Phaeochromycins A−E, Anti-inflammatory Polyketides Isolated from the Soil Actinomycete *Streptomyces phaeochromogenes* LL-P018

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Five new polyketide metabolites, phaeochromycins A−E (**1**−**5**), were isolated from an actinomycete designated *Streptomyces phaeochromogenes* LL-P018, cultured from a soil sample collected from a riverbank in Westevenger, Germany. Phaeochromycins A and C were found to be weak inhibitors of MAPKAP kinase-2 (IC_{50} = 39 and 130 μM, respectively). The structures of the compounds were determined by spectroscopic analysis, primarily two-dimensional NMR, and revealed that phaeochromycins A, B, C, and E were octaketides, elaborated from a C4 starter unit, related to shunt products of the actinorhodin pathway, namely, mutactin, dehydromutactin, SEK34b, and BSM1. Phaeochromycin D (**4**) is an unusual partially cyclized degraded octaketide intermediate.

Rheumatoid arthritis is a chronic, debilitating inflammatory disease that affects up to 1% of the population with a 3:1 prevalence in women over men and has no known cure. While a number of small-molecule drug therapies exist to ease symptoms (nonsteroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs), they often demonstrate limited efficacy and many adverse side effects.[1,2] Recent protein-based therapies, including anti-tumor necrosis factor (anti-TNF-α) agents such as etanercept (Enbrel), have been shown to be relatively safe and effective therapies for the treatment of various inflammatory diseases. On the basis of these successful therapies, it is highly desirable to develop the next generation of small-molecule therapeutic agents targeting TNF-α production and alleviate the issues associated with the usage and production of biologic agents.[3] One such molecular target implicated in the regulation of TNF-α translation is a downstream substrate of mitogen-activated protein (MAP) kinase p38, namely, MAP kinase-activated kinase 2 (MAPKAP-2 or MK-2). TNF-α and other cytokine levels have been shown in MK-2 knockout mice to be reduced to various degrees under LPS challenging conditions.[4]

During the course of screening fermentation extracts of microbial fermentations from our culture collection for inhibitory activity in an in vitro enzyme assay for MK-2, we were able to rapidly identify the known kinase inhibitors equisetin and staurosporine as the active principles from the majority of active extracts. In contrast, inhibitory activity was observed for material generated from an isolate designated *Streptomyces phaeochromogenes* LL-P018. When dereplication of the mass spectral information from the associated LC/MS trace failed to yield previously reported compounds, the active compound(s) were isolated via bioassay-guided fractionation. We herein report the structures and biological activities of the new compounds and propose that the biogenesis of the compounds proceeds via type II polyketide synthase (PKS) pathways analogous to those delineated for the production of a number of actinorhodin and enterocin PKS shunt metabolites and intermediates.[5−9]

Fermentation broths (1 L) of *S. phaeochromogenes* LL-P018 were extracted with ethyl acetate, and the resulting extract was chromatographed by reversed-phase HPLC employing a slow gradient of acetonitrile (5% to 40%) in water with 0.025% trifluoroacetic acid as modifier. Analysis of the resultant active fractions indicated the presence of a number of previously unidentified metabolites that exhibited characteristic UV absorbances for α-pyrones. This material was rechromatographed to yield pure samples of phaeochromycins A (**1**, 12.5 mg), B (**2**, 3.0 mg), C (**3**, 5.0 mg), D (**4**, 2.0 mg), and E (**5**, 6.0 mg).

The high-resolution Fourier transform inductively coupled resonance (FT-ICR) mass spectrum of phaeochromycin A (**1**) gave a parent ion at $(M + H)^+$ = 313.1067 to yield a molecular formula of $C_{18}H_{16}O_5$ (calculated = 313.1070). Analysis of the 2D NMR spectral data for **1** revealed a pattern of correlations consistent with the structural assignment. The three aromatic proton resonances at δ 6.75, 6.95, and 7.28 were straightforwardly assigned as contiguous (H-8, H-9, H-10) based on the observed correlations in the COSY spectrum of **1**. The HMBC spectrum of **1** showed strong correlations from a carbon at δ 112.1 to both proton signals at δ 6.75 and 6.95, indicating that this carbon must be meta to both protons (C-12). Similarly, the remaining carbons of the ring were assigned from three-bond HMBC correlations to the resonance at δ 7.28 (H-9) from δ 135.2 (C-7) and 154.0 (C-11). An additional three-bond HMBC correlation from C-12 was observed to an aromatic proton signal at δ 6.69 that was assigned to H-14 and to an additional phenolic proton resonance at δ 11.14 that was partially overlapping with that for the C-11 hydroxyl proton at δ 11.11. Additional three-bond HMBC correlations to the signal assigned to H-14 include a quaternary carbon resonance at δ 120.0 and an upfield methylene resonance at δ 35.1. The assignment of the resonance at δ 120.0 to C-6 was further supported by the observed three-bond HMBC correlation from this carbon to the proton resonance at δ 6.95 that had already been assigned to H-8. The carbon resonance at δ 35.1 (C-16) was shown to be part of an *n*-propyl group via HMBC and COSY correlations with signals for an additional methylene carbon at δ 23.9 (C-17) and a methyl carbon at δ 13.9 (C-18). That the *n*-propyl group was attached at the position ortho to C-14 was demonstrated by HMBC correlations to

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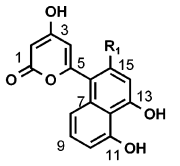
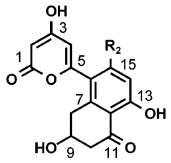
H-16 (δ 2.51) from C-14 and C-6 and a resonance at δ 141.1 that was therefore assigned to C-15.

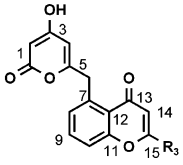
An additional three-bond HMBC correlation was observed from the carbon resonance at δ 120.0 (C-6) to a proton signal at δ 6.18. The HSQC spectrum of **1** revealed that this proton was attached to a carbon with a signal at δ 105.1 that showed an HMBC correlation to a phenolic proton signal at δ 11.9. This in turn showed HMBC correlations from carbon resonances at δ 170.1 and 89.5. Since the carbon resonance at δ 170.1 must bear the phenol group due to its chemical shift, it necessarily follows that the carbons assigned to the resonances at δ 89.5, 170.1, and 105.1 must be contiguous and were thus assigned to C-2, C-3, and C-4, respectively. HMBC correlations from the carbon resonance at δ 164.2 to H-2 and from the carbon signal at δ 161.1 to H-4 confirmed the assignments of C-1 and C-5, respectively. All carbon and proton assignments for **1** were in complete agreement with those for the aromatic rings of wailupemycin G[5] (**6**) and dehydromutactin[6] (**7**).



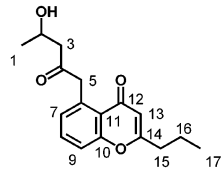
(**1**) phaeochromycin A R_1 = ^{n}Pr
(**6**) wailupemycin G R_1 = phenyl
(**7**) dehydromutactin R_1 = Me



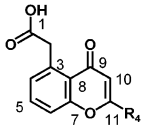
(**2**) phaeochromycin B R_2 = ^{n}Pr
(**8**) wailupemycin F R_2 = phenyl
(**9**) mutactin R_2 = Me



(**3**) phaeochromycin C R_3 = ^{n}Pr
(**10**) SEK34b R_3 = Me



(**4**) phaeochromycin D



(**5**) phaeochromycin E R_4 = ^{n}Pr
(**11**) BSM1 R_4 = Me

The high-resolution FT-ICR mass spectrum of phaeochromycin B (**2**) gave a parent ion at $(M + H)^+ = 331.1180$ to yield a molecular formula of $C_{18}H_{18}O_6$ (calculated = 331.1176). Analysis of the 2D NMR spectra of **2** quickly revealed that the compound was an *n*-propyl analogue of mutactin (**8**); assignments for C-8 to C-10 were made using a combination of COSY, HSQC, and HMBC data, and all assignments were in good agreement with literature data for wailupemycin F[5] (**8**) and mutactin[7] (**9**).

The high-resolution FT-ICR mass spectrum of phaeochromycin C (**3**) gave a parent ion at $(M + H)^+ = 313.1071$ to yield a molecular formula of $C_{18}H_{16}O_5$ (calculated = 313.1070). The absence of observable phenolic proton resonances and the presence of both a carbonyl carbon resonance at δ 181.8 and an aliphatic methylene resonance at δ 39.2 immediately suggested that **3** be the product of an alternate folding analogous to the octaketide SEK34b (**10**).[8] This was confirmed by the observation of HMBC correlation from the carbon resonance at δ 167.8 (C-5) to the methylene proton signals at δ 4.50 (H-6) and to the aromatic doublet at δ 7.35 (H-8). Similarly, the assignments of the benzopyranone carbons C-11 to C-15 were supported by the expected HMBC correlations and are in good agreement with the literature.[8]

The high-resolution FT-ICR mass spectrum of phaeochromycin D (**4**) gave a parent ion at $(M + H)^+ = 289.1437$ to yield a molecular formula of $C_{17}H_{20}O_4$ (calculated = 289.1434). Initial examination of the 2D NMR spectral data for **4** revealed that the assignments for the *n*-propylbenzopyranone portion of the compound (C-6 to C-17) were identical to that for phaeochromycin C (**3**, C-7 to C-18). The presence of a methylene group attached at C-6 of **4**, analogous to **3**, was indicated by the observed HMBC correlation from the carbon resonance at δ 131.8 (C-7) to a proton signal at δ 4.22 (H-5) that integrated to two protons. This signal showed an additional HMBC correlation from a carbon resonance at δ 205.7, suggestive of a neighboring ketone carbonyl. Furthermore, this carbonyl resonance at δ 205.7 (C-4) showed additional correlations to a pair of methylene proton resonances at δ 2.75 and 2.60 that were attached to a carbon resonance at δ 52.1 (C-3). The H-3 protons also showed HMBC correlations from carbons at δ 62.8 and 23.7, the latter resonance correlating to a methyl doublet signal at δ 1.00 in the me-HSQC spectrum of **4**. The remaining hydroxyl moiety dictated by the molecular formula of **4** was easily accommodated at C-2 (δ 62.8) and is supported by the observed chemical shifts of C-1 to C-3.

The high-resolution FT-ICR mass spectrum of phaeochromycin E (**5**) gave a parent ion at $(M + H)^+ = 247.0965$ to yield a molecular formula of $C_{14}H_{14}O_4$ (calculated = 247.0965). The molecular formula of **5** indicated that the compound was a truncated hexaketide (assuming a C4 starter unit, vide infra), and the 2D NMR data confirmed that the *n*-propyl-substituted benzopyranone portion of the compound (C-3 to C-14) was identical to that of phaeochromycin C (**3**). The C-2 methylene protons (δ 4.18) were identified via an HMBC correlation from C-4 (δ 167.8). An additional HMBC correlation to the H-4 signals from the sole remaining unassigned carbon resonance at δ 174.4 indicated that **5** must be a carboxylic acid analogous to the minor actinorhodin shunt metabolite, BSM1 (**11**).[9] Again, NMR assignments were in excellent agreement with the literature.

MK-2 activity of compounds was assessed using human recombinant MK-2-containing residues 41 through 353 in an ELISA-based assay. The kinase reaction was performed on 96-well streptavidin-coated plates using a biotinylated

13-mer peptide derived from LSP1 in 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 3 mM DTT, and 1 μM ATP. The reaction was stopped after 30 min incubation at room temperature and washed in PBS 0.05% Tween 20. Polyclonal anti phospho-LSP1 antibodies were then added to the plate along with goat anti-rabbit labeled with europium in 20 mM MOPS, 150 mM NaCl, 0.025% Tween 20, 0.02% gelatin, and 1% BSA for 1 h at room temperature. The plate was then washed in PBS 0.05% Tween 20, and enhancement solution from Perkin-Elmer was added before counting on a Victor 2 reader from Perkin-Elmer. Phaeochromycins A (**1**) and C (**3**) were found to be weak inhibitors of MK-2 (IC_{50} = 39 and 130 μM, respectively), whereas the other compounds isolated were inactive.

The biogenesis of the phaeochromycins is presumed to follow the well-studied precedent for type II polyketide synthesis.[10] Phaeochromycins A, B, C, and E are direct analogues, with a butyrate starter unit, of the shunt products of the actinorhodin pathway, namely, mutactin, dehydromutactin, SEK34b, and BSM1, respectively.[5−9] Phaeochromycin D is an unusual partially cyclized degraded intermediate, and while it is likely that the terminal methyl group (C-1) of **4** is derived from C-2 of malonate, the mechanism by which this occurs remains obscure. Future experiments are planned to investigate this mechanism as well as to confirm the proposed butyrate starter unit via feeding experiments with labeled precursors and molecular biological methods.

In conclusion, in an effort to identify novel anti-inflammatory compounds that inhibit MAPKAP-2 kinase for use as leads in the development of new agents for treating rheumatoid arthritis, we have isolated and characterized five novel compounds, phaeochromycins A−E. Phaeochromycins A and C were found to be weak inhibitors of MAPKAP kinase-2 (IC_{50} = 39 and 130 μM, respectively).

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker Avance DPX-400 spectrometer using a 3 mm broadband detect probe; FT-ICR MS spectra were obtained using a Bruker Daltonics 7 T system. Optical rotations were measured on a Jasco P-1020 polarimeter. Preparative chromatography was accomplished on a Varian HPLC system equipped with dual Dynamax SD-300 pumps and a Prostar diode array detector (DAD) under the control of Varian Star software. Analytical chromatography was carried out using an Agilent HP1100 HPLC system equipped with a DAD using Chemstation software. All HPLC solvents were EM Omnisolv quality and used without further purification. Water used in chromatography was doubly distilled using a Millipore Milli-Q purifier.

Fermentation of *S. phaeochromogenes* LL-P018. Strain LL-P018 was maintained at 28 °C on agar medium containing (g/L) dextrose (5), yeast extract (2.5), agar (15), soluble starch (10), NZ-amine A (2.5), and calcium carbonate (0.5). For production of the phaeochromycins, strain LL-P018 was grown in a 250 mL Erlenmeyer flask containing 50 mL of seed medium. Seed medium contained (g/L) dextrose (10), soluble starch (20), yeast extract (5), NZ-amine A (5), and calcium carbonate (1). This seed culture was incubated at 28 °C with shaking at 200 rpm and 2 in. throw for 3 days. Production medium contained (g/L) magnesium sulfate 7-hydrate (0.5), potassium chloride (0.5), potassium phosphate dibasic (2.5), sodium chloride (5), glycerol (10), and soy peptone (5). One liter (1 L) of production medium was inoculated with 20 mL (2% inoculum) of seed culture and incubated at 28 °C with shaking at 200 rpm and 2 in. throw for 6 days.

Extraction and Isolation. Fermentation broths (1 L) of *S. phaeochromogenes* LL-P018 were centrifuged, and the supernatant was extracted with three 300 mL portions of ethyl acetate. The combined organic extracts were dried over anhydrous $MgSO_4$ and reduced in vacuo to yield 350 mg of an orange oil. The crude organic extract was suspended in 1:1 acetonitrile/methanol and chromatographed by reversed-phase HPLC (YMC ODS-A 20 × 250 mm C_{18} column, MeCN/H_2O 5% to 40% in 25 min with 0.0025% trifluoroacetic acid as modifier) to yield pure samples of **1** (12.5 mg), **3** (5.0 mg), **4** (2.0 mg), and **5** (6.0 mg), obtained as colorless glasses. Material eluting between 20 and 22 min was collected and further purified by reversed-phase HPLC (YMC ODS-A 10 × 250 mm C_{18} column, acetonitrile/water 45% to 60% in 20 min) to yield pure **2** as a colorless glass (3.0 mg).

Table 1. ^{13}C NMR Data for Phaechromycins A−E (**1**−**5**), 100 MHz, DMSO-d_6

position	**1**	**2**[a]	**3**	**4**	**5**[b]
1	164.2	168.2	166.0	23.7	174.4
2	89.5	89.6	90.9	62.8	41.4
3	170.1	173.0	173.5	52.1	136.2
4	105.1	107.2	101.6	205.7	129.2
5	161.1	162.1	167.8	48.8	133.6
6	120.0	125.2	39.2	136.4	118.4
7	135.2	143.2	137.3	131.8	158.5
8	115.8	37.0	130.3	136.4	122.1
9	128.2	66.7	134.9	117.4	181.4
10	108.5	47.4	119.6	157.3	110.9
11	154.0	204.9	159.8	121.1	170.0
12	112.1	116.3	122.5	178.5	36.2
13	155.6	164.7	181.8	110.4	20.6
14	109.4	117.3	111.6	168.2	13.7
15	141.1	152.7	171.2	34.7	
16	35.1	37.2	36.7	19.6	
17	23.9	25.2	21.3	13.3	
18	13.9	14.4	13.9		

[a] Data obtained in CD_3OD. [b] Data obtained in 2:1 CD_3OD/$CDCl_3$.

Phaeochromycin A (1): colorless amorphous glass; UV (MeOH), λ_{max} 236, 312, 338sh; IR ($CDCl_3$) ν_{max} 2870, 2790, 905, 740 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.90 (1H, s, 3-OH), 11.14 (1H, s, 13-OH), 11.11 (1H, s, 11-OH), 7.28 (1H, dd, J = 8.3, 8.0 Hz, H-9), 6.95 (1H, d, J = 8.3 Hz, H-8), 6.75 (1H, d, J = 8.0 Hz, H-10), 6.69 (1H, s, H-14), 6.18 (1H, d, J = 1.8 Hz, H-4), 5.43 (1H, d, J = 1.6 Hz, H-2), 2.51 (2H, H_2-16), 1.56 (2H, m, H_2-17), 0.85 (3H, t, J = 8.0 Hz, H_3-18); ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; HRFT-ICRMS m/z 313.1067 $(M + H)^+$, calcd for $C_{18}H_{16}O_5$ 313.1070.

Phaeochromycin B (2): colorless amorphous glass; $[\alpha]_D^{22}$ −1.0° (c 0.2 MeOH); UV (MeOH), λ_{max} 208, 232, 275sh, 304; IR ($CDCl_3$) ν_{max} 2850, 2790, 1650, 905, 735 cm^{-1}, ^1H NMR (DMSO-d_6, 400 MHz) δ 6.76 (1H, s, H-14), 6.20 (1H, s, H-4), 5.50 (1H, d, J = 2.0 Hz, H-2), 4.30 (1H, m, H-9), 3.07 (1H, dd, J = 16.5, 3.5 Hz, H-8), 2.94 (1H, dd, J = 17, 3.5 Hz, H-10), 2.85 (1H, dd, J = 16.5, 6.8 Hz, H-8′), 2.72 (1H, dd, J = 17, 7.1 Hz, H-10′), 2.52 (2H, t, J = 7.5 Hz, H_2-16), 1.58 (2H, m, H_2-17), 0.90 (3H, t, J = 7.3 Hz, H_3-18); ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; HRFT-ICRMS m/z 331.1180 $(M + H)^+$, calcd for $C_{18}H_{18}O_6$ 331.1176.

Phaeochromycin C (3): colorless amorphous glass, UV (MeOH), λ_{max} 208, 232, 275sh, 302; IR ($CDCl_3$) ν_{max} 1700, 1640, 1580, 905, 725 cm^{-1}, ^1H NMR (DMSO-d_6, 400 MHz) δ 11.52 (1H, s, H-2), 7.73 (1H, dd, J = 8.7, 7.7 Hz, H-9), 7.57 (1H, d, J = 8.7 Hz, H-10), 7.30 (1H, d, J = 7.7 Hz, H-8), 6.15 (1H, s, H-14), 5.47 (1H, s, H-4), 5.17 (1H, s, H-2), 4.43 (2H, s, H_2-6), 2.59 (2H, t, J = 7.4 Hz, H_2-16), 1.69 (2H, m, H_2-17), 0.94 (3H, t, J = 7.4 Hz, H_3-18); ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; HRFT-ICRMS m/z 313.1071 $(M + H)^+$, calcd for $C_{18}H_{16}O_5$ 313.1070.

Phaeochromycin D (4): colorless amorphous glass; $[\alpha]_D^{22}$ −5.3° (c 0.2, methanol); UV (MeOH), λ_{max} 236, 254sh, 306; ^1H NMR (DMSO-d_6, 400 MHz) δ 7.64 (1H, dd, J = 8.3, 7.8 Hz, H-8), 7.48 (1H, d, J = 7.8 Hz, H-9), 7.12 (1H, d, J = 8.3 Hz, H-7), 6.09 (1H, s, H-13), 4.22 (2H, s, H_2-5), 4.11 (1H, m, H-2), 2.75 (1H, dd, J = 15.2, 6.4 Hz, H-3), 2.60 (1H, m, H-3′), 2.58 (2H, t, J = 7.3 Hz, H_2-15), 1.68 (2H, m, H_2-16), 1.00 (3H, d, J

= 6.8 Hz, H_3-1), δ 0.94 (3H, t, J = 8.3 Hz, H_3-17); ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; HRFT-ICRMS *m/z* 289.1437 $(M + H)^+$, calcd for $C_{17}H_{20}O_4$ 289.1434.

Phaeochromycin E (5): colorless amorphous glass, UV (MeOH), λ_{max} 232, 258sh, 270sh, 304; IR ($CDCl_3$) ν_{max} 3700, 2900, 1645, 1390, 1080, 1040 cm^{-1}, ^1H NMR (DMSO-d_6, 400 MHz) δ 7.65 (1H, dd, J = 8.5, 7.3 Hz, H-5), 7.48 (1H, d, J = 8.5 Hz, H-6), 7.21 (1H, d, J = 7.3 Hz, H-4), 6.13 (1H, s, H-10), 4.18 (2H, s, H_2-2), 2.64 (2H, t, J = 7.3 Hz, H_2-12), 1.78 (2H, m, H_2-13), 1.02 (3H, d, J = 7.3 Hz, H_3-14); ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; HRFT-ICRMS *m/z* 247.0965 $(M + H)^+$, calcd for $C_{14}H_{14}O_4$ 247.0965.

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