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Short communication

Preparative separation of alkaloids from *Nelumbo nucifera* leaves by conventional and pH-zone-refining counter-current chromatography

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ABSTRACT

Two modes of high-speed counter-current chromatography (HSCCC) were successfully applied to the separation of alkaloids from crude extract of *Nelumbo nucifera* leaves. The conventional HSCCC separations were performed with a two-phase solvent system composed of tetrachloromethane–$CHCl_3$–methanol–0.1 M HCl at a volume ratio of 1:3:3:2 (v/v/v/v), and 120 mg crude extract could be successfully separated. pH-Zone-refining CCC was performed with a two-phase solvent system composed of petroleum ether (60–90 °C)–ethyl acetate–methanol–water (5:5:2:8, v/v/v/v) where triethylamine (10 mM) was added to the upper organic stationary phase as a retainer and hydrochloric acid (5 mM) to the aqueous mobile phase as an eluent. From 4.0 g of the crude extract, 120 mg *N*-nornuciferine, 1020 mg nuciferine and 96 mg roemerine were obtained in a single run each with a purity of over 98% as determined by HPLC. The structures of the isolated compounds were identified by ESI-MS, ^{1}H NMR and ^{13}C NMR.

1. Introduction

Nelumbo nucifera GAERTN (commonly known as lotus), a perennial aquatic herb, is used not only as an ornamental plant and a dietary staple, but also as a medicinal herb in Eastern Asia [1]. Almost all parts of *N. nucifera* Gaertn, i.e. leaves, flowers, seeds and rhizomes, have been utilized for various medicinal purposes in Chinese herbal medicine [1]. In particular, the leaves are known for diuretic and astringent properties, and are used to clean heat, resolve summer heat and stop bleeding [2]. It is also officially listed in the Chinese Pharmacopoeia. The major alkaloids components of lotus leaves are aporphine alkaloids such as *N*-nornuciferine, nuciferine, and roemerine (see Fig. 1) which have received considerable attention because of their reputation of various biological activities. *N*-Nornuciferine has been reported to inhibit CD45 protein tyrosine phosphatase [3]. Nuciferine exhibited inhibitory activity toward acetylcholine in rat Renshaw cells [4] and anti-HIV activity [2]. Roemerine has been shown to exhibit antimicrobial activity [5]. Total alkaloids from lotus leaves are effective in lowering hyperlipemia [6,7] and level of cholesterol [8], resisting karyokinesis and exhibiting antimicrobial activity [9,10].

In order to further study the biological activities of these aporphine alkaloids and to control the quality of this traditional Chinese medicine and their products, a large quality of pure compounds are urgently needed. However, the preparative separation and purification of these bioactive compounds from the lotus leaves by classical methods are very difficult, tedious and usually require multiple chromatography steps.

High-speed counter-current chromatography (HSCCC) is a support-free liquid–liquid chromatographic technique that has been successfully applied to the separation and isolation of many natural products [11,12]. pH-Zone-refining CCC, a variation of HSCCC, was developed in the mid-1990s as a novel preparative-scale separation technique [13–15]. It allows the separation of organic acids and bases into a succession of highly concentrated rectangular peaks that elute according to their pK_a values and hydrophobicities. This protocol is restricted to solutes that are ionisable and show a dramatic difference in solubility between their neutral and ionized forms [15]. Alkaloids are thus good candidates for pH-zone-refining CCC purification. However, no report has been seen on the use of HSCCC for the isolation and purification of these aporphine alkaloids from natural resource as far as we known. In the present paper, HSCCC in its two forms, conventional and pH-zone-refining CCC, was successfully applied to the preparative

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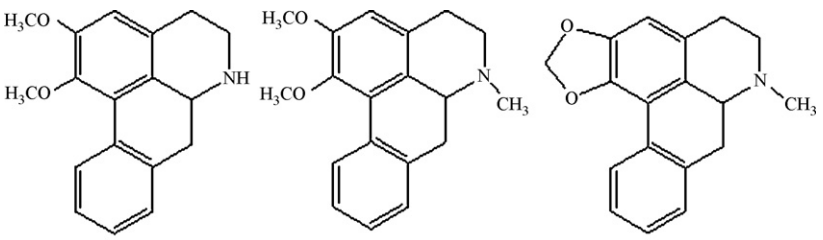

Fig. 1. Chemical structures of alkaloids from *Nelumbo nucifera* leaves.

separation of alkaloids from the crude extract of lotus leaves for the first time.

2. Experimental

2.1. Reagents and materials

Methyl *tert*-butyl ether (MtBE), hydrochloric acid (HCl), triethylamine (TEA), petroleum ether (60–90 °C), ethyl acetate, methanol, $CHCl_3$, and ethanol were of analytical grade (Jinan Juye Chemical Factory, Jinan, China). Acetonitrile used for HPLC analysis was of chromatographic grade (Tianjin Siyou Special Reagent Factory, Tianjin, China). Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions.

The raw material, a commercial extract of *N. nucifera* leaves, was purchased from Chaozhou Zerun Pharmaceutical Co. Ltd., China. The content of nuciferine is about 5%.

2.2. Apparatus

Counter-current chromatography was carried out using a Model GS10A-2 apparatus (Beijing Institute of New Technology Application, Beijing, China), with a multilayer coil of 1.6 mm i.d. and 110 m in length with a total capacity of 230 mL. The β values of this preparative column range from 0.5 at internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R (R = 8 cm) is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm and a Model 320 pH meter (Mettler Toledo Instruments (Shanghai) Co., China). A manual sample injection valve with 30 mL loop (Tianjin High New Science Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

HPLC was carried out on a Waters Millennium^{32} system including a model 996 photodiode array detector (PAD), a model 600 multisolvent delivery system, a model 600 system controller, a model 600 pump, and a Millennium^{32} work-station (Milford, MA, USA).

2.3. Preparation the sample

100 g commercial extract of lotus leaves was extracted two times by $CHCl_3$ (5 L) using an ultrasound bath (25 MHz, 250 W) for 30 min. After filtration, the extract was combined and evaporated to dryness by rotary vaporization under reduced pressure at 50 °C. 18 g of light yellow crude extract was obtained which was used for further isolation and separation.

2.4. Separation procedure

In the conventional HSCCC separation, the selected solvent system (tetrachloromethane–$CHCl_3$–methanol–0.1 M HCl, 1:3:3:2, v/v/v/v) was thoroughly equilibrated in a separation funnel by repeated vigorous shaking at room temperature. The two phases were separated shortly prior to use. The upper phase was used as the stationary phase, while the lower phase was used as the mobile phase. The sample solution was prepared by dissolving the crude extract in a solution composed of the upper and lower phases (1:1, v/v) of the solvent system used HSCCC separation. The separation was initiated by filling the entire column with the stationary phase using the pump, and then loading the sample dissolved in a mixture of stationary and mobile phases. The mobile phase was then pumped into the column at 1.5 mL/min while the column was rotated at 800 rpm in the combined head to tail elution mode. The absorbance of the eluter was continuously monitored at 254 nm. The fractions were collected according to the chromatogram and were analyzed by HPLC.

The pH-zone-refining CCC separations followed previously established procedures [16]. The two-phase solvent system used consisted of petroleum ether–ethyl acetate–methanol–water (5:5:2:8, v/v/v/v). The solvent system was equilibrated in a separatory funnel, and the two phases were separated before use. The lower phase was acidified with HCl resulted at a 5 mM solution in HCl. The upper phase was rendered basic by addition of TEA resulted at a 10 mM solution in TEA. The basic organic phase was used as the stationary phase and the acidic lower phase was used as the mobile phase. The separation was initiated by filling the entire column with the stationary phase using the pump, and then loading the sample dissolved in a mixture of stationary and aqueous phases (in the ratio of 3:1, e.g. 30 mL:10 mL for a 4.0 g sample portion). The mobile phase was then pumped into the column at 1.5 mL/min while the column was rotated at 800 rpm in the combined head to tail elution mode. The absorbance of the eluent was continuously monitored at 254 nm and 5-mL fractions were collected. The pH of each eluted fraction was measured with a pH meter. The fractions collected were brought to dryness using a rotary vaporization under reduced pressure and were analyzed by HPLC. The HCl and traces of triethylamine were removed from target compounds by water washing.

After the separation was completed, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas.

2.5. HPLC analysis

The crude sample and each purified fraction from the CCC separation were analyzed by HPLC with a Welch Materials C_{18} (250 mm × 4.6 mm, i.d., 5 μm) at 270 nm and column temperature of 25 °C. The mobile phase, a solution of acetonitrile: 0.1% TEA in gradient elution mode (0–15 min, 40% acetonitrile to 70% acetoni-

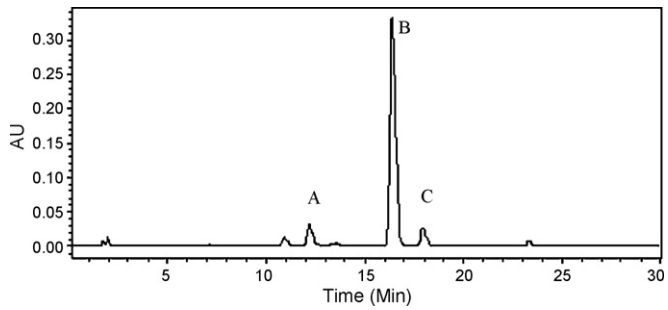

Fig. 2. The results of HPLC analyses of the crude alkaloids from *Nelumbo nucifera* leaves.

trile, 15–20 min, 70% acetonitrile to 100% acetonitrile), was set at a flow-rate of 1 mL/min. The effluent was monitored by a photodiode array detector.

3. Results and discussion

3.1. Optimisation of HPLC conditions

The optimum HPLC condition was aimed at chromatograms with good resolution of adjacent peaks within a short analysis time, especially when numerous samples were to be analyzed. Several elution systems were tested in HPLC separation of crude sample, such as gradient elution of methanol–0.1%TEA, acetonitrile–0.1%TEA, methanol–acetonitrile–0.1%TEA, etc. The results indicated that when acetonitrile–0.1%TEA was used as mobile phase in gradient elution mode (0–15 min, 40% acetonitrile to 70% acetonitrile, 15–20 min, 70% acetonitrile to 100% acetonitrile), the major peaks were obtained and each peak achieved baseline separation. The HPLC chromatogram of crude extract is shown in Fig. 2, which contained several peaks, and peaks A, B and C correspond to *N*-nornuciferine, nuciferine and roemerine, respectively.

3.2. Separation of the crude extract of N. nucifera leaves by conventional HSCCC

Because the sample solvent was extracted by $CHCl_3$, the target compounds mainly behave hydrophobic. Preliminary HSCCC studies were carried out with the two-phase solvent system composed of $CHCl_3$–methanol–0.1 M HCl (4:3:2, v/v/v), which has been used for many kinds of alkaloids [17–19]. Experiments showed that the target compounds mainly partitioned in the lower phase. By adding tetrachloromethane to the $CHCl_3$–methanol–0.1 M HCl, the partition could be improved. When the solvent system tetrachloromethane–$CHCl_3$–methanol–0.1 M HCl at the volume ratio of 1:3:3:2 was used, good separation could be achieved. Fig. 3 shows the preparative HSCCC separation of 120 mg of the crude sample using the optimized solvent system. The retention of the stationary phase was 78%, and the separation time was about 7 h in each separation run. Three kinds of compounds were obtained, yielding ~3 mg of A, 28 mg of B and ~2 mg of C with the purity of 99.0%, 98.7%, 98.1%, respectively. The HPLC chromatograms of these compounds are also shown in Fig. 3. Attempts to separate larger quantities of this mixture by conventional HSCCC failed mainly due to the poor retention of the stationary phase and resulted in a poor resolution.

3.3. Separation of the crude extract of N. nucifera leaves by pH-zone-refining CCC

In order to separate a large amount of target compounds from the crude extract of lotus leaves, a different approach was necessary. pH-Zone-refining CCC has been successfully applied to the separation of ionizable compounds at the multigram level [11]. According to the rule for the selection of

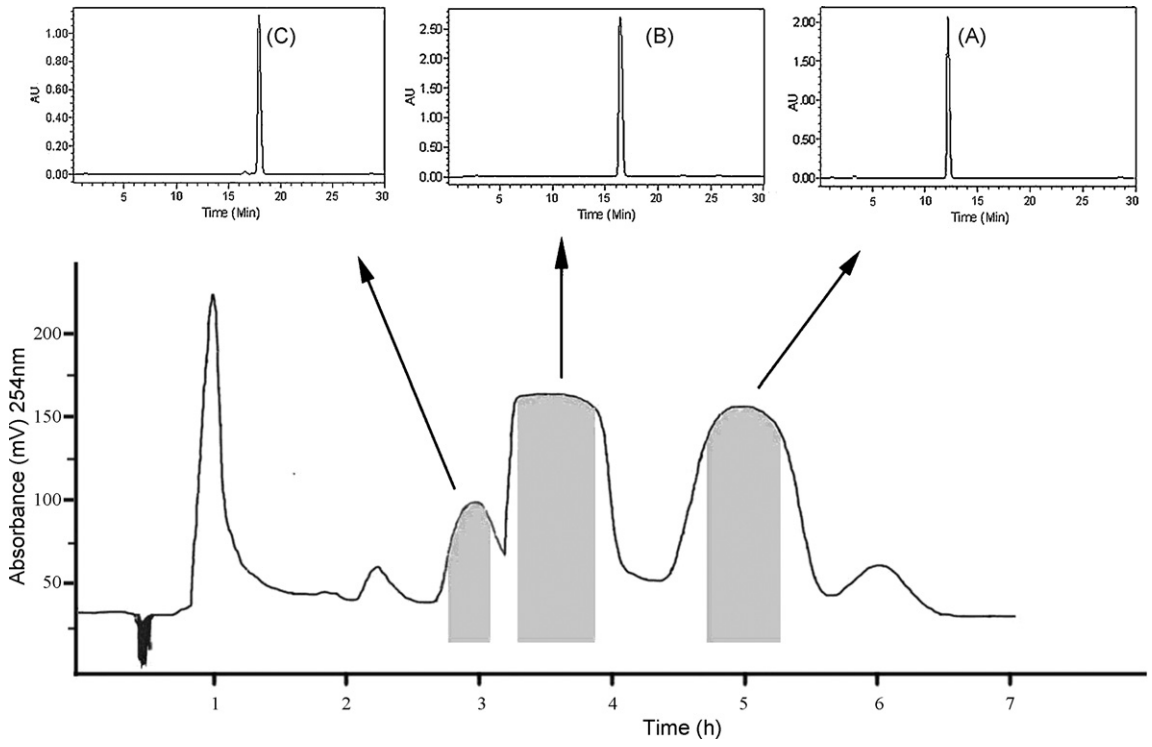

Fig. 3. High-speed counter-current chromatogram and HPLC control for the separation of 120 mg of alkaloid extract from *Nelumbo nucifera* leaves. Experiment condition: solvent system: tetrachloromethane–$CHCl_3$–methanol–0.1 M HCl (1:3:3:2, v/v/v/v); retention of stationary phase: 78%; flow-rate: 1.5 mL/min; detection: 254 nm; revolution speed: 800 rpm.

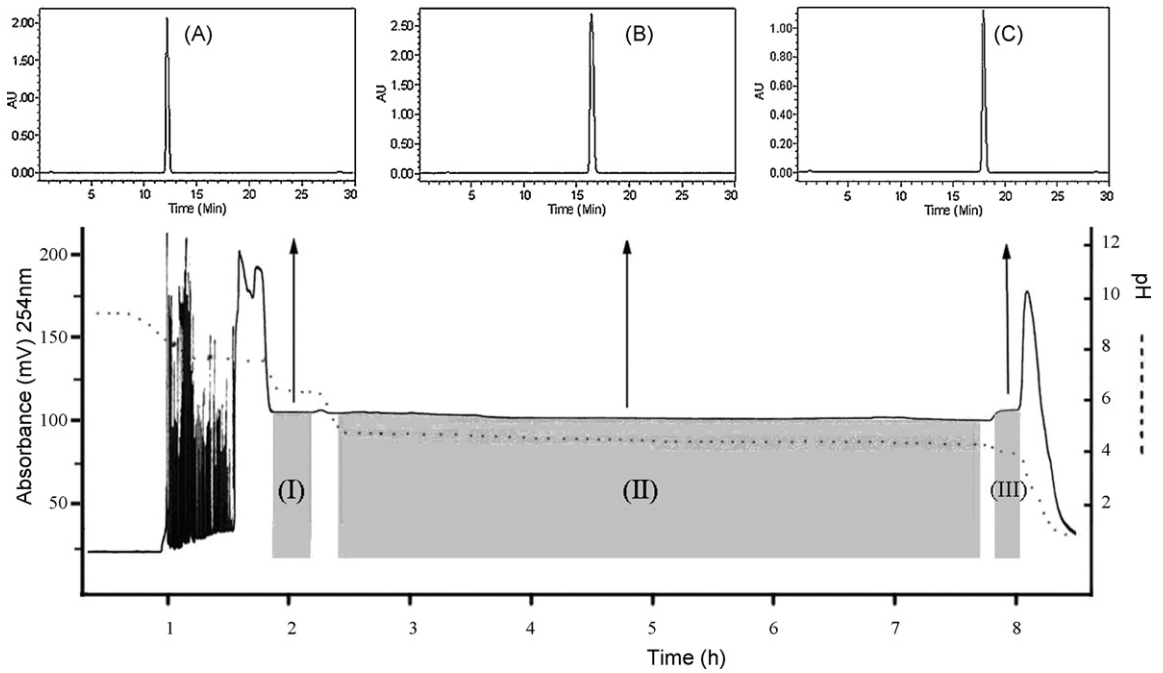

Fig. 4. pH-Zone-refining counter-current chromatogram and HPLC control for the separation of 4.0 g of alkaloid extract from *Nelumbo nucifera* leaves. Experiment condition: solvent system: petroleum ether–ethyl acetate–methanol–water (5:5:2:8, v/v/v/v), 10 mM TEA in the upper organic stationary phase and 5 mM HCl in the lower phase; retention of stationary phase: 60%; flow-rate: 1.5 mL/min; detection: 254 nm; revolution speed: 800 rpm.

the solvent system described in the references [11], the new quaternary biphasic solvent systems composed of petroleum ether–ethyl acetate–methanol–water at volume ratios of 5:5:5:5 and 5:5:2:8 were tested. After trying both of the above solvent systems, we found the solvent system petroleum ether–ethyl acetate–methanol–water 5:5:2:8 (v/v/v/v) where triethylamine (10 mM) was added to the upper organic stationary phase as a retainer and hydrochloric acid (5 mM) to the aqueous mobile phase as an eluent was suitable for the preparative separation.

Fig. 4 shows a typical chromatogram obtained for the separation of 4.0 g of the crude extract by pH-zone-refining CCC. The retention of the stationary phase was 60%, and the total separation time was about 8 h. Alkaloids were eluted as an irregular rectangular peak where three absorbance plateaus were observed. The measurement of the collected fractions also revealed three flat pH zones, I, II and III which respectively correspond to the above absorbance plateaus, suggesting the successful separation of three components. Considerable amounts of impurities were eluted in the front and the back of the main peak, forming multiple peaks.

Based on the HPLC analysis and the elution curve of the pH-zone-refining CCC, all collected fractions were combined into different pooled fractions. The three main fractions obtained from CCC separations were evaporated under reduced pressure and lyophilized to dryness. 120 mg compound A (fraction I in Fig. 4), 1020 mg compound B (fraction II in Fig. 4), and 96 mg compound C (fraction III in Fig. 4) were obtained in one step separation after an injection of 4.0 g of a crude extract, with the purity of 98.7%, 99.0% and 98.0%, respectively, as determined by HPLC (Fig. 4).

3.4. Identification of the isolated compounds

Identification of pH-zone-refining CCC purified fractions was carried out by ESI-MS (operated in the positive ion mode in the range of *m*/*z* 100–1000), ^{1}H NMR and ^{13}C NMR spectra with TMS as internal standard.

N-nornuciferine (compound A): Positive ESI–MS, *m*/*z* 282 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 8.40 (1H, d, J = 8.4 Hz, H-11), 7.21–7.33 (3 H, m, H-8, 9, 10), 6.66 (1H, s, H-3), 3.89 (3 H, s, C_1–OCH_3), 3.66 (3 H, s, C_2–OCH_3), 3.50, 3.51 (N–H, dd, J = 5.4, 6.0 Hz, N–H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 145.5 (C-1), 126.7 (C-1a), 152.6 (C-2), 111.7 (C-3), 28.2 (C-4), 128.5 (C-4a), 42.6 (C-5), 60.3 (C-6a), 36.5 (C-7), 135.3 (C-7a), 128.0 (C-8), 127.6 (C-9), 127.2 (C-10), 128.0 (C-11), 132.0 (C-11a), 53.4 (OCH_3), 56.0 (OCH_3).

Nuciferine (compound B): Positive ESI–MS, *m*/*z* 296 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 8.36 (1H, d, J = 8.4 Hz, H-11), 7.21–7.32 (3 H, m, H-8, 9, 10), 6.63 (1H, s, H-3), 3.89 (3 H, s, C_1–OCH_3), 3.66 (3 H, s, C_2–OCH_3), 2.54 (3H, s, N–CH_3). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 145.1 (C-1), 126.8 (C-1a), 151.9 (C-2), 111.2 (C-3), 29.3 (C-4), 128.3 (C-4a), 53.3 (C-5), 44.0 (N-CH_3), 62.3 (C-6a), 35.2 (C-7), 136.5 (C-7a), 127.9 (C-8), 127.3 (C-9), 127.0 (C-10), 128.0 (C-11), 132.2 (C-11a), 55.9 (OCH_3), 60.2 (OCH_3).

Romerine (compound C): Positive ESI–MS, *m*/*z* 280 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 8.06 (1H, d, J = 7.8 Hz, H-11), 7.22–7.33 (3H, m, H-8, 9, 10), 6.66 (1H, s, H-3), 6.09 (1H, d, J = 1.2 Hz, OCH_2), 5.94 (2H, d, J = 1.8 Hz, OCH_2), 2.54 (3H, s, N–CH_3). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 142.6 (C-1), 116.4 (C-1a), 146.7 (C-2), 107.5 (C-3), 29.2 (C-4), 128.2 (C-4a), 53.6 (C-5), 44.0 (N–CH_3), 62.1 (C-6a), 34.7 (C-7), 135.4 (C-7a), 127.0 (C-8), 126.9 (C-9), 126.6 (C-10), 131.1 (C-11a), 127.5 (C-11), 100.7 (OCH_2).

After comparing the data with spectral information from literature [20–23], compounds A–C were confirmed as *N*-nornuciferine, nuciferine, and roemerine, respectively.

4. Conclusion

This work presents a successful application of CCC, in both its conventional and pH-zone-refining modes, to the preparative separation of *N*-nornuciferine, nuciferine and roemerine from the crude extract of *N. nucifera* leaves. Conventional HSCCC is useful for separating up to hundred milligram quantities of this sample, while pH-zone-refining CCC results in excellent separations at the multigram level. Comparing with the two modes, it is clearly demonstrated that pH-zone-refining CCC has many advantages over conventional CCC, for example an over 10-fold

increase in sample-loading capacity, high purity, and high concentration of the collected fractions. The present method may be applied to purification of various other alkaloids from natural products.

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