This open access document is posted as a preprint in the Beilstein Archives at https://doi.org/10.3762/bxiv.2023.58.v1 and is considered to be an early communication for feedback before peer review. Before citing this document, please check if a final, peer-reviewed version has been published.

This document is not formatted, has not undergone copyediting or typesetting, and may contain errors, unsubstantiated scientific claims or preliminary data.

Preprint Title	Chemical and Biosynthetic Potential of *Penicillium shentong* XL-F41
Authors	Ran Zou, Xin Li, Xiaochen Chen, Yue-Wei Guo and Baofu Xu
Publication Date	07 Dez. 2023
Article Type	Full Research Paper
Supporting Information File 1	Supporting Information-re.pdf; 2.8 MB
ORCID® iDs	Baofu Xu - https://orcid.org/0000-0002-0144-2455

License and Terms: This document is copyright 2023 the Author(s); licensee Beilstein-Institut.

This is an open access work under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0). Please note that the reuse, redistribution and reproduction in particular requires that the author(s) and source are credited and that individual graphics may be subject to special legal provisions.
The license is subject to the Beilstein Archives terms and conditions: https://www.beilstein-archives.org/xiv/terms.
The definitive version of this work can be found at https://doi.org/10.3762/bxiv.2023.58.v1

Chemical and Biosynthetic Potential of *Penicillium shentong* XL-F41

Ran Zou[1,3], Xin Li[1], Xiaochen Chen[1], Yue-Wei Guo[1,2], Baofu Xu[*1,2]

[1]Shandong Laboratory of Yantai Drug Discovery, Bohai Rim Advanced Research Institute for Drug Discovery, Yantai, Shandong 264117, China.

[2]State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Zhangjiang Hi-Tech Park, Shanghai, 201203, China.

[3]School of Life Sciences, Ludong University, Yantai 264025, China.

Baofu Xu*: bfxu@simm.ac.cn

* Corresponding author

Abstract

Penicillium strains are renowned for producing diverse secondary metabolites with unique structures and promising bioactivities. Our chemical investigations, accompanied by fermentation media optimization, of a newly isolated fungus, *Penicillium shentong* XL-F41, led to the isolation of twelve compounds. Among these are two novel indole terpene alkaloids, Shentonin A and B (**1** and **2**), and a new fatty acid (**3**). **1** is distinguished by an unusual methyl modification at the oxygen atom of the typical succinimide ring, a feature not seen in the structurally similar brocaeloid D. Additionally, **1** exhibits a *cis* configuration between H-3 and H-4, as opposed to the *trans* configuration in brocaeloid D, suggesting a divergent enzymatic ring-expansion

24 process in their respective fungi. Both **1** and **2** also feature a reduction of a ketone to
25 a hydroxyl group within the succinimide ring. All isolated compounds were subjected
26 to antimicrobial evaluations, and compound **12** was found to have moderate inhibitory
27 activity against *Candia albicans*. Moreover, genome sequencing of *Penicillium*
28 *shentong* XL-F41 uncovered abundant silent biosynthetic gene clusters, indicating the
29 need for future efforts to activate these clusters and unlock the full chemical potential
30 of the fungus.

31 **Keywords**

32 *Penicillium*; natural products; indole terpene alkaloid; structure elucidation; genome
33 analysis;

34 Introduction

35 *Penicillium*, a genus within the *Ascomycota* phylum, is a type of critical saprophytic
36 fungus with over 400 strains identified in diverse environments such as mountains,
37 oceans, and the gut [1]. The landmark discovery of penicillin from a *Penicillium* strain
38 in 1929 revolutionized medicinal research, and since then, *Penicillium* has been
39 important in drug development. Researchers have identified numerous compounds
40 with anticancer properties, including mycophenolic acid, brefeldin A, wortmannin, and
41 emodin [2], as well as compounds with antibacterial properties like xestodecalactones
42 A–C, penicifurans A, and anthraquinonecitrinin [3]. From 2010 to 2022, researchers
43 have identified over 260 secondary metabolites from *Penicillium* [4], exhibiting not only
44 antibacterial and anticancer activities but also potent antioxidant properties, inhibition
45 of GSK-3β and α-glucosidase activities, and interaction with the pregnane X receptor
46 (PXR). These compounds are categorized into polyketides, alkaloids, sterol

47 derivatives, terpenoids, and macrolides, with polyketides and alkaloids comprising
48 40% and 32% of the total, respectively.
49 Alkaloids are a diverse group of compounds with multiple pharmacological
50 activities, including anti-inflammatory, antibacterial, antiviral, insecticidal, and
51 anticancer properties [5-9]. Historically, most alkaloids were isolated from higher
52 plants, with a significant number found in the Compositae family. Notable examples
53 such as vinblastine, vinorelbine, vincristine, and vindesine have gained prominence as
54 effective anticancer drugs [5]. Recent studies have revealed that certain fungi are also
55 prolific sources of indole alkaloids, which are among the largest classes of nitrogen-
56 containing secondary metabolites. Characterized by at least one indole moiety and
57 derived from tryptophan or tryptamine, indole alkaloids are known for their diverse
58 structures, electron-donating capabilities, and excellent biocompatibility, contributing
59 to their potent antibacterial and anticancer activities [8, 9]. Over 4000 species [7]
60 producing indole alkaloids have been identified, and many of these compounds are
61 now successfully employed in clinical applications.
62 Despite the extensive catalog of secondary metabolites discovered, the pace of
63 new findings has decelerated. However, the advent of bioinformatics analysis tools has
64 reinvigorated the search for fungal secondary metabolites. The estimated number of
65 non-redundant clusters in *Penicillium* is around 25,000 [1], yet the number of isolated
66 compounds is significantly lower, indicating the presence of many unexpressed gene
67 clusters. This suggests a wealth of undiscovered compounds with potentially novel
68 structures and significant biological activities. To stimulate the expression of
69 biosynthetic gene clusters (BGCs), several methods can be utilized, for instance,
70 epigenetic regulation, co-culture, precursor feeding, heterologous expression, and
71 changing fermentation parameters [10-13].

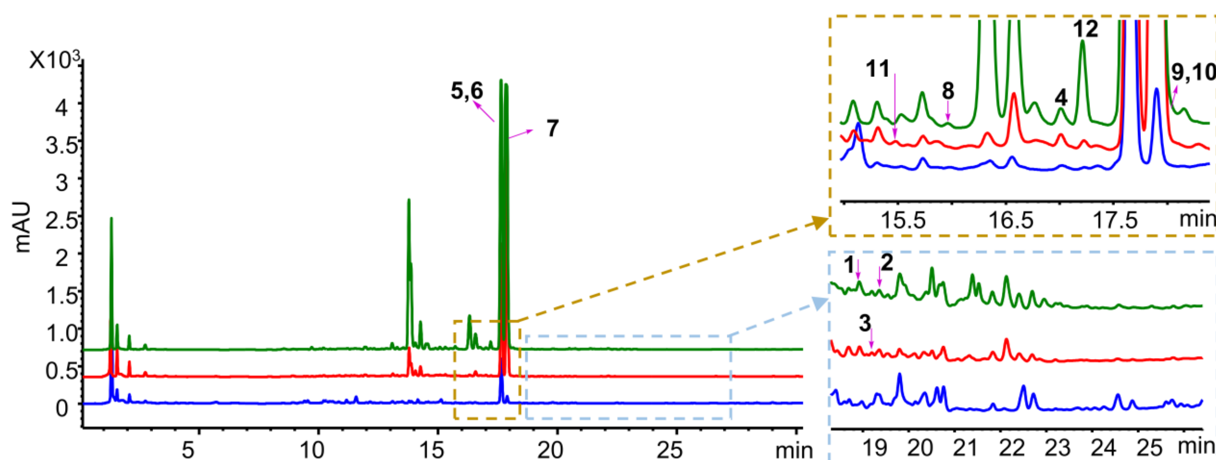
In the present study, we focused on a newly identified *Penicillium* strain, *Penicillium shentong* XL-F41. To activate the BGCs of this strain, we employed a combination of elicitors in our fermentation media, including histone deacetylase inhibitors and DNA methyltransferase inhibitors. We developed two specialized media, XISR I and XISR I II, which outperformed the traditional potato dextrose broth (PDB) in stimulating the production of a greater number of metabolite peaks, as shown in **Figure 1**. Scaled-up fermentation allowed us to isolate and characterize two new indole terpene alkaloids, Shentonin A and B (**1** and **2**), a new fatty acid (**3**), and nine previously identified compounds (**4**-**12**), among which were gram quantities of curvularin analogs. Our bioactivity assays identified one compound, **12**, with promising antimicrobial properties. Subsequent genome sequencing analysis pinpointed the likely BGCs associated with our isolated compounds and suggested a vast potential for the production of additional compounds, given the application of suitable activation techniques.

Results and Discussion

Compound isolation and structure elucidation

To activate the silent BGCs in *Penicillium shentong* XL-F41, we conducted small-scale fermentations using various media. Analysis revealed that HPLC peaks, which correspond to fermentation products, showed a lower number and abundance in the PDB medium than in the XISR I and XISR III media, as illustrated in **Figure 1**. Consequently, we chose XISR I and XISR III media for further fermentation.

93



94 **Figure 1. HPLC analysis of small-scale fermentation with different media.** More
95 details of media, XISR I and XISR III can be found in the methods section.

96 The fermentation broth was exhaustively extracted with ethanol, after which the
97 ethanol extract was partitioned between EtOAc and H2O. The EtOAc fraction was
98 chromatographed repeatedly over silica gel and reverse-phase high-performance
99 liquid chromatography (RP-HPLC), resulting in the isolation of pure compounds (**1**-**12**).
100 According to literature reports of known compounds, some of them were identified as
101 fusarindoles B (**4**) [14], dehydrocurvularin (**5**) [15], hydroxycurvularin (**6**) [16], curvularin
102 (**7**) [17], curvulopyran (**8**) [18], (*S*)-6- (sec-butyl)-3-isobutylpyrazin-2 (1H)-one (**9**) [19],
103 3, 6-di-sec-butyl-2 (1H)-pyrazinone (**10**) [19], daidzein (**11**) [20], and genistein (**12**) [21].
104 Notably, compound **7**, corresponding to the major peak in our optimized fermentation
105 (**Figure 1**), was obtained at the gram level.

106

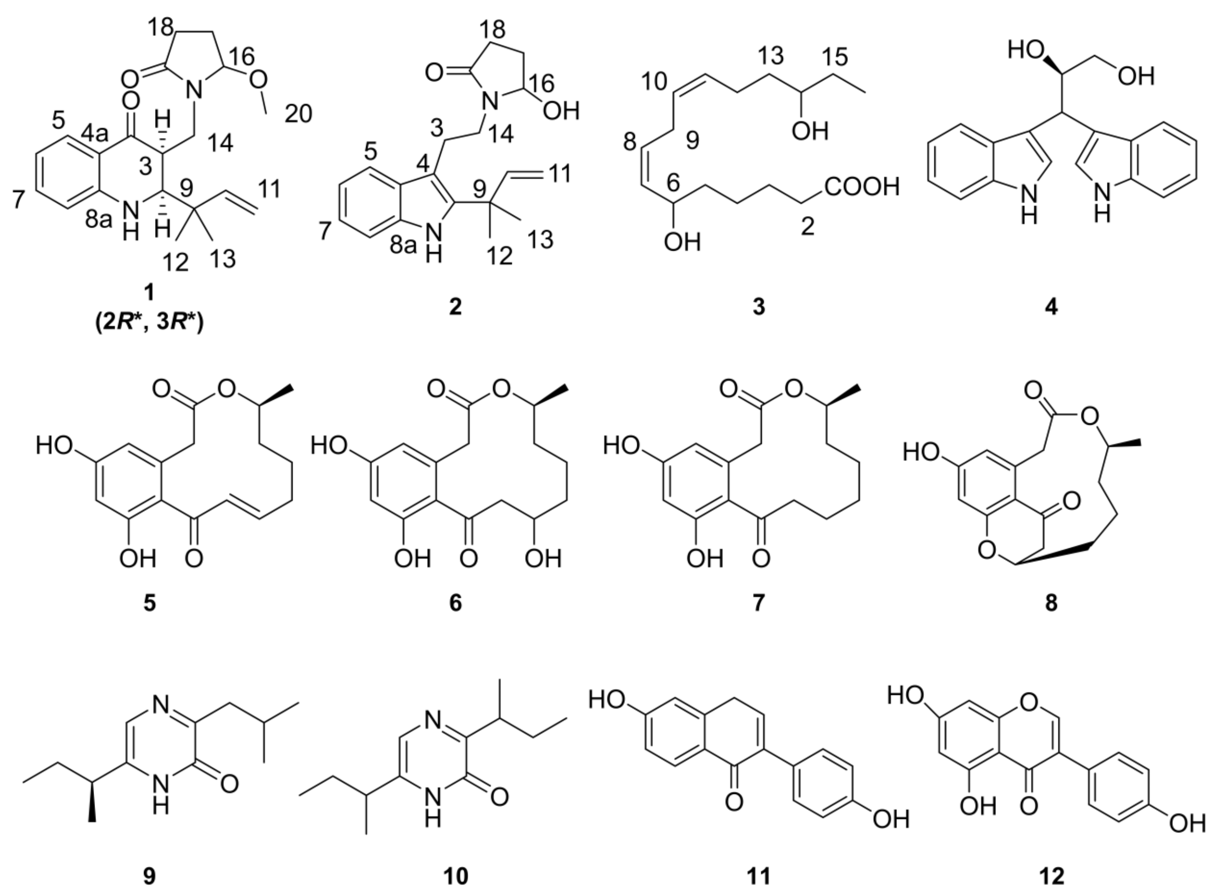


Figure 2. Chemical structures of compounds 1-12

Compound **1** (Shentonin A) was obtained as a light green crystal with a chemical formula of $C_{20}H_{26}N_2O_3$, as determined by HRMS m/z 365.1828 $[M+Na]^+$ (calcd. for $C_{20}H_{26}N_2O_3Na^+$, 365.1835) and HRMS m/z 341.1862 $[M-H]^-$ (calcd. for $C_{20}H_{25}N_2O_3$, 341.1870). Spectroscopic analysis, including ^{1}H-NMR, ^{13}C-NMR, and DEPT, revealed that compound **1** contains three methyl groups, one of which is oxygenated, four methines, three saturated non-protonated carbons, and two ketone carbonyl carbons (δC175.94, δC194.36). Its NMR data closely resemble those of brocaeloid D, with the notable addition of a methoxy group (δH 3.20/δC 53.92). HMBC correlations confirmed the presence of an isoprene group and differentiated compound **1** from brocaeloid D by the substitution of a succinimide substructure at C-14 with a methine at C-16, indicated by the methoxy group. The methoxy group's position was established by HMBC correlations, and the ^{13}C NMR data suggested that compound **1** includes a 2,

121 3-dihydroquinoline-4(1H)-one fragment. The planar structure was established from
122 HMBC correlations linking three different fragments.

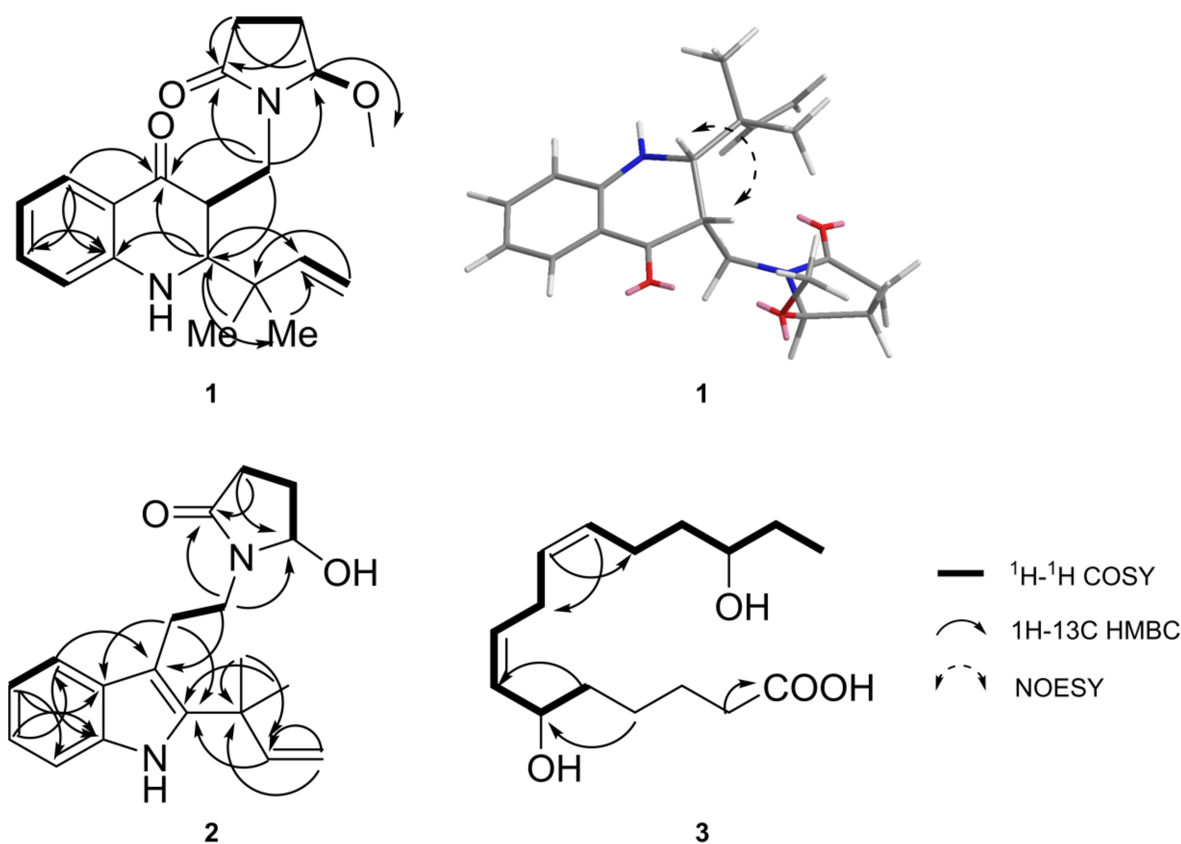
123 Compound **1** features three chiral centers at C-2, C-3, and C-16. The relative
124 configuration of C-2 and C-3 was determined as (2*R**, 3*R**) by ^{1}H-^{1}H NOESY
125 correlations, while the relative configuration of C-16 remains unresolved due to the
126 inapplicability of the NOESY experiment. Further, a low content of **1** precludes direct
127 methods for absolute configuration determination.

128 **Table 1. ^{1}H and ^{13}C data of compound 1 (recorded in $CDCl_3$)**

	δH mult (*J* in Hz)	δC mult
1	-	NH
2	3.09 (dd, 3.9, 1.0)	61.42, CH
3	2.92 (t, 7.6)	45.22, CH
4	-	194.36, qC
4a	-	116.93, qC
5	7.68 (dd, 7.9, 1.6)	127.22, CH
6	6.61, m	114.92, CH
7	7.28 (d, 1.7)	136.06, CH
8	6.61, m	116.75, CH
8a	-	149.93, qC
9	-	43.15, qC
10	5.65 (dd, 17.5, 10.8)	144.38, CH
11	5.02, m	114.37, CH2
12	0.97 (d, 7.2)	23.25, CH3
13	0.97 (d, 7.2)	23.52, CH3
14a	3.16, m	41.85, CH2
14b	3.90 (dd, 13.9, 8.8)	-
15	-	N
16	4.77 (dd, 6.1, 1.2)	89.77, CH
17a	1.99 (ddd,13.5,9.5)	24.32, CH2
17b	2.12, m	-
18a	2.37 (ddd, 17.2, 9.9)	28.94, CH2
18b	2.51 (dt, 17.8, 9.2)	-
19	-	175.94, qC
20	3.20, s	53.92, CH3

129

130



131 **Figure 3. Key 2D NMR correlations of compounds 1-3**

132 Compound **2** (Shentonin B) was isolated as a light green crystal. Its chemical
133 formula, $C_{19}H_{24}N_2O_2$, was confirmed by HRMS with m/z 335.1719 $[M+Na]^+$ (calcd. for
134 $C_{19}H_{24}N_2O_2Na^+$, 335.1730) and m/z 311.1755 $[M-H]^-$ (calcd. for $C_{19}H_{23}N_2O_2$,
135 311.1765). Spectroscopic analysis using ^{1}H-NMR, ^{13}C-NMR, and DEPT (**Table 2**)
136 indicated that compound **2** comprises two methyl groups, five methines, five saturated
137 non-protonated carbons, and one ketone carbonyl carbon (δC 175.94, δC 194.36). Its
138 NMR profile is similar to brocaeloid C[23], with the distinction of an added succinimide
139 substructure at N-15, where the ketone carbonyl carbon at C-16 is replaced by a
140 hydroxyl carbon. The isoprene group is consistent with that in compound **1**. HMBC
141 cross-peaks from H-2 to C-10 and C-13 connect the indole and isoprene units, while
142 HMBC correlations from H-14 to C-4, C-16, and C-19, and from H-3 to C-4a and C-9,
143 elucidate the connectivity of three fragments. These data collectively establish the
144 planar structure of compound **2**.

145 **Table 2. ^1H and ^{13}C NMR data of compound 2 (recorded in $CDCl_3$)**

	δH mult (J in Hz)	δC mult
1	7.90, s	NH
2	-	140.07, qC
3	3.09 (ddd, 14.1, 9.3, 5.6)	23.98, CH2
3a	3.16 (ddd, 14.1, 9.5, 6.5)	-
4	-	108.14, qC
4a	-	129.69, qC
5	7.61 (dp, 7.8, 0.7)	118.38, CH
6	7.09 (ddd, 8.1, 7.0, 1.1)	119.66, CH
7	7.14 (ddd, 8.1, 7.0, 1.2）	121.73, CH
8	7.29 (dt, 8.0, 1.0)	110.66, CH
8a	-	134.20, qC
9	-	39.11, qC
10	6.14 (dd, 17.4, 10.5）	146.00, CH
11	5.16 (d, 1.1)	112.24, CH2
11a	5.18 (dd, 2.5, 1.1)	-
12	1.56 (d, 1.5)	27.77, CH3
13	-	27.80, CH3
14	3.51 (m)	41.54, CH2
14a	3.67 (ddd, 13.7, 9.5, 5.6)	-
15	-	N
16	4.98 (s)	84.32, CH
17	2.30 (ddd, 17.1, 10.1, 4.3)	28.96, CH2
17a	2.54 (ddd, 16.9, 9.7, 7.2)	-
18	1.76 (dddd, 13.8, 9.7, 4.3, 2.4)	28.76, CH2
18a	2.19 (dddd, 13.7, 10.1, 7.3, 6.4)	-
19	-	174.67, qC

146 Compound **3** was isolated as a transparent oily liquid, and its chemical formula,
147 $C_{16}H_{28}O_4$, was confirmed by LC-MS with m/z 283.2 $[M-H]^-$ (calcd. for $C_{16}H_{27}O_4$, 283.2)
148 (**Figure S12**). Spectroscopic analyses, including ^1H-NMR, ^{13}C-NMR, DEPT, HSQC,
149 COSY, and HMBC (**Table 3**, **Figure 3**), identified compound **3** as a sixteen-carbon
150 fatty acid. Notably, two methylene carbons overlapped in the ^{13}C NMR spectrum. The
151 COSY correlations facilitated the determination of the carbon chain fragments from C-
152 11 to C-16 and C-2 to C-10, despite two methylene signals overlapping. The carboxyl
153 group's position at C-1 was confirmed by HMBC correlations from H-2/3. Furthermore,
154 HMBC cross-peaks from H-12 to C-10, H-11 to C-9, and H-10 to C-12 indicated that

155 the fragments are connected through C-11 and C-10, establishing the structure of

156 compound **3**.

157 **Table 3. ^{1}H and ^{13}C NMR data of compound 3 (recorded in CDCl3)**

	δH mult (J in Hz)	δC mult
1	-	177.2, qC
2	2.41, m; 1.96 (dp, 12.9, 9.3)	29.35, CH2
3	2.57, m	29.08, CH2
4	2.57, m	29.08, CH2
5	2.41, m; 1.96 (dp, 12.9, 9.3)	29.35, CH2
6	5.30, m	76.4, CH
7	5.47, m	127.53, CH
8	5.66 (dt, 11.0, 7.5)	134.02, CH
9	2.86 (dt, 15.3, 7.3)	26.32, CH2
9a	2.96 (dt, 13.5, 7.6)	-
10	5.36, m	126.92, CH
11	5.47, m	131.04, CH
12	2.19 (q, 7.6)	23.75, CH2
13	1.49, m	36.57, CH2
14	3.53 (tt, 8.3, 4.4)	72.84, CH
15	1.49, m	30.54, CH2
16	0.94 (t, 7.5)	10.03, CH3

158 **Biological activities**

159 In our bioassays, we evaluated the inhibitory activity of all isolated compounds

160 against a panel of microorganisms, including *Escherichia coli*, *Candida albicans*,

161 *Staphylococcus aureus*, *Pseudomonas fulva*, and *Enterobacter hormaechei*. The

162 results indicated that compounds **3**, **5**, **6**, **7**, and **12** were active against *Candida*

163 *albicans*. Notably, compound **12** showed particularly promising inhibitory activity

164 against this fungal pathogen.

165 **Table 4. Antimicrobial activity of compounds 1-12.** Minimum inhibitory

166 concentrations were shown in µg/mL.

No.	*Escherichia coli*	*Candida albicans*	*Staphylococcus aureus*	*Pseudomonas Fulva*	*Enterobacter hormaechei*
1	—	—	>100	—	—

3	—	50-100	—	>100	>100
4	—	>100	—	—	—
5	>100	25-50	>100	>100	>100
6	>100	25-50	>100	>100	>100
7	>100	64-128	>100	>100	>100
8	—	—	—	>100	>100
9	>100	>100	—	>100	>100
11	—	—	—	—	—
12	>100	12.5-25	>100	>100	>100

Genome sequencing analysis

The genome sequencing yielded 7,118,236 reads with an average read length of 1,858.7 bp. The assembled genome is 34,621,366 bp long, comprising 9 contigs with a mean contig length of 3,846,818.44 bp, and the longest contig is 5,975,444 bp. The genome's GC content is 46.43%. Annotation of the genome sequence of *Penicillium shentong* XL-F41 identified 11,235 coding sequences and 172 tRNA genes.

Upon utilizing the fungal version of AntiSMASH 5.0 software for the analysis of the *Penicillium shentong* XL-F41 genome, we identified 46 BGCs. These include 13 NRPS-like fragments, 6 NRPS, 13 Type I PKS, 2 PKS/NRPS hybrids, 1 NI-siderophore, 2 NRP-metallophore/NRPS hybrids, 1 fungal RiPP with POP or UstH peptidase types, 1 fungal-RiPP-like/T1PKS, 1 betalactone, 1 PKS type I/NRPS/indole hybrid, 1 fungal-RiPP-like/T1PKS hybrid, 1 NRP-metallophore/NRPS hybrid, NRPS-like/terpene/phosphonate hybrids, 3 terpenes, and 1 indole-related cluster (**Table 5**).

BGC 7.3, identified as an indole-type gene cluster, includes genes for cytochrome P450, pyridoxal-dependent decarboxylase, glutamine synthase, and tryptophan dimethyltransferase (**Figure 4**). These genes are likely crucial for the biosynthesis of

183 the newly isolated alkaloids, **1** and **2**. In examining the XL-F41 genome for
184 methyltransferase domain-containing BGCs, we found a methyltransferase near BGC
185 7.3, suggesting its involvement in adding a methoxy group at the C16 position of
186 compound 1. From these key enzyme genes, we propose a hypothetical biosynthetic
187 pathway (**Figure 4**).

188 Compounds **1** and **2** are hypothesized to be synthesized from a tryptophan
189 precursor via a shared biosynthetic pathway (**Figure 4**). Briefly, the prenyl group is
190 attached to tryptophan through a prenylation reaction catalyzed by ShnA, followed by
191 the decarboxylation of the carboxy group by ShnC. Subsequently, compound **2** is
192 formed by the addition of succinimide to N15 in **1** via a reaction catalyzed by ShnB.
193 The transformation of the five-membered pyrrole ring in compound **2** to the six-
194 membered ring in compound **1** is particularly intriguing. For this transformation, three
195 hypotheses are considered. One suggests that the methyl modification at the oxygen
196 atom of the succinimide ring occurs first, which is then followed by a ring-opening
197 rearrangement. Alternatively, it is proposed that the ring-opening rearrangement
198 precedes the methyl modification at the oxygen atom of the succinimide ring.

199 We aim to confirm the initial step of this pathway, where tryptophan and DMAPP
200 are catalyzed by the enzyme ShnA to form a reverse prenylated tryptophan. However,
201 attempts to express the protein in various *Escherichia coli* hosts were unsuccessful,
202 suggesting that eukaryotic hosts might be more suitable for future studies. We plan to
203 conduct further experiments to substantiate the hypothesis regarding the biosynthetic
204 pathways in the future.

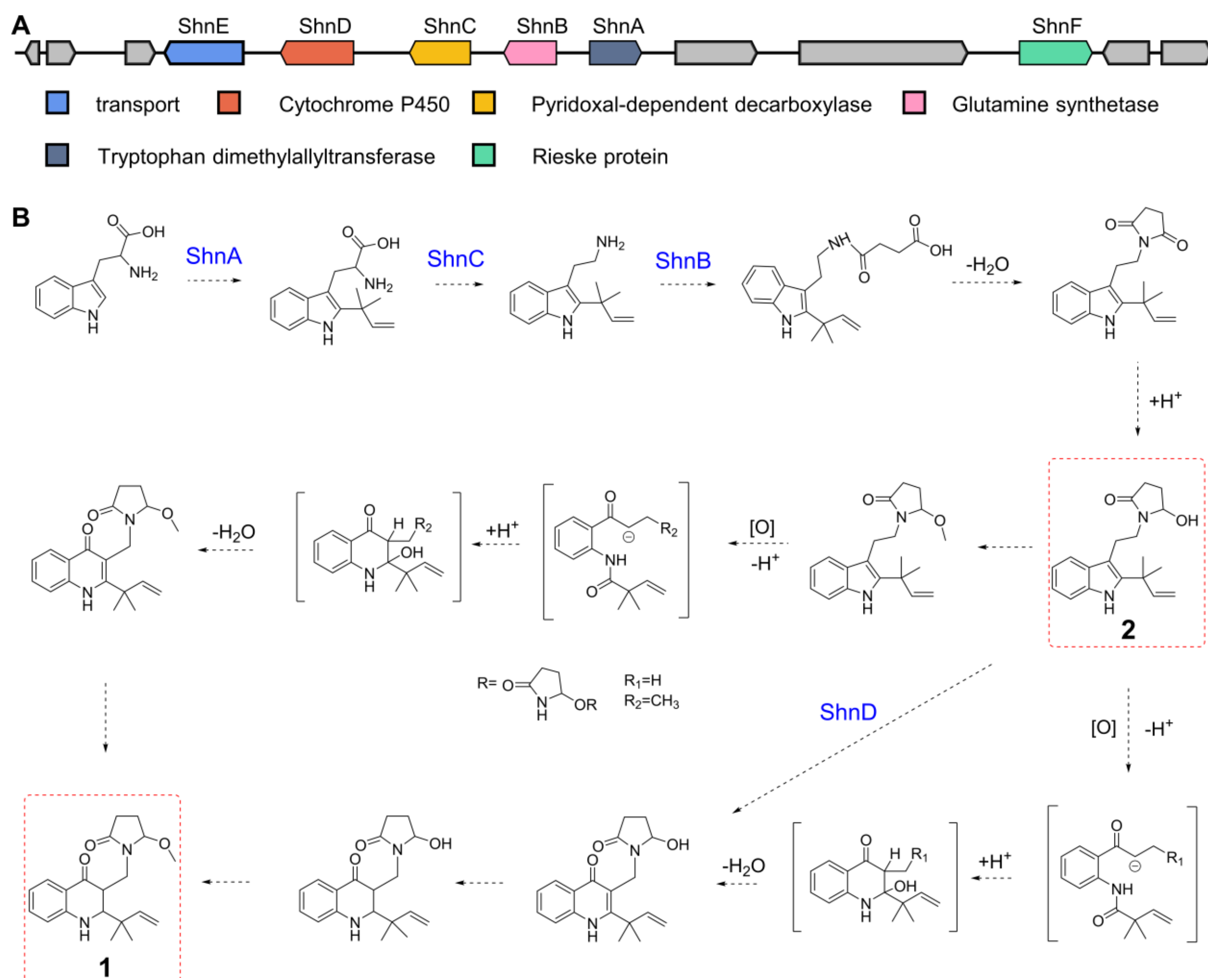


Figure 4. Biosynthetic exploration of compounds 1 and 2. A: The schematic presents the biosynthetic gene cluster for compounds **1** and **2**, highlighting ShnA, ShnB, ShnC, ShnD, and ShnE as core genes. **B**: The diagram proposes biosynthetic pathways for compounds **1** and **2**, detailing three potential mechanisms that could convert the five-membered ring structure of compound **2** into the six-membered ring structure of compound **1**.

Table 5. Biosynthetic gene clusters of the *Penicillium shentong* XL-F41

BGC	Type	Putative product
1.1	NRPS-like	
1.2	NRPS-like	
1.3	NI-siderophore	
1.4	NPR-metallophore, NRPS	
1.5	NRPS	
1.6	PKS type I	
1.7	PKS type I	
1.8	PKS type I	
1.9	PKS type I	
1.10	NRPS-like	
2.1	NRPS-like	

2.2	NRPS-like	
2.3	PKS type I	
2.4	NRPS-like	
3.1	PKS type I	
3.2	terpene	
3.3	fungal-RiPP	
3.4	PKS type I	
3.5	PKS type I	
4.1	PKS type I	
4.2	NRPS-like	
4.3	NRPS	
4.4	NRPS-like	
5.1	PKS type I, NRPS	
5.2	NRPS-like	
5.3	PKS type I, NRPS, indole	
5.4	PKS type I	
5.5	betalactone	
6.1	NRPS	
6.2	fungal-RiPP-like, T1PKS	
6.3	PKS type I	
6.4	terpene	
6.5	NRP-metallophore, NRPS	
7.1	NRPS	
7.2	NRPS-like, terpene, phosphonate	
7.3	indole	Shentonin A-B
7.4	terpene	
7.5	NRPS-like	
8.1	NRPS-like	
8.2	NRPS, PKS type I	
9.1	PKS type I	dehydrocurvularin
9.2	NRPS	
9.3	NRPS-like	
9.4	NRPS	
9.5	NRPS-like	
9.6	PKS type I	

213 # Conclusion and Discussion

214 In the present study, we fermented *Penicillium shentong* XL-F41 by adding a
215 series of elicitors in the medium, which led us to identify twelve compounds, including
216 two new indole alkaloids, Shentonin A and B (**1** and **2**), and a new fatty acid (**3**).

217 Notably, compound **1** differs from the known brocaeloid D by the addition of a
218 methyl group, and there is a change in the relative stereochemistry at C2 and C3. In

219 addition, the conversion of the five-membered pyrrole ring in compound **2** to the six-
220 membered piperidine ring in compound **1** is intriguing.

221 Moreover, to address the low yields that hindered the determination of absolute
222 stereochemistry, we attempted to boost the production of compounds **1** and **2** by
223 supplementing the medium with the precursor tryptophan. Contrary to our
224 expectations, this approach did not increase their production. Our next step is to plan
225 the heterologous expression of core genes in proper fungal hosts to improve
226 production and investigate the biosynthetic pathways of compounds **1** and **2**.

227 Furthermore, we conducted a genome sequencing analysis of *Penicillium*
228 *shentong* XL-F41, which allowed us to pinpoint the biosynthetic gene clusters (BGCs)
229 associated with our isolated compounds and reveal the biosynthetic capabilities of this
230 strain. Despite the addition of various elicitors to the fermentation medium, numerous
231 BGCs remain uncharacterized, indicating that additional strategies are required to fully
232 elucidate the compounds encoded by all BGCs.

233 Experimental

234 General experimental procedures

235 HRESIMS spectra were acquired using a Waters ACQUITY UPLC I-Class-Vion
236 IMS Q-Tof Liquid Chromatograph Mass Spectrometer. For NMR analysis, we utilized
237 an AVANCE II 600 spectrometer, referencing residual solvent peaks at δH/δC
238 7.27/77.0 ppm in $CDCl_3$ for chemical shift calibration. We utilized commercial silica gel
239 from Yantai Xinnuo New Material Technology Co., Ltd, Yantai, China, available in 100–
240 200 and 200–300 mesh sizes. Reversed-phase HPLC analyses were conducted on an
241 Agilent 1260 instrument equipped with a DAD detector and an Agilent ZORBAX SB-

242 C18 column (5 μm, 4.6 × 150 mm). The solvents used for HPLC were supplied by
243 Yantai Huisente New Material Technology Co., Ltd, Yantai, China.

244 Fungus isolation and characterization

245 The fungus *Penicillium shentong* XL-F41 was isolated from soil collected in
246 Shentong Mountain, Shandong Province, China, in June 2022. It was cultured on
247 potato dextrose agar (PDA) at 28 ^{o}C. Sequencing and comparison with the GenBank
248 database confirmed its identification as *Penicillium* sp. This strain is preserved at the
249 Shandong Laboratory of Yantai Drug Discovery.

250 Fermentation in shaking flasks

251 For large-scale fermentations, fresh mycelia of *Penicillium shentong* XL-F41 were
252 first cultivated in liquid potato dextrose broth at 28 ^{o}C for 2 days. Subsequently, they
253 were inoculated into XISR I and XISR III liquid media (total volume 30 L) with a 20%
254 inoculum dose. The cultures were further fermented for 14 days at 28 ^{o}C and 200 rpm.
255 Media recipes:
256 XISR I medium (yeast extract 4 g/L； malt extract 10 g/L； glucose 4 g/L； $MgCl_2$
257 1 μM； $FeSO_4$ 1 μM； KI 2 g/L, KCl 2 g/L, KBr 2 g/L, $NaNO_2$ 2 g/L; H_2O_2 20 μM；
258 (Methyl Jasmonate) MeJA 10 μM)
259 XISR III medium (yeast extract 4 g/L; soy flour 10 g/L; glucose 30 g/L; $MgCl_2$ 1
260 μM; $FeSO_4$ 1 μM; KI 2 g/L, KCl 2 g/L, KBr 2 g/L, $NaNO_2$ 2 g/L; H_2O_2 20 μM; MeJA 10
261 μM; 5-azacytidine 6 μM; suberoylanilide hydroxamic acid 6 μM; sodium butyrate 6 μM)

262 Extraction

263 The mycelium was separated from the fermentation broth using a centrifuge and
264 subsequently extracted with ethanol in a 1:1 ratio using ultrasound, three times for 20
265 minutes each. The combined organic solvents were dried with a rotary evaporator to

266 yield an ethanol extract. This extract was further processed with ethyl acetate (EtOAc)
267 three times. The combined EtOAc phase was then dried using a rotary evaporator to
268 obtain the EtOAc extract, which was stored at -80 ^{o}C until further purification process.
269 The above fermentation broth was adsorbed onto macroporous resin for 4 hours
270 or left overnight. It was then eluted with deionized water and ethanol through a
271 chromatography column. The ethanol eluate was concentrated to dryness using a
272 rotary evaporator. Subsequently, the ethanol extract underwent a triple extraction with
273 EtOAc. The combined EtOAc extracts were dried using a rotary evaporator to obtain
274 the final EtOAc extract, which was stored at -80 ^{o}C until further isolation process.

275 Compounds purification

276 The EtOAc extract (3.5 g) obtained from the fermentation broth of XISR I medium
277 underwent column chromatography on silica using a gradient of petroleum ether-ethyl
278 acetate (PE-EA) and ethyl acetate-methanol (EA-MeOH) to yield 22 fractions (Fr.A-
279 Fr.N) as determined by TLC analysis. Fraction Fr.J (62.32 mg) was further purified
280 using silica gel column chromatography with a PE-EtOAc gradient to obtain nine
281 subfractions (Fr.J1-Fr.J9). Subfraction Fr.J7 (17 mg) was then subjected to RP-HPLC
282 with an acetonitrile-water gradient, resulting in the isolation of compounds **1** (1.36 mg)
283 and **2** (0.36 mg).
284 The EtOAc extract (9.3 g) from the fermentation broth of XISR I II medium was
285 subjected to column chromatography on silica of PE-EA (100/0, 90/10, 80/20,
286 70/30,50/50, 30/70, 20/80, 10/90, 0/100) and EA-MeOH (90/10, 80/20, 0/100) to yield
287 22 fractions (Fr.1- Fr.22) based on TLC analysis. Fr.12 (200mg) was purified by silica
288 gel column chromatography using a gradient of PE- EtOAc (3/1, 1/1, 1/5, 0/1) to obtain
289 7 fractions (Fr.a- Fr.g) according to TLC analysis. Fr.e (23.19 mg) was extracted using
290 RP-HPLC (flow rate 20 ml/min) gradient from acetonitrile-H2O (5% acetonitrile for 5

291 min, 5% -100% acetonitrile for 5-30 min, 100% acetonitrile for 30-50 min). Then,
292 compound **3** (2.19 mg) was obtained.

293 Similarly, the EtOAc extract (9.3 g) from the fermentation broth of XISR III medium
294 was fractionated by column chromatography on silica with PE-EA and EA-MeOH
295 gradients to yield 22 fractions (Fr.1-Fr.22) based on TLC analysis. Fraction Fr.12 (200
296 mg) was purified using a PE-EtOAc gradient to produce seven subfractions (Fr.a-Fr.g).
297 Subfraction Fr.e (23.19 mg) was processed using RP-HPLC with an acetonitrile-water
298 gradient, leading to the isolation of compound **3** (2.19 mg).

299 Antimicrobial activity evaluation

300 All isolated compounds were dissolved in 1% DMSO and introduced to pathogenic
301 bacteria or fungi in LB or PDB media. The 96-well plates were incubated at 37 ^{o}C for
302 18 hours, with 1% DMSO serving as the negative control. After incubation, the OD_{600}
303 of the bacterial cultures was measured using a Microplate Reader.

304 Supporting Information

305 File Name: Supplementary Information
306 File Format: pdf
307 Title: Supplementary Information for Chemical and Biosynthetic Potential of *Penicillium*
308 *shentong* XL-F41

309 Acknowledgements

310 We thank Congcong Guo and the Pharmaceutical Analysis and Quality Research
311 Platform of Bohai Rim Advanced Research Institute for Drug Discovery for all the
312 technical support in NMR data collection.

313 # Funding

314 B.X. thanks for the financial support of the National Key Research and Development
315 Program of China (No. 2022YFC2804100), the Taishan Scholars Program, the
316 Shandong Laboratory Program (SYS202205), the Shanghai Pujiang Program
317 (23PJ1415300), and the start-up funding provided by the Shanghai Institute of Materia
318 Medica, Chinese Academy of Sciences.

319

320 # References

321 1. El Hajj Assaf C; Zetina-Serrano C; Tahtah N; Khoury AE; Atoui A; Oswald IP, Puel
322 O; Lorber S. *Int. J. Mol. Sci.* **2020**, *21,* 9462. doi: 10.3390/ijms21249462

323 2. Koul M; Singh S. *Anticancer Drugs*. **2017**, *28*, 11-30. doi:
324 10.1097/CAD.0000000000000423

325 3. Ma HG; Liu Q; Zhu GL; Liu HS; Zhu WM. *J. Asian Nat. Prod. Res.* **2016**, *18*, 92-115.
326 doi: 10.1080/10286020.2015.1127230.

327 4. Zhang X; Yin Q; Li X; Liu X; Lei H; Wu B. *Fitoterapia* **2022**, *163,* 105349. doi:
328 10.1016/j.fitote.2022.105349

329 5. Mondal A; Gandhi A; Fimognari C; Atanasov AG; Bishayee A. *Eur. J. Pharmacol.*
330 **2019**, *858*, 172472. doi: 10.1016/j.ejphar.2019.172472

331 6. Qiu S; Sun H; Zhang AH; Xu HY; Yan GL; Han Y; Wang XJ. *Chin. J. Nat. Med.* **2014**,
332 *12*, 401-406. doi: 10.1016/S1875-5364(14)60063-7

333 7. Rosales PF; Bordin GS; Gower AE; Moura S. *Fitoterapia* **2020**, *143*, 104558. doi:
334 10.1016/j.fitote.2020.104558

335 8. Song J; Zhang B; Li M; Zhang J. *Fitoterapia* **2023**, *165*, 105430. doi:
336 10.1016/j.fitote.2023.105430

337 9. Liu Y; Cui Y; Lu L; Gong Y; Han W; Piao G. *Arch. Pharm*. **2020**, *353*, 2000120. doi:
338 10.1002/ardp.202000120
339 10. Zou R; Chen B; Sun J; Guo YW; Xu B. *Fitoterapia* **2023**, *167*, 105503. doi:
340 10.1016/j.fitote.2023.105503
341 11. Brakhage AA. *Nat. Rev. Microbiol*. **2012**, *11*, 21-32. doi: 10.1038/nrmicro2916
342 12. Brakhage AA; Schroeckh V. *Fungal Genet. Biol*. **2011**, *48*, 15-22. doi:
343 10.1016/j.fgb.2010.04.004
344 13. Avnish Kumar; Monika Asthana; Ankur Gupta; Darshika Nigam; S. Mahajan. *New*
345 *and Future Developments in Microbial Biotechnology and Bioengineering*. Elsevier:
346 *USA*, **2018**, pp 47-68. doi: 10.1016/B978-0-444-63501-3.00003-X
347 14. Dai XM; Pan HL; Lan WJ; Chen LP; Feng GK; Deng R; Zhu XF; Li HJ.
348 *Phytochemistry* **2022**, *204*, 113456. doi: 10.1016/j.phytochem.2022.113456
349 15. K. Arai; B. Rawlings; Y. Yoshizawa; J. Vederas. *J. Am. Chem. Soc*. **1989**, *111*,
350 3391-3399. doi: 10.1021/JA00191A042
351 16. Yaoquan Liu; Li Z; Vederas JC. *Tetrahedron* **1998**, *54*, 15937-15958. doi:
352 10.1016/S0040-4020(98)01003-5
353 17. E. Ghisalberti; D. Hockless; C. Rowland; Allan H. White. *Aust. J. Chem.* **1993**, *46*,
354 571-575. doi: 10.1002/CHIN.199330293
355 18. Jutatip Choochuay; Xinrui Xu; V. Rukachaisirikul; Piyada Guedduaythong; S.
356 Phongpaichit; J. Sakayaroj; J. Chen; Xu Shen. *Phytochem. Lett*. **2017**, *22*, 122-127.
357 doi: 10.1016/J.PHYTOL.2017.09.011
358 19. L. Shaala; D. Youssef; J. Badr; S. Harakeh. *Molecules* **2016**, *21*, 1116. doi:
359 10.3390/molecules21091116
360 20. S. Lee; H. Baek; Chang Hee Lee; Hyun Pyo Kim. *Arch. Pharm. Res*. **1994**, *17*, 31-
361 35. doi: 10.1007/BF02978244
362 21. M. A. Selepe; S. Drewes; F. V. van Heerden. *J. Nat. Prod*. **2010**, *73*, 1680–1685.

363 doi: 10.1021/np100407n

364 22. Jingyu Zhang; Zhenzhen Wang; Zhijun Song; L. Karthik; Chengjian Hou; Guoliang
365 Zhu; Lan Jiang; Jianying Han; Rong Ma; Li Li; Lixin Zhang; Xueting Liu; T. Hsiang.
366 *Synth. Syst. Biotechnol.* **2019**, *4*, 173-179. doi: 10.1016/j.synbio.2019.09.001

367 23. Peng Zhang; L. Meng; A. Mándi; T. Kurtán; Xiao-Ming Li; Yang Liu; Xin Li;
368 Chunshun Li; Bin-Gui Wang. *Eur. J. Org. Chem.* **2014**, *2014*, 4029-4036. doi:
369 10.1002/ejoc.201400067

370