ISOLATION OF (-)-STEPHOLIDINE, AN ALKALOID OF ANTISEROTONERGIC-LIKE ACTIVITY FROM SINOMENIUM ACUTUM

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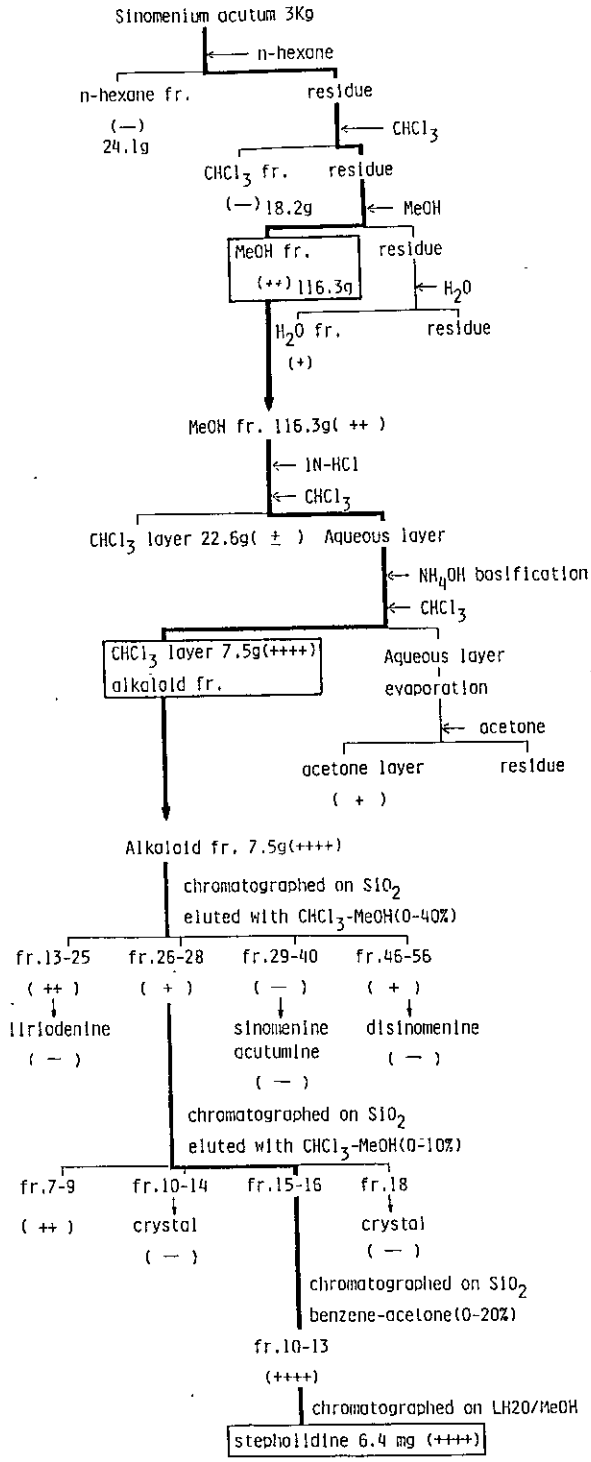
Abstract——A tetrahydroprotoberberine alkaloid, (-)-stepholidine (5), was isolated as an active principle showing antiserotonergic-like activity from Sinomenium acutum Rehder et Wilson (Menispermaceae) which has been used as an oriental medicinal drug (Japanese name, Bohi; Chinese name, Fang-Ji) in Japan. An aporphine type alkaloid, liriodenine (4), was isolated first time from this plant along with known alkaloids hitherto obtained from this plant.

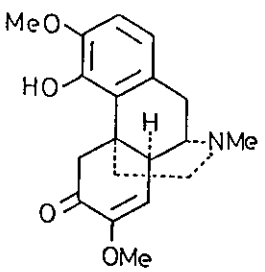
Oriental medicine has been used in health care of people in China, Japan and Southeast Asian countries for many years. Theoretical basis of oriental medicine is completely different from that of western medicine and this poses considerable difficulty to the studies of active principles contained in oriental medicinal drugs with modern pharmacological methods. In order to clarify the active principles of oriental medicinal drugs, we have introduced in vitro random screening methods, in which animal tissues, organs and enzymes are used to monitor biologically active constituents. In our previous papers, we reported the isolation of the inhibitors of phosphodiesterase[1] and a prostaglandin synthesizing enzyme sytem.[2] During the course of our screening work to find compounds showing antiserotonergic-like effect, Chinese medicinal drugs originating from Citrus plants were found to show strong inhibitory activity to rat uterus contraction induced by serotonin (5-hydroxytryptamine; 5-HT). An active principle was isolated and identified as (-)-synephrine which had been known as a synthetic sympathomimetic agent.[3] In this paper we describe the isolation of an alkaloid showing antiserotonergic like-activity from the root of Sinomenium acutum Rehder et Wilson (Menispermaceae), which has been used as a Chinese medicinal drug (Japanese name, Bohi; Chinese name, Fang-Ji) in Japan.

Hot aqueous extracts of more than 150 Chinese medicinal drugs were tested for their antiserotonergic-like activity by using rat uterus as described in a previous paper.[3] A hot aqueous extract of the title plant showed considerable strong activity in the screening bioassay test and an alkaloid fraction was found to contain active compounds. The alkaoid fraction was further subjected to chromatographic separation with silica gel column as shown in scheme 1. All the eluted fractions in the first column chromatography did not show any higher activity than the original alkaloid fraction and hence highly active compounds contained in the alkaloid fraction seemed to decompose during the chromatographic separation. Although sinomenine (1)[4], disinomenine (2)[5] and acutumine (3)[5,6] were isolated as known alkaloids of the plant, they showed no significant activity in the bioassay test. One of the active fractions obtained in the column chromatography

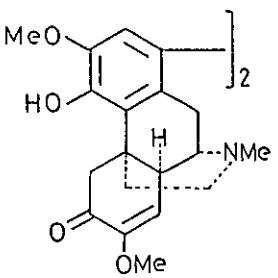


Scheme 1. Isolation of stepholidine (5) and other alkaloids.

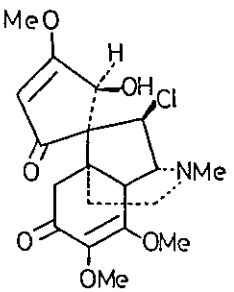
gave a yellow crystalline alkaloid, however its antiserotonergic-like activity was not so high as it was expected from the activity of the fraction. Therefore, a small amount of more active compound should be contained in the fraction, though it was not otained from the fraction. The yellow crystalline compound showed spectral data corresponding to liriodenine (4), an aporphine type alkaloid reported to occurr in a large number of plants.[7] This alkaloid was also reported to show antitumor activity in in vitro test with human nasopharinx carcinoma cells.[8] This is the first time that liriodenine (4) was isolated from Sinomenium acutum. Active fractions obtained in the silica gel column chromatography were further separated by second silica gel column chromatography. Although two crystalline compounds were isolated from the chromatographic fractions, they showed no significant activity in the bioassay test. Fractions which showed significant activity were further separated by third silica gel column chromatography to give crude crystals highly active in the bioassay test. The alkaloid was finally purified by a LH-20 column and obtained as dark orange coloured crystals. It gave molecular formula of $C_{19}H_{21}NO_4$ in the high resolution mass spectrum and and showed (-) ORD curve. The ^{1}H-NMR spectrum of the alkaloid indicated the presence of two methoxy groups, methylene groups and four aromatic protons. Fragment peaks at m/z 178 and 150 indicated retro-Diels-Alder type fragmentation of tetrahydroprotoberberine alkaloid having one hydroxy and one methoxy in both A and D rings.



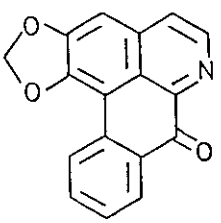
sinomenine (1)



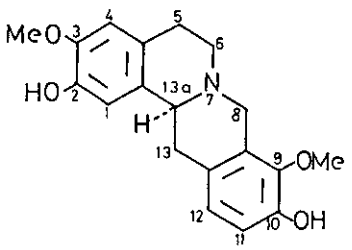
disinomenine (2)



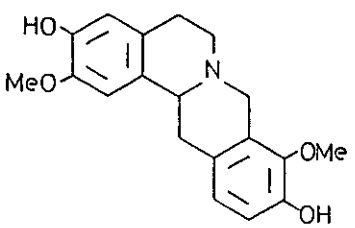
acutumine (3)



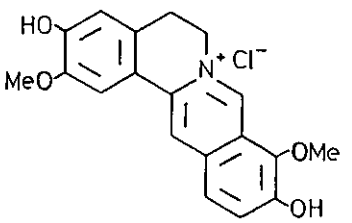
liriodenine (4)



(-) stepholidine (5)



discretamine (6)



dehydrodiscretamine chloride (7)

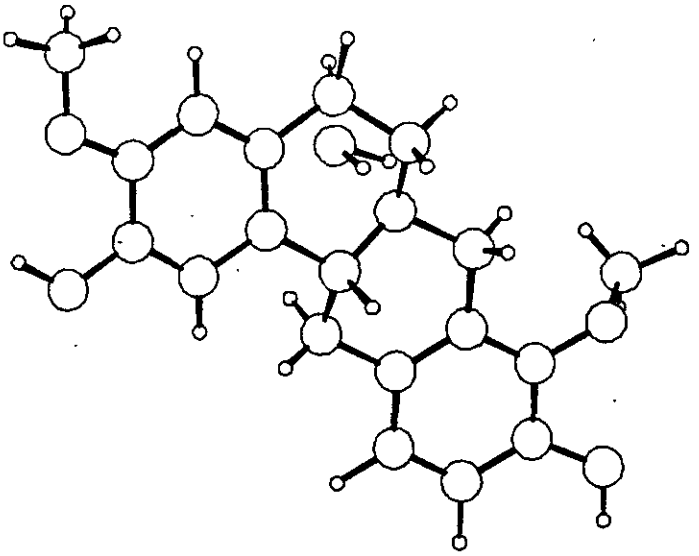
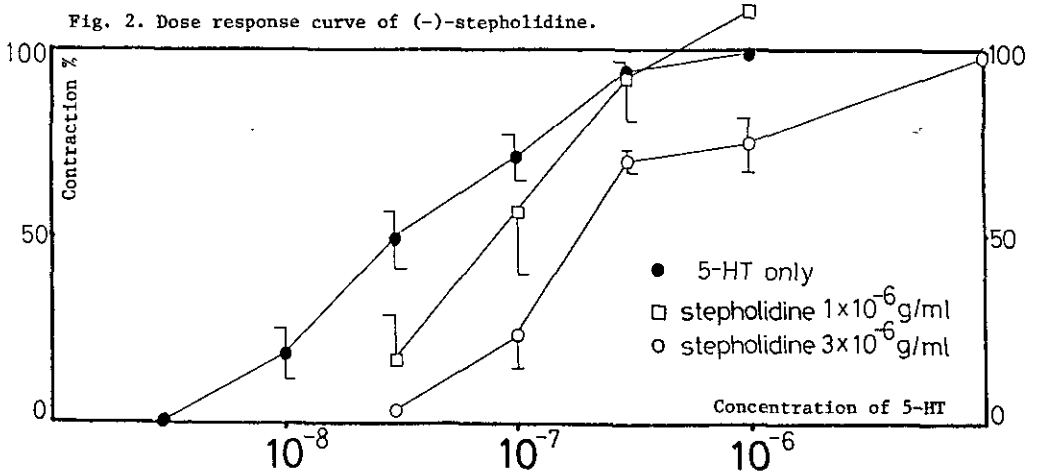


Fig. 1 PLUTO drawing of stepholidine.

Richter and Brochmann-Hanssen reported that tetrahydroprotoberberine alkaloids having 9-methoxy group showed relatively strong fragment peaks corresponding to M-OCH_3 in mass spectra.[9] Since M-31(OCH_3) fragment in the mass spectrum of the alkaloid was observed at m/z 296 and its intensity relative to that of molecular ion was 14%, indicating the alkaloid being a tetrahydroprotoberberine alkaloid with a methoxy group at C-9. Therefore, the alkaloid should be either (-)-stepholidine (5) or (-)-discretamine (6). A sample of discretamine (6) prepared by $NaBH_4$ reduction of dehydro-discretamine (7) showed the same Rf value on TLC, however the colour reaction upon heating was not identical to that of the alkaloid obtained from Sinomenium acutum. The alkaloid was finally identified to be (-)-stepholidine (5) by a X-ray analysis. It gave crystals suitable for a X-ray analysis; space group $P2_12_12_1$, Z=4. The structure was solved by the direct method and refined by block diagonal least squares to give a final R value of 0.0479 with anisotropic temperature factors for non-hydrogen atoms and isotropic temperature factors for hydrogen atoms. The crystal was found to contain one molecule of water per molecule of the alkaloid. (Fig. 1)

Fig. 2. Dose response curve of (-)-stepholidine.



From dose response curve of (-)-stepholidine (5) (Chart. 1) pA_2 value was calculated to be 6.26. Anti-acetylcholine activity of tetrahydroprotoberberine alkaloids have been reported by Kitabatake et al.[10] and this is not incompatible with our observation. Fig. 2 shows the pA_2 values of several tetrahydroprotoberberine alkaloids indicating their antiserotonergic-like action is common to this type of alkaoids.

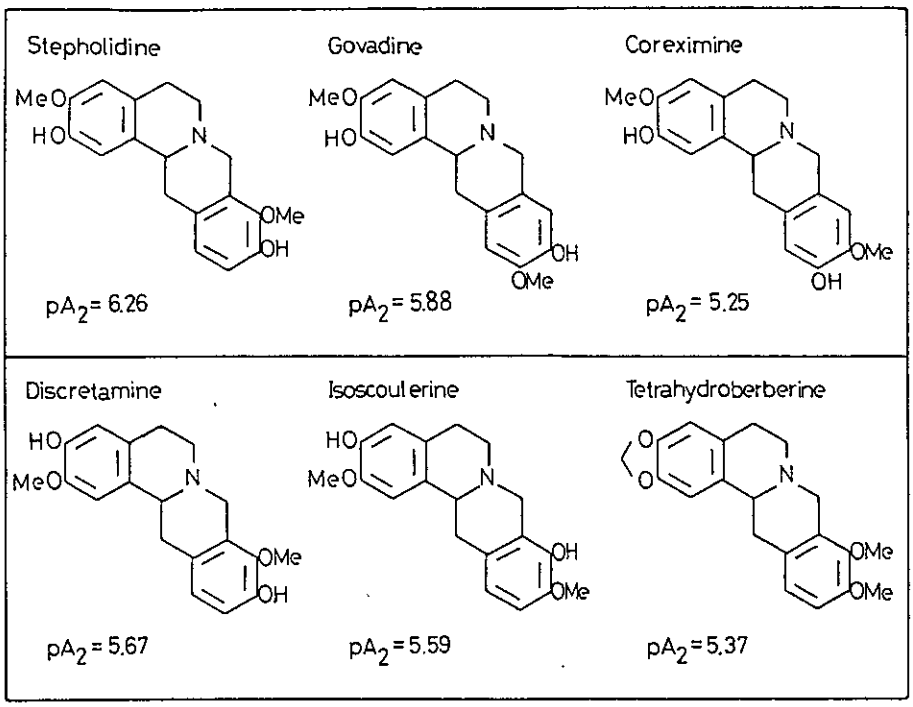


Fig. 3 Antiserotonergic-like activity of tetrahydroprotbererine alkaloids.

EXPERIMENTAL

Melting points were determined by a Yanagimoto hot stage instrument and are uncorrected. UV spectra were measured on a Hitachi Model 100-60 spectrometer and ^{1}H-NMR spectra were recorded at 100 MHz on a JEOL FX-100 system with TMS as an internal standard. Low and high resolution mass spectra were recorded with a JEOL JMS-DX 300 spectrometer. ORD spectrum was measured on JASCO J-20 Spectrometer. -Estradiol was obtained from SIGMA and serotonin creatinine sulphate from Wako Pure Chem. Ind. Ltd.

Determination of in vitro antiserotonergic-like activity - All the fraction was evaluated for in vitro antiserotonergic-like activity using isolated rat uterus. -Estradiol (200 g in olive oil) was injected subcutaneously to virgin Sprague-Dawley rats weighing 170 to 250 g 24 h prior to sacrifice. Half of the isolated uterine horn was mounted in a 10 ml organ bath containing low Ca^{2+} concentration solution of Kumagai et al[11] at 30° C and aerated with 95% O_2 and 5% CO_2. Isometric contraction was recorded on a Rectigraph 8K11 (San-ei Sokki Co.) through a Shinko U-Gage tension transducer and a strain amplifier (6M62, San-ei Sokki Co.). The uterus was allowed to stand for 30 min and the dose-response curve of serotonin (5-HT) was determined before every experiments to obtain the concentration of 5-HT inducing 70 % of maximum contraction. This concentration of 5-HT was applied at ten-minute intervals and the samples were added 5 min before the addition of 5-HT.

The doses necessary to produce 50 % reduction of the 5-HT response, i.e. ED50, were obtained from the dose-response curve of the sample. The relative activity of each sample was expressed by comparing with the activity of crude MeOH oxtract. The MeOH extract was dissolved in saline and applied into the organ bath, while other non polar extracts and fractions were suspended in 5 % aqueous arabia gum solution.

Plant Material - The roots of Sinomenium acutum Rehder et Wilson (Menispermaceae) were purchased from Uchida Wakanyaku Co., Tokyo. In this work, 3 kg of material was used from the same lot.

Extraction and Fractionation. - The plant material was successively extracted with n-hexane, chloroform, methanol and water under reflux. Although n-hexane and chloroform extracts showed no activity, methanol and water extracts showed significant activity. The methanol extract was two times more potent than the water extract. The methanol extract (116 g) was dissolved in 2 l of 1 N-HCl and extracted with chloroform for 5 times. The chloroform layer was evaporated in vacuo to give 22.6 g extract which showed relative activity of 0.98 to the starting MeOH extracts. The aqueous layer was made alkaline by the addition of c.NH_4OH and extracted repeatedly with chloroform. Evaporation of chloroform gave an alkaloid fraction (7.5 g) of relative activity of 6.11, while an aqueous layer showed a relative activity of 1.22.

The alkaloid fraction (7.5 g) was chromatographed on silica gel column made in chloroform. Seventy fractions were collected by elution with chloroform and chloroform-methanol (0 - 40 %). Monitering with the bioassay test, the relative activity of the fractions 13-25 were 2.70, the fractions 26-28 1.12, the fraction 29-40 0.0, the fractions 46-56 1.36 and the fracion 57-70 1.00. None of the fractions showed higher activity than the alkaloid fraction, indicatig highly active compounds were unstable for the chromatographic separation.

Isolation of sinomenine (1), disinomenine (2), acutamine (3) and liriodenine (4)- Considerably active fractions (13 - 25) gave crystals on standing which were recrystallized from chloroform to gave yellow needles of liriodenine (4), mp 280° C (decomp.). High MS m/z: Calcd for $C_{17}H_9NO_3$ 275.0580; Observed 275.0580. ^{1}H-NMR (d_6-DMSO) δ: 6.51 (s, 2H), 7.57 (s, 1H), 8.04 (d, 1H, J=5.2 Hz), 8.82 (d, 1H, J=5.2 Hz), 7.6-8.8 (m, 4H). The fractions 29 - 40 which showed no activity in the bioassay test gave sinomenine (1) and acutamine (3) which were identified by the direct comparison with authentic samples. Another fractions (46 - 56) of relatively low activity (relative activity 1.26) gave white rods of disinomenine (2), mp. 215 - 216 ($CHCl_3$). MS m/z: 656(M^+), 641, 598, 505, 328, 192. ^{1}H-NMR (d_6-DMSO) δ: 1.85 (br.s, 2H), 2.25 (s, 3H), 2.29 (s, 3H), 2.92 (br.s, 1H), 3.09 (br.s), 3.40 (s, 3H), 3.70 (s, 3H), 4.21 (d, 1H, J=15 Hz), 5.72 (d, 1H, J=5.2 Hz), 7.6 - 8.8 (4H).

Isolation of stepholidine (5) - A fraction showed considerable activity (26 -28, 1.3 g) in the second chromatography was rechromatographed on silica gel and 27 fractions were collected by elution with $CHCl_3$ and then $CHCl_3$-MeOH (0 - 10 %). The frations (15 -16, 353 mg) were further fractionated by silica gel column chromatography using benzene-acetone (0 - 20 %) as an eluting solvent. Remarkable increase of ralative activity was observed in fractions 10 - 13 (relative activity 9.65) which gave crude crystals on standing. Final purification was performed with Sephadex LH-20 column chromatography to give (-)-stepholidine (5) as brownish coloured rods which were recrystallized from acetone (6.4 mg), mp 125 - 126° C. High MS m/z: Calcd for $C_{19}H_{21}NO_4$ 327.1495, Observed 327.1483. ^{1}H-NMR (d_4-MeOH) δ: 2.72(m, 3H), 3.43 (d, 1H, J=16 Hz), 4.17 (d, 1H, J=16Hz), 3.80 (s, 3H), 3.82 (s, 3H), 6.68 - 6.83 (m, 4H). ORD (c=0.001, ethanol) [α] (nm): -1.13×10^6 (239) (trough), -2.26×10^5 (270).

X-ray analysis of (-)-stepholidine (5) - Crystals of (-)-stepholidne (5) obtained from acetone were found suitable for X-ray analysis. The crystal is orthorhombic, space group $P2_12_12_1$, with 4

molecules in a cell of dimensions a = 10.7830, b = 15.1481, c = 10.0410 Å, D_x = 1.325 g/cm^3, V = 1640.116 Å^3. A total of 1806 reflections were recorded using a Philips PW 1100 diffractometer with monochromated CuK radiation. The structure was solved by the direct method (MULTAN) and refined by the block-diagonal least-squares. The final R value was 0.048 including anisotropic temperature factors for C, N and O atoms and isotropic temperature factors for H atoms.[12]

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