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Two New Cytotoxic Compounds from a Deep-Sea *Penicillum citreonigrum* XT20-134

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Received: 28 July 2019; Accepted: 27 August 2019; Published: 29 August 2019

Abstract: *Penicillum citreonigrum* XT20-134 (MCCC 3A00956) is a fungus with cytotoxic activity, derived from deep-sea sediment. Five new compounds, adeninylpyrenocine (**1**), 2-hydroxyl-3-pyrenocine-thio propanoic acid (**2**), ozazino-*cyclo*-(2,3-dihydroxyl-trp-tyr) (**3**), 5,5-dichloro-1-(3,5-dimethoxyphenyl)-1,4-dihydroxypentan-2-one (**4**), and 2,3,4-trihydroxybutyl cinnamate (**5**), together with 19 known compounds (**6**–**24**), were isolated from an ethyl acetate (EtOAc) extract of its fermentation. The structures of the new compounds were comprehensively characterized by high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS), 1D and 2D nuclear magnetic resonance (NMR). All isolates were evaluated for their cytotoxic activities. The heteroatom-containing new compounds **2** and **4** showed potent cytotoxicity to the human hepatoma tumor cell Bel7402 with IC_{50} values of 7.63 ± 1.46, 13.14 ± 1.41 μM and the human fibrosarcoma tumor cell HT1080 with IC_{50} values of 10.22 ± 1.32, 16.53 ± 1.67 μM, respectively.

Keywords: *Penicillum citreonigrum* XT20-134; MCCC 3A00956; deep-sea fungus; cytotoxic activity

1. Introduction

Considering the extreme environment of high salinity, darkness, high pressure, and high/low temperature [1], the discovery of new lead compounds from deep-sea microorganisms has become a hot topic in natural products research. Deep-sea fungi are attracting great interest because of their relatively large genome size, which may produce more second metabolites compared with bacteria. Cancer is the leading lethal disease in the world and deep-sea microorganism-originated compounds are thought to be the new anti-tumor drugs repository [2,3]. Recently, the new antitumor compounds diketopiperazine, cytochalasan alkaloids, chromone-derived polyketides, etc., have been isolated from deep-sea fungi [4–6].

During the previous studies, several new cytotoxic compounds were characterized from deep-sea microbial resources [7–11]. In the present study, *Penicillum citreonigrum* XT20-134 (MCCC 3A00956), a fungal strain that originated from the deep-sea sediment in the southeast Indian Ocean, was found to possess cytotoxic activity. From its ethyl acetate (EtOAc) extract, five new compounds, adeninylpyrenocine (**1**), 2-hydroxyl-3-pyrenocine-thio propanoic acid (**2**), ozazino-*cyclo*-(2,3-dihydroxyl-*trp-tyr*) (**3**), 5,5-dichloro-1-(3,5-dimethoxyphenyl)-1,4-dihydroxypentan-2-one (**4**), and 2,3,4-trihydroxybutyl cinnamate (**5**) were isolated together with 19 known compounds (**6**–**24**) (Figure 1). The structures of the new compounds were comprehensively characterized by high-resolution

electrospray ionization-mass spectrometry (HR-ESI-MS), 1D and 2D nuclear magnetic resonance (NMR). Their cytotoxic activities were studied. The new heteroatom-containing compounds **2** and **4** showed potent cytotoxicity to the human hepatoma tumor cell Bel7402 with IC_{50} values of 7.63 ± 1.46 and 13.14 ± 1.41 μM, and the human fibrosarcoma tumor cell HT1080 with IC_{50} values of 10.22 ± 1.32 and 16.53 ± 1.67 μM, respectively.

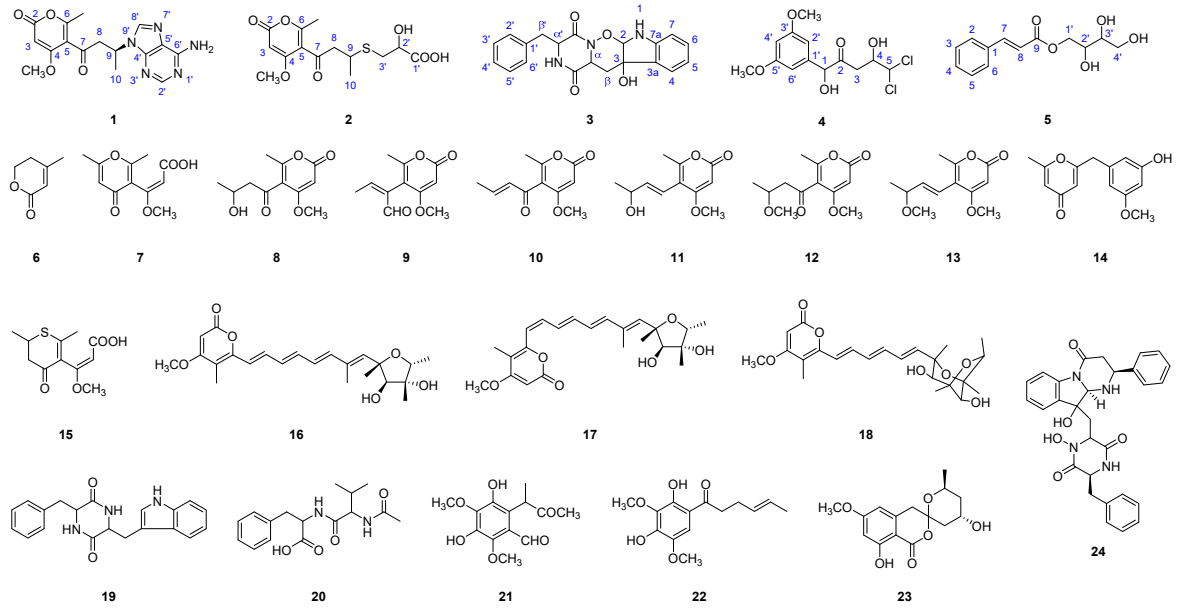

Figure 1. Structures of compounds **1**–**24** isolated from an extract of *Penicillum citreonigrum* XT20-134.

2. Results

2.1. Structural Identification of New Compounds

Adeninylpyrenocine (**1**) was isolated as a white amorphous powder. The infrared (IR) spectrum of **1** indicated the presence of conjugated ketone and conjugated lactone carbonyl signals at 1695 and 1642 cm^{-1}, respectively. Its molecular formula of $C_{16}H_{17}N_5O_4$ was established by HR-ESI-MS at 344.1357 $[M + H]^+$ (calcd. for 344.1353, $C_{16}H_{18}N_5O_4$). The unsaturation degree of 11 indicated the presence of heteroaromatic rings. With the aid of heteronuclear single quantum coherence (HSQC) spectra, three singlet signals in the low field of ^1H NMR at δ_H 8.21 (1H, s, H-8′), 8.11 (1H, s, H-2′), and 5.62 (1H, s, H-3) were attributed to olefinic protons. Their corresponding carbon signals were found in ^{13}C NMR at δ_C 140.3, 152.6, and 87.8, respectively. The signal at δ_H 7.16 (2H, br.s, 6′-NH_2) should be assigned to the primary amino group due to the absence of carbon correlation in the HSQC spectrum. In the sp^3 region of the ^1H NMR, two singlet signals, belonging to a methoxyl at δ_H 3.74 (3H, s, 4-OCH_3) and a methyl at δ_H 1.85 (3H, s, 6-CH_3), could be found. In addition, a CH_2–CH–CH_3 fragment could be established, based on the $ABXC_3$ spin system at δ_H [3.78 (1H dd, J = 17.6, 7.9 Hz) and 3.35 (1H dd, J = 17.6, 6.0 Hz), H-8], δ_H 5.06 (1H, m, H-9), and the doublet methyl signal and 1.53 (3H, d, J = 6.0 Hz, H-10). Considering the five nitrogen atoms in the molecular formula and the carbon signals at δ_C 156.4, 152.6, 149.5, 140.3, and 119.5, an adenine moiety is present in **1**. With the help of distortionless enhancement by polarization transfer (DEPT-135) along ^{13}C NMR, the four quaternary carbons at δ_C 156.4, 152.6, 149.5, 140.3, and the methine at δ_C 87.8 were attributed to an α-pyrone structure unit. In the ^1H–^1H homonuclear chemical shift correlation spectroscopy (COSY) spectrum, only the correlations in the CH_2CH–CH_3 were presented. In the ^1H detected heteronuclear multiple bond correlation (HMBC) spectrum, key correlations were found to reveal the total structure. The correlations between H-9 (δ_H 5.06) and C-4′ (δ_C 149.5) and C-8′ (δ_C 140.3) indicated that the adenine moiety was connected to C-9 at N-9′. The conjugated ketone signal at δ_C 198.8 (C-7) in ^{13}C NMR, is considered to link to C-8, due

to the HMBC correlation between H-8 and C-7. The other two methyl groups were also assigned, as shown in Figure 2. The theoretical electronic circular dichroism (ECD) spectra of *9R*-**1** and *9S*-**1** were further calculated and compared with the experimental ones to determine the absolute configurations. As shown in Figure 3, the experimental ECD spectrum was similar to the calculated ECD spectrum of *9S*-**1** and the absolute configuration of **1** was determined as *9S*.

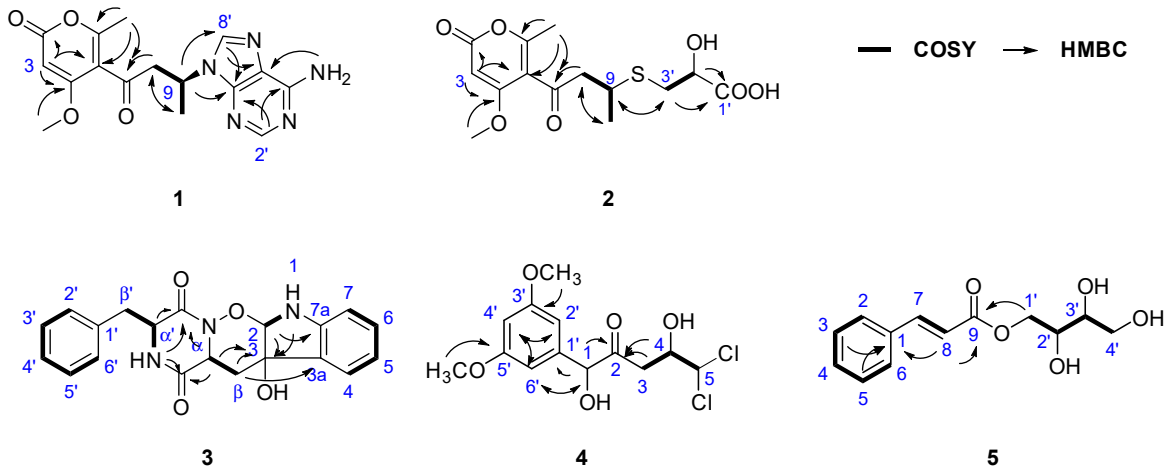

Figure 2. Key ^{1}H–^{1}H COSY, HMBC correlations of compounds **1**–**5**.

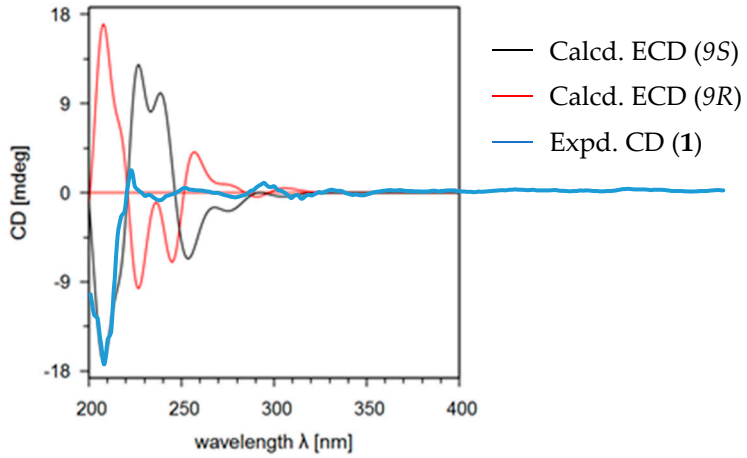

Figure 3. Calculated (*9R* and *9S*) and experimental electronic circular dichroism (ECD) spectra of compound **1**.

We isolated 2-hydroxyl-3-pyrenocine-thio propanoic acid (**2**, a pair of epimers with a ratio of 1:2) as a light yellow powder. In the IR spectrum of **2**, unconjugated carbonyl and α-pyrone ketone signals emerged at 1714 and 1626 cm^{-1}, respectively. Its molecular formula of $C_{14}H_{18}O_7S$, which gave six degrees of unsaturation, was established by the positive HR-ESI-MS ion peaks at *m*/*z* 331.0845 [M + H]$^+$, 353.0670 [M + Na]$^+$ and negative ion peaks at *m*/*z* 329.0706 [M − H]$^-$, respectively. The presence of a sulfur atom was supported by the isotope quasi-molecular ion peaks at *m*/*z* 333.0808 [M(^{34}S) + H]$^+$, 355.0627 [M(^{34}S) + Na]$^+$ and 331.0662 [M(^{34}S) – H]$^-$, respectively. In the ^{1}H NMR spectrum of **2**, an α-pyrone olefinic proton, a methyl and a methoxyl signal were found at δ_H 5.69 (1H, s, H-3), 1.23 (1H, d, J = 6.8 Hz, H-10) and 3.87 (3H, s, 4-OCH$_3$), respectively. In the ^{1}H–^{1}H COSY spectrum, the correlations between δ_H 5.69 (H-10), δ_H 3.30 (H-9) and δ_H 2.99 & 2.90 (H-8) revealed the presence of a CH$_2$CH–CH$_3$ structure unit. In the ^{13}C NMR, signals belong to the pyrenocine moiety were similar to those in **1**, except for the C-9 signal at δ_C 36.3, indicating that they differed in the substituent at C-9 (Table 1). The adeninyl signals were absent in the NMR data of **2**. Except for the adeninyl signals, the ^{1}H-NMR

of **2** presented other ABX system signals at δ_H 4.09 (1H, m, H-2′) and δ_H [2.83 (1H, dd, J = 13.5, 5.1 Hz) and 2.72 (1H, dd, J = 13.5, 9.5 Hz), H-3′]; and three additional carbon signals were at δ_C 174.5 (C-1′), 71.1 (C-2′) and 34.7 (C-3′). With the help of ^{1}H–^{1}H COSY and HMBC spectra, they were attributed to a –CH_2–CH(OH)–COOH structure fragment. The HMBC correlations between H-3′ (δ_H 2.83, 2.71) and C-9 (δ_C 36.3), and between H-9 (δ_H 3.30) and C-3′ (δ_C 34.7) revealed that the two structure units connected at C-9 and C-3′. The ^{1}H-NMR and ^{13}C-NMR signals at positions C-1′, 2′, 3′ and C-8, 9, whose peak intensities were halved and appeared in pairs, indicated that compound **2** is a pair of epimers (Figure 2). Considering the similar structure unit in **1**, the absolute configuration of C-9 is *S*, while C-2′ contains the pair of *R* and *S* configurations.

Table 1. ^{1}H NMR and ^{13}C NMR (DMSO-d_6) data of compounds **1** and **2**.

Positions	**1**		**2**		**2′**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2	162.0		162.2		162.2	
3	87.8	5.62, s	87.9	5.69, s	87.9	5.69, s
4	168.3		168.5		168.5	
5	115.1		115.4		115.4	
6	162.8		163.2		163.2	
7	198.8		199.5		199.5	
8	49.3	3.78, dd (17.6, 7.9) 3.35, dd (17.6, 6.0)	51.7	2.99, dd (17.1, 6.6) 2.90, dd (17.1, 3.7)	51.6	2.99, dd (17.1, 6.6) 2.89, dd (17.1, 3.7)
9	47.7	5.06, m	36.3	3.30, m	36.2	3.30, m
10	21.1	1.54, br. d (6.6)	21.8	1.23, d (6.8)	21.8	1.23, d (6.8)
6-CH_3	18.0	1.85, s	18.5	2.18, d (2.6)	18.5	2.18, d (2.6)
4-OCH_3	57.4	3.74, s	57.5	3.87, s	57.5	3.87, s
2′	152.6	8.11, br. s				
4′	149.5					
5′	119.5					
6′	156.4					
8′	140.3	8.21, br. s				
6′-NH_2		7.16, br. s				
1′			174.5		174.5	
2′			71.1	4.09, m	71.0	4.09, m
3′			34.7	2.83, dd (13.5, 5.1) 2.72, dd (13.6, 9.5)	34.6	2.82, dd (13.5, 5.1) 2.71, dd (13.6, 9.5)

Ozazino-*cyclo*-(2,3-dihydroxyl-*trp*-*tyr*) (**3**) was isolated as a white powder. The HR-ESI-MS cationized ion peaks revealed the presence of three nitrogen atoms. In the IR spectrum of **3**, amide carbonyl signal was found at 1669 cm^{-1}. The ultravoilet (UV) maximum absorption wavelengths at λ_{max} (log ε) 240 (1.92) nm and 299 (1.55) nm, belong to the amide carbonyl and aromatic rings, respectively. In the ^{1}H NMR spectrum of **3**, a set of *mono*-substituted benzene ring signals could be found at δ_H 6.98 (2H, br. d, J = 7.2 Hz, H-2′, 6′), 6.86 (2H, br. t, J = 7.4 Hz, H-3′, 5′) and 6.75 (1H, m, H-4′), respectively. Another *ortho*-substituted benzene ring was elucidated by the signals at δ_H 6.90 (1H, br. d, J = 7.2 Hz, H-4), 6.56 (1H, br. t, J = 7.0 Hz, H-5), 7.00 (1H, br. t, J = 7.5 Hz, H-6), and 6.56 (1H, br. d, J = 7.7 Hz, H-7). In addition, two ABX spin system signals were present at δ_H 4.19 (1H, dd, J = 5.0, 2.8 Hz, H-α′), [2.87 (1H, dd, J = 13.9, 2.8 Hz) and 2.74 (1H, dd, J = 13.8, 5.0 Hz), H-β′], and at δ_H

4.17 (1H, dd, *J* = 9.1, 4.8 Hz, H-α), [1.91 (1H, dd, *J* = 13.6, 4.8 Hz) and 1.03 (1H, dd, *J* = 13.5, 9.1 Hz) H-β], and were attributed to two –CH–CH_2– structure units. All these fragments were confirmed by the ^{13}C NMR and DEPT spectra. In addition, two amide carbonyls at δ_C 166.1 and 161.5, together with a quaternary carbon at δ_C 74.7 and a methylidyne δ_C 99.3, indicated that **3** is a compound comprising two amino acid units, one of which is phenylalanine and the other one is a tryptophane-like structure unit. Similar structures have been isolated from *P. citroviride* [12] and from *Penicillium* sp. [13]. In the HSQC spectrum of **3**, the proton signals at δ_H 7.96 (α′-NH) and δ_H 6.57 (1-NH) were attributed to two exchangeable protons, for the absence of correlation with carbon. With the help of the ^{1}H–^{1}H COSY spectrum, the signals belonging to the phenylalanine unit were attributed (Figure 2). The COSY correlation between δ_H 6.57 (1-NH) and δ_H 5.10 (1H, d, *J* = 2.8 Hz, H-2), together with the HMBC correlation between H-2 and C-3 (δ_C 74.6), revealed that C-2 and C-3 of the tryptophane unit were oxygen-connected. The HMBC correlations between δ_H 7.96 (α′-NH), 4.17 (H-α), 4.19 (H-α′), and δ_C 166.1 (C=O), 161.5 (C=O′) indicated the *cyclo*-dipeptide structure. Comparing the NMR data of **3** with those of *cyclo*-(L-tryptophyl-L-phenylalanyl) (**19**), a known compound reported previously [14], the chemical shift of 1-NH was significantly deduced from δ_H 10.83 to δ_H 6.57, indicating that the double bond between C-2 and C-3 in the indole ring disappeared. Moreover, absence of the α-NH signal (δ_H 7.96 in **19**), and the high-field shifting of C=O′ (δ_C 167.3 in **19** and δ_C 161.5 in **3**) were observed. Considering that the degree of unsaturation in **3** is 13, a six-membered ring between α-N and 2-O, connected by an ozazino bond, is prefered. Thus, the structrue of 3 is elucidated as ozazino-*cyclo*-(2,3-dihydroxyl-trp-tyr).

We isolated 5,5-dichloro-1-(3,5-dimethoxyphenyl)-1,4-dihydroxypentan-2-one (**4**) as a white powder. The HR-ESI-MS cationized ion peaks indicated a molecular formula of $C_{13}H_{16}Cl_2O_5$. The presence of the two chlorine atoms was confirmed by the isotope ion peak relative high ratio of 9:6:1. In the IR spectrum of **4**, an unconjuated ketone carbonyl signal was at 1714 cm^{-1}. A symmetrical 1,3,5-trisubstituted benzene ring was deduced by the ^{1}H NMR signal at δ_H 6.37 (2H, d, *J* = 2.2 Hz, H-2′, 6′) and δ_H 6.27 (1H, d, *J* = 2.2 Hz, H-4′). The methoxyl signal, identical at δ_H 3.57 (6H, s), was attributed to 3′, 5′-OCH_3. A –CH_2–CH–CH– structural unit could be revealed by the peak splitting and coupling constants of the proton signals at δ_H [2.66 (1H, dd, *J* = 17.4, 8.8 Hz) and 2.52 (1H, dd, *J* = 17.4, 2.8 Hz), H-3], 4.07 (1H, ddd, *J* = 8.8, 3.3, 2.8 Hz, H-4), and 5.96 (1H, d, *J* = 3.3 Hz, H-5). The fragments were confirmed by the ^{1}H–^{1}H COSY correlations of **4**. The ^{13}C NMR of **4** showed an unconjuated ketone carbonyl at δ_C 207.8. The correlation between δ_H 4.85 (1H, s, H-1) and δ_C 79.7 (C-1), found in the HSQC spectrum, revealed an oxygen-linked methylidyne. The HMBC correlations between δ_H 4.85 (H-1) and δ_C 141.7 (C-1′), 207.8 (C-2), and between δ_H 2.66 and 2.52 (H-3) and δ_C 207.8 (C-2), allowed the elucidation of the structure of **4** (Figure 2).

We isolated 2,3,4-trihydroxybutyl cinnamate (**5**) as a white powder. The molecular formula of $C_{13}H_{16}O_5$ was revealed by the HR-ESI-MS cationized ion peaks at *m*/*z* 275.0882 $[M + Na]^+$ (calcd. for 275.0890 $C_{13}H_{16}O_5Na$) in positive mode, and *m*/*z* 251.0922 $[M - H]^-$ (calcd. for 251.0925, $C_{13}H_{14}O_5$) in negative mode. A conjugated ester carbonyl IR signal was present at 1700 cm^{-1}. A *mono*-substituted benzene ring was deduced by the ^{1}H NMR signal at δ_H 7.72 (2H, dd, *J* = 6.3, 3.0 Hz, H-2, 6) and δ_H 7.74 (3H, overlapped, H-3,5 and H-4). A pair of *trans*-alkene proton signals were exhibited at δ_H 7.69 (1H, d, *J* = 16.0 Hz, H-7) and δ_H 6.64 (1H, d, *J* = 16.0 Hz, H-8). With the help of ^{1}H–^{1}H COSY spectrum, two ABX spin systems at δ_H [4.33 (1H, dd, *J* = 11.3, 2.7 Hz) and 4.11 (1H, dd, *J* = 11.3, 7.1 Hz), H-1′], 3.66 (1H, m, H-2′), and at δ_H [3.58 (1H, br. d, *J* = 9.2 Hz) and 3.42 (1H, br. d, *J* = 9.2 Hz), H-4′], 3.44 (1H, m, H-3′), were connected to a structure fragment as O–CH_2–CH(O)–CH(O)–CH_2–O (Figure 2). In the HMBC spectrum, correlations from H-1′ (δ_H 4.33 & 4.11), H-8 (δ_H 6.64) to C-9 (δ_C 166.9), and from H-3, 5 (δ_H 7.44), H-8 (δ_H 6.64) to C-1 (δ_C 134.5), allowed the elucidation of 2,3,4-trihydroxybutyl cinnamate. The chlorogenic acid contained in the potato medium may be the original precursor of this compound.

The structures of compounds **6**–**24** were elucidated by the comparison of their MS and NMR data with those reported in the literature, and they were identified as: 4-methyl-5,6-dihydropyren-2-one (**6**) [15], citreo-g-pyrone (**7**) [16], pyrenocine B (**8**) [17], pyrenocine D (**9**) [18], pyrenocine A (**10**) [19],

pyrenocine I (**11**) [20], pyrenocine E (**12**) [18], pyrenocine J (**13**) [21], citreovirenone (**14**) [12], citroethiolactone (**15**) [16], citreoviridin A (**16**) [22], isocitreoviridin A (**17**) [22], aurovertin U (**18**) [14], cyclo (*phe-trp*) (**19**) [14], *N*-(*N*-acetyl-valyl)-phenylalanine (**20**) [23], 2′,3′-dihydrosorbicillin (**22**) [24], citreoviranol (**23**) [25], and haenamindole (**24**) [26]. Most of the compounds have been isolated from the genus of *Penicillum*. Compound **21**, a known compound without reported NMR data, was identified by MS and 1D, 2D NMR spectra data as 3,5-dihydroxy-2,4-dimethyl-6-(3-oxobutan-2-yl)benzaldehyde.

2.2. Cytotoxicity Evaluation

All of the isolated compounds were evaluated for their cytotoxic effects on four types of tumor cells; Bel7402, HT1080, CNE2 and A549. Compounds **2** and **4** showed potent cytotoxicity to the cell lines Bel7402 and HT1080, while they showed no obvious effects on Cne2 and A549 (Table 2). All other compounds exhibited much lower cytotoxicity to the four tumor cell lines, with IC_{50} values larger than 100 μM.

Table 2. Cytotoxic activities of compounds **2** and **4** (IC_{50}, μM).

Compd.	Bel7402	HT1080	Cne2	A549
2	7.63 ± 1.46	10.22 ± 1.32	73.14 ± 5.32	87.08 ± 7.32
4	13.14 ± 1.41	16.53 ± 1.67	83.56 ± 6.49	92.47 ± 6/33
paclitaxel	<1	<1	<1	<1
DMSO	None	None	None	None

3. Discussion

In this study, five new compounds, including two new heteroatom-containing compounds were isolated from the ethyl acetate extract of a deep-sea fungus *P. citreonigrum* XT20-134 (MCCC 3A00956). Chemically, the relative configuration of these compounds was confirmed by their NOESY spectra; the absolute configuration of compound **1** was revealed by comparison of its CD spectra with the calculated ECD. All of the compounds were evaluated for cytotoxic activity. The new heteroatom-containing compounds **2** and **4** showed potent cytotoxicity to the tumor cell lines Bel7402 and HT1080.

Marine microorganisms can utilize chloride ions or sulfate ions, the two most abundant anions in seawater, and produce heteroatom-containing compounds, with diverse chemical structures and various bioactivities [27]. The bioactive halogenated, mainly referring to chlorinated, natural products from microorganisms mainly manifest with cytotoxic and antibacterial activity, indicating that halogenated compounds produced from microorganisms, due to adaptability or defense from the extreme environment, have cytotoxic and antibacterial activity [28]. In this study, compound **4** that contained two choline atoms was also found to have potent cytotoxicity. The most abundant source of sulfur-containing natural products is also marine organisms. Sulfur can appear in a multitude of combinations and oxidation states: thiol, sulfide, disulfide, sulfoxide, sulfonate, etc. The diversity of sulfur-containing chemical structures leads to their various bioactivities [29]. Among them, psammaplin A, has been found to have a broad bioactive spectrum, especially in terms of antimicrobial and antiproliferative activities [30]. With a sulfur atom, compound **2** exhibited potent cytotoxic activity. Similar compounds without sulfur (**1**, **8**, and **10**) did not shown cytotoxic activity. However, compound **16** show no activity, indicating that the type of sulfur bond is important to the activity.

4. Materials and Methods

4.1. General Experimental Procedures

An electrospray ionization source (ESI)-equipped Q-Exactive mass spectrometer (Thermo Fisher Scientific Corporation, Waltham, MA, USA) was used to analyze the HR-ESI-MS data. A Shimadzu UV-260 spectrometer (Shimadzu Corporation, Tokyo, Japan) and a Perkin-Elmer 683 infrared

spectrometer (PerkinElmer, Inc., Waltham, MA, USA) were used to obtain the UV and IR spectra, respectively. A JASCO P-200 polarimeter (JASCO Corporation, Tokyo, Japan) with a 5 cm cell was applied to measure the optical rotation value. The NMR spectra with TMS as the internal standard were taken on a Brucker Avance III 600 FT NMR spectrometer (Bruker Corporation, Billerica, MA, USA).

4.2. Eletronic Circular Dichroism (ECD) Calculations

The theoretical electronic circular dichroism (ECD) spectra of the isolated compounds were calculated on the basis of the relative configurations determined by their NOESY spectra and *J* values in ^{1}H NMR. Conformational analyses and density functional theory (DFT) calculations were used to generate and optimize the conformers with energy. The ECD calculations were performed as previously described [31,32].

4.3. Fungal Strain and Fermentation

The strain *Penicillium* sp. XT20-134 was isolated from southeast Indian Ocean sediments at 2910 m by the tablet pour method. The internal transcribed spaces (ITS) region was amplified and sequenced using the general primers ITS1 and ITS4. The ITS region of the fungi is a 573 bp DNA sequence (GenBank Accession Number: KY 978587) that showed 99% identity to *P. citreonigrum*. The strain was deposited at the China Center for Type Culture Collection (CCTCC) as accession number M2017125 and Marine Culture Collection of China (MCCC) as accession number MCCC 3A00956. The fungus grew well on the rice medium in artificial seawater. Carbohydrate fermentation was conducted by subculturing the fungus in rice medium in artificial seawater and incubating at 28 °C for 30 days in a standing position.

4.4. Extraction and Isolation

The rice medium (10 kg) of *P. citreonigrum* XT20-134 was extracted with ethyl acetate (20 L) trice and concentrated under reduced pressure at 40 °C to yield 19.2 g of the residue.

The EtOAc extract (18 g) was fractionated over a column packed with silica gel (360 g, Yantai Chemical Industry Research Institute, Yantai, China), eluted with petroleum ether-ethyl acetate (*v*/*v*) (5:1, 1.0 L) and $CHCl_3$–CH_3OH (*v*/*v*) (5;1, 1.0 L) and CH_3OH (1.0 L), to afford **PE** eluent (0.5 g), **CM** eluent (12.3 g), and methanol eluent (3.4 g). The **PE** eluent was purified by a silica gel (30 g) column and eluted with petroleum ether-ethyl acetate (*v*/*v*) (10:1, 5:1, and 3:1, each 200 L) to give compound **22** (13 mg). The **CM** eluent was separated over a Cosmosil reversed-phase C18 (300 g, 75 μm, Nakalai Tesque Co. Ltd., Kyoto, Japan) column and eluted with CH_3OH/H_2O (10%–100%, each 1.5 L) to provide 12 fractions (Fr. 1–Fr. 12). Compound **24** (83 mg) was obtained from Fr. 11 after recrystallization. Other fractions were purified over a preparative Cosmosil ODS column (250 mm × 20.0 mm i.d., 5 μm, Cosmosil, Nakalai Tesque Co. Ltd., Kyoto, Japan), and isocratically eluted with a mobile phase system of acetonitrile–H_2O in different ratios. Eluting with 15% acetonitrile, preparative HPLC separation on Fr. 1, Fr. 2, and Fr. 3 resulted in the isolation of: compound **6** (6 mg) from Fr. 1, compounds **7** (8 mg), **8** (57 mg), **9** (5 mg) from Fr. 2, and compounds **1** (50 mg), **10** (90 mg), **11** (15 mg), **12** (8 mg) from Fr. 3, respectively. Preparative HPLC purification of Fr. 5, eluted with acetonitrile–H_2O (30:70, *v*/*v*), led to the isolation of compound **2** (15 mg) and compound **15** (150 mg). Fr. 4, Fr. 6, and Fr. 7 were separated with 30% acetonitrile. As a result, compounds **5** (5 mg), **13** (4 mg), and **14** (5 mg) were isolated from Fr. 4; compound **19** (6 mg) from Fr. 6, and compounds **20** (13 mg) and **21** (20 mg) from Fr. 7, respectively. Fr. 8 was isolated with 35% acetonitrile to obtain compound **4** (4 mg). Separation of Fr. 9 and Fr. 10, eluted with 40% acetonitrile, obtained compound **3** (8 mg) and compound **23** (13 mg), respectively. The other three compounds, **16** (15 mg), **17** (18 mg) and **18** (13 mg), were obtained from Fr. 12, by eluting with 45% acetonitrile.

*4.5. Structrural Elucidation of the New Compounds **1–5***

Adeninylpyrenocine (**1**): white amorphous powder; $[\alpha]_D^{25}$ − 14° (c = 0.1, CH_3OH); IR (KBr) (ν_{max}): 3441, 1695, 1642, 1402 and 1256 cm^{-1}; UV (MeOH) λ_{max} (log ε): 204 (2.49) and 261 (2.22) nm. HR-ESI-MS: *m/z* 344.1357 $[M + H]^+$ (calcd. for 344.1353 $C_{16}H_{18}N_5O_4$) and 366.1172 $[M + Na]^+$ (calcd. for 366.1173 $C_{16}H_{17}N_5O_4Na$) in positive mode, and *m/z* 342.1220 $[M - H]^-$ (calcd. for 342.1208, $C_{16}H_{16}N_5O_4$) in negative mode. ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) spectra data are listed in Table 1, and Figures S1–S9 in supporting materials.

2-Hydroxyl-3-pyrenocine-thio propanoic acid (**2**): light yellow powder; $[\alpha]_D^{25}$ + 26° (c = 0.1, CH_3OH); IR (KBr) (ν_{max}): 3435, 2927, 1714, 1626, 1453, 1400, 1260, and 1096 cm^{-1}; UV (MeOH) λ_{max} (log ε): 203 (2.10) nm and 260 (2.52) nm. HR-ESI-MS: *m/z* 331.0845 $[M + H]^+$ (calcd. for 331.0859 $C_{14}H_{19}O_7S$) and 353.0670 $[M + Na]^+$ (calcd. for 353.0665 $C_{14}H_{18}O_7SNa$) in positive mode, and *m/z* 329.0706 $[M - H]^-$ (calcd. for 329.0700, $C_{14}H_{17}O_7S$) in negative mode. ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) spectra data are listed in Table 1, and Figures S10–S17 in supporting materials.

Ozazino-*cyclo*-(2,3-dihydroxyl-trp-tyr) (**3**): white powder; $[\alpha]_D^{25}$ + 43° (c = 0.1, CH_3OH); IR (KBr) (ν_{max}): 3437, 1669, 1623, 1460, 1396, and 1081 cm^{-1}; UV (MeOH) λ_{max} (log ε): 204 (2.55) nm, 240 (1.92) nm, and 299 (1.55) nm. HR-ESI-MS: *m/z* 366.1449 $[M + H]^+$ (calcd. for 366.1448 $C_{20}H_{20}N_3O_4$) and 388.1265 $[M + Na]^+$ (calcd. for 388.1268 $C_{20}H_{19}N_3ONa$) in positive mode, and *m/z* 364.1303 $[M - H]^-$ (calcd. for 364.1303, $C_{20}H_{18}N_3O$) in negative mode. ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6) spectra data are listed in Table 3, and Figures S18–S25 in supporting materials.

Table 3. ^1H NMR and ^{13}C NMR (DMSO-d_6) data of compounds **3** and **19**.

Positions	**3**		**19**	
	δ_C	δ_H	δ_C	δ_H
2	99.3	5.10, d (2.8)	121.4	6.88, br. s
3	74.6		109.2	
4	123.1	6.90, br. d (7.2)	118.9	7.43, br. d (7.2)
5	118.3	6.56, br. t (7.0)	119.2	6.93, br. t (7.0)
6	129.1	7.00, br. t (7.5)	124.9	7.01, br. t (7.4)
7	109.6	6.56, br. d (7.7)	111.8	7.26, br. d (7.7)
3a	131.9		128.0	
7a	148.1		136.5	
1′	136.0		137.0	
2′, 6′	130.4	6.98, br. d (7.2)	130.2	7.10, overlapped
3′, 5′	128.2	6.86, br. t (7.4)	128.5	6.64, br. t (7.4)
4′	126.5	6.75, m	126.9	7.12, overlapped
C=O	166.1		166.7	
C=O′	161.5		167.3	
α	54.6	4.17, dd (9.1, 4.8)	55.8	3.92, m
α′	54.8	4.19, dd (5.0, 2.8)	56.1	3.79, m
β′	37.7	2.87, dd (13.9, 2.8)	39.5	2.40, overlapped
		2.74, dd (13.8, 5.0)		1.78, dd (13.5, 7.0)
β	36.0	1.91, dd (13.6, 4.8)	30.2	2.74, dd (14.0, 4.4)
		1.03, dd (13.5, 9.1)		2.44, overlapped
1-NH		6.57, br. s		10.83, br. s
α-NH				7.85, br. s
α′-NH		7.96, br. s		7.64, br. s

5,5-Dichloro-1-(3,5-dimethoxyphenyl)-1,4-dihydroxypentan-2-one (**4**): white powder; $[\alpha]_D^{25}$ − 23° (c = 0.1, CH_3OH); IR (KBr) (ν_{max}): 3447, 1608, 1396, and 1159 cm^{-1}; UV (MeOH) λ_{max} (log ε): 204 (2.36) nm and 275 (1.23) nm. HR-ESI-MS: *m*/*z* 323.0449 $[M + H]^+$ (calcd. for 323.0448 $C_{13}H_{17}O_5Cl_2$) and 345.0261 $[M + Na]^+$ (calcd. for 345.0267 $C_{13}H_{16}O_5Cl_2Na$) in positive mode, and *m*/*z* 321.0304 $[M - H]^-$ (calcd. for 321.0302, $C_{13}H_{15}O_5Cl_2$) in negative mode. ^1H NMR (600 MHz, DMSO-d_6) δ_H: 6.37 (2H, d, *J* = 2.2 Hz, H-2′, 6′), 6.27 (1H, t, *J* = 2.2 Hz, H-4′), 5.96 (1H, d, *J* = 3.3 Hz, H-5), 4.85 (1H, s, H-1), 4.07 (1H, ddd, *J* = 8.8, 3.3, 2.8 Hz, H-4), 3.57 (6H, s, H-3′, 5′-OCH_3), [2.66 (1H, dd, *J* = 17.4, 8.8 Hz) & 2.52 (1H, dd, *J* = 17.4, 2.8 Hz), H-3]; ^{13}C NMR (150 MHz, DMSO-d_6) δ_C: 207.8 (C-2), 160.9 (C-3′, 5′), 141.7 (C-1′), 105.3 (C-2′, 6′), 100.0 (C-4′), 79.7 (C-1), 77.7 (C-5), 71.1 (C-4), 55.6 (C-3′, 5′-OCH_3), 40.9 (C-3). The spectra are provided in Figures S26–S33 in supporting materials.

2,3,4-Trihydroxybutyl cinnamate (**5**): white powder; $[\alpha]_D^{25}$ − 23° (c = 0.1, CH_3OH); IR (KBr) (ν_{max}): 3421, 1700, 1634, 1396, 1186 and 1085 cm^{-1}; UV (MeOH) λ_{max} (log ε): 216 (1.93) nm and 276 (1.99) nm. HR-ESI-MS: *m*/*z* 275.0882 $[M + Na]^+$ (calcd. for 275.0890 $C_{13}H_{16}O_5Na$) in positive mode, and *m*/*z* 251.0922 $[M - H]^-$ (calcd. for 251.0925, $C_{13}H_{14}O_5$) in negative mode. ^1H NMR (600 MHz, DMSO-d_6) δ_H: 7.69 (1H, d, *J* = 16.0 Hz, H-7), 7.44 (1H, overlapped, H-4), 7.44 (2H, overlapped, H-3, 5), 7.72 (2H, dd, *J* = 6.3, 3.0 Hz, H-2, 6), 6.64 (1H, d, *J* = 16.0 Hz, H-8), 3.44 (1H, m, H-3′), 3.66 (1H, m, H-2′), [4.33 (1H, dd, *J* = 11.3, 2.7 Hz) and 4.11 (1H, dd, *J* = 11.3, 7.1 Hz), H-1′], [3.58 (1H, br. d, *J* = 9.2 Hz) and 3.42 (1H, br. d, *J* = 9.2 Hz), H-4′]; ^{13}C NMR (150 MHz, DMSO-d_6) δ_C: 166.9 (C-9), 144.8 (C-7), 134.5 (C-1), 130.9 (C-4), 129.4 (C-3, 5), 128.8 (C-2, 6), 118.8 (C-8), 72.8 (C-3′), 69.9 (C-2′), 67.0 (C-1′), 63.5 (C-4′). The spectra are provided in Figures S34–S42 in supporting materials.

4.6. Cytotoxicity Assay

Cytotoxicity assay was carried out according to the instructions of CCK-8 kit [7]. Briefly, compounds at different concentrations were added into the culture medium containing 10^5 /mL HT1080, Cne2 and Bel7402 cells and incubated for 24, 48, 72 h. Then, 10 μL of CCK-8 solution was added into each well of the 96-well plate, incubated for 2 h and then the absorbance was measured at 450 nm using a microplate reader (BIO-RAD, Hercules, California, USA). The inhibition rate = $(A_{control} - A_{treated})/A_{control} \times 100$. The IC_{50} was the concentration at which it caused 50% inhibition of cell proliferation (50% reduction in the absorbance value in the treated cells, in respect to the control).

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/17/9/509/s1, Figures S1–S42: Supporting NMR, IR, and HR-ESI-MS spectra.

Author Contributions: X.-X.T. identified the fungus and performed the cytotoxicity activity assays. X.Y. and S.-Z.L. isolated the compounds. B.-W.T., M.-J.F., and X.-M.W. were responsible for the structural elucidation. Y.-K.Q. and Z.W. supervised the project.

Funding: The project was supported by the COMRA Project of China (DY135-B2-08), National Basic Research Program of China (973 Program) (No. 2015CB755901), Xiamen Ocean Economic Innovation and Development Demonstration Project (16PZP001SF16) and Xiamen Science and Technology Program (3502Z20182029), the Natural Science Foundation of Fujian Province of China (No. 2018J01132), and the Fundamental Research Funds for the Central Universities (No. 20720180051). The project was supported by the Natural Science Foundation of Fujian Province of China (No. 2018J01131).

Conflicts of Interest: The authors have no conflict of interest to declare. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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