NEW ANTIBIOTICS, METHYLENOMYCINS A AND B

II. STRUCTURES OF METHYLENOMYCINS A AND B

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The structure of methylenomycin A was studied physico-chemically and finally established by X-ray crystallographic analysis as 2-methylene-cyclopentane-3-one-4,5-epoxy-4,5-dimethyl-1-carboxylic acid. The structure of methylenomycin B was also determined by a comparative study of the nuclear magnetic resonance spectra of methylenomycins A and B to be 4,5-epoxy-4,5-dimethyl-2-methylene-cyclopentane-3-one, which makes it a decarboxylated derivative of methylenomycin A.

Methylenomycins A and B, produced by *Streptomyces violaceoruber* No. 2416, are new antibiotics with inhibitory activity against gram-positive and gram-negative bacteria, and especially, against *Proteus*. The taxonomy of the producing organism and production and characteristics of these antibiotics were reported in the previous paper.[1] The present paper deals with the structural elucidation of methylenomycins A and B.

Structural Elucidation of Methylenomycin A

Methylenomycin A (**I**), $C_9H_{10}O_4$(M^+182), is an acidic (pKa′ 3.65), lipophilic, colorless crystalline substance; m.p. 115°C (dec.), $[\alpha]_D^{20}$+42.3°(*c* 1, $CHCl_3$). The IR spectrum of methylenomycin A showed absorptions at 3500~2500 and 1720 cm^{-1} due to a carboxyl group. An enol or cisoid carbonyl group absorption was found at 1740 cm^{-1} and an absorption band at 1650 cm^{-1} indicated the existence of an unsaturated double bond. The NMR spectrum taken at 100 MHz in $CDCl_3$ showed two methyl signals at δ 1.58 and 1.48, two protons due to an ethylenic double bond at δ 5.65 (doublet J=1.7 Hz) and 6.27 (doublet, J=1.9 Hz), one proton coupled with two protons of the terminal methylene group at δ 3.82 (multiplet, J=1.9 and 1.7 Hz) and one proton exchangeable with D_2O, due to the carboxyl group, at δ 9.95. The UV spectrum of methylenomycin A showed a maximum at 224 mμ (ε 6,300), which indicated the possible existence of an α,β-unsaturated carbonyl function. The relationship between the carbonyl group and the terminal methylene in the molecule was provided by the reduction of each functional group.

Hydrogenation of methylenomycin A on palladium-carbon yielded dihydroderivative (**III**), $C_9H_{12}O_4$, *m/e* 184, in which the presence of three methyl groups was noted in the NMR spectrum (δ=1.55, 1.44 and 1.05) and characteristics of methylenomycin A, such as its UV absorption maximum at 224 nm and IR absorption band at 1650 cm^{-1} due to the conjugated double bond, disappeared.

On $NaBH_4$ reduction, compound **III** afforded quantitatively a crystalline monohydroxy derivative (**VII**), $C_9H_{14}O_4$, *m/e* 186, which showed only the IR absorption band in the carbonyl

region at 1710 cm^{-1}, attributable to the carboxyl group. This compound **VII** was readily regenerated to compound **III** by JONES oxidation. In the NMR spectrum of compound **VII**, the C_2-H multiplet, centered at δ 2.25, was coupled to the C_3-H, at δ 3.92 with a coupling constant J=5.0 Hz, and to C_1-H, at δ 3.07 with coupling constant J=7.5 Hz. A similar relationship was also observed between C_1-H (δ 3.44) and C_2-H (δ 2.83) in compound **III** (J=8.0 Hz).

Reaction of methylenomycin A with diazomethane provided a methyl ester C_2-2,3-pyrazoline derivative (**IV**). The diazomethane adduct was further converted to C_2-ethylidene methyl ester (**XI**) and to C_2-cyclopropane methyl ester (**XII**) on heating the adduct in toluene at 120°C for 30 minutes.

Treatment of methylenomycin A with bromine yielded crystalline dibromo-methylenomycin A (**VI**). Its NMR spectrum in $CDCl_3$ showed that addition of bromine to the terminal methylene resulted in alteration of two protons of the terminal methylene to an A B type quartet and also gave a sharp singlet signal corresponding to a proton attached to the carbon atom bearing the carboxyl group.

These facts indicated that methylenomycin A possessed a terminal methylene conjugated to a ketone group and the NMR splitting patterns of C_2-H in hydrogenated derivative (**III**) indicated the presence of partial structural unit $O=\overset{|}{C}-CH(CH_3)-\overset{|}{C}H-COOH$ in the molecule. Meanwhile, the product derived from compound **III** by alkaline degradation, $C_5H_6O_4$, m.p. 202~204°C was identified with mesaconitic acid (methyl fumaric acid) (**XXX**) by comparative IR and NMR spectra studies with an authentic preparation.

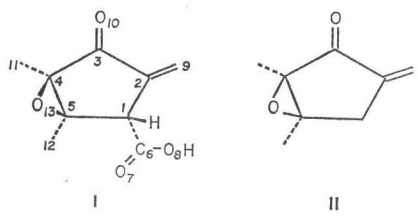
These results also supported the presence of a partial structural unit

$$O=\overset{|}{C}-C(=CH_2)-\overset{|}{C}H-COOH$$

in methylenomycin A.

To account for the remaining two unsaturations, methylenomycin A was examined to ascertain the arrangement of the remaining one oxygen atom, two carbon atoms bearing no hydrogen atom, and two methyl groups.

Chart 1



I II

By treatment of compound **III** with 85% phosphoric acid at 60°C for 30 minutes, a neutral crystalline compound, **X**, $C_9H_{12}O_4$ was obtained. Its IR spectrum showed a characteristic absorption band at 1800 cm^{-1}, which was consistent with the presence of a β-lactone. The NMR spectrum showed a proton at C_1 of δ 3.00 (doublet, J=4.5 Hz), a proton at C_2 of δ 3.05 (multiplet J=4.5 and 7.0 Hz), a methyl group at C_2 of δ 1.20 (doublet, J=7.0 Hz), and two singlet methyl groups of δ 1.33 and 1.56. On the other hand, compound **VIII** treated with alkaline aqueous methanol provided compound **XXVII** ($C_{10}H_{14}O_4$). Its NMR spectrum showed the absence of a proton at C_1 observed in the starting material as a doublet, and the alteration of multiplet to quartet on a proton at C_2 (J=8.0 Hz).

The formation of these two compounds suggested cleavage of an epoxide ring in methylenomycin A by treatment with acid or base. Therefore, taking into consideration the remain-

ing group to be placed: two methyl groups, two carbon atoms bearing no hydrogen atom, and one unsaturation, structure **I** was deduced as the most possible for methylenomycin A.

Independently, methylenomycin A was submitted to X-ray crystallographic analysis. The crystal data of this compound are: a=7.34, b=10.00, c=12.22 Å, space group $P2_12_12_1$, D_{obs}=1.34 g/cm^3, D_{calc}=1.35 g/cm^3. Reflection data were collected on a Rigaku four-circle auto diffractometer up to a 2θ limit of 60° using Mo-Kα radiation. A total of 875 reflections were used for the subsequent structure analysis. By the application of the tangent formula of HAUPTOMAN and KARLE[2], the structure was satisfactorily determined. The five reflections taken as starting phases were as follows: 5,0,14=0°, 7,0,11=90°, 7,7,0=−90°, 6,1,0=90°, and 0,7,3=a. The 6 peaks of the 13 highest peaks on the resulting E-map (as a=90°), corresponded to a part

Table 1. Fractional coordinates of methylenomycin A

Atom	X/a	Y/b	Z/c
C 1	0.2226	0.3151	0.3368
C 2	0.2656	0·4341	0.2667
C 3	0.3065	0.3895	0.1536
C 4	0.2548	0.2430	0.1445
C 5	0.2033	0.1980	0.2554
C 6	0.3725	0.2826	0.4191
C 7	0.3440	0.2475	0.5108
C 8	0.5371	0.2955	0.3777
C 9	0.2615	0.5550	0.2968
O 10	0.3690	0.4557	0.0808
C 11	0.3158	0.1546	0.0529
C 12	0.2054	0.0562	0.2923
O 13	0.0636	0.2360	0.1772
H 1	0.103	0.322	0.378
H 8	0.617	0.248	0.433
H 9A	0.300	0.616	0.274
H 9B	0.244	0.588	0.367
H 11A	0.433	0.177	0.040
H 11B	0.255	0.205	0.012
H 11C	0.239	0.078	0.029
H 12A	0.341	0.053	0.346
H 12B	0.211	0.000	0.245
H 12C	0.110	0.054	0.336

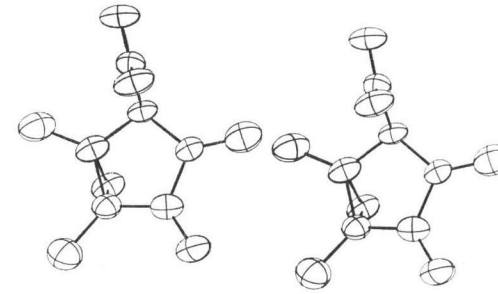
Table 2. Bond lengths (a) and angles (b) of methylenomycin A

(a) Bond lengths	
C 1—C 2	1.500
C 2—C 3	1.484
C 3—C 4	1.518
C 4—C 5	1.477
C 5—C 1	1.543
C 1—C 6	1.526
C 6—C 7	1.192
C 6—O 8	1.316
C 2—C 9	1.264
C 3—O 10	1.200
C 4—C 11	1.494
C 4—O 13	1.461
C 5—C 12	1.488
C 5—O 13	1.452

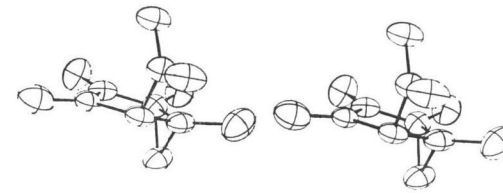
(b) Bond angles	
C 2—C 1—C 5	104.7
C 2—C 1—C 6	113.2
C 5—C 1—C 6	109.3
C 1—C 2—C 3	109.6
C 1—C 2—C 9	126.0
C 3—C 2—C 9	124.3
C 2—C 3—C 4	108.0
C 2—C 3—O 10	127.1
C 4—C 3—O 10	125.0
C 3—C 4—C 5	106.9
C 3—C 4—C 11	123.4
C 3—C 4—O 13	105.4
C 5—C 4—C 11	125.7
C 5—C 4—O 13	59.2
C 11—C 4—O 13	117.7
C 1—C 5—C 4	109.7
C 1—C 5—C 12	121.8
C 1—C 5—O 13	106.9
C 4—C 5—C 12	124.4
C 4—C 5—O 13	59.8
C 12—C 5—O 13	117.1
C 1—C 6—O 7	123.7
C 1—C 6—O 8	112.8
O 7—C 6—O 8	123.4
C 4—O 13—C 5	61.0

of the molecular feature with reasonable bond lengths and angles. Successive FOURIER and least-squares refinements using these peaks, established the whole molecular structure as **I**, and the R-factor dropped to 7.9%. The final atomic parameters and the bond lengths and angles are listed in Tables 1 and 2 (a), (b), respectively. Figure 1 (a), (b) shows the stereographic views of this compound in which the relation between the carboxylic acid group and the epoxide group is trans. The carboxylic acid group is hydrogen-bonded to another carboxylic acid group of the neighboring molecule along the b-axis with a value of 2.67 Å. Very short intermolecular atomic contact is observed at C9....O13, with a value of 3.01 Å. The dihedral angle between the five membered ring and the epoxide group is 86°. The molecular structure of methylenomycin A thus determined explains reasonably all its physical and chemical properties.

Fig. 1. Stereodiagram of methylenomycin A
(a) Viewed along the a axis.



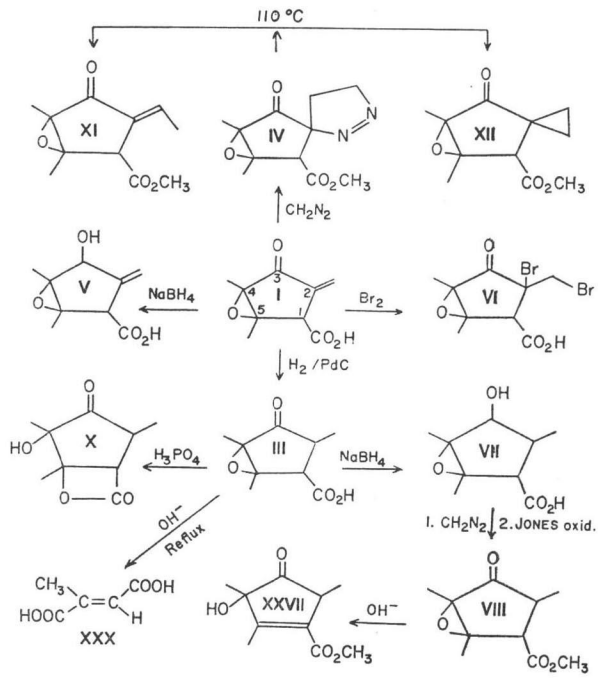
(b) Viewed along the c axis.



Structural Elucidation of Methylenomycin B

The structural investigation of methylenomycin B was mainly carried out by comparative studies of its NMR spectral data with those of methylenomycin A. Two signals, a multiplet

Chart 2



at δ 3.82 due to a proton on a carbon atom bearing carboxyl group and a singlet at δ 9.95 due to carboxyl group were only found in methylenomycin A, while a methylenic proton at δ 3.08 was only observed in methylenomycin B. All of the other eight protons (terminal methylene, and two methyl groups) were common to these two antibiotics.

From these results and its other physical and chemical properties, the structure of methylenomycin B was determined as **II**, a decarboxylated form of methylenomycin A.

Experimental

Melting points were measured by a Yamato micro-melting point apparatus and were uncorrected. UV spectra were run on a Hitachi 124 recording spectrophotometer and IR spectra on a Hitachi infrared spectrophotometer. Optical rotations were determined with Perkin-Elmer 141 polarimeter.

All NMR spectra were run on a Varian model NMR spectrometer and chemical shifts were measured to an internal standard, TMS, and were recorded as δ values.

Compound **III**. Hydrogenation of compound **I** (550 mg) in 20 ml of acetone over Pd-carbon (200 mg) was carried out at atmospheric pressure for 30 minutes at room temperarure. Approximately one equivalent of hydrogen was absorbed. The reaction mixture was filtered to remove the catalyst and concentrated to dryness. The residue was purified on a preparative silica gel thin-layer plate (Merck Co., Ltd., Silica gel plate 2.0 mm, F_{254}) using a mixture of benzene-MeOH-AcOH (45:8:4) as a solvent system and the band was eluted with the same solvent mixture. On evaporation of the solvents, 420 mg of **III** was obtained as colorless plates; mp 78~79°C, *m/e* 184, IR $\nu_{max.}^{CHCl_3}$ 1750, 1710 cm^{-1}; UV, end absorption; NMR, $\delta_{ppm}^{CDCl_3}$ 1.05 (d, 3H, $-\underset{|}{C}H-CH_3$, J=8.0 Hz), 2.83 (m, 1H, $-\underset{|}{C}H-CH_3$, J=7.5 and 8.0 Hz), 3.44 (d, 1H, $HOOC-\underset{|}{C}H-\underset{|}{C}H-CH_3$, J=7.5 Hz) and 9.74 (bs, 1H, HOOC–). Found: C, 59.21; H, 6.75 %. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57 %.

Compound **IV**. Compound **I** (1.2 g) was dissolved in 10 ml of ethylacetate and ethereal diazomethane was added until bubbling ceased and the solution was allowed to stand for 2 hours at room temperature with occasional stirring. After removal of the solvent under reduced pressure, the residue was washed with *n*-hexane, and then it was purified by preparative silica gel thin-layer plate using a solvent system of EtOAc-acetone (9:1) and elution with acetone. Finally **IV** was obtained as colorless needles (890 mg); mp 95~96°C; *m/e* 238; IR $\nu_{max.}^{KBr}$ 1745, 1735, 1555, 1348 and 1170 cm^{-1}; NMR $\delta_{ppm}^{CDCl_3}$ 3.77 (s, 3H, $-COOCH_3$), 4.53 (m, 2H, $-CH_2-CH_2-N=$, J=18, 8 and 8 Hz), 1.75 (m, 2H, $-CH_2-CH_2-N=$, J=18, 8 and 8 Hz) and 3.25 (s, 1H, $CH_3OOC-\underset{|}{C}H-$). Found: C, 55.58; H, 5.80; N, 11.93 %. Calcd. for $C_{11}H_{14}O_4N_2$: C, 55.45, H, 5.92; N, 11.76 %.

Compound **V**. Compound **I** (3.0 g) dissolved in 20 ml of EtOH, was reduced with 0.6 g of $NaBH_4$ at 0°C. After addition of 10 ml of distilled water, the reaction mixture was concentrated to 5 ml, acidified with dil. HCl to pH 2.0, saturated with sodium chloride and extracted with EtOAc. The extract was concentrated to yield 2.9 g of **V** as colorless oil; *m/e* 184, UV end absorption; NMR, $\delta_{ppm}^{CDCl_3}$ 3.48 (s, 1H, $-\underset{|}{C}H-COOH$), 4.58 (bs 1H, $-\underset{|}{C}H-OH$). Found: C, 59.0; H, 6.65 %. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57 %.

Compound **VI**. To compound **I** (200 mg) dissolved in 20 ml of $CHCl_3$ was added an appropriate aliquot of bromine solution at room temperature and the reaction mixture was stirred for one hour. After evaporation of the solvent under vacuum, the resultant crystalline residue was recrystallized from benzene as colorless needles (210 mg); mp12 4°C; IR $\nu_{max}^{CHCl_3}$ 3500~2500 cm^{-1}, 1705 cm^{-1}. Found: C, 32.08; H, 2.93; Br, 46,57 %. Calcd. for $C_9H_{10}Br_2O_4$: C, 31.58; H, 2.92; Br, 46.78 %.

Compound **VII**. Comopund III (1.0 g), dissolved in 30 ml of EtOH, was reduced with 400 mg of $NaBH_4$ at 0°C. After addition of 30 ml of distilled water to the reaction mixture, it was concentrated to 15 ml under vacuum and acidified with dil. HCl to pH 2.0 and extracted with EtOAc. The extract was washed with water saturated with sodium chloride and concentrated to dryness. The crude compound **VII** thus obtained was purified by column chromatography on silica gel packed with benzene, eluted with the same solvent to obtain compound **VII** as colorless needles (420 mg); mp, 123~124°C; *m/e* 186; IR $\nu_{max}^{CHCl_3}$ 3500~2500, 1700 cm^{-1}; NMR $\delta_{ppm}^{CDCl_3}$ 1.0 (d, 3H, -CH-$\underline{CH_3}$, J=7.5 Hz), 2.25 (m, 1H, -CH(OH)-$\underline{CH}$(CH_3)-CH(COOH)-, J=7.5, 7.5 and 5.0 Hz), 3.07 (d, 1H, -$\underline{CH}$(COOH)-CH(CH_3)-, J=7.5 Hz), and 3.92 (d, 1H, -$\underline{CH}$(OH)-CH(CH_3)-, J=5.0 Hz). Found: C, 57.94, H, 7.58 %. Calcd. for $C_9H_{14}O_4$: C, 58.05; H, 7.58 %.

Compound **VIII**. Ethereal diazomethane was added to compound **VII** (400 mg) dissolved in 10 ml of $CHCl_3$ until the end of bubbling. The resultant methyl ester of compound **VII** was further treated with JONES reagent to obtain compound **VIII**. JONES oxidation was carried out by addition of JONES reagent (2 ml) to the acetone solution of methyl ester of compound **VII** (3 ml). The upper layer of the reaction mixture was separated and washed with water saturated with sodium chloride. The product was purified on a silica gel column packed with benzene followed by elution with the same solvent. Compound **VIII** was obtained as colorless oil (305 mg); *m/e* 198; IR $\nu_{max}^{CHCl_3}$ 1745, 1710 cm^{-1}; NMR $\delta_{ppm}^{CDCl_3}$ 3.78 (s, 3H, -$COOCH_3$). Found: C, 60.55; H, 7.14 %. Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12 %.

Compound **X**. Compound **III** (1.0 g) was treated with 85 % phosphoric acid at 80°C for 20 minutes and the reaction mixture was extracted with EtOAc. The extract was concentrated to dryness. Then compound **X** was purified on the preparative thin-layer plate of silica gel, developing with EtOAc. Elution of the thin-layer plate with EtOAc gave colorless needles of **X** (272 mg); mp 86~89°C; *m/e* 184; IR $\nu_{max}^{CHCl_3}$ 3600, 3400, 1800, 1775, 1040, 915 cm^{-1}; NMR $\delta_{ppm}^{CDCl_3}$ 1.20 (d, 3H, -CH-$\underline{CH_3}$, J=7.0 Hz), 3.00 (d, 1H, -CH(CH_3)-$\underline{CH}$-, J=4.5 Hz), 3.05 (m, 1H, -$\underline{CH}$(CH_3)-CH-, J=7.0 and 4.5 Hz). Found: C, 58.11; H, 6.63 %. Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57 %.

Compound **XI** and **XII**. Compound **IV** (700 mg) was treated with toluene at 110°C for 30 minutes. Compound **XI** and **XII** (1:1 mixture) was obtained with simultaneous liberation of nitrogen. They were separated on a preparative thin-layer plate of silica gel by developing with the solvent system of EtOAc-benzene (1:9). Finally 260 mg of compound **XI** and 244 mg of compound **XII** were obtained, respectively.

Compound **XI**. Oil; *m/e* 210; IR ν_{max}^{Liq} 1740, 1720, 1625 cm^{-1}; UV λ_{max}^{MeOH} 240 nm (ε 7600); NMR $\delta_{ppm}^{CDCl_3}$ 2.14 (dd, 3H, -CH($\underline{CH_3}$)=C(CH-$COOCH_3$)), J=7.5 and 2.0 Hz), 3.74 (bs, 1H, $CH(CH_3)$=C-$\underline{CH}$-$COOCH_3$), 4.30 (m, 1H, $\underline{CH}$(CH_3)=C-, J=7.5 and 1.5 Hz). Found: C, 63.26; H, 6.79 %. Calcd. for $C_{11}H_{14}O_4$: C, 62.84; H, 6.71 %.

Compound **XII**. Oil; *m/e* 210; IR ν_{max}^{Liq} 1730, 1260 cm^{-1}; UV λ_{max}^{MeOH} 210 nm shoulder at 240 nm; NMR $\delta_{ppm}^{CDCl_3}$ two methylene groups at cyclopropane ring 0.86 and 1.20 (m, each 2H, J= 10.0, 4.0 and 3.0 Hz), 3.08 (s, 1H, -C-$\underline{CH}$-$COOCH_3$), and 3.73 (s, 3H, -$COO\underline{CH_3}$). Found: C, 62.55; H, 6.79 %. Calcd. for $C_{11}H_{14}O_4$: C, 62.84; H, 6.71 %.

Compound **XXVII**. Alkaline degradation of compound **VIII** afforded compound **XXVII**. Compound **VIII** (250 mg) was dissolved in 10 ml of 1 N NaOH in 50 % aq. MeOH and left for 30 minutes at 50°C. After evaporation of the reaction mixture under reduced pressure, the concentrate was extracted with ether and purified on a preparative thin-layer plate of silica gel developed with benzene and eluted with EtOAc (148 mg); colorless oil *m/e*; 198; IR $\nu_{max}^{CHCl_3}$ 1750, 1710 cm^{-1}; NMR $\delta_{ppm}^{CDCl_3}$ 1.03 (d, 3H, -CH-$\underline{CH_3}$, J=8.0 Hz), 1.67 (s, 3H,

$-\underset{|}{C}(OH)-\underline{CH_3}$) 2.00 (s, 3H, $-C(\underline{CH_3})=$), 2.66 (q, 1H, $-\underline{CH}-CH_3$, J=8.0 Hz), 3.66 (s, 3H, $-COO\underline{CH_3}$). Found; C, 60.33; H, 7.21 %. Calcd. for $C_{10}H_{14}O_4$: C, 60.59; H, 7.12 %.

Compound **XXX**. Compound **III** was refluxed in 30 % NaOH solution for 3 hours and the pH was adjusted to 8.0. The reaction mixture was applied to a Dowex 1×1 ($HCOO^-$ form) column and eluted with 0.5 N HCOOH. The eluate was concentrated *in vacuo*. The residue was crystallized from a mixture of EtOH and EtOAc. Thus compound **XXX** was obtained as colorless plates; mp 204~205°C; IR $\nu_{max}^{CHCl_3}$ 1700, 1650, 900 cm^{-1}; UV λ_{max}^{MeOH} 222 nm; NMR $\delta_{ppm}^{DMSO-d_6}$ 2.14 (3H), 6.64 (1H). Found: C, 46.42, H, 4.72 %. Calcd. for $C_5H_6O_5$: C, 46.16; H, 4.65 %.

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