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Reengineering an Azaphilone Biosynthesis Pathway in *Aspergillus nidulans* to create Lipoxygenase Inhibitors

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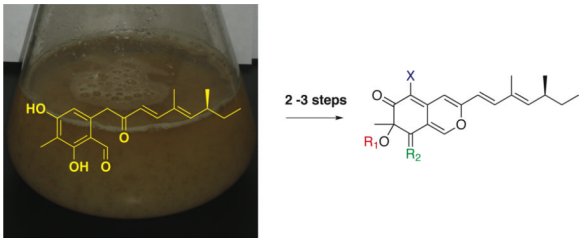
Abstract



Sclerotiorin, an azaphilone polyketide, is a bioactive natural product known to inhibit 15-lipoxygenase and many other biological targets. To readily access sclerotiorin and analogs, we developed a 2–3 step semisynthetic route to produce a variety of azaphilones starting from an advanced, putative azaphilone intermediate (5) over-produced by an engineered strain of *Aspergillus nidulans*. The inhibitory activities of the semisynthetic azaphilones against 15-lipoxygenase were evaluated with several compounds displaying low micromolar potency.

Lipoxygenases (EC 1.13.11.12) are ubiquitous enzymes widely distributed within plants, fungi and mammals.[1] They are non-heme iron dioxygenases that catalyze the addition of molecular oxygen to polyunsaturated fatty acids with a *cis, cis*-1,4 pentadiene to generate a hydroperoxydiene formed through a radical, regio- and stereoselective mechanism.[2] Reaction products of lipoxygenases are involved in several common human disorders such as allergies, inflammation and asthma. Lipoxygenases are responsible for the oxidation lipids in foods subsequently reducing the foods' nutritional value.[3]

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Supporting Information Available

The *Aspergillus nidulans* strain used in the study, in addition to experimental details and characterization of compounds **7–17** (^{1}H NMR, ^{13}C NMR, HRMS, and FT-IR) is provided in supporting information.

Several natural products from microbial sources inhibit 15-lipoxgyenase (15-LOX).[4] More recently the fungal pigment, (+)-sclerotiorin (**1**) was found to inhibit lipoxygenase-1, also known as 15-LOX.[5] Sclerotiorin was first isolated from *P. sclerotiorum* in 1940.[6] Since then **1** has been found to inhibit multiple therapeutic targets.[7]

Sclerotiorin belongs to an important class of natural products called azaphilones. Azaphilones are structurally diverse polyketides that share a highly oxygenated bicyclic core and chiral quaternary center. These polyketides are known for their 4H-pyran motif, which reacts with amines to produce the corresponding vinylogous γ-pyridones.[8] Early synthetic studies by Whalley and coworkers reported the total synthesis of several azaphilones, which included compound **8** prepared in 14 steps.[9] Recent synthetic efforts by Porco and coworkers have shown assembly of the azaphilone core through a copper-mediated enantioselective dearomatization route. The application of their asymmetric methodology was demonstrated on (−)-S-15183a (**2**), (−)-mitrorubin (**3**), and more recently with **1** (Figure 1).[10]

Although many azaphilones have been isolated and identified, their biosynthetic pathways remained unknown until our recent identification of the asperfuranone (**4**) biosynthetic pathway in *Aspergillus nidulans*.[11] A mutant strain from the previous study provided aldehyde **5** as a stable intermediate, which has been isolated from other azaphilone-producing organisms. Our work aims to enhance the production of the putative azaphilone intermediate (**5**) and use synthetic chemistry to structurally diversify **5** into natural and non-natural azaphilones.

In this study, the fungal strain used to over-produce compound **5** contains two genetic alterations (Scheme 1). The native promoter of *afoA*, the gene that codes for the pathway-specific transcription activator of the asperfuranone pathway was replaced with the inducible alcohol dehydrogenase promoter, *alcA*. The *afoD* gene, that codes for the hydroxylase in the asperfuranone pathway, was deleted to enable the accumulation of intermediate, compound **5**.[11] It should be noted that the afo cluster is silent under normal laboratory growing conditions. The wild type strain, thus, did not produce detectable quantities of compound **5** and asperfuranone (**4**) by LC/MS analysis. The mutant strain was initially cultured in a liquid lactose minimal medium under inducing conditions (refer to Supporting Information) at 37°C for three days to produce nearly 200 mg/L of **5** without need for further purification since **5** is poorly dissolved in aqueous media.

We altered culture conditions in several ways to optimize the titer of compound **5**. First, culture time prior to the induction of *alcA* was investigated. Cultures were incubated from 12 to 36 hours before the chemical inducer cyclopentanone, necessary to induce the *alcA* promoter, was introduced. Thereafter the culture remained under inducing conditions for an additional 72 hours. The experiment revealed the production of **5** was enhanced by growth 30-36 hours before induction (Fig S1). We next examined a second parameter, the culture time post induction. The *A. nidulans* strain was cultured for seven days after induction and samples were collected at one-day intervals from day three through day seven (Fig S2). An increase and then decline was observed over the period, with the accumulation of **5** peaking on day five. Under optimized expression conditions our engineered strain produced the polyketide (**5**) abundantly, providing a titer of 900 mg/L. The elevated production of this advanced metabolite allowed us to employ it for the preparation of a small library of azaphilones.

We focused on applying our semisynthetic route to prepare (+)-sclerotiorin by treating **5** with *p*-toluenesulfonic acid (Scheme 2) to form the 2-benzopyrilium salt (**6**), which is then oxidized by lead tetraacetate to generate the non-halogenated azaphilone (**7**).[9] Although the

acetoxylation at C-7 would be non-stereospecific, the diastereomers were indistinguishable (t.l.c., ^{1}H and ^{13}C NMR) nor could they be separated by HPLC.

Electrophilic chlorination of azaphilone **7** introduces a chlorine atom at C-5 by using a slight excess of Nchlorosuccinimide to provide the natural product (+)-sclerotiorin and 7-*epi*-sclerotiorin (**8**) in 61% yield. Despite the recalcitrant purification of **8**, the diastereomers were separated by analytical chiral HPLC to reveal close to a 1:1 ratio of (+)-sclerotiorin and 7-*epi*-sclerotiorin.

Additionally, several azaphilone analogs were also prepared from **5** (Scheme 3). To create a more efficient synthetic route, we were interested in hypervalent-iodine-mediated phenol oxidative dearomatization with *o*-iodoxybenzoic acid (IBX), a method developed by Pettus and coworkers.[12] The reaction proceeds with the formation of **6**, which subsequently is treated with IBX and catalyst Bu_4NI at room temperature to form **9**. We observed **14** as the major side product of the reaction. It is plausible that the generation of **14** could arise from tetrabutylammonium triiodide or IOH formed in the presence of the residual acetic acid with adventitious water.[13,14] To assist in the regioselective halogenation of **11**, the corresponding N-halosuccinimides were employed to produce compounds **12, 13** and **14**. Then to further functionalize the scaffold of the azaphilone core, a wittig olefination was performed with carbethoxymethylenetriphenylphosphorane. It was observed the ylide selectively coupled with the less hindered ketone and produced a mixture of E:Z isomers (1:1.05) as determined by ^{1}H NMR data. Due to the difficulties in separation, the isomeric mixtures of **15–17** were tested towards the inhibition lipoxygenase-1.

Based on a report that (+)-sclerotiorin has potent LOX-1 inhibition, [5] the biological activities of all semisynthetic azaphilones were evaluated for soybean LOX-1 inhibition to provide a preliminary structure-activity relationship (SAR). In screening for inhibition, azaphilones **7** and **13** displayed the highest lipoxgyenase-1 inhibition (Table 1). Azaphilones **5, 6, 8**, and **9** showed very similar inhibition activities. Iodinated azaphilones displayed less inhibition toward LOX-1, which may be due, in part to putative chemical instability. The other compounds (**15–17**), showed no appreciable LOX-1 inhibition.

The LOX-1 inhibition screening suggested that the C-8 ketone might be an important structural feature for activity against lipoxygenase targets. The azaphilone analogs also indicated that halogenation at C-5 is not essential to maintain low micromolar activity, except that when C-5 was iodinated a loss of inhibition was observed. Since (+)-sclerotiorin has similar IC_{50} with **8** containing both (+)-sclerotiorin and 7-*epi*-sclerotiorin, the chiral center C-7 is not critical for the LOX-1 inhibition.

It has been suggested that **1** inhibits LOX-1 by trapping lipid radicals formed at the active site of the enzyme-substrate complex. Although, we have not measured the reductive properties of our semisynthetic azaphilones, it is reasonable that they have similar antioxidant properties to (+)-sclerotiorin.

The metabolic engineering of a biosynthetic pathway in the filamentous fungus, *Aspergillus nidulans*, demonstrates the feasibility of producing copious amounts of the advanced polyketide (**5**). Coupled with existing synthetic methodology, this provides facile synthetic access to derivatives of the natural product sclerotiorin. Azaphilone analogs **7** and **13** were the most effective to inhibit the therapeutic target, LOX-1. Preliminary SAR indicates the importance of the C-8 ketone for inhibition of lipoxygenase. This may also provide insight into the further development of more potent LOX-1 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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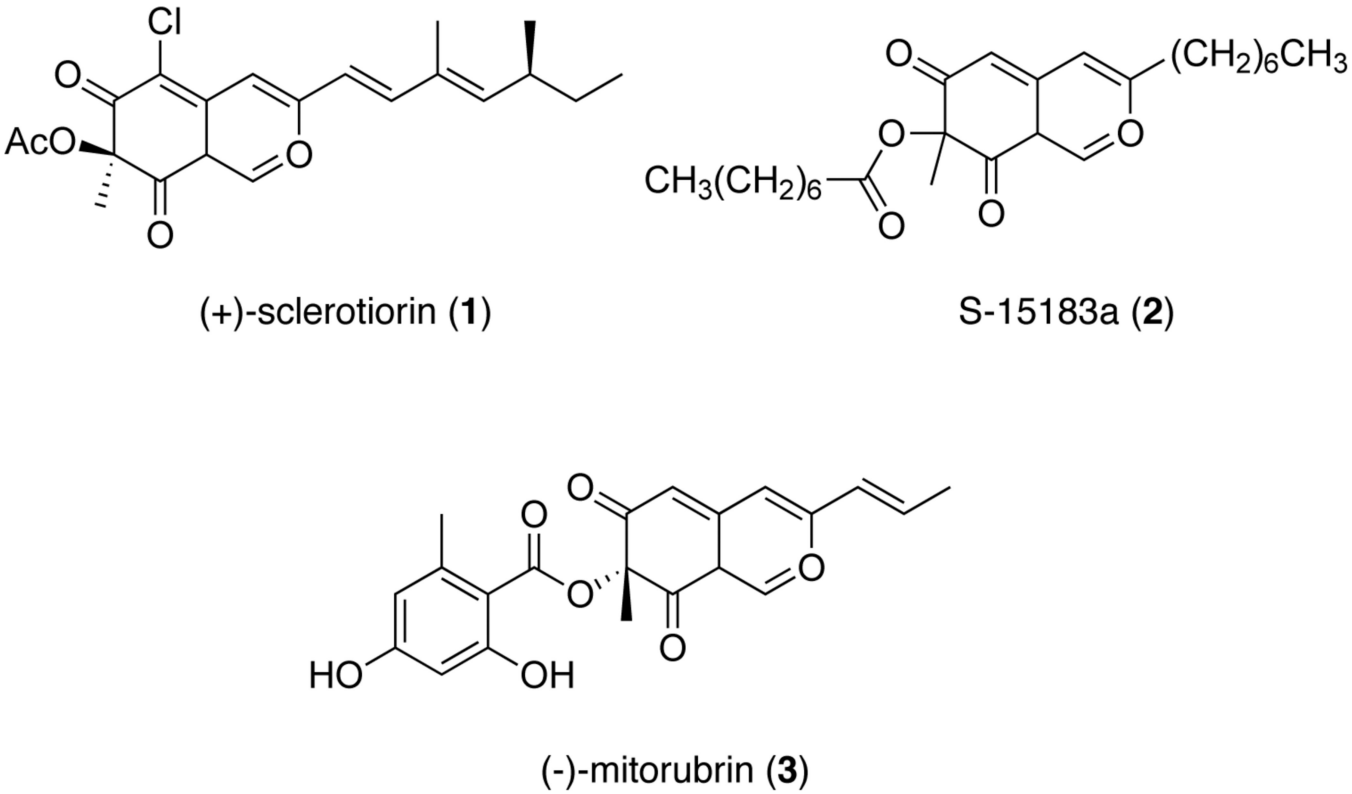
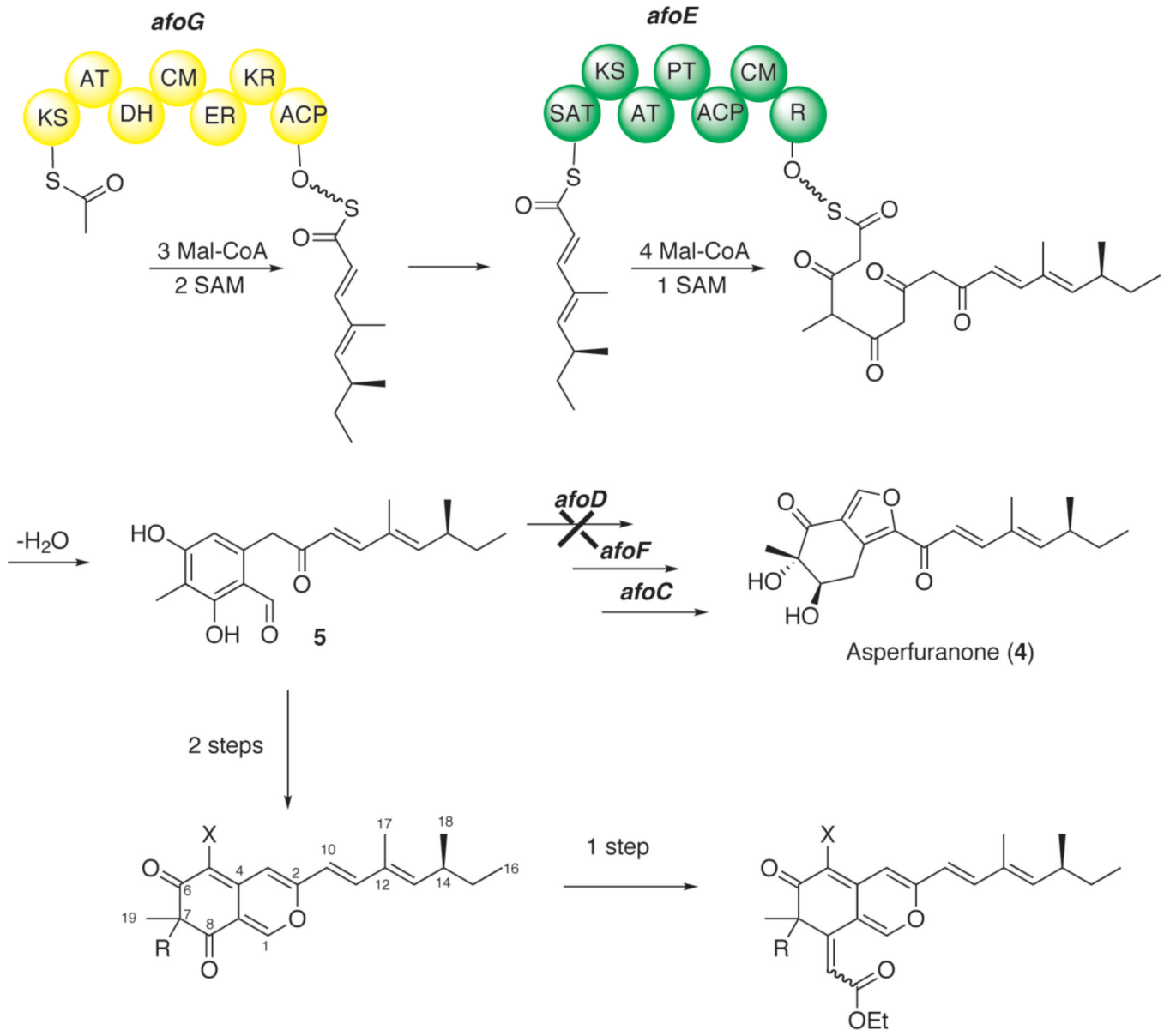
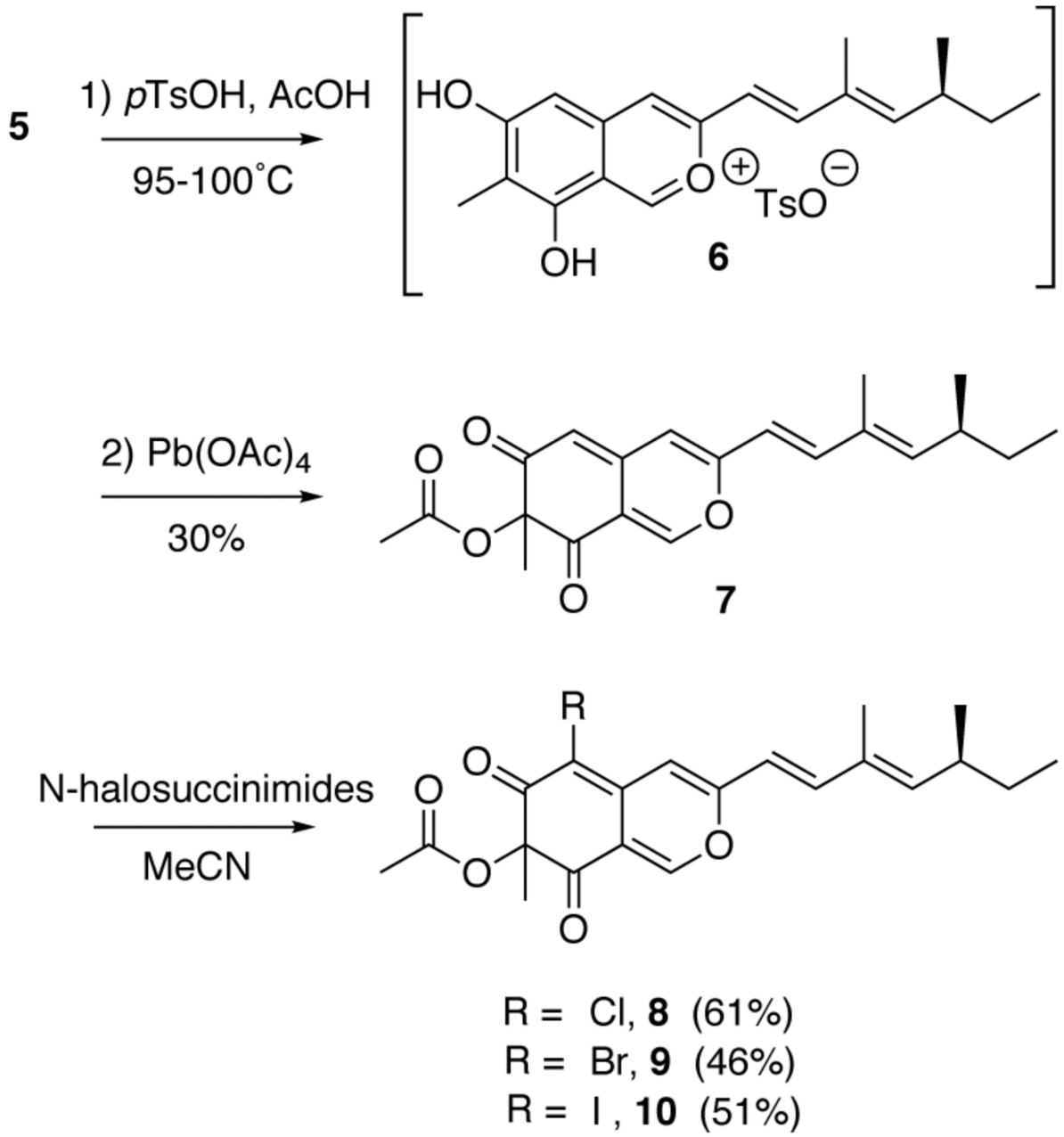


Figure 1.
Azaphilone natural products



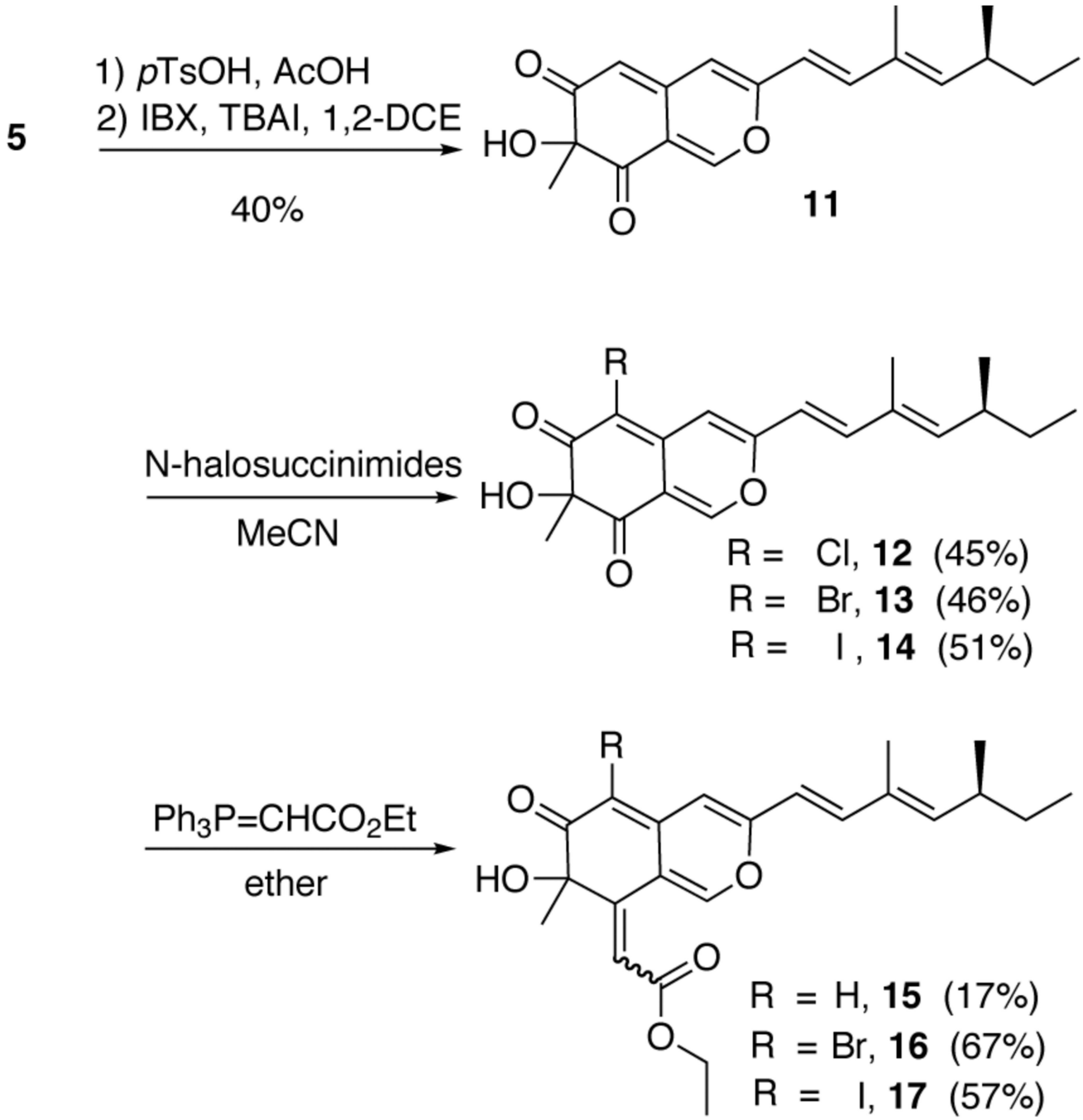
Scheme 1.

The reengineered biosynthetic pathway for the synthesis of (+)-sclerotiorin and 7-*epi*-sclerotiorin (**8**) and non-natural azaphilone polyketides.



N-halosuccinimides: NCS, NBS and NIS

Scheme 2.
Concise synthesis of (+)sclerotiorin and 7-*epi*-sclerotiorin (**8**) and analogs.



Scheme 3.
A short route to azaphilone analogs.

Table 1

Lipoxygenase-1 Inhibitory Activity.

Compound	IC_{50} (μM) ± s.d.
1	7.8 ± 2.4
4	>100
5	97.2 ± 2.0
7	4.9 ± 3.3
8	2.3 ± 0.9
9	6.8 ± 4.5
10	17.4 ± 8.1
11	10.7 ± 6.6
12	7.9 ± 3.9
13	3.2 ± 1.5
14	19.6 ± 11.9
15	>100
16	>100
17	>100

All assays performed in triplicate with the average value reported.