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Precursor-Directed Biosynthesis of Phenylbenzoisoquinolindione Alkaloids and the Discovery of a Phenylphenalenone-Based Plant Defense Mechanism

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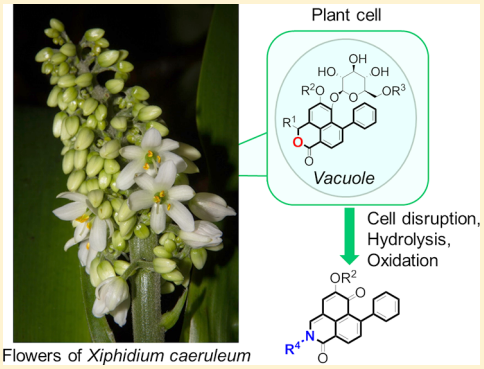
Supporting Information

ABSTRACT: Phenylbenzoisochromenone glucosides (oxa-phenylphenalenone glucosides) occurring in some phenylphenalenone-producing plants of the Haemodoraceae undergo conversion to phenylbenzoisoquinolindiones (aza-phenylphenalenones) in extracts of *Xiphidium caeruleum*. Precursor-directed biosynthetic experiments were used to generate a series of new phenylbenzoisoquinolindiones from native phenylbenzoisochromenone glucosides and external amines, amino acids, and peptides. Intermediates of the conversion were isolated, incubated with cell-free extracts, and exposed to reactions under oxidative or inert conditions, respectively, to elucidate the entire pathway from phenylbenzoisochromenones to phenylbenzoisoquinolindiones. An intermediate in this pathway, a reactive hydroxylactone/aldehyde, readily binds not only to amines in vitro but may also bind to the *N*-terminus of biogenic peptides and proteins of herbivores and pathogens in vivo. The deactivation of biogenic amino compounds by *N*-terminal modification is discussed as the key reaction of a novel phenylphenalenone-based plant defense mechanism. According to these data, the ecological function of phenylphenalenone-type compounds in the Haemodoraceae, subfamily Haemodoroideae, has been substantiated.



Phenylphenalenones and their oxa- and aza-derivatives (Figure 1) belong to a group of polycyclic phenylpropanoid-derived natural products[1] that occur mainly in the monocotyledonous plant families Haemodoraceae[2] and Musaceae.[3] In banana (*Musa* species), the role of phenylphenalenones as inducible plant defense metabolites (phytoalexins) has been well documented.[4]

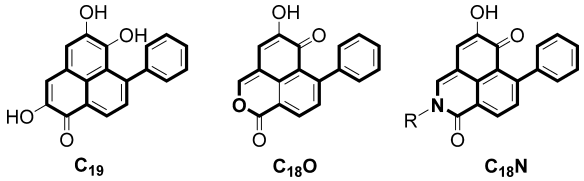


Figure 1. Classification of phenylphenalenones (C_{19}), their oxa-derivatives (phenylbenzoisochromenones, PBICs, $C_{18}O$), and aza-derivatives (phenylbenzoisoquinolindiones, PBIQs, $C_{18}N$).

Owing to the relatively high levels of phenylphenalenones that occur in some Haemodoraceae species, plants and root cultures of this plant family have served as model systems for biosynthetic studies[5] and for locating phenylphenalenone-type compounds in the plant tissue.[6] Just as in the family Musaceae, phenylphenalenones in some species of the Haemodoraceae may function as phytoalexins. Alternatively, the accumulation of phenylphenalenone derivatives reported for the roots[7] and the stamens[6a] suggests that these compounds could be constitutive and therefore may be considered as phytoanticipins rather than phytoalexins. The hypothesis that these are phytoanticipins seems valid, especially for plants of the Haemodoroideae, because this subfamily is rich in glucosidic phenylphenalenones and phenylbenzoisochromenones (PBICs, oxa-phenylphenalenones, $C_{18}O$ skeleton).[7,8] Other constitutive glycosidic plant defense compounds, such as the glucosinolates, cyanogenic glycosides, benzoxazinoid glucosides, and avenacosides,[9] are stored in the vacuole. During an attack by herbivores or pathogens, vacuoles are mechanically damaged, and defensive glucosides are exposed to hydrolytic enzymes present in the cytosol or neighboring cells. Owing to their hydrophilicity, phenylphenalenone- and PBIC glucosides are also stored in the vacuole and, after cell disruption, may undergo hydrolytic cleavage. After the hydrolysis of glucosides, phenylphenalenone aglucones could, as photodynamic agents or as Michael acceptors, act to deter pathogens or predators. The photodynamic activity of phenylphenalenone-producing *Lachnanthes*

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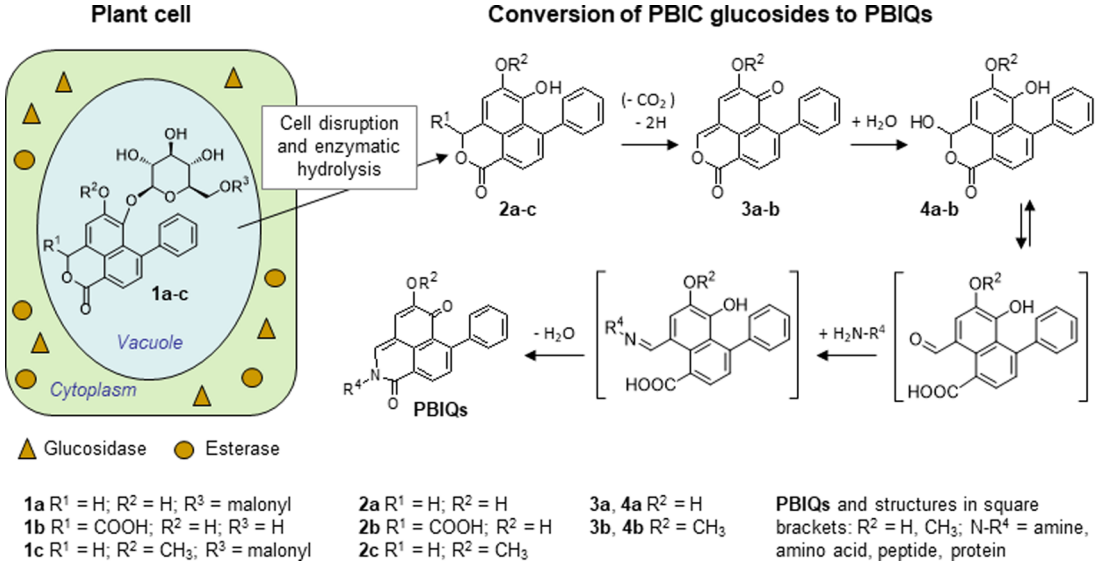

Figure 2. Conversion of intravacuolar phenylphenalenone-derived phenylbenzoisochromenone (PBIC) glucosides (**1a**–**c**) from *X. caeruleum* to phenylbenzoisoquinolindiones (PBIQs) by a series of enzymatic and spontaneous reactions. The occurrence of the ß-glucosidase and the esterase in the same cell is still hypothetical; alternatively, they may be located in adjacent cells. 1-Carboxy-5,6-dihydroxy-4-phenylnaphthalen-8-aldehyde is in equilibrium with its cyclic isomer, the hydroxylactone (**4a**). The aldehyde represents a highly reactive defense compound that binds to primary amines such as amino acids and proteins.

tinctoria plants in pigs was first described by Darwin.[10] The phenalenones are reported to act as photosensitizers with the formation of singlet oxygen as the active agent against pathogens.[11] The ability of phenalenone-type compounds to function as Michael acceptors has been discussed as another possible mode of action.[12]

The occurrence of phenylphenalenone-related compounds such as PBICs and phenylbenzoisoquinolindiones (PBIQs, aza-phenylphenalenones, $C_{18}N$ skeleton) in *Xiphidium caeruleum*[2b] and the ease with which hydrolytic and oxidative conversion of such compounds[13] occurs prompted us to investigate whether the bioactivation of phenylphenalenone and PBIC glucosides results in chemically reactive derivatives. Reactive derivatives and biosynthetic intermediates may act as defense compounds against herbivores and pathogens. On the basis of the recent observation of the co-occurrence of phenylphenalenone glucosides, PBIC glucosides, and PBIQs in *X. caeruleum*,[8] especially in the stamens,[6a] and on the basis of the results of further phytochemical and metabolic studies on phenyl-phenalenone derivatives in *X. caeruleum*,[6c] herein we propose a novel defense mechanism (Figure 2) that may apply not only to the studied species and the Haemodoroideae subfamily but also to other phenylphenalenone-producing plants.

To substantiate the hypothetical pathway shown in Figure 2 and demonstrate precursor-product relationships, we conducted phytochemical studies and isotopic labeling experiments. Isolated compounds **1a**–**4b** were incubated with cell-free extracts to observe metabolic reactions, and spontaneous conversions were tracked under oxidative and inert conditions.

PBIQ alkaloids ($C_{18}N$ scaffold) were first reported from *L. tinctoria*[14] and later found in *X. caeruleum*[6a,8] and in its sister species, *Wachendorfia thyrsiflora*.[7] Until now, their biosynthesis had not been investigated. On the basis of the chemical structures of PBIQs,[6a] it is reasonable to hypothesize that nitrogen-containing side chains are derived from plant proteinogenic amino acids. If the hypothesis shown in Figure 2 is correct, the amino acid should react with an aldehyde, which could be derived from a native PBIC. To test this possibility, excised flowers of *X. caeruleum*, which are rich in PBIC glucosides (see Figure S3 and Scheme S1), were suspended in aqueous solutions of (3-^{13}C)-L-phenylalanine and (1-^{13}C)-L-leucine, respectively. After incubation at ambient temperature for 12 h, the ^{13}C-labeled PBIQs 2-[(1″*S*)-1″-carboxy-2″-phenylethyl]-5-hydroxy-7-phenyl-2*H*-benzo[*de*]-isoquinoline-1,6-dione (**5**) and 2-[(1″*S*)-1″-carboxy-3″-methyl-*n*-butyl]-5-hydroxy-7-phenyl-2*H*-benzo[*de*]isoquinoline-1,6-dione (**6**) were isolated from the flower extract (Figure S1). Unlike the ^{13}C NMR spectra of nonlabeled compounds, the spectra of labeled **5** and **6** displayed enhanced signals at δ 35.5 and 172.5, respectively, corresponding to ^{13}C-enriched C-2″ of **5** and ^{13}C-enriched C-5″ of **6** (Figure S2). The isotopic enrichments of labeled carbon atoms were 91.8 and 90.9% for **5** and **6**, respectively. This finding indicated that the nitrogen-containing side chain of PBIQs originated from amino acids.

To examine whether PBIQ alkaloids are formed from a spontaneous reaction or an enzyme-catalyzed reaction, we incubated various amino acids with acetone extracts of macerated aerial plant material instead of a suspension of fresh flowers. Using this precursor-directed biosynthetic approach, 10 new PBIQs (**7**–**16**) (Figure 3) and nine known PBIQs (**5**, **6**, **17**, **27**–**32**) (Table S1) were obtained.[6a,8] For MS and NMR data, see Tables S2–S5. Two of the new compounds, **7** and **8**, are enantiomers of compounds **5** and **6** generated by incubation of the plant material with D-phenylalanine and D-leucine instead of the natural L-amino acids. Likewise, the new 5-*O*-methyl derivatives **9** and **10** were obtained from D-phenylalanine and D-leucine. In the case of L-lysine, the two primary amino groups both reacted with the aldehyde form of compound **4a** to give bis-PBIQ **15**. The formation of the latter compound and of (2-hydroxyethyl)-lachnanthopyridone (**17**) shows that the reaction is not restricted to the amino group of α-amino acids but is generally accessible for primary amines. Incubation with L-cysteine resulted in the formation of another unusual compound, **16**,

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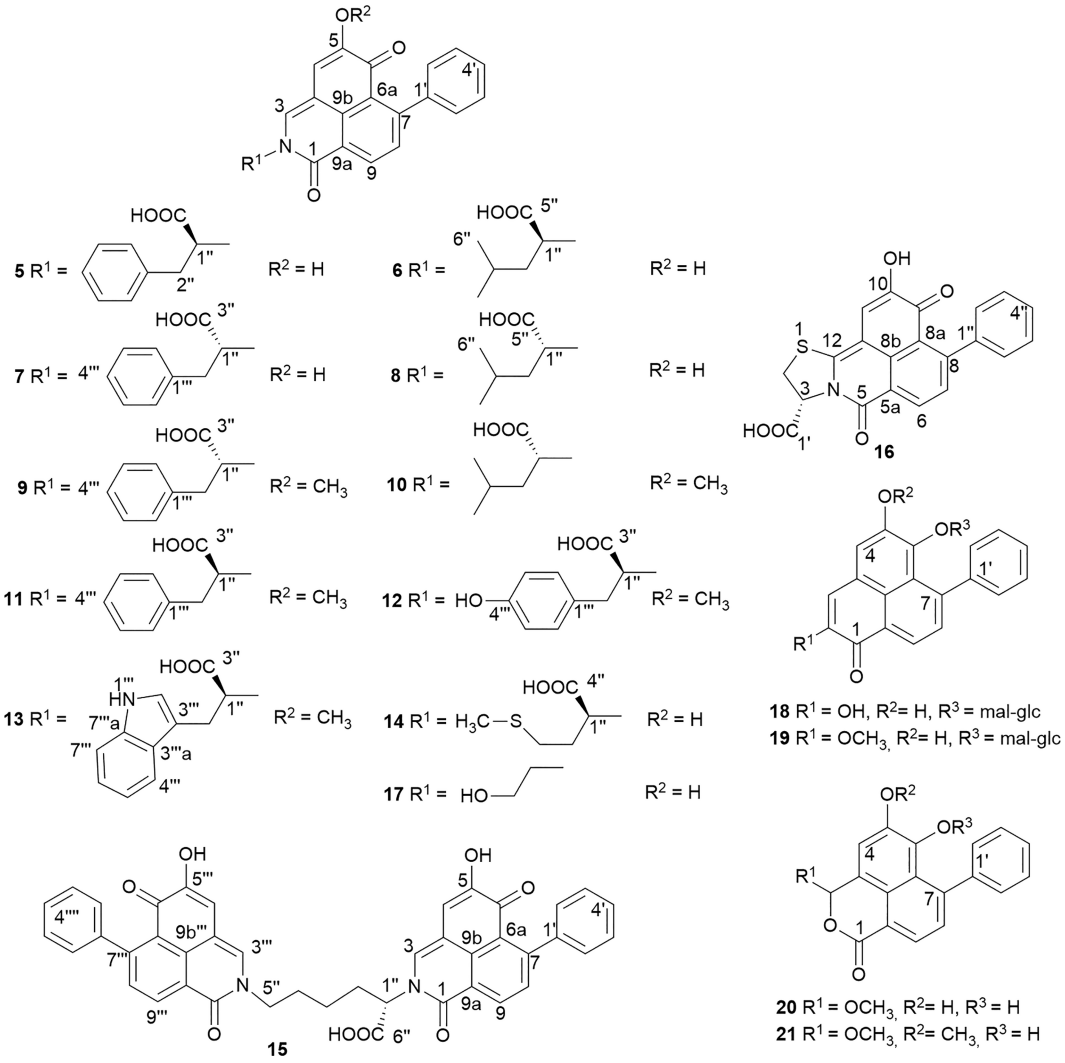

Figure 3. Structures of PBIQs **5**–**17**, phenylphenalenone glucosides **18** and **19**, and PBICs **20** and **21**.

which has a thiazolo[3,2-*b*]isoquinoline backbone. The use of acetone as a solvent and the preparation of D-amino acid derivatives demonstrated that PBIQs are spontaneously formed by a nonenzymatic reaction. Starting from primary amines and excised flowers or extracts of aerial plant material afforded these alkaloids in one preparative step. It is noteworthy that extracts from green plant parts resulted in a mixture of 5-hydroxy- and 5-methoxy-PBIQs (Table S1), whereas flower material gave only the 5-hydroxy-PBIQs, suggesting the plant parts contained tissue-specific PBICs as precursors (see below). Because *X. caeruleum* can be hydroponically grown and vegetatively propagated, plant material is accessible for the precursor-directed biosynthesis of PBIQs on a preparative scale.

To explore the biosynthetic precursors of PBIQs, HPLC-ESIMS analyses and further phytochemical investigation of different lyophilized tissues of *X. caeruleum* were performed. The results showed that phenylphenalenones mainly occur in the form of the five glucosides (**1a** - **1c**, **18**, **19**) (Figure S3, Supporting Information). PBIC glucosides **1a** and **1b** were the two major constituents in flowers, while **1a** - **1c** were the three major glucosides in green leaves. The C_{19}-phenylphenalenone glucosides (**18**, **19**) (Figure 3) are only present in the roots. However, no PBIQ was found in the acetone extracts of lyophilized plant material, which supports the finding that PBIQs are artifacts generated during extraction and isolation.

Incubation experiments using cell-free extracts demonstrated that **1a** and **1b** were converted to their aglycones (**2a**, **2b**) (paragraph S4) by hydrolytic enzymes from the green leaves of *X. caeruleum* when oxidation was simultaneously suppressed using $(NH_4)_2SO_3$ (Scheme 1). It should also be noted that the purification of **1b** required the compound to be collected in an $(NH_4)_2SO_3$ solution to prevent oxidation. The hydrolysis of glucosides could be blocked by castanospermine, a glucosidase inhibitor, which suggested that the hydrolysis of the glucosidic bond, as well as of the malonyl ester bond, was one of the first steps operating during the metabolic conversion of malonyl-glucoside **1a**. In the absence of $(NH_4)_2SO_3$, both **1a** and **1b** were transformed into the same oxidized product, lachnanthopyrone (**3a**) (Scheme S3.1–3). The transformation from **1a** to **3a** was shown to be time- and temperature-dependent (Scheme S4). The oxidation of **2a** in air gave **3a** (Scheme S6), which indicated that this conversion happens spontaneously and without the catalytic activity of an oxidase. Aglycone **2b**, though too unstable to be isolated, was clearly detected by HPLC-HRESIMS. Thus, **2b** underwent spontaneous decarboxylation followed by oxidation to generate **3a**. Our investigation

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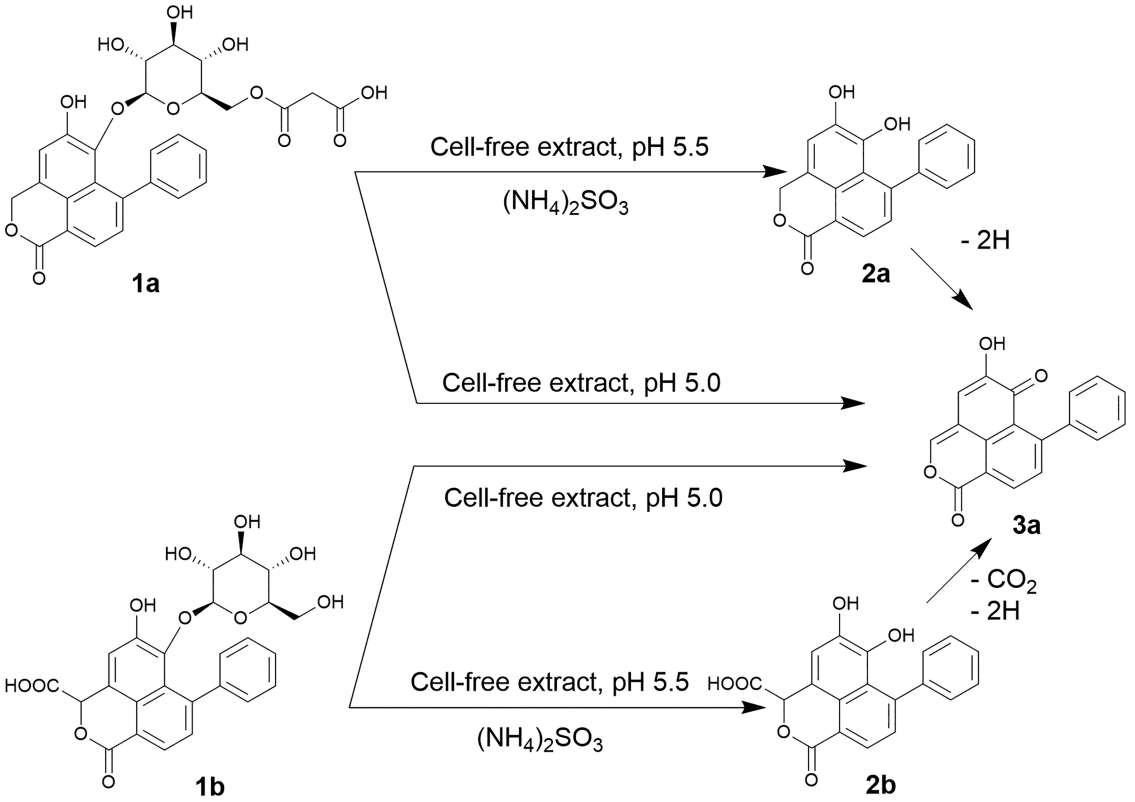
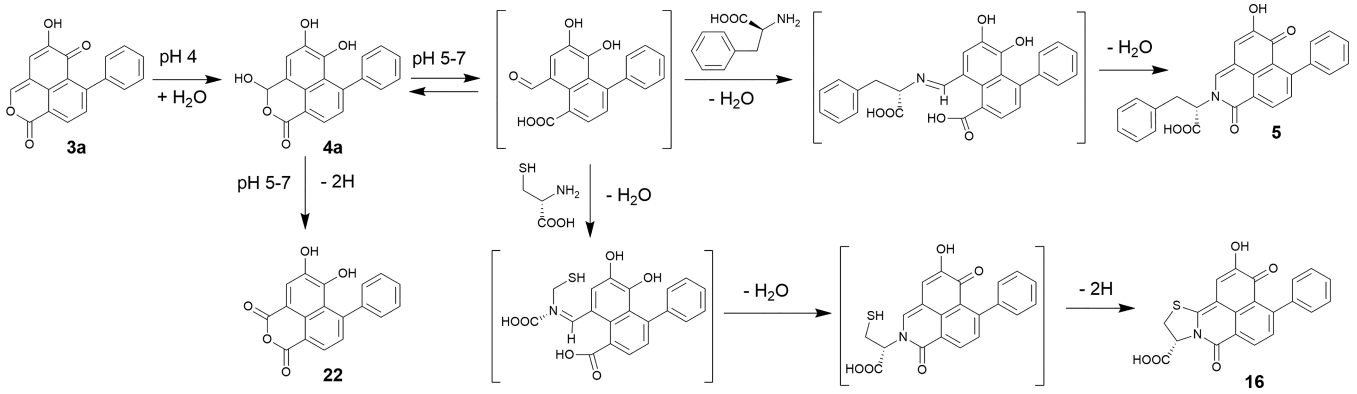
Scheme 1. Conversions of Glucosides 1a and 1b to 3a by a Cell-Free Extract from *X. caeruleum*



Scheme 2. pH-Dependent Conversion of Compound 3a to PBIQs 5 and 16



unambiguously establishes the early steps of the metabolic pathway **1a**/**1b** → **2a**/**2b** → **3a** (Scheme 1).

Further experiments were performed to identify the pathway from **3a** to PBIQ **5** (Scheme 2). As demonstrated for the conversions in buffer solutions of different acidities (pH 4.0−7.0), **3a** was readily hydrated to **4a** at pH 4.0−5.0 (Scheme S7A−C), which was confirmed by a parallel experiment in weakly acidic solution (Scheme S8). When the pH of the buffer was increased to pH 5.0−6.0, **4a** was oxidized to **22** (Scheme S7D). In the presence of an amine (e.g., L-phenylalanine) in the above mixtures, PBIQ **5** was obtained from **4a** at pH 5.0 (Scheme S9A−C), and its yield increased with the basicity of the buffer (Scheme S9D and E). 1-Carboxy-5,6-dihydroxy-4-phenylnaphthalen-8-aldehyde was postulated to be the reactive intermediate during the formation of **5**. These results clearly indicated that, after the hydrolysis of the flower glucosides **1a** and **1b**, PBIQs (Table S1) are formed via intermediate **3a** through a series of pH-dependent nonenzymatic reactions. This pathway was verified by the detection of **5**, which was generated by directly incubating L-phenylalanine with **1a** and **1b** in cell-free extracts at pH 8.0 (Scheme S5). Compound **16**, formed from 1-carboxy-5,6-dihydroxy-4-phenylnaphthalen-8-aldehyde with L-cysteine, is a special case because the reaction did not stop after formation of the isoquinolindione but continued in annelation of a thiazole ring involving both a 1,6-Michael addition reaction and an oxidation step.

Likewise, methoxy-PBIQs arose from the methoxy malonyl-glucoside (**1c**) via a parallel pathway comprising its aglycone (**2c**) (Scheme S2.3), 5-methoxylachnanthopyrone (Scheme S3.4) (**3b**), and 3,6-dihydroxy-5-methoxy-7-phenyl-3*H*-benzo-[*de*]isochromen-1-one (**4b**) as the intermediates. This "5-OMe" pathway was also supported by the organ-specific occurrence of 5-OMe PBICs in aerial green plant parts (Scheme S1).

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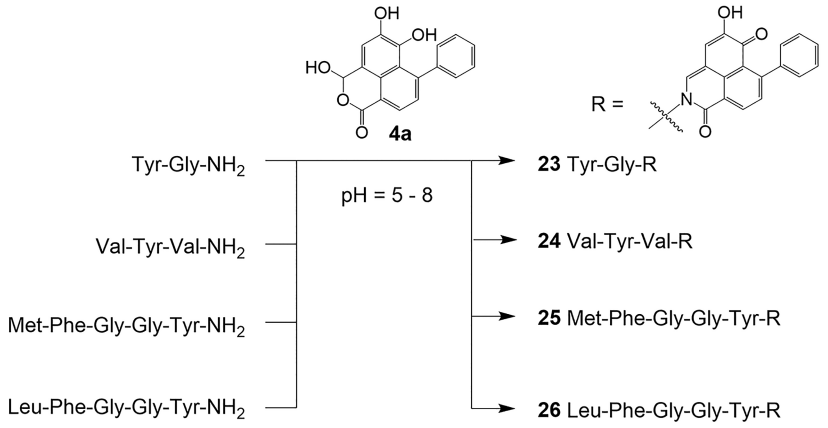
Scheme 3. PBIC 4a Coupling with Various Peptides



The formation of PBIQs from PBICs prompted the question of whether PBICs will react with proteins or peptides to mimic the interaction with biomolecules of pathogens or herbivores. Therefore, a standard peptide mixture of Tyr-Gly, Val-Tyr-Val, Met-Phe-Gly-Gly-Tyr (methionine enkephalin), and Leu-Phe-Gly-Gly-Tyr (leucine enkephalin), all of which have a free terminal amino group, was added to **4a** as a PBIC prototype in different buffer solutions (pH from 4 to 8) at 30 °C for 2 h (one-pot reactions). HPLC-HRESIMS analysis was used to identify the adducts **23**–**26** (Scheme 3). The results revealed that each peptide was bound via the free amino group to a PBIQ moiety originating from **4a**. The yields of adducts **23**–**26** increased as the basicity of the reaction mixture changed from pH 5.0 to 7.0 (Scheme S10A–E). However, at pH 8.0, **23** and **24** became the major products (Scheme S10 F). From these data, it is evident that plant-derived PBICs are involved in the *N*-terminal modification of peptides and proteins. The reaction mechanism may be effective in a chemical environment, as it exists in parts of the insect gut that have appropriate pH values. Thus, this reaction is thought to be part of the phenylphenalenone-based defense against florivores, herbivores, and plant cell-disrupting microorganisms. This mild conjugation reaction also represents a new preparative approach to chemically modify proteins in a site-selective way.

The naturally occurring PBICs, their transformed aglycones, and the PBIQs derived from precursor-directed biosynthetic experiments were subjected to antimicrobial tests (Table S8). Compared to ciprofloxacin and amphotericin B, all tested PBICs and their aglycones showed moderate activity against *E. coli* and *S. salmonicolor*. In addition, **3a**, **4a**, **20**, and **21** (Figure 3) showed activity against multiresistant *Staphylococcus aureus* strains. Tested PIBQs showed no bioactivity.

The present study reveals a novel phenylphenalenone-based plant defense mechanism operating in *X. caeruleum* and other species of the Haemodoraceae, subfamily Haemodoroideae. The hydroxylactone **4a** and its acyclic aldehyde form, which is oxidatively generated from plant PBICs, have been identified as the reactive structures that bind to primary amines, amino acids, and peptides. Hence, biogenic peptides and proteins of herbivores and pathogens may be deactivated by *N*-terminal modification. The reaction was also used for precursor-directed biosynthetic experiments to prepare PBIQs, being substituted at the ring nitrogen with amino acid-, peptide-, and protein-derived side chains. In contrast to PBIQs, which were shown to be inactive metabolic end products, PBIC glucosides provided evidence via bioassays of their antimicrobial activity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00885.

Experimental procedures, plant materials, extraction and isolation of PBICs from *X. caeruleum* and PBIQs from precursor-directed biosynthetic experiments, metabolite analysis by LC-ESIMS and NMR, NMR spectra, and bioassay data (PDF)

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Notes

The authors declare no competing financial interest.

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