO_2) and the CPE data were assessed. The reduction of virus multiplication was calculated as % virus control = $CPE_{sample}/CPE_{virus\ control} \times 100$. The concentration that reduced CPE by 50% with respect to virus control was estimated from graphic plots, and was defined as 50% inhibited concentration (IC_{50}). Both IC_{50} and TC_{50} were expressed in μg mL^{-1}. The selectivity against RSV virus was characterized by the selectivity index (SI) = TC_{50}/IC_{50}. Both the cytotoxicity assay and the CPE reduction assay were repeated three times.

Acknowledgements

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