A New Quinazolinone Alkaloid from *Rhodiola tibetica* Endophytic Fungus *Penicillium* sp. HJT-A-6

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Abstract: A new quinazolinone alkaloid, named peniquinazolinone A (**1**), as well as eleven known compounds, 2-(2-hydroxy-3-phenylpropionamido)-*N*-methylbenzamide (**2**), viridicatin (**3**), viridicatol (**4**), (±)-cyclopeptin (**5a/5b**), dehydrocyclopeptin (**6**), cyclopenin (**7**), cyclopenol (**8**), methyl-indole-3-carboxylate (**9**), 2,5-dihydroxyphenyl acetate (**10**), methyl *m*-hydroxyphenylacetate (**11**), conidiogenone B (**12**), was isolated from the endophytic *Penicillium* sp. HJT-A-6 derived from the stem of *Rhodiola tibetica*. The chemical structures of all compounds were elucidated by comprehensive spectroscopic analysis, including 1D, 2D NMR and HRESIMS. The absolute configuration at C-13 of peniquinazolinone A (**1**) was established by applying the modified Mosher's method. Compounds **2**, **3**, and **7** exhibited an optimal promoting effect on the seed germination of *Rhodiola tibetica* at a concentration of 0.01 mg/mL, while the optimal concentration for compounds **4** and **9** to promote *Rhodiola tibetica* seed germination is 0.001 mg/mL. Compound **12** showed optimal seed-germination-promoting activity at a concentration of 0.01 mg/mL. Compared with the positive drug 6-benzyladenine (6-BA), compounds **2**, **3**, **4**, **7**, **9**, and **12** could extend the seed-germination period of *Rhodiola tibetica* up to the 11th day.

Keywords: *Rhodiola tibetica*; *Penicillium* sp. HJT-A-6; quinazolinone; seed-germination-promoting activity

1. Introduction

Significant mutualistic relationships have been established between endophytic fungi and their host plants, attracting considerable attention due to their ecological and biotechnological potential [1]. Currently, the structural types of secondary metabolites isolated from endophytic fungi fermentation products, including alkaloids, polyketides, terpenes, etc [2,3]. Some compounds exhibit biological activities such as antibacterial [4], antiviral [5], anti-tumor [6], and some even possess activity in promoting seed germination [7]. Endophytic fungi can both independently produce various structural types of secondary metabolites and participate in the biosynthesis or biotransformation of secondary metabolites in host plants, making them an important source of active natural products. Active natural products derived from endophytic fungi hold vast potential applications in biopharmaceuticals, agricultural production, and industrial fermentation [2,8–10].

Quinazolinones have great development prospects in medicinal chemistry [11], originating from a wide range of their antibacterial [12], anti-inflammatory [13,14], antiviral [15,16], and antituberculosis [17] properties. As of now, only a limited number of quinazolinones have been reported from endophytic fungi, including neosartoryadins and glyantrypines, antiviral agents from mangrove-derived fungi *Neosartorya udagawae* and *Cladosporium* sp., respectively [18,19]; chaetominine, a cytotoxic agent from an endophytic fungus *Chaetomium* sp. [20]; aniquinazolines A–D, antibacterial and cytotoxic molecules from a mangrove-derived fungus *Aspergillus nidulans* [21];

and (–)-(1*R*,4*R*)-1,4-(2,3)-Indolmethane-1-methyl-2,4-dihydro-1*H*-pyrazino-[2,1-*b*]-quinazoline-3,6-dione, an antifungal agent from an endophytic fungus *Penicillium vinaceum* [22]. In a sense, mining novel bioactive quinazoline molecules from endophytic fungi holds great promise.

As a part of our ongoing studies on the bioactive secondary metabolites from *Rhodiola tibetica* endophytic fungi, we have isolated more than 30 polyketides with antiviral or anti-inflammatory activities [23–25]. Global Natural Products Social (GNPS) molecular networking has been widely applied in the analysis of natural products to cluster compounds with similar MS/MS spectra, expediting the dereplication process of known natural products [26–28]. We performed a GNPS molecular networking analysis of the EtOAc extract of endophytic fungus *Penicillium* sp. HJT-A-6, leading to the discovery of a new quinazolinone alkaloid, peniquinazolinone A (**1**), together with eleven known secondary metabolites including 2-(2-hydroxy-3-phenylpropionamido)-*N*-methylbenzamide (**2**) [29], viridicatin (**3**) [30], viridicatol (**4**) [31], (±)-cyclopeptin (**5a**/**5b**) [32,33], dehydrocyclopeptin (**6**) [33], cyclopenin (**7**) [34], cyclopenol (**8**) [35], methyl-indole-3-carboxylate (**9**) [36], 2,5-dihydroxylbenzeneacetic acid (**10**) [37], methyl *m*-hydroxyphenylacetate (**11**) [38] and conidiogenone B (**12**) [39] in Figure 1. Herein, we describe the molecular networking analyss of the EtOAc extract, isolation and structural elucidation of the new compound, as well as the seed-germination-promoting activities of compounds **2**, **3**, **4**, **7**, **9**, and **12**.
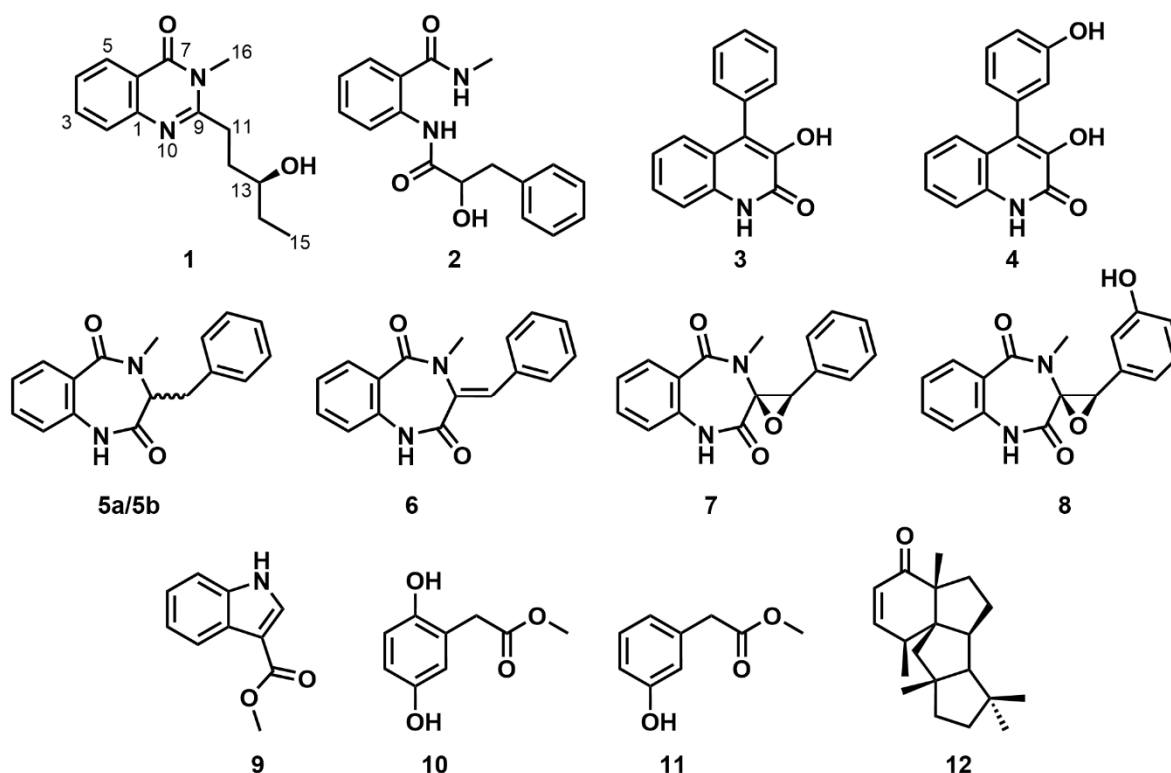
Figure 1. Chemical structures of compounds **1–12**.

2. Results and Discussion

2.1. Molecular Networking-Guided   Isolation Workflow

To target the isolation of quinazolinone alkaloid, the crude EtOAc extract of *Penicillium* sp. HJT-A-6 was subjected to full scan HPLC-Q-TOF-MS/MS analysis. The obtained MS/MS data was used to generate the initial molecular network using the Global Natural Products Social Molecular Networking (GNPS) platform following the established protocol. This initial network was visualized and analyzed via Cytoscape 3.7.1 software.

As shown in Figure 2, the annotated nodes of total secondary metabolites were displayed, the color of the node indicates the *m/z* of the parent ion. This process generated a network featuring 2373

nodes and 755 clusters, those representative highlighted clusters indicated several known natural products and a promising new compound. In cluster 1 highlighted in light red, the node (*m/z* 276.062) was connected to the node (*m/z* 260.069), which were supposed to be two known compounds, viridicatin (**3**) and viridicatol (**4**).   Clusters 2 and 3 highlighted in light blue and light purple, respectively, identifying two classes of natural products, cyclopeptin analogues (**5a/5b**–**7**) and phenyl acetate derivates (**10**, **11**). The orphan cluster 4 (*m/z* 247.144) in light green, which was not associated with any structurally known compound, guiding the targeted isolation of a new quinazolinone alkaloid, peniquinazolinone A (**1**).
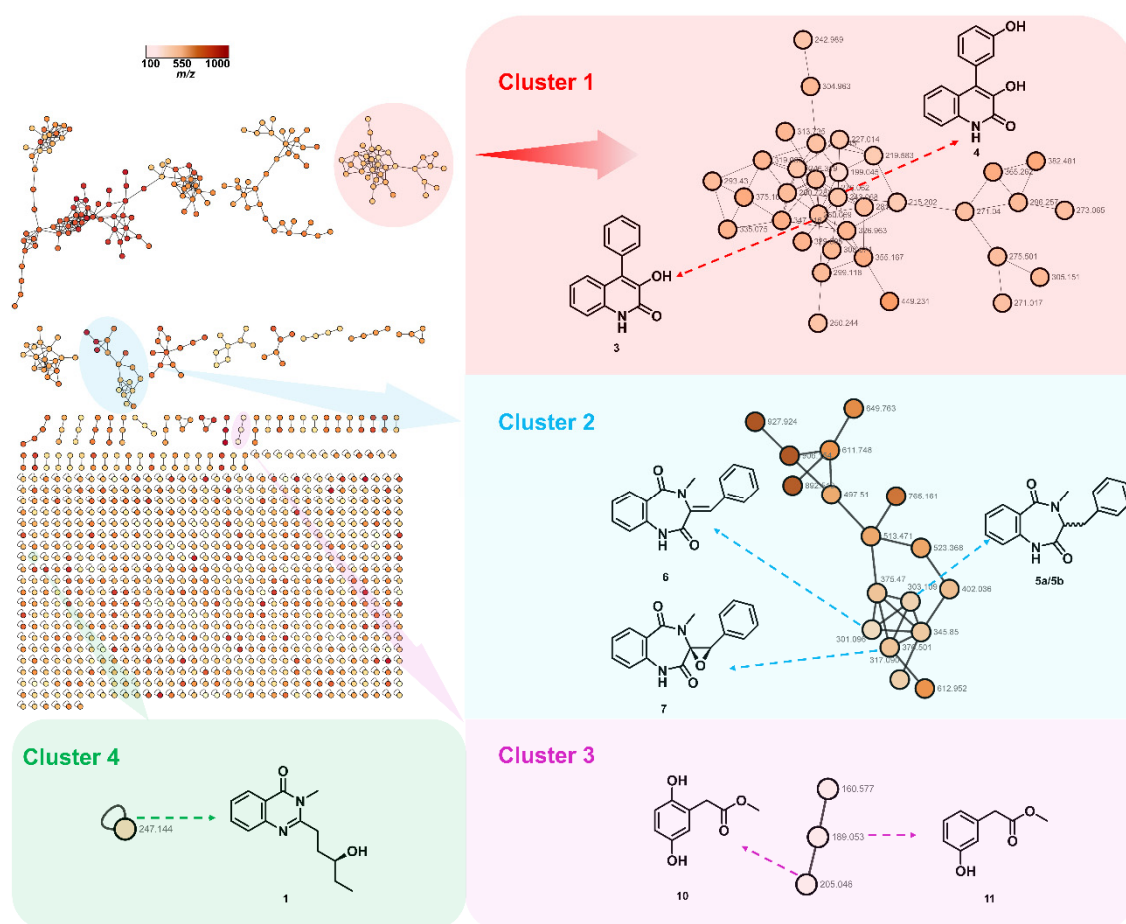
Figure 2. Molecular networking-guided chemometric discovery of peniquinazolinone A (**1**) and several known compounds (**3**–**7**, **9**, **10**).

*2.2. Structural Elucidation of Compound **1***

Compound **1** was obtained as a yellow oil. Its molecular formula was determined as $C_{14}H_{18}N_2O_2$ based on HRESIMS at *m/z* 269.1271 [M + Na]+ (calcd for 269.1266) (Figure S1), indicating seven degrees of unsaturation. The ¹H NMR spectrum (Table 1 and Figure S3) revealed the resonances of four aromatic protons at δ_H 8.10 (d, *J* = 7.5 Hz, H-5), 7.77 (t, *J* = 7.5 Hz, H-3), 7.59 (d, *J* = 7.5 Hz, H-2) and 7.47 (d, *J* = 7.5 Hz, H-4), which indicated the presence of one 1,2-disubstituted phenyl group in **1**; three methylene protons at δ_H 1.43–2.99; one oxygenated methine proton at δ_H 3.48 (m, H-13); two methyl protons at δ_H 3.56 (s), 0.90 (t, *J* = 7.5 Hz, H-15). The ¹³C NMR and HSQC spectra (Table 1 and Figures S6–S7) of **1** displayed the presence of fourteen carbons, including one carbonyl carbon, seven aromatic/olefinic carbons, three methylenes, one oxygenated methine, and two methyls.

Table 1. ^{1}H and ^{13}C NMR data for compound **1** in DMSO-d_6.

Position	δ_H (mult., J in Hz)	δ_C, Type
1	—	147.4, C
2	7.59 (d, 7.5)	127.1, C
3	7.77 (t, 7.5)	134.6, C
4	7.47 (t, 7.5)	126.6, C
5	8.10 (d, 7.5)	126.6, C
6	—	120.2, C
7	—	161.9, C
8	—	—
9	—	158.6, C
10	—	—
11	2.99 (m)	31.6
	2.85 (m)	
12	1.92 (m)	33.7
	1.73 (m)	
13	3.48 (m)	71.0
14	1.43 (m)	30.4
15	0.90 (t, 7.5)	10.6
16	3.56 (s)	30.4
13-OH	4.57 (s)	—

The planar structure of compound **1** was deduced by HMBC and ^{1}H–^{1}H COSY spectra (Figure 3 and Figures S8–S9). The HMBC correlations from H-2 to C-6, from H-3 to C-1, from H-4 to C-6, from H-5 to C-3 and C-7, as well as from H-16 to C-7 and C-9, established the structural skeleton of quinazolinone. The proton spin systems of H-11/H-12/H-13/H-14/H-15 and H-13/13-OH, coupled with the HMBC correlations from H-11 to C-9, C-12, and C-13, from H-14 to C-13, and from H-15 to C-13 and C-14, confirmed the linkage of the quinazolinone group and the pentan-3-ol moiety at C-9. Consequently, the planar structure of compound **1** was constructed, named peniquinazolinone A.
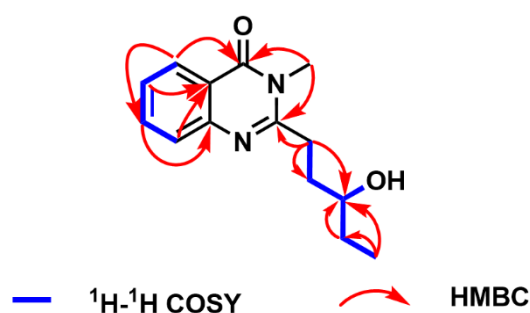
Figure 3. Key ^{1}H-^{1}H COSY and HMBC correlations of **1**.

The absolute configuration of C-13 was defined by the application of the Mosher method. Compound **1** was reacted with both (*R*)-(–) and *S*-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chlorides (MTPA-Cl) to afford the corresponding (*S*)- and (*R*)-Mosher esters, respectively (**1a** and **1b**), respectively. The absolute configuration at C-13 in **1** was determined to be *R* by the observed chemical shift differences $\Delta\delta(\delta_S\text{-}\delta_R)$ (Figure 4 and Figures S4–S5).
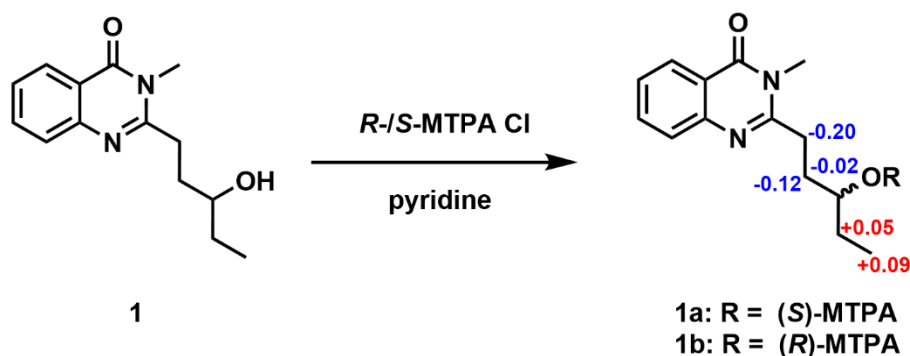
Figure 4. Δδ(δ$_S$-δ$_R$) values for MTPA esters of **1**.

2.3. Seed-Germination-Promoting Activity of the Isolated Compounds

Compounds **2**, **3**, **4**, **7**, **9**, and **12** were tested for their seed-germination-promoting activity. As shown in Figure 5, Compounds **2**, **3**, and **7** showed an optimal promoting effect on the seed germination of *Rhodiola tibetica* at a concentration of 0.01 mg/mL, with a germination rate of about 62%, 70%, and 62%, respectively, compared to the germination rate of about 52% of the positive drug 6-BA. Compounds **4** and **9** exhibited a germination rate of about 72% and 58%, respectively, at the optimal concentration of 0.001 mg/mL, indicating a negative correlation between concentration and germination rate. Compared with other tested compounds, compound **12** showed the best germination rate of about 62% at the same concentration level (0.1 mg/mL). In addition, compounds **2**, **3**, **4**, **7**, **9**, and **12** could prolong the seed-germination period of *Rhodiola tibetica* up to the 11th day, which was relatively better than the positive drug 6-BA (Figure 6). Compound **1** was not tested for its seed-germination-promoting activity due to the trace amount. Compound **2** is a structural analogue of compound **1**, generated by ring-opening of the quinazoline moiety of compound **1**, further seed germination assays of compound **1** may verify whether the existence of the quinazoline moiety affects its seed-germination-promoting activity.
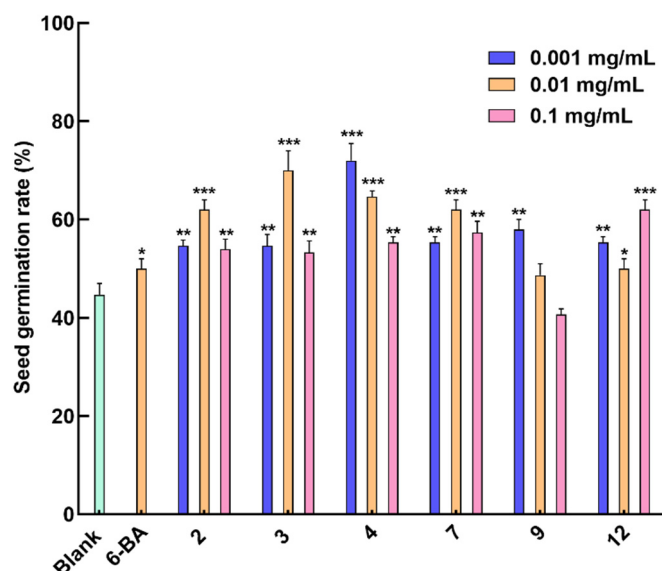
Figure 5. *Rhodiola tibetica* Seed germination rate of **2**, **3**, **4**, **7**, **9**, and **12**. Columns represent the mean ± SD, n = 3, ***P < 0.001, **P < 0.01 and *P < 0.05 vs. the blank control water.
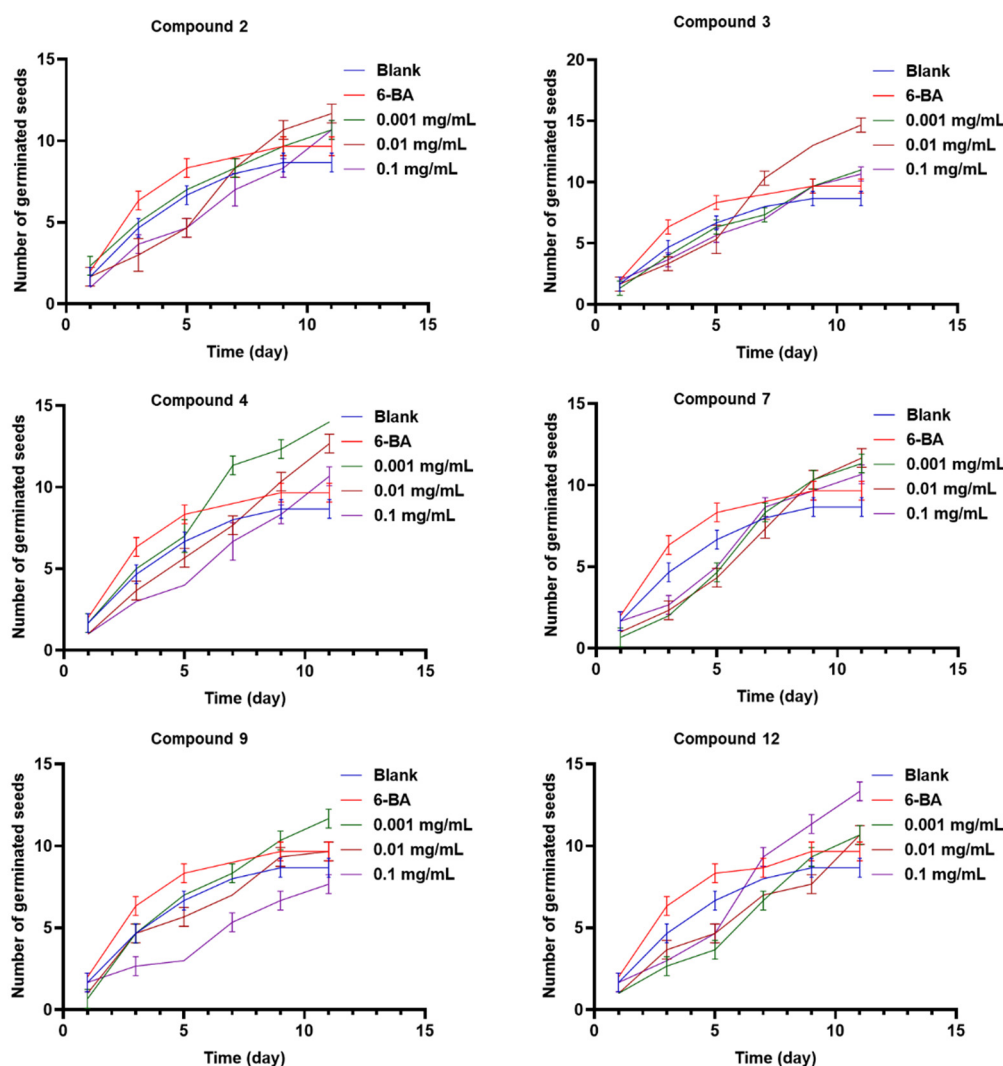
Figure 6. Effects of all the tested compounds, 6-BA, and the blank control water on the seed-germination period of *Rhodiola tibetica*.

3. Materials and Methods

3.1. General Experimental Procedures

UV spectrum was recorded on a Jasco V-560 spectrophotometer (JASCO Corporation, Japan). Optical rotation was obtained on an Autopol IV Polarimeter (Rudolph Research Analytical, Flanders, NJ, USA). CD spectrum was acquired on a Jasco J-810-150S spectropolarimeter (JASCO Corporation, Japan). High-resolution electrospray ionization mass spectrometry (HRESIMS) data was collected on an AB Sciex Triple TOF 4600 mass spectrometer (AB SCIEX, Framingham, MA, USA). NMR spectra were carried out on a Bruker Avance II 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) with tetramethylsilane (TMS) as an internal standard. Agilent 1260 Infinity (Agilent Technologies Inc., Santa Clara, CA, USA), Waters 2535 (Waters Corporation, Milford, MA, USA) and Shimadzu LC-20AR (Shimadzu Corporation, Kyoto, Japan) semi-preparative HPLC systems were performed using a Welch Ultimate XB-C18 column, (250 mm × 10.0 mm, 5 μm). Silica gel (100−200 mesh & 200−300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were used for column chromatography. The silica gel GF254 (Qingdao Marine Chemical Co., Ltd., Qingdao, China) was used for analytical and preparative thin-layer chromatography (TLC).

3.2. Fungal Material

The fungus strain *Penicillium* sp. HJT-A-6 was obtained from the stem of *Rhodiola tibetica* collected in Langkazi County, Shannan City, Tibet, China, in July 2021. It was identified based on its morphological characteristics and its sequence of the internal transcribed spacer (ITS) analysis of rDNA, and the BLAST search result showed that the sequence was the most similar (99%) to the sequence of *Penicillium* sp. (compared to MN634462.1), The sequence data of the fungus was submitted to GenBank database, accession number: OR346333.1. The fungus was deposited in the College of Life and Health, Dalian University, Dalian, China.

3.3. Fermentation and Isolation

The fungal strain was cultured on autoclaved rice medium (one hundred 500 mL Erlenmeyer flasks, each containing 80 g rice, 110 mL water) in the stationary phase at 28 °C for 40 days. After 40 days, the fermentation was cut into small pieces, and extracted with 95% EtOH three times. The extract was concentrated under reduced pressure to afford an aqueous solution, and then partitioned with petroleum ether, EtOAc, and *n*-BuOH to obtain the EtOAc-soluble extract (64 g). The extract was subjected to silica gel column chromatography with CH_2Cl_2/MeOH (100:0–0:100) to afford fourteen fractions (Fr. A–N).

Fr. B (10.5 g) was chromatographed on a silica gel column with gradient elution (PE/EtOAc, 5:1–1:2) to yield 8 subfractions (Fr. B1–Fr. B8). Fr. B5 was further purified by semi-preparative HPLC with MeOH/H_2O (40:60, 0–35 min, 3 mL/min) to obtain compound **11** (1.6 mg, t_R = 29 min).

Fr. C (6.3 g) was eluting with gradient petroleum (PE/EtOAc, 5:1–1:1) to afford 9 subfractions (Fr. C1–Fr. C9). Fr. C1 and Fr. C4 were subjected to Sephadex LH-20 gel column and preparative TLC, respectively, to obtain compounds **3** (250 mg) and **12** (4.6 mg). Fr. C5 was chromatographed by Sephadex LH-20 gel column using isocratic elution with CH_2Cl_2/MeOH (1:1), yielding Fr. C5b, which was purified by semi-preparative HPLC with CH_3CN/H_2O (30:70, 0–30 min, 3 mL/min) to give compound **6** (13.6 mg, t_R = 22 min). Fr. C7 was purified by semi-preparative HPLC with MeOH/H_2O (60:40, 0–40 min, 3 mL/min) to obtain compound **7** (193 mg, t_R = 14 min) and the mixture of **5a** and **5b** in a 1:1.7 molar ratio (13.8 mg, t_R = 17 min). Fr. C8 was applied to Sephadex LH-20 gel column with CH_2Cl_2/MeOH (1:1) to give Fr. C8e, which was purified by semi-preparative HPLC with gradient MeOH/H_2O (20:80–60:40, 0–30 min, 3 mL/min) to obtain compound **9** (20 mg, t_R = 25 min).

Fr. E (6.3 g) was chromatographed on a silica gel column with gradient elution (PE/EtOAc, 7:1–1:2), affording 20 fractions (Fr. E1–Fr. E20). Fr. E13 was purified by semi-preparative HPLC with gradient MeOH/H_2O (20:80–95:5, 0–40 min, 3 mL/min) to yield compound **1** (1.6 mg, t_R = 26 min). Fr. E14 was purified by semi-preparative HPLC with MeOH/H_2O (40:60, 0–40 min, 3 mL/min) to obtain compound **2** (3.4 mg, t_R = 16 min) and compound **4** (120 mg, t_R = 32 min). Fr. E16 was purified by semi-preparative HPLC with CH_3CN/H_2O (45:55, 0–25 min, 3 mL/min) to afford compound **8** (6 mg, t_R = 12 min).

Fr. G (6.7g) was chromatographed on a silica gel column with gradient elution (PE/EtOAc, 10:1–1:1) to afford 5 subfractions (Fr. G1–Fr. G5). Fr. G4 was applied to Sephadex LH-20 gel column with CH_2Cl_2/MeOH (1:1) to yield Fr. G4d, which was purified by semi-preparative HPLC with gradient MeOH/H_2O (20:80–95:5, 0–30 min, 3 mL/min) to obtain compound **10** (5.8 mg, t_R = 10 min).

Peniquinazolinone A (**1**): yellow oil; $[\alpha]_D^{20}$ +20 (*c* 0.2, MeOH); UV (CH_3OH) λ_{max} (log ε) 223 (4.24), 267 (3.74) nm ; ECD (CH_3OH) λ_{max} ($\Delta\varepsilon$) 220 (–7.0) nm; ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) data (see Table 1); HRESIMS *m/z* 269.1271 [M + Na]$^+$ (calculated for $C_{14}H_{18}N_2O_2Na$, 269.1266).

3.4. Mosher Esterification of Compound **1**

Compound **1** (0.5 mg) was dissolved in 100 μL $CDCl_3$ in an NMR tube, sequentially added 9 μL pyridine and 15 μL (*R*)-(–)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride ((*R*)-(–)-MTPA-Cl). The mixture was stirred at room temperature for 1 h to afford the corresponding (*S*)-Mosher ester

(**1a**), and subsequently diluted with 300 μL CDCl₃ to acquire ¹H NMR spectrum. The (*R*)-Mosher ester (**1b**) of **1** was prepared from (*S*)-(+)-MTPA-Cl using the same method.

3.5. Seed-Germination-Promoting Assay

Compounds **2**, **3**, **4**, **7**, **9** and **12** was dissolved in 0.2% DMSO aqueous solution to yield the stock solution with a concentration of 0.06 mg/mL. 5 μL, 50 μL and 500 μL of compounds **2**, **3**, **4**, **7**, **9** and **12** was added to the 30 mm filter paper placed in the 6-well plate. After evaporation of the solvent, the filter paper was immersed in 300 μL of distilled water, and then 20 seeds of *Rhodiola tibetica* were displayed in each 30 mm filter paper and incubated at a light-dark regime of 16:8 h condition, 20 °C for 7 days. The germination rate of the seeds was calculated after incubation. The experimental data were collected from three independent experiments. Further experiments of the relationship between the number of germinated seeds and germination time for compounds **2**, **3**, **4**, **7**, **9** and **12** were also conducted, the germination period was set up to 11 days.

4. Conclusions

In this work, the chemical investigation of the *Rhodiola tibetica* endophytic fungus *Penicillium* sp. HJT-A-6 led to the isolation and identification of a new quinazolinone alkaloid, named peniquinazolinone A (**1**), and eleven known compounds, 2-(2-hydroxy-3-phenylpropionamido)-*N*-methylbenzamide (**2**), viridicatin (**3**), viridicatol (**4**), (±)-cyclopeptin (**5a/5b**), dehydrocyclopeptin (**6**), cyclopenin (**7**), cyclopenol (**8**), methyl-indole-3-carboxylate (**9**), 2,5-dihydroxyphenyl acetate (**10**), methyl *m*-hydroxyphenylacetate (**11**), and conidiogenone B (**12**). Compound **1** was identified by extensive spectroscopic analysis and the modified Mosher's method. Compounds **2**, **3**, **4**, **7**, **9**, and **12** were evaluated for their seed germination activity. Compounds **2**, **3**, and **7** showed an optimal promoting effect on the seed germination of *Rhodiola tibetica* at a concentration of 0.01 mg/mL. Compounds **4** and **9** optimal seed-germination-promoting activity at a concentration of 0.001 mg/mL, while the optimal concentration for compound **12** to promote *Rhodiola tibetica* seed germination is 0.1 mg/mL. Additionally, all the tested compounds assuredly prolonged the seed-germination period of the host plant. These results not only broadened the structural diversity of quinazoline metabolites derived from fungi, but also provided data support for understanding the interactive relationship between endophytic fungi and host plants.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Positive mode HRESIMS spectrum of **1**; Figure S2: UV spectrum of **1**; Figure S3: ¹H NMR (DMSO-*d₆*, 500 MHz) spectrum of **1**; Figure S4: ¹H NMR (DMSO-*d₆*, 500 MHz) spectrum of **1a**; Figure S5: ¹H NMR (DMSO-*d₆*, 500 MHz) spectrum of **1b**; Figure S6: ¹³C NMR (DMSO-*d₆*, 125 MHz) spectrum of **1**; Figure S7: HSQC (DMSO-*d₆*, 500 MHz) spectrum of **1**; Figure S8: HMBC (DMSO-*d₆*, 500 MHz) spectrum of **1**; Figure S9: ¹H-¹H COSY (DMSO-*d₆*, 500 MHz) spectrum of **1**; Figure S10: NOESY (DMSO-*d₆*, 500 MHz) spectrum of **1**; Figure S11: CD spectrum of **1**.

Author Contributions: Conceptualization, D.X., X.L. and B.F.; methodology, D.X., Y.W., X.L. and B.F.; validation, D.X., Y.W., X.L. and B.F.; formal analysis, D.X., X.L. and B.F.; investigation, D.X., Y.W., C.G., X.Z., W.F., X.L. and B.F.; resources, X.L. and B.F.; data curation, D.X., X.L. and B.F.; writing—original draft preparation, D.X. and Y.W.; writing—review and editing, D.X. and X.L.; visualization, X.L. and Y.W.; supervision, D.X., X.L. and B.F.; project administration, X.L. and B.F.; funding acquisition, D.X., C.G. and X.L. All authors have read and agreed to the published version of the manuscript.

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