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Application of combinatorial biocatalysis for a unique ring expansion of dihydroxymethylzearalenone

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ABSTRACT

Combinatorial biocatalysis was applied to generate a diverse set of dihydroxymethylzearalenone analogs with modified ring structure. In one representative chemoenzymatic reaction sequence, dihydroxymethylzearalenone was first subjected to a unique enzyme-catalyzed oxidative ring opening reaction that creates two new carboxylic groups on the molecule. These groups served as reaction sites for further derivatization involving biocatalytic ring closure reactions with structurally diverse bifunctional reagents, including different diols and diamines. As a result, a library of cyclic bislactones and bislactams was created, with modified ring structures covering chemical space and structure activity relationships unattainable by conventional synthetic means.

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Zearalenone[1] and hypothemycin[2,3] are closely related resorcylic acid 14-membered lactones. They possess a variety of activities including antifungal activity.[3] Two related compounds, dihydroxymethylzearalenone (L-783279) and a dehydro compound (L-783277) were recently isolated from various *Phoma* spp. by bioassay guided using a kinase screen.[4,5] L-783277 is a potent and irreversible inhibitor of MEK (MAP kinase, a threonine/tyrosine specific kinase) (IC_{50} 4 nM) and a slightly weaker inhibitor of Lck kinase (IC_{50} 750 nM).[5] L-783279 is a fourfold better and reversible inhibitor of Lck kinase (IC_{50} 200 nM). The overall better activity profile of L-783279 prompted us to investigate this compound for Lck SAR using a combinatorial biocatalysis approach.

Combinatorial biocatalysis is a high throughput synthetic biocatalytic approach particularly suited to optimizing potentially labile, complex, or polyfunctional leads. This divergent strategy can be a powerful complement for classical lead discovery approaches, enabling access to unique derivatives and structure activity relationships.[6] The combination of multi-step biocatalytic reactions with complementary chemical reactions over multiple iterative rounds of derivatization results in divergent 'branches', each originating from key biocatalytic modifications on different regions of a lead optimization candidate.

As one branch of a combinatorial biocatalysis program aiming to expand chemistry space and generating lead candidates with improved properties, dihydroxymethylzearalenone **1** was subjected to a series of consecutive enzymatic and chemical derivatization steps. A key derivatization strategy utilized a mild enzymatic oxidative ring opening to enable a chemoenzymatic cascade, as outlined in Figure 1. In the first step of this branch of the derivatization process, dihydroxymethylzearalenone was treated with lipase from *Candida antarctica* (Biocatalytics) in a reaction medium composed predominantly of ethyl acetate and containing hydrogen peroxide. Under these reaction conditions, the enzyme catalyzes a perhydrolysis reaction between hydrogen peroxide and ethyl acetate under mild conditions, resulting from the continuous, in situ formation of peroxyacetic acid.[7] This oxidative reagent reacted in situ with **1** to generate the diacid derivative **2** with an opened ring system, as identified by NMR analysis. The enzyme-catalyzed perhydrolysis reaction offered clear advantages over chemical oxidation with peroxyacetic acid,[7a] resulting in a significantly cleaner reaction with less by-products and improved yields.

In a typical reaction, 1 g of **1** was dissolved in 3 mL of DMF. This solution was added to 150 mL of ethyl acetate containing 7.5 g of the enzyme. The reaction was initiated by addition of 2 mL H_2O_2 (30% in water). The reaction mixture was incubated in an orbital shaker (225 rpm) at 29 °C. Periodically, samples were removed and analyzed by LC/MS.[8] An additional 1 mL portion of H_2O_2 was added during the course of the reaction to further drive the

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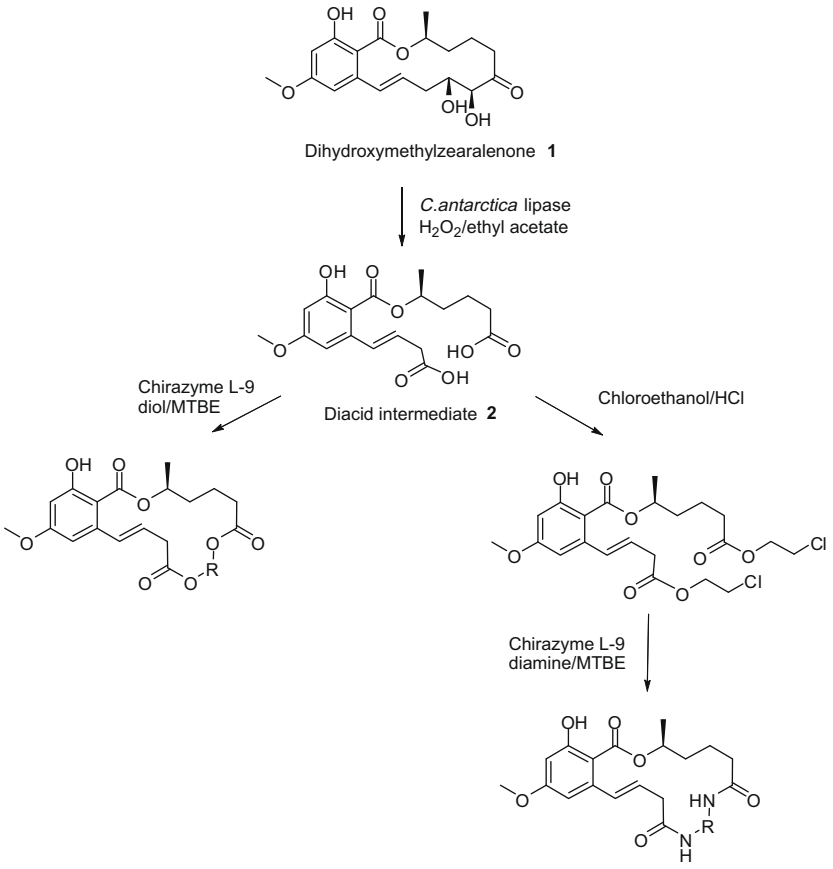


Figure 1. Synthetic strategy for chemoenzymatic preparation of the library of dihydroxymethylzearalenone analogs with expanded ring system.

conversion to the desired product. After 30 h incubation, the reaction was stopped by removal of the enzyme by filtration, and filtered enzyme was washed consecutively with 3×20 mL of acetonitrile and 20 mL methanol. All solutions were pooled and concentrated under vacuum. Preparative HPLC of the dried reaction mixture gave 366 mg (37% yield) of the diacid product.[9a] ^{1}H NMR analysis of the diacid product was consistent with the proposed structure.[10]

The diacid intermediate **2** was used as a branching point for creating a diverse library of dihydroxymethylzearalenone analogs with an expanded ring system using the combinatorial biocatalysis strategy summarized in Figure 1. The essential design of this chemoenzymatic strategy involved cyclization of **2** by means of enzyme-catalyzed esterification or amidation of the diacid intermediates with a variety of bifunctional linking reagents, including diols and diamines to produce corresponding bislactones and bislactams.

In order to implement the lactonization route, thirteen commercially available lipases were screened for their ability to cyclize the diacid intermediate **2** using 1,6-hexanediol as a nucleophile. The reaction involved dissolving 2 mg of **2** (2 mM) in dry *tert*-butyl methyl ether (MTBE) containing 50 mM 1,6-hexanediol in a 4 mL screw cap vial. The dry enzyme powder was added and the reaction mixture was incubated at 45 °C under shaking at 250 rpm. Periodically, samples were removed and analyzed by LC/MS.[8] After 72 h incubation, Chirazyme L-9 (immobilized lipase from *Mucor miehei,* Biocatalytics) showed the highest conversion (50%) of the starting diacid to bislactone. This enzyme was used for the synthesis of a library of bislactones from **2** and a range of structurally diverse diols.

In a typical synthetic reaction, 2 mM of **2** was dissolved in dry MTBE containing 50 mM corresponding diol, in a total volume of 20 mL. Chirazyme L-9 and anhydrous sodium sulfate (200 mg each) were added to initiate the reaction, and reaction mixture was incubated at 45 °C under shaking at 250 rpm. After 120 h incubation, the reaction was terminated by removing the enzyme by filtration. The filtrate was dried under vacuum and the reaction products were isolated and purified by preparative HPLC.[9b]

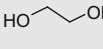
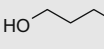
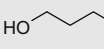
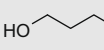
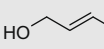
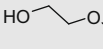
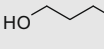
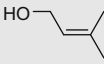
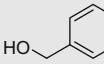
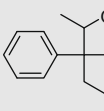
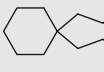
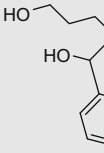
Structures of synthesized cyclic bislactones and corresponding reaction yields are shown in Table 1. In most cases, the corresponding diester derivatives were also generated by separate esterification of both carboxylic groups in **2** with corresponding diols, with yields ranging approximately from 5% to 55%. Structures of all purified bislactones were confirmed by NMR.

Screening of lipases to identify biocatalysts capable of the direct synthesis of cyclic bislactams starting from the diacid intermediate **2** and diamines was unsuccessful. None of the tested enzymes were able to catalyze amide bond formation between free carboxylic groups and amines. Therefore, an alternative chemoenzymatic strategy was implemented, involving chemical esterification of carboxylic groups with chloroethanol followed by biocatalytic amide bond formation between the activated ester groups with diamines (Fig. 1).

For the synthesis of esterified diacid intermediate, 100 mg of **2** was dissolved in 10 mL of chloroethanol and HCl gas was bubbled through the solution for 30 min at room temperature, followed by evaporation of solvent under reduced pressure. LC/MS analysis of the product showed essentially complete conversion of **2** to the diester, with trace amounts of monoester.[8] The diester intermediate was used in the subsequent enzyme-catalyzed cyclic bislactam formation without additional purification.

In a typical enzymatic synthesis of cyclic bislactams, 1 mM of esterified diacid intermediate was dissolved in dry MTBE containing 2 mM corresponding diamine, in a total volume of 80 mL.

Table 1
Cyclic bislactone analogs of dihydroxymethylzearalenone

Compound ID	Diol	Yield (%)
3		72
4		70
5		70
6		65
7		45
8		40
9		44
10		48
11		20
12[a]		48
13		55
14[a]		90

[a] Esterification occurred at the primary hydroxyls.

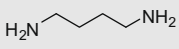
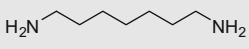
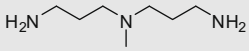
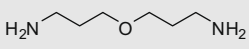
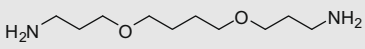
Chirazyme L-9 (0.5 g) and anhydrous sodium sulfate (6 g) were added to initiate the reaction, and reaction mixture was incubated at 60 °C under shaking at 250 rpm. After 72 h incubation, the reaction was terminated by removing the enzyme by filtration. The filtrate was dried under vacuum and the reaction products were isolated and purified by preparative HPLC.[9c]

Structures of synthesized cyclic bislactams and corresponding reaction yields are shown in Table 2. In most cases, the corresponding amide (either mono- or di-) derivatives were also generated by amidation of one or both carboxylic groups in **2** with corresponding diamines, with yields ranging approximately from 10 to 25%. Structures of all purified bislactams were confirmed by NMR.[10]

All compounds in Tables 1 and 2 (**3**-**19**) were evaluated as inhibitors of Lck activity. None of these compounds showed Lck inhibitory activity at 10 μM suggesting that the presence of the 14-membered macrocyclic lactone is critically important for inhibition of the kinase activity.

In summary, a combinatorial chemoenzymatic strategy was applied to generate a diverse set of dihydroxymethylzearalenone analogs with modified ring structure. The reaction cascade was initiated with a unique enzyme-catalyzed oxidative ring opening reaction of the parent compound, resulting in generation of new reactive sites on the molecule amenable to further derivatization via a combination of chemical and enzymatic steps. This general biocatalytic strategy is applicable to a wide variety of pharmaceutical compounds with complex structures that may be difficult to approach using conventional synthetic methods, thus allowing expansion into otherwise inaccessible chemical space.

Table 2
Cyclic bislactam analogs of dihydroxymethylzearalenone

Compound ID	Diol	Yield (%)
15		46
16		23
17		57
18		74
19		83

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8. LC/MS analysis was performed on an Applied Biosystems MDS SCIEX API 2000 system operated in ESI positive mode. Chromatography was accomplished using a Luna C8 (Phenomenex) column (50 × 2 mm, 5 μm) with the mobile phase initially composed of 80% water and 20% acetonitrile (both containing 0.4% acetic acid) at a flow rate of 1.0 mL/min. After an initial isocratic hold for 0.5 min, concentration of acetonitrile was increased linearly to 100% in 3 min, followed by isocratic hold for 0.5 min.
9. (a) Purifications were performed on a Shimadzu LC8A preparative HPLC system. Columbus C18 (Phenomenex) column (21 × 60 mm, 5 μm) with the mobile phase initially composed of 67% water and 33% acetonitrile at a flow rate of 20 mL/min. After an initial isocratic hold for 16 min, concentration of acetonitrile was increased linearly to 100% in 3 min, followed by isocratic hold for 4 min.; (b) SymmetryPrep C8 (Waters) column (40 × 200 mm, 7 μm), with the mobile phase initially composed of 60% water and 40% acetonitrile at a flow rate of 40 mL/min, with linear gradient to 100% acetonitrile over 30 min, followed by isocratic hold for 3 min, with UV detection at 230 nm.; (c) Zorbax RX-C8 (Agilent) column (21 × 250 mm, 7 μm), with the mobile phase initially composed of 80% water and 20% acetonitrile (both containing 0.1% trifluoroacetic acid) at a flow rate of 15 mL/min, with linear gradient to 100% acetonitrile over 40 min, followed by isocratic hold for 3 min, with UV detection at 230 nm.
10. All compounds were fully characterized by 1D and where necessary by 2D NMR analysis. The compounds showed expected NMR spectra. Data for representative compounds are listed here. Compound **5**: ^{1}H NMR 500 MHz (CD_3OD–$CDCl_3$, 1:1) δ 7.06 (1H, d, J = 15.5 Hz), 6.46 (1H, d, J = 2.5 Hz), 6.37 (1H, d, J = 2.5 Hz), 5.93 (1H, dt, J = 15.5, 8.0 Hz), 5.16 (1H, m), 4.16 (4H, m), 3.87 (3H, s), 3.31 (1H, m), 3.24 (1H, dd, 15.5, 7.5 Hz), 2.37 (2H, m), 1.77 (2H, m), 1.66 (6H,

m), 1.44 (4H, m), 1.34 (3H, d, J = 6.5 Hz). Compound **6**: ^{1}H NMR 500 MHz (CD_3OD–$CDCl_3$, 1:1) δ 7.04 (1H, d, J = 15.5 Hz), 6.46 (1H, d, J = 2.5 Hz), 6.37 (1H, d, J = 2.5 Hz), 5.93 (1H, dt, J = 15.5, 7.0 Hz), 5.17 (1H, m), 4.16 (2H, m), 4.10 (2H, m), 3.81 (3H, s), 3.28 (1H, ddd, J = 17, 7.5, 1.5 Hz), 3.20 (1H, ddd, J = 17, 7.5, 1.5 Hz), 2.36 (2H, t, J = 7.5 Hz), 1.78–1.60 (8 H, m), 1.37 (8H, m), 1.34 (3H, d, J = 6.5 Hz). Compound **13**: ^{1}H NMR 500 MHz (CD_3OD–$CDCl_3$, 1:1) δ 7.01 (1H, d, J = 15.5 Hz), 6.44 (1H, d, J = 2.5 Hz), 6.34 (1H, d, J = 2.5 Hz), 5.92 (1H, ddd, J = 15.5, 8.0, 1.5 Hz), 5.13 (1H, m), 4.16 (4H, m), 3.77 (3H, s), 3.23 (1H, m), 3.16 (1H, m), 2.30 (2H, m), 1.67 (9H, m), 1.37 (9H, m), 1.30 (3H, d, J = 6.5 Hz). Compound **15**: ^{1}H NMR 500 MHz (CD_3OD–$CDCl_3$, 1:1) δ 7.01 (1H, d, J = 15.5 Hz), 6.47 (1H, d, J = 2.5 Hz), 6.38 (1H, d, J = 2.5 Hz), 6.03 (1H, dt, J = 15.5, 8.0 Hz), 5.22 (1H, m), 3.80 (3H, s), 3.23 (2H, m), 3.14 (3H, m), 3.04 (1H, ddd, J = 15.5, 8.0, 1.0 Hz), 2.20 (2H, m), 1.70 (3H, m), 1.58–1.51 (5H, m), 1.34 (3H, d, J = 6.0 Hz).