Concise synthesis of artemisinin from a farnesyl diphosphate analogue

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Abstract

Artemisinin is one of the most potent anti-malaria drugs and many often-lengthy routes have been developed for its synthesis. Amorphadiene synthase, a key enzyme in the biosynthetic pathway of artemisinin, is able to convert an oxygenated farnesyl diphosphate analogue directly to dihydroartemisinic aldehyde, which can be converted to artemisinin in only four chemical steps, resulting in an efficient synthetic route to the anti-malaria drug.

Keywords: Malaria, Artemisinin, Amorphadiene synthase, Dihydroartemisinic aldehyde, Dihydroartemisinic acid, Farnesyl diphosphate

1. Introduction

Malaria affects almost 50% of the world’s population and causes hundreds of thousands of deaths each year. Isolated from the plant Artemisia annua (qinghaosu), the sesquiterpene artemisinin (1) exhibits excellent anti-malaria activity and kills the parasite at most of its asexual stages of development in human blood. Although several synthetic routes to artemisinin (1) have been developed, the chemical synthesis is lengthy and low yielding due to the highly complex structure of the sesquiterpene endoperoxide. The worldwide supply of artemisinin (1) relies predominantly on the extraction of (1) from the plant Artemisia annua and as a consequence the world market price is highly volatile ranging from US $350 to $1700 per kilogram. Most countries affected by malaria epidemics are in the developing world, and therefore a stable and affordable supply of artemisinin (1) is highly desirable.

Currently the most efficient synthetic route to produce artemisinin is the combination of a biosynthetic process with several chemical steps (Scheme 1). The biosynthesis of artemisinin is well understood and the key step to this synthesis involves the class I sesquiterpene cyclase amorphadiene synthase (ADS). This enzyme catalyses the cyclisation of (E,E)-farnesyl diphosphate (FDP, 2) to amorpha-4,11-diene (3), a bicyclic intermediate with four stereo-centres. 3 can be converted to the advanced synthetic intermediate dihydroartemisinic acid (DHAA, 4) either chemically or enzymatically using engineered yeast (Scheme 1). The latter method has been developed into a semi-synthetic production of artemisinin (1). Engineered yeast containing ADS and five other enzymes produce artemisinic acid (5), which is subsequently reduced to DHAA (4) by a transition metal-catalysed hydrogenation. DHAA (4) can then be converted to artemisinin (1) in three well-established steps. The pharmaceutical company Sanofi developed a commercial route for biosynthetically produced artemisinin in 2014, but this process has now discontinued due to strong market forces. Alternative routes for the low-cost production of artemisinin (1) are therefore urgently required.

Here we report a novel synthetic route to artemisinin (1) starting from the oxygenated farnesyl diphosphate analogue 12-hydroxynorfarneyl diphosphate (6) (Scheme 2). Amorphadiene synthase (ADS) is able to convert 6 in a single step to dihydroartemisinic aldehyde (DHAAl, 7), an advanced intermediate of artemisinin. This route does not proceed via amorpha (3) and therefore avoids several redox steps. Increasing the oxidation state at the linear precursor stage produces a two-step synthesis of 4, which significantly shortens the synthesis of artemisinin (1).

2. Enzymatic reaction

ADS catalyses the Mg 2+ -dependent, highly chemo- and stereoselective cyclisation of FDP (2) to amorpha (3) (Scheme 3). ADS cleaves the C–O bond in 2 and generates diphosphate and a carbocation, which rearranges through a series of ring closures.

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and hydride transfer processes. The last step in the enzymatic sequence is the deprotonation of amorphyl cation (8) to yield amorphadiene (3) (Scheme 3).

The enzyme’s remarkable ability to convert a linear precursor to a structurally and stereochemically complex cyclic product offers a very efficient synthetic route to terpenes. Sesquiterpene synthases are not only highly effective and often stereospecific in the reactions they catalyse, many of them also display some degree of substrate promiscuity and some analogues of FDP (2) can be converted to modified terpenoids.\(^{14}\) As an example, it has been shown that ADS catalyses the cyclisation of 12-hydroxy FDP (6) to produce dihydroartemisinic aldehyde (7) (Scheme 4) with a 34% yield.\(^{12}\) The seemingly moderate yield is common for sesquiterpene cyclases as the reaction is limited by the release of the hydrocarbon products from the aqueous incubation media.\(^{15}\) Aldehyde 7 is a well-established intermediate in the biosynthesis of artemisinin (1).\(^{7}\) In contrast to the three redox steps required to convert (3)
to (7), consisting of an allylic oxidation of amorphadiene to dihydroartemisinic alcohol, further oxidation to the corresponding aldehyde and a final reduction to dihydroartemisinic aldehyde. In our approach yields aldehyde 7 in a single step starting from a linear FDP precursor.

3. Synthesis of 12-hydroxyfarnesyl diphosphate (7)

12-Hydroxyfarnesyl diphosphate (6) was synthesised in three steps starting from commercially available (EE)-farnesol (10) (Scheme 5). Chlorination of 10 gave farnesyl chloride (11) in a quantitative yield, which was carried forward without purification. The following step was a selenium dioxide-catalysed oxidation at C12 of 11. The reaction conditions for the allylic oxidation were optimised, but the yield was still moderate due to the instability of the product 12-hydroxyfarnesyl chloride (12) and the formation of a by-product resulting from the allylic oxidation at C8. Compound 12 was finally diphosphorylated under standard conditions to afford 6.

4. Synthesis of artemisinin

The single step production of 7 provided a great opportunity to shorten the synthesis of artemisinin. The key intermediate DHAA (4) is the starting point for many syntheses developed for artemisinin (1). The conversion of 4 to 1 can be achieved by reaction with singlet oxygen followed by air oxidation. The commercial route developed by Sanofi used engineered yeast to produce arte-misinic acid from the plant Artemisia annua. Our approach exploits the promiscuity of terpene synthases. This new route may have potential to be developed into a low-cost supply of this important antimalarial drug.

5. Conclusion

A novel concise synthetic route to artemisinin (1) was developed. The process benefits from a new chemoenzymatic reaction between amorphadiene synthase and 12-hydroxyfarnesyl diphosphate (6). Due to its relaxed substrate selectivity, ADS accepts the oxygenated FDP analogue 6 to generate dihydroartemisinic aldehyde (7), which can be converted to artemisinin (1) in four steps. Different from any known synthetic route for artemisinin (1), this approach exploits the promiscuity of terpene synthases. Oxidation of FDP prior to cyclisation allows the ADS catalysed formation of a much-advanced intermediate on the pathway to artemisinin. The whole process only utilised one enzyme combined with known chemistry. This new route may have potential to be developed into a low-cost supply of this important antimalarial drug.

6. Experimental section

6.1. General remarks

All chemicals were purchased from Sigma-Aldrich, Acros Chemicals, Fluorochem or Alfa Aesar and used without further purification unless otherwise stated. Anhydrous acetonitrile was obtained from a MBraun SPS800 solvent purification system unless otherwise stated. Anhydrous acetonitrile was obtained from a MBraun SPS800 solvent purification system unless otherwise stated. Anhydrous acetonitrile was obtained from a MBraun SPS800 solvent purification system unless otherwise stated. Anhydrous acetonitrile was obtained from a MBraun SPS800 solvent purification system unless otherwise stated. Anhydrous acetonitrile was obtained from a MBraun SPS800 solvent purification system unless otherwise stated. Anhydrous acetonitrile was obtained from a MBraun SPS800 solvent purification system unless otherwise stated.

6.2. Preparation of ADS

6.2.1. General methods

LB media was prepared by dissolving tryptone (10 g), yeast extract (5 g) purchased from Fluka and NaCl (10 g) in 1 L of deionised water. Cell lysis buffer for ADS was prepared by dissolving trizma-HCl (50 mM), NaCl (500 mM), 2-mercaptoethanol (20 mM) and glycerol (10% v/v) in deionised water. The final pH was adjusted to 8.0. Dialysis buffer for ADS was prepared by dissolving HEPES (25 mM), NaCl (100 mM) and dithiothreitol (1 mM) in deionised water. The final pH was adjusted to 7.5.
6.2.2. Transformation of E. coli BL21 with cDNA for wild-type ADS

Cloning of the ADS gene into pET21d plasmid. The gene coding for amorphanediene synthase (ADS) from *Artetmisia annua* was obtained from gene bank (JF951730). It was supplied in a pTrc99a vector between the Ncol and BamHI restriction sites (pTrc-ADS). pET21d and pTrc-ADS were digested with the endonucleases Ncol and BamHI (0.1 μL of each enzyme, 1 μL of buffer, 10 μL of plasmid, 1 h, 37 °C) and the fragments ligated using T4 DNA ligase (1:2 M ratio of pET: ADS, 0.1 μL enzyme, 2 μL of buffer, H2O to make total volume to 20 μL) to give a new plasmid pET21d-ADS. Supercompetent *E. coli* XL1-blue cells were transformed with 5 μL of ligated DNA and stored on ice (30 min) before being heat shocked in a water bath at 40 °C for 40 s and placed on ice for 2 min. LB medium (1 mL) was added and the solution shaken for 60 min (37 °C, 150 rpm). The cells were harvested by centrifugation (3400g, 1 min) and spread on an agar plate containing ampicillin (100 μg/mL) after resuspending in a minimum amount of buffer. Plates were incubated overnight at 37 °C and then stored at 4 °C. A single colony from the agar plate was used to inoculate 15 mL of LB medium containing ampicillin (100 μg/mL). The culture was incubated at 37 °C and then stored at 4 °C. The cells were harvested by centrifugation (3400 g) (2.0 mL, 8 mmol) was dissolved in anhydrous DMF (11 mL, 8 mmol). The sequence was confirmed by SDS-PAGE electrophoresis. All the fractions with protein were combined and dialyzed in dialysis buffer at 4 °C. The crude was purified by flash chromatography (Biotage SNAP Ultra 10 g, CV = 15 mL, Pet Ether/ EtO, 0% to 10% EtO over 10 CV).

6.3. 12-Hydroxyfarnesyl chloride (12)

SeO2 (226 mg, 2.4 mmol), salicylic acid (331 mg, 2.4 mmol) and tBuOOh (70%, 5.5 mL, 40 mmol) were dissolved in CH2Cl2 (40 mL) and stirred for 30 min at room temperature. The reaction mixture was then cooled to 0 °C and (1 mL, 8 mmol) in CH2Cl2 (20 mL) was added and stirred at 0 °C for 1 h. The reaction was quenched with Na2SO3 (sat.) at 0 °C. The mixture was extracted with EtO (50 mL × 3). The combined organic phases were dried over anhydrous Na2SO4 and concentrated under reduced pressure. The crude oil was purified by flash chromatography on silica gel (CH2Cl2 with 1% NEt3, the eluent used for the column chromatography was cooled on ice before use and fractions were kept on ice. Compound 12 was obtained as a yellow oil (1.02 g, 48% over two steps). 1H NMR (300 MHz, CDCl3); δ (ppm) 5.46–5.40 (1H, m), 5.41–5.36 (1H, m) 5.10 (1H, m), 4.10 (2H, d, J = 8.0), 3.99 (2H, s), 2.16–1.99 (8H, m), 1.73 (3H, s), 1.66 (3H, s), 1.63 (3H, s); 13C NMR (75 MHz, CDCl3); δ (ppm) 142.7, 135.3, 134.7, 125.0, 123.7, 120.3, 69.0, 41.2, 39.4, 39.3, 26.2, 26.1, 16.1, 16.1, 13.7. HRMS (ES+): calcd for C15H20ClO[Na]+: 279.1483; found: 279.1492.

6.3.2. 12-Hydroxyfarnesyl diphtaphosphate (6)

12 (102 mg, 0.4 mmol) and (BuN+)[H][Na]+ (720 mg, 0.8 mmol) were dissolved in anhydrous MeCN (10 mL). The reaction was stirred at room temperature for 17 h. After removing solvent under reduced pressure, the crude oil was loaded onto an ion-exchange resin DOWEX 40-W, which was received from Aldrich in H+ form. The resin was converted into NH4 form by washing with concentrated NH4OH, followed by deionised water until the pH dropped to 7 and finally equilibrated with ion-exchange buffer (25 mM NH4HCO3 containing 2% i-ProOH). The fractions were collected and lyophilized for 18–24 h. The resulting yellow solid was purified by reverse phase column chromatography (Biotage KP-C18-HS 12 g column, CV = 15 mL, H2O/MeCN, 0% to 5% MeCN over 10 CV, 10% to 90% MeCN over 5 CV, 90% MeCN for 5 CV, UV collection 210 nm & 220 nm). The fractions were collected and lyophilized for 18–24 h to give compound 6 as a light white powder (94 mg, 52% yield). M.p. 132–136 °C. 1H NMR (500 MHz, D2O); δ (ppm) 5.41–5.33 (2H, m), 5.15 (1H, t, J = 6.5), 4.40 (2H, t, J = 6.5), 3.88 (2H, s), 2.16–1.96 (8H, m), 1.65 (3H, s), 1.57 (3H, s) and 1.56 (3H, s); 31P NMR (121 MHz, D2O); δ (ppm) –7.93, –7.53. HRMS (ES+): calced for C15H20O2P[H][Na]+: 419.1001, found: 419.0983.

6.4. Synthesis of artemisinin (1)

6.4.1. Dihydroartemisinic acid (aldehydes (7))

Purified ADS (8.8 mL of 90 μL, 2 μm) and 6 (72 mg, 0.4 mmol) were added to incubation buffer (400 mL, pH = 9.4, Glycine/NaOH) containing MgCl2 (190 mg, 5 mM). The mixture was overlaid with pentane (600 mL). The resulting two phased solution was slowly stirred at 4 °C for 2 days. The pentane phase was separated, dried over anhydrous Na2SO4 and concentrated under reduced pressure. The crude was purified by flash chromatography (Biotage SNAP Ultra 10 g, CV = 15 mL, Pet Ether/EtO, 0% to 10% EtO over 10 CV,
10% to 90% Et₂O over 5 CV, 90% Et₂O for 5 CV, UV collection 210 nm & 220 nm). The fractions were collected and concentrated under reduced pressure. Pure compound 7 were obtained as colourless oil (12 mg, 34% yield). Compound 7 was isolated as a mixture of epimers at C11. Ratio of the two epimers were 5:1 with (11S)-7 as the major product. (11S-7): 1H NMR (500 MHz, CDCl₃): δ (ppm) 9.62 (1H, d, J = 3.5), 5.26 (1H, bs), 2.48 (1H, m), 2.39 (1H, m), 1.91–1.25 (11H, m), 1.63 (3H, s), 1.08 (3H, d, J = 7.0), 0.87 (3H, d, J = 6.5). (11S-7): 13C NMR (125 MHz, CDCl₃): δ (ppm) 177.4, 135.0, 120.4, 51.4, 45.1, 41.6, 39.2, 35.4, 30.3, 27.5, 26.5, 25.5, 24.8, 23.8, 19.7, 16.2; (11R-7): 1H NMR (500 MHz, CDCl₃): δ (ppm) 7.56 (1H, s), 4.67 (1H, d, J = 5.4 Hz). HRMS calcd for C₁₇H₁₇O₂ (M⁺) 283.1554, found 283.1559.

References

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1. 10.1016/j.bmc.2013.03.068

14. (a) Cane DE. Chem Rev. 1990;90:1089;
   (g) Faraldos JA, Zhao Y, O’Maille PE, Noel JP, Coates RM. ChemBioChem. 2007;8:1826;
18. (a) Roth RJ. J Nat Prod. 1989;52:1183;
19. (a) Kühnel E, Laffan DDP, Lloyd-Jones GC, Martínez del Campo T, Shepperson IR, Slaughter JL. Angew Chem Int Ed. 2007;46:7075;