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Naturally produced isocoumarins: inhibitors of calmodulin-sensitive cyclic guanosine 3′,5′-monophosphate phosphodiesterase

V.R. Hegde[a], H. Wittreich[a], M.G. Patel[a], A.C. Horan[a], R.F. Hart[a], J.J. Troyanovich[a], M.S. Puar[b] and V.P. Gullo[a]

[a]*Microbial Products Division and* [b]*Molecular Spectroscopy Division, Schering Corporation, Bloomfield, NJ, U.S.A.*

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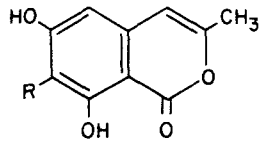
SUMMARY

Three isocoumarins have been isolated from a strain of *Streptoverticillium* sp. and all inhibit the calmodulin-sensitive cyclic guanosine 3′,5′-monophosphate phosphodiesterase (EC 3.1.4.17, Boehringer Mannheim). Two of the compounds, 6,8-dihydroxy-7-methoxy-3-methyl isocoumarin and 6,7,8-trihydroxy-3-methyl isocoumarin have previously been isolated from *Streptomyces*. The third fermentation product, 6,8-dihydroxy-3-methyl isocoumarin, was also found as a metabolite of *Ceratocystis minor*, a fungal species associated with the blue stain disease of pine [2,3].

INTRODUCTION

In the course of screening for novel pharmacologically active microbial products from the fermentations of soil microorganisms, we have isolated three isocoumarins (Fig. 1) from a *Streptoverticillium* sp. Two of these isocoumarins, 6,8-dihydroxy-7-methoxy-3-methyl isocoumarin (**1**) [6,10,11] and a trihydroxy analog (**2**) [6] have been isolated previously from a *Streptomyces* sp. A third fermentation product, 6,8-dihydroxy-3-methyl isocoumarin (**3**) has recently been isolated from static grown cultures of the fungus *Ceratocystis minor* [2,3]. These compounds inhibit the calmodulin-sensitive cyclic guanosine-3′,5′-monophosphate phosphodiesterase (Ca-PDE) and may promote relaxa-



1 R = -OCH3
2 R = -OH
3 R = H

Fig. 1. Structures of **1**, **2** and **3**.

Correspondence: V.R. Hegde, Microbial Products Division, Schering Corporation, Bloomfield, NJ 07003, U.S.A.

tion of vascular smooth muscle by this mechanism. Previously, 6,8-dihydroxy-7-methoxy-3-methyl isocoumarin was isolated as an inhibitor of cyclic adenosine-3',5'-monophosphate phosphodiesterase (EC 3.1.4c) [8]. This paper describes the producing culture, fermentation, isolation, structure and biological activity of these isocoumarins.

MATERIALS AND METHODS

Microorganisms

The producing culture, SCC 1864, was isolated from a Mexican soil sample by suspending 1 g of sieved soil in 10 ml of sterile tap water, plating the resulting suspension on y/s medium (yeast extract, 0.1%; soluble starch, 0.1%; Difco agar, 1.5%; in tap water) containing 5 μg/ml spectinomycin (Sigma) and incubating for 7–10 days at 30°C. Macroscopic and microscopic observations were performed using ATCC medium 172 [1] and water agar (Difco agar, 1.5% in tap water) after 21 days incubation at 28–30°C. Whole cell analysis was by the method of Lechevalier [9].

Fermentation

Two and a half milliliters of the producing culture, maintained at –20°C, was inoculated into 50 ml sterile seed medium consisting of (w/v): beef extract 0.3%, tryptone 0.5%, yeast extract 0.5%, cerelose 0.1%, potato starch (insoluble) 2.4%, and $CaCO_3$ 0.2% in a 250 ml Erlenmeyer flask. The resulting broth was incubated for 48 h at 30°C on a rotary shaker operating at 300 rpm, then used to inoculate (5% v/v) a second seed stage using the same conditions as the first seed stage.

The second seed culture (25 ml) was inoculated into 350 ml of the sterile production medium composed of (w/v): yeast extract 0.5%, corn steep liquor 0.1%, South African fish solubles 0.1%, potato starch (soluble) 3%, and $CaCO_3$ 0.1% in a 2 liter Erlenmeyer flask. Fermentation proceeded for 90 h at 30°C on a 300 rpm shaker.

Measuring phosphodiesterase activity

Phosphodiesterase (PDE) activity was measured as previously described [4]. Briefly, reaction mixtures that contained 20 mM imidazole pH 7.5, 5 mM $MgCl_2$, 200 μM EGTA (ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid), 300 μM $CaCl_2$, 1.6 nM calmodulin, 25 μM [^{3}H]cGMP (150 000 cpm, New England Nuclear), 25 μg bovine heart (Boehringer Mannheim), 5.6 units alkaline phosphatase (Sigma) and various concentrations of inhibitor were incubated at 37°C for 30 min. The reaction was stopped by addition of 500 μl AG1X8 anion exchange resin suspension; 500 μl isopropanol and 3 ml Aquasol were added prior to liquid scintillation counting. Samples were submitted for testing in either methanol or dimethyl sulfoxide with final solvent concentrations in the reaction mixture limited to less than 5%. At these concentrations, solvent-mediated inhibition of PDE activity was less than 10%.

Isolation

The whole broth (4 liters) was extracted twice with equal volumes of ethyl acetate. The extract was concentrated to an oil, dissolved in small volumes of acetone and precipitated with hexane. The resulting crude complex was dissolved in methanol (25 ml), applied to a column of Sephadex LH-20 (500 ml) and eluted with methanol. The fractions were monitored by Ca-PDE inhibition and the analytical reverse phase HPLC system described below. The major component **1** was further purified by preparative reverse phase HPLC on a Waters 30 × 2.5 cm μBondapak C-18 (10 μ) using methanol/water (60:40, v/v) as the eluting solvent system. The LH-20 fractions containing the minor components (components **2** and **3**) were separated on a Waters 30 × 0.46 cm, μBondapak C-18 column (10 μ), using 0.01 M NaH_2PO_4 (pH 3)/methanol (54:46, v/v) as the eluting system (flow rate, 1 ml/min; detection at 245 nm).

Ultraviolet spectra were obtained by using a Hewlett Packard '8450 A' UV-vis spectrophotometer equipped with HP-9872B plotter. Infrared spectra were recorded by using a Nicolet FTIR model 10-MX instrument. All FAB mass spectra were obtained by using a Varian MAT-312 mass spectrometer in a glycerol-thioglycerol matrix. NMR spectra were measured on a Varian XL-300

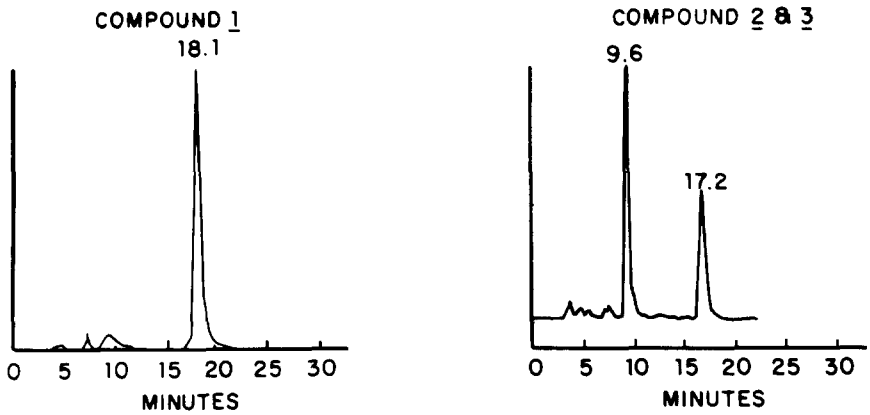


Fig. 2. Analytical HPLC profiles of the pure component **1** and the mixture of minor components **2** and **3**, as eluted from the LH-20 column. See Materials and Methods for the chromatographic conditions.

instrument operating at 300 and 75 MHz for ^{1}H- and ^{13}C-NMR respectively. Spectra were obtained in deuteriochloroform solution.

RESULTS AND DISCUSSION

Strain SCC 1864 forms yellow-brown to brown vegetative mycelial pigments, and yellow-brown soluble pigments. The aerial mycelia are gray-white and form characteristic branches in whorls at regular intervals along its length. The branches fragment into chains of spores. Whole cells contain LL-diaminopimelic acid. Based on the producing cultures distinctive morphology and the presence of the LL-isomer of diaminopimelic acid, SCC 1864 was identified as a species of *Streptoverticillium*.

Fermentation of SCC 1864 proceeded as described in the Materials and Methods section. The pH at harvest for different fermentations ranged from 6.6 through 6.9 and the inhibition of Ca-PDE ranged from 52 to 74%. From a 4 liter fermentation which inhibited Ca-PDE at 70%, 58.2, 4.8 and 3.2 mg of **1**, **2** and **3** were obtained, respectively. Fig. 2 shows the analytical HPLC profiles of the pure component **1** and the mixture of minor components, **2** and **3**, as eluted from the LH-20 column.

The physico-chemical properties of the isolated compounds are tabulated in Table 1. The UV absorption maxima (245, 278, 326 nm) are all similar

Table 1

Physico-chemical data of **1**, **2** and **3**

	1	**2**	**3**
UV (MeOH) λ_{max}, nm	245,278,325	242,278,326	249,279,327
IR (Nujol)ν_{max}, cm^{-1}	3450,1680,1650,1080,720	3360,3260,1680,1180	3250,1680,1630,1180
FAB MS $(M + 1)^+$	223	209	193
^{1}H-NMR ($CDCl_3$), δ	2.25 (S,3H), 4.03 (S,3H), 6.17 (S,1H), 6.40 (S,1H), 6.5 (S,1H)*, 11.3 (S,1H)*	2.25 (S,3H), 5.6 (bS,1H),* 6.1 (bS,1H)*, 6.18 (S,1H), 6.45 (S,1H), 11.05 (S,1H)*	2.25 (S,3H), 6.17 (S,1H), 6.3 (d,J = 2 Hz,1H), 6.45 (d,J = 2Hz, 1H), 8.77 (S,1H)*, 11.20 (S,1H)*

* These protons are exchangeable by D_2O.

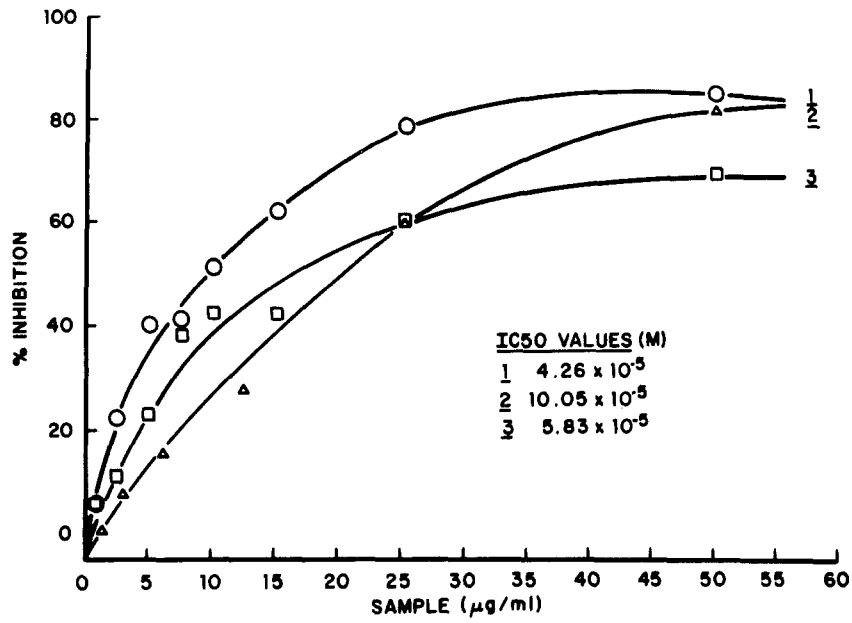


Fig. 3. Inhibition of calmodulin-sensitive cyclic nucleotide phosphodiesterase by various isocoumarins. Phosphodiesterase activity was determined in the presence of various concentrations of **1** (○), **2** (△) and **3** (□) as described in Materials and Methods. IC_{50} values were obtained by linear regression.

and are characteristic of coumarin type compounds. The IR absorption band at 1680 cm^{-1} is characteristic of α,β-unsaturated lactones and confirmed the coumarin nature. Comparison of the melting point and all the spectral data of **1** with naturally occurring known coumarins identified **1** as 6,8-dihydroxy-7-methoxy-3-methyl isocoumarin [6,10,11]. Similarly compound **2**, which has a molecular weight 14 mass units less than **1**, was identified as 6,7,8-trihydroxy-3-methyl isocoumarin [6]. Compound **3** showed a protonated molecular ion at 193, corresponding to a molecular weight of 192, which is 30 and 16 mass units less than **1** and **2** respectively, suggesting it to be a dihydroxy compound. The 200 MHz NMR showed proton peaks at δ 6.3 and δ 6.45 that are coupled by 2 Hz, which is indicative of meta coupling. The compound was assigned structure **3**, i.e. 6,8-dihydroxy-3-methyl isocoumarin [2,3,5,12].

A comparison of the potencies of **1, 2** and **3** as inhibitors of Ca-PDE is shown in Fig. 3. Compound **1**, reticulol, is the most potent compound. Reticulol has been reported previously to be an inhibitor of cAMP-PDE, $IC_{50} = 4.1 \times 10^{-5}$ M [7,8].

REFERENCES

1 American Type Culture Collection Catalogue of Strains I, 15th Edn. 1982. ATCC, Rockville, M.D.

2 Ayer, W.A., B. Kratochvil, E. Allen, L.M. Browne, C. Dufresne, D. Figueroa and A. Szenthe. 1986. The chemistry of the blue stain fungi, Part 2. Some essential metal levels of diseased and healthy lodgepole pine. Can. J. Chem. 64: 910–913.

3 Ayer, W.A., B. Kratochvil, E. Allen, L.M. Browne, C. Dufresne, D. Figueroa and A. Szenthe. 1987. The chemistry of the blue stain fungi, Part 3. Some metabolites of *Ceratocystis minor* (Hedgcock) Hunt. Can. J. Chem. 65: 765–769.

4 Brooker, G., L.J. Thomas and M.M. Appleman. 1968. The assay of adenosine 3′,5′-cyclic monophosphate and guanosine 3′,5′-cyclic monophosphate in biological materials by enzymatic radioisotopic displacement. Biochemistry 7: 4177–4181.

5 Curtis, R.F., P.C. Harries and C.H. Hassall. 1964. Biosynthesis of phenols, Part VIII. The synthesis of (2-carboxy-3,5-dihydroxyphenyl) propane-2-one (c-acetyl-o-oosellinic acid). J. Chem. Soc., 5382–5385.

6 Eaton, M.A.W. and D.W. Hutchinson. 1971. Isocoumarins from *Streptomyces mobaraensis*. Tetrahedron Lett. 18: 1337–1340.

7 Furutani, Y., M. Shimada, M. Hamada, T. Takeuchi and H. Umezawa. 1977. Reticulol, an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. Agric. Biol. Chem. 4: 989–993.

8 Furutani, Y., M. Shimada, M. Hamada, T. Takeuchi and H. Umezawa. 1977. Reticulol, an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. J. Antibiot. 28: 558–560.

9 Lechevalier, M.P. 1968. Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934–944.

10 Lin, J., S. Yoshida and N. Takahashi. 1971. Metabolites produced by *Streptomyces mobaraensis*. Agric. Biol. Chem. 35: 363–369.

11 Mitscher, L.A., W.W. Andres and W. McCrae. 1962. Reticulol, a new metabolic isocoumarin. Experientia 20: 258–259.

12 Money, T., I.H. Qureshi, G.B. Wibster and A.I. Scott. 1965. Chemistry of polypyrones, a model for acetogenin biosynthesis. J. Am. Chem. Soc. 87: 3004–3005.