In Vitro Leishmanicidal Activity of Benzophenanthridine Alkaloids from *Bocconia pearcei* and Related Compounds

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Received March 24, 2010; accepted May 15, 2010; published online May 18, 2010

Leishmanicidal activities of benzophenanthridine alkaloids isolated from fruits of *Bocconia pearcei* and their derivatives were examined. Seven benzophenanthridine compounds were isolated from the methanolic extracts of *B. pearcei*. Among them, dihydrosanguinarine showed the most potent leishmanicidal activities (IC_{50} value: 0.014 μg/ml, respectively). To examine the structure–activity relationship of the benzophenanthridine skeleton, the leishmanicidal activities for 32 synthetic samples were examined. The existence of bulky groups at the C_7–C_8 position was found to enhance the activity. On the other hand, the bulkiness at the C_2–C_3 position on the D-ring, a carbonyl group at C-6, substitution at C-6 and cleavage or saturation of the C_5–C_6 bond reduced activity. A methyl group on nitrogen of the C-ring was thought to be necessary for significant activity.

Key words leishmaniasis; *Bocconia pearcei*; Papaveraceae; benzophenanthridine; alkaloid

Bocconia pearcei Hutchinson (Papaveraceae) is indigenous to the highlands of Central and South America and its fruits are commonly found in markets in Peru under the local name "Yanali." The sap of *B. pearcei* has been used as a dye, while extracts of various tissues are used medicinally to control mange, lice, and intestinal worms, and to treat ulcers of the eyes, wounds, edema and jaundice.[1,2] Furthermore, the fruits are used to pray for a good harvest by sowing them in fields. However, the details of the chemical components have not been investigated.[3—5]

Leishmaniasis is endemic in tropical regions and currently affects 12 million people in 88 countries.[6] The disease is transmitted by small biting sandflies (*Phlebotomus* spp.). The first-line drugs for the treatment of leishmaniasis are pentavalent antimonials such as *N*-methylglucamine antimonate (Glucantime®) and sodium stilbogluconate (Pentostam®). However, these drugs are toxic and generally expensive. Therefore, we have been searching for new medicines from plant sources that grow in areas where leishmaniasis is distributed. We have already reported several leishmanicidal constituents of medicinal plants in tropical and subtropical regions (Peru, Myanmar, Bolivia, Pakistan, *etc.*).[7—11] In the first screening of hundreds of Peruvian medicinal plant extracts for leishmanicidal activity, we found that the extract of *Bocconia pearcei* fruit exhibited potent activity.

In this paper, we describe the chemical components of 'Yanali' and their leishmanicidal activities *in vitro* and the structure–activity relationships using several derivatives of benzophenanthridine alkaloids.

Results and Discussion

The methanolic extract of *B. pearcei* fruit exhibited the most potent leishmanicidal activity *in vitro* [Minimum Lethal Concentration (MLC): 3.1 μg/ml] in a screening with hundreds of Peruvian plant extracts. Therefore, we investigated the active compounds against *Leishmania major* using activity-guided fractionation. After successive partitioning with *n*-hexane, chloroform, *n*-butanol and water, the MLC of each layer was measured to be 0.4, 0.2, 6.3 and 200 μg/ml, respectively. Purification of the *n*-hexane and chloroform layers afforded compounds **1**—**4** from the *n*-hexane layer and compounds **1**, **3**, **5**—**7** from the chloroform layer. Compounds **1**—**7** were identified to be dihydrosanguinarine (**1**),[12] dihydrochelirubine (**2**),[12] dihydrochelerythrine (**3**),[12] oxychelerythrine (**4**),[13] norchelerythrine (**5**),[14,15] 12-methoxynorchelerythrine (**6**)[16] and oxysanguinarine (**7**),[17] respectively, based on spectral findings (Fig. 1). This is the first time that these compounds have been isolated from *B. pearcei*.

The leishmanicidal activities of **1**—**3** were examined. **4**—**7** were not examined because the amounts available were insufficient. Among them, dihydrosanguinarine (**1**) showed the most potent activity (IC_{50}: 0.014 μg/ml), and **2** was as effective as **3** (IC_{50}: 0.166 μg/ml). To determine the structure–activity relationship of the benzophenanthridine skeleton, we examined the leishmanicidal activities of 32 synthetic samples, including compounds **4** and **5**.

Compounds in which the substituents on the A-ring and D-ring were modified were mainly used to examine the structure–activity relationships (Figs. 2, 3).

As a result, the replacement of a proton by a methoxyl group at C-7 (**9** and **16**), or the replacement of a hydroxyl by a methoxyl or ethoxyl group at C-8 (**24**—**26**; **9** and **12**) enhanced the activity. Furthermore, the replacement of a methylenedioxyl by a dimethoxyl group at C-7 and C-8 (**19** and **20**; **21** and **22**; **23** and **24**) enhanced the activity. Therefore, bulkiness at C-7 and C-8 apparently contributed to the potent activity. On the other hand, the same bulkiness at C-8 and C-9, or C-2 and C-3 on the D-ring clearly reduced the activity (**8** and **9** for C-8 and C-9; **19** and **21**, **20** and **22** for C-2 and C-3). A carbonyl group (*i.e.*, oxidation of C-6; **24**

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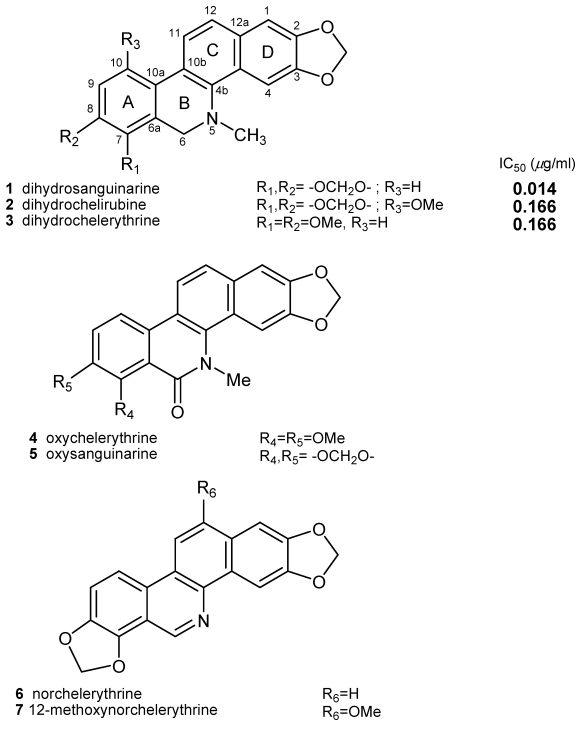


Fig. 1. Chemical Structures of Compounds Isolated from *B. pearcei*

and **33**; **9** and **27**) or substitution at C-6 obviously reduced the activity. The unsaturation of the C–C bond between C-5 and C-6 seemed to be necessary for this activity (**30** and **24**; **2** and **19**). However, several derivatives showed no change in activity (**31** and **9**; **1** and **23**). The methyl group on the nitrogen atom of the C-ring seemed to be important for remarkable activity (**24** and **35**). Ring-cleavage of the bond between C-5 and C-6 caused a complete loss of activity (**36**—**39**).

Considering these relationships, the bulkiness on the A-ring was thought to help enhance leishmanicidal activity, while bulkiness on the C-ring (**17** and **19**) and D-ring reduced this activity.

Several benzophenanthridine alkaloids including **8** and **9** have been reported to have antimalarial activity (IC_{50} 1.8 μg/ml for **9**; 11.7 μg/ml for **8**). If we compare our results to those for antimalarial activity, the effects of bulkiness at C-8 and C-9 for antileishmanial activity are opposite those for antimalarial activity.[18]

Interestingly, the structure–activity relationships for leishmanicidal activity were almost the same as those for antitumor activity against the Sarcoma 180 cell line.[19]

Conclusion

We found that several benzophenanthridine compounds had remarkable growth–inhibitory activity against *L. major* promastigotes *in vitro*.

B. pearcei has been used as a crude drug, but not for the treatment of leishmania. In this study, we found that methanolic extracts of *B. pearcei* had significant leishmanicidal activity, and this led us to isolate four benzophenanthridine alkaloids as active constituents.

Benzophenanthridine alkaloids have been reported to have various bioactivities, such as antimicrobial activity,[20] inhibition of tubulin polymerization,[21] suppression of angiogenesis,[22] induction of apoptosis in OCM-1 cells,[23] *etc.* Benzophenanthridine alkaloids have been reported to have antimalarial activity, but this is the first report of leishmanicidal activity. In some countries, a toothpaste containing sanguinarine is commercially available because sanguinarine inhibits dental plaque and improves gingival health.[24,25] However, it has been generally difficult to use benzophenanthridine alkaloids as medical products because of their strong toxicity. Although they may be difficult to apply in oral or injectable form, application as an ointment for cutaneous leishmanisis might be a breakthrough for this problem. The effects of these compounds against leishmania amastigotes have not been investigated, and the mechanism of their action toward protozoa will be examined in the near future.

Experimental

Plant Material Air-dried fruits of *B. pearcei* Hutchinson were purchased at Cusco, Peru in 2001, and were identified by Dr. M. Satake (Ochanomizu University). A voucher specimen (No. 01PR019) was deposited at the Research Center of Medicinal Plant Resources, National Institute of Biomedical Innovation (1–2 Hachimandai, Tsukuba, Ibaraki 305–0843, Japan).

Chemicals TetraColor ONE was purchased from Seikagaku Kogyo Co., Ltd. Medium 199 and ES Cell Qualified Fetal Bovine Serum were obtained from Invitrogen Co., Ltd. Synthetic benzo[*c*]phenanthridine alkaloids (**8**—**39**)[19] were provided by Dr. Tsutomu Ishikawa (Chiba University).

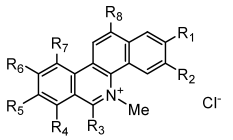
General Experimental Procedures HPLC was performed on a Shimadzu LC-10A apparatus with a UV detector (Shimadzu SPD-M10A, Japan). To purify extracts, Silica gel (Merck, Germany), Mega Bond Elut® (C_{18}, Si, Almina) (Varian, U.S.A.), Sephadex LH-20 (GE Healthcare U.K. Ltd., U.K.), and Chromatorex NH-DM1020 (Fuji Silysia Chemical Ltd., Japan) were used.

Cultivation of Leishmania Promastigotes Medium 199 was used for the cultivation of promastigotes of *L. major* (MHOM/SU/73/5ASKH). Promastigotes were cultured in the medium [supplemented with heat-inactivated (56 °C for 30 min) fetal bovine serum (10%)] at 27 °C, 5% CO_2 in an incubator.

Leishmanicidal Activity Assay The leishmanicidal effects of each fraction and compound were assessed by the improved 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) method described in the previous paper.[8—11] Amphotericin B was used as a positive control for assay (IC_{50} 0.04 μg/ml).

Isolation Procedure Air-dried fruits of *B. pearcei*, Yanali, (498 g) were powdered and extracted with hot methanol (1 l×1, 0.6 l×2), and the extract was then filtered. The filtrate was concentrated under reduced pressure to yield a residue (65 g), 21 g of which were suspended in 10% methanol/water and then partitioned successively with *n*-hexane, chloroform and *n*-butanol. Each layer was evaporated under reduced pressure to afford an *n*-hexane fraction (Fr. H) (4.0 g), chloroform fraction (Fr. C) (2.6 g), *n*-butanol fraction (Fr. B) (8.9 g) and water fraction (Fr. W) (3.5 g). Half of Fr. H (2.0 g) was subjected to column chromatography on silica gel with *n*-hexane/ethylacetate as an eluent to give 32 fractions (Fr. H1—H32). Fr. H7—H9 (eluted with *n*-hexane/ethylacetate=15 : 1) were combined and purified by Bond elut C_{18}® while eluting with acetonitrile/chloroform to give **1** (12 mg). Fr. H10—H15 (eluted with *n*-hexane/ethylacetate=10 : 1) were combined and fractionated by silica gel column chromatography while eluting with a chloroform–ethylacetate–methanol solvent system. Fractions eluted with chloroform were combined and rechromatographed on LH-20 using chloroform/methanol (1 : 1) as an eluent to give 20 fractions. Concentrated fractions 13 and 14 (10 mg) were purified by Bond elut Alumina® eluted with *n*-hexane–ethylacetate to obtain **2** (2 mg) as colorless needles. Fr. H16—H22 (eluted with *n*-hexane/EtOAc=5 : 1) were purified by Bond elut C_{18}® eluted with acetonitrile–methanol to give **3** (6 mg) as needle crystals. Fr. 3 of this column was purified again by Bond elut C_{18}® eluted with acetonitrile/water to give linoleic acid (43 mg), and HPLC purification of fr. 12 using an ODS column (mobile phase: methanol–water solvent system) gave **4** (1 mg).

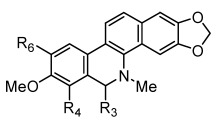
Part of Fr. C (1.2 g) was subjected to HPLC on a NH_2-silica gel column using an *n*-hexane–chloroform solvent system as a mobile phase to give 48 fractions (Fr. C1—C48). Fr. C13—C16 were combined and concentrated to afford **1** (11 mg). Fr. C23—C28 (eluted with *n*-hexane/chloroform=4 : 1) were combined and rechromatographed on a NH_2-silica gel column while eluting with an *n*-hexane–chloroform system to give 48 fractions. **3** (11 mg)

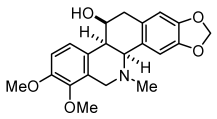


	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	IC_{50} (μg/ml)
8 (avicine)	OCH_2O		H	H	OCH_2O		H	H	**0.079**
9 (nitidine)	OCH_2O		H	H	OMe	OMe	H	H	**0.48**
10 (6-Me-nitidine)	OCH_2O		Me	H	OMe	OMe	H	H	**1.8**
11 (isoterihanine)	OCH_2O		H	H	OMe	OH	H	H	**>20**
12 (terihanine)	OCH_2O		H	H	OH	OMe	H	H	**3.6**
13 (fagaronine)	OH	OMe	H	H	OMe	OMe	H	H	**0.090**
14 (^{i}Pr-fagaronine)	O^{i}Pr	OMe	H	H	OMe	OMe	H	H	**0.49**
15 (10-methoxynitidine)	OCH_2O		H	H	OMe	OMe	OMe	H	**0.008**
16 (9-methoxychelerythrine)	OCH_2O		H	OMe	OMe	OMe	H	H	**0.13**
17 (macarpine)	OCH_2O		H	OCH_2O		H	OMe	OMe	**0.022**
18 (10-isopropoxysanguinarine)	OCH_2O		H	OCH_2O		H	OPr^i	H	**0.010**
19 (chelirubine)	OCH_2O		H	OCH_2O		H	OMe	H	**0.015**
20 (chelilutine)	OCH_2O		H	OMe	OMe	H	OMe	H	**0.009**
21 (sanguirubine)	OMe	OMe	H	OCH_2O		H	OMe	H	**0.050**
22 (sanguilutine)	OMe	OMe	H	OMe	OMe	H	OMe	H	**0.016**
23 (sanguinarine)	OCH_2O		H	OCH_2O		H	H	H	**0.011**
24 (chelerythrine)	OCH_2O		H	OMe	OMe	H	H	H	**0.006**
25 (Et-decarinium)*	OCH_2O		H	OMe	OEt	H	H	H	**0.003**
26 (decarinium)	OCH_2O		H	OMe	OH	H	H	H	**0.24**
27	OCH_2O		H	OMe	H	OCH_2O		H	**0.002**

* N-Et

	R_3	R_4	R_6	IC_{50} (μg/ml)
28 (*dl*-bocconoline)	CH_2OH	OMe	H	**0.29**
29 (chelerythrine φ-cyanide)	CN	OMe	H	**0.019**
30 (dihydrochelerythrine)	H	OMe	H	**0.069**
31 (dihydronitidine)	H	H	OMe	**0.48**

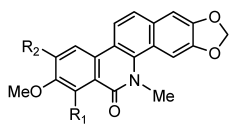




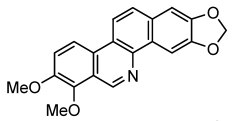
32 (*dl*-homochelidonine) **1.9**

Fig. 2. Chemical Structures of Synthetic Benzophenanthridine Alkaloids and Their Leishmanicidal Activities

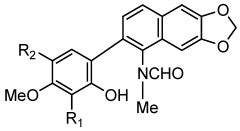
	R_1	R_2	IC_{50} (μg/ml)
33 (oxychelerythrine)	OMe	H	**>100**
34 (oxynitidine)	H	OMe	**13**



35 (norchelerythrine) **>100**



	R_1	R_2	
36 (arnottianamide)	OMe	H	**>20**
37 (isoarnottianamide)	H	OMe	**23**



	R_1	R_2	R_3	R_4	
38	OMe	H	OCH_2O		**>100**
39	CO_2Me	OMe	OMe	H	**9.3**

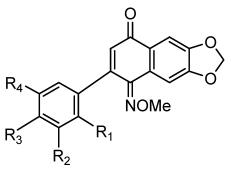


Fig. 3. Chemical Structures of Synthetic Benzophenanthridine Alkaloids and Their Leishmanicidal Activities

was obtained from Fr. 11. Fr. 16 was further purified by HPLC using NH_2-silica gel (*n*-hexane/chloroform=7 : 2) to give **5** (2 mg) and **6** (1 mg). Fr. C29—C38 (eluted with *n*-hexane/chloroform=75 : 25) were combined and rechromatographed on a silica gel column eluted with *n*-hexane/ethylacetate to give **7** (2 mg).

1—**3**,[12] **4**—**5**,[13—15] **6**,[16] **7**[7] were characterized by comparison of their respective spectroscopic properties with values in the literature.

Acknowledgement This research was financially supported by The Japan Human Science Foundation (Research on Health Sciences focusing on Drug Innovation; Grant Number SH24404) and Ministry of Health, Labour and Welfare (Health and Labour Sciences Research Grants; Grant Number H19-Seibutsushigen-ippan-008).

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