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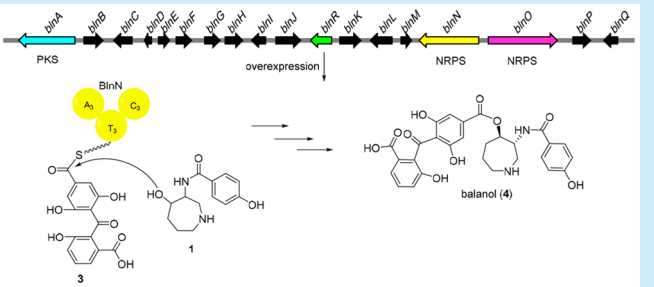
Revelation of the Balanol Biosynthetic Pathway in *Tolypocladium ophioglossoides*

Xian He, Min Zhang, Yuan-Yang Guo, Xu-Ming Mao, Xin-Ai Chen,* and Yong-Quan Li*

Institute of Pharmaceutical Biotechnology, Zhejiang University, Hangzhou 310058, China

Supporting Information

ABSTRACT: A cryptic gene cluster, *bln*, was activated by genome mining in *Tolypocladium ophioglossoides*. This activation led to the production of balanol and eight other metabolites. Gene disruption and metabolite profile analysis showed that the biosynthesis of balanol involved the convergence of independent PKS and NRPS pathways, and a biosynthetic pathway for balanol was proposed.



Balanol (**4**) was first isolated from *Tolypocladium ophioglossoides*[1] in 1977 as ophiocordin.[2−4] This compound is known to be a potent natural protein kinase C (PKC) inhibitor that can bind to the ATP-binding pockets of PKC and protein kinase A (PKA) with an affinity 3000 times greater than that of ATP due to its three-dimensional structural resemblance to ATP.[5] As a hybrid polyketide-nonribosomal peptide (PK-NRP) compound, balanol consists of a benzophenone, an azepine, and a *p*-hydroxybenzamide unit, and thus, balanol is unlikely to be synthesized by commonly integrated PKS-NRPS enzymes. Since the yield of balanol is very low when extracted from the fungus under laboratory conditions,[3] many studies have focused on the chemical synthesis of balanol and its analogues.[6−13] The chemical synthesis and structural modification of balanol are complex and challenging, and the biosynthetic pathway of balanol has not yet been reported.

Recently, we sequenced the genome of the previously isolated *T. ophioglossoides* strain.[14] Bioinformatic analysis indicates that the genome has the potential to generate 13 polyketides, 8 nonribosomal peptides, 6 hybrid PK-NRP metabolites and 4 terpenes (Tables S2−S5). Among these biosynthetic gene clusters, we found an intriguing cluster, namely, *bln*, which contains a PKS and two NRPS genes. Some genes, such as the PKS gene, in the *bln* cluster exhibited relatively high homology with genes from the monodictyphenone biosynthetic cluster in *Aspergillus nidulans*[15] (Figure 1). As balanol contains moieties derived from amino acids and a benzophenone moiety that is structurally similar to monodictyphenone (Figure 1), we speculated that this gene cluster could be associated with balanol biosynthesis. Transcriptional analysis showed that the expression of genes within the *bln* cluster could not be detected (data not shown), which suggests that *bln* was a cryptic gene cluster under the laboratory conditions used.

Techniques have been developed to activate silent gene clusters, including variation in growth conditions, manipulation of regulatory genes, epigenetic perturbation, promoter engineering, and heterologous expression.[16] *BlnR* is a pathway-specific Zn_2Cys_6 transcriptional regulator in the *bln* gene cluster and is homologous to *mdpE* from the *mdp* cluster. Since replacing the promoter of *mdpE* with a regulatable promoter has allowed the induction of expression of the *mdp* cluster in *A. nidulans*,[15,17] we constructed a vector for the overexpression of *bln R*, which was under the control of the strong promoter *ApTEF*. The obtained transformants (*blnROE*) were verified by RT-PCR (Figure S2). The culture broth of the *blnROE* strain was a darker shade of yellow than that of the wt strain (Figure S1), and HPLC analysis of the *blnROE* culture showed a distinctly different metabolite profile from that of the wt strain (Figure 2). To characterize the new compound produced by the *blnROE* strain, large-scale fermentation was carried out. Nine metabolites were isolated and identified (Figure 3), including two nonribosomal peptides (**1**, **2**), five polyketide compounds (**3**, **5**, **7**, **8**, **9**), and two compounds containing both polyketide and amino acid derived moieties (**4**, **6**). Among these compounds, compound **7** has not been reported to date, and **1**, **2**, **3**, and **6** were isolated from nature for the first time.

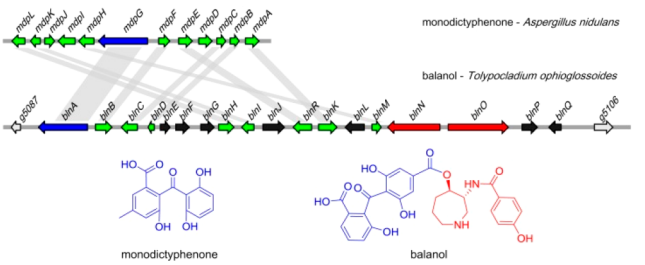


Figure 1. Comparison of the balanol (*bln*) and monodictyphenone (*mdp*) gene clusters from *T. ophioglossoides*.

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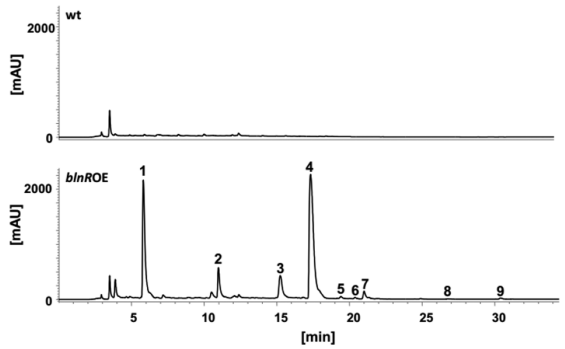

Figure 2. HPLC profiles (λ = 254 nm) of the culture broths of the wt and *blnROE* strains of *T. ophioglossoides*.

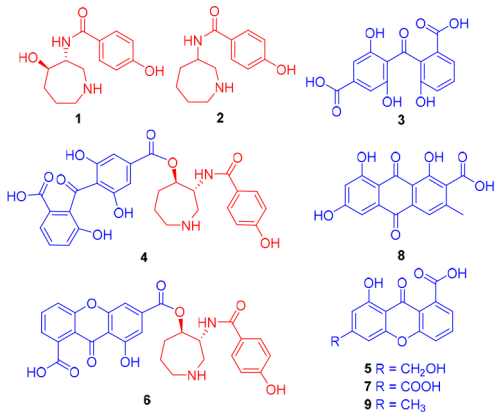

Figure 3. Structures of compounds **1**–**9** from *blnROE*.

The molecular formula of compound **1** was established as $C_{13}H_{18}N_2O_3$ by high-resolution mass spectrometry (HRMS) (Table S7). The structure of **1** was elucidated by extensive analysis of one- and two-dimensional (2D) NMR spectroscopic data (Table S8 and Figures S3–S6). Compound **1** was previously synthesized to confirm the structure of balanol.[18] The molecular formula of **2** was established as $C_{13}H_{18}N_2O_2$ by HRMS (m/z 233.1299 $[M - H]^-$). Compared to the known compound **1**, compound **2** lacked a hydroxyl group. Therefore, the planar structure of **2** was further determined via combination of the ^1H, ^{13}C, and 2D NMR spectroscopic data (Table S9 and Figures S7–S10).

The structures of **3**, **5**, **8**, and **9** were elucidated by spectroscopic analysis (Tables S7, S10, S12, S15, and S16 and Figures S11–S14, S19–S22, and S31–S33). Compound **3** was formerly synthesized together with **1** to confirm the structure of balanol.[18] The molecular formula of **7** was established as $C_{15}H_8O_7$ by HRMS (m/z 299.0189 $[M - H]^-$), and the UV absorption data suggested that compounds **5**, **7**, and **9** had the same xanthone skeleton (Table S7). Compound **7** seems to have a carboxyl group instead of a hydroxymethyl group at the C-6 position, in contrast to **5**. In addition, the structure of **7** was identified by ^1H, ^{13}C, and 2D NMR spectroscopic data (Table S14 and Figures S27–S30) as a previously unreported structure.

The structure of **4** (balanol) was identified by HRMS and 1D and 2D NMR spectroscopy (Table S11 and Figures S15–S18). Compound **4** has been previously purified from *T. ophioglossoides*,[2] but we obtained this compound only after overexpression of *blnR*, as shown in the HPLC analysis (Figure 2). The molecular formula of **6** was deduced by HRMS (m/z 531.1409 $[M - H]^-$) as $C_{28}H_{24}N_2O_9$, and the structure of this compound was further elucidated by extensive analysis of 1D and 2D NMR spectroscopic data (Table S13 and Figures S23–S26). Compound **6** was identified to be a known analog of balanol, which has been chemically synthesized previously for examination of its inhibitory activity toward protein kinases.[19] Compound **4** can be synthesized by esterification of **1** and **3**.

To elucidate the biosynthetic pathway of balanol, we further determined the borders of the *bln* gene cluster via analysis of the expression of putative genes by qRT-PCR based on antiSMASH[20] prediction. Compared with the expression in the wt strain, the expression of 18 genes in the *blnROE* strain, from *blnA* to *blnQ*, was significantly upregulated, while there was no significant change in the expression of other genes (Figure 4).

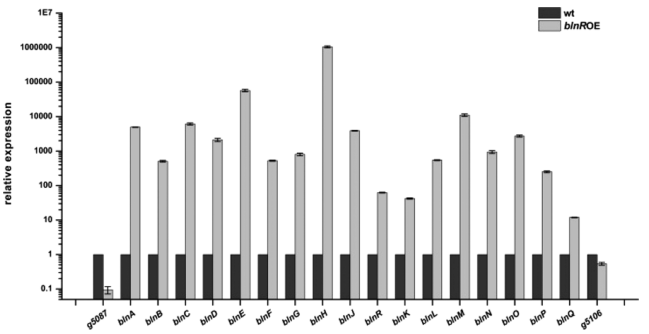

Figure 4. Determination of the borders of the balanol cluster by qRT-PCR.

This result demonstrates that the *bln* biosynthetic cluster spans the region from *blnA* to *blnQ*, comprising 18 genes (approximately 42 kilobases in length), including one PKS gene and two NRPS genes; the detailed annotations are shown in the Supporting Information (Table S6).

In addition to several genes of the *bln* cluster exhibiting similarity with genes from the *mdp* cluster of *A. nidulans*, five monodictyphenone analogs (**3**, **5**, **7**, **8**, and **9**) have been isolated from the *blnROE* strain. Since the *mdp* cluster had been elucidated,[15] we focused on the genes in the *bln* cluster that were not homologous to the *mdp* cluster. To further investigate the functions of these genes, several genes in the *bln* cluster were disrupted by homologous recombination in the *blnROE* background. The genotypes of the deletion strains were confirmed by PCR (Figure S34–S40). The effect of gene deletion on the production of compounds **1**–**9** in fermented broth was analyzed by HPLC (Figure S41) and LC–MS (Table S17 and Figures S42–S51), as summarized in Table 1.

Table 1. Effects of the Deletion of *bln* Genes on the Production of Compounds 1–9[a]

	effect on the production of compounds **1**–**9**								
strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
bln ROE	+	+	+	+	+	+	+	+	+
bln ROEΔ*bln E*	+	+	+	+	–	+	–	+	–
bln ROEΔ*bln F*	–	+	+	–	+	–	+	+	+
bln ROEΔ*bln J*	$+^R$	$+^R$	+	+	+	+	+	+	+
bln ROEΔ*bln L*	+	+	–	–	–	–	–	+	+
bln ROEΔ*bln N*	+	+	+	–	+	–	+	+	+
bln ROEΔ*bln O*	–	–	+	–	$+^R$	–	+	+	+

[a] + indicates production. – indicates absence of production. R indicates reduced production level.

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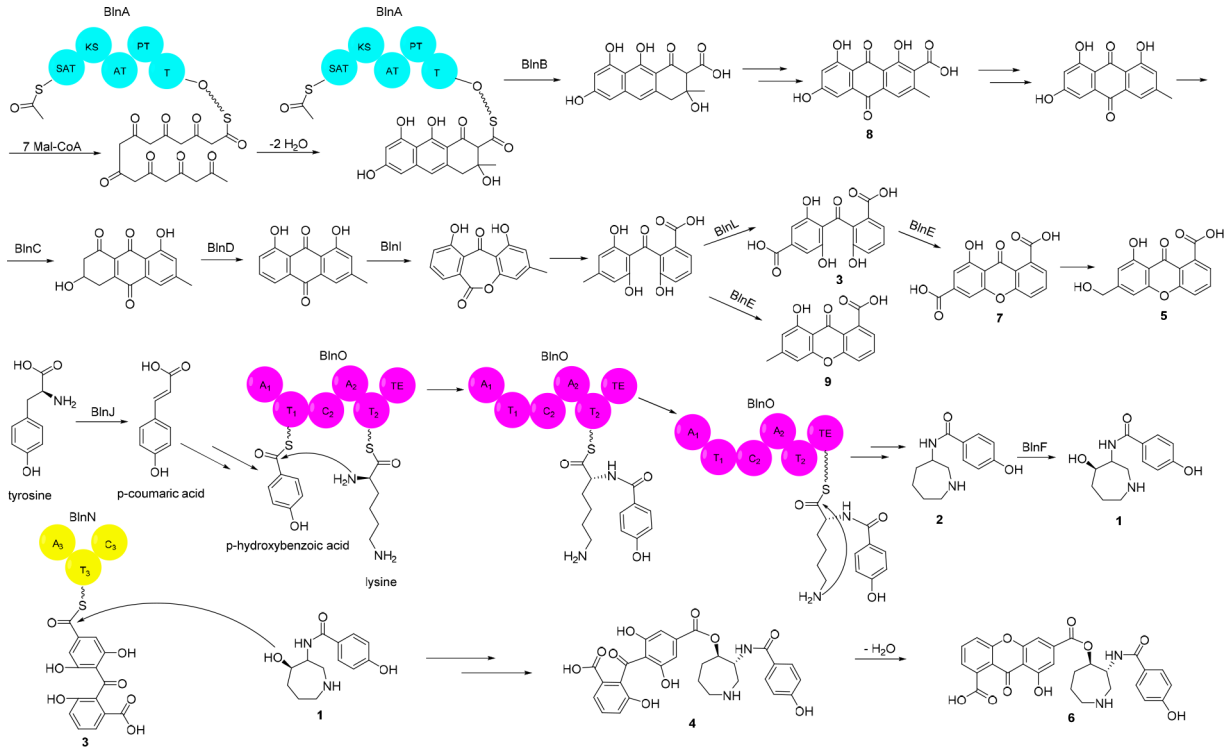

Figure 5. Putative biosynthetic pathway of balanol in *T. ophioglossoides*.

Bln E is a hypothetical protein-encoding gene. Deletion of *bln E* led to the absence of xanthones **5**, **7**, and **9**, suggesting that *bln E* is involved in the cyclodehydration of upstream benzophenones to produce xanthones. The fact that **6** could still be produced in the absence of **7** suggests that **6** is more likely produced by cyclization of **4** than by an esterification reaction between **7** and **1**. When the PKS gene was disrupted, production of the nonribosomal peptides **1** and **2** remains unaffected, indicating the existence of a relatively independent NRPS pathway during balanol biosynthesis.

Bln L is a cytochrome-P450-encoding gene, and deletion of *bln L* abolished the production of **3**, **4**, **5**, **6**, and **7**. Since **3** is an essential precursor of **4**, the absence of **3** seemed to result in elimination of the hybrid PK-NRP compounds **4** and **6**. However, the xanthone compound **9** was still produced, implying that Bln *L* is involved in catalyzing the generation of **3** from the upstream benzophenone of **9** via oxidation of the methyl group to a carboxylic acid group.

There are two NRPS genes in the *bln* cluster, namely, *blnN* and *blnO*. *BlnN* encodes three NRPS domains, namely, A_3-T_3-C_3, in which A_3 is predicted to activate Hyv/Orn/Leu/Gly/Glu (Table S20). Upon deletion of *blnN*, the hybrid PK-NRP compounds **4** and **6** could not be detected, and the biosynthesis of other compounds was not affected. Both **4** and **6** have an intermolecular ester bond that connects the PK and NRP moieties. It has been reported that NRPS can catalyze ester bond formation by utilizing thioesterase (TE) or C domains.[21−24] Taken together, our data show that NRPS Bln *N* is responsible for catalyzing ester bond formation between the polyketide **3** and the nonribosomal peptide **1** to produce **4**.

Upon deletion of *bln O*, the production of compounds **1**, **2**, **4**, and **6** was not observed. The absence of **4** and **6** could be a result of **1** deficiency. This result, together with the fact that the biosynthesis of the nonribosomal peptides **1** and **2** are not affected in the *bln* ROEΔ*bln N* strain, suggests that Bln *O* plays a major role in the synthesis of **1** and **2**. There are six NRPS domains in Bln *O*, namely, A_1-T_1-C_2-A_2-T_2-TE, as revealed by analysis of the amino acid sequence of this protein. The A_1 and A_2 domains are predicted to activate Phe/Tyr/Arg/Leu and Arg/Glu/Val/Leu/Trp/Lys/Gln, respectively (Tables S18 and S19). Based on the structures of **1** and **2**, it can be deduced that **2** likely originates from intramolecular cyclization of L-lysine linked via an amide bond with p-hydroxybenzoic acid, which is followed by carbonyl reduction. This speculation was supported by feeding experiment of labeled $^{13}C_6$-L-lysine in culture broth of *bln* ROE strain, which led to the formation of labeled **4** with a mass shift of 6 Da to higher masses (Figure S55). The additional hydroxyl group in **1** compared with **2** suggests that **1** is produced by oxidation of **2**. While the production of the nonribosomal peptides **1** and **2** was eliminated by *bln O* deletion, the biosynthesis of polyketides was not affected, suggesting that the biosyntheses of polyketides and nonribosomal peptides from the *bln* cluster are processed in parallel.

Bln *F* is a predicted dioxygenase. In the *bln*ROEΔ*blnF* strain, while the biosynthesis of **2** was not affected, the hydroxylated derivative **1** was absent, which eliminated the production of **4** and **6** due to loss of ester bond formation. This result suggests that Bln *F* could catalyze the hydroxylation of **2** to produce **1**. To further verify our speculation, *blnF* was cloned and expressed in *E. coli*. Formation of **1** is observed when Bln *F* was incubated with **2**, α-KG, $Fe(NH_4)_2(SO_4)_2$ and L-ascorbate (Figure S53).

Bln *J* is predicted to be a phenylalanine/tyrosine ammonia-lyase. In the *bln* ROEΔ*bln J* strain, the production of compounds **1** and **2** was significantly reduced, which suggests that Bln *J* might be related to the biosynthesis of p-hydroxybenzoic acid as the substrate of Bln *O*. We expressed *bln J* in *E. coli* and performed in vitro biochemical reaction, the result verified that Bln *J* could catalyze the deamination of tyrosine to produce *p*-coumaric acid (Figure S54), and *p*-coumaric acid could be further catabolized to generate *p*-hydroxybenzoic acid.[25] The production of

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compounds **1** and **2** was only reduced but not eliminated since *p*-hydroxybenzoic acid could also be originated from the shikimic acid pathway.[26]

These results show that the biosynthesis of balanol involves two convergent biosynthetic pathways, in which PKS is responsible for biosynthesis of benzophenone and other polyketides and the NRPS Bln *O* produces the dipeptides **1** and **2**. Coupling of the parallel products is achieved by esterification of **3** and **1** to generate balanol (**4**), which is catalyzed by the NRPS Bln *N*. This result is consistent with the hypothesis introduced by Molnár.[27] Based on a combination of the studies conducted on the homologous *mdp* cluster[15] and the findings of our study, a biosynthetic pathway for balanol was proposed (Figure 5). The mechanism for the removal of the ketone of the amide bond during balanol biosynthesis will require further study.

In summary, we identified the balanol biosynthetic gene cluster *bln* for the first time by genome mining. By overexpression of the pathway-specific regulatory gene *blnR* from the *bln* cluster, the biosynthesis of balanol and other intermediate compounds was activated. By gene deletion and analysis of the metabolite profile, the biosynthetic pathway of balanol, containing independent PKS and NRPS pathways, was proposed. Convergence of these pathways is achieved by intramolecular ester bond formation between parallel products, which is catalyzed by the NRPS Bln *N*. The discovery of the balanol biosynthetic pathway will provide improved access to balanol and its analogues and could also improve our understanding of the mechanisms of fungal hybrid PK-NRP metabolite biosynthesis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.8b01543.

Supplementary methods, figures, tables, compound characterization, and NMR spectra of compounds **1**-**9** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: lyq@zju.edu.cn.
*E-mail: biolab@zju.edu.cn.

ORCID

Yong-Quan Li: 0000-0001-6013-4068

Notes

The authors declare no competing financial interest.

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