Inhibition of the Human Chemokine Receptor CCR5 by Variecolin and Variecolol and Isolation of Four New Variecolin Analogues, Emericolins A−D, from *Emericella aurantiobrunnea*

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Received May 3, 2004

An extract from the fungus *Emericella aurantiobrunnea* was found to compete with macrophage inflammatory protein (MIP)-1α for binding to human CCR5 in a scintillation proximity assay (SPA). Bioassay-guided fractionation led to the isolation of variecolin (**1**) and variecolol (**2**), which had IC50 values of 9 and 32 μM, respectively. An X-ray crystal structure of variecolin (**1**) was obtained for the first time. Also isolated were four new inactive analogues, emericolin A (**3**), B (**4**), C (**5**), and D (**6**), and the relative stereochemistry of these compounds was determined by NMR methods using ROESY spectra and $^1H/^1H$ coupling constants.

The human immunodeficiency virus Type 1 (HIV-1) uses chemokine receptors (principally CCR5 and CXCR4) as co-receptors with CD4 to gain entry into target cells.[1] As a consequence, molecules that bind to the CCR5 receptor could potentially prevent HIV-1 entry into cells and retard viral growth, making CCR5 an attractive drug target.[2] A review on synthetically derived small molecule CCR5 inhibitors has recently been published,[3] while previous reports from our own and other laboratories have described other natural product CCR5 inhibitors.[4]

An extract from the fungus *Emericella aurantiobrunnea* was found to compete with macrophage inflammatory protein (MIP)-1α for binding to human CCR5 in a scintillation proximity assay (SPA). Bioassay guided fractionation led to the purification of two known sesterterpenes, variecolin (**1**) and variecolol (**2**), and four new analogues, emericolin A (**3**), B (**4**), C (**5**), and D (**6**). Only variecolin (**1**) and variecolol (**2**) competed for MIP-1α binding to human CCR5 with IC_{50} values of 9 and 32 μM, respectively.

Variecolin (**1**) was first reported in 1991 from the fungus *Aspergillus variecolor* by Merck & Co. as an angiotensin II receptor binding inhibitor, and the relative stereochemistry was determined using $^1H/^1H$ coupling constants and NOE.[5] Variecolin (**1**) was isolated subsequently, along with emindole PA, from *Emericella purpurea* in 1994.[6] The next reports of variecolin-type sesterterpenes were described in two 1998 Japanese patents. One disclosed the isolation of S-1977 (**7**) (identical to AB5362-B and variecolactone) as an endothelin agonist, along with variecolin (**1**), from *E. aurantiobrunnea*.[7] The other disclosed the isolation of AB5362-B (**7**) (identical to S-1977 and variecolactone), AB5362-A (**8**), and AB5362-C (identical to variecolin (**1**)) as fungicides from a *Phoma* sp.[8] In 1999, Kawai and co-workers reported the isolation of variecolol (**2**) and variecolactone (**7**) from the *E. purpurea*.[9] The structure of **7** was confirmed by X-ray crystal analysis, while reduction of variecolin (**1**) with $NaBH_4$ gave a product that was identical to the natural variecolol (**2**).[9] These results confirmed the relative stereochemistry originally defined by the Merck group.[5] Two further variecolin analogues, variecoacetal A (**9**) and B (**10**), were reported along with variecolin (**1**) and variecolactone (**7**) as immunomodulatory constituents from *E. aurantiobrunnea* by Fujimoto and co-workers.[10] The absolute stereochemistry of variecolin (**1**) was established for the first time by derivatization with (2*R*,3*R*)-(−)-butane-2,3-diol.[10] To date, two articles have reported work toward the total synthesis of variecolin (**1**).[11,12]

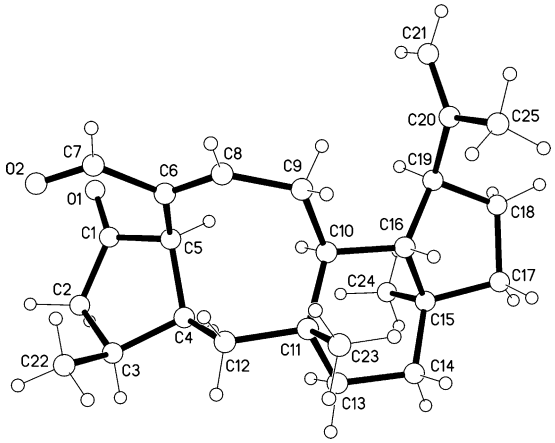

Figure 1. X-ray crystal structure of variecolin (**1**) (atoms numbered according to X-ray data).

Results and Discussion

CCR5 bioassay-guided fractionation of a 1:1 CH_2Cl_2−CH_3OH extract of a 2 L freeze-dried fermentation broth from the filamentous fungus *E. aurantiobrunnea* (F31149) gave variecolin (**1**) as the active component. Variecolin (**1**) was identical in all respects to that previously reported[5,8,10] and had an IC_{50} of 9 μM in the CCR5 assay. Further confirmation of the structure of **1** was obtained by X-ray crystallography on crystals of **1** obtained from MeOH (Figure 1).[13] Also isolated were variecolol (**2**)[9] and four new variecolin analogues, emericolin A (**3**), B (**4**), C (**5**), and D (**6**). Emericolin A (**3**), B (**4**), C (**5**), and D (**6**) were inactive in the CCR5 assay, while variecolol (**2**) was weakly active (IC_{50} 32 μM).

The molecular formula of **3** was determined to be $C_{25}H_{38}O_2$, 2H more than variecolin (**1**), on the basis of [M

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10.1021/np049844c CCC: $27.50
Published on Web 09/14/2004

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data of Emericolin A (**3**), B (**4**), C (**5**), and D (**6**) in $CDCl_3$

	3		**4**		**5**		**6**	
no.	^{13}C δ	^{1}H δ, m, J	^{13}C δ	^{1}H δ, m, J	^{13}C δ	^{1}H δ, m, J	^{13}C δ	^{1}H δ, m, J
1	39.5	1.15, m	42.6	0.95, m	38.0	0.94, m	41.9	1.19, m
1′		1.24, m		1.54, dd, 14.2, 11.1		1.79, m		1.67, dd, 14.6, 12.2
2	41.9	2.43, m	43.6	2.62, ddd, 11.4, 11.1, 6.6	43.5	2.64, ddd, 10.6, 7.1, 6.9	42.9	2.61, m
3	38.7	2.29, m	38.7	2.10, m	38.3	2.17, m	44.3	2.56, m
4	42.2	1.73, m	42.3	1.81, m	44.3	1.82, m	137.0	5.82, ddd, 5.7, 2.7, 2.1
4′				1.83, m				
5	76.9	4.64, m	76.5	4.75, ddd, 13.8, 6.8, 6.8	77.0	4.59, m	132.8	6.00, dd, 5.7, 3.2
6	53.8	3.05, m	52.1	3.10, m	53.8	3.10, m	48.0	3.96, br d, 9.2
7	143.2	–	136.7	–	133.1	–	133.6	–
8	162.4	6.77, td, 5.4, 1.6	134.9	5.84, ddd, 7.0, 2.3, 2.3	128.9	5.56, br d, 6.8	144.3	6.84, ddd, 7.6, 3.1, 3.1
9	31.5	2.25, m	31.2	1.78, m	31.3	1.65, m	31.8	2.03, dd, 12.6, 7.6
9′		2.75, m		2.47, m		2.39, m		2.61, m
10	41.3	2.04, m	40.9	2.09, m	41.8	2.18, m	42.2	2.29, m
11	39.2	–	39.7	–	39.5	–	40.2	–
12	35.2	0.98, dm, 13.6	37.4	0.93, m	42.7	0.95, m	37.1	1.00, m
12′		1.79, td, 13.6, 4.4		1.72, m		1.63, m		1.89, ddd, 13.8, 13.8, 4.6
13	36.3	1.42, m	36.7	1.36, m	36.8	1.36, m	36.5	1.41, m
13′		1.49, m		1.45, m		1.45, m		1.48, m
14	44.5	–	44.3	–	44.3	–	44.3	–
15	49.1	1.46, m	50.5	1.31, m	51.0	1.31, m	50.2	1.39, m
16	49.3	2.43, m	49.5	2.42, ddd, 11.1, 11.1, 5.3	49.6	2.44, m	49.5	2.45, ddd, 11.0, 11.0, 5.3
17	30.9	1.39, m	31.2	1.35, m	31.0	1.34, m	31.1	1.39, m
17′		1.99, m		1.99, m		1.98, m		1.99, m
18	40.9	1.25, m	41.1	1.20, m	41.1	1.19, dd, 11.6, 10.7	41.0	1.22, m
18′		1.45, m		1.42, m		1.40, m		1.42, m
19	16.4	0.84, d, 6.3	16.9	0.74, d, 7.2	17.2	0.73, d, 7.6	17.2	0.72, d, 6.9
20	200.5	9.30, s	71.1	3.94, d, 11.5	24.4	1.71, s	173.7	–
20′				4.30, br d, 11.5				
21	22.8	0.90, s	22.8	0.86, s	22.9	0.88, s	23.0	0.95, s
22	19.2	0.85, s	19.2	0.86, s	19.2	0.86, s	19.0	0.87, s
23	151.5	–	151.9	–	152.1	–	151.7	–
24	111.5	4.66, m	110.9	4.59, m	110.7	4.58, br s	111.2	4.61, d, 2.0
24′		4.77, br d, 2.2		4.71, br d, 2.2		4.69, br s		4.72, d, 2.0
25	20.0	1.73, m	20.1	1.70, br s	20.1	1.70, br s	20.1	1.71, br s

$- H]^-$ in the HRESIMS. Comparison of the NMR data of **3** (Table 1) and variecolin **1** indicated that the extra 2H in **3** were from reduction of the C-5 ketone present in **1** to an alcohol. Evidence for this structure was observed with NMR signals at δ 4.64 (H-5) and δ 76.9 (C-5), which were indicative of a secondary alcohol. COSY correlations from H-5 to H-4 and H-4′, along with HMBC correlations to C-3, C-4, and C-7, confirmed the position of the alcohol on ring A. The relative stereochemistry about ring A was determined by a ROESY correlation from H-6 to H-5. ROESY correlations and ^{1}H/^{1}H coupling constants were similar to those observed in variecolin (**1**), which allowed the relative stereochemistry to be assigned as shown, and the trivial name of emericolin A was assigned to **3**.

The molecular formula of emericolin B (**4**) was determined to be $C_{25}H_{40}O_2$, 2H more than emericolin A (**3**), on the basis of $[2M + Na]^+$ in the HRESIMS. NMR signals (Table 1) at δ 3.94 and δ 4.30 (^{1}H) and δ 71.1 (^{13}C) gave evidence for reduction of the C-20 aldehyde in **3** to a primary alcohol. COSY correlations from H_2-20 to H-8 and HMBC correlations to C-6, C-7, and C-8 confirmed the position of the alcohol group. A ROESY correlation from H-5 to H-2 indicated that ring A of **4** had the same relative stereochemistry as **3**.

Examination of the HRESIMS of emericolin C (**5**) indicated a molecular formula $C_{25}H_{40}O$. Comparison of the NMR data of **5** (Table 1) and emericolin B (**4**) showed the absence of resonances associated from the primary alcohol in **4** and the appearance of an olefinic methyl in **5**. Placement of the olefinic methyl at C-20 was confirmed by HMBC correlations to C-6, C-7, and C-8. A ROESY correlation from H-5 to H-2 indicated that **5** had the same relative stereochemistry around ring A as **3** and **4**.

Emericolin D (**6**) had a molecular formula of $C_{25}H_{36}O_2$, as determined by a HRESIMS on the $[M - H]^-$ mass ion peak, which was isomeric with variecolin (**1**). However, **6** differed from **1** by the presence of an acid (^{13}C: δ 173.7) instead of an aldehyde group and an extra double bond (^{1}H: δ 5.82, ddd, J = 5.7, 2.7, 2.1 Hz; 6.00, dd, J = 5.7, 3.2 Hz; ^{13}C: δ 137.0, 132.8) instead of a ketone. The ^{1}H/^{1}H coupling constant of 5.7 Hz between the double bond protons suggested that the double bond was in a five-membered ring,[14] which would most likely have originated from dehydration of the 5-OH group present in **3**, **4**, and **5**. This was confirmed by COSY correlations from H-2 and H-6 to δ 5.82 and δ 6.00, which placed the double bond at C-4/C-5 of ring A. This assignment was supported by HMBC correlations from δ 5.82 and δ 6.00 to C-2, C-3, C-6, and C-19 and the corresponding correlations from the ring A protons to C-4 and C-5. The acid group was assigned to C-20 by the observation of HMBC correlations from H-6 and H-8 to δ 173.7.

The 2,6-disubstituted 3-methyl-cyclopent-4,5-ene moiety present in ring A of **6** has been reported previously in the natural products literature in cylindramide (**11**)[15] and in the tentative structure of karatavin (**12**).[16] NMR spectra of **6** acquired using C_6D_6 as solvent were used for determination of the relative stereochemistry of ring A, as key resonances were overlapping in $CDCl_3$. ROESY correlations between H-2 and H-6 and between H_3-19 and H-1/H-1′ indicated a cis relationship between H-2 and H-6 and a trans relationship between H-2 and H_3-19. This assignment was consistent with the relative stereochemistry of variecolin but differed from that reported for cylindramide (**11**),[15] which has a cis ring junction with a cis relationship to the methyl group (Figure 2). The ^{1}H/^{1}H coupling constants of

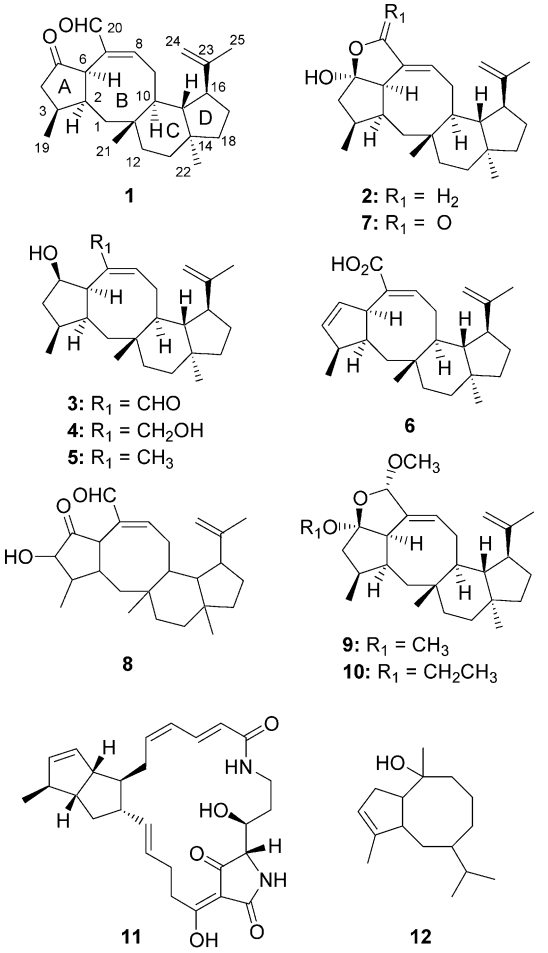

Figure 2.

H-2/H-6 in **6** and **11** were similar (9.2 and 9.5 Hz, respectively) as expected, while H-2/H-3 was 7.5 Hz in **6** and 2.3 Hz in **11**, which supported the different relative orientation of the methyl groups. The relative stereochemistry of the remaining part of the molecule was identical to that of variecolin (**1**).

Variecolin (**1**) and variecolol (**2**) had IC_{50} values of 9 μM and 32 μM, respectively, in the CCR5 assay, while emericolin A (**3**), B (**4**), C (**5**), and D (**6**) were inactive at the highest dose tested (33 μM). These data suggest that the C-4 ketone on ring A and the C-20 aldehyde on ring B in variecolin **1** are important for activity against the human CCR5 receptor.

Experimental Section

General Experimental Procedures. The melting point was uncorrected and was obtained on a Bausch and Lomb hot-stage microscope. NMR spectra were collected on a Bruker Avance DRX-500 NMR spectrometer, using 5-mm inverse (^{1}H, G-COSY, multiplicity-edited G-HSQC, G-HMBC, and G-ROESY spectra) or normal (^{13}C spectra) probeheads equipped with z-gradients. Spectra were calibrated to residual protonated solvent signals. HRESIMS values were collected on an Applied Biosystems Mariner TOF mass spectrometer, using sodium trifluoroacetate as an internal standard for both positive and negative ionization modes. Preparative HPLC was performed on a Gilson system complete with UniPoint software, 170 DAD detector, dual 306 pumps, 811C dynamic mixer, Gilson 202 fraction collector, and a Rheodyne 7125 injector with a 5-mL injection loop. Semipreparative HPLC was performed on a Waters system complete with Waters Millenium software, Waters 996 PDA detector, Waters 600 gradient controller and pump, Waters 717plus autosampler, and Waters Fraction Collector II fraction collector. TLC was carried out using Merck aluminum-backed silica gel 60 F_{254} plates. UV spectra were scanned on a Pharmacia Biotech Ultrospec 2000 and IR spectra on a Perkin-Elmer BioRad FT-IR spectrophotometer. Optical rotations were recorded on a Jasco DIP-1000 digital polarimeter.

Microorganism and Fermentation. The fungal strain was supplied by an external collaborator and is deposited in the MerLion Pharmaceuticals culture collection as strain F31149. The strain was identified by the collaborator as *E. aurantiobrunnea* (Atkins, Hindson, Russell) Malloch & Cain, and this identification was confirmed by us using morphological characteristics.[17] The strain was subcultured on malt extract agar plate (CM057B, Oxoid) for 7 days at 24 °C. It was used to inoculate 250-mL Erlenmeyer flasks, each containing 50 mL of seed medium composed of 0.4% glucose, 1% malt extract, and 0.4% yeast extract. The pH of the medium was adjusted to 5.5 before sterilization. The seed culture was incubated for 5 days at 24 °C on a rotary shaker at 200 rpm. A volume of 5 mL of seed culture was used to inoculate 50 mL of liquid medium in a 250-mL flask. The liquid medium was composed of 1.5% glucose, 2% cane molasses, 4% soluble starch, 0.8% $CaCO_3$, 2.5% Pharmamedia (Traders Protein), and 2% freshwater solution (Sigma W2004, ×50) and was autoclaved at 121 °C for 30 min. The fermentation was carried out for 9 days at 24 °C at 200 rpm.

X-ray Crystallography. The diffraction experiments were carried out on a Bruker AXS SMART CCD diffractometer at −50 °C. The program SMART[18] was used for collecting the intensity data, for indexing reflections, and for determining lattice parameters; SAINT[17] was used for integration of the intensity of reflections and scaling; SADABS[19] was used for absorption correction; and SHELXTL[20] was used for space group and structure determination and least-squares refinements on F^2. Anisotropic thermal parameters were refined for all the non-hydrogen atoms. All the hydrogen atoms were located in the difference Fourier; however, they were placed in their calculated positions for the purpose of the structure factor calculations only. Although **1** crystallized in the chiral space group, the absolute stereochemistry could not be determined reliably.[13]

Biological Assays. CCR5 receptor binding activity was determined in a 96-well SPA assay format[20] using [^{125}I]-human MIP-1α and membranes prepared from Chinese hamster ovary (CHO) cells overexpressing the human CCR5 receptor. The samples were dissolved in 12.5% aqueous DMSO and incubated with 12 μg of membranes, 0.17 nM [^{125}I]-MIP-1α, and 0.25 mg of Wheat Germ Agglutinin-SPA beads in assay buffer (50 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA, and a protease inhibitor cocktail) for 5 h at room temperature with shaking. Radioactivity (total binding) was measured after a 2-h bead settling period. Nonspecific binding was defined in the presence of 1 μM recombinant human MIP-1α. Human MIP-1α was also used as a reference compound and had an IC_{50} of 2.7 nM.

Extraction and Isolation. The freeze-dried fermentation broth (2 L) was extracted three times with CH_2Cl_2−CH_3OH (1:1) and evaporated to dryness under vacuum. The extract (3 g) was subjected to reverse-phase preparative HPLC (isocratic; 20 mL/min; (0.1% HCOOH in CH_3CN)/(0.1% HCOOH in H_2O) (7/3); Waters NovaPak radial cartridge column, 40 × 100 mm) to give emericolin B (**4**) (5 mg, RT 36 min) and variecolol (**2**) (10 mg, RT 68 min) and three semipure fractions. Fraction one (RT 45 min) was purified on reversed-phase semipreparative HPLC (isocratic; 4 mL/min; (0.1% HCOOH in CH_3CN)/(0.1% HCOOH in H_2O) (59/41); Thermoquest Phenyl column, 150 × 10 mm) to yield variecolin (**1**) (40 mg, RT 11 min) and emericolin C (**5**) (4 mg, RT 14 min). Fractions two (RT 41 min) and three (RT 75 min) were separated by preparative TLC using the solvent system CH_3OH/CH_2Cl_2 (0.5/99.5) to generate compounds emericolin A (**3**) (3 mg) and emericolin D (**6**) (4 mg), respectively.

Variecolin (1): colorless needles (CH_3OH); mp 158−159 °C; $[\alpha]_D$ −82° (*c* 1.16, CH_3CN) [lit. $[\alpha]_D$ −11.5° (*c* 0.50, CH_3CN);[5] $[\alpha]_D$ −72° (*c* 1.0, CH_3CN);[8] $[\alpha]_D$ −110.5° (*c* 0.50, CH_3CN);[10] $[\alpha]_D$ −84.4° (CH_3CN)];[10] identical in other respects to that previously reported.[5,8,10]

Variecolol (2): colorless powder; $[\alpha]_D$ −22° (*c* 0.95, $CHCl_3$); UV (CH_3OH) λ_{max} (log ϵ) 203 (3.62) nm; identical in other respects to that previously reported.[9]

Emericolin A (3): colorless powder; $[\alpha]_D$ +60° (*c* 0.17, CH_3CN); UV (CH_3OH) λ_{max} (log ϵ) 203 (3.78) and 241 (4.05) nm; IR (film) 3384, 2932, 1666, 1443 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; HR-ESI-MS (*m*/*z*): 369.2803 $[M - H]^-$ (calcd for $C_{25}H_{37}O_2$, 369.2794).

Emericolin B (4): colorless powder; $[\alpha]_D$ +36° (*c* 0.78, CH_3CN); UV (CH_3OH) λ_{max} (log ϵ) 203 (3.92) nm; IR (NaCl) 3350, 2942, 1642 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; HR-ESI-MS (*m*/*z*): 767.5916 $[2M + Na]^+$ (calcd for $C_{50}H_{80}O_4Na$, 767.5954).

Emericolin C (5): colorless powder; $[\alpha]_D$ +82° (*c* 0.18, CH_3CN); UV (CH_3OH) λ_{max} (log ϵ) 204 (3.81) nm; IR (NaCl) 3358, 2941, 1641, 1448 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; HR-ESI-MS (*m*/*z*): 357.3171 $[M + H]^+$ (calcd for $C_{25}H_{41}O$, 357.3157).

Emericolin D (6): colorless powder; $[\alpha]_D$ +37° (*c* 0.55, $CHCl_3$); UV (CH_3OH) λ_{max} (log ϵ) 203 (3.84) and 226 (3.53) nm; IR (film) 3100 (br), 2940, 1686, 1449 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; ^1H NMR (C_6D_6) δ 1.16 (H-1, m), 1.79 (H-1′, m), 2.65 (H-2, m), 2.51 (H-3 dq, J = 7.5, 7.0 Hz), 5.84 (H-4, m), 6.23 (H-5, dd, J = 5.5, 2.4 Hz), 3.98 (H-6, br d, J = 9.2 Hz), 6.85 (H-8, br d, J = 7.3 Hz), 1.91 (H-9, m), 2.59 (H-9′, m), 2.24 (H-10, m), 0.88 (H-12, m), 1.77 (H-12′, m), 1.34 (H-13, m), 1.35 (H-13′, m), 1.30 (H-15, m), 2.36 (H-16, ddd, J = 11.0, 11.0, 5.3 Hz), 1.34 (H-17, m), 1.91 (H-17′, m), 1.11 (H-18, m), 1.35 (H-18′, m), 0.86 (H-19, d, J = 7.0 Hz), 0.82 (H-21, s), 0.68 (H-22, s), 4.68 (H-24, br s), 4.78 (H-24′, br s), 1.67 (H-25, br d, J = 7.3 Hz); ^{13}C NMR (C_6D_6): δ 41.6 (C-1), 42.6 (C-2), 44.2 (C-3), 136.1 (C-4), 132.9 (C-5), 47.9 (C-6), 133.9 (C-7), 143.4 (C-8), 31.4 (C-9), 41.9 (C-10), 39.7 (C-11), 36.7 (C-12), 36.2 (C-13), 43.8 (C-14), 49.9 (C-15), 49.0 (C-16), 30.8 (C-17), 40.7 (C-18), 17 (C-19), 174.6 (C-20), 22.4 (C-21), 18.4 (C-22), 151.1 (C-23), 110.9 (C-24), 19.6 (C-25); HR-ESI-MS (*m*/*z*): 367.2648 $[M - H]^-$ (calcd for $C_{25}H_{36}O_2$, 367.2637).

Acknowledgment. We express our gratitude to GlaxoSmithKline, the Economic Development Board of Singapore, and the Institute of Molecular and Cell Biology for financial support.

Supporting Information Available: Variecolin (**1**) numbering system and crystallographic data and bond lengths and angles for variecolin (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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NP049844C