Apoptosis, or programmed cell death, is a highly regulated biological process involved in maintaining normal tissue homeostasis. 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. Caspase-3 is one of the executioners in caspase-dependent apoptosis, and is activated in nearly every model of apoptosis, including those with different signaling pathways. 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.

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. 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 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-anthrаниlamide (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

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 20274T 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 dianomipemic 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 μM/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-isobutyl-anthranylarnide (1) was obtained as colorless needles (MeOH); its molecular formula was established as C11H12N2O2 according to the [M−H]− at m/z 221.0939 (Calcd for C11H11N2O2, 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 (δi 11.32, 9.98, 6.16, 5.71) and 4H quaternary carbon (δq 179.19 (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-
three substituted phenyl skeleton in 1; \( \delta_H 2.72 \) (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 \( \delta_H 9.98 \) (–OH)//\( \delta_C 124.4 \) (C-4) indicated that the hydroxyl group should be attached to C-3, combining the signals of \( \delta_H 7.14 \) (H-5)//\( \delta_C 150.8 \) (C-3) and \( \delta_H 9.98 \) (–OH)//\( \delta_C 150.8 \) (C-3). On the other hand, the HMBC correlation from \( \delta_H 1.30 \) (9-CH\(_3\)) to \( \delta_C 178.6 \) (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\(_2\) in solution, according to the chemical shift value of exchangeable proton (\( \delta_H 11.32 \) in CDCl\(_3\), \( \delta_H 10.37 \) in DMSO-d\(_6\)). Because the bond between nitrogen and carbon in –N–CO– can not rotate freely, the two protons in 7-NH\(_2\) 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 C\(_{12}\)H\(_8\)N\(_2\)O\(_3\) according to the [M−H]\(^{-}\) at \( m/z \) 151.0510 (Calcd for C\(_{12}\)H\(_8\)N\(_2\)O\(_3\), 151.0513) in HR-ESI-MS. The \(^1\)H-NMR data of 2 (Table 2) showed the presence of four exchangeable protons (\( \delta_H 9.38, 7.64, 7.00, 6.05 \)); \( \delta_H 7.07 \) (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 \( \delta_H 6.36 \) (H-5)/\( \delta_C 114.1 \) (C-1) and \( \delta_H 7.07 \) (H-6)/\( \delta_C 171.4 \) (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 \( \delta_H 6.36 \) (H-5)/\( \delta_C 144.6 \) (C-3), \( \delta_H 6.75 \) (H-6)/\( \delta_C 139.4 \) (C-2) and \( \delta_H 9.38 \) (–OH)/\( \delta_C 139.4 \) (C-2). These data in conjunction 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 C\(_{12}\)H\(_8\)N\(_2\)O\(_3\) according to the [M−H]\(^{-}\) at \( m/z \) 177.0313 (Calcd for C\(_{12}\)H\(_8\)N\(_2\)O\(_3\), 177.0306) in HR-ESI-MS. \(^1\)H-NMR data of 6 (Table 3) showed the presence of three exchangeable protons (\( \delta_H 11.18, 10.27, 10.27 \)); \( \delta_H 7.35 \) (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 $^1$H-NMR (400 MHz) Data of Compounds 2, 3 (in DMSO-$d_6$)

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_{C}$, mult.</th>
<th>$\delta_{H}$ (J in Hz)</th>
<th>HMBC</th>
<th>$\delta_{C}$, mult.</th>
<th>$\delta_{H}$ (J in Hz)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>114.1, s</td>
<td></td>
<td></td>
<td>113.7, s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>139.4, s</td>
<td></td>
<td></td>
<td>150.1, s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>144.6, s</td>
<td></td>
<td></td>
<td>114.3, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>115.5, d</td>
<td>6.75 (d, 7.9)</td>
<td>C-2, 6</td>
<td>131.8, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>113.9, d</td>
<td>6.36 (pseudo t, 7.9)</td>
<td>C-1, 3</td>
<td>116.3, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>119.2, d</td>
<td>7.07 (d, 7.9)</td>
<td>C-2, 4, 7</td>
<td>128.7, d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>171.4, s</td>
<td></td>
<td></td>
<td>171.3, s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_{C}$, mult.</th>
<th>$\delta_{H}$ (J in Hz)</th>
<th>HMBC</th>
<th>$\delta_{C}$, mult.</th>
<th>$\delta_{H}$ (J in Hz)</th>
<th>HMBC</th>
<th>$\delta_{H}$ (J in Hz)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-NH</td>
<td>11.18 (br s)</td>
<td></td>
<td></td>
<td>149.8, s</td>
<td>11.18 (br s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>143.4, d</td>
<td>8.04 (s)</td>
<td>C-4, 8a</td>
<td>152.5, s</td>
<td>12.11 (br s)</td>
<td>C-4a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-NH</td>
<td>12.20 (br s)</td>
<td></td>
<td></td>
<td>161.6, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>160.7, s</td>
<td></td>
<td></td>
<td>161.6, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>115.5, d</td>
<td>7.54 (dd, 8.0, 1.4)</td>
<td>C-4, 7, 8a</td>
<td>115.4, d</td>
<td>7.50 (dd, 8.0, 1.2)</td>
<td>C-4, 7, 8a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>127.2, d</td>
<td>7.32 (pseudo t, 8.0)</td>
<td>C-4a, 8</td>
<td>126.1, d</td>
<td>7.24 (pseudo t, 8.0)</td>
<td>C-4a, 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>118.3, d</td>
<td>7.18 (dd, 8.0, 1.4)</td>
<td>C-5, 8a</td>
<td>118.0, d</td>
<td>7.15 (dd, 8.0, 1.2)</td>
<td>C-5, 8a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>152.9, s</td>
<td></td>
<td></td>
<td>152.2, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>123.4, s</td>
<td></td>
<td></td>
<td>121.4, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8a</td>
<td>137.6, s</td>
<td></td>
<td></td>
<td>137.9, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_3$</td>
<td>—</td>
<td>21.3, q</td>
<td></td>
<td>2.37 (s)</td>
<td></td>
<td>C-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH</td>
<td>9.68 (br s)</td>
<td></td>
<td></td>
<td>9.33 (br s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Tab. 3) displayed five quaternary carbons (three of quater-

nary carbons bonded to oxygen or nitrogen) and three me-thines. These signals suggested that 6 possessed the same phenyl group as in 8-hydroxy1-2-methyl-4(3$H$)-quinazoline (5). This deduction was confirmed by HMBC analysis. These data in conjunction with molecular formula and comparing with $^1$H-NMR data of 8-hydroxyl-2,4-dioxoquinazoline$^{13}$ 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 vitr. 2 and 6 showed inhibitory effect on Caspase-3 with IC$_{50}$ values of 32±7 and 36±2 $\mu$m, respectively. 1, 3–5 did not show any significant inhibitory activity, and both had IC$_{50}$ values greater than 100 $\mu$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 posi-
tive control in this assay. Although 2 and 6 were not effective as Ac-DEVD-CHO (24–22 nM) in vitr., their effect on Cas-
pase-3 was close to reported small molecular Caspase-3 in-
hibitors 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 $\mu$m).

Some peptide-based Caspase-3 inhibitors are effective in vitr. 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 quina-
zolines, especially 2 and 6, were artificial.$^{2–11,14}$ 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-FT-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/MS TOF mass spectrometer. NMR spectra were measured on a Bruker AV 400 (equipped with a 5 mm broad band observe (BBB) z-gradient probe) at 400 MHz for 1H- and 100 MHz for 13C-NMR assignments were made using 1H, 13C, correlation spectroscopy (COSY), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum coherence (HSQC), and HMBBC 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 (4.6×250 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 Altech (Grace) 2000ES evaporative light scattering detector (ELSD) (Altech, U.S.A.). The preparative HPLC was carried on a Welch Materials XB-C18 column (21.2×250 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 (Toyopearl), Diaion HP 20 (200—300 mesh) (Qingdao Haiyang Chemical Group Corp., Qingdao, China) and ODS (60—80 China), 21.2-mm bore columns were used for column chromatography was carried out on silica gel (Qingdao Haiyang Chemical Group Corp., Qingdao, China). The GenBank accession number for C.9H7N2O2, 175.0513).

Aktinomyces Material. The Streptomyces sp. strain (No. 061316) was isolated from mangrove soils at Wenchang (N19°36′50′′, E110°47′46′′), Hainan province, P. R. China, on Gause No. 1 medium (soluble starch 1g/l, casein 0.03% NaCl, 0.5 g/l, K2HPO4, 0.5 g/l, MgSO4·7 H2O, 0.5 g/l, FeSO4·7H2O, 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°C for 7 d. Biomass was obtained from the ISP2 culture for cell wall amino acid analysis and 16S rRNA sequencing analysis. 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.

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), CaCO3 (4 g/l), sea salt (18 g/l); pH 7.0) at 28 °C for 7 d.

The enzymatic activity of Caspase-3 at 35 °C was determined by measuring the change in absorbance at 405 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.

A voucher specimen was deposited at the China Center for Type Culture Collection, Wuhan, P. R. China (CCTCC M209152). The GenBank accession number for C.9H7N2O2, 175.0513).

The enzymatic activity of Caspase-3 at 35 °C was determined by measuring the change in absorbance at 405 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 μM recombinant enzyme, 100 μM Ac-DEVD-pNA in 50 mM Heps 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.

Acknowledgment We thank Doctor Herman Ho-Yung Sung (Department of Chemistry, The Hong Kong University of Science and Technology, Hong Kong, China) for X-ray crystal diffraction analysis. This project was supported by grants from the National Natural Science Foundation of China (U0633008), the Ministry of Science and Technology of China (2009CB522300, 2008ZX09401-05 and 2009ZX09302-004), the Ministry of Education of China/Fok Ying Tung Education Foundation (121039), and State Key Laboratory of Drug Research (SIMM0812KF-02).

References and Notes
18) 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.
19) 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.