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The chemoenzymatic synthesis of usnic acid

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ABSTRACT

Usnic acid, a highly functionalized dibenzofuran, is a polyketide secondary metabolite produced by several species of lichens. Synthesis of usnic acid from commercially available starting material was accomplished in two steps. The synthesis involves the methylation of phloracetophenone followed by oxidation with horseradish peroxidase. This work will lay the foundation for further biosynthetic studies on usnic acid.

Lichens are the result of a symbiotic association between a fungal partner (mycobiont) and an algal partner (photobiont). These ubiquitous organisms thrive in a variety of geographical niches but are particularly common in the harsh climate of the sub-arctic north. Possibly because of their adaptation to these harsh environments lichens have proven to be a rich source of biologically active secondary metabolites. One such example is usnic acid (**1**) a common metabolite in lichen genera such as *Usnea*, *Cladonia*, *Alectoria*, *Evernia*, *Ramalina*, and *Lecanora*. This compound was first described in 1844 and has been subjected to extensive study of its biological activity ever since. The antibacterial activity of usnic acid was recognized early, whereas findings of anticancer, antiviral, antioxidant, anti-inflammatory, and analgesic properties have been more recent.[1] In the United States, pure usnic acid and extracts containing usnic acid are available over-the-counter as dietary supplements to assist in weight loss. However, numerous reports to the US Food and Drug Administration have linked these weight loss products to liver damage.[2] Despite this apparent toxicity there are still clinical applications of usnic acid (**1**) including uses such as an additive to toothpaste.[1] Because of the wide array of biological activity displayed by usnic acid (**1**) we have become interested in examining the biosynthesis of this natural product in more detail. A clear understanding of the biosynthesis of usnic acid (**1**) in lichens may lead to the tools necessary to develop analogues that retain the beneficial bioactivity while mitigating the hepatotoxicity.

Early work by Shibata confirmed the biosynthetic origin of usnic acid as being derived from acetic acid, presumably via the polyketide pathway.[3] Feeding experiments with ^{14}C labeled methylphloracetophenone (**2**) resulted in the isolation of radioactive **1**, confirming that **2** is the biosynthetic precursor for usnic acid (**1**). A proposed biosynthesis of usnic acid (**1**) based on the results of these experiments is summarized in Scheme 1.[4]

Although there has been recent interest in lichen secondary metabolites,[5] little work has been focused on usnic acid biosynthesis. The biosynthetic proposal in Scheme 1 suggests that there are two enzymes involved in the production of **1**. It appears likely that a polyketide synthase (PKS) is responsible for the biosynthesis of the key intermediate on the pathway, methylphloracetophenone (**2**). The final step in the biosynthesis of **1** is an oxidative homocoupling of two molecules of **2** as established by Shibata.[3] To date there have been no reports in the literature regarding characterization of either the PKS or oxidative enzyme responsible for biosynthesis of **1** in lichens. We decided, therefore, as a first step in our efforts to investigate the biosynthesis of lichen secondary metabolites to attempt to characterize the oxidative enzyme involved in the formation of usnic acid (**1**). Required for this work is a way to access the key intermediate **2** in a straightforward manner. We anticipate it will be necessary to include an isotopic label in the structure of **2** for use in bioassay-guided isolation of the oxidative enzyme. Here, we report the one-step synthesis of **2** and its conversion to usnic acid (**1**) by a model enzymatic oxidation using horseradish peroxidase.

We began our efforts by examining the previously reported Friedel–Crafts acylation of trihydroxytoluene (**3**) using boron trifluoride and acetic acid.[6] Under several different conditions the major product we isolated from this reaction was compound **4**, the result of double acylation of the aromatic ring (Scheme 2).

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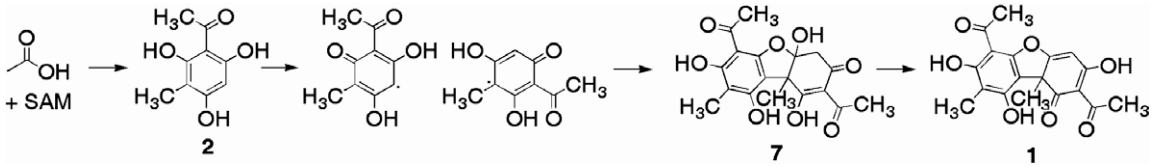
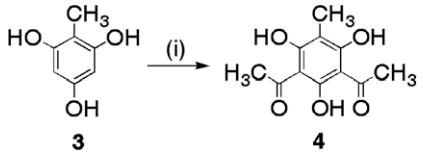


Scheme 1. Proposed biosynthesis of usnic acid (**1**) based upon Shibata.[3] Methylphloracetophenone (**2**) is produced by a polyketide synthase and then oxidized first to **7**, followed by elimination of H_2O to produce **1**. S-adenosyl methionine (SAM) is the source of the methyl group bonded directly to the aromatic ring.



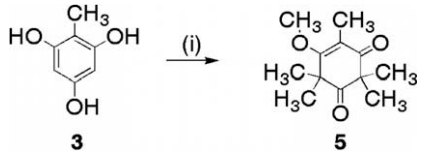
Scheme 2. Acylation of trihydroxytouene (**3**) forming **4**. Reagents and conditions: (i) BF_3–OEt_2, HOAc, Ac_2O, 100 °C, 3 h, anhydrous.

In an effort to deactivate the aromatic ring towards acylation, we decided to attempt to protect the hydroxyl groups by methylation. We treated trihydroxytoluene (**3**) with an excess of iodomethane, anticipating trimethoxytoluene as the sole product. However, somewhat unexpectedly, the only product isolated from this reaction was compound **5** (Scheme 3) with no trace of trimethoxytoluene detected. Compound **5** is the result of extensive carbon methylation of the aromatic ring of **3** by iodomethane. Trihydroxytoluene (**3**) is an ambident nucleophile whose alcohol oxygen atoms are stronger nucleophiles than the carbon atoms in the ring. Methylation with iodomethane occurs at the carbon atom rather than the oxygen atom since iodide is a weak base and a good leaving group and thus considered a soft anion.

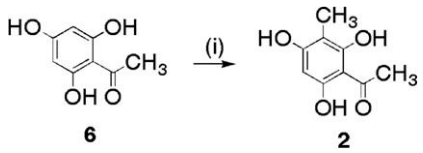
We used the result in Scheme 3 as a suggestion that it might be possible to use a similar reaction to alkylate the aromatic ring of phloracetophenone (**6**) (Scheme 4). The direct alkylation of **6** with iodomethane would provide an efficient one-step synthesis of **2** from an atom economy view. In addition, **6** is commercially available and considerably less expensive than **3**. This would improve the overall attractiveness of this approach. Initial attempts at this reaction, at room temperature, were successful in producing a small amount of **2** (<10%). The major side products of this reaction are the result of methylation of the oxygen atoms producing mono-, di- and tri methoxy versions of **2**. By reducing the temperature to 0 °C, with slow addition (5 min) of the excess iodomethane (4 equiv), we could improve our yield of **2** while minimizing the production of the oxygen methylated products. Trihydroxyacetophenone (**6**) is sold as the monohydrate and a key step in improving our yield was to dissolve **6** in anhydrous acetone and then remove the solvent by rotary evaporation. We found it necessary to repeat this process at least twice to optimize the yield. On a 1 mmol scale our conditions gave a 17% yield of **2**, with the side products being the result of oxygen methylation. However, when we increased our reaction to a 10 mmol scale, the yield improved to a more preparatively useful 45% (Scheme 4).[7] Again the major side products were the result of methylation at the oxygen atoms. Reducing the number of equivalents of iodomethane resulted in an overall erosion of the isolated yield of **2**, without any significant change in the ratio of this desired product to the side products.

It was also possible to recover more than 20% of the starting material under the reaction conditions described in Scheme 4 implying that recycling of starting material could further increase our yield. Furthermore, although we have not yet demonstrated this, it should be possible to improve the overall yield of (**2**) from (**6**) by removing the *O*-methyl groups from the side products. We have developed a straightforward methodology for the synthesis of the key usnic acid (**1**) intermediate, **2**, in one-step from commercially available precursors.

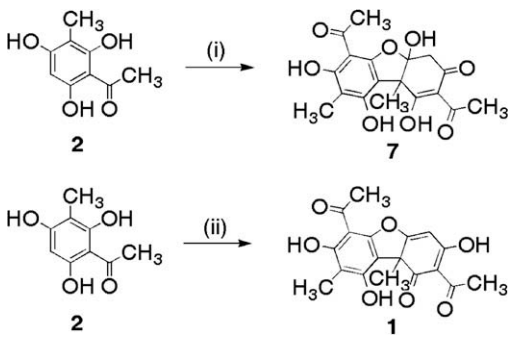
Our next efforts focused on attempting to convert our synthetic methylphloracetophenone (**2**) to usnic acid (**1**) by using horseradish peroxidase (HRP) as a model system for the oxidative enzyme. Using HRP with H_2O_2 as oxidant and 5 mg of **2** we were able to detect the production of usnic acid (**1**) (Scheme 5) using a combination of LC–MS and ^{1}H NMR. We chose to use HRP as a model system as this had been successfully used in a previous synthesis of usnic acid (**1**)[8] and is commercially available. Key to our success in this effort was the realization that it was critical to add the H_2O_2 stepwise over an extended period of time (2.5 h). After a total incubation time of 5.5 h at 37 °C a 40% yield, of hydrated usnic acid (**7**) was obtained from the enzyme assay mixture as determined by ^{1}H NMR.[9] This identity of this molecule was confirmed by LC–MS and by comparison of the ^{1}H NMR with the spectral data for a commercial standard sample of usnic acid (**1**). A modified enzyme assay work-up[10] using acetic anhydride followed by the addition of sulfuric acid[11] resulted in the detection of **1** in our enzyme assay in addition to a significant amount of **7**. A significant amount of unreacted starting material was recovered and we could not detect any other oxidation products in our HRP enzyme assay mixture. In



Scheme 3. Methylation of trihydroxytoluene. Reagents and conditions: (i) CH_3I, K_2CO_3, DMF, 100 °C.



Scheme 4. Methylation of phloracetophenone (**6**) with excess ioodomethane. Reagents and conditions: (i) CH_3I, K_2CO_3, acetone, 0 °C, 9 h (45%) see Ref. 7.



Scheme 5. Oxidation of methylphloracetophenone (**2**) by horseradish peroxidase (HRP). Reagents and conditions: (i) See Ref. 9; (ii) See Ref. 10.

addition we carried out several control assays in order to confirm that both enzyme and H_2O_2 were necessary for the production of usnic acid (**1**). Furthermore, it does not appear that **1** undergoes oxidation in the presence of HRP and H_2O_2.

We were also able to prepare usnic acid (**1**) and hydrated usnic acid (**7**) by inorganic oxidation of methylphloracetophenone (**2**) with potassium ferricyanide as an oxidant.[11] This inorganic oxidation lead to a complex mixture of reaction products, however we could isolate a small amount of **1** (approx. 4% yield) along with a trace of **7**.

In summary, we have demonstrated the ability to produce usnic acid (**1**) in a simple two step process from commercially available trihydroxyacetophenone (**6**). This route involves an unusual direct carbon methylation of the aromatic ring of **6** by iodomethane to produce the key biosynthetic intermediate methylphloracetophenone (**2**). We have also demonstrated that our synthetic methylphloracetophenone (**2**) can be oxidized by horseradish peroxidase (HRP) and H_2O_2 to produce usnic acid (**1**). Our synthetic scheme is attractive as it is amenable to the incorporation of an isotopic tracer (such as ^{13}C or ^{14}C) in methylphloracetophenone (**2**). This should easily be accomplished by carrying out the methylation reaction with appropriately labeled iodomethane. We anticipate that tracer labeled **2** will be a valuable tool that will assist in identifying the oxidative enzyme responsible for the dimerization of **2** to produce **1**. This will be a necessary step in establishing the full biosynthetic machinery in lichens that produces usnic acid (**1**).

Acknowledgments

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7. Reagents and conditions (i): 10 mmol of trihydroxyacetophenone monohydrate (1.862 g) was dried three times with acetone under vacuum. 2.5 equiv K_2CO_3 (3.45 g) and 100 mL acetone were added and mixture was brought to 0 °C with stirring. 4 equiv CH_3I (2.50 mL) was added over 5 min and the reaction was allowed to proceed for 9 h at 0 °C with stirring. The reaction was quenched with 100 mL H_2O and 1.5 mL concd HCl before extraction with DCM and drying under vacuum. Silica gel column chromatography (1:99 MeOH/DCM) provided 781 mg (45% yield) methylphloracetophenone (**2**) and 419 mg (23% recovery) trihydroxyacetophenone (**6**).
8. Penttila, A.; Fales, H. M. *Chem. Commun.* **1966**, *18*, 656.
9. Reagents and conditions (i): 5 mg methylphloracetophenone, 100 μL MeOH, 20 Activity units (AU)/mL horseradish peroxidase (type VI, 254 AU/mg, where one AU is the amount of HRP that forms 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C), 0.883 M H_2O_2 over 2.5 h so the final amount of H_2O_2 was 25 μmol, phosphate buffer 0.1 M pH 6.5 to bring reaction to 1 mL total, 37 °C, 5.5 h (40%).
10. Reagents and conditions (ii): 5 mg methylphloracetophenone, 100 μL MeOH, 20 Activity units (AU)/mL horseradish peroxidase (type VI, 254 AU/mg, where one AU is the amount of HRP that forms 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C), 0.883 M H_2O_2 over 2.5 h so the final amount of H_2O_2 was 25 μmol, phosphate buffer 0.1 M pH 6.5 to bring reaction to 1 mL total, 37 °C, 5.5 h reaction followed by workup with Ac_2O and H_2SO_4 (20:1), 40 °C, 30 min, quenched with cold H_2O, extracted with $CHCl_3$, H_2SO_4, 0 °C, 5 min, extracted with $CHCl_3$
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