Isolation and Structure of New Isocoumarins[†]Yoshio FURUTANI,* Hiroshi NAGANAWA, Tomio TAKEUCHI
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Received December 23, 1976

Besides reticulol, the strain MD611-C6 produced two compounds which inhibited cyclic nucleotide phosphodiesterases [EC 3.1.4.C.] These substances were isolated and their structures were elucidated to be 8-hydroxy-6, 7-dimethoxy-3-hydroxymethylisocoumarin (II) and 6, 8-dihydroxy-7-methoxy-3-hydroxymethylisocoumarin (III). Concentrations of II and III for 50% inhibition of cAMP phosphodiesterase were 3.97×10^{-4} M and 1.26×10^{-3} M, respectively.

As reported in our previous paper,¹⁾ an inhibitor of cAMP phosphodiesterase (PDE) were isolated and identified as reticulol (6,8-dihydroxy-7-methoxy-3-methylisocoumarin). Furthermore, the strain MD611-C6 which produced reticulol was found to produce other two active substances. These active substances were isolated and their structures were determined by derivation to dimethyl ether of reticulol. In this paper, isolation and structural determination of these active substances are reported.

MATERIALS AND METHODS

Assay methods of cyclic AMP phosphodiesterase which were reported in the previous paper¹⁾ were employed. The culture method of MD611-C6 was also reported in the previous paper.¹⁾ UV spectra were recorded on Hitachi EPS-3T spectrophotometer. IR spectra were measured on Hitachi EPI 510 spectrophotometer. NMR spectra were taken on Varian XL-100 NMR spectrometer. Reticulol (70 mg/0.6 ml) and compound III (58 mg/0.6 ml) dissolved in DMSO-*d*₆ were run in 5 mm tube at 33°C. The NOE were observed after those solutions were warmed at 60°C for 3~5 min.

RESULTS

Isolation of active substances

[†] Inhibitors of Cyclic Nucleotide Phosphodiesterases Produced by Streptomyces. Part II.

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The strain MD611-C6 was cultured in the medium described in the previous paper.¹⁾ The culture filtrate was extracted with the same volume of butyl acetate at pH 2.0. The extract was concentrated under reduced pressure to a brown syrup. The syrup was submitted to a silica gel column chromatography. Elution was conducted with chloroform and chloroform-methanol mixture with increasing content of methanol. Elution pattern and amounts of active substances are summarized in Table I. Compound I, II, III were further purified by recrystallization and colorless needles were obtained. Amounts of purified compounds I, II, III were 210, 21.0 and 37.5 mg, respectively, as shown in Table I.

TABLE I. ELUTION PATTERN AND AMOUNTS OF ACTIVE SUBSTANCES

Active substances and order of elution	Solvent system	Amounts of substances (mg)
Compound I	CHCl ₃	446
Reticulol	"	690
Compound II	CHCl ₃ : CH ₃ OH (100: 1)	88.4
Compound III	" (100: 2)	84.2

UV spectra of compound I, II, III

UV spectra of compound I, II, III and reticulol in neutral, acidic and basic solutions are shown in Fig. 1. Since the spectra of compound I, II, III are similar to that of reticulol, it was presumed that compound I,

FIG. 1. UV Absorption Spectra of Reticulol, Compound I, Compound II and Compound III.

Panel A: Compound I, B: Reticulol, C: Compound II, D: Compound III. Strait line: 0.4 $\mu\text{g/ml}$ in methanol and in 0.01 N HCl-methanol, dotted line: 0.4 $\mu\text{g/ml}$ in 0.01 N NaOH-methanol.

II, III also had isocoumarin skeleton. But, when their spectra were examined more in detail, there were two distinctive characters. First, compound III and reticulol showed a shoulder at 240 nm, but compound I and II didn't. Second, the spectra of compound III and reticulol showed red shift in basic solution, compound I and II showed blue shift. From these results, it was suggested that compound III had partially the same structure with reticulol, and compound I did with compound II. Furthermore, the red shifts (about 10 nm) of the main absorption bands of reticulol and compound III were also observed in sodium acetate-ethanol solution, but no significant changes were given in the spectra of compound I and compound II. The same spectral changes as described above were

reported in isoflavones and flavones with or without free hydroxy group at 7-position. From these observations, it was assumed that compound III has free hydroxy group at 6-position as reticulol, but 6-hydroxy group of compound I and compound II are masked.

NMR spectra of reticulol and compound III

The chemical shifts of signals in PMR spectrum of compound III are very similar to those of reticulol except that the signal assigned to methyl protons (τ : 7.82 (3H, s)) in reticulol is substituted by methylene protons (τ : 5.70 (2H, b)) and hydroxy proton (τ : 4.40 (1H, b)) which was assumed to be hydroxy-methyl group at 3-position. Especially, it was characteristic that the two signals assigned to hydroxy protons had almost the same chemical shift (τ : -0.7 (1H, bs), τ : -1.0 (1H, s)) in both compounds (reticulol and compound III).

TABLE II. NOE OF RETICULOL AND COMPOUND III

	Irrad. H. (τ value)	Observed H ^{a)}	
		4-H	5-H
Reticulol	3-CH ₃ (7.82)	39.2	—
	6-OH (-0.63)	—	13.4
Compound III	3-CH ₂ (5.70)	11.5	—
	6-OH (-0.70)	—	26.2

^{a)} Percent value of single enhancement.

Further confirmation of the structure was achieved by observation of the NOE of reticulol and compound III. As shown in Table II, the integral value of the aromatic proton (τ : 3.40) was increase of 26% by irradiation of the hydroxy proton (τ : -0.70) of compound III but that of the other aromatic proton (τ : 3.46) was not affected. The NOE of the former proton was not observed by irradiation of the methylen protons of compound III but 11.5% NOE of the latter proton was. From these results, the signal at τ : 3.46 of compound III was assigned to 4-H which was at ortho position to the methylen group, and the signal of τ : 3.40 assigned to 5-H adjacent to 6-hydroxy group. On the other hand, reticulol showed 39.2% or 13.4% NOE of the aromatic proton (τ : 3.60 (1H, s) or τ : 3.58 (1H, s)) by irradiation of the 6-hydroxy proton (τ : -0.63).

tion of the methyl protons or 6-hydroxy proton (τ : -0.63), respectively. According to these results and UV data, it was elucidated that compound III had the same structure except 3-hydroxymethyl group as reticulol.

Methylation of reticulol and compound III

Monomethyl ether of reticulol was obtained by methylation with diazomethane. This compound showed the same IR spectrum with compound I and did not depress the melting point of compound I. Hence it was determined that compound I was 8-hydroxy-6,7-dimethoxy-3-methylisocoumarin. This compound was already isolated by Lin *et al.*²⁾ and by Eaton *et al.*³⁾ from streptomyces culture filtrates. Monomethyl ether of compound III was obtained by methylation in methanol-diethylether (1:1) with diazomethane. IR spectrum of this compound was identical with that of compound II but was not with those of compound I and reticulol. Therefore it was confirmed that compound II was the monomethyl ether of compound III.

Derivation from compound II to dimethylether of reticulol

From the data of UV, NMR, mass spectroscopy and elemental analysis, it was suggested that compound II had the same structure with

reticulol except for the substitution of the 3-methyl residue by hydroxymethyl. Therefore, as shown in Fig. 2, the hydroxymethyl group of compound II was tried to methyl group of reticulol. In the first step, compound II was methylated to compound IV with diazomethane. In the second step, compound IV was chlorinated to compound V in chloroform solution with thionylchloride.⁴⁾ In the last step, compound V was reduced to compound VI in dimethyl sulfoxide solution with sodiumborohydride.⁵⁾ Compound VI obtained by reduction gave the same IR spectrum and melting point with dimethyl ether of reticulol. As described above, it was elucidated that compound III had the same structure except 3-methyl residue as reticulol from UV and NMR data. Thus, it was confirmed that the structures of compound II and compound III were 8-hydroxy-6,7-dimethoxy-3-hydroxymethylisocoumarin and 6,8-dihydroxy-7-methoxy-3-hydroxymethylisocoumarin respectively. Both compounds are new isocoumarins. Concentrations required for 50% inhibition of PDE (Ic 50) in our assay system are shown in Table III. Compound II showed the same Ic 50 value as theophylline and compound III did ten times larger value. Compound I also showed low inhibition percent but its Ic 50 value could not be measured, since its solu-

FIG. 2. Derivation of Compound II, III to Compound VI.

TABLE III. I_c 50 FOR cAMP PDE

Inhibitor	I_c 50
Reticulol	$3.38 \times 10^{-5}(M)$
Compound II	3.97×10^{-4}
Compound III	1.26×10^{-3}
Theophylline	2.8×10^{-4}

I_c 50, inhibitor concentration in the reaction mixture for 50% inhibition; substrate, 0.1 mM cAMP; enzyme, 27 μ g prot./ml.

bility to water was very low. It is interesting that the activity is influenced by small structural variations.

8-Hydroxy-6,7-dimethoxy-3-methylisocoumarin (I)

mp 196~196.5°C. λ_{max}^{MeOH} nm (ϵ): 243 (41,900), 280 (8,260), 336 (4,370). ν_{max}^{KBr} (cm^{-1}): 1678.

8-Hydroxy-6,7-dimethoxy-3-hydroxymethylisocoumarin (II)

mp 158.5~160°C. λ_{max}^{MeOH} (ϵ): 243 (45,700), 281 (6,930), 335 (5,800). NMR (DMSO- d_6) τ : 6.25 (3H, s), 6.07 (3H, s), 5.70 (2H, d), 4.36 (1H, t), 3.33 (1H, s), 3.18 (1H, s), -0.86 (1H, s). Ms: M^+ , m/e , 252. Found: C, 56.27; H, 4.66; O, 35.81; Calcd. for $C_{12}H_{12}O_6$: C, 57.14; H, 4.80; O, 38.06%.

6,8-Dihydroxy-7-methoxy-3-hydroxymethylisocoumarin (III)

mp 186~188°C. λ_{max}^{MeOH} nm (ϵ): 247 (41,600), 281 (5,950), 333 (5,360). NMR (DMSO- d_6) τ : 6.20 (3H, s), 5.70 (2H, b), 4.40 (1H, b), 3.45

(1H, s), 3.40 (1H, s), -0.70 (1H, b), -1.03 (1H, s). Ms: M^+ , m/e , 238. Found: C, 55.29; H, 4.13; O, 38.39. Calcd. for $C_{11}H_{10}O_6$: C, 55.46; H, 4.23; O, 40.30%.

Derivation from reticulol to compound I

Reticulol (40 mg) was kept at room temperature for 30 min in methanol-diethyl ether (1:1) containing excess of diazomethane. Monomethyl ether (38 mg) of reticulol was isolated by tlc (thin-layer chromatography) on silica gel plates using chloroform. IR spectrum of this compound was identical with that of compound I and did not depress the melting point of compound I.

6,7,8-Trimethoxy-3-hydroxymethylisocoumarin (IV)

Compound III (105 mg) was kept at room temperature for 16 hr in methanol-diethyl ether (1:1) containing excess of diazomethane. Dimethyl ether (IV, 105 mg) of compound III was isolated by silica gel column chromatography developed with chloroform. mp 141~142°C. λ_{max}^{MeOH} nm (ϵ): 247 (40,600), 280 (4,389), 328 (2,930). ν_{max}^{KBr} (cm^{-1}): 1688. NMR (chloroform- d_1) τ : 7.40 (1H, t), 6.08 (3H, s), 6.00 (3H, s), 5.98 (3H, s), 5.50 (2H, d), 3.80 (1H, s), 3.40 (1H, s). Ms: M^+ , m/e , 266.

6,7,8-Trimethoxy-3-chloromethylisocoumarin (V)

Compound IV (74.5 mg) was chlorinated at room temperature for 23 hr in chloroform-pyridine (6:1) with 0.5 ml of thionylchloride.

FIG. 3. IR Spectrum of Compound II in KBr Disk.

FIG. 4. IR Spectrum of Compound III in KBr Disk.

After the solution was washed with water, compound V (43.9 mg) was isolated by tlc on silica gel plates using chloroform-ethylacetate (9:1). mp 147~147.6°C. $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ): 247 (64,300), 280 (8,580), 320 (4,070). $\nu_{\text{max}}^{\text{KBr}}$ (cm^{-1}): 1710. NMR (chloroform- d_1) τ : 6.08 (3H, s), 6.00 (3H, s), 5.98 (3H, s), 5.62 (2H, s), 3.53 (1H, s), 3.36 (1H, s) Ms: M^+ , m/e , 284.

Reduction of compound V

Compound V (22.4 mg) was reduced at 20°C for 4 hr in 1 ml of dimethyl sulfoxide with 21.5 mg of sodium borohydride. The solution was washed with water after small volume of chloroform was added. Reduced compound (14.8 mg) was obtained by tlc on silica gel plates using benzene-ethyl acetate (7:3). This compound showed the identical IR spectrum and melting point with dimethyl ether of reticulol.

Dimethyl ether of reticulol (VI)

Reticulol (38.2 mg) was heated under reflux

for 6 hr in 20 ml of dry acetone containing 160 mg of potassium carbonate and 0.2 ml of dimethyl sulfate. After small volume of water was added to the reaction mixture, dimethyl ether of reticulol was extracted with chloroform. The extract was dried up and the reaction product (23 mg) was purified by silica gel column chromatography developed with chloroform. mp 117~118°C. $\lambda_{\text{max}}^{\text{MeOH}}$ nm (ϵ): 247 (49,800), 278 (6,250), 330 (4,150). $\nu_{\text{max}}^{\text{KBr}}$ (cm^{-1}): 1710. NMR (chloroform- d_1) τ : 7.72 (3H, s), 6.05 (3H, s), 6.00 (3H, s), 5.97 (3H, s), 3.88 (1H, s), 3.46 (1H, s). Ms: M^+ , m/e , 250.

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