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ORIGINAL ARTICLE

Alkyldihydropyrones, new polyketides synthesized by a type III polyketide synthase from *Streptomyces reveromyceticus*

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Genome sequencing allows a rapid and efficient identification of novel catalysts that produce novel secondary metabolites. Here we describe the catalytic properties of dihydropyrone synthase A (DpyA), a novel type III polyketide synthase encoded in a linear plasmid of *Streptomyces reveromyceticus*. Heterologous expression of *dpyA* led to the accumulation of alkyldihydropyrones A (1), B (2), C (3) and D (4), which are novel dihydropyran compounds that exhibit weak cytotoxicity against the leukemia cell line HL-60. DpyA catalyzes the condensation of β-hydroxyl acid thioester and methylmalonyl-CoA to yield a triketide intermediate that then undergoes lactonization of a secondary alcohol and a thioester to give alkyldihydropyrone.
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INTRODUCTION

Type III polyketide synthase (PKS), which is widely distributed among higher plants, fungi and bacteria, catalyzes the assembly of primary metabolites such as acyl-CoA compounds to synthesize structurally complex polyketides.[1] The reaction catalyzed by type III PKS starts with the formation of a thioester bond between the catalytic center cysteine and an acyl group derived from a starter substrate. Decarboxylative condensation of an extender substrate toward a thioester linkage between the acyl group and the catalytic center cysteine extends the growing polyketide chain. With a few exceptions such as diketide synthase, which catalyzes hydrolysis of a polyketide chain,[2] typical type III PKS enzymes catalyze cyclization of a polyketide chain to yield a monocyclic compound. The variety in the reactions catalyzed by type III PKSs can be attributed to the selectivity of starter and extender substrates, the condensation times and the pattern of cyclization.[1]

A number of type III PKSs have been identified from the genus *Streptomyces*. For example, RppA catalyzes condensation of five molecules of malonyl-CoA to synthesize 1,3,6,8-tetrahydroxynaphthalene, which is a key intermediate of hexahydroxyperylenequinone melanin biosynthesis in *S. griseus*.[3,4] Germicidin synthase synthesizes germicidin in *S. coelicolor* A3(2) via the condensation of an acyl carrier protein ester of β-keto acid and ethylmalonyl-CoA.[5] SrsA synthesizes alkylresorcinols by sequential and order-controlled condensation of malonyl-CoA, malonyl-CoA and methylmalonyl-CoA toward long-chain acyl-CoA in *S. griseus*.[6] Ken2, a homolog of DpgA,[7–9] which synthesizes 3,5-dihydroxyphenylacetyl-CoA, was found in a kendomycin biosynthetic gene cluster of *S. violaceoruber*.[10] Very recently, Tang *et al.*[11] discovered that presulficidin, which is synthesized by Cpz6, a type III PKS from the caprazamycin biosynthetic cluster, relays sulfonate from 3′-phosphoadenine-5′-phosphosulfate to caprazamycin.

Genome sequence analyses of *Streptomyces* species have shown that the number of biosynthetic gene clusters encoded on the chromosome is much higher than the number of secondary metabolites isolated from each species.[12,13] The genome of *S. reveromyceticus* SN-593 was sequenced in the course of the studies of the biosynthesis of reveromycin A, which inhibits bone resorption and bone metastases of tumor cells.[14–16] Analysis of the draft genome data of *S. reveromyceticus* revealed that a type III PKS named DpyA is encoded on a linear plasmid derived from this bacterium. In this study, we demonstrate that DpyA catalyzes the synthesis of the novel dihydropyran compounds alkyldihydropyrone A–D (Figure 1) from β-hydroxyl acid thioesters and methylmalonyl-CoA, both *in vivo* and *in vitro*. DpyA preferentially uses the β-hydroxyl acid thioester rather than the β-keto acid thioester as a starter substrate. A unique feature of DpyA is an ability to catalyze lactonization of secondary alcohols and thioesters. In addition, alkyldihydropyrones showed weak cytotoxicity against the leukemia cell line HL-60.

MATERIALS AND METHODS

General

Media, growth conditions and general recombinant DNA techniques of *Escherichia coli* and *Streptomyces* were described by Sambrook *et al.*[17] and Kieser *et al.*,[18] respectively. *E. coli* HST04 and HST08, restriction enzymes and other DNA-modifying enzymes used for DNA manipulation were purchased

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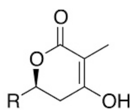

alkyldihydropyrone A (**1**): R = isopentyl
alkyldihydropyrone B (**2**): R = *n*-pentyl
alkyldihydropyrone C (**3**): R = 3-methylpentyl
alkyldihydropyrone D (**4**): R = isohexyl

Figure 1 Structures of alkyldihydropyrone A–D (**1**–**4**).

from Takara Biochemicals (Shiga, Japan). *E. coli* ASKA clone (−) JW1079 was obtained from the National BioResource Project (National Institute of Genetics of Japan). HR-MS were measured using a JEOL AccuTOF-DART mass spectrometer (JEOL, Tokyo, Japan). ^{1}H, ^{13}C and 2D NMR spectra of alkyldihydropyrones were measured in CD_3OD on a Bruker BioSpin AV400N FT-NMR spectrometer (Bruker, Billerica, MA, USA). Optical rotations of alkyldihydropyrones were measured on a JASCO P-2200 digital polarimeter (JASCO, Tokyo, Japan). IR spectra of alkyldihydropyrones were measured on a JASCO FT/IR-550 spectrometer (JASCO). UV spectra of alkyldihydropyrones were measured on a JASCO V-630BIO spectrophotometer (JASCO).

Construction of pIJ6021-SRE2_11 and pIJ4123-SRE2_11

An *Nde*I site was introduced at the start codon of *dpyA* by PCR with primer I: 5′-GCGGAATTC*CATATG*GCTGC**C**TATGTGAGCTG-3′ (the *Eco*RI site is underlined; the *Nde*I site is italicized; the boldface letter indicates the silent mutation introduced to abolish the native *Nde*I site), and primer II: 5′-GCG*GGATCC*GACCTGGACTGACCATCA-3′ (the *Bam*HI site is italicized). The amplified fragment was cloned between the *Eco*RI and *Bam*HI sites of pUC19, resulting in pUC19-SRE2_11. DNA sequencing of the plasmid confirmed the correct sequence. The *Nde*I–*Bam*HI fragment excised from pUC19-SRE2_11 was cloned between the *Nde*I and *Bam*HI sites of pIJ6021 and pIJ4123,[19] resulting in pIJ6021-SRE2_11 and pIJ4123-SRE2_11, respectively.

HPLC analysis of alkyldihydropyrones produced by *Streptomyces*

S. coelicolor M1146/pIJ6021-SRE2_11 was used to inoculate 50 ml of yeast extract–malt extract liquid medium containing 5 μg ml^{-1} of kanamycin, and the resultant culture was grown at 30 °C. After 24 h, 5 μg ml^{-1} of thiostrepton was added to induce the *tip* promoter, and the culture was continued for a further 48 h. A 2 ml aliquot of the supernatant from the broth culture was acidified with 30 μl of 6 M HCl and extracted with ethyl acetate. The ethyl acetate phase was evaporated and the residue was dissolved in a small amount of methanol. Reverse-phase HPLC analysis was carried out using a Docosil B column (4.6 × 250 mm; Senshu Scientific, Tokyo, Japan). Fractions eluted with a gradient of 10–90% acetonitrile in water (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 ml min^{-1} at 40 °C for 30 min. UV absorbance was detected at 254 nm.

Large-scale preparation of polyketides produced by *S. coelicolor* M1146/pIJ6021-SRE2_11

S. coelicolor M1146/pIJ6021-SRE2_11 was used to inoculate 2 × 1 liter of yeast extract–malt extract liquid medium containing 5 μg ml^{-1} of kanamycin, and grown at 30 °C. After 24 h, 5 μg ml^{-1} of thiostrepton was added and the culture was continued for a further 72 h. The pH of the culture supernatant was adjusted to 2.0 with 6 M HCl, and then extraction was carried out using ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated until dry. The crude materials were dissolved in a small amount of acetone and flash chromatographed on silica gel using benzene/acetone (1:1, vol/vol) as an eluent. The eluate was evaporated and dissolved in methanol for reverse-phase preparative HPLC. The crude materials were purified using a reverse-phase preparative HPLC apparatus equipped with a 5C_{18}-AR-II column (20 × 250 mm^2; Nacalai Tesque, Kyoto, Japan) by elution with a gradient of methanol in water containing 0.1% trifluoroacetic acid, at a flow rate of 5 ml min^{-1} at ambient temperature. The elution conditions were as follows: 10–70% methanol for 50 min, followed by 70–100% methanol for 40 min. After lyophilization, the compounds were further purified using a recycle HPLC system equipped with a Docosil SP-100 column (10 × 250 mm; Senshu Scientific). Compounds **1** and **2** were eluted with 45% acetonitrile and 0.1% trifluoroacetic acid in water, whereas compounds **3** and **4** were eluted with 55% methanol and 0.1% trifluoroacetic acid in water, at a flow rate of 3 ml min^{-1} at ambient temperature. The collected fractions were lyophilized to give **1** to **4** as white solids. The yields of **1**, **2**, **3** and **4** were 19, 18, 7 and 4 mg, respectively.

Physicochemical properties of alkyldihydropyrones

*5,6-dihydro-4-hydroxy-6-isopentyl-3-methyl-2H-pyran-2-one (**1**).* ^{1}H NMR (400 MHz, CD_3OD): δ 0.92 (d, 6H, J = 6.6 Hz, C4′H_3 and C3′-CH_3), 1.26 (m, 1H, C2′Ha), 1.41 (m, 1H, C2′Hb), 1.57 (m, 1H, C3′H), 1.61–1.75 (m, 2H, C1′H_2), 1.71 (s, 3H, C3-CH_3), 2.41 (ddd, 1H, J = 0.7, 4.3 and 17.1 Hz, C5Ha), 2.52 (ddd, 1H, J = 1.8, 11.8 and 17.1 Hz, C5Hb), 4.30 (m, 1H, C6H); ^{13}C NMR (100 MHz, CD_3OD): δ 8.6 (C3-$\underline{C}H_3$), 22.8 (C4′ and C3′-$\underline{C}H_3$), 29.1 (C3′), 33.7 (C1′), 34.2 (C5), 35.1 (C2′), 76.8 (C6), 99.0 (C3), 168.1 (C4) and 172.5 (C2); positive mode direct analysis in real time-time of flight-mass spectrum (DART/TOF-MS), m/z 199.13163 (calculated for $C_{11}H_{19}O_3$,199.13342); $[\alpha]_D^{20}$-41 (c 0.64, CH_3OH); IR ν_{max}^{KBr} 3102, 2955, 2863 and 1602 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 249 nm (1.12). These underlined 'C' mean that the signals are assigned for the underlined 'C' but not for C3.

*5,6-dihydro-4-hydroxy-3-methyl-6-pentyl-2H-pyran-2-one (**2**).* ^{1}H NMR (400 MHz, CD_3OD): δ 0.91 (t, 3H, J = 6.9 Hz, C5′H_3), 1.34 (m, 4H, C3′H_2 and C4′H_2), 1.38 (m, 1H, C2′Ha), 1.51 (m, 1H, C2′Hb), 1.62 (m, 1H, C1′Ha), 1.70 (s, 3H, C3-CH_3), 1.73 (m, 1H, C1′Hb), 2.40 (ddd, 1H, J = 0.8, 4.3 and 17.1 Hz, C5Ha), 2.50 (ddd, 1H, J = 1.8, 11.8 and 17.1 Hz, C5Hb), 4.31 (m, 1H, C6H); ^{13}C NMR (100 MHz, CD_3OD): δ 8.6 (C3-$\underline{C}H_3$), 14.3 (C5′), 23.6 (C4′), 25.7 (C2′), 32.7 (C3′), 34.5 (C5), 35.8 (C1′), 76.6 (C6), 98.7 (C3), 168.8 (C4) and 172.7 (C2); positive mode DART/TOF-MS, m/z 199.13169 (calculated for $C_{11}H_{19}O_3$,199.13342); $[\alpha]_D^{20}$-19 (c 0.72, CH_3OH); IR ν_{max}^{KBr} 3111, 2955, 2934, 2863 and 1597 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 248 nm (0.81).

*5,6-dihydro-4-hydroxy-3-methyl-6-(3′-methylpentyl)-2H-pyran-2-one (**3**).* ^{1}H NMR (400 MHz, CD_3OD): δ 0.89 (t, 3H, J = 7.1 Hz, C5′H_3), 0.89 (d, 3H, J = 6.5 Hz, C3′-CH_3), 1.18 (m, 1H, C4′Ha), 1.27–1.46 (m, 3H, C2′Ha, C3′H and C4′Hb), 1.50–1.65 (m, 2H, C1′Ha and C2′Hb), 1.70 (s, 3H, C3-CH_3), 1.75 (m, 1H, C1′Hb), 2.41 (ddd, 1H, J = 0.7, 4.3, 17.1 Hz, C5Ha), 2.51 (ddd, 1H, J = 1.6, 13.1 and 17.1 Hz, C5Hb), 4.30 (m, 1H, C6H); ^{13}C NMR (100 MHz, CD_3OD): δ 8.6 (C3-$\underline{C}H_3$), 11.7 (C5′), 19.4 (C3′-$\underline{C}H_3$), 30.5 (C4′), 32.7 (C2′), 33.3 (C1′), 34.2 (C5), 35.5 (C3′), 76.9 (C6), 99.0 (C3), 168.1 (C4) and 172.6 (C2); positive mode DART/TOF-MS, m/z 213.14548 (calculated for $C_{12}H_{21}O_3$, 213.14907); $[\alpha]_D^{20}$-52 (c 0.52, CH_3OH); IR ν_{max}^{KBr} 3111, 2959, 2926, 2870 and 1717 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 246 nm (0.444).

*5,6-dihydro-4-hydroxy-3-methyl-6-(4′-methylpentyl)-2H-pyran-2-one (**4**).* ^{1}H NMR (400 MHz, CD_3OD): δ 0.91 (d, 6H, J = 6.6 Hz, C5′H_3 and C4′-CH_3), 1.22 (m, 2H, C3′H_2), 1.41 (m, 1H, C2′Ha), 1.51–1.57(m, 2H, C2′Hb and C4′H), 1.60 (m, 1H, C1′Ha), 1.70 (s, 3H, C3-CH_3), 1.72 (m, 1H, C1′Hb), 2.41 (ddd, 1H, J = 0.7, 3.8 and 17.1 Hz, C5Ha), 2.51 (ddd, 1H, J = 1.4, 11.7 and 17.1 Hz, C5Hb), 4.33 (m, 1H, C6H); ^{13}C NMR (100 MHz, CD_3OD): δ 8.6 (C3-$\underline{C}H_3$), 22.9 (C5′ and C4′-$\underline{C}H_3$), 23.8 (C2′), 29.1 (C4′), 34.2 (C5), 36.0 (C1′), 39.9 (C3′), 76.6 (C6), 99.0 (C3), 168.2 (C4) and 172.6 (C2); positive mode DART/TOF-MS, m/z 213.14623 (calculated for $C_{12}H_{21}O_3$, 213.14907); $[\alpha]_D^{20}$-59 (c 0.47, CH_3OH); IR ν_{max}^{KBr} 3111, 2954, 2926, 2862 and 1725 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 245 nm (0.546).

Production and purification of recombinant DpyA protein

For production of N-terminus His-tagged DpyA, *S. lividans* TK21 harboring pIJ4123-SRE2_11 was used to inoculate 50 ml of yeast extract–malt extract liquid medium containing 5 μg ml^{-1} of kanamycin, and the resultant culture was grown at 30 °C. After 24 h, 5 μg ml^{-1} of thiostrepton was added and the culture was continued for a further 24 h. The cells were collected by centrifugation, and a crude cell lysate was prepared by sonication and removal of cell debris by centrifugation at 20 000 *g* for 10 min. Recombinant DpyA

was purified to homogeneity (Supplementary Figure S1) by using a Ni-nitrilotriacetic acid column (Qiagen, Hilden, Germany), according to the manual from the manufacturer. The recombinant protein was dialyzed two times against 2 l of 10 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol. The protein concentration was measured by Bradford assay with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

Synthesis of (±)-3-hydroxyoctanoic acid *S*-(2′-acetamidoethyl) ester (5)

A solution of (±)-3-hydroxyoctanoic acid (100 mg, 0.624 mmol), 4-methylaminopyridine (20.1 mg, 0.164 mmol), *N*-acetylcysteamine (81.8 mg, 0.686 mmol) and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (132 mg, 0.689 mmol) in 5 ml dichloromethane was stirred at room temperature overnight. The reaction was quenched with ice, and the organic phase was separated. The aqueous phase was extracted three times with a portion of dichloromethane. The combined organic phase was washed with 1 M HCl, and then with brine, dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The residue was chromatographed by silica gel using hexane/ethyl acetate (10:1, vol/vol) as a solvent to give 74 mg (0.283 mmol) of (±)-**5** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, 3H, J = 6.8 Hz, $C8H_3$), 1.20–1.53 (m, 8H, $C4H_2$, $C5H_2$, $C6H_2$ and $C7H_2$), 1.95 (s, 3H, CH_3 of acetyl), 2.66 (dd, 1H, J = 8.6, 15.4 Hz, C2H), 2.74 (dd, 1H, J = 3.4, 15.4 Hz, C2H), 3.03 (m, 2H, $C1'H_2$), 3.44 (m, 2H, $C2'H_2$), 4.04 (m, 1H, C3H) and 5.78 (br s, 1H, NH). Positive mode DART/TOF-MS, *m/z* 262.14910 (calculated for $C_{12}H_{24}NO_3S$, 262.14769).

Synthesis of 3-oxooctanoic acid *S*-(2′-acetamidoethyl) ester (6)

A solution of Meldrum's acid (1.00 g, 6.94 mmol) and pyridine (1.10 g, 13.9 mmol) in 5 ml of ice-cooled dichloromethane was added hexanoyl chloride (1.03 g, 7.65 mmol). The reaction mixture was stirred for 1 h at 0 °C, and then allowed to warm to room temperature for 1 h. The reaction was quenched with ice, and the organic phase was separated. The aqueous phase was extracted two times with dichloromethane. The combined organic phase was washed with brine, dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The resultant oil was chromatographed on a silica gel using hexane/ethyl acetate (10:1, vol/vol) as a solvent to give 887 mg (3.66 mmol) of 5-hexanoyl-2,2-dimethyl-1,3-dioxane-4,6-dione as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.84 (t, 3H, J = 7.0 Hz, $C6'H_3$), 1.30–1.35 (m, 4H, $C4'H_2$ and $C5'H_2$), 1.59 (m, 2H, $C3'H_2$), 1.67 (s, 6H, C2-$(CH_3)_2$) and 3.00 (t, 2H, J = 7.6 Hz, $C2'H_2$). A solution of *N*-acetylcysteamine (236 mg, 1.98 mmol) and 5-hexanoyl-2,2-dimethyl-1,3-dioxane-4,6-dione (698 mg, 2.88 mmol) in anhydrous benzene (20 ml) was refluxed for 5 h. After evaporation under reduced pressure, the resultant oil was chromatographed on a silica gel using hexane/ethyl acetate (10:1, vol/vol) as a solvent to give 229 mg (0.88 mmol) of **6** as a white solid. NMR analysis indicates that the product exists predominantly as a keto form. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.83 (t, 3H, J = 7.0 Hz, $C8H_3$), 1.20–1.28 (m, 4H, $C6H_2$ and $C7H_2$), 1.53 (m, $C5H_2$), 1.92 (s, 3H, CH_3 of acetyl), 2.47 (t, 2H, J = 7.4 Hz, $C4H_2$), 3.03 (t, 2H, J = 6.3 Hz, $C1'H_2$), 3.38 (t, 2H, J = 6.3 Hz, $C2'H_2$), 3.64 (s, 1H, $C2H_2$) and 6.25 (br s, 1H, NH). Positive mode DART/TOF-MS, *m/z* 260.12904 (calculated for $C_{12}H_{22}NO_3S$, 260.12867).

Preparation of (*S*)- and (*R*)-3-hydroxyoctanoic acid *S*-(2′-acetamidoethyl) ester (5)

Recombinant FabG was prepared from *E. coli* ASKA clone (−) JW1079. JW1079 was inoculated into 100 ml of LB (Lysogeny Broth) liquid medium containing 12.5 μg ml^{-1} of chloramphenicol, and grown at 37 °C. After 6 h, 0.1 mM of isopropyl β-D-1-thiogalactopyranoside was added, and the culture was continued for a further 24 h. The cells were collected by centrifugation, and a crude cell lysate was prepared by sonication and removal of cell debris by centrifugation at 20 000 *g* for 10 min. Recombinant FabG protein was purified using a Ni-nitrilotriacetic acid column (Qiagen). The solution, which contained 100 mM Tris-HCl (pH 7.5), 4.2 μM of recombinant FabG, 500 μM NADPH and 500 μM 3-oxooctanoic acid *S*-(2-acetamidoethyl) ester (**6**), was stirred overnight at 30 °C. The reaction mixture was extracted with ethyl acetate, and the organic layer was evaporated under reduced pressure to give a residue that was chromatographed on silica gel as described above. The formation of (*R*)-**5** was confirmed by HR-MS analysis; positive mode DART/TOF-MS, *m/z* 262.14939 (calculated for $C_{12}H_{24}NO_3S$, 262.14769). The optical purity of (*R*)-**5** was confirmed by chiral chromatography (Supplementary Figure S2). (*S*)-**5** was prepared by chiral chromatography of (±)-**5** (Supplementary Figure S2).

In vitro reaction of DpyA

The reactions, which contained 100 mM Tris-HCl (pH 7.5), 1.1 μM of recombinant DpyA, 10 μM of starter substrate and 10 μM of methylmalonyl-CoA, were performed in a total volume of 500 μl. The reaction mixtures were incubated at 30 °C for 1 h before being quenched by 30 μl of 6 M HCl, and extracted with 500 μl of ethyl acetate. The organic layer was evaporated, and the residue was dissolved in 20 μl of methanol for HPLC analysis. The conditions for analytical and chiral HPLC were as described above.

Determination of kinetic parameters of DpyA

The reactions, which contained 100 mM Tris-HCl (pH 7.5), (*R*)-**5** or **6**, 200 μM methylmalonyl-CoA and 2 μM DpyA, were performed in a total volume of 200 μl. The concentrations of (*R*)-**5** and **6** were varied between 20–300 and 10–400 μM, respectively. The reactions were initiated by adding (*R*)-**5** or **6** and continued for 4–32 min, and stopped with 30 μl of 6 M HCl, and the material in the mixture was extracted with ethyl acetate. The organic layer was collected and evaporated. The residual material was dissolved in 20 μl of methanol for HPLC analysis. Reverse-phase HPLC analysis was carried out by using a Docosil B column (4.6 × 250 mm; Senshu Scientific), and fractions were eluted with a gradient of acetonitrile in water (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 ml min^{-1} at 40 °C. The conditions of the gradient were 10–63% acetonitrile for 20 min. UV absorbance was detected at 254 and 320 nm for **2** and **7**, respectively; **2** and **7** were used to generate the standard curve for the quantification of the product. Steady-state parameters were determined by Hanes–Woolf plot (Supplementary Figure S3).

Cytotoxicity assay

A human promyelocytic leukemia cell line, HL-60 (RIKEN Cell Bank, Saitama, Japan), was cultured in RPMI 1640 (Life Technologies, Irvine, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere that contained 5% CO_2. HL-60 cells (1.5×10^4 cells per well) were seeded in 96-well culture plates and exposed to test compounds for 48 h. Cell growth was measured using Cell Count Reagent SF (Nacalai Tesque) as per the manufacturer's instructions. Briefly, the WST-8 solution was added to each well, and the plates were incubated at 37 °C for 1.5 h. Cell growth was then measured at 450 nm on a microplate reader (Perkin-Elmer, Boston, MA, USA).

RESULTS

Heterologous expression of *dpyA* in *S. coelicolor*

DpyA forms a distinct clade in a phylogenetic tree constructed from type III PKS enzymes, which were selected by BLAST search using the DpyA sequence as a query (Supplementary Figure S4). This indicates that DpyA is evolutionarily distinct from type III PKS enzymes, the catalytic properties of which have been characterized. To clarify its *in vivo* role, *dpyA* was overexpressed in *S. coelicolor* M1146[20] by using a high-copy-number plasmid pIJ6021.[19] *S. coelicolor* M1146/pIJ6021-SRE2_11, a *dpyA*-overexpressing strain, accumulated compounds **1**–**4**, as revealed by HPLC analysis (Figure 2a). Compounds **1**–**4** were not detected in a negative control culture, *S. coelicolor* M1146/pIJ6021 (Figure 2b). NMR and MS analyses of compounds **1**–**4** revealed that these compounds were alkyldihydropyrones with side chains that differed from each other (Figure 1). ^{1}H NMR-, ^{13}C NMR-, HMBC-, HSQC spectra and key HMBC coupling of compounds **1**–**4** are shown in Supplementary Figures S5–21.

In vitro analysis of recombinant DpyA

In keeping with polyketide structure, alkyldihydropyrones would be expected to be composed of short-chain fatty acid, malonate and methylmalonate building blocks. We assume that fatty acid and malonate moieties of alkyldihydropyrones are derived from acyl carrier protein thioesters of β-hydroxyl acids, which are intermediates of fatty acid biosynthesis.[21] It is known that germicidin synthase uses the acyl carrier protein thioester of β-keto acid as a starter substrate to synthesize 4-hydroxy-3-methyl-2-pyrone derivatives.[22] *N*-acetylcysteamine is an analogue of the phosphopantethienyl arm of holo-acyl carrier protein and CoA. The *N*-acetylcysteamine ester is well accepted by PKSs as a substrate analogue of acyl carrier protein thioesters.[23,24] Therefore, we used 3-hydroxyoctanoic acid *S*-(2′-acetamidoethyl) ester (**5**) as an analogue of the corresponding acyl carrier protein thioester (Figure 3). We also prepared 3-oxooctanoic acid *S*-(2′-acetamidoethyl) ester (**6**) to compare the reactivity of DpyA against β-hydroxyl and β-keto substrates (Figure 3). As expected, 10 μM (*R*)-**5** was readily accepted as a starter substrate to give (*R*)-**2** (Figure 4a), although (*S*)-**5** was a poor substrate for DpyA (Figure 4b). We also analyzed the optical purity of **5** to ascertain the proportion that remained unreacted. As expected, the consumption rate of (*R*)-**5** was larger than that of (*S*)-**5** (Supplementary Figure S22). We therefore conclude that (*R*)-**5** is a native substrate of DpyA. Consistent with the *in vivo* result, which showed that **7** was not produced by *S. coelicolor* M1146/pIJ6021-SRE2_11 (Figure 2a), 10 μM **6** was not used as a substrate by DpyA (Figure 4c). At higher concentrations (100 μM), **6** was accepted as a substrate to give 4-hydroxy-3-methyl-6-pentyl-2*H*-pyran-2-one (**7**) (Supplementary Figure S23). The apparent K_m, k_{cat} and k_{cat}/K_m values of (*R*)-**5** for **2** synthesis were 159 ± 14 μM, 3.54 ± 0.16 min^{-1} and 22.5 ± 1.3 min^{-1} mM^{-1} (mean ± s.e., $n = 3$), respectively. Whereas the apparent K_m, k_{cat} and k_{cat}/K_m values of **6** for **7** synthesis were 310 ± 22 μM, 1.45 ± 0.032 min^{-1} and 4.69 ± 0.24 min^{-1} mM^{-1} (mean ± s.e., $n = 3$), respectively. The k_{cat}/K_m value of (*R*)-**5** is five times higher than that of **6**, indicating that DpyA preferentially uses the β-hydroxyl substrate over the β-keto substrate. It is known that type III PKS is promiscuous and capable of reacting with several substrates.[1] Similarly, when hexanoyl-CoA, malonyl-CoA and methylmalonyl-CoA were used as substrates, the reaction yielded very small amount of **7** with three unknown products at the substrate concentration of 100 μM but not at 10 μM (Supplementary Figure S24). These data do not indicate that **7** or triketide **8** is an intermediate of **2** *in vivo*, but merely reflect the promiscuity of DpyA.

Biological activities of alkyldihydropyrones

The cytotoxic activity of **1**–**4** was evaluated *in vitro*. Compounds **1**, **2**, **3** and **4** showed cytotoxicity against the leukemia cell line HL-60 with half-maximal inhibitory concentration values of 46.0, 52.1, 15.3 and 10.0 μg ml^{-1}, respectively. The difference in activity level among alkyldihydropyrones may have resulted from the structure of the acyl side chain.

DISSCUSSION

In this study, we have characterized the catalytic properties of DpyA. We found that DpyA catalyzes the condensation of β-hydroxyacyl thioesters and methylmalonyl-CoA to synthesize dihydropyran compounds **1**–**4**. While a number of type III PKSs are shown to catalyze the synthesis of pyrone ring compounds,[4–6] there are no reports of type III PKS synthesizing dihydropyran compounds. DpyA was identified from the genome sequence data of *S. reveromyceticus*, and its function was characterized by heterologous expression and *in vitro* studies. This genome data-based approach for discovering a novel

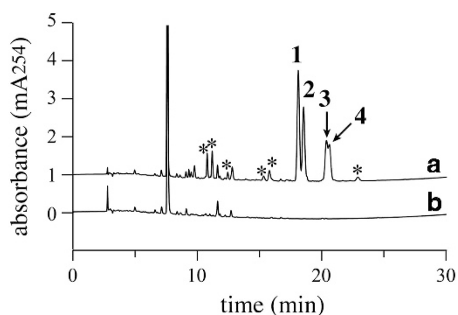

Figure 2 HPLC chromatograms of *S. coelicolor* M1146/pIJ6021-SRE2_11 (**a**) and *S. coelicolor* M1146/pIJ6021 (**b**). Uncharacterized minor peaks are marked with asterisks.

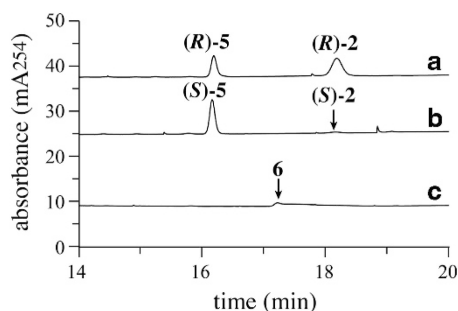

Figure 4 HPLC chromatograms of *in vitro* reactions of dihydropyrone synthase A (DpyA). (*R*)-**5** (**a**), (*S*)-**5** (**b**) and **6** (**c**) were used as starter substrates at a concentration of 10 μM.

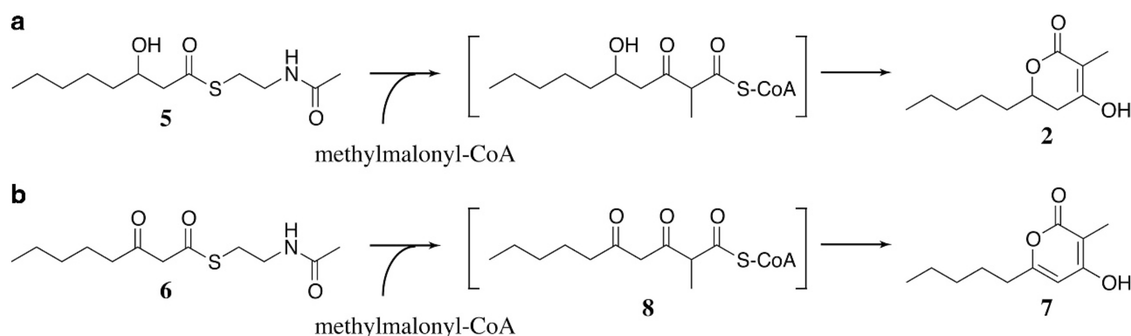

Figure 3 Reactions of dihydropyrone synthase A (DpyA) from **5** (**a**) and **6** (**b**).

biocatalyst is sometimes called 'genome mining', and has been used to characterize functionally several type III PKSs.[5,6] In some cases, the catalyst identified by genome mining is not expressed under native conditions. This is the case for DpyA: we could not detect **1–4** in the culture of *S. reveromyceticus* (unpublished data). Alternatively, **1–4** may be transferred to other compounds by endogenous enzymes.

We suggest that the ring formation catalyzed by DpyA will proceed via nucleophilic attack at the thioester bond by the β-hydroxyl group. The possibility of dehydration between the β-hydroxyl group and the carboxylic acid, which is derived from hydrolysis of the thioester bond, was ruled out, as **2** was detected by HPLC analysis without acidifying the reaction mixture (data not shown). Furthermore, we could not detect any carboxylic acid compounds by HPLC (Figure 4a).

We could not separate **2** by chiral HPLC, hindering the determination of the enantiomeric purity of alkyldihydropyrones. However, we found that DpyA preferentially utilizes (*R*)-**5** over (*S*)-**5**, as 42% of (*R*)-5 was consumed compared with 29% of (*S*)-5 (Supplementary Figure S21). Furthermore, (*S*)-**5** showed barely detectable amounts of the product (*S*)-**2** (Figure 4b). FabG in the fatty acid biosynthesis pathway is known to catalyze 3-keto-acyl-CoA to (*R*)-3-hydroxyacyl-CoA.[21] These data and evidence strongly suggest that the absolute configuration of the lactone moiety of alkyldihydropyrones is the (*R*)-form. Comparison of the optical rotation of **2** ($[\alpha]_D^{20}$-19) and the reported optical rotation ($[\alpha]_D^{25}+39$)[25] of (*S*)-5,6-dihydro-4-hydroxy-3-methyl-6-undecyl-2*H*-pyrane-2-one, a structurally related compound of **2**, supports the idea that the absolute configuration of **2** is the (*R*)-form.

Compound **3** contains another chiral center in its 3-methylpentyl group, which is an intermediate of anteiso branched-chain fatty acids (Figure 1). It is known that anteiso branched-chain fatty acids are biosynthesized from L-isoleucine via (*S*)-α-methylbutyryl-CoA in *Streptomyces*.[26] Therefore, we suggest that the absolute configuration of C3′ position of **3** is the (*S*)-form. In summary, we have demonstrated that DpyA is a novel catalyst synthesizing alkyldihydropyrones, which exhibit weak cytotoxicity against the leukemia cell line HL-60.

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