

## ISOLATION AND STRUCTURE OF TWO NOVEL MUSCARINIC RECEPTOR ANTAGONISTS

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**ABSTRACT.**—The structures of two novel muscarinic receptor antagonists, **1** and **2**, were determined by their spectral data and high-resolution mass measurements of their degradation products. Both are aliphatic long-chain compounds and contain amide and keto functionalities. The major microbial metabolite [**1**] contains three terminal guanidino groups and the minor compound [**2**] has two terminal guanidino groups.

Muscarinic receptors are localized in both brain and peripheral tissues. Various subtypes of these receptors have been identified (1–3). They have been attributed to a broad range of physiological activities including regulation of cardiac contraction, modulation of vascular smooth muscle tone, memory, and nociception (4). A selective muscarinic receptor antagonist would have utility in the treatment of Alzheimer's disease (5).

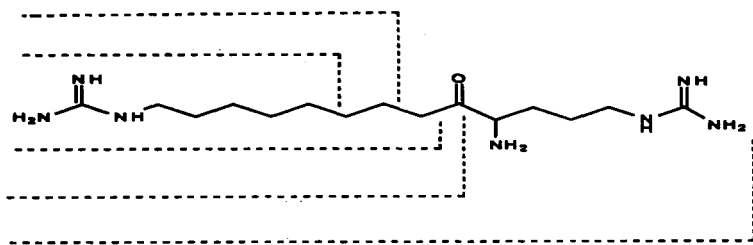
In the course of screening extracts from a wide variety of soil microorganisms for muscarinic receptor activity, we have isolated two novel compounds, **1** and **2**, from the fermentation broth of an actinomycete (SCC 2268). The isolation of these compounds was accomplished by assay-directed fractionation on a polymeric reversed-phase resin followed by chromatography on fractogel and reversed-phase hplc.

### RESULTS AND DISCUSSION

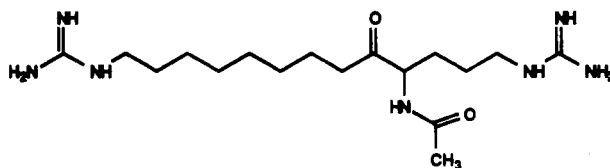
Compound **1** was isolated as a white solid,  $[\alpha]_D^{26} - 30^\circ$  ( $c=0.3$ ,  $H_2O$ ). The  $Cs^+$  ion sims displayed an intense protonated ion at  $m/z$  525  $(M+H)^+$  and a sodiated species at  $m/z$  547  $(M+Na)^+$ . Peak matching measurements using hrms showed the elemental composition to be  $C_{25}H_{53}N_{10}O_2$  (observed  $m/z$  525.4328, calcd 525.4353) suggesting five degrees of unsaturation. The uv spectrum of this compound showed only end absorption, and the ir spectrum displayed peaks at 3360, 3290, 2935, 1685, 1675, 1640, 1205, and 1140  $cm^{-1}$ . The  $^{13}C$ -nmr spectrum measured in  $DMSO-d_6$  at 75 MHz revealed the presence of 25 carbon atoms and the  $^{13}C$ -nmr APT experiment showed no methyl carbon signals, 19 methylene carbon signals, one methine carbon signal, and five quaternary carbon signals. The chemical shifts of three of the methylenes at 38.62, 40.21, and 40.57 ppm suggested they may be linked to nitrogen. The methine carbon chemical shift at 57.35 ppm is similar to the C-2 chemical shift in  $\alpha$ -amino acids. Three of the quaternary carbon signals (156.60, 156.65, and 156.70 ppm) were characteristic of guanidino, ureido, or oxime functionalities. These observations along with the presence of ten nitrogens indicated the presence of three guanidino groups in the molecule. This was further confirmed by the strong basic nature of **1**, a strong absorption band (1685  $cm^{-1}$ ) in the ir spectrum (6), a negative color reaction in the ninhydrin test, and a positive reaction with Sakaguchi reagent (7). The fourth quaternary carbon, with a chemical shift of 172.41 ppm, appeared to be an amide which was further confirmed by the presence of an amide absorbance in the ir spectrum at 1640  $cm^{-1}$ . The fifth quaternary carbon at 209.44 ppm is a characteristic aliphatic carbonyl function. The three guanidino groups, one amide carbonyl, and an aliphatic carbonyl together account for the required degree of unsaturation for the molecule, suggesting it to be a straight-chain compound with an amide linkage.

The 300 MHz  $^1\text{H}$ -nmr spectrum in  $\text{D}_2\text{O}$  of **1** was not well resolved in the high-field region, where there was considerable overlap of the saturated methylene signals. There were eleven well-resolved downfield protons. The two, two-proton signals at  $\delta$  2.12 and  $\delta$  2.43 were due to two methylenes linked to the carbons attached to carbonyl (1 amide, 1 ketone) groups. The six proton signals at  $\delta$  3.08 were due to three methylenes attached to guanidino groups. The lone proton signal at  $\delta$  4.18 was correlated to the carbon at 57.35 ppm and appears similar to protons on C-2 in  $\alpha$ -amino acids. The 2D ( $^1\text{H}$ - $^1\text{H}$ ) correlation studies in DMSO revealed a correlation of the amide proton at  $\delta$  8.16 with the proton signal at  $\delta$  4.18 which was further coupled to a geminally coupled methylene signal at  $\delta$  1.7 and  $\delta$  1.45. The three imine carbon signals were also coupled to the six-proton signal at  $\delta$  3.08 due to three methylenes. Also, when the proton signal at  $\delta$  4.18 was irradiated, intense carbon signals at 24.9, 26.5, 172.4, and 209.4 ppm were observed indicating the proximity of amide carbonyl and keto groups to this proton.

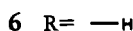
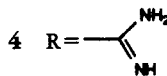
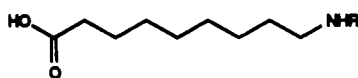
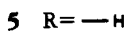
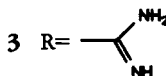
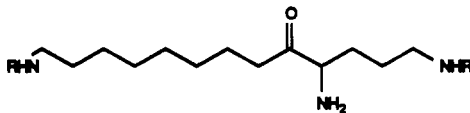
Compound **1** is stable in dilute mineral acids. However, when heated with 6 N HCl



**1**



**2**



at 100° for 8 h, it gave two hydrolysis products, **3** and **4**. Compound **4** showed a molecular ion at  $m/z$  216 ( $M+H$ )<sup>+</sup> in the fabms and a molecular formula of C<sub>10</sub>H<sub>22</sub>N<sub>3</sub>O<sub>2</sub> as determined by hrms. This compound showed two degrees of unsaturation which can be accounted for by the presence of a carboxylic acid and a guanidino group. The structure of this compound was obvious from the molecular formula and from the spectral data of the parent. Compound **3** showed a molecular ion peak at  $m/z$  328 ( $M+H$ )<sup>+</sup>, and a molecular formula of C<sub>15</sub>H<sub>34</sub>N<sub>7</sub>O. It was apparent from the molecular formula that **3** still possesses two guanidino groups and an amino group, along with the ketone in the aliphatic chain. The fabms of **3** displayed a molecular ion peak at  $m/z$  328 ( $M+H$ )<sup>+</sup>; and other fragment peaks at  $m/z$  216, 198, 170, 156, and 128. The elemental compositions of the fragment ions were determined by high-resolution mass measurement (Figure 1). The mass spectral fragmentation provided the critical information for the placement of the keto group in the chain. This is also in agreement with the chemical shift of the proton attached to the carbon which is  $\alpha$ - to the amino and keto groups. Compound **3**, when oxidized with concentrated HNO<sub>3</sub>, showed arginine among other products. This reaction, in addition to the spectral data, revealed the structure as **3**.

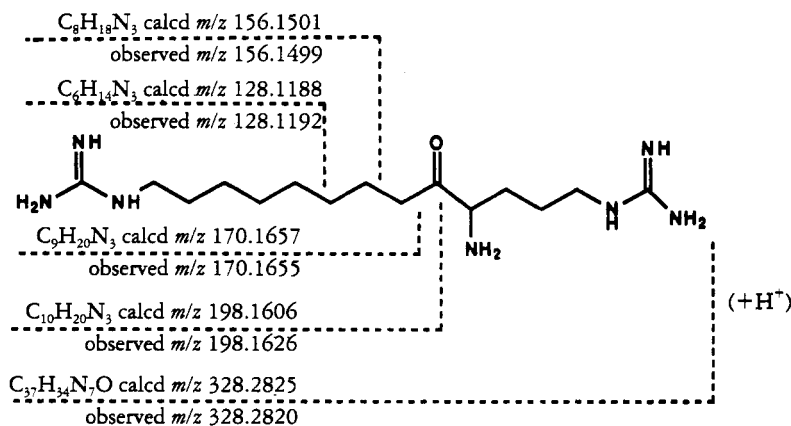


FIGURE 1

The hydrolysis of **1** by barium hydroxide (8,9) provided further evidence for the above structure. When refluxed with 0.5 M Ba(OH)<sub>2</sub> in a sealed tube for 20 h, compound **1** yielded **5** and **6**, which showed molecular ions at  $m/z$  174 and 244 in the fabms, respectively.

Compound **2** showed physicochemical properties, such as uv and ir spectra, similar to those of compound **1**. The Cs<sup>+</sup> sims displayed a molecular ion at  $m/z$  370 ( $M+H$ )<sup>+</sup>, 155 mass units fewer than **1**. Hrms revealed a molecular formula of C<sub>17</sub>H<sub>36</sub>N<sub>7</sub>O<sub>2</sub>, C<sub>8</sub>H<sub>17</sub>N<sub>3</sub> less than compound **1**. The <sup>1</sup>H-nmr spectrum in DMSO-*d*<sub>6</sub> showed an additional singlet at  $\delta$  1.87 due to a methyl group, and no methylene triplet at  $\delta$  2.28. It also showed two imine protons at  $\delta$  6.8 and  $\delta$  7.65, and only four protons at  $\delta$  3.08. This evidence suggested that **2** possesses only two guanidino groups and a methyl group. The hydrolysis of **2**, with 6 N HCl, afforded only one compound, with a molecular ion at  $m/z$  328 in the fabms which was identical to **3**. These results revealed **2** to be the acetyl derivative of **3**.

Compound **1** inhibited the binding of quinuclidinyl benzilate (QNB) to the muscarinic m<sub>1</sub>, m<sub>2</sub>, and m<sub>4</sub> receptors (10) with IC<sub>50</sub> (50% inhibitory concentration) values of 0.007, 0.006, and 0.010  $\mu$ M. Analogous data for compound **2** were 1.41, 0.96,

and 0.91  $\mu\text{M}$ , respectively. Both compounds are therefore muscarinic-receptor antagonists.

## EXPERIMENTAL

**GENERAL EXPERIMENTAL PROCEDURES.**—Solvents employed for chromatography were obtained from Fisher Scientific, Fair Lawn, NJ. The polymeric adsorbent XAD-16 was supplied by Rohm and Haas, Philadelphia, PA. The cellulose-based weak cation-exchange resin, CM-Sephadex C-25 ( $\text{Na}^+$ ) (40–120  $\mu\text{m}$ ), was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. TSK-Gel Toyopearl HW-40, a hydrophilic macroporous gel filtration medium with a methacrylic backbone, was supplied by Toso Haas, Philadelphia, PA. All ir spectra were obtained on a Nicolet Ft-ir model 10-MX instrument. Uv spectra were run on a Hewlett-Packard 8450A uv-vis spectrophotometer equipped with a HP-9872B plotter. All fabms were obtained on a Finnigan MAT-312 mass spectrometer.  $\text{Cs}^+$  sims and hrms measurements were obtained on a VG-ZAB-SE mass spectrometer using a glycerol-thioglycerol or *m*-nitrobenzyl alcohol matrix with the sample dissolved in DMSO. Nmr spectra were measured on a Varian XL-300 instrument operating at 300 and 75 MHz for  $^1\text{H}$ - and  $^{13}\text{C}$ -nmr measurements, respectively.  $^1\text{H}$ - and  $^{13}\text{C}$ -nmr spectra were recorded relative to TMS as internal standard. COSY spectra were measured on a Varian XL-400 instrument. The  $m_1$ ,  $m_2$ , and  $m_4$  muscarinic assay procedures have been reported recently (10).

**MICROORGANISM.**—The producing culture was identified as an actinomycete probably belonging to the genus *Streptomyces* based on the formation of abundant yellow aerial mycelia, which fragments into long, straight to flexous, spore chains; yellow-brown substrate mycelia; and the production of a yellow-brown soluble pigment. The culture has been deposited in the Schering Central Culture Collection under accession number SCC 2268.

**EXTRACTION AND ISOLATION.**—A 20-liter portion of fermentation broth was filtered and the filtrate passed through a column (2.5"  $\times$  15" i.d.) packed with XAD-16 resin. The column was washed with  $\text{H}_2\text{O}$  (2 liters) and eluted with 50% MeOH/ $\text{H}_2\text{O}$  (2 liters), and MeOH (3 liters). The muscarinic receptor-active compounds which eluted with MeOH were collected, the solvent removed, dissolved in  $\text{H}_2\text{O}$ , and loaded onto a CM-Sephadex C-25 ( $\text{Na}^+$ ) weak cation-exchange column (2"  $\times$  3" i.d.). The resin was washed with  $\text{H}_2\text{O}$  (1 liter) and eluted with 1.0 M NaCl (1 liter). The muscarinic receptor-active fractions (monitored by bioassay) were combined and passed through a TSK-GEL Toyopearl HW-40(S) column (2"  $\times$  12" i.d.). The column was washed with  $\text{H}_2\text{O}$ , gradient MeOH, and then again with  $\text{H}_2\text{O}$  (downward gradient). The active compounds that were tightly bound to the stationary phase were finally eluted with 0.02 N HCl. The active aqueous fractions were combined and lyophilized to obtain 40 mg of a mixture of **1** and **2**. These were finally separated on a polymer-based prep. reversed-phase column [Polymer Lab., PLRPS 2.5  $\times$  15 cm i.d., 100  $\text{\AA}$ , 10  $\mu\text{m}$ , mobile phase: 0.1% TFA- $\text{CH}_3\text{CN}$  (8:2), detection: uv (205 nm)]. The active eluate of **1** on freeze-drying afforded 27 mg of **1**. Compound **2** was further purified on the same hplc column using a mixture of 0.1% aqueous TFA and  $\text{CH}_3\text{CN}$  (9:1). The active eluate on freeze-drying afforded 5 mg of **2**.

**Compound 1.**—Mp  $>250^\circ$ ;  $[\alpha]_D^{26} -30^\circ$  ( $c=0.3$ ,  $\text{H}_2\text{O}$ ); uv (MeOH)  $\lambda$  max end absorption; ir (KBr)  $\nu$  max 3360, 3290, 2935, 1685, 1640, 1205, 1140  $\text{cm}^{-1}$ ; sims  $m/z$  525 ( $\text{M}+\text{H}^+$ ); hrsims  $m/z$   $\text{C}_{25}\text{H}_{33}\text{N}_{10}\text{O}_3$  ( $\text{M}+\text{H}^+$ ) (observed 525.4327, calcd 525.4353);  $^1\text{H}$  nmr (DMSO)  $\delta$  1.25 (14H, m), 1.45 (10H, m), 1.7 (1H, m), 2.12 (2H, t,  $J=3.8$  Hz), 2.43 (2H, dt), 3.08 (6H, m), 4.18 (1H, dt), 6.8–7.6 (12H, br), 7.7 (2H, m), 7.81 (1H, t), 8.16 (1H, d,  $J=6.1$  Hz);  $^{13}\text{C}$  nmr (DMSO)  $\delta$  22.85, 24.91, 25.10, 25.92, 25.93, 26.51, 28.29, 28.36, 28.40, 28.49, 28.55, 28.59, 28.69, 34.89, 38.02, 38.62, 40.21, 40.57, 57.35, 156.60, 156.65, 156.70, 172.41, 209.44.

**Compound 2.**—Mp  $>250^\circ$ ; uv (MeOH)  $\lambda$  max end absorption; ir (KBr)  $\nu$  max 3394, 2915, 1685, 1675, 1640  $\text{cm}^{-1}$ ; sims  $m/z$  370 ( $\text{M}+\text{H}^+$ ); hrsims  $m/z$   $\text{C}_{17}\text{H}_{36}\text{N}_7\text{O}_2$  ( $\text{M}+\text{H}^+$ ) (observed 370.2921, calcd 370.2930);  $^1\text{H}$  nmr (DMSO)  $\delta$  1.23 (7H, m), 1.45 (6H, m), 1.68 (1H, m), 1.87 (3H, s), 2.45 (2H, m), 3.05 (4H, m), 4.20 (1H, m), 6.7–7.6 (8H, m), 7.75 (1H, t), 7.81 (1H, t), 8.25 (1H, d,  $J=6.8$  Hz);  $^{13}\text{C}$  nmr (DMSO- $d_6$ ) 22.18, 22.87, 24.93, 25.91, 26.59, 28.32, 28.37, 28.37, 28.72, 37.81, 38.09, 38.09, 57.47, 156.71, 156.71, 169.43, 209.48.

**ACID HYDROLYSIS OF 1.**—A 10-mg sample of **1** was refluxed overnight with 6 N HCl in a sealed tube at  $100^\circ$ , diluted with  $\text{H}_2\text{O}$ , and freeze-dried to yield a mixture of **3** and **4**. These were separated by semi-prep. hplc (column: styrene divinylbenzene polymeric column PLRPS, 7.8  $\times$  250 mm i.d., flow rate: 5 ml/min, 45 min, gradient from 0.1% aqueous trifluoroacetic acid to MeCN, detection 205 nm,  $R_s$  for **3** and **4** were 10.4 min and 12.1 min, respectively) to yield 4.3 and 3.9 mg of **3** and **4**, respectively.

**Compound 3.**—Fabms  $m/z$  328 ( $\text{M}+\text{H}^+$ ); hrsims  $\text{C}_{15}\text{H}_{34}\text{N}_7\text{O}$  (observed 328.2820, calcd 328.2825);  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ )  $\delta$  1.4 (8H, br s), 1.65 (5H, m), 1.8 (1H, m), 2.05 (1H, m), 2.2 (1H, m), 2.75 (2H, dq,  $J=7$  Hz),

3.25 (2H,  $t, J=7$  Hz), 3.35 (2H, m), 4.4 (1H, m);  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) 23.48, 24.43, 26.57, 27.03, 28.63, 28.91, 28.93, 29.14, 39.58, 41.13, 41.99, 59.22, 157.64, 157.64, 209.69.

**Compound 4.**—Fabms  $m/z$  216 ( $\text{M}+\text{H}^+$ ); hrsims  $m/z$   $\text{C}_{10}\text{H}_{22}\text{N}_3\text{O}_2$  (observed 216.1726, calcd 216.1712);  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ )  $\delta$  1.4 (8H, br s), 1.65 (4H, m), 2.45 (2H,  $t, J=7$  Hz), 3.22 (2H,  $t, J=7$  Hz);  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) 25.13, 26.40, 26.40, 28.54, 28.76, 28.83, 28.89, 41.93, 157.53, 180.85.

**ACID HYDROLYSIS OF 2.**—A 2-mg sample of **2** was refluxed overnight with 6 N HCl in a sealed tube at  $100^\circ$ , diluted with  $\text{H}_2\text{O}$ , and freeze-dried to yield 1.6 mg of a crude solid, which was purified by hplc as described for the separation of **3** and **4**. This was identical with compound **3** isolated from the acid hydrolysis of **1**.

**OXIDATION WITH CONCENTRATED  $\text{HNO}_3$ .**—A 5-mg sample of **3** was dissolved in about 2 ml of cold concentrated  $\text{HNO}_3$ . The solution was stirred for 2 h at room temperature and then heated at  $100^\circ$  for 3 h. The contents of the cooled solution were diluted with  $\text{H}_2\text{O}$  (25 ml) and freeze-dried to yield 4.5 mg of solid. The presence of arginine was revealed by hplc amino acid analysis, comparison on tlc against an authentic sample [Analtech, Si gel plates, solvent system  $n\text{-BuOH-EtOAc-AcOH-H}_2\text{O}$  (1:1:1:1), detection by ninhydrin spray], and by the presence of a peak at  $m/z$  157 ( $\text{M}+\text{H}^+$ )<sup>+</sup> in the cims.

**HYDROLYSIS WITH  $\text{Ba}(\text{OH})_2$ .**—A 10-mg sample of **1** was refluxed in 5 ml of 0.5 N  $\text{Ba}(\text{OH})_2$  for 20 h. The aqueous hydrolysate was adjusted to pH 5.0 with  $\text{H}_2\text{SO}_4$ , the precipitated  $\text{BaSO}_4$  was filtered, and the filtrate was lyophilized to give 8 mg of a crude solid. Compounds **5** and **6** were identified by  $\text{Cs}^+$  ion sims.

#### ACKNOWLEDGMENTS

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