

Benzamides and Quinazolines from a Mangrove Actinomycetes *Streptomyces* sp. (No. 061316) and Their Inhibiting Caspase-3 Catalytic Activity *in Vitro*

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One new benzamide, 3-hydroxyl-2-*N*-iso-butyryl-anthranilamide (1), together with two known benzamides (2, 3) and three known quinazolines (4–6), was isolated from a mangrove actinomycetes *Streptomyces* sp. (No. 061316), which displayed inhibiting Caspase-3 activity *in vitro*. The structure of 1 was elucidated by electrospray ionization (ESI)-MS, NMR spectroscopies and X-ray crystal diffraction. After evaluation of all compounds for their inhibitory effect on Caspase-3 *in vitro*, 3-hydroxyl-anthranilamide (2) and 8-hydroxyl-2,4-dioxoquinazoline (6) showed activity against Caspase-3 with IC₅₀ values of 32 and 36 μM, respectively.

Key words *Streptomyces* sp.; benzamide; quinazoline; Caspase-3

Apoptosis, or programmed cell death, is a highly regulated biological process involved in maintaining normal tissue homeostasis.¹⁾ However, deregulation of the apoptotic process can contribute to many human diseases, including neurodegenerative diseases (such as Alzheimer disease and Huntington disease), ischemic damage, autoimmune disorders, and several forms of cancer.²⁾ Caspase-3 is one of executioners in caspase-dependent apoptosis, and is activated in nearly every model of apoptosis, including those with different signaling pathways.^{3,4)} Furthermore, Caspase-3 is expressed in almost all tissues at relatively high levels and has high catalytic activity compared with other executioner caspases, so it has been seen as a promising therapeutic target, which received particular attention in finding its inhibitors.⁵⁾ Both reversible and irreversible peptide-based inhibitors have been reported to be effective in animal models of stroke, myocardial ischemia/reperfusion injury, liver disease, and traumatic brain injury.^{6–8)} Research on Caspase-3 inhibitors can develop drugs against excessive apoptosis-related diseases. During investigation on bioactive components from microorganisms, one MeOH extract of a mangrove actinomycetes *Streptomyces* sp. (No. 061316) showed inhibition of Caspase-3 with IC₅₀ value of 5.2±0.9 mg/ml. 16S RNA analysis identified the producing organism as *Streptomyces* sp. (Genbank accession no. GU980133). With the help of various chromatographic methods, one new compound, 3-hydroxyl-2-*N*-iso-butyryl-anthranilamide (1), was isolated from the mangrove actinomycetes *Streptomyces* sp. (No. 061316), along with five known compounds 3-hydroxyl-anthranilamide (2), anthranilamide (3),⁹⁾ 8-hydroxyl-4(3*H*)-quinazoline (4), 8-hydroxyl-2-methyl-4(3*H*)-quinazoline (5) and 8-hydroxyl-2,4-dioxoquinazoline (6). Herein, the identification and characterization of compounds from *Streptomyces* sp. (No. 061316) were described in this paper, as well as their inhibitory effect on Caspase-3 *in vitro*.

Results and Discussion

The strain (No. 061316) was isolated from a mangrove soil sample collected at Wenchang, and was identified to belong to *Streptomyces* by the 16S ribosomal RNA (rRNA) gene sequence analysis result and its morphological and cell wall chemical components (e.g., the highest 16S rRNA gene sequence similarity was similar to *Streptomyces tanashiensis* LMG 20274^T at 99.385% with 9 nt difference at 1463 nt; aerial mycelia developed well, long and ranged in color from white to grey on yeast extract-malt extract (ISP2) medium; it contained diaminopimelic acids (DAP) as cell wall amino acid). The strain was cultured with FM3 media at 28 °C for 7 d, and the fermentation broth was concentrated to brown gum, and refluxed three times with MeOH. After filtration and evaporation of the solvent *in vacuo*, the crude extract was diluted with H₂O and then successively partitioned with cyclohexane and chloroform. The Caspase-3 bioassays showed that the chloroform extract (C) and aqueous residue (W) displayed inhibition on Caspase-3 with IC₅₀ value of 2.3±1.0 and 3.2±0.7 μg/ml, respectively. The chloroform extract was subjected to silica gel column chromatography and preparative HPLC to yield 1 (3.4 mg). The aqueous residue (W) was subjected to macroporous resin HP20 to yield 3 fractions (W0, W1 and W2). Then fraction W1 was separated by column chromatography over octadecyl silane chemically bonded to silica gel (ODS), sephadex LH20, HW40 and preparative HPLC to yield 2 (69 mg), 3 (4.2 mg), 4 (3.0 mg), 5 (17.4 mg) and 6 (2.9 mg).

3-Hydroxyl-2-*N*-iso-butyryl-anthranilamide (1) was obtained as colorless needles (MeOH); its molecular formula was established as C₁₁H₁₄N₂O₃ according to the [M–H][–] at *m/z* 221.0939 (Calcd for C₁₁H₁₃N₂O₃, 221.0932) in high resolution-electrospray ionization-mass spectra (HR-ESI-MS). The ¹H-NMR data of 1 (Table 1) showed the presence of four exchangeable protons (δ_H 11.32, 9.98, 6.16, 5.71); δ_H 7.19 (1H, dd, *J*=7.8, 1.8 Hz), 7.14 (1H, pseudo t, *J*=7.8 Hz) and 7.10 (1H, dd, *J*=7.8, 1.8 Hz) showed the presence of a 1,2,3-

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Table 1. ^{13}C -NMR (100 MHz) and ^1H -NMR (400 MHz) Data of Compound **1** and Key Signals of HMBC

Position	1					
	$\delta_{\text{C}}^{\text{a)}$ mult	δ_{H} (J in Hz) ^{a)}	$\delta_{\text{C}}^{\text{b)}$ mult	δ_{H} (J in Hz) ^{b)}	HMBC	
1			123.3, s			
2	124.6, s		127.4, s			
3	151.1, s		150.8, s			
4	119.9, d	7.00 (dd, 6.7, 2.9)	124.4, d	7.19 (dd, 7.8, 1.8)	C-2, 6	
5	126.1, d	7.12 (m, overlap)	126.1, d	7.14 (pseudo t, 7.8)	C-1, 3	
6	119.3, d	7.12 (m, overlap)	119.0, d	7.10 (dd, 7.8, 1.8)	C-2, 4	
7	169.9, s		171.4, s			
8	177.0, s		178.6, s			
9	34.7, d	2.66 (sep, 6.8)	36.5, d	2.72 (sep, 6.8)		
9-CH ₃	19.3, q	1.14 (d, 6.8)	19.6, q	1.30 (d, 6.8)	C-8, 9	
-CONH _a		7.81 (br s)		6.16 (br s)		
-CONH _b		7.55 (br s)		5.71 (br s)		
ArNH				11.32 (br s)		
-OH				9.98 (br s)	C-3, 4	

a) Measured in DMSO-*d*₆. b) Measured in CDCl₃.

three substituted phenyl skeleton in **1**; δ_{H} 2.72 (1H, sep, $J=6.8$ Hz) and 1.30 (each 3H, d, $J=6.8$ Hz) suggested that there was a geminal dimethyl group in **1**. The ^{13}C -NMR spectrum of **1** combined with the DEPT-135 spectrum displayed 10 signals, but 11 carbons were shown to be contained from its molecular formula (two of carbons were chemical equivalence): five quaternary carbons (three of quaternary carbons bonded to oxygen or nitrogen), four methines and two chemically equivalent methyl groups.

The heteronuclear multiple bond correlation (HMBC) signal found at δ_{H} 9.98 (-OH)/ δ_{C} 124.4 (C-4) indicated that the hydroxyl group should be attached to C-3, combining the signals of δ_{H} 7.14 (H-5)/ δ_{C} 150.8 (C-3) and δ_{H} 9.98 (-OH)/ δ_{C} 150.8 (C-3). On the other hand, the HMBC correlation from δ_{H} 1.30 (9-CH₃) to δ_{C} 178.6 (C-8) revealed the connection of the geminal dimethyl group to C-8 in **1**. These data in conjunction with molecular formula indicated the planar structure of **1** as shown in Fig. 1. Furthermore, 1-CO may form hydrogen bond with 2-NH in solution, according to the chemical shift value of exchangeable proton (δ_{H} 11.32 in CDCl₃, δ_{H} 10.37 in DMSO-*d*₆). Because the bond between nitrogen and carbon in -N-CO- can not rotate freely, the two protons in 7-NH₂ were not chemical equivalence. The X-ray crystal diffraction analysis (Fig. 2) allowed the determination of the structure of **1**.

3-Hydroxyl-anthranilamide (**2**) was obtained as red needles (MeOH); its molecular formula was determined as C₇H₈N₂O₂ according to the [M-H]⁻ at m/z 151.0510 (Calcd for C₇H₇N₂O₂, 151.0513) in HR-ESI-MS. The ^1H -NMR data of **2** (Table 2) showed the presence of four exchangeable protons (δ_{H} 9.38, 7.64, 7.00, 6.05); δ_{H} 7.07 (1H, d, $J=7.9$ Hz), 6.75 (1H, d, $J=7.9$ Hz) and 6.36 (1H, pseudo t, $J=7.9$ Hz) showed the presence of a 1,2,3-three substituted phenyl group in **2**. ^{13}C -NMR data of **2** (Table 2) showed four quaternary carbons (two of quaternary carbons bonded to oxygen or nitrogen) and three methines.

The HMBC correlations found at δ_{H} 6.36 (H-5)/ δ_{C} 114.1 (C-1) and δ_{H} 7.07 (H-6)/ δ_{C} 171.4 (C-7) suggested that an amido group was attached to C-1, while a hydroxyl group was bonded to C-3 according to the HMBC correlations of δ_{H} 6.36 (H-5)/ δ_{C} 144.6 (C-3), δ_{H} 6.75 (H-6)/ δ_{C} 139.4 (C-2) and δ_{H} 9.38 (-OH)/ δ_{C} 139.4 (C-2). These data in conjunc-

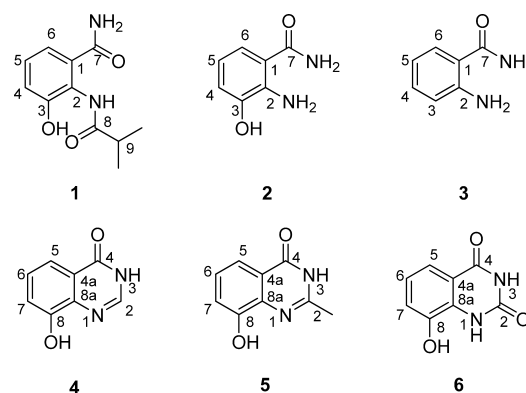


Fig. 1. Structures of **1**–**6**

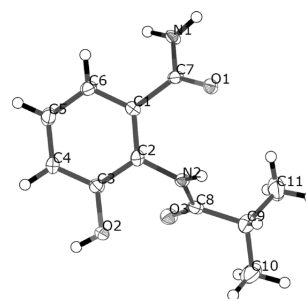


Fig. 2. ORTEP Drawing of 3-Hydroxyl-2-*N*-iso-butryl-anthranilamide (**1**)

tion with molecular formula indicated the planar structure of **2** as shown in Fig. 1. **2** was determined as 3-hydroxyl-anthranilamide, which was usually synthetic in the past⁽¹⁰⁾ and was first time in being from nature in this report.

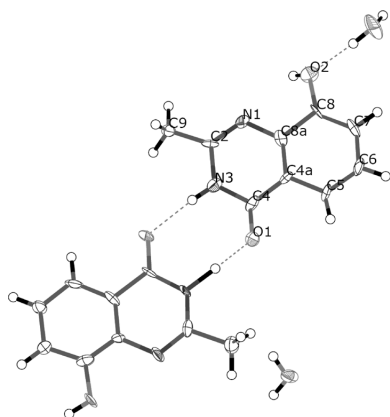
8-Hydroxyl-2,4-dioxoquinazoline (**6**) was obtained as white amorphous powder; its molecular formula was determined as C₈H₆N₂O₃ according to the [M-H]⁻ at m/z 177.0313 (Calcd for C₈H₅N₂O₃, 177.0306) in HR-ESI-MS. ^1H -NMR data of **6** (Table 3) showed the presence of three exchangeable protons (δ_{H} 11.18, 10.27, 10.27); δ_{H} 7.35 (1H, dd, $J=7.8, 1.2$ Hz), 7.07 (1H, dd, $J=7.8, 1.2$ Hz) and 6.99 (1H, pseudo t, $J=7.8$ Hz) showed the presence of a 1,2,3-three substituted phenyl skeleton in **6**. ^{13}C -NMR data of **6**

Table 2. ^{13}C -NMR (100 MHz) and ^1H -NMR (400 MHz) Data of Compounds **2**, **3** (in $\text{DMSO}-d_6$)

Position	2		HMBC	3	
	δ_{C} , mult.	δ_{H} (J in Hz)		δ_{C} , mult.	δ_{H} (J in Hz)
1	114.1, s			113.7, s	
2	139.4, s			150.1, s	
3	144.6, s			114.3, d	6.67 (dd, 7.8, 1.3)
4	115.5, d	6.75 (d, 7.9)	C-2, 6	131.8, d	7.12 (pseudo td, 7.8, 1.3)
5	113.9, d	6.36 (pseudo t, 7.9)	C-1, 3	116.3, d	6.47 (pseudo td, 7.8, 1.3)
6	119.2, d	7.07 (d, 7.9)	C-2, 4, 7	128.7, d	7.52 (dd, 7.8, 1.3)
7	171.4, s			171.3, s	
-CONH _a		7.64 (br s)			7.68 (br s)
-CONH _b		7.00 (br s)			7.01 (br s)
ArNH ₂		6.05 (br s)			6.53 (br s)
-OH		9.38 (br s)	C-2		

Table 3. ^{13}C -NMR (100 MHz) and ^1H -NMR (400 MHz) Data of Compounds **4**, **5**, **6** and Key Signals of HMBC (in $\text{DMSO}-d_6$)

Position	4			5			6		
	δ_{C} , mult.	δ_{H} (J in Hz)	HMBC	δ_{C} , mult.	δ_{H} (J in Hz)	HMBC	δ_{C} , mult.	δ_{H} (J in Hz)	HMBC
1-NH								11.18 (br s)	
2	143.4, d	8.04 (s)	C-4, 8a	152.5, s			149.8, s		
3-NH		12.20 (br s)			12.11 (br s)	C-4a		10.27 (br s)	
4	160.7, s			161.6, s			162.8, s		
5	115.5, d	7.54 (dd, 8.0, 1.4)	C-4, 7, 8a	115.4, d	7.50 (dd, 8.0, 1.2)	C-4, 7, 8a	116.6, d	7.35 (dd, 7.8, 1.2)	C-4, 7, 8a
6	127.2, d	7.32 (pseudo t, 8.0)	C-4a, 8	126.1, d	7.24 (pseudo t, 8.0)	C-4a, 8	122.2, d	6.99 (pseudo t, 7.8)	C-4a, 8
7	118.3, d	7.18 (dd, 8.0, 1.4)	C-5, 8a	118.0, d	7.15 (dd, 8.0, 1.2)	C-5, 8a	118.9, d	7.07 (dd, 7.8, 1.2)	C-5, 8a
8	152.9, s			152.2, s			144.2, s		
4a	123.4, s			121.4, s			115.3, s		
8a	137.6, s			137.9, s			130.1, s		
CH ₃	—	—	21.3, q	2.37 (s)		C-2	—	—	
-OH		9.68 (br s)			9.33 (br s)			10.27 (br s)	

Fig. 3. ORTEP Drawing of 8-Hydroxyl-2-methyl-4(3H)-quinazoline (**5**)

(Table 3) displayed five quaternary carbons (three of quaternary carbons bonded to oxygen or nitrogen) and three methines. These signals suggested that **6** possessed the same phenyl group as in 8-hydroxyl-2-methyl-4(3H)-quinazoline (**5**). This deduction was confirmed by HMBC analysis. These data in conjunction with molecular formula and comparing with ^1H -NMR data of 8-hydroxyl-2,4-dioxoquinazoline¹¹ indicated the planar structure of **6** as shown in Fig. 1. **6** was determined as 8-hydroxyl-2,4-dioxoquinazoline, which was firstly discovered from the nature.

All the isolated compounds were examined for their

inhibitory effect on Caspase-3 *in vitro*. **2** and **6** showed inhibitory effect on Caspase-3 with IC_{50} values of 32 ± 7 and $36 \pm 7 \mu\text{M}$, respectively. **1**, **3**—**5** did not show any significant inhibitory activity, and both had IC_{50} values greater than $100 \mu\text{M}$. Ac-DEVD-CHO, which was a selective peptide inhibitor of Caspase-3 and competitively inhibits Caspase-3 by binding to the catalytic active site,²⁾ was prepared as a positive control in this assay. Although **2** and **6** were not effective as Ac-DEVD-CHO ($24 \pm 2 \text{ nM}$) *in vitro*, their effect on Caspase-3 was close to reported small molecular Caspase-3 inhibitors from nature (such as F03ZA-673A,¹²⁾ which was from metabolites of a Dematiaceae fungi, also have ability to inhibit the activation of Caspase-3 with IC_{50} value of $21 \mu\text{M}$).

Some peptide-based Caspase-3 inhibitors are effective *in vitro* such as Ac-DEVD-CHO, but the pharmacokinetics of these inhibitors prevents their use in clinical environments. Small molecules that inhibit Caspase-3 activity would be valuable for treatment of diseases involving excessive cell death.¹³⁾ Most Skeletons of small-molecule inhibitors against Caspase-3 were *N*-nitrosoanilines, dithiocarbamate, isatin sulfonamide and quinolines, but benzamides have not been ever reported. Furthermore, most of benzamides and quinazolines, especially **2** and **6**, were artificial.^{9–11,14)} Therefore, the isolation of compounds from *Streptomyces* sp. (No. 061316) not only provided another approach to obtaining benzamides and quinazolines, but also may offer new source in finding Caspase-3 inhibitor.

Experimental

General UV spectra were recorded on a JASCO V-550 UV/vis spectrometer. IR spectra were recorded on a JASCO FT/IR-480 plus spectrometer. ESI-IT-MS spectra were performed on a Finnigan LCQ Advantage MAX mass spectrometer and HR-ESI-MS spectra were obtained on a Micromass Q-time-of-flight (TOF) and an Agilent 6210 LC/MSD TOF mass spectrometers. NMR spectra were measured on a Bruker AV 400 (equipped with a 5 mm broad band observe (BBO) z-gradient probe) at 400 MHz for ^1H - and 100 MHz for ^{13}C -NMR assignments were made by using ^1H , ^{13}C , correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC), and HMBC experiments. Chemical shifts were given in δ (ppm) with the solvent signals as an internal standard. The analytical HPLC was performed on a Welch Materials XB-C18 column (4.6 \times 250 mm, 5 μm) and a HPLC system, equipped with a Dionex Ultimate 3000 pump, a Dionex Ultimate 3000 diode array detector (DAD), a Dionex Ultimate 3000 Column Compartment, a Dionex Ultimate 3000 autosampler (Dionex, U.S.A.) and a Alltech (Grace) 2000ES evaporative light scattering detector (ELSD) (Alltech, U.S.A.). The preparative HPLC was carried on a Welch Materials XB-C18 column (21.2 \times 250 mm, 5 μm) and a VARIAN Prostar 210, equipped with UV detectors (U.S.A.). Column chromatography was carried out on silica gel (200–300 mesh) (Qingdao Haiyang Chemical Group Corp., Qingdao, China), Sephadex LH-20 (Pharmacia), HW40 (Toyoperal), Diaion HP 20 (Mitsubishi) and ODS (60–80 μm , YMC), respectively. Silica gel GF254 (Qingdao Haiyang Chemical Group Corp., Qingdao, China).

Actinomycetes Material The *Streptomyces* sp. strain (No. 061316) was isolated from mangrove soils at Wenchang (N19 $^{\circ}$ 36.506', E110 $^{\circ}$ 47.746'), Hainan province, P.R. China, on Gause No. 1 medium (soluble starch 1 g/l, casein 0.03 g/l, NaCl 0.5 g/l, K_2HPO_4 , 0.5 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l, agar 20 g/l, pH 7.2–7.4). The strain was grown on ISP2 medium (yeast extract 4 g/l, malt extract 10 g/l, glucose 4 g/l, agar 20 g/l, pH 7.2–7.4) at 28 $^{\circ}\text{C}$ for 7 d. Biomass was obtained from the ISP2 culture for cell wall amino acid analysis and 16S rRNA sequencing analysis. Amino acid of whole-cell hydrolysates were analyzed by thin layer chromatography (TLC) as described by Hasegawa *et al.*¹⁵ Genomic DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out using procedure described as Hong *et al.*¹⁶ The values for pairwise 16S rRNA gene sequence similarity among the closest strains were determined using the EzTaxon server (<http://www.Eztaxon.org>).¹⁷ A voucher specimen was deposited at the China Center for Type Culture Collection, Wuhan, P.R. China (CCTCC M209152). The GenBank accession number was GU980133.

Fermentation, Extraction and Isolation The strain (No. 061316) was cultured in 500 ml shake-flasks with FM3 media (soluble starch (20 g/l), soybean extract (15 g/l), yeast powder (5 g/l), peptone (2 g/l), CaCO_3 (4 g/l), sea salt (18 g/l); pH 7.0) at 28 $^{\circ}\text{C}$ for 7 d.

The fermentation broth of strain (No.061316) was concentrated to brown gum, and refluxed three times with 5 l MeOH at 70 $^{\circ}\text{C}$ for 1 h. After filtration and evaporation of the solvent *in vacuo*, the crude extract (904.5 g) was diluted with H_2O and then successively partitioned with cyclohexane and chloroform three times with the same volume. After solvent removed, the crude extract was divided into cyclohexane extract (A, 10.1 g), chloroform extract (C, 3.1 g) and aqueous residue (W, 836.7 g). The Caspase-3 bioassays showed that the chloroform extract (C) and aqueous residue (W) displayed inhibition on Caspase-3 with IC_{50} value of 2.3 ± 1.0 and 3.2 ± 0.7 $\mu\text{g}/\text{ml}$, respectively. The chloroform extract (C, 3.1 g) was subjected to silica gel column chromatography, eluted with a cyclohexane–ethyl acetate gradient (100 : 0, 98 : 2, 70 : 30, 0 : 100 and MeOH) to yield 5 fractions (C1, 13.7 mg; C2, 191.8 mg; C3, 69.7 mg; C4, 283.2 mg; C5, 946.5 mg). The fraction C4 was isolated by preparative HPLC with MeOH : H_2O (50 : 50, v/v), repeatedly, and then yield **1** (3.4 mg). The aqueous residue (W) was subjected to macroporous resin HP20, eluted with a H_2O –MeOH gradient (100 : 0, 50 : 50 and 0 : 100, v/v) to yield 3 fractions (W0, 747.8 g; W1, 23.8 g; W2, 4.4 g). Then fraction W1 (23.8 g) was separated by ODS column chromatography, eluted with a gradient of increasing MeOH (5–100%, v/v) in H_2O to afford seven subfractions (W1a–g). Subfraction W1a (15.1 g) was separated by sephadex LH20 using 100% H_2O to yield **2** (69 mg). Subfraction W1b (1.91 g) was separated by HW40 with a gradient of increasing MeOH (0–50%, v/v) in H_2O to yield **6** (2.9 mg). Subfraction W1c (1.16 g) was separated by HW40 with a gradient of increasing MeOH (0–50%, v/v) in H_2O , and then purified by RP-HPLC using MeOH : H_2O (25 : 75, v/v) to yield **3** (4.2 mg). Subfraction W1d (1.10 g) was separated by HW40 with a gradient of increasing MeOH (0–50%, v/v) in H_2O , and then purified by RP-HPLC using MeOH : H_2O (30 : 70, v/v) to yield **4** (3.0 mg). Subfraction W1e

(1.56 g) was separated by HW40 with a gradient of increasing MeOH (0–50%, v/v) in H_2O to yield **5** (17.4 mg).

3-Hydroxy-2-N-iso-butyryl-anthranilamide (1): Colorless needles (MeOH); UV (MeOH) λ_{max} (log ϵ) 245 nm (4.10), 316 nm (3.75); IR (KBr) ν_{max} 2920, 1683, 1618, 1475, 1451, 1387, 1230, 761 cm^{-1} ; ^1H - and ^{13}C -NMR data, see Table 1; ESI-MS m/z 221 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 221.0939 $[\text{M}-\text{H}]^-$ (Calcd for $\text{C}_{11}\text{H}_{13}\text{N}_2\text{O}_3$, 221.0932).

3-Hydroxy-anthranilamide (2): Red needles (MeOH); UV (MeOH) λ_{max} (log ϵ) 219 nm (4.38), 316 (3.68); IR (KBr) ν_{max} 3415, 3387, 3219, 1660, 1627, 1550, 1407, 1277, 1206, 1119, 753 cm^{-1} ; ^1H - and ^{13}C -NMR data, see Table 2; ESI-MS m/z 153 $[\text{M}+\text{H}]^+$, m/z 151 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 151.0510 $[\text{M}-\text{H}]^-$ (Calcd for $\text{C}_7\text{H}_7\text{N}_2\text{O}_2$, 151.0513).

Anthranilamide (3): Colorless needles (MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.48), 238 (4.55), 281 (4.24), 318 (4.14) nm; IR (KBr) ν_{max} 3411, 3325, 3192, 1660, 1627, 1401, 1315, 1257, 745 cm^{-1} ; ^1H - and ^{13}C -NMR data, see Table 2; ESI-MS m/z 159 $[\text{M}+\text{Na}]^+$, 295 $[\text{M}+\text{Na}]^+$; HR-ESI-MS m/z 159.0526 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_7\text{H}_8\text{N}_2\text{O}_3\text{Na}$, 159.0529).

8-Hydroxy-4(3H)-quinazoline (4): Colorless needles (MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.57), 238 (4.70), 284 (4.23), 316 (4.21) nm; IR (KBr) ν_{max} 3411, 3324, 3179, 1697, 1656, 1615, 1577, 1483, 1453, 1400, 1349, 1296, 1234, 980 cm^{-1} ; ^1H - and ^{13}C -NMR data, see Table 3; ESI-MS m/z 163 $[\text{M}+\text{H}]^+$, 161 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 163.0504 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_8\text{H}_7\text{N}_2\text{O}_2$, 163.0502).

8-Hydroxy-2-methyl-4(3H)-quinazoline (5): Colorless needles (MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.10), 237 (4.23), 279 (3.84), 314 (3.75) nm; IR (KBr) ν_{max} 3444, 3047, 2910, 1677, 1627, 1578, 1476, 1306, 1237, 1065, 757 cm^{-1} ; ^1H - and ^{13}C -NMR data, see Table 3; ESI-MS m/z 177 $[\text{M}+\text{H}]^+$, 199 $[\text{M}+\text{Na}]^+$, 175 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 175.0511 $[\text{M}-\text{H}]^-$ (Calcd for $\text{C}_9\text{H}_7\text{N}_2\text{O}_2$, 175.0513).

8-Hydroxy-2,4-dioxoquinazoline (6): White amorphous powder; UV (MeOH) λ_{max} (log ϵ) 219 (4.22), 235 (4.24), 314 (3.64) nm; IR (KBr) ν_{max} 3171, 3101, 1719, 1659, 1600, 1515, 1427, 1320, 1284, 1179, 742 cm^{-1} ; ^1H - and ^{13}C -NMR data, see Table 3; ESI-MS m/z 177 $[\text{M}-\text{H}]^-$, 355 $[\text{M}-\text{H}]^-$; HR-ESI-MS m/z 177.0313 $[\text{M}-\text{H}]^-$ (Calcd for $\text{C}_8\text{H}_5\text{N}_2\text{O}_3$, 177.0306).

X-Ray Crystallographic Analysis of 1¹⁸ Upon crystallization from MeOH using the vapor diffusion method, colorless crystals of **1** were obtained. Data were collected using a OXFORD CCD with a graphite monochromator and Mo $K\alpha$ radiation, $\lambda = 0.71073$ \AA at 150(2) K. Crystal data: $\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_3$, $M_r = 222.24$, space group monoclinic, $P2_12_1$; unit cell dimensions were determined to be $a = 7.155(2)$ \AA , $b = 7.546(8)$ \AA , $c = 20.458(8)$ \AA , $\alpha = 90.00^\circ$, $\beta = 90.00^\circ$, $\gamma = 90.00^\circ$, $V = 1104.5(12)$ \AA^3 , $Z = 4$, $D_x = 1.337$ mg/m^3 , $F(000) = 472$, $\mu(\text{MoK}\alpha) = 0.098$ mm^{-1} . 2150 unique reflections were collected to $\theta_{\text{max}} = 26.49^\circ$, in which 1918 reflections were observed [$F^2 > 4\sigma(F^2)$]. The structure was solved by direct methods using the SHELXS-97 program, and refined by the program SHELXL-97 and full-matrix least-squares calculations. In the structure refinements, nonhydrogen atoms were placed on the geometrically ideal positions by the “ride on” method. Hydrogen atoms bonded to oxygen were located by the structure factors with isotropic temperature factors. The final refinement gave $R = 0.0696$, $R_w = 0.1771$, and $S = 1.175$.

X-Ray Crystallographic Analysis of 5¹⁹ Upon crystallization from MeOH using the vapor diffusion method, colorless crystals of **5** were obtained. Data were collected using a OXFORD CCD with a graphite monochromator and Mo $K\alpha$ radiation, $\lambda = 0.71073$ \AA at 150(2) K. Crystal data: $\text{C}_9\text{H}_8\text{N}_2\text{O}_2$, $M_r = 176.17$, space group monoclinic, P_n ; unit cell dimensions were determined to be $a = 5.0458(3)$ \AA , $b = 25.0498(13)$ \AA , $c = 7.2978(5)$ \AA , $\alpha = 90.00^\circ$, $\beta = 106.921(7)^\circ$, $\gamma = 90.00^\circ$, $V = 882.48(9)$ \AA^3 , $Z = 4$, $D_x = 1.326$ mg/m^3 , $F(000) = 368$, $\mu(\text{MoK}\alpha) = 0.096$ mm^{-1} . 4020 unique reflections were collected to $\theta_{\text{max}} = 30.17^\circ$, in which 2208 reflections were observed [$F^2 > 4\sigma(F^2)$]. The structure was solved by direct methods using the SHELXS-97 program, and refined by the program SHELXL-97 and full-matrix least-squares calculations. In the structure refinements, nonhydrogen atoms were placed on the geometrically ideal positions by the “ride on” method. Hydrogen atoms bonded to oxygen were located by the structure factors with isotropic temperature factors. The final refinement gave $R = 0.0849$, $R_w = 0.0755$, and $S = 0.809$.

Caspase-3 Enzymatic Assay and Inhibition of Catalytic Activity¹³ The enzymatic activity of Caspase-3 at 35 $^{\circ}\text{C}$ was determined by measuring the change in absorbance at 460 nm caused by the accumulation of pNA from hydrolysis of Ac-DEVD-pNA. A typical 100 μl assay mixture contained 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 100 μM Ac-DEVD-pNA, and recombinant Caspase-3. Enzymatic activity was monitored continuously and the initial rate of hydrolysis was determined from the early linear region of the enzymatic reaction curve.

Ac-DEVD-CHO solution was prepared as a positive control and inhibition assays were performed with 20 nM recombinant enzyme, 100 μ M Ac-DEVD-pNA in 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. Dilutions of inhibitors were based on estimated IC₅₀ values. The IC₅₀ was calculated from a nonlinear curve of percent inhibition vs. inhibitor concentration [I] using the equation, percentage inhibition = 100/[1 + (IC₅₀/[I])^k], where k is the Hill coefficient.

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- The crystallographic data of **1** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 804262. Copies of the data can be obtained, free of charge, at http://www.ccdc.cam.ac.uk/data_request/cif.
- The crystallographic data of **5** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 804263. Copies of the data can be obtained, free of charge, at http://www.ccdc.cam.ac.uk/data_request/cif.