Cremastrine, a Pyrrolizidine Alkaloid from *Cremastra appendiculata*

Yoshitaka Ikeda,*,† Hikaru Nonaka,† Tamotsu Furumai,‡ and Yasuhiro Igarashi‡

Pharmaceuticals Research Unit, Mitsubishi Pharma Corporation, 2-25-1, Shodai-Ohtani, Hirakata, Osaka 573-1153, Japan, and Biotechnology Research Center, Toyama Prefectural University, 5180, Kurokawa, Kosugi, Toyama 939-0398, Japan

A new pyrrolizidine alkaloid, cremastrine (**1**), was isolated from the bulbs of *Cremastra appendiculata*. Its configuration was determined by spectroscopic and chemical analyses. Compound **1** inhibited the binding of tritium-labeled *N*-methylscopolamine to the muscarinic M3 receptor with a K_i value of 126 nM.

Anticholinergic alkaloids such as atropine have been used for a century for the treatment of obstructive airway diseases, although they have unfavorable peripheral and central nervous system effects such as tachycardia, mydriasis, and dementia.[1] To date, five muscarinic acetylcholine receptor subtypes, m1−m5, have been identified and cloned.[2] Of these, M3 receptors, localized in smooth muscle and mucosal glands, mediate contraction and mucus secretion, respectively. Subtype-selective surrogate ligands that recognize the different localizations and functions of these receptor subtypes provide the possibility of developing drugs since they may avoid the occurrence of adverse effects. It has been hypothesized that the selective blockade of muscarinic M3 receptors may be therapeutically useful in the treatment of respiratory disorders such as chronic obstructive pulmonary disease, gastrointestinal disorders such as irritable bowel syndrome, and urinary tract disorders such as urinary incontinence.[3] Plants and microorganisms have been reliable sources of new drugs, and much emphasis has been placed on finding novel drug candidates from these sources. Despite extensive screening studies, however, there is still no compound that can bind selectively to M3 receptors.

In the course of our screening for muscarinic M3 receptor binding inhibitors from natural sources, the extract of Santsigu Tuber, a dried bulb of *Cremastra appendiculata* (D.Don) Nakai (Orchidaceae), was found to be highly active. Santsigu Tuber is used for the treatment of burns, chaps, and frostbite in Japan and used as an antidote for snake and insect bites in China. This plant is widely distributed in Japan and China, but its chemical constituents and their bioactivities have not been studied. Bioassay-guided chromatography of the extract of Santsigu Tuber provided a new pyrrolizidine alkaloid, cremastrine (**1**), as an active principle. This paper describes the isolation, structure determination, and its inhibition of the muscarinic M3 receptor.

The dried bulbs of *C. appendiculata* were extracted with 70% aqueous EtOH, and the extract was concentrated in vacuo. The residual aqueous solution was acidified with HOAc and extracted with EtOAc. The aqueous layer was then basified with aqueous ammonia solution and extracted successively with EtOAc and *n*-BuOH. The alkaline EtOAc extract was fractionated by preparative HPLC using an ODS column. The active fractions were further purified to afford **1** by preparative HPLC using a C_{30} column. Com-

pound **1** was also obtained from the *n*-BuOH extract in a similar manner.

Compound **1** was isolated as colorless syrup. It is alkaline but was negative in the ninhydrin color reaction test, indicating the presence of a tertiary amine group. The molecular formula of **1** was determined as $C_{14}H_{25}NO_3$ on the basis of the high-resolution LC/MS ([M + H]+, *m/z* 256.1900) and 13C NMR spectroscopic data.

The 1H NMR spectrum of **1** showed the presence of one hydroxyl group (δ 6.90) and two methyl groups (δ 1.08 and 0.95, H-5′ and -6′). The 13C NMR and DEPT spectra revealed the presence of 14 carbon signals comprising one ester carbonyl carbon (δ 174.0, C-1′), one oxymethine carbon (δ 72.8, C-2′), one oxymethylene carbon (δ 63.0, C-9), one nitrogen-bearing methine carbon (δ 66.5, C-8), and two nitrogen-bearing methylene carbons (δ 54.3 and 52.4, C-3 and -5). The remaining carbon signals were assigned to two methyls, four methylenes, and two methines. Further analyses of COSY, HMQC, and HMBC spectra suggested that **1** consisted of a pyrrolizidine (C-1−C-8) moiety and a 2-hydroxy-3-methylpentanoic acid (Hmp, C-1′−C-6′) moiety connected through hydroxymethyl (C-9) at C-1. In addition, HMBC correlations from the hydroxymethylene protons (H-9) to the carbonyl carbon (C-1′) and to C-1, -2, and -8 indicated the position of the ester attachment at C-9. Thus, **1** was characterized as the Hmp ester of 1-hydroxymethylpyrrolizidine.
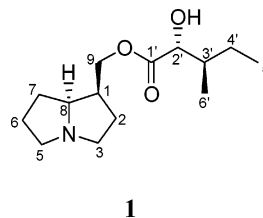
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Alkaline hydrolysis of **1** gave Hmp and 1-hydroxymethylpyrrolizidine. The absolute configuration of the Hmp moiety was determined to be (2*R*, 3*R*) by comparison with four synthetic stereoisomers of Hmp in a chiral HPLC analysis.[4] The 1-hydroxymethylpyrrolizidine moiety was estimated as (−)-isoretronecanole (1*S*, 8*S*-form) or its antipode based on the NOESY spectrum of **1** that showed a positive NOE between the protons at H-1 and H-8, indicating the *cis*-configuration for these protons. As the optical rotation of the 1-hydroxymethylpyrrolizidine, [α]20D −75.9 (*c* 0.027, EtOH), was in good agreement with that of (−)-isoretronecanole, [α]20D −77.0 (*c* 1.0, EtOH),[5] the absolute configuration of the 1-hydroxymethylpyrrolizidine

* To whom correspondence should be addressed. Tel: +81-72-856-9205. Fax: +81-72-868-9597. E-mail: Ikeda.Yoshitaka@mc.m-pharma.co.jp.
† Mitsubishi Pharma Corporation.
‡ Toyama Prefectural University.

Table 1. K_i Values of **1** to Five Subtypes, M1−M5

	K_i (nM)		K_i (nM)
M1	505	M4	498
M2	>5517	M5	1220
M3	126		

was determined as (1*S*, 8*S*). There are many reports on the esters of 1-hydroxymethylpyrrolizidine[6] such as the (−)-trachelanthic acid ester (heliocurassavicine) and the (−)-curassavic acid ester (heliocurassavine) of (−)-isoretronecanole isolated from *Heliotropium curassavicum* L.[7] Cremastrine (**1**) is the first example of the Hmp ester of 1-hydroxymethylpyrrolizidine including (−)-isoretronecanole.[8]

The inhibition activities of cremastrine (**1**) to muscarinic receptor subtypes are summarized in Table 1. Compound **1** showed selective inhibition of the muscarinic M3 receptor. It inhibits the binding of tritium-labeled *N*-methylscopolamine ([³H]-NMS) to the muscarinic M3 receptor with an IC$_{50}$ of 594 nM (K_i = 126 nM). Some pyrrolizidine alkaloids such as cynaustraline [viridifloric acid ester of (+)-isoretronecanole] showed antagonistic activity against the muscarinic receptor in the guinea-pig ileum, but their selectivity for M1−M5 receptors is unknown.[9] Further studies on the pharmacological functions of cremastrine (**1**) are in progress.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 digital polarimeter using a 5 cm cell. NMR spectra were measured on a Bruker AMX-500 spectrometer using standard Bruker pulse programs. Chemical shifts are given in δ values with reference to tetramethylsilane as an internal standard. IR specra were recorded on a Perkin Elmer 1725X FT-TR spectrophotometer. The MS spectra were measured on an Agilent MSD spectrometer, and high-resolution MS spectra were measured on a JEOL JMS-700 spectrometer.

Plant Material. The plant material was purchased from Alps Pharmaceutical Ind. Co. Ltd. (Gifu, Japan). A small amount of the sample is preserved in our laboratory.

Extraction and Isolation. The plant material (150 g) was extracted with 70% aqueous EtOH (1.5 L) overnight at room temperature, and the EtOH solution was concentrated in vacuo. The residual aqueous solution (ca. 400 mL) was adjusted to pH 2.8 with HOAc and extracted with EtOAc (250 mL, ×2), and the EtOAc layer was discarded. Then, the aqueous layer was adjusted to pH 9.4 with aqueous ammonia solution and extracted with EtOAc (250 mL ×3) and *n*-BuOH (250 mL ×3). The EtOAc and *n*-BuOH layers were separately evaporated in vacuo. The alkaline EtOAc extract (274.3 mg) was fractionated by preparative HPLC using an ODS column (Delta-Pak C$_{18}$, 40 mm i.d. × 100 mm, Waters, MA) with the eluent of CH$_3$CN−0.05% TFA (a linear gradient from 5% to 50% of CH$_3$CN over 20 min) at a flow rate of 80 mL/min. On the basis of results of a muscarinic M3 receptor binding assay, the active fractions were combined and evaporated in vacuo. The active fraction was further purified to afford **1** (15.4 mg) by preparative HPLC using a C$_{30}$ column (Develosil C30-UG, 20 mm i.d. × 150 mm, Nomura Chemical Co. Ltd., Japan) with CH$_3$CN−0.2% HOAc (a linear gradient from 0% to 50% of CH$_3$-CN over 30 min) at a flow rate of 20 mL/min. Compound **1** (10.1 mg) was also obtained from the *n*-BuOH extract (998.0 mg) in a manner similar to that described above.

Cremastrine (1): colorless syrup; [α]$^{25}_D$ −26.8 (*c* 1.0, EtOH); IR (film) ν_{max} 3412, 2967, 2879, 1732, 1681, 1463, 1203, 1141 cm^{-1}; ¹H NMR (pyridine-d_5, 500 MHz) δ 6.90 (1H, br s, −O*H*), 4.50 (1H, d, *J* = 3.9 Hz, H-2′), 4.33 (1H, dd, *J* = 6.7, 11.1 Hz, H-9a), 4.24 (1H, dd, *J* = 7.9, 11.1 Hz, H-9b), 4.17 (1H, m, H-8), 3.64 (1H, br t, *J* = 7.5 Hz, H-5a), 3.45 (1H, dt, *J* = 6.4, 11.1 Hz, H-3a), 2.93 (1H, br t, *J* = 8.3 Hz, H-3b), 2.76 (1H, m, H-1), 2.66 (1H, dt, *J* = 6.4, 10.3 Hz, H-5b), 2.03 (1H, m, H-3′), 1.89 (1H, m, H-2a), 1.85−1.75 (3H, m, H-6, H-7a),

1.75−1.70 (2H, m, H-2b, H-4′a), 1.59 (1H, m, H-7b), 1.43 (1H, m, H-4′b), 1.08 (3H, d, *J* = 7.5 Hz, H-6′), 0.95 (3H, t, *J* = 6.8 Hz, H-5′); ¹³C NMR (pyridine-d_5, 125 MHz) δ 174.0 (C, C-1′), 72.8 (CH, C-2′), 66.5 (CH, C-8), 63.0 (CH$_2$, C-9), 54.3 (CH$_2$, C-5), 52.4 (CH$_2$, C-3), 39.1 (CH, C-1), 38.1 (CH, C-3′), 25.9, 25.4, 25.3, 25.0 (CH$_2$, C-2, -6, -7, -4′), 13.3 (CH$_3$, C-6′), 10.9 (CH$_3$, C-5′); HRMS (positive mode) *m/z* 256.1900 [M + H]$^+$ (calcd for C$_{14}$H$_{26}$-NO$_3$, 256.1913).

Alkaline Hydrolysis of 1. A solution of **1** (2.3 mg) in 0.5 N NaOH (0.4 mL) was refluxed for 1 h. After cooling, the reaction mixture was neutralized with HOAc and further purified to afford Hmp (1.1 mg) and 1-hydroxymethylpyrrolizidine (0.27 mg) by preparative HPLC under the following conditions: column, XTerra Prep RP18 column (10 mm i.d. × 150 mm, Waters, MA); eluent, CH$_3$CN−aqueous ammonia solution (pH 10.9, a linear gradient from 2.5% to 50% of CH$_3$-CN over 20 min); flow rate, 4.0 mL/min; oven temperature, 40 °C; detection, UV at 220 nm.

Synthesis of Hmp Stereoisomers. A solution of NaNO$_2$ (103 mg) in water (1.0 mL) was added to a stirred and ice-cooled solution of L-isoleucine (L-Ile, 131 mg) in 1 N H$_2$SO$_4$ (2.0 mL). The mixture was stirred for 13 h and then extracted with Et$_2$O (6.0 mL). The Et$_2$O solution was washed with brine, dried over Na$_2$SO$_4$ (anhydrous), and evaporated in vacuo to give 2*S*,3*S*-Hmp (52.5 mg, 40%): ¹H NMR (pyridine-d_5, 500 MHz) δ 10.56 (2H, br s, −O*H*), 4.48 (1H, d, *J* = 4.4 Hz, H-2), 2.21 (1H, dddq, *J* = 4.4, 4.5, 7.0, 9.0 Hz, H-3), 1.86 (1H, ddq, *J* = 4.5, 7.5, 13.5 Hz, H-4a), 1.54 (1H, ddq, *J* = 7.5, 9.0, 13.5 Hz, H-4b), 1.21 (1H, d, *J* = 7.0 Hz, H-6), 0.96 (3H, t, *J* = 7.5 Hz, H-5); ¹³C NMR (pyridine-d_5, 125 MHz) δ 176.4 (C, C-1), 74.6 (CH, C-2), 38.4 (CH, C-3), 23.5 (CH$_2$, C-4), 15.0 (CH$_3$, C-6), 11.0 (CH$_3$, C-5). In the same manner as described above, d-Ile, allo-L-Ile, and allo-D-Ile afforded 2*R*,3*R*-Hmp, 2*S*,3*R*-Hmp, and 2*R*,3*S*-Hmp, respectively.

Chiral HPLC Analysis of Hmp. Chiral HPLC analysis was achieved using SUMICHIRAL OA-5000 [4 mm i.d. × 150 mm (Osaka, Japan); flow rate, 0.7 mL/min; eluent, *i*-PrOH−H$_2$O containing 2.0 mM of CuSO$_4$ (15:85); oven temperature, 40 °C; detection, UV at 238 nm]. The retention times of the synthetic Hmp stereoisomers were as follows; 2*S*,3*R*-Hmp (37.0 min), 2*S*,3*S*-Hmp (42.4 min), 2*R*,3*R*-Hmp (58.9 min), and 2*R*,3*S*-Hmp (67.8 min). The retention time of the Hmp derived from **1** was 58.9 min.

Muscarinic M3 Receptor Binding Assay. The binding affinities (K_i) to five subtypes were determined by inhibition of specific binding of [³H]-NMS using the human receptor membranes at MDS Pharma Services (Taiwan). In competitive experiments, membranes from insect Sf9 cells stably expressing cloned human m1−m5 were incubated with 0.29 nM [³H]-NMS in the medium consisting of a buffer containing 50 mM Tris-HCl, 10 mM MgCl$_2$, and 1 mM EDTA at pH 7.4 and 25 °C for 1 h. Nonspecific binding was determined in the presence of 1 μM atropine. IC$_{50}$ values were determined from competition binding curves and converted to apparent K_i values using the Cheng−Prusoff equation.[10]

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