Studies on the Biosynthesis of the Fungal Metabolite Oudenone. 2. Synthesis and Enzymatic Cyclization of an \( \alpha \)-Diketone, Open-Chain Precursor into Oudenone in Cultures of Oudemansiella radicata

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The \( \alpha \)-diketone 4 was shown to be the open-chain biosynthetic precursor of the fungal metabolite oudenone (1a and 1b). Intact incorporation of 4 into 1 was achieved upon incubation of a \( ^{1}H \)-labeled, N-acetylcycteamine thioester derivative of 4 with growing cultures of Oudemansiella radicata. A biosynthetic scheme for the formation of the hexaketide 4 and its enzymatic cyclization into oudenone (1), consistent with the experimental data, is described. The proposed mechanism for the cyclization of 4 to 1 is analogous to the “polyepoxide cascade” model, which has been previously implicated in the biosynthesis of polyether antibiotics.

Introduction

Over the last 10 years, polyketide synthase (PKS) enzymes associated with the biosynthesis of macrolides (bacterial PKS Type I; e.g., erythromycin A,† niddamyacin,‡ FR-008,§ rapamycin,¶ FK506), and aromatic polyketides (bacterial PKS Type II; e.g., tetracyclic PKS C,‖ actinorhodin,¶ frenolycin,¶ and fungal PKS Type I; e.g., aflatoxin§§) have been identified and studied extensively. Furthermore, major advances in the field of microbial genetics have illuminated our knowledge and ability to manipulate the gene clusters encoding for these enzymes.‖

Although it is generally believed that the biosynthetic steps associated with the skeletal construction of polyketides are catalyzed by modular, multifunctional PKS type I enzymes analogous to those known in the cases of macrolides,†–§ little is yet reported on the specific enzymes and genetics associated with this family of natural products.

An idea that was initially proposed by Westley for the biogenesis of lasalocid,‖ and later modified by Cane, Celmer, and Westley as a unified hypothesis for the biosynthesis of all polyether polyketides [e.g., monesin A (§§)], is the “polyepoxide cascade” mechanism (Scheme 1, path A).12 In this three-step process, the open-chain oligo ketide precursor 9 is formed from acetate, propionate, and butyrate via repeated decarboxylative Claisen condensations and subsequent modifications of the \( \gamma, \beta \)-keto group; all of these steps are believed to be catalyzed by the multifunctional PKS enzyme(s). The second step was proposed to be an enzymatic polyepoxidation reaction of the acyclic hydroxypropene precursor 9, followed by a cascade of intramolecular nucleophilic attacks on the keto and epoxide moieties of intermediate 10, eventually leading to the formation of the tetrahydrofuran and tetrahydropyran rings of the final product 8 (Scheme 1, path A).

More recently, the alternative mechanism of “syn-oxidative polycyclization” of the hydroxypropene precursor 9 was proposed by Townsend, Basak, and McDonald (Scheme 1, path B).13 In this later model, an alkoxide-bound oxo metal derivative 11 was proposed to undergo a series of consecutive intramolecular [2 + 2] cycloadditions with the double bonds to give the corresponding metallo-oxetene intermediate 12, followed by reductive elimina-

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tion of the metal and closure of the tetrahydrofuran or tetrahydropyran ring (Scheme 1, Path B).

In addition to the polyether antibiotics, both of these elegant models may also be relevant in the biogenesis of some marine natural products that share common structural features with the polyether antibiotics. Among these metabolites, the best known examples are the marine toxins typified by brevetoxin A (13). Nonetheless, to date both of these proposals remain unproven, mainly due to the chemical complexity and instability of the precursors and intermediates associated with the biosynthesis of such structurally complex molecules as monensin A (8) and brevetoxin A (13).

Results and Discussion

In our initial studies on the biosynthesis of oudenone (1), we showed that its carbon skeleton is derived from six acetate units, and we proposed that the \( \alpha \)-diketone 4 was the most likely open-chain hexaketide intermediate (Scheme 2).\(^{14}\) To test this hypothesis and further probe the mechanism of cyclization, the synthesis of the deuterium labeled 4 as its \( \text{N-acetylcysteamine (NAC) thioester derivative 20} \) was undertaken (Scheme 3).

In this paper, we report the progress of our ongoing investigations into the biosynthesis and enzymology of polyether-type microbial metabolites. Recently, we reported our studies on the biosynthesis of the fungal metabolite oudenone (1a and 1b),\(^{14}\) a structurally unique hexaketide characterized by a tetrahydrofuran and a 1,3-cyclopentadione moiety (1a).\(^{15}\) We speculated that due to the small molecular structure of 1, this metabolite could serve as a model for our exploration into the general biogenesis of tetrahydrofuranyl and tetrahydropyranyl moieties of polyether-type polyketides. In our previous report, we showed that the N-acetylcysteine thioester derivative of (SS)-5-hydroxyoctanoic acid (2) can serve as a substrate of the PKS enzyme(s) catalyzing the biosynthesis of 1 in cultures of Oudemansiella radicata. Furthermore, the enzymatic incorporation of 2 into the tetrahydrofuran moiety of 1 was shown to proceed without any change in the absolute stereochemistry of the C9 chiral center. On the basis of these results, plausible hexaketide open-chain precursors and cyclization mechanisms analogous to the "polyepoxide cascade" and the "oxidative polycyclization" models were proposed.\(^{14}\) In this paper, we present the synthesis and in vivo transformation of the \( \alpha \)-diketone 4 into oudenone (1), strongly suggesting that the cyclization mechanism leading to the formation of 1 is very likely analogous to the "polyepoxide cascade" model (Scheme 2).


\(^{(15)}\) The structure of oudenone in anhydrous organic solvents is that of 1a; however, in aqueous solvents 1a is in dynamic equilibrium with the \( \beta \)-trione anion 1b, via the simple addition of \( \text{H}_2\text{O} \) (pH 4.1).
the DIBAL-H reduction of the corresponding lactone 14,16 the preparation of 14 from commercially available ethyl-2-oxocyclopentanecarboxylate was described previously.14 Lactol 15 was subsequently reacted with 1,3-propanedithiol in the presence of BF$_3$-etherate in order to protect the masked aldehyde,17 and the 4-hydroxy group of the crude dithiane 16 was converted to the tert-butyldimethylsilyl ether 17 in greater than 90% overall yield (Scheme 3).

Deprotonation of compound 17 (at C1) was carried out with the careful addition of 1 equiv of n-butyllithium at −78 to −20 °C.18 To avoid the addition of excess base, extra care was taken to introduce the solution of n-butyllithium into the reaction mixture in small aliquots. The progress of the reaction was monitored by the $^1$H NMR of the crude reaction mixture after quenching with D$_2$O. Once the deprotonation was complete, the anionic solution of 17 was transferred through a precooled cannula at −78 °C to an anhydrous solution of succinic anhydride (or $[2,3-^2$H$_4$]succinic anhydride) at the same temperature. Both the deuterium-labeled and unlabeled compounds 18 were prepared using the same protocol and fully characterized by $^1$H, COSY, DEPT, HETCOR, $^{13}$C and $^2$H NMR, and MS. The yield of this condensation ranged between 35 and 55%, based on the isolated pure compound 18 ($>$80% based on the recovery of unreacted compound 17, however, the unreacted $[2,3-^2$H$_4$]succinic anhydride could not be recovered). It is worth mentioning that any attempt to remove the 1,3-dithiane protecting group of 18 using standard literature procedures led primarily to decomposition of the starting material and the isolation of the TBDMS silyl ether of 4,5-dioxo-9-
hydroxydodecanoic acid in less than 15% yield. Thus, we coupled the protected carboxylic acid 18 with N-acetyl-
cysteamine in the presence of DCC and DMAP to obtain the thioester 19 in 50% yield after chromatography.

Finally, the removal of both the 1,3-dithiane and the silyl ether protecting groups were achieved in one step with [bis(trifluoroacetoxy)iodo]benzene following Stork's methodology.19 The final product 20 (20a and 20b) was isolated in 40% yield after chromatography and, not surprisingly, was found to be very chemically unstable. Therefore, feeding experiments had to be timed carefully so that the labeled final product, $[2,3-^2$H$_4$]α-diketone NAC thioster 20, could be used as soon as it was prepared.

The production ofoudenone (1) in growing cultures of O. radicata, as well as the appropriate timing for the feeding of labeled 20, were monitored by the changes in UV absorption of the liquid culture, as described previously.14 Initially, unlabeled 20 was fed to cultures of O. radicata at various concentrations in order to establish the maximum amount tolerated by the culture without substantially inhibiting the production ofoudenone (1). Pulse feeding of $[2,3-^2$H$_4$]α-diketone NAC thioster 20 to three cultures of O. radicata, in the presence of the $\beta$-oxidase inhibitor 3-(tetradecylthio)propanoic acid,20 led to the isolation of metabolite 1, labeled with deuterium at C2/C3 as predicted (Figure 1). All three samples of labeled oudonone (1) were purified by semipreparative C18 reversed-phase HPLC and analyzed by analytical HPLC and NMR.

In one of the feeding experiments, deuterium incorporation (albeit with very low % incorporation) was ob-

C10 than at C12. Therefore, these results strongly suggest that the cyclization of oudenone (1) proceeds via an intramolecular β-addition, followed by a Claisen-type intramolecular condensation and dehydration to give 1 (Scheme 2). This type of mechanism bears many similarities to the "polyepoxide cascade" model, as well as the catalytic mechanism leading to the formation of the 2-amino-3-hydroxy-cyclopent-2-enone structural unit. 22

Experimental Section

General Procedures. NMR spectra were obtained at 20–22 °C, 1H, 2H, and 13C NMR chemical shifts are given in ppm and are referenced to the internal deuterated solvent (D2O NMR spectra were recorded in CDCl3). All reactions carried out under anhydrous conditions were performed under a nitrogen atmosphere using oven-dried syringes and glassware. THF was distilled from sodium-benzophenone ketyl, CHCl3 was distilled from P2O5, MeOH was distilled from Mg turnings, toluene was distilled from CaH2, and DMF was distilled from CaO. Reagents and solvents were purchased from Aldrich Chemical Co. and VWR Scientific of Canada, respectively. Isotopically labeled reagents were purchased from Cambridge Isotope Laboratories. Stock cultures of O. radiata ATCC 20295 were maintained on PDY (ATCC medium No 337) with 1.5% agar in slant tubes at 4 °C. All culture media and glassware were autoclaved prior to use, and all biological manipulations were conducted in a sterile hood. O. radiata cultures were grown at 26.5 °C and 140 rpm, in a rotary shaker. Flash column chromatography was carried out on Merck Kieselgel 60, 230–400 mesh. Reversed-phase flash column chromatography was carried out on silica gel reacted with n-octadecyltrichlorosilane, following previously reported procedures. 26

Fermentation of O. radiata ATCC 20295, Precursor Feeding, and Isolation of Oudenone. Cultures of O. radiata were grown on both solid and liquid media as previously described. 26 Oudenone (1) production, before and after feeding of labeled precursors, was monitored by UV. 24 In all cases, 2H-labeled compound 40 was dissolved in absolute ethanol (~1.5 mg sample/100 μL of ethanol) and administered in 100 μL aliquots, every 6–12 h (3x/day) for a period of 48 h, to growing cultures of O. radiata (100 mL of fermentation broth). Between feedings, the ethanolic solution of compound 40 was stored at ~85 °C to prevent decomposition. At the same time, the β-oxidation inhibitor 3-(tetradecylthio)propanoic acid (1.5 mg in 30 μL of ethanol) was also administered in order to suppress degradation of compound 40. 20 All cultures were grown in slant tubes at 4 °C at pH 7.0–7.5

Figure 1. (a) 46.6 MHz 1H NMR spectrum of oudeanone (1a) derived from the intact incorporation of 2H-labeled compound 40, feeding experiment I. (b) 46.6 MHz 1H NMR spectrum of oudeanone (1a) derived from the feeding of 2H-labeled compound 40 to cultures of O. radiata; experiments II and III. (c) 300 MHz 1H NMR spectrum of oudeanone (1a).

Figure 1. (a) 46.6 MHz 1H NMR spectrum of oudeanone (1a) derived from the intact incorporation of 2H-labeled compound 40, feeding experiment I. (b) 46.6 MHz 1H NMR spectrum of oudeanone (1a) derived from the feeding of 2H-labeled compound 40 to cultures of O. radiata; experiments II and III. (c) 300 MHz 1H NMR spectrum of oudeanone (1a).
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previously. All final samples of 2H-labeled oudone (obtained in 90% yield and in high enough purity to be used as a source of the final product) were further purified by reversed-phase HPLC to ensure greater than 99% purity before they were analyzed by 1H NMR.

3-Propyl-4-valerolactone (15). An anhydrous solution of 3-propyl-4-valerolactone (14) (12.51 g, 58.06 mmol) in THF was cooled to 0 °C and stirred for ~30 min before a solution of Dibal-H (176.2 mL of 1 M solution in anhydrous THF, 2 equiv) was added over a period of 30 min via a dropping funnel and under nitrogen. The reaction mixture was quenched at ~70 °C for 1 h and then at ~20 °C for an additional period of 5 h. The reaction was quenched by pouring the solution (slowly) into a mixture of 10 g of ice and 10 mL of acetic acid. The mixture was then extracted with diethyl ether (3 × 200 mL). The organic layers were combined, washed with brine (150 mL), saturated aqueous NaOH (15 mL) and then dried (anhydrous MgSO4), the solvents were evaporated to obtain the crude product as an oil. The pure lactol 17 (7.998 g) was isolated in 63% yield after purification by flash column chromatography, using a solvent mixture of hexanes–EtOAc (linear gradient from 15:1 to 10:1).

TLC (silica, hexane/EtOAc (4:1)): Rf = 0.22.

1H NMR (mixture of lactol conformers, CDCl3, 270 MHz): 0.80 (t, J = 6.1 Hz, 3H), 1.15–1.48 (m, 10H (several overlapping signals)), 2.64–2.67 (m, 4H, 3.55 (dd, J = 4.4, 2.0 Hz, 1H)), 4.00 (t, J = 6.1 Hz, 1H).

13C NMR (CDCl3, 75 MHz): 12.7, 18.2, 18.5, 22.6, 30.1, 35.5, 37.0, 39.7, 47.6, 71.5.

HRFABMS (glycerol at rt) m/z: 429.225157, calcd mass for C21H25O5SiH+ = 429.225157.

To a cooled (78 °C) solution of 17 (3.72 g, 10.7 mmol) in anhydrous THF (10 mL) was added a solution of n-butylthiium (1.6 M solution in hexanes, 16.0 mmol, 1.5 equiv), followed by tetramethylethylenediamine (2.4 mL, 1.86 g, 16 mmol, 1.5 equiv). The reaction mixture was allowed to warm to ~20 °C and stirred at that temperature for ~1 h. An aliquot of the reaction mixture was quenched with D2O and analyzed by 1H NMR to confirm complete deprotonation of the starting material before continuing with the addition of the electrophile. Succinic anhydride (1.6 g, 16.0 mmol, 1.5 equiv) was dissolved in anhydrous THF (18 mL) and cooled to ~78 °C. The anionic solution of 17 was then cooled to ~78 °C before it was transferred through a precooled cannula to the solution of deuterated succinic anhydride. Once again, the mixture was allowed to warm to ~20 °C and stirred at that temperature for an additional 5 h. Finally, the reaction was quenched with the addition of acidified water (50 mL, pH ~3). The crude product was extracted into EtOAc (3 × 150 mL), the organic layers were dried over MgSO4, and the EtOAc was evaporated to dryness. The pure lauroic acid derivative 18 was isolated after flash column chromatography (2.65 g, 82% yield based on the recovery of unreacted starting material) using a mixture of hexane/EtOAc (25:1) containing 0.3% acetic acid as the eluent.

TLC (silica, hexane/EtOAc (1:1) plus 0.3% AcOH): Rf = 0.37.

1H NMR (CDCl3, 300 MHz): δ = 0.01 (s, 6H), 0.85 (overlapping signals and t, 12H), 1.21–1.55 (m, 8H), 1.70–2.10 (m, 4H), 2.59–2.64 (m, 4H), 2.92–3.07 (m, 4H, 3.55 (m, 1H).

13C NMR (CDCl3, 75 MHz) δ = 4.4–4.6, 14.2, 18.0, 18.6, 20.1, 24.8, 25.9, 27.8, 28.6 30.7, 37.3, 39.0, 39.2, 60.8, 71.6, 176.8, 184.2, 197.2.

HRFABMS (glycerol at rt) m/z: 449.22158, calcd mass for C21H25O5SiH+ = 449.221557.

[2,3,4,5,6-2H5]-20-oxo-20-siropentadecanoic Acid (18). The deuterium-labeled analogue 18 was obtained following the same synthetic procedure as described for the unlabeled compound 18, with the exception that the anion of 17 was coupled with [2,3,4,5,6-2H5]succinic anhydride.

1H NMR (CDCl3, 300 MHz): δ = 0.01 (s, 6H), 0.85 (overlapping signals and t, 12H), 1.21–1.55 (m, 8H), 1.70–2.10 (m, 4H, 2.66 (dt, J = 3.6, 1.2 Hz, 2H), 2.97–3.07 (m, 2H), 3.55 (m, 1H).

13C NMR (CDCl3, 75 MHz) δ = 3.06 (s, 2H) ~1:1 ratio.

1C NMR (CDCl3, 75 MHz) δ = 4.3–4.4, 14.2, 18.3, 18.8, 20.3, 24.9, 26.1, 27.9, (resonance at ~28.6 could not be clearly observed), 37.5, 39.1, 39.4, 60.9, 71.8, 178.3, 202.8.

HRFABMS (glycerol at rt) m/z: 453.24664, calcd mass for C21H25O5SiH+ = 453.246646.

N-(4-Benzylthiobutylidimethylsilyloxy)-5-(1,3-dithiane)-4-oxo-Nacetyl-N-acetylcysteamine (19). A solution of 18 (134 mg, 0.3 mmol) in anhydrous CH2Cl2 (4 mL) was cooled to 0 °C under nitrogen. 1,3-Dicyclohexylcarbodiimide (1 mL, 86 mg, 0.42 mmol, 1.4 equiv) and 4-(dimethylaminopyridine (73 mg, 0.6 mmol, 1.4 equiv), dissolved in 7 mL of anhydrous CH2Cl2) were added, followed by a solution of N-acetyl-L-cysteine (142 mg, 1.2 mmol, 0.42 equiv, dissolved in 7 mL of anhydrous CH2Cl2) was added. The reaction mixture was stirred at 0 °C for 30 min before it was allowed to warm to rt and stirred for an additional 2 h. The precipitated urea was subsequently removed by filtration, the solid material was rinsed with EtOAc, and the combined organic solvents were evaporated to dryness. The crude residue was chromatographed on silica gel using EtOAc/hexane (1:1) as the eluent providing pure NAC-derivative 19 (128 mg) in 78% yield.

TLC (silica, EtOAc): Rf = 0.17.

1H NMR (CDCl3, 300 MHz): δ = 0.01 (s, 6H), 0.85 (overlapping signals and t, 12H), 1.2–1.5 (m, 8H), 1.7–2.1 (m, 4H), 1.97 (s, 3H), 2.6 (dt, J = 3.6, 1.2 Hz, 2H), 2.84 (t, J = 5.2 Hz, 2H, H3), 2.92 (d, J = 9.2 Hz, 2H), 3.04 (t, J = 6.0 Hz, 2H, H2), 3.08 (t, J = 5.2 Hz, 2H, H3), 3.38 (q, J = 5.7 Hz, 2H), 3.58 (m, 1H), 5.95 (bs, NH).

1C NMR (CDCl3, 75 MHz) δ = 4.3–4.5, 14.2, 18.3, 18.8, 20.3, 23.4, 24.9, 26.1, 27.9, 28.8, 31.4 (C2), 37.4, 38.5 (C3), 39.1, 39.4, 39.7, 61.0, 71.8, 170.5, 198.6, 202.7.

HRFABMS (glycerol at rt) m/z: 550.25174, calcd mass for C15H24N2O4SiH+ = 550.251478.

(27) 1H and 13C resonances at C2 and C3 were assigned from the combined 1H, 13C, COSY, HETCOR, and DEPT NMR data of the unlabeled and labeled compound.
[2,3-2H4]-9-(tert-Butyldimethylsilyloxy)-5-(1,3-dithiane)-4-oxo lauryl NAC Thioester (19). [2,3-2H4]-9-(tert-Butyldimethylsilyloxy)-5-(1,3-dithiane)-4-oxoa uric acid (18) was converted to the deuterium-labeled NAS-derivative 19 following the same synthetic procedure as described for the unlabeled compound 19.

1H NMR (CDCl3, 300 MHz) δ: -0.01 (s, 6H), 0.85 (overlapping s and t, 12H), 1.2-1.5 (m, 8H), 1.7-2.1 (m, 4H), 1.97 (s, 3H), 2.66 (dt, J = 3.6, 1.2 Hz, 2H), 2.92 (d, J = 12.6 Hz, 2H), 3.04 (t, J = 6.3 Hz, 2H), 3.38 (q, J = 6.3 Hz, 2H), 3.58 (m, 1H), 5.95 (s, NH).

2H NMR (CDCl3, 46.6 MHz) δ: 2.87 (s), 3.14 (s) 1:1 ratio.

13C NMR (CDCl3, 75 MHz) δ: -4.3, -4.2, 14.5, 18.3, 18.8, 20.3, 23.4, 24.9, 26.1, 27.9, 28.8, 31.4 (m, C2), 37.4, (resonance at δ 38.5 could not be clearly observed, C3), 39.1, 39.4, 39.7, 61.0, 71.8, 170.5, 198.6, 202.7.

HRFAB+ MS (glycerol at rt) m/z: 554.27674, calcd mass for C25H43NO4S3SiD4+H+ 554.276585.

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Supporting Information Available: Copies of 1H, 2H, 13C, COSY, DEPT, and HETCOR NMR data for key synthetic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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