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Novel Alkaloids from the Red Ascidian *Botryllus leachi*

Rosario Durán, Eva Zubía, María J. Ortega, Santiago Naranjo† and Javier Salvá*

Departamento de Química Orgánica, Facultad de Ciencias del Mar, Apdo. 40, 11510 Puerto Real, Cádiz, SPAIN

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Abstract

The red ascidian *Botryllus leachi* contains two novel pyrazine alkaloids, botryllazine A (**1**) and botryllazine B (**2**) together with the new imidazole alkaloid 2-(*p*-hydroxybenzoyl)-4-(*p*-hydroxyphenyl)imidazole (**3**). The structures of compounds **1-3** were elucidated by interpretation of spectral data, with special emphasis on the analyses of ^{1}H-^{13}C couplings. Botryllazine A (**1**) represents the first example of a marine alkaloid containing a pyrazine nucleus derived from three tyrosine precursors. It is proposed that the biogenetic pathway leading from two tyrosine precursors to botryllazine B (**2**) involves amide formation and, subsequently, cyclization *via* imine formation. The cytotoxicity assay results of the new compounds **1-3** against four tumor cell lines are presented. © 1999 Elsevier Science Ltd. All rights reserved.

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Marine ascidians (tunicates) are a rich source of diverse amino acid derived metabolites many of which are of biomedical interest. In particular, ascidians have given rise to a great array of structurally diverse aromatic amino acid derived metabolites in which the biogenetic precursor is either phenylalanine, tyrosine, or both. Key examples found among marine natural products include the pyrrole alkaloids lamellarins,[1] the benzopentathiepin varacin,[2] or the botryllamides.[3]

As a part of the project initiated in our laboratories directed to explore the biomedical potential of ascidians from the south coast of Spain, we have studied specimens of the red ascidian *Botryllus leachi* Savigny (Ascidiidae) collected off Tarifa Island (Cádiz, Spain). Previous studies of this genus afforded the botryllamides A-D, a series of brominated tyrosine derivatives from two independent collections of *Botryllus schlosseri* from Australia and *Botryllus* sp. from the Philippines,[3] and the cadiolides, non nitrogenous metabolites isolated from *Botryllus* sp. from Indonesia.[4] In the chemical

*Corresponding author. E-mail: javier.salva@uca.es.
†Present address: Laboratorio Biología Marina, Dpto. Biología Animal, Univ. de Sevilla, Apdo. 1095, 41080 Sevilla, Spain.

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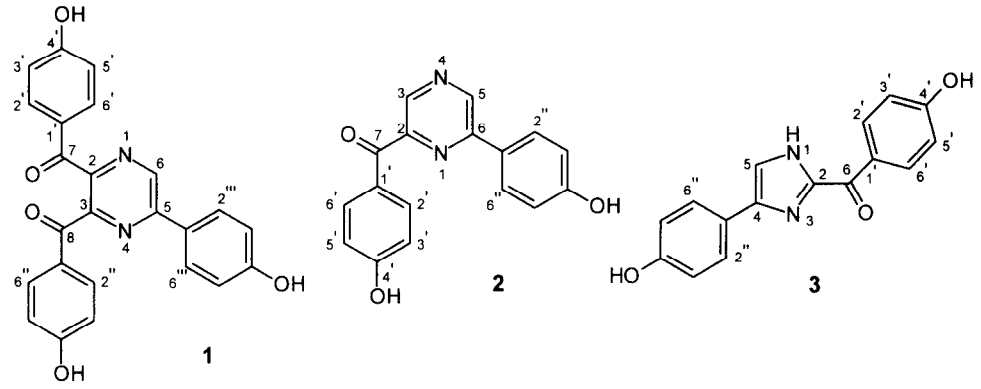
study of *B. leachi* reported in this paper we describe the isolation and structure elucidation of the botryllazines (**1**, **2**) which are novel pyrazine alkaloids together with the new 2-(*p*-hydroxybenzoyl)-4-(*p*-hydroxyphenyl)imidazole (**3**). The structures of botryllazine A (**1**), botryllazine B (**2**) and of the imidazole **3** suggest a tyrosine biosynthetic origin.



Specimens of *B. leachi* were collected by hand using SCUBA and immediately frozen. The frozen material was extracted with an acetone-methanol mixture to afford, after solvent evaporation, an aqueous residue that was subsequently extracted with Et_2O and *n*-BuOH. The *n*-BuOH soluble material was chromatographed on silica gel and final purification using HPLC allowed isolation of the following compounds (in order of increasing polarity): 2-(*p*-hydroxybenzoyl)-4-(*p*-hydroxyphenyl)imidazole (**3**, 0.005% dry wt), botryllazine B (**2**, 0.004% dry wt), and botryllazine A (**1**, 0.009% dry wt).

Botryllazine A (**1**) was isolated as a yellow powder. The molecular formula, $C_{24}H_{16}N_2O_5$, was obtained from the high resolution mass measurement. The infrared absorptions at 3290 and 1650 cm^{-1} were consistent with the presence of OH and/or NH and carbonyl functionalities. The ^1H NMR spectrum of **1** looked quite simple and only a singlet at δ 9.18 (s, 1H) and six doublets (J = 8.9 Hz) at δ 6.8-8.1 integrating for two protons each were present. The COSY spectrum showed cross peaks between the doublets at δ 8.06, 7.87, and 7.84 with those at δ 6.92, 6.84, and 6.85, respectively. These COSY connectivities together with a careful inspection of the shape of these signals, typical of AA'XX' aromatic spin systems,[5] indicated that three *p*-disubstituted phenyl rings were present in the structure of **1**.

The ^{13}C NMR spectrum of **1** contained eighteen signals, six of them attributable to the twelve methine carbons of the *p*-disubstituted rings mentioned above. The singlets at δ 164.6, 164.5, and 161.8 were assigned to three quaternary sp^2 carbons bearing oxygen and the singlets at δ 128.8, 128.7, and 127.5 were attributed to the remaining three carbons of the *p*-disubstituted phenyl rings. These data together with the presence of two conjugated carbonyl signals at δ 193.2 (s) and 192.7

(s) and the peaks in the MS at *m/z* 121 and 93 suggested the presence of two *p*-hydroxybenzoyl and a *p*-hydroxyphenyl substituent in the structure of **1**. The assignment of the ^{1}H and ^{13}C NMR signals either to the *p*-hydroxyphenyl or to the *p*-hydroxybenzoyl rings was accomplished with the aid of COSY, HETCOR, LR HETCOR, HMBC (J = 10 Hz) and HMBC (J = 5 Hz) experiments. The key correlation in the rationale was established upon observation of the cross peaks in the HMBC (J = 5 Hz) between the carbonyl signals at δ 193.2 and 192.7 with the proton signals at δ 7.84 and 7.87, respectively, which allowed assignment of these signals to the protons *ortho* to the carbonyl groups.

Two *p*-hydroxybenzoyl and a *p*-hydroxyphenyl substituents accounted for $C_{20}H_{15}O_5$ of the molecular formula. The remaining C_4HN_2 moiety was assigned to a 2,3,5-trisubstituted pyrazine ring as follows. The ^{1}H NMR signal at δ 9.18 (s, 1H), attributed to a methine proton adjacent to nitrogen in a diazine nucleus, was correlated in the HETCOR experiment with the ^{13}C NMR signal at δ 140.6 (d) consistent with a methine carbon of a pyrazine nucleus.[6] In addition, the signal at δ 9.18, exhibited mutual NOE enhancements with the signal at δ 8.06 (d, 2H, J = 8.9) assigned to the H-2‴/H-6‴ protons of the *p*-hydroxyphenyl substituent which therefore must be located on a neighboring carbon to the methine group of the pyrazine nucleus. The remaining three carbons of the heterocyclic system gave rise to the ^{13}C NMR singlets at δ 153.8, 152.9, and 149.9.

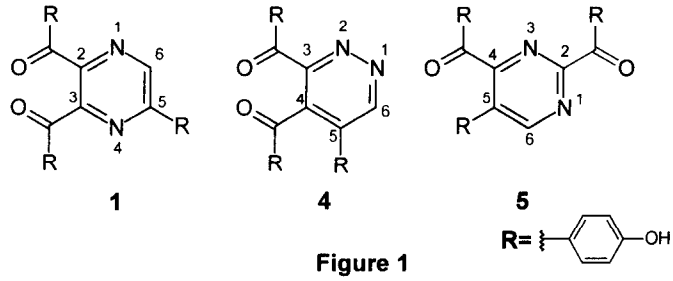


Figure 1

In Figure 1 the 2,3,5-trisubstituted pyrazine **1**, consistent with the data mentioned above, is shown together with the alternatives 3,4,5-trisubstituted pyridazine **4** and 2,4,5-trisubstituted pyrimidine **5**. However these latter two possibilities can be fully discarded with the aid of the analyses of the two and three bonds ^{1}H-^{13}C couplings. In the pyridazine nucleus **4** the values ^{2}J(C-5, H-6) = 6.7 Hz and ^{3}J(C-4, H-6) = 5.2 should be expected. The pyrimidine nucleus **5** would give rise to ^{2}J(C-5, H-6) = 9.5 Hz, ^{3}J(C-4, H-6) = 5.3 Hz, and ^{3}J(C-2, H-6) = 10.3 Hz. Finally, a pyrazine nucleus as depicted in formula **1** would exhibit ^{2}J(C-5, H-6) = 10.4 Hz and ^{3}J(C-2, H-6) = 9.8 Hz.[6,7] The two HMBC experiments carried out for botryllazine A (**1**) were diagnostic. The HMBC (J = 10 Hz) experiment showed correlations between the quaternary carbon signals at δ 152.9 and 149.9 and the methine proton signal at δ 9.18 whereas the HMBC (J = 5 Hz) experiment exhibited a cross peak between the carbon signal at δ 152.9 and the proton signal at δ 8.06 of the H-2‴/H-6‴. These data allowed us to assign the ^{13}C NMR singlets at δ 153.8, 152.9, and 149.9 to the C-3, C-5, and

C-2, respectively, of a pyrazine nucleus as depicted in formula **1**. The structure of 2,3-bis(*p*-hydroxybenzoyl)-5-(*p*-hydroxyphenyl)pyrazine was therefore proposed for botryllazine A (**1**).

Botryllazine B (**2**) was isolated as a yellow powder of molecular formula $C_{17}H_{12}N_2O_3$, as indicated by the high resolution mass measurement. The infrared spectrum exhibited absorptions at 3450 and 1645 cm^{-1}, assigned to the presence of OH and/or NH and carbonyl functionalities. The ^1H NMR spectrum of **2** contained four doublets (J = 8.9 Hz) in the range δ 6.8-8.1, accounting for two protons each, and two singlets at δ 9.17 (s, 1H) and 8.81 (s, 1H). The HRMS and ^1H NMR data mentioned suggest that the structure of compound **2** is closely related to that of **1** but lacked one of the *p*-hydroxybenzoyl substituents that were present in the structure of botryllazine A (**1**). Furthermore the ^{13}C NMR spectrum of **2** contained thirteen signals with a single singlet in the carbonyl region at δ 192.2. The COSY and HETCOR experiments allowed the complete assignments of the ^1H and ^{13}C NMR signals due to the *p*-hydroxybenzoyl and *p*-hydroxyphenyl groups which account for $C_{13}H_{10}O_3$ of the molecular formula. The remaining $C_4H_2N_2$ unit was assigned, among the different possibilities, to a 2,6-disubstituted pyrazine nucleus employing a similar rationale to that followed in the structural elucidation of **1**. The ^1H NMR singlets at δ 9.17 and 8.81 were correlated in the HETCOR experiment with the ^{13}C NMR doublets at δ 143.2 and 142.6, respectively, and were assigned to two methine groups of the heterocyclic nucleus which in addition gave rise to the singlets at δ 152.4 and 151.8. Irradiation of the signal at δ 9.17 caused a significant NOE enhancement of the signal at δ 8.01 (d, 2H, J = 8.9 Hz) assigned to the H-2"/H-6" protons of the *p*-hydroxyphenyl substituent. Finally, an LR HETCOR (J = 10 Hz) experiment showed correlations between the proton signal at δ 9.17 with the carbon signals at δ 152.4 and at δ 142.6, and between the proton signal at δ 8.81 with the carbon signals at δ 151.8 and at δ 143.2. These data are in agreement with a structure of 2-(*p*-hydroxybenzoyl)-6-(*p*-hydroxyphenyl)pyrazine for botryllazine B (**2**).

Although two tyrosine units are likely implied in the biosynthesis of botryllazine B (**2**), the biogenetic pathway through which this compound might be derived from the amino acid precursors deserves attention. The double condensation of two amino acid units *via* amide formation leads to a diketopiperazine system 3,6-disubstituted by the α-chains of the aminoacid precursors. Thus several 3,6-disubstituted diketopiperazines from marine sponges, mainly of the *Dysidea* genus,[8] from an ascidian,[9] and more recently from a marine yeast,[10] have been described. Another group of alkaloids containing a central nucleus either of 3,6-disubstituted pyrazinone like dragmacidin D,[11] or of 2,5-disubstituted piperazine, like the metabolites isolated from *Didemnum candidum*[12] or from a sponge of the genus *Dragmacidon*,[13] can be derived from two tryptamine units in a process that implies the linkage between each amino group with the C-2 of the other tryptamine precursor, likely *via* benzylic oxidation and imine formation. The novel structure of botryllazine B (**2**) can be explained neither by a condensation of two tyrosine units *via* diketopiperazine nor by the condensation proposed for the pyrazinones and piperazines. However a plausible explanation

involves a tyrosine condensation with tyramine to afford an amide followed by cyclization *via* imine formation as shown in Figure 2. This condensation proposed for the aromatic units is uncommon. A survey among the structures of marine alkaloids reveals that a similar pathway would be required to explain the biosynthetic origin of the dihydropyrazine portion of the bioluminiscent compounds *Cypridina* luciferin, coelenterazin and its disulfate.[14]

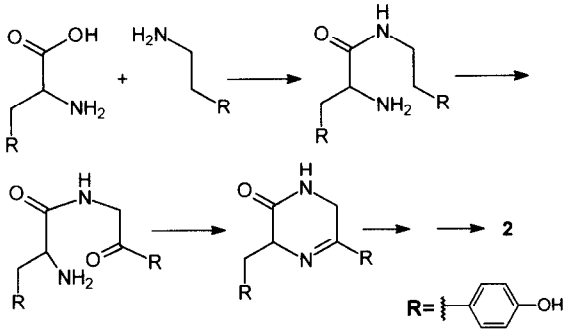


Figure 2

The structure of botryllazine A (**1**) is unprecedented since three tyrosine derived units are involved in the formation of the pyrazine nucleus. However, the way in which these units condense with each other is not evident specially with respect to the formation of the C-2, C-3 bond between two of the aminoacid precursors.

Compound **3** was isolated as an amorphous yellow solid. The molecular formula, $C_{16}H_{12}N_2O_3$, was obtained from the high resolution mass measurement. The ^{1}H NMR spectrum showed four doublets at δ 8.38, 7.71, 6.89, and 6.84 (J = 8.9 Hz) integrating for two protons each, attributable to the proton signals of two aromatic *p*-disubstituted rings, together with a singlet at δ 7.59 (s, 1H). The four doublets mentioned above were correlated in the HETCOR experiment with the signals at δ 134.8 (d), 128.1 (d), 117.1 (d), and 117.2 (d), respectively, which account for the eight methine carbons of the *p*-disubstituted phenyl rings. These data together with the presence of the singlets at δ 167.7 and 160.8, assigned to two quaternary sp^2 carbons bearing oxygen, at δ 178.2, assigned to a carbonyl carbon, and at δ 126.8 and 119.5, indicated that the substituents *p*-hydroxyphenyl and *p*-hydroxybenzoyl were present in the structure of **3**. The aromatic proton signals were unambiguously attributed to each aromatic ring since the singlet at δ 126.8, due to C-1' of the *p*-hydroxybenzoyl substituent showed coupling in the LR HETCOR (J = 8 Hz) experiment with the signal at δ 6.84 which, in addition, showed a correlation in the COSY spectrum with the doublet at δ 8.38. The aromatic substituents account for $C_{13}H_{10}O_3$ of the molecular formula. The remaining $C_3H_2N_2$ moiety was assigned to a 2,4-disubstituted imidazole nucleus as follows. The ^{1}H NMR singlet at δ 7.59 was correlated in the HETCOR experiment with the ^{13}C NMR signal at δ 122.9

(d), typical of a carbon adjacent to nitrogen in an imidazole nucleus rather than in the alternative pyrazole ring,[7] and upon irradiation of this proton signal a NOE enhancement of the doublet at δ 7.71, assigned to H-2"/H-6" of the *p*-hydroxyphenyl ring, was observed. These data indicated that compound **3** was 2-(*p*-hydroxybenzoyl)-4-(*p*-hydroxyphenyl)imidazole.

It is well known that imidazole systems exist as mixtures of annular tautomers in equilibrium. However in the spectra of compound **3** neither duplicity nor broadening of signals were observed indicating a fast interconversion of tautomers. Our data do not afford any information about the composition of the tautomeric equilibrium and therefore compound **3** is depicted as the 2,4-disubstituted rather than the alternative 2,5-disubstituted tautomer, arbitrarily.

The structure of compound **3** is related to those presented by the sponge metabolites topsentins,[15] bis(indolyl)imidazoles derived from two tryptamine units. Imidazole **3** might be derived in *B. leachi* from two tyramine units through a similar biogenetic pathway.

The new alkaloids **1-3** isolated from *Botryllus leachi* were tested against P-388 mouse lymphoma, A-549 human lung carcinoma, HT-29 human colon carcinoma, and MEL-28 human melanoma to detect *in vitro* cytotoxicity. Botryllazine A (**1**) was inactive with ED_{50} values over 10 μg/mL in all cases. Botryllazine B (**2**) exhibited a low cytotoxicity (ED_{50} = 5 μg/mL) against A-549 and MEL-28 cell lines. Imidazole **3** was slightly active against the four tumor cell lines with ED_{50} of 5 μg/mL in all cases.

Experimental Section

General. ^1H NMR and ^{13}C NMR spectra were made at 399.952 MHz and 100.577 MHz respectively using CD_3OD as solvent. The resonance of residual methanol at δ_H 3.30 and of CD_3OD at δ_C 49.00 were used as internal reference for ^1H and ^{13}C spectra, respectively. In High Performance Liquid Chromatography separations LiChrosorb silica 60 was used in normal phase mode using an UV detector. All solvents were spectral grade or distilled from glass prior to use.

Collection, Extraction, and Isolation Procedures. The tunicate *Botryllus leachi* (141 g dry wt) was collected by hand using SCUBA off Tarifa Island (Cádiz, Spain) in May 1996 and immediately frozen. The frozen tissue was extracted exhaustively with acetone-methanol (1:1) at room temperature.The filtered solution was evaporated under reduced pressure and the aqueous residue was extracted sequentially with Et_2O (6 x 500 mL) and n-BuOH (3 x 500 mL). The butanolic layer was evaporated under reduced pressure yielding an orange residue (3.24 g) that was chromatographed on SiO_2 column eluted with mixtures of increasing polarities from $CHCl_3$ to MeOH. Fractions eluted with $CHCl_3$/MeOH (9:1) were subjected to normal phase HPLC separation using a preparative LiChrosorb Si 60 column eluted with $CHCl_3$/MeOH (93:7) to afford, 2-(*p*-hydroxybenzoyl)-4-(*p*-hydroxyphenyl)imidazole (**3**, 6.9 mg, 0.005% dry wt), botryllazine B (**2**, 5.6 mg, 0.004% dry wt) and botryllazine A (**1**, 12.5 mg, 0.009% dry wt). Final purification of each compound was accomplished by HPLC on normal phase mode using $CHCl_3$/MeOH (98:2), (96:4)

and (95:5) for the compounds **3**, **2** and **1**, respectively.

Botryllazine A (1): yellow powder; $[\alpha]_D^{25}$ 0 (*c*= 0.12 MeOH)[16];IR (film) 3290, 2925, 1650, 1595, 1250, 845, 760 cm^{-1}; UV (MeOH) λ_{max} 228 (ϵ=18500), 310 (ϵ=27500) nm; ^{1}H NMR (CD_3OD) 9.18 (s, H-6), 8.06 (d, *J*= 8.9 Hz, H-2''' and H-6'''), 7.87 (d, *J*= 8.9 Hz, H-2'' and H-6''), 7.84 (d, *J*= 8.9 Hz, H-2' and H-6'), 6.92 (d, *J*= 8.9 Hz, H-3''' and H-5'''), 6.85 (d, *J*= 8.9 Hz, H-3' and H-5'), 6.84 (d, *J*= 8.9 Hz, H-3'' and H-5''); ^{13}C NMR (CD_3OD) 193.2 (s, C-7),* 192.7 (s, C-8),* 164.6 (s, C-4'), 164.5 (s, C-4''), 161.8 (s, C-4'''), 153.8 (s, C-3), 152.9 (s, C-5), 149.9 (s, C-2), 140.6 (d, C-6), 134.5 (d, C-2'' and C-6''), 134.4 (d, C-2' and C-6'), 130.3 (d, C-2''' and C-6'''), 128.8 (s, C-1''), 128.7 (s, C-1'), 127.5 (s, C-1'''), 117.1 (d, C-3''' and C-5'''), 116.3 (d, C-3' and C-5'), 116.2 (d, C-3'' and C-5''); EIMS (70 eV) *m/z* (rel. int.) 412 (70), 319 (48), 291 (70), 121 (100), 93 (85); HREIMS Obsd. *m/z*= 412.1067 $(M)^+$, $C_{24}H_{16}N_2O_5$ requires *m/z*= 412.1059. Assignments marked with an asterisk may be interchanged.

Botryllazine B (2): yellow powder; $[\alpha]_D^{25}$ 0 (*c*= 0.14 MeOH)[16]; IR (film) 3450, 1645, 1575, 1250, 840 cm^{-1}; UV (MeOH) λ_{max} 228 (ϵ=14700), 298 (ϵ=22800) nm; ^{1}H NMR (CD_3OD) 9.17 (s, H-5), 8.81 (s, H-3), 8.02 (d, *J*= 8.9 Hz, H-2' and H-6'), 8.01 (d, *J*= 8.9 Hz, H-2'' and H-6''), 6.92 (d, *J*= 8.9 Hz, H-3'' and H-5''), 6.83 (d, *J*= 8.9 Hz, H-3' and H-5'); ^{13}C NMR (CD_3OD) 192.2 (s, C-7), 167.6 (s, C-4'), 161.4 (s, C-4''), 152.4 (s, C-6), 151.8 (s, C-2), 143.2 (d, C-5), 142.6 (d, C-3), 135.1 (d, C-2' and C-6'), 129.8 (d, C-2'' and C-6''), 128.0 (s, C-1'), 127.1 (s, C-1''), 117.2 (d, C-3'' and C-5''), 117.1 (d, C-3' and C-5'); EIMS (70 eV) *m/z* (rel. int.) 292 (60), 171 (45), 121 (100), 93 (60); HREIMS Obsd. *m/z*= 292.0844 $(M)^+$, $C_{17}H_{12}N_2O_3$ requires *m/z*= 292.0848.

2-(*p*-Hydroxybenzoyl)-4-(*p*-hydroxyphenyl)imidazole (3): amorphous yellow solid; $[\alpha]_D^{25}$ 0 (*c*= 0.08 MeOH);[16] IR (film) 3380, 2920, 1590, 1470, 1260, 800 cm^{-1}; UV (MeOH) λ_{max} 248 (ϵ=7600), 305 (ϵ=6000), 360 (ϵ=13700) nm; ^{1}H NMR (CD_3OD) 8.38 (d, *J*= 8.9 Hz, H-2' and H-6'), 7.71 (d, *J*= 8.9 Hz, H-2'' and H-6''), 7.59 (s, H-5), 6.89 (d, *J*= 8.9 Hz, H-3'' and H-5''), 6.84 (d, *J*= 8.9 Hz, H-3' and H-5'); ^{13}C NMR (CD_3OD) 178. 2 (s, C-6), 167. 7 (s, C-4'), 160.8 (s, C-4''), 158.3 (s, C-2), 155.9 (s, C-4), 134.8 (d, C-2' and C-6'), 128.1 (d, C-2'' and C-6''), 126.8 (s, C-1'), 122.9 (d, C-5), 119.5 (s, C-1''), 117.2 (d, C-3' and C-5'), 117.1 (d, C-3'' and C-5''); EIMS (70 eV) *m/z* (rel. int.) 281 (15), 121 (100), 93 (13), 65 (14); HREIMS Obsd. *m/z*= 281.0924 $(M+1)^+$, $C_{16}H_{13}N_2O_3$ requires *m/z*= 281.0926.

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