Properties and Substrate Specificity of RppA, a Chalcone Synthase-related Polyketide Synthase in *Streptomyces griseus*\(^*\)

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RppA, a chalcone synthase-related polyketide synthase (type III polyketide synthase) in the bacterium *Streptomyces griseus*, catalyzes the formation of 1,3,6,8-tetrahydroxynaphthalene (THN) from five molecules of malonyl-CoA. The \(K_m\) value for malonyl-CoA and the \(k_{cat}\) value for THN synthesis were determined to be 0.93 ± 0.1 \(\mu\)M and 0.77 ± 0.04 min\(^{-1}\), respectively. RppA accepted aliphatic acyl-CoAs with the carbon lengths from C\(_4\) to C\(_8\) as starter substrates and catalyzed sequential condensation of malonyl-CoA to yield \(\alpha\)-pyrones and phloroglucinols. In addition, RppA yielded a hexaketide, 4-hydroxy-6-(2′,4′,6′-trioxotridecyl)-2-pyrene, from octanoyl-CoA and five molecules of malonyl-CoA, suggesting that the size of the active site cavity of RppA is larger than any other chalcone synthase-related enzymes found so far in plants and bacteria. RppA was also found to synthesize a C-methylated pyrone, 3,6-dimethyl-4-hydroxy-2-pyrene, by using acetoacetyl-CoA as the starter and methylmalonyl-CoA as an extender. Thus, the broad substrate specificity of RppA yields a wide variety of products.

Bacteria synthesize a number of polyketides, and their huge structural diversity reflects the variety of pharmacological and veterinary properties (1). The bacterial polyketide synthases (PKS)\(^1\) are divided into three categories. The first category of PKS is type I PKS, a giant assembly of multifunctional polypeptides, which is mechanistically related to type I fatty acid synthase, typically found in yeast and mammals (2). Type II PKS is a large multi-enzyme complex of discrete enzymes with different functions (3), and its pivotal component responsible for the condensing activity resembles \(\beta\)-ketoacyl synthase II of type II fatty acid synthase found in bacteria and plants (4). The third type of PKS is a small homodimeric protein that possesses overall sequence homology to the chalcone synthase (CHS) family (5), which is related in structure and mechanism to \(\beta\)-ketoacyl synthase III of type II fatty acid synthase (6, 7). RppA, which was found in the Gram-positive and soil-inhabiting bacterium *Streptomyces griseus*, is the first bacterial PKS identified as a member of the CHS superfamily and categorized in type III PKS (8). Phloracetophenone, a precursor in the 2,4-diacylphloroglucinol biosynthesis in *Pseudomonas fluorescens*, is synthesized by PhlD that shares 49% identity in amino acid sequence with RppA (9). DpgA, which is encoded in the balhimycin (a vancomycin derivative) biosynthetic gene cluster in *Amycolatopsis mediterranei*, possesses 22% similarity to RppA and catalyzes the 3,5-dihydroxyphenylacetic acid synthesis solely from malonyl-CoA (10). Together with the finding that RppA is concerned with melanin production in *S. griseus* (8), we suppose that RppA-type enzymes are involved in the biosynthesis of a wide variety of secondary metabolites not only in filamentous *Streptomyces* but also in single-cell bacteria.

RppA catalyzes polyketide synthesis by selecting malonyl-CoA as the starter, carrying out four successive extensions of malonyl-CoA, and cyclizing the resulting pentaketide to THN (1) (Fig. 1).\(^2\) The final ring closure to THN (1) is accompanied presumably by decarboxylation of the carboxyl group of the malonyl-CoA used as the starter (8). CHSs found exclusively in plants catalyze sequential condensation of three malonyl-CoAs to p-coumaroyl-CoA to form an enzyme-bound tetraketide intermediate that is subsequently folded into chalcone. A wide variety of chemical structures of the CHS family-catalyzed products results from the differences in starter unit, extension unit, number of condensation, and position specificity of ring closure (11–19). In plants, CHS is a key enzyme to give the central intermediate, naringenin chalcone, for anthocyanins, used for pigmentation (20) and protection against UV irradiation (21). In *S. griseus*, however, RppA yields THN (1) as an intermediate for melanin biosynthesis (8). The most striking difference between RppA and CHS is that the former condenses malonyl-CoA four times to give a pentaketide and the latter condenses malonyl-CoA three times to give a tetraketide. Recent advances in three-dimensional structure studies of the CHS family have revealed that the size of the cavity in the active site determines starter molecule selectivity and the upper limit of the chain length in polyketide products (22, 23). Although the crystal structure of RppA has not been solved, the difference in the volume of the active site cavity might reflect the product profile of RppA and CHS.

We thus expected that site-directed mutants of RppA might be useful in the synthesis and conversion of various compounds once the properties of RppA catalysis are understood. For this purpose, it is essential to determine its kinetic parameters,  

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\(^\ddagger\) The abbreviations used are: PKS, polyketide synthase; CHS, chalcone synthase; THN, 1,3,6,8-tetrahydroxynaphthalene; HPLC, high-performance liquid chromatography; LC/APCIMS, liquid chromatography/atmospheric pressure chemical ionization mass spectrometry; RppA, enzyme that causes *S. griseus* to produce a red-brown pigment.

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specificity of starters and extension units, and the number of condensation with starters different from malonyl-CoA. We optimized the reaction conditions of THN synthesis from malonyl-CoA and measured the $K_m$ value for malonyl-CoA and the $k_{cat}$ values for THN synthesis. Under these conditions, the substrate specificity and chemical structure of resultant products were determined. For structural determination of the products, we chemically synthesized several compounds, expected as products, and used them as authentic samples.

**EXPERIMENTAL PROCEDURES**

**Materials**

[2-14C]Malonyl-CoA (2.2 GBq/mmol), dl-2-[methyl-14C]methylmalonyl-CoA (2.2 GBq/mmol), and [1-14C]acetyl-CoA (2.2 GBq/mmol) were purchased from PerkinElmer Life Sciences. All unlabeled CoA esters were purchased from Sigma, except for octanoyl-CoA used in large scale reactions. Octanoyl-CoA was synthesized by the method of Stöckigt and Zenk (24).

**Polyketide Synthesis Assay**

Histidine-tagged RppA was purified as described (8). The standard reaction mixture contained 200 μM starter-CoA, 20 μM [2-14C]malonyl-CoA (88,000 dpm) or 200 μM dl-2-[methyl-14C]methylmalonyl-CoA (88,000 dpm), 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 17 μg of RppA in a total volume of 600 μL. For the reaction of methylmalonyl-CoA, 130 μg of RppA was used. After the reaction mixture had been preincubated at 30°C for 3 min, the reaction was initiated by adding the substrate(s) and was continued for a further 10 min. When methylmalonyl-CoA was used as the substrate, the reaction period was 2.5 h. Reactions were stopped by adding 120 μL of 6 M HCl, and the products were extracted with 600 μL of ethyl acetate. The organic layer was dried up by N$_2$ flush, and the residual material was dissolved in 15 μL of CH$_3$OH for HPLC analysis. Reverse-phase HPLC conditions were as follows: ODS-SiTs (C18) column (4.6 × 150 mm; Tosoh), maintained at 40°C; mobile phase for reactions A, B, C, D, G, and H (see Fig. 4), linear from 5% CH$_3$CN in H$_2$O (each contained 2% acetic acid) to 40% CH$_3$CN in H$_2$O over 30 min and then 100% CH$_3$CN within 10 min with detection at 280 nm; flow rate, 0.8 ml/min. The mobile phase for reactions E and F (see Fig. 4) were: linear from 30% CH$_3$CN in H$_2$O (each contained 2% acetic acid) to 60% CH$_3$CN in H$_2$O over 20 min and then 100% CH$_3$CN within 5 min with detection at 280 nm; flow rate, 1.0 ml/min. UV spectra were detected on a Waters 996 photodiode array detector. The eluate was collected every minute and measured directly by liquid scintillation counting. Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC/APCIMS) was performed on an LCQ (ThermoQuest). Samples prepared for LC/MS were scaled up 10-fold, and nonradioactive malonyl-CoA was used.

**Determination of Kinetic Parameters**

The standard reaction in a total volume of 100 μL contained 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, [2-14C]malonyl-CoA (the concentration was varied between 0.33 and 4.2 μM), and 0.57 μg of RppA. After the reaction mixture had been preincubated at 30°C for 3 min, the reaction was initiated by adding the substrate and was continued for 10 or 15 s. The reaction was stopped with 20 μL of 6 M HCl, and the mixture in the material was extracted with 200 μL of ethyl acetate. The organic layer was combined with nonlabeled authentic THN [1] and dried up by N$_2$ flush for HPLC analysis. The THN [1] produced was separated by HPLC and its amount was quantified by means of [2-14C]malonyl-CoA incorporation.

**Chemical Synthesis of Phloroglucinols**

To a stirring solution of 1,3,5-trimethoxybenzene (1.65 g, 9.98 mmol) in 10 ml of dry diethyl ether, octanoyl chloride (12.9 g; 79 mmol) or hexanoyl chloride (10.8 g; 80 mmol) was added. The mixture was stirred for 10 min at room temperature, and 2 ml of concentrated H$_2$SO$_4$ was added dropwise. The reaction was allowed to be stirred at room temperature for 18 h before being diluted with ice. The products were extracted with ethyl acetate, washed with a saturated solution of NaHCO$_3$, and brine, dried with Na$_2$SO$_4$, and concentrated under reduced pressure. The crude products were flash chromatographed (25–50% ethyl acetate in hexane as an eluant) to provide phloroglucinol trimethyl esters as orange oils. Phlorocaprophenone trimethyl ether (2.5 g, 84% yield): 1H NMR (500 MHz, CD$_3$OD) δ 3.87 (s, 6H, OCH$_3$), 2.78 (t, J = 7.0 Hz, 2H, CH$_2$), 1.64 (m, 2H, C3H), 0.99 (t, J = 7.0 Hz, 3H, CH$_3$). Phlorocaprophenone trimethyl ether (2.4 g, 89% yield): 1H NMR (500 MHz, CD$_3$OD) δ 6.10 (s, 2H, ArH), 3.82 (s, 6H, OCH$_3$), 3.77 (s, 6H, OCH$_3$), 2.72 (t, J = 7 Hz, 2H, C2H), 1.64 (m, 2H, C3H), 1.29 (m, 6H, CH$_2$), 0.97 (t, J = 7.0 Hz, 3H, CH$_3$), Phlorocaprophenone trimethyl ether (2.4 g, 89% yield): 1H NMR (500 MHz, CD$_3$OD) δ 6.10 (s, 2H, ArH), 3.82 (s, 3H, OCH$_3$), 3.77 (s, 6H, OCH$_3$), 2.72 (t, J = 7.0 Hz, 2H, C2H), 1.65 (m, 2H, C3H), 1.31 (m, 4H, CH$_2$), 0.89 (t, J = 7.0 Hz, 3H, CH$_3$).

To a stirring solution of phloroglucinol trimethyl ethers (1.9 mmol) in 20 ml of dry CH$_2$Cl$_2$ at −78°C, BBr$_3$ (2.65 g, 10.4 mmol) was added dropwise. The mixture was allowed to warm to room temperature for 5 h and was then recooled to 0°C. The reaction was quenched with water and extracted with ethyl acetate. The organic layer was washed with brine, dried with Na$_2$SO$_4$, and concentrated in vacuo. The crude products were flash chromatographed (30–50% ethyl acetate in hexane as an eluant) to provide phloroglucinols as orange solids. Phlorocaprophenone [5a] (286 mg, 60% yield): 1H NMR (500 MHz, CD$_3$OD) δ 5.79 (s, 2H, ArH), 3.00 (t, J = 7.5 Hz, 2H, C2H), 1.64 (m, 2H, C3H), 1.31 (m, 8H, CH$_2$), 0.89 (t, J = 7.0 Hz, 3H, CH$_3$). Phlorocaprophenone [5b] (214 mg, 50% yield): 1H NMR (500 MHz, CD$_3$OD) δ 5.79 (s, 2H, ArH), 3.01 (t, J = 7.5 Hz, 2H, C2H), 1.65 (m, 2H, C3H), 1.34 (m, 4H, CH$_2$), 0.91 (t, J = 7.0 Hz, 3H, CH$_3$).

**Chemical Synthesis of 4-Hydroxy-6-(2’-oxoheptyl)-2-pyrene**

Benzyl bromide (204 mg, 1.19 mmol) was added to a solution of 4-hydroxy-6-methyl-2-pyrene (100 mg, 0.794 mmol) and K$_2$CO$_3$ (329 mg, 2.38 mmol) in 4 ml of dry N,N-dimethylformamide, and the resulting mixture was stirred for 1 h and then recooled to 0°C. The reaction was quenched with water and extracted with ethyl acetate. The organic layer was washed with brine, dried with Na$_2$SO$_4$, and concentrated in vacuo. The crude products were flash chromatographed (30% ethyl acetate in hexane as an eluant) to provide 4-benzoyloxy-6-methyl-2-pyrene (110 mg, 84% yield): 1H NMR (500 MHz, CD$_3$OD) δ 7.37 (m, 6H, J= 7.0 Hz, 3H, CH$_3$).
the mixture had been stirred for 1 h at 78 °C, solution of 50 mg (0.231 mmol) of 4-benzoyloxy-6-methyl-2-pyrene in 4 ml of dry tetrahydrofuran. After was stirred for 4 h at 78 °C, solution. 4-hydroxy-6-(2,5-dimethyl)-2-pyrone (3a): retention time was 18.4 min on LC; UV, λ max 266 nm. LC/APCIMS positive, 215(M + H) –; LC/APCIMS negative, 213(M + H) –; MS (precursor ion at m/z 215), 207(M − CO2 − H) –. 4-Hydroxy-6-(2,4,6-trimethoxy)-2-pyrone (3a): retention time was 15.4 min on LC; LC/APCIMS positive, MS 235(M + H) –; LC/APCIMS negative, MS 233(M + H) –; MS/MS (precursor ion at m/z 235), 207(M − CO2 − H) –, 215(C6H4O3H) –. 4-Hydroxy-6-isobutyl-2-pyrone (3b): retention time was 19.7 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 169(M + H) –; LC/APCIMS negative, MS 167(M + H) –; MS/MS (precursor ion at m/z 167), 123(M − CO2 − H) –. 4-Hydroxy-6-(4-methyl-2-oxopentyl)-2-pyrone (4a): retention time was 21.8 min on LC; LC/APCIMS positive, MS 223(M + H) –; LC/APCIMS negative, MS 221(M + H) –; MS/MS (precursor ion at m/z 223), 179(M − CO2 − H) –, 125(C6H4O3H) –. Products from Isobutyryl-CoA (3a): 4-Hydroxy-6-isobutyl-2-pyrone (3a); retention time was 22.7 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 169(M + H) –; LC/APCIMS negative, MS 167(M + H) –; MS/MS (precursor ion at m/z 167), 123(M − CO2 − H) –. 4-Hydroxy-6-(3-methyl-2-oxopropyl)-2-pyrone (4a): retention time was 21.8 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 217(M + H) –; LC/APCIMS negative, MS 215(M + H) –; MS/MS (precursor ion at m/z 223), 151(M − CO2 − H) –, 125(C6H4O3H) –. Products from Butyryl-CoA (3a): 4-Hydroxy-6-propyl-2-pyrone (3a); retention time was 20.0 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 155(M + H) –; LC/APCIMS negative, MS 153(M + H) –; MS/MS (precursor ion at m/z 153), 109(M − CO2 − H) –. 4-Hydroxy-6-(3-methyl-2-oxopropyl)-2-pyrone (4a): retention time was 21.8 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 217(M + H) –; LC/APCIMS negative, MS 215(M + H) –; MS/MS (precursor ion at m/z 223), 151(M − CO2 − H) –, 125(C6H4O3H) –. Products from Acetoacetyl-CoA (3a): 4-Hydroxy-6-methyl-2-pyrone (3a); retention time was 19.7 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 155(M + H) –; LC/APCIMS negative, MS 153(M + H) –; MS/MS (precursor ion at m/z 153), 109(M − CO2 − H) –. 4-Hydroxy-6-(3-methyl-2-oxopropyl)-2-pyrone (4a): retention time was 21.8 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 217(M + H) –; LC/APCIMS negative, MS 215(M + H) –; MS/MS (precursor ion at m/z 223), 151(M − CO2 − H) –, 125(C6H4O3H) –. Products from Isobutyryl-CoA (2a): 4-Hydroxy-6-iso-proply-2-pyrone (3a); retention time was 19.7 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 155(M + H) –; LC/APCIMS negative, MS 153(M + H) –; MS/MS (precursor ion at m/z 153), 109(M − CO2 − H) –. 4-Hydroxy-6-(3-methyl-2-oxopropyl)-2-pyrone (4a): retention time was 21.8 min on LC; UV, λ max 286 nm. LC/APCIMS positive, MS 217(M + H) –; LC/APCIMS negative, MS 215(M + H) –; MS/MS (precursor ion at m/z 223), 151(M − CO2 − H) –, 125(C6H4O3H) –. Results Determination of the Kinetic Parameters of RppA—In previous work (8) we constructed plasmid pET-RppA that directed the synthesis of histidine-tagged RppA with a structure of Met-Gly-His10-Ser2-Gly-His-Ile-Glu-Gly-Arg-His-RppA in the soluble fraction of Escherichia coli. We purified the His-tagged RppA with a nickel-nitrilotriacetic acid column to near homogeneity and used for the following assays. Incubation of RppA with [2-14C]malonyl-CoA gave THN [1] as a product. However, the product THN [1] readily polymerized, giving structurally unknown compounds, and was auto-oxidized to flavilolin because of the chemical instability of THN [1] (8). This instability hampered quantitative analysis of the RppA catalysis. To determine the kinetic parameters of RppA, we improved the conditions of reaction by shortening the reaction period to avoid the oxidation and polymerization of THN [1]. When [2-14C]malonyl-CoA was used as a substrate and the reaction was subjected to HPLC analysis, all of the radioactive substances were comigrated with authentic THN [1] (Fig. 2A), indicating that under the improved conditions THN [1] was the only radioactive product from [14C]malonyl-CoA by the action of RppA. We measured the amount of THN [1] to determine the initial reaction velocities at various concentrations of [14C]malonyl-CoA. RppA exhibited a normal Michaelis-Menten satura-
tion pattern, i.e., a straight line on the [malonyl-CoA]/v against [malonyl-CoA] plot with the $K_m$ value of 0.93 ± 0.1 μM (an average of the values obtained from three independent experiments) (Fig. 3). The $k_{cat}$ value for THN formation was calculated to be 0.77 ± 0.04 min$^{-1}$. A slight substrate inhibition, which was about a 10% decrease in velocity against the maxi-

![Figure 4: HPLC analysis of the products synthesized from various starters and extenders by RppA.](image)

The substrates used were $^{14}$C-malonyl-CoA (A), $^{14}$C-malonyl-CoA + butyryl-CoA (B), $^{14}$C-malonyl-CoA + isobutyryl-CoA (C), $^{14}$C-malonyl-CoA + isovaleryl-CoA (D), $^{14}$C-malonyl-CoA + hexanoyl-CoA (E), $^{14}$C-malonyl-CoA + octanoyl-CoA (F), $^{14}$C-malonyl-CoA + acetoacetyl-CoA (G), and $^{14}$C-methylmalonyl-CoA + acetoacetyl-CoA (H). Symbols connected by broken lines represent radioactivity measurements, and those connected by solid lines represent the UV absorbance detected at 280 nm. See "Experimental Procedures" for the assay and HPLC conditions. The inset shows the ratio of the amounts of products as determined by radioactivity measurements.
Substrate Specificity of RppA

In the fungus *Colletotrichum lagenarium*, PKSI PKS, the type I PKS, is responsible for melanin production and synthetizes THN [1] solely from malonyl-CoA (25). In this case, the aldol reaction for the first ring closure might occur between the C-2 and C-7 positions of the pentaketide intermediate. This pathway was inferred from the chemical structure of the de-riennal product, \( \alpha \)-acytlersellinic acid, that was detected in the reaction in vitro. In the reaction mixture of RppA, however, no ion peak corresponding to the molecular mass of \( \alpha \)-acytlersellinic acid or its isomer was observed by LC/MS, and thus the ring-folding pattern of the pentaketide intermediate in the RppA reaction remains unclear. Another ion chromatogram for the pseudo-molecular ion \([M – H]^-\) : 125 revealed that triacetic acid lactone was also synthesized by RppA (retention time 8.6 min on HPLC) in a negligible amount, although it was hardly detected by the autoradiogram experiment (Fig. 2A). We also examined the possibility of incorporation of \([1-\text{\textsuperscript{14}C}]\text{acyt-CoA}\) into triacetic acid lactone or \( \alpha \)-acytlersellinic acid and its isomer formed as a result of different ring folding. However, the presence of \([\text{\textsuperscript{14}C}]\text{acyt-CoA}\) in the assay did not give any radioactive products (Fig. 2B).

Tempera and pH Dependence of RppA—We examined temperature and pH dependence of RppA by using \([2-\text{\textsuperscript{14}C}]\text{malonyl-CoA}\) at a final concentration of 100 \( \mu \text{M} \). The optimal pH was about 7.5 in 100 \( \text{mM} \) Tris-HCl buffer (data not shown). Raising or lowering the pH by 1.0 caused an ~25% loss of activity. The highest enzyme activity was obtained at about 30 \( ^\circ \)C at pH 7.5 in 50–200 \( \text{mM} \) Tris-HCl buffer.

Starter Substrate Specificity of RppA—The starter specificity of RppA was examined by monitoring the incorporation of \([1-\text{\textsuperscript{14}C}]\text{malonyl-CoA}\) into the condensation products. We used various acyl-CoA compounds with 4–10 carbon lengths. At a low concentration (3.6 \( \mu \text{M} \)) of the acyl-CoA compounds, only THN [1] was formed from malonyl-CoA, and none of the unnatural starter CoAs tested here were used as a starter substrate by RppA, which suggested a clear preference for malonyl-CoA to other acyl-CoAs as the starter bound to the active site pocket. Efficient incorporation of the unnatural starter CoAs was observed when the concentrations of the starter CoAs were increased to 200 \( \mu \text{M} \), which is 10-fold higher than that of malonyl-CoA as the extender substrate.

When butyryl-CoA \([2e]\) was incubated with RppA along with \([1-\text{\textsuperscript{14}C}]\text{malonyl-CoA}\), two radioactive products, \([3e]\) and \([4e]\), were obtained in addition to THN [1] (Fig. 4B). Comparison of the UV absorption and radiochromatograms in Fig. 4, A and B, showed that these two products represented the products formed from butyryl-CoA. The molecular weights (\( M \)) obtained by LC/APCIMS analysis of \([3e]\) and \([4e]\) were 154 (corresponding to the mass of a triketide) and 196 (a tetraketide), respectively. Similar product patterns were observed when isobutyryl-CoA \([2d]\) and isovaleryl-CoA \([2c]\) were incorporated as starter substrates (Fig. 4, C and D). The \( M \) of \([3d]\) and \([4d]\) containing isobutyryl-CoA as the starter were 154 (a triketide) and 196 (a tetraketide), respectively. The \( M \) of \([3c]\) and \([4c]\) containing isovaleryl-CoA as the starter were 168 (a triketide) and 210 (a tetraketide), respectively. The ratio of the triketide \([3c]\) and the tetraketide \([4c]\) was 24:14 in the butyryl-CoA-primed products, when the radioactivity levels were quantified, whereas that of the triketide \([3e]\) and the tetraketide \([4e]\) was 5:16 in the isovaleryl-CoA-primed products.

When hexanoyl-CoA \([2b]\) was incorporated, an additional tetraketide \((M, 224; 5b)\), which migrated as a less polar compound than \([4b]\), was observed in addition to a triketide \((M, 182; 3b)\) and a tetraketide \((M, 224; 4b)\) (Fig. 4E). Surprisingly, octanoyl-CoA \([2a]\) as a starter yielded a small amount of a hexaketide \((6a)\), which resulted from seven cycles of condensation (Fig. 4F), which was confirmed by its ion peaks \([M + H]^+\) at 337 and \([M – H]^–\) at 335 by LC/APCIMS analysis. In addition to \([6a]\), the octanoyl-CoA-primed reaction yielded a triketide \((120; 3a)\) and two tetraketides \((M, 252; 4a\) and \([5a]\) RppA also accepted acetoacetyl-CoA \([2f]\), leading to formation of triacetic acid lactone \([3f]\) (Fig. 4G). Thus, RppA exhibited broad starter substrate specificity toward aliphatic acyl-CoA compounds as summarized in Table I. On the other hand, under the assay conditions used in this study, no activity was detected with acetyl-CoA, decanoyl-CoA, benzoyl-CoA, phenylacetyl-CoA, crotonyl-CoA, or tiglyl-CoA. Of the all acyl-CoAs examined, acetoacetyl-CoA gave the corresponding products in the greatest amount. The relative order of acyl-CoAs to be incorporated as a starter was determined to be acetoacetyl-CoA > hexanoyl-CoA > butyryl-CoA > isovaleryl-CoA > isobutyryl-CoA > octanoyl-CoA (Table I). A longer chain acyl-CoA, such as decanoyl-CoA, was not used as the starter, indicating that the upper limit of carbon chain length of the starter substrate is 8.

Incorporation of Methylmalonyl-CoA as an Extender Unit—When 200 \( \mu \text{M} \) starter CoAs and 20 \( \mu \text{M} \) \([\text{\textsuperscript{14}C}]\text{methylmalonyl-CoA}\) were incubated with 21 \( \mu \text{g} \) of RppA in a total of 200 \( \mu \text{L} \) at 30 \( ^\circ \)C for 2 h, a radioactive product was obtained only from the reaction with acetoacetyl-CoA \([2f]\), although at a very low level (data not shown). Optimization of the reaction conditions, under which 200 \( \mu \text{M} \) methylmalonyl-CoA, 200 \( \mu \text{M} \) acetoacetyl-CoA, and 130 \( \mu \text{g} \) RppA in a total of 600 \( \mu \text{L} \) were incubated at 30 \( ^\circ \)C for 2.5 h, led to production of \([7]\) as a major peak (Fig. 4H). LC/APCIMS analysis of \([7]\) gave a \([M + H]^+\) peak at 141 and a \([M – H]^–\) peak at 139, suggesting that \([7]\) is a triketide expected from acetoacetyl-CoA as the starter and one methylmalonyl-CoA molecule as the extender.

Identification of Reaction Products—Three major cyclization patterns of tetraketides are known for CHS-related PKSs; these are aldol condensation observed for stilbene synthase producing \( \beta \)-resorcylic acid, Claissen condensation observed for CHS producing phloroglucin (5), and lactonization observed for p-coumaroyl triatomic acid synthase producing \( \alpha \)-pyrone (16). To identify the tetraketides formed by the action of RppA, we chemically synthesized hexanoyl-CoA-derived tetraketides as authentic samples for LC/APC tandem MS analysis. As shown in Fig. 5, 4-hydroxy-6-\((\text{2-oxoheptyl})\)-2-pyrole \([4b]\), expected from the p-coumaroyl triatomic acid synthase-type ring folding, gave the fragment at \( m/z \) 125 corresponding to \([\text{C}_{6}\text{H}_{9}\text{O}_{2}]^–\) in addition to \([\text{C}_{7}\text{H}_{10}\text{O}_{2}]^–\) at 179 (Fig. 5B). The fragment ion at \( m/z \) 125 was hardly detected in phloracpro-

| Table I: Activities of RppA with various starter substrates |
|-----------------------------|-----------------------------|
| Acetyl-CoA                  | <1                          |
| Acetoacetyl-CoA             | 100                         |
| Butyryl-CoA                 | 21 ± 6                      |
| Isobutyryl-CoA              | 7 ± 3                       |
| Isovaleryl-CoA              | 13 ± 3                      |
| Hexanoyl-CoA                | 43 ± 12                     |
| Octanoyl-CoA                | 6 ± 2                       |
| Decanoyl-CoA                | <1                          |
| Benzoyl-CoA                 | <1                          |
| Phenylacetyl-CoA            | <1                          |
| Crotonyl-CoA                | <1                          |
| Tiglyl-CoA                  | <1                          |

\( ^\text{a} \) In each experiment, the amount of starter substrate reacted was determined by the total sum of the values, which are calculated from a division of the measured dpm by the number of \([2-\text{\textsuperscript{14}C}]\text{methylmalony-CoA}\) incorporated in each product. The deviations are based on two independent experiments.
Substrate Specificity of RppA

**DISCUSSION**

The broad substrate specificity of RppA, belonging to type III PKS, implies that the supply of the starter substrate is a significant factor in determining the substrate in *vivo*. However, considering the remarkably low value of the apparent $K_m$ (0.93 ± 0.1 μM) for malonyl-CoA, together with the facts that RppA prefers malonyl-CoA and does not utilize other acyl-CoAs as a primer at a low substrate concentration, we suppose that malonyl-CoA is the natural substrate of RppA in the cell. This is consistent with the observation that *S. griseus* overexpressing RppA accumulates flavinol, an auto-oxidized product of THN [1], as a single peak on HPLC.3

The present study has demonstrated that RppA can accept C4 to C8 aliphatic acyl-CoAs as a starter substrate. RppA contained all of the key amino acid residues that are responsible for reaction priming and chain elongation (Cys-138, His-270, and Asn-303) (26–28). Of these three residues, Cys-138 was shown to be essential for polyketide synthesis (8). It is widely accepted that the initial reaction step involves the loading of a starter acyl-CoA onto the thiol group of an active site cysteine, giving rise to an enzyme-bound acyl molecule via a thioester bond. This acyl transfer reaction is clearly a decisive step in determining the starter specificity of CHS-related enzymes. RppA is unusual among CHS-related enzymes in that it prefers malonyl-CoA as a starter unit. The difference of the residues surrounding the active site presumably accounts for this unique feature of RppA. Interestingly, RppA uses branched chain acyl-CoAs (isobutyryl-CoA [2d] and isovaleryl-CoA [2e]) but not aromatic acyl-CoAs (benzoyl-CoA and phenylacetyl-CoA) (Table I), although plant CHSs, in contrast, use both branched and aromatic acyl-CoAs (29–34). The electronic and/or steric hindrance caused by a bulkier residue present in the substrate binding pocket in RppA could explain the differences in substrate specificity between RppA and CHS. Consistent with this idea, RppA accepted butyryl-CoA [2e] but not crotonoyl-CoA. Site-directed mutagenesis experiments to clarify the starter specificity are in progress.

RppA synthesizes several α-pyrones when some acyl-CoAs are used as starters. The mechanism of the final ring closure,
yielding pyrone, is the second feature that distinguishes RppA from CHS. CHSs produce bisnoryangonin and p-coumaroyltriacetic acid lactone as derailment products, as a result of hydrolysis of the polyketide chain from active site cysteine (or CoA) and subsequent non-enzymatic pyrone formation (35). However, the reaction mechanism by which RppA yields pyrone is supposed to be quite different from that of CHS. For generation of a carboxylic acid of the polyketide chain through the hydrolysis of the thioester bond from Cys-138 (or CoA) before the pyrone formation, an acidic condition to accomplish the dehydration of hemiacetal is necessary (Fig. 7A). All of the pyrones produced by RppA were detected by HPLC analysis without acidifying the reaction mixture (data not shown), although pyrone is poorly extracted with ethyl acetate under the assay condition used (pH 7.5). Furthermore, we could not detect any hemiacetal or carboxylic acid compounds by either HPLC/autoradiogram (Fig. 4) or LC/MS ion scanning analysis. We thus conclude that the release of the final product from Cys-138 (or CoA) occurs by intramolecular lactonization, as in the case of animal fatty acid synthase (36, 37), or by Claisen condensation of the polyketide chain. Nucleophilic attacking of the carbonyl carbon of the thioester by the oxyanion at the C-5 position leads to the formation of /H9251/ pyrones, whereas that by the carbanion at the C-6 position yields phloroglucinols (Fig. 7B). This could proceed when the polyketide is attached to CoA, because there is no evidence of retransfer of the polyketide onto CHS.

**Fig. 6. Summary of RppA reactions with various starter substrates.** The extender substrate used were malonyl-CoA (A) and methylmalonyl-CoA (B).
the active site cysteine after the final chain extension (22). It is possible that lactonization occurs after the release of the CoA-attached polyketide from the enzyme. Type I PKSs possess thiosterase domains, which are responsible for release of the polyketide chain from the enzyme surface (38–40). In CHS-related enzymes, however, no such residues capable of hydration or intermolecular Claisen condensation of the polyketide have been identified (22, 23), and therefore the mechanism of product release remains mysterious. Our results also show that the structure of the starter unit of the nascent polyketide chain contribute much toward the folding of the final product, because the reaction from isovaleryl-CoA (24) yielded pyrone (4d) with no apparent formation of phloroglucinol, whereas the octanoyl-CoA (2a) primed reaction produced phloroacrylophenone (5a) (Fig. 6).

Interestingly, hexaketide 6a was produced from octanoyl-CoA (2a) and five molecules of malonyl-CoA. This is the first observation that CHS-related enzymes possess the ability to catalyze condensation of more than five units of malonyl-CoA. The size of the active site cavity physically limits the number of malonyl-CoA condensations, as implied from the x-ray crystal structures of 2-pyrene synthase and CHS (22, 23). The carbon length (C13) of 6a suggests that the cavity volume of RppA is quite large, although malonyl-CoA (natural substrate) cannot utilize the maximum potential of RppA. The stability of the active site pocket could be altered depending on the structure of the starter substrate incorporated into the growing chain, and therefore the variety in the chain length of products would arise. In the case of acetoacetyl-CoA (2f) primed reaction, a short starter moiety, such as a methyl group, may be insufficient to be retained in the pocket and released before further condensation of malonyl-CoA.

In conclusion, RppA exhibits high activity toward 4- to 8-carbon straight and branched chain acyl-CoAs but little activity toward aromatic or unsaturated acyl-CoAs. The broad substrate specificity of RppA leads to production of tri- to hexaketide pyrones and tetra-ketide phloroglucinols, presenting a diverse product profile. The availability of the knowledge of substrate specificity of RppA is promising for the production of single-ring pyrones, phloroglucinols, or their derivatives by overexpressing mutant RppA enzymes in Streptomyces and E. coli cells supplemented with various starter compounds.

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