Biosynthesis of Brevinic Acid from Lawsone

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Abstract

The menaquinone-pathway (men) is widespread in bacteria and key to the biosynthesis of intriguing small molecules such as the essential vitamin menaquinone and the natural dye lawsone. The violet molecule brevinic acid is another proposed product of men, but its direct biosynthetic precursor has remained doubtful. In this study, we isolated brevinic acid from E. coli and confirmed its non-enzymatic formation from lawsone and homocysteine involving an intermediate acetylation or phosphorylation step. We furthermore compared our proposed substrates in a non-enzymatic assay against the previously hypothesized precursor DHNA and showed that the reaction with activated lawsone derivatives proceeded faster, more selective, and with complete turnover. This supports our proposed biosynthesis of brevinic acid from lawsone and enables a cost effective, larger-scale synthesis of brevinic acid.

Introduction

Brevinic acid (1) is a violet molecule first patented in 1976 by Eguchi et al. as a dye.¹ In 1984, 1 was isolated from Brevibacterium finum, thus establishing it as a natural product and its chemical synthesis from 2,3-dichloro-1,4-naphthoquinone and homocysteine (2) was reported.² Biosynthetically, 1 consists of a 1,4-naphtoquinone moiety fused to a 7-membered heterocycle, most likely incorporating 2. The mechanism of this condensation and the biosynthetic origin of the 1,4-naphtoquinone moiety have remained unclear. Recently, 1 has been re-isolated from a broad range of bacteria including marine bacterium Agrococcus sp.,³ intestinal Enterobacter amnigenus,⁴ and Escherichia coli.⁵,⁶ Its biological

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significance has, however, remained unsure despite extensive bioactivity testing. In a study from 2022, 1 was identified in a range of Gram-negative and -positive pathogens from the human gut capable of menaquinone biosynthesis and was furthermore found to be present in fecal samples from mice.5 A non-enzymatic biosynthetic pathway from DHNA (3) through addition of homocysteine to the cytotoxic oxidation product of DHNA, 1,4-naphthoquinone-2-carboxylic acid (4), was proposed (Figure 1A). It was argued that 1 would be part of a cellular detoxification strategy, since it was not found to be cytotoxic against HCT116 cells. This is, however, questioned by another 2022 study, which discovered cytotoxicity of 1 against HL-60 cells with an IC50-value of 21.5 μM.3 Furthermore, the suggested spontaneous oxidation of 3 to 4 appears unlikely under the prevailing anaerobic conditions in the gut.

Here, we propose that 1 is most likely formed by addition of homocysteine to lawsone (5), another colorful molecule originating from the menaquinone pathway, requiring only intermediate activation of 5, e.g., by acetylation or phosphorylation, for quantitative turnover.

**Results and Discussion**

During our heterologous expression experiments of cryptic biosynthetic gene clusters (BGCs) from the gut microbiota,7 we discovered expression of a violet molecule in *E. coli*. The violet color was attributed to a mass peak of 290.0483 m/z eluting at around 17.5 min, which was identified in ethyl acetate extracts of acidified culture broth. Notably, production of this compound seemed to be boosted by tetracycline-induced expression of several cryptic BGCs. However, 1 was identified in all extracts of *E. coli* expressions in small amounts, independent of the presence of an expression vector. This suggested that the biosynthesis of this molecule was entirely encoded within *E. coli*.

For isolation of 1, cultivation of *E. coli* BAP1 was carried out in 1 L TB medium for 3 days at 20 °C with supplementation of the media with 0.5 μg/mL tetracycline. The culture broth was separated from the cells by centrifugation and extracted with ethyl acetate. The crude extract was pre-purified by silica column chromatography with dichloromethane/methanol (30:1), which yielded a violet fraction. Final purification was conducted by preparative HPLC. 1H and 13C NMR showed the presence of fourteen 13C-signals, which, in combination with the exact mass, led to an assumed chemical formula of C14H11NO4S. After literature research, the 1H and 13C NMR spectra were found to be identical to brevinic acid (1), a metabolite with a reported intense purple color.3
Figure 1. Different mechanisms proposed for the biosynthesis of 1. A. Biosynthesis of 1 from 3 through oxidation to 5, decarboxylation and dehydrogenation, as proposed by Gatsios et al. B. Condensation between 6 and 2 as proposed by Surup. C. Stepwise addition mechanism starting from 5 proposed in this study. This was however found to yield 7 as main product and not quantitatively proceed to 1.

Two studies from 2022 independently proposed 3 as a precursor of 1: Ding et al. stated that 2, originating as a byproduct of a radical-SAM-enzyme, and 3 could form 1 but did not state a mechanism to facilitate such product formation. Concurrently, Gatsios et al. isolated 1 from intestinal E. coli as “MK-hCys”, referring to the structural similarity to menaquinones without acknowledging that the molecule had been identified before. In this study, it was proposed that 1 would be synthesized in Nature by spontaneous, non-enzymatic addition of 2 to 1,4-naphthoquinone-2-carboxylic acid (4), which should be formed by spontaneous oxidation of 3 (Figure 1A). While the oxidation of 3 to 4 seems unlikely under anaerobic conditions such as found in the gut, the mechanism of a non-enzymatic addition of homocysteine to 4 can be called into question because it involves further steps, such as spontaneous decarboxylation and oxidation, which were not properly explained in the cited literature.

Apart from the pathway originating from 3 (Figure 1A), only one alternative biosynthetic mechanism to 1 had been proposed in the literature: a double condensation reaction between 2 and 2,3-dihydroxy-1,4-naphthoquinone (6), as proposed in a PhD thesis by Surup et al. (Figure 1B). Compound 6 was suggested to originate from the shikimate-pathway by oxidation. This molecule, however, has not yet been isolated from Nature.
We now propose that the naphthoquinone precursor of 1 is 2-hydroxy-1,4-naphthoquinone (5), a red pigment known as lawsone. This molecule is believed to originate from 3 through oxidative decarboxylation, although the involved enzyme has not been identified so far.\textsuperscript{11} Compound 5 is abundantly found in plants, such as walnut and henna, and thus might also be biosynthesized in bacteria. For biosynthesis of 1, the first step would be a Michael-addition-type reaction between the nitrogen in 2 and 5, leading to intermediate 7. This type of reactivity of 5 has been described before and also occurs with keratin in traditional hair-dying techniques with henna.\textsuperscript{12,13} Afterwards, intramolecular condensation using the side-chain thiol as a nucleophile could yield brevinic acid (1, Figure 1C).

To test this hypothesis, 5 and 2 were incubated in water under basic conditions. However, primarily production of 7 with a monoisotopic mass of 308.0588 m/z was observed, while the mass of 1 was only detected in traces. This generally supported the suspected reactivity of 5 for the initial addition step, but indicated that the hydroxyl group of 5, which is not a good leaving group, might have to be activated to enable straightforward elimination. Candidate activation reactions \textit{in vivo} are, e.g., phosphorylation or acetylation with ATP or Acetyl-CoA, respectively, two building blocks readily available in cells. To test this hypothesis, phosphorylated (8) and acetylated lawsone (9) were synthesized chemically. Phosphorylation was achieved with diethylchlorophosphate using triethylamine as the base in dichloromethane, which after preparative HPLC-purification yielded 8 in sufficient 22% yield. Acetylation was performed according to literature\textsuperscript{14} with acetic acid anhydride as solvent under acidic conditions, delivering 9 in 38% yield after silica column-purification.
Figure 2. Derivatization of 5 by phosphorylation (8) or acetylation (9) both enables subsequent formation of 1 under physiological conditions.

With compounds 8 and 9 in hand, we moved on to non-enzymatic condensation reaction assays with reaction partner 2 under physiological conditions and found that both were readily converted to 1 under neutral pH. This was compared to identical reaction conditions in which 8/9 were replaced by 3 or 5 as a control. To reliably follow the course of and quantify the reactions, samples were taken at 0, 15, 30, 60, 120, 180 and 240 minutes and immediately quenched in 0.01 M HCl to stop further reaction prior to HPLC-measurement. This caused the decomposition of substrates 8 and 9 to 5, which was quantified by HPLC in their stead. The reaction turnover was determined by comparison of the integrals of the UV peaks of 3 (16.5 min) and 5 (11 min) and 1 (17.5 min) and identified superior performance of 8 and 9 as substrates compared to 3 (Figure 3A). While 3 also formed 1 under the tested conditions, this reaction was noticeably slower, stopped after about 45% conversion and yielded several uncharacterized side products (see Figure 3B). This contrasts compared to the selective reactions from 8 and 9 (Figures 3C and D), further strengthening the hypothesis for our proposed mechanism towards 1 from 5.
Figure 3. Comparison of non-enzymatic synthesis of 1 with different substrates. A. While 8 and 9 proceed rapidly and show complete turnover after 60 min, the reaction of 3 proceeds slower and seems to stop after about 40% turnover. Turnover was calculated as ratio of integrated peak areas of product to starting material. B. HPLC-chromatogram of quenched reaction of 3 showed about 50% turnover of substrate while forming multiple side products (*). C and D. Reactions of 8 and 9 showed no remaining starting material after 60 min, 9 with little to no observed side products. Upon quenching in 0.01 M HCl, 8 and 9 were both hydrolyzed to 5.

Conclusion

In conclusion, we isolated brevinic acid (1) from E. coli, supporting its proposed origin from the menaquinone pathway. Furthermore, we synthesized lawsone-derivatives 8 and 9 and employed them in a non-enzymatic assay to synthesize 1 with 100% turnover after 60 min and little to no side reactions. We showed that the same reaction with 3 as a substrate progressed slower, with significantly less turnover, and inferior selectivity, supporting our claim that the biosynthesis of 1 does not progress directly from 3 but from its downstream product 5. Finally, we present a new way to chemically access 1 in a two-step process from 5, which is a significantly less expensive starting material than 2,3-dichloro-1,4-naphthoquinone (10), paving the way for larger-scale synthesis and functional studies on 1.
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Material and Methods

Bacterial Strains

*E. coli* BAP1 were cultivated at 37 °C in LB medium supplemented with a suitable selection antibiotic while shaking at 180 rpm, or on LB-Agar supplemented with selection antibiotic at 37 °C unless otherwise specified. DNA was kept in MilliQ water for short-term storage.

Analytical and preparative HPLC

Analytical high-performance liquid chromatography (HPLC) was performed on an Azura HPLC device manufactured by Knauer, consisting of the following components: AS 6.1L sampler, P 6.1L pump, DAD 2.1L detector. Components were separated on a Phenomenex Luna 3u C-18 column (150 × 4.6 mm) at a flowrate of 1 mL/min with the eluents water (A) and acetonitrile (B), both supplemented with 0.05% trifluoroacetic acid. The elution method consisted of equilibration at 5% B for 2 min, followed by a gradient of 5–100% B over 28 min. Column washing was performed at 100% B for 5 min and the column was re-equilibrated at 5% B for 2 min before the next measurement.

Preparative HPLC was performed on a Jasco HPLC system consisting of an UV-1575 Intelligent UV/Vis detector, two PU-2068 Intelligent preparation pumps, a Mika 1000 dynamic mixing chamber (1,000 μl; Portmann Instruments AG Biel-Benken) and a LC-NetII/ADC and a Rheodyne injection valve. The system was controlled by Galaxie software. Chromatographic separation was performed on a Eurospher II 100-5 C18 A (250 × 16 mm) column with precolumn (30 × 16 mm) provided by Knauer at a flow-rate of 10 ml/min and the eluents were water (A) and acetonitrile (B). The gradient was adjusted depending on the polarity of the compounds. Collected product fractions were combined, the organic solvent was evaporated under reduced pressure at 40 °C and water was removed by lyophilization.

HR LC-MS measurement

For liquid chromatography (LC) coupled to high resolution mass spectrometry (HR-MS), a Bruker Elute UHPLC-system with an Intensity Solo 2 C18-column (100 × 2.1 mm) coupled to a Bruker Impact II ultra-high resolution Q-TOF mass spectrometer with electron-spray ionization (ESI) were used. For LC, water (A) and acetonitrile (B) were used as eluents, both supplemented with 0.1% formic acid, at a flowrate of 1 mL/min.
0.3 mL/min. The elution method consisted of equilibration at 5% B for 2 min, a gradient of 5–95% B over 23 min, washing at 95% B for 3 min and re-equilibration at 5% B for 2 min.

**NMR-measurement**

$^1$H and $^{13}$C Nuclear Magnetic Resonance spectra (NMR) were recorded on Bruker AVANCE 300 and AVANCE 600 spectrometers at room temperature. The chemical shifts are given in $\delta$-values (ppm) downfield from TMS and are referenced on the residual peak of the deuterated solvent (CDCl$_3$: $\delta_H = 7.26$ ppm, $\delta_C = 77.2$ ppm; D$_2$O: $\delta_H = 4.80$ ppm). The coupling constants $J$ are given in Hertz [Hz].

**Production and Isolation of Brevinic Acid (1)**

*E. coli* BAP1 were chemically transformed with an expression vector as described previously or plated from a cryostock in the case of plasmid-free *E. coli* BAP1. A single positive clone was selected on agar-plates and was used to inoculate a preculture. An expression culture of 1 L terrific broth in a 2 L Erlenmeyer flask was inoculated with 1% preculture (vol/vol) and was incubated while shaking at 180 r.p.m. at 37 °C until an OD$_{600}$ of 0.8 was reached. The culture was cooled to 4 °C for 60 min and expression was induced by adding 0.5 µg/mL tetracycline. The cultures were incubated at 20 °C while shaking at 180 r.p.m in darkness.

After incubation, cultures were centrifuged (6,000 g for 15 min) to separate *E. coli* biomass from growth medium. The culture supernatants were adjusted to a pH of 3–4 by addition of conc. HCl and extracted with ethyl acetate (2 × 700 mL per 1 L of growth medium). The combined extracts were washed with saturated brine, dried over MgSO$_4$ and filtered. The solvent was removed under reduced pressure at 40 °C. The crude extract was purified on a silica column (dichloromethane/methanol = 30:1), which yielded a crude violet product. Final purification ensued on a preparative HPLC-system with a gradient of 25–45% B over 15 min, where 1 eluted between 8.5 and 9 min. Brevinic acid (1) was isolated as purple crystals (4.8 mg).

$^1$H NMR (600 MHz, CDCl$_3$): $\delta = 8.06$ (ddd, $J = 7.6$, 1.2, 0.4 Hz, 1H), 8.02 (ddd, $J = 7.6$, 1.3, 0.4 Hz, 1H), 7.68 (ddd, $J = 7.6$, 7.5, 1.3 Hz, 1H), 7.61 (ddd, $J = 7.6$, 7.5, 1.3 Hz, 1H), 6.42 (d, $J = 3.8$ Hz, 1H), 5.54 (ddd, $J = 11.5$, 5.0, 3.8 Hz, 1H), 3.73 (ddd, $J = 14.9$, 11.7, 5.8 Hz, 1H), 3.10 (ddd, $J = 14.9$, 6.8, 1.4 Hz, 1H), 2.54 (ddd, $J = 13.3$, 11.7, 6.8, 5.0 Hz, 1H), 2.29 (ddd, $J = 13.3$, 11.5, 5.8, 1.4 Hz, 1H) ppm;

$^{13}$C NMR (151 MHz, CDCl$_3$): $\delta = 181.7$, 179.1, 174.9, 145.6, 134.6, 133.0, 132.9, 130.4, 126.7, 126.6, 117.6, 54.6, 30.9, 30.5 ppm; HRMS (m/z): [M]$^+$ calcd. for C$_{14}$H$_{12}$NO$_3$S$^+$, 290.0482; found, 290.0483.
Synthesis of (3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)homocysteine (7)

Lawsone (5, 40.0 mg, 0.23 mmol, 1.0 eq.) and homocysteine (2, 31.1 mg, 0.23 mmol, 1 eq.) are dissolved in 8 mL water and stirred at room temperature. Pyridine (74.2 µL, 0.92 mmol, 4 eq.) is slowly added and the reaction is stirred for 3 h. The reaction mixture is lyophilized, and the product is purified on a preparative HPLC with a gradient of 20–45% B over 20 min, where 7 elutes between 8 and 9 min. Compound 7 is obtained as orange crystals (27.6 mg, 0.090 mmol, 39%).

1H NMR (300 MHz, D2O): δ = 7.82 (t, J = 7.9 Hz, 2H), 7.70 (t, J = 7.3 Hz, 1H), 7.65 (t, J = 7.1 Hz, 1H), 4.11 (t, J = 6.3 Hz, 1H), 3.01 (bs, 2H), 2.22–2.04 (m, 2H) ppm; 13C NMR (151 MHz, D2O): δ = 184.0, 183.9, 172.3, 162.3, 135.1, 133.7, 132.3, 129.7, 126.6, 126.5, 52.3, 30.1, 28.4 ppm; HRMS (m/z): [M]+ calcd. for C14H14NO5S+, 308.0587; found, 308.0588.

Synthesis of 2-diethylphosphate-1,4-naphthoquinone (8)

In a round-bottom flask, 5 (40.0 mg, 0.23 mmol, 1.0 eq.) is dissolved in 6 mL dichloromethane and triethylamine (95.5 mL, 0.69 mmol, 3 eq.) is added under stirring. Diethylchlorophosphate (134 µL, 0.92 mmol, 4 eq.) is dissolved in 3 mL dichloromethane and slowly added to the reaction. The mixture is stirred over night at room temperature and finally heated to 40 °C for 30 min, before the solvent is removed under reduced pressure. The product is purified on a preparative HPLC with a gradient of 35–55% B over 25 min, where 8 elutes at 14 min. Compound 8 is obtained as a red oil (15.7 mg, 0.050 mmol, 22%).

1H NMR (600 MHz, CDCl3): δ = 8.14–8.07 (m, 2H), 7.79–7.74 (m, 2H), 6.89 (d, J = 1.5 Hz, 1H), 4.33 (dq, J = 7.1, 8.2 Hz, 4H), 1.42 (td, J = 1.0, 7.1 Hz, 6H) ppm; 13C NMR (151 MHz, CDCl3): δ = 184.7, 179.0, 152.7, 134.5, 133.9, 131.8, 130.9, 126.8, 126.4, 122.1, 65.7, 16.0 ppm; 31P NMR (242 MHz, CDCl3): δ = −7.66 ppm; HRMS (m/z): [M]+ calcd. for C14H16O6P+, 311.0679; found, 311.0675.

Synthesis of 2-acetoxy-1,4-naphthoquinone (9)

In a round-bottom flask, 5 (40.0 mg, 0.23 mmol, 1.0 eq.) is dissolved in 5 mL acetic acid anhydride. After addition of 5 drops of concentrated sulfuric acid, the reaction is stirred at room temperature for 5 h. The reaction is stopped by addition of 100 mL aqueous NaHCO3-solution (5%), followed by extraction with dichloromethane (3 × 40 mL) and washing with brine. The solvent is removed under reduced pressure and the crude product is purified on a silica column (cyclohexane:ethyl acetate 10:1 → 7:1). Compound 9 is obtained as yellow crystals (18.9 mg, 0.087 mmol, 38%).
\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta = 8.14–8.07 \) (m, 2H), \(7.80–7.75 \) (m, 2H), \(6.76 \) (s, 1H), \(2.39 \) (s, 3H) ppm;

HRMS (m/z): [M]+ calcd. for C\(_{12}\)H\(_9\)O\(_4\)+, 217.1995; found, 217.1998. The spectroscopic data was in agreement with those stated in literature.\(^15\)

**Brevinic Acid (1) Synthesis Assays**

Four substrates are tested for their performance in enzyme-free formation of 1 under physiological conditions. In four different reaction flasks, L-homocysteine (2, 5 mg, 0.037 mmol, 1 eq.) is dissolved in 10 mL PBS (8.0 g NaCl, 0.20 g KCl, 1.42 g Na\(_2\)HPO\(_4\), 0.27 g KH\(_2\)PO\(_4\), MilliQ-Water to 1 L, pH adjusted to 7.4 with HCl). Compounds 3 (5 mg, 0.037 mmol, 1 eq.), 5 (negative control, 6 mg, 0.037 mmol, 1 eq.), 8 (10.8 mg, 0.037 mmol, 1 eq.) and 9 (7.5 mg, 0.037 mmol, 1 eq.) are separately dissolved in 200 \(\mu\)L DMSO each and individually introduced into the four flasks containing 2. The assays are incubated at 37 °C at 200 r.p.m. and samples for HPLC-measurement are taken at 0, 30, 60, 120 and 600 min timepoints. To prevent continued reaction, the samples were quenched in 0.01 M HCl and stored at –80 °C prior to measurement.
References

1. JPS5284220A.


