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Anti-*Helicobacter pylori* activity of steroidal alkaloids obtained from three *Veratrum* plants

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Abstract

Anti-*Helicobacter pylori* (HP) activities were examined, by disc method, on three total alkaloid fractions and fourteen steroidal alkaloids obtained from three *Veratrum* plants (*V. maackii, V. nigrum* var. *ussuriense* and *V. patulum*), which are used as a name of "Li-lu (藜蘆)" to treat aphasia arising from apoplexy, wind type dysentery, jaundice, headache, scabies, chronic malaria, *etc.* Among them, verapatulin (**12**) and veratramine (**13**) revealed anti-HP activities, and the disc-minimum inhibitory concentration (disk-MIC) value (10 μg/ml) of **12** against two standard HP strains, NCTC11637 and NCTC11916, was higher than that of a clinically used antibiotic, erythromycin ($\leq$0.013 μg/ml), but was comparable to those of penicillin G (3.1 μg/ml and 1.6 μg/ml, respectively).

Key words *Helicobacter pylori,* steroidal alkaloid, ri-ro (li-lu, 藜蘆), verapatulin, antibacterial agent, *Veratrum patulum.*

Abbreviations BSA, bovine serum albumin; cfu, colony forming units; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; HP, *Helicobacter pylori*; Ri-ro(Li-lu), 藜蘆.

Introduction

Helicobacter pylori (HP) is a recently recognized human pathogen causing chronic active gastritis in association with duodenal ulcers.[1] Furthermore, it was postulated that HP infection increased the risk of gastric carcinoma.[2,3] An eradication of HP in the gastric mucosa of patients with gastritis of peptic ulcer has been tried to cure such stomach diseases.[4] Recent therapy with a combination of a proton pump inhibitor, antibiotics and metronidazole has resulted in a high eradication rate of HP.[5,6] Resistant strains against these antibiotics and/or metronidazole, however, have appeared,[7] and thus more efficient and less expensive drugs are demanded.

As one of our studies on bioactive natural products, we have reported the anti-HP activities of trichorabdal A from *Rhabdosia trichocarpa* and lupulone from *Humulus lupulus* together with activities of 27 natural medicines.[8,9] On the other hand, we also examined the constituents of three *Veratrum* plants (*V. maackii* REG.,[10] *V. nigrum* L. var. *ussuriense* NAKAI[11,12] and *V. patulum* LOES.[13]) and reported five new steroidal alkaloids[maackinine (**1**), verussurinine (**6**), verapatuline (**12**), verussurine, 20-isoveratramine] and thirteen known ones [germanitrine (**2**), germidine (**3**), germerine (**4**), 15-*O*-(2-methylbutyroyl)germine (**5**), neogermbudine (**7**), angeloylzygadenine (**8**), verazine (**9**), verazinine (**10**), jervine (**11**), veratramine (**13**), veratrosine (**14**), zygadenine, verabenzoamine].

Dried roots and rhizomes of several *Veratrum* species, such as *V. nigrum* L., *V. maackii, V. dahuricum* LOES. f. and *V. patulum* (Liliaceae), are used as a name of "Li-lu (藜蘆)" to treat aphasia arising from apoplexy, wind type dysentery, jaundice, headache,

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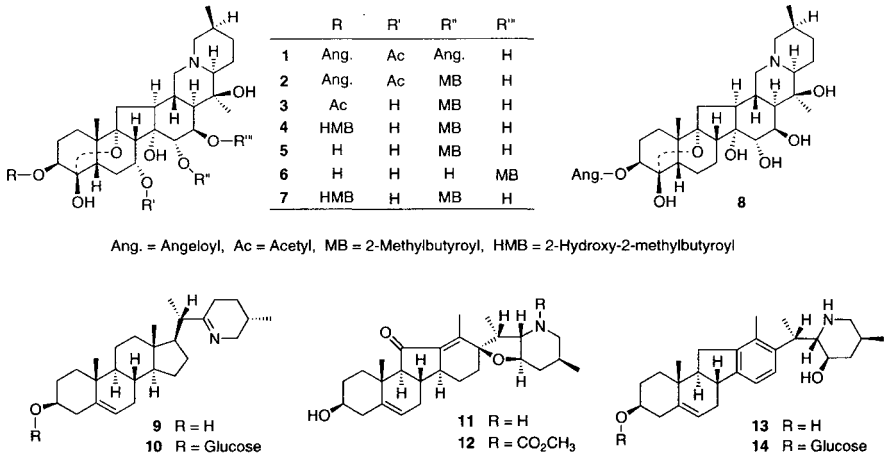

	R	R'	R"	R"'
1	Ang.	Ac	Ang.	H
2	Ang.	Ac	MB	H
3	Ac	H	MB	H
4	HMB	H	MB	H
5	H	H	MB	H
6	H	H	H	MB
7	HMB	H	MB	H

Fig. 1 Structure formulas of *Veratrum* alkaloids **1**-**14**.

scabies, chronic malaria, *etc.*[14,15] These uses suggest that *Veratrum* plants and/or their constituents may have antibacterial activity, and indeed insecticidal,[16] cytotoxic,[17] anti-herpetic[18] and antimicrobial[19] activities of *Veratrum* alkaloids have been reported. On the anti-HP activity, however, there is no report, and we thus examined the anti-HP activities of the total alkaloid fractions and fourteen steroidal alkaloids (**1**-**14**) of the three *Veratrum* plants (Fig. 1).

Materials and Methods

Strains used and culture conditions : Two standard HP strains, NCTC11637 and NCTC11916, were used in this study. They were grown on Brucella agar (Becton Dickinson Microbiol. Systems, USA) supplemented with horse defibrinated blood at 5 % (blood agar), or with bovine serum albumin (BSA) fraction V (Sigma Chemical Co., USA. A-4503) sterilized with a 0.2 μm filter at 0.5 mg/ml (albumin agar). They were cultured at 37°C in a glove box under a humidified microaerobic atmosphere consisting of 80 % N_2, 15 % CO_2 and 5 % O_2 for 3 d. They were harvested and suspended in Brucella broth supplemented with BSA fraction V at 0.5 mg/ml.

Sample preparation : Steroidal alkaloids used in this study (Fig. 1) were isolated from three *Veratrum* plants, *V. maackii, V. nigrum* var. *ussuriense* and *V. patulum*, as reported previously.[10,13] Each sample was dissolved in dimethyl sulfoxide (DMSO) at 1000 μg/ml and then diluted with DMSO to 100, 10 and 1 μg/ml.

Anti-HP activity by disc method : One million colony forming units (cfu) of HP were inoculated onto blood agar and albumin agar (20 ml) in 90 mm Petri dishes. On the agar plates 8 mm thin paper disc (Advantec Ltd., Japan) charged with 20 μl of sample solution was put and the plates were incubated at 37°C under microaerobic condition for 3-5 d. The inhibitory zone from the edge of the disc was measured and the minimum inhibitory concentration (MIC) of the samples required to produce the inhibitory zone more than 1 mm was expressed as disc-MIC. No inhibitory zone around the disc charged with DMSO alone was observed against HP strains.

Results and Discussion

Three total alkaloid fractions and fourteen steroidal alkaloids obtained from three *Veratrum* plants (*V. maackii, V. nigrum* var. *ussuriense* and *V. patulum*) were examined for their anti-HP activity by disc method. Their anti-HP activities are presented in Table I with disc-MIC values along with that of clinically-used antibiotics, erythromycin and penicillin G, as a reference.

Three total alkaloid fractions showed no activity at a high concentration of 1 mg/ml with both blood

Table I Anti-HP activity (disc-MIC value in μg/ml) or steroidal alkaloids.

Sample	*H. pylori* Strain number			
	NCTC 11637		NCTC 11916	
	Blood agar	Albumin agar	Blood agar	Albumin agar
Total alkaloid fraction				
V. maackii	>1000	1000	>1000	1000
V. nigrum var. *ussuriense*	>1000	1000	>1000	1000
V. patulum	>1000	1000	>1000	1000
Steroidal alkaloid				
1	>1000	1000	>1000	1000
2	>1000	1000	>1000	1000
3	>1000	1000	>1000	1000
4	>1000	>1000	>1000	1000
5	>1000	>1000	>1000	1000
6	>1000	>1000	>1000	1000
7	>1000	>1000	>1000	1000
8	>1000	1000	>1000	1000
9	>1000	1000	>1000	1000
10	>1000	1000	>1000	1000
11	>1000	>1000	>1000	1000
12	1000	10	1000	10
13	>1000	100	>1000	100
14	>1000	>1000	>1000	>1000
Erythromycin	13	0.78	6.3	0.78
(agar dilution)[a]	0.20	≦0.013	0.20	≦0.013
Penicillin G	6.3	3.1	6.3	1.6
(agar dilution)[a]	0.050	0.025	0.10	0.025

a) MIC values by agar dilution method (reference 23).

agar and albumin agar, while almost all steroidal alkaloids, except for veratrosine (**14**), showed the anti-HP activity at 1 mg/ml with the albumin agar. Among the 14 steroidal alkaloids, verapatulin (**12**) and veratramine (**13**) revealed anti-HP activity with the albumin agar. Several natural products were previously reported to have anti-HP activity,[8,9,20-22] but **12** and **13** were the first example of the anti-HP compounds of the steroidal alkaloids. The steroidal alkaloids could be classified into five groups; *i.e.*, germine esters (**1-7**), zygadenine ester (**8**), verazine derivatives (**9** and **10**), jervine derivatives (**11** and **12**) and veratramine derivatives (**13** and **14**). The two compounds (**12** and **13**) which showed anti-HP activities belong to the different groups (jervine derivative and veratramine), and thus the anti-HP activity could not be related to the structure.

The disc-MIC value of **12** against both NCTC11637 and NCTC11916 with the albumin agar was 10 μg/ml, which was weaker than that of a clinically-used antibiotic, erythromycin (0.78 μg/ml), but was comparable to those of penicillin G (3.1 μg/ml and 1.6 μg/ml, respectively). The disc-MIC value of **12** with the albumin agar was 1/100 fold of the value with the blood agar, a tendency which was observed in the cases of trichorabdal A[8] and macrolide antibiotics (*e.g.* erythromycin) but not in other antibiotics (*e.g.* penicillin G) (Table I).[23] The difference was speculated according to the same reason as macrolide antibiotics; *i.e.* specific combining of BSA with **12** without inactivating it.[23] Protein such as serum albumin is essential for grotwh of HP as a nutrient, and the albumin agar contains only BSA as a protein in only minimum amount needed for growth of HP. In the albumin agar, thus, HP would take in BSA combined with **12** for the growth, and **12** taken in may damage HP by the antibacterial action. In the blood agar containing much proteins including serum

albumin, on the other hand, HP may use free serum albumin for the growth, and thus the anti-HP activity of **12** can be weaker.

The natural products, previously reported to have anti-HP activity, had the MIC values of 2.5-64 μg/ml by agar dilution method. Because the amount of **12** obtained was small and **12** was insoluble in water, the MIC value of **12** by agar dilution method was estimated from the disc-MIC value. As indicated in Table I, the disc-MIC value of erythromycin against NCTC11637 and NCTC11916 strains with the albumin agar was 0.78 μg/ml and those of penicillin G were 3.1 and 1.6 μg/ml, respectively. On the other hand, the values against both strains by agar dilution method were≦0.013 μg/ml (erythromycin) and 0.025 μg/ml (penicillin G), respectively. Thus the ratio of MIC by disc method to it by agar dilution method is 60-124. From this ratio, when the MIC value by agar dilution method is assumed to be 1/10-1/100 fold of disc-MIC,[9] MIC of **12** by agar dilution method can be estimated to be 0.1-1 μg/ml, suggesting that **12** may be a very strong anti-HP compound.

Conclusion

In the present study, it was found that a steroidal alkaloid, verapatulin (**12**), has anti-HP activity. The anti-HP activity of steroidal alkaloids has not been examined and thus anti-HP drugs related to steroidal alkaloids are unknown. As mentioned in the Introduction, appearance of resistant strains against antibiotics and/or metronidazole used in a triple therapy method is a serious problem for an eradication of HP. Under this circumstance, a more efficient and/or different type of drug is demanded. Thus, **12** should give a new type of lead compound for the anti-HP drugs.

和文抄録

漢薬“藜蘆”として用いられている3種のヴェラトラム属植物（*V. maackii, V. nigrum* var. *ussuriense* and *V. patulum*）から得た総アルカロイドフラクション3種及びステロイドアルカロイド14種について，抗ヘリコバクター・ピロリ活性をディスク法で測定した。調べたステロイドアルカロイドの中で，ヴェラパツリン（**12**）及びヴェラトラミン（**13**）が抗ヘリコバクター・ピロリ活性を示した。ヴェラパツリン（**12**）のヘリコバクター・ピロリ標準株2種（NCTC11637及びNCTC11916)に対するdisk-MIC値は10 μg/mlであり，臨床で用いられる抗生物質のエリスロマイシン（≦0.013 μg/ml）よりは弱いが，ペニシリンG（各標準株に対して3.1 μg/ml, 1.6 μg/ml）と同程度であった。

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