Communication

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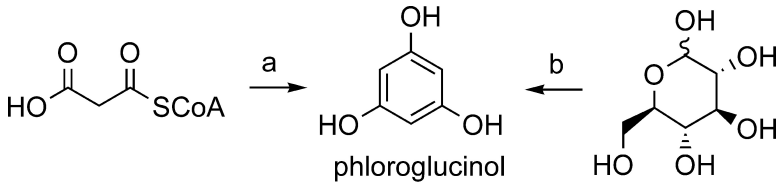

(a) PhlD; (b) *E. coli* JWF1(DE3)/pJA3.131A

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Biosynthesis of Phloroglucinol

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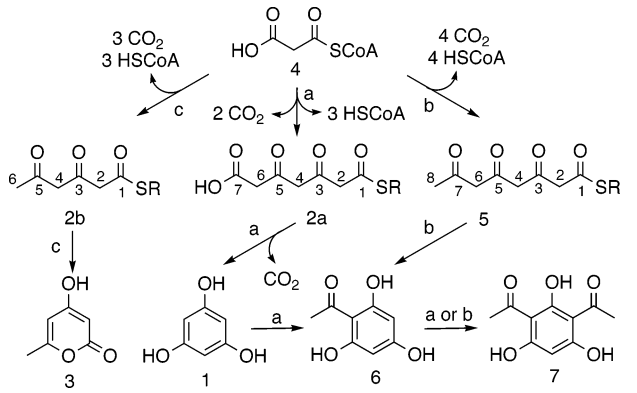
Phloroglucinol **1** (Scheme 1) is found as a substituent in a variety of natural products. However, biosynthesis of phloroglucinol **1** as a free-standing molecule has not been delineated. As part of a search for such biosynthetic activity, biosynthesis of acetylphloroglucinols encoded by the *phlACBDE* gene cluster found in *Pseudomonas fluorescens* Pf-5 was examined.[1] In addition to 2,4-diacetylphloroglucinol **7** and 2-acetylphloroglucinol **6** (Scheme 1), formation of phloroglucinol **1** was detected. Subsequent heterologous expression of *phlD* led to accumulation of phloroglucinol **1** in *Escherichia coli* cultures. PhlD-catalyzed formation of phloroglucinol **1** suggests an alternative to the previously proposed route for the biosynthesis of acetylphloroglucinols.[2] In addition, PhlD activity expressed by intact microbes provides the basis for the formulation of new syntheses (Scheme 2) of phloroglucinol **1** and resorcinol **11**.

The condensation of three malonyl-CoA molecules required for the biosyntheses of phloroglucinol **1** and triacetic acid lactone **3** (Scheme 1) may differ only in the timing of a single decarboxylation. Decarboxylation of the priming malonyl-CoA may lead to 3,5-diketohexanoate **2b** (Scheme 1), while retention of the carboxylate of the priming malonyl-CoA may lead to 3,5-diketoheptanedioate **2a** (Scheme 1).[11] A stabilized C-4,5 enolate in **2b** may cyclize to triacetic acid lactone **3**, while decarboxylation of **2a** and cyclization of a C-6 carbanion may lead to phloroglucinol **1**. Triacetic acid lactone **3** has been synthesized by *Gerbera hybrida* 2-pyrone synthase,[3] mutated *Brevibacterium ammoniagenes* fatty acid synthase B,[4] and mutated *Penicillium patulum* 6-methylsalicylic acid synthase.[5] Phloroglucinol was not formed by any of these enzymes.

Prospecting for the biosynthesis of phloroglucinol **1** led to *P. fluorescens* Pf-5 and the biosynthesis of 2,4-diacetylphloroglucinol **7** (Scheme 1).[1] Acetylphloroglucinol biosynthesis is encoded by a gene cluster consisting of *phlACBD*, a protein for product export encoded by *phlE*, and a divergently transcribed *phlF*-encoded regulator.[2] PhlD has been suggested to be involved in the formation and cyclization of an activated 3,5,7-triketooctanoate **5** (Scheme 1).[2] The resulting intermediate 2-acetylphloroglucinol **6** is then presumably acetylated to form 2,4-diacetylphloroglucinol **7** (Scheme 1).[2] Biosynthesis of phloroglucinol **1** is not an activity that has been assigned to PhlD.

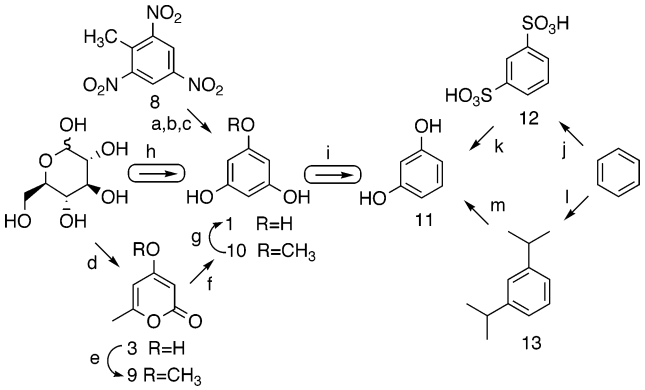
P. fluorescens Pf-5/pME6031 was examined for products that accumulated in its culture supernatants. In addition to accumulation of 2,4-diacetylphloroglucinol **7** and 2-acetylphloroglucinol **6**, formation of phloroglucinol **1** was discovered (entry 1, Table 1). To increase the concentration of biosynthesized phloroglucinols, *P. fluorescens* Pf-5 was transformed with pJA2.232, a plasmid derived from the insertion of the *phlACBDE* gene cluster into pME6031. The goal was to evade regulation by genomically encoded PhlF by presenting multiple copies of the biosynthetic gene cluster. This approach resulted in large increases in the concentrations of synthesized phloroglucinols **1**, **6**, and **7** (entry 2 vs entry 1, Table 1).

Scheme 1[a]



[a] (a) Biosynthesis of acetylphloroglucinols **6** and **7** via phloroglucinol **1**. (b) Previously proposed biosynthesis of acetylphloroglucinols **6** and **7**.[2] (c) Biosynthesis of triacetic acid lactone **3**.

Scheme 2[a]



[a] (a) $Na_2Cr_2O_7$, H_2SO_4; (b) Fe, HCl; (c) H_2SO_4, 108 °C; (d) see ref 4; (e) Dowex 50 H^+, MeOH; (f) Na, MeOH, 185 °C; (g) 12 N HCl; (h) *phlD*-expressing microbe; (i) i. H_2, Rh on Al_2O_3, ii. 0.5 M H_2SO_4, reflux; (j) SO_3, H_2SO_4; (k) NaOH, 350 °C; (l) HZSM-12, propene; (m) i. O_2, ii. H_2O_2, iii. H^+.

Further analysis followed from heterologous expression from a *T7* promoter of *phlACBDE* genes in *Escherichia coli* (entry 3−7, Table 1). All *E. coli* constructs also carried a chromosomal *gene1* insert encoding the *T7* RNA polymerase. *E. coli* BL21(DE3)/pJA3.085, which carried a *phlACBDE* plasmid insert, synthesized phloroglucinol **1** and 2-acetylphloroglucinol **6** but no 2,4-diacetylphloroglucinol **7** (entry 3, Table 1). The absence of the *phlE*-encoded product exporter in *E. coli* BL21(DE3)/pJA3.156 had only a modest impact on the concentrations of biosynthesized phloroglucinol **1** and 2-acetylphloroglucinol **6** (entry 4, Table 1). Product formation attendant with heterologous expression of only *phlD* was

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Table 1. Maximum Concentrations of Phloroglucinol **1**, 2-Acetylphloroglucinol **6**, and 2,4-Diacetylphloroglucinol **7** Biosynthesized by Constructs Expressing *phlACBDE* Genes

entry	host/ plasmid	plasmid inserts	phloroglucinols (mg/L) **1**	**6**	**7**
1	*P. fluorescens* Pf-5/ pME6031[a]	none	10	23	35
2	*P. fluorescens* Pf-5/ pJA2.232[a]	*phlACBDE*	470	500	790
3	*E. coli* BL21(DE3)/ pJA3.085[b]	*phlACBDE*	32	14	0
4	*E. coli* BL21(DE3)/ pJA3.156[b]	*phlACBD*	22	13	0
5	*E. coli* BL21(DE3)/ pJA2.042[b]	*phlD*	720	0	0
6	*E. coli* JWF1(DE3)/ pJA3.131A[c]	*phlD*	780	0	0
7a	*E. coli* BL21(DE3)/ pJA3.169	*phlACB*	0[b]	0[b]	0[b]
7b			37[d]	28[d]	3[d]
7c			29[e]	16[e]	1[e]
7d			22[f]	9[f]	0[f]

[a] Cells were cultured in YM medium under shake-flask conditions. [b] Cells were cultured under shake-flask conditions in TB medium and harvested. Following resuspension in M9 minimal salts medium, cells were cultured under shake-flask conditions. [c] Cells were cultured in M9 minimal salts medium under fermentor-controlled conditions. Concentrations of phloroglucinols 48 h after addition of [d]**1** (100 mg/L), [e]**6** (100 mg/L), or [f]**7** (100 mg/L) to cells cultured in M9 medium under shake-flask conditions.

then evaluated using *E. coli* BL21(DE3)/pJA2.042 (entry 5, Table 1). Only phloroglucinol **1** formation was observed. Synthesis of phloroglucinol **1** from glucose in minimal salts medium under fermentor-controlled conditions was examined using *E. coli* JWF1-(DE3)/pJA3.131A (entry 6, Table 1). Under these culture conditions, synthesis of phloroglucinol **1** occurred only during the log phase and not during the stationary phase of growth. Triacetic acid lactone **3** (Scheme 1) was not observed in the culture supernatants in any of the experiments summarized in Table 1.

PhlD was purified to homogeneity, and its in vitro enzymology was examined. No activity was observed when acetyl-CoA alone was employed as a substrate. Approximately equal specific activities were observed when malonyl-CoA and acetyl-CoA were incubated with PhlD relative to incubation of PhlD with only malonyl-CoA. A $K_m = 5.6\ \mu M$ for malonyl-CoA and a $k_{cat} = 10\ min^{-1}$ were determined for PhlD. No triacetic acid lactone **3** or 2-acetylphloroglucinol **6** was observed when purified PhlD was incubated with malonyl-CoA.

The products formed by microbes expressing *phlD* and during incubation of purified PhlD with malonyl-CoA suggest that cyclization of an activated 3,5-diketoheptanedioate **2a** (Scheme 1) leads to phloroglucinol **1**. Stepwise acetylation of **1** might then lead to acetylphloroglucinols **6** and **7** (Scheme 1). No phloroglucinols were synthesized (entry 7a, Table 8) by *E. coli* BL21(DE3)/pJA3.169, which carried plasmid-localized *phlACB*. However, addition of phloroglucinol **1** to the culture medium of *E. coli* BL21-(DE3)/pJA3.169 led to formation of acetylphloroglucinols **6** and **7** (entry 7b, Table 1). Deacetylase activity was also observed with the conversion of 2-acetylphloroglucinol **6** into phloroglucinol **1** (entry 7c, Table 1) and the conversion of 2,4-diacetylphloroglucinol **7** into both phloroglucinol **1** and 2-acetylphloroglucinol **6** (entry 7d, Table 1).

PhlD is of particular importance in establishing the outline of new syntheses of phloroglucinol **1** and resorcinol **11** (Scheme 2). Phloroglucinol is currently synthesized (Scheme 2) from 2,4,6-trinitrotoluene **8** by a route involving an oxidation utilizing $Na_2Cr_2O_7$.[6] Beyond the explosion hazard, environmentally problematic chromates are generated along with other salts as waste streams during synthesis of phloroglucinol **1** from 2,4,6-trinitrotoluene **8**. Recently, an alternate route (Scheme 2) to phloroglucinol **1** has been elaborated involving microbe-catalyzed synthesis of triacetic acid lactone **3**.[4] Multiple chemical steps are needed to convert triacetic acid lactone **3** into phloroglucinol **1** via intermediacy of the methyl ethers **9** and **10** (Scheme 2).[7] In contrast to these chemical and chemoenzymatic routes to phloroglucinol, heterologous expression of PhlD in *E. coli* allows phloroglucinol **1** to be made in a single microbe-catalyzed step from glucose (Scheme 2).

Resorcinol **11** is currently manufactured (Scheme 2) by alkali fusion of 1,3-benzenedisulfonic acid **12** or hydroperoxidation of 1,3-diisopropylbenzene **13**.[8] Alkali fusion requires high temperatures and generates large salt waste streams.[8] Acetone hydroperoxide formed during hydroperoxidation is an explosion hazard.[8] In addition, both 1,3-benzenedisulfonic acid **12** and 1,3-diisopropylbenzene **13** are produced from petroleum-derived, carcinogenic benzene (Scheme 2). The new route to resorcinol **11** is based on the Rh-catalyzed hydrogenation[7] (Scheme 2) of microbe-synthesized phloroglucinol **1**. Acid-catalyzed dehydration of the resulting dihydroresorcinol intermediate leads to resorcinol **11**. Since phloroglucinol **1** can now be synthesized from glucose, resorcinol joins catechol[9] and hydroquinone[10] as a dihydroxy aromatic that is amenable to synthesis from nontoxic, plant-derived glucose (Scheme 2).

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Supporting Information Available: Plasmid maps; strain construction; culture conditions; enzyme assays; pH optimum for PhlD activity (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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