ISOLATION AND IDENTIFICATION OF A NOVEL MICROBIAL ALKALOID

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(Received for publication July 12, 1993)

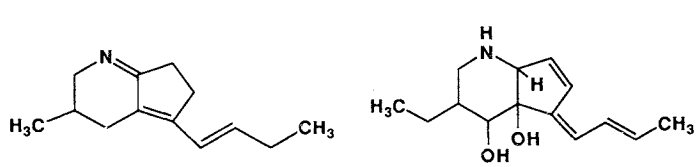
In the course of screening for unique microbial products with pharmacological activity, we have isolated two metabolites from the fermentation filtrate of an unidentified *Streptomyces* sp. SCC 2313, which exhibited an affinity for muscarinic receptors. The assay directed separation of the active ingredients provided two compounds **1** and **2**. Based on physico-chemical characteristics, we have elucidated the structure of both the compounds. While microbial product **1** was found to be a known compound and was identical to pyrindicin[1,2], a microbial alkaloid, compound **2**, has not previously been reported in the literature. This paper describes the production, isolation, physico-chemical properties and structure elucidation of these novel natural products.

Streptomycetes sp. SCC 2313 was isolated from a cultured loam soil sample obtained from Thailand. Two and one half milliliters of the producing culture, maintained at −80°C, were inoculated in a 250-ml Erlenmeyer flask to a 70 ml sterile seed medium consisting of (w/v): glucose 1.0%, trehalose 1.0%, EHC (enzyme hydrolyzed casein) 0.5%, soyflour 0.5%, yeast extract 0.5%, $CaCO_3$ 0.2%, and Dow Corning Antifoam Type B emulsion 1 ml/liter. The pH was adjusted to 7.2, prior to sterilization. The resulting broth was incubated for 48 hours at 30°C on a rotary shaker operating at 300 rpm and then used to inoculate (5% v/v) a second germination stage using the same conditions. Five milliliters of the second seed culture were inoculated into 100 ml of sterile production medium consisting of (w/v): soluble starch 2%, cerelose 1%, Bactopeptone 0.5%, and malt extract 0.5%, with presterilization pH adjustment to 7.8, in a 500-ml Erlenmeyer shake flask. Fermentation proceeded for 90 hours at 30°C on a rotary shaker operating at 300 rpm.

The steps leading to the isolation and purification of **1** and **2** are outlined in Fig. 2. A 4 liters fermentation broth was filtered to remove the cells and the muscarinic active ingredients from the filtrate were adsorbed onto Amberlite XAD-2 resin. The charged resin was separated from the inactive spent filtrate, washed with water and the active ingredients were eluted with a mixture of water and methanol (1 : 1). Further purification included ion exchange chromatography on a strong cation exchanger SP-Sephadex C-25 (H^+) and eluted with 1.0 N NaCl. The active eluate was desalted on a polymeric reverse phase CHP-20 column and eluted with a mixture of methanol-water (1 : 1). Final purification and separation of **1** and **2** was achieved by preparative HPLC on a PLRP-S reverse phase column (styrene divinyl benzene polymeric column, 3 × 25 cm) and elution with 0.08% TFA-acetonitrile (8 : 2). The biologically active eluates upon lyophillization afforded 30 and 28 mg of **1** and **2**, respectively.

The physico-chemical properties of compound **1** and **2** are shown in Table 1. The compound **1** was isolated as a pale yellow solid, soluble in water, $[\alpha]_D^{21.5} = -38.8$ (H_2O, *c* 0.05). It is basic in nature but showed a negative Ninhydrin color reaction indicating the absence of primary or secondary amine groups. The low resolution FAB mass spectrum displayed an intense protonated $(M+H)^+$ peak at 190. Peak matching, using high resolution mass measurements, showed the elemental composition to be $C_{13}H_{19}N$ (obsd *m/z* 190.1596; calcd for $C_{13}H_{20}N$ 190.1598) suggesting five degrees of unsaturation. The UV spectrum of this compound showed absorption maxima at 310 nm, and the IR spectrum displayed peaks at 3430, 1680, 1635, 1205, and 1135 cm^{-1}. The chemical shifts of verious proton signals in ^1H NMR spectra are

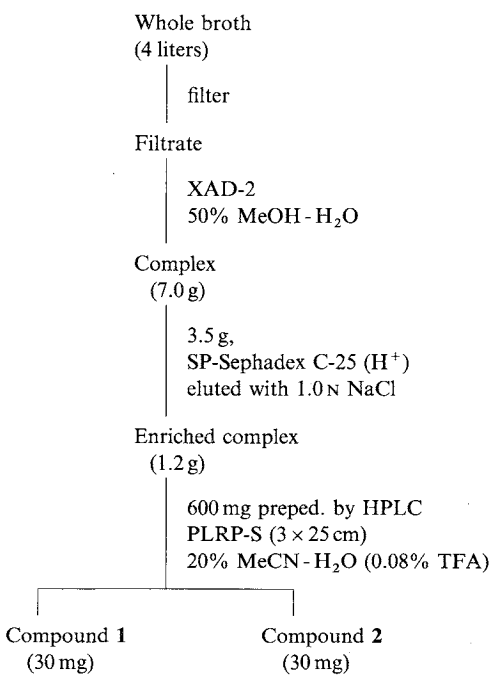
Fig. 1. Structures of compounds **1** and **2**.



1 **2**

shown in Table 1. The 300 MHz ^{1}H NMR spectrum showed one primary methyl group and a secondary methyl group, eleven protons attached to saturated carbons (two of which on a carbon attached to nitrogen) and two olefinic protons. The ^{13}C NMR spectrum was measured in D_2O at 75 MHz, and revealed the presence of 13 carbon atoms, supporting the above elemental composition. The ^{13}C NMR APT experiments revealed two methyl carbon signals (14.0, 19.1 ppm), five methylene carbon signals (28.6, 29.1, 30.4, 31.5 and 51.4 ppm) one saturated methine (27.5 ppm) two unsaturated methine carbon signals (124.5, 152.6 ppm) and three unsaturated quaternery carbon signals (130.8, 144.8, 173.3 ppm). The methylene at 51.4 ppm is linked to nitrogen and is responsible for the two proton signals at δ 3.16 and δ 3.70. The quaternary carbon chemical shift at 173.3 appears to be due to an unsaturated carbon attached to nitrogen.^{13}C NMR chemical shifts indicate three double bonds and hence the structure should contain two rings. Based on 2D(^{1}H-^{1}H) studies, we arrived at structure **1**. This structure is identical to the structure reported for pyrindicin[1,2], a microbial alkaloid. However we could not obtain a sample of pyrindicin for direct comparison.

Fig. 2. Isolation and purification scheme for compounds **1** and **2**.

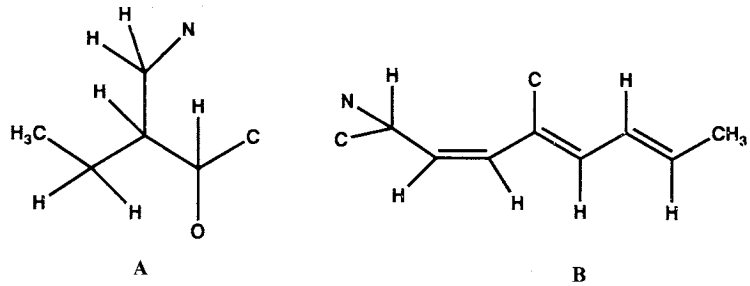


Compound **2** is also a pale yellow solid, soluble in water, $[\alpha]_D^{21.5} = -68.6$ (H_2O, c 0.04) and showed a positive Ninhydrin color reaction. The UV spectrum of **2** was different from that of **1**, and showed a maximum at 275 nm. The IR spectrum displayed peaks at 3440, 1665, 1615, 1205, 1135 cm^{-1}. The FAB mass spectrum showed an intense protonated peak at 236 $(M+H)^+$. High resolution mass measurements revealed the molecular formula to be $C_{14}H_{21}NO_2$ (calcd for $C_{14}H_{22}NO_2$, 236.1650,

Table 1. Physico-chemical properties of compounds **1** and **2**.

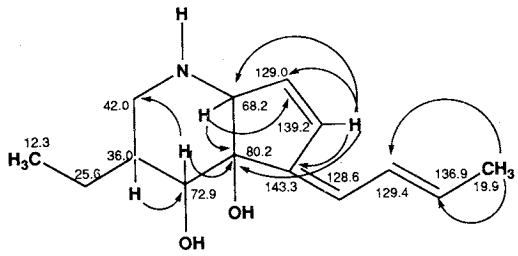
$[\alpha]_D^{21.5}$ (Water)	−38.8	−68.6
UV (MeOH) λ_{max} nm	310	275
IR (KBr) ν_{max} cm^{-1}	3430, 1680, 1635, 1205, 1135	3440, 1665, 1615, 1205, 1135
CI-MS	190 $(M+H)^+$	236 $(M+H)^+$
HR-MS	$C_{13}H_{20}N_2$ Calcd: 190.1596 Found: 190.1598	$C_{14}H_{22}NO_2$ Calcd: 236.1650 Found: 236.1645
^{1}H NMR (D_2O) δ	0.98 (d, J=6.5 Hz, 3H), 1.01 (t, J=6 Hz, 3H), 2.00 (m, 2H), 2.06 (dd, J=15, 9 Hz, 1H), 2.26 (dt, J=7.5, 5 Hz, 2H), 2.69 (dd, J=15, 2 Hz, 1H), 2.90 (br s, 2H), 2.92 (br d, J=15 Hz, 1H), 3.16 (dd, J=15, 5 Hz, 1H), 3.70 (dd, J=15, 3Hz, 1H), 6.60 (d, J=13 Hz, 1H), 6.70 (dt, J=12.5, 5 Hz, 1H)	0.76 (d, J=7.5 Hz, 3H), 1.32 (dq, J=7.5, 7 Hz, 2H), 1.50 (dt, J=7, 6 Hz, 1H), 1.70 (dd, J=1, 6 Hz, 3H), 2.75 (dd, J=11, 6 Hz, 1H), 3.00 (dd, J=11, 6 Hz, 1H), 3.90 (d, J=1 Hz, 1H), 4.20 (s, 1H), 5.90 (dq, J=11, 6 Hz, 1H), 5.95 (d, J=6 Hz, 1H), 6.06 (d, J=11 Hz, 1H), 6.40 (ddd, J=15, 10, 2 Hz, 1H), 6.98 (d, J=6 Hz, 1H)
^{13}C NMR (D_2O) ppm	14.0 (−CH_3), 19.1 (−CH_3), 27.5 (−CH<), 28.6 (−CH_2−), 29.1 (−CH_2−), 30.4 (−CH_2−), 31.5 (−CH_2−), 51.4 (−CH_2−), 124.5 (−CH=), 130.8 (>C=), 144.8 (>C=), 152.6 (−CH=), 173.3 (C=N)	12.3 (−CH_3), 19.9 (−CH_3), 25.6 (−CH_2−), 36.0 (−CH<), 42.0 (−CH_2−), 68.2 (−CH<), 72.9 (−CH<), 80.2 (>C<), 128.6 (−CH=), 129.0 (−HC=), 129.4 (−CH=),136.9 (−CH=), 139.2 (−HC=), 143.3(>C=)

Fig. 3. Fragments established by COSY expt.



A B

Fig. 4. Long-range ^{1}H-^{13}C coupling observed in the SINEPT spectra of compound **2**.



obsd 236.1645) indicating five degrees of unsaturation. The 300 MHz ^{1}H NMR in D_2O of **2** showed two methyl groups, one primary methyl at δ 0.76 and another secondary methyl at δ 1.70. The primary methyl group is coupled to the methylene signal at δ 1.32, which is in turn coupled to the methine signal at δ 1.50. The methine signal at δ 1.50 is also coupled with the proton signals at δ 2.75, 3.00 and 3.90. The peaks at δ 2.75 and δ 3.00 are geminally coupled to each other and they represent the methylene group attached to nitrogen. The 2D(^{1}H-^{1}H) correlation studies established the following fragments (Fig. 3):

The ^{13}C NMR spectrum showed 14 carbon atoms in agreement in the molecular formula. The ^{13}C APT experiment revealed two methyl, two methylenes, eight methines and two quaternary carbons signals. Five olefinic methines, two methines due to carbons linked to a heteroatom and an apliphatic methine accounted for the eight methine signals. Based on this spectral information and SINEPT studies (Fig. 4); the structure of two was given as **2**.

Both compounds **1** and **2** are weak antimicrobials and inhibit the binding of QNB-^{3}H to the muscarinic receptor with an IC_{50} of 12 and 43 μM, respectively. The greater affinity of **1** may be due to the increased basicity of the tertiary nitrogen.

Acknowledgments

The authors are indebted to the following personnel from our laboratory: Dr. A HORAN and Mrs. M. SHEARER for the isolation of the microorganism and Dr. R. BRYANT and Dr. R. MCQUADE for the muscarinic assay support.

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