STUDIES ON CELL GROWTH STIMULATING SUBSTANCES OF LOW MOLECULAR WEIGHT

PART 3. RESORCININ, A MAMMALIAN CELL GROWTH STIMULATING SUBSTANCE PRODUCED BY *Cytophaga johnsonae*†

Sir:

In the course of our screening program for mammalian cell growth stimulating substances[1,2], a gliding bacterium, *Cytophaga johnsonae* AJ No. 12589, isolated from Kanagawa Prefecture, Japan, was found to produce a substance stimulating the growth of NIH 3T3 mouse fibroblast cells in the presence of only 1% of fetal calf serum (FCS). Without addition of this substance, the cells did not show any growth under the same conditions. In this communication, we report isolation, characterization, and structural elucidation of the compound named resorcinin.

Cytophaga johnsonae AJ No. 12589 was cultivated at 28°C in a 30-liter jar fermentor containing the medium composed of casitone 2% and $MgCl_2$ 0.2% with agitation rate of 210 rpm and air flow of 0.25 v/v/m. After cultivation for 24 hours, the mycelium was collected by centrifugation from 20 liters of the fermentation broth and was extracted with acetone. The extract was concentrated to a small volume and the aqueous residue was extracted three times with EtOAc. The solvent layer was evaporated and the residue was subjected to silica gel column chromatography developed with $CHCl_3$. The active fraction was concentrated to dryness and further subjected to silica gel column chromatography (hexane - EtOAc, 15 : 1). The active fraction thus obtained was purified by Sephadex LH-20 column chromatography ($CHCl_3$ - MeOH, 1 : 1).

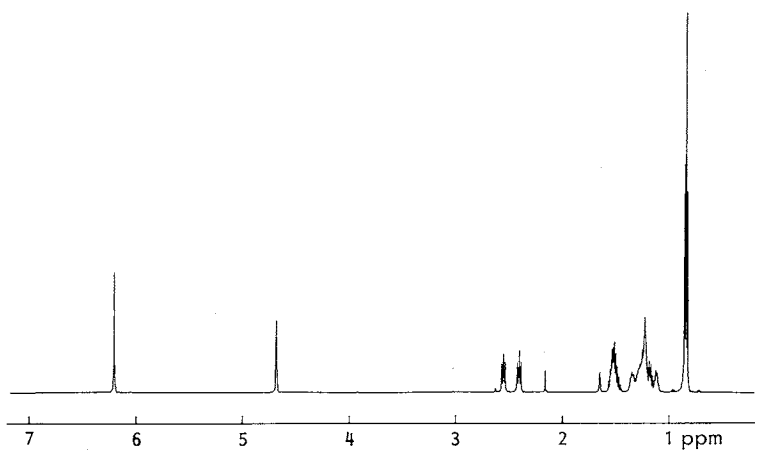
Evaporation of the active fraction gave resorcinin as a pale yellow powder (70 mg).

The physico-chemical properties of resorcinin were as follows: MP 75~78°C; UV λ_{max}^{hexane} nm (ε) 270 (1,500), 274 (1,400), 278 (1,300); $[\alpha]_D^{25}$ −5° (*c* 0.5, $CHCl_3$); IR (KBr) cm^{-1} 3400, 2925, 1630, 1580, 1465, 1445, 1385, 1365, 1165, 837. HRFAB-MS of resorcinin showed the molecular ion peak at *m/z* (M+H) 349.3095 corresponding to the molecular formula $C_{25}H_{40}O_2$ (calcd for $C_{25}H_{41}O_2$, 349.3107).

The 500 MHz ^1H NMR spectrum of resorcinin taken in $CDCl_3$ is shown in Fig. 1. There were observed two identical aromatic protons (6.220 ppm, 2H, s), two equivalent hydroxyl functions (4.700 ppm, 2H, s, disappeared with D_2O), two triplet methylenes (2.569 ppm, 2H, and 2.423 ppm, 2H), unresolved methines and methylenes (1.1 ppm to 1.6 ppm, total 20H) and two pairs of equivalent methyls (0.854 ppm, 6H, d, and 0.866 ppm, 6H, d). The presence of equivalent hydroxyl functions and two identical aromatic methines, the UV absorptions at 274 nm and 278 nm (characteristic for the dihydroxybenzene moiety)[3,4] and the IR absorptions at 1630, 1580 and 837 cm^{-1} suggested that resorcinin was a 2,5-dialkylresorcinol derivative[5].

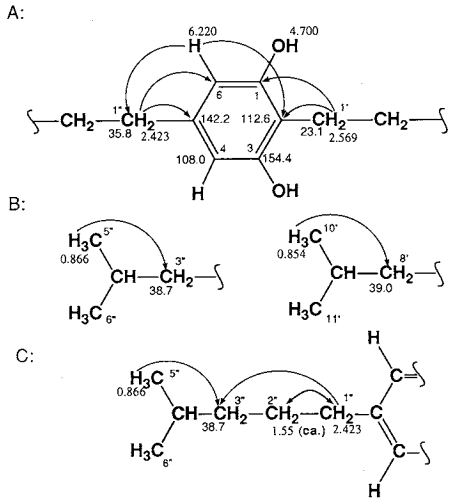
The ^1H-^1H COSY, ^1H-^{13}C COSY and heteronuclear multiple-bond correlation[6] (HMBC) ex-

Fig. 1. 500 MHz ^1H NMR spectrum of resorcinin in $CDCl_3$.



† For part 2[2].

Fig. 2. Partial structures of resorcinin.



$\longrightarrow$ ^{13}C-^{1}H long range couplings.
$\longleftrightarrow$ ^{1}H-^{1}H coupling.

periments confirmed this structure. Partial structures, revealed by the 2D NMR techniques, are summarized in Fig. 2. Thus, ^{13}C-^{1}H long range couplings from 1′-H benzylic protons (2.569 ppm, t) to C-1, C-3 (two equivalent signals, 154.4 ppm) and C-2 (112.6 ppm), from two equivalent 4-H and 6-H aromatic protons (6.220 ppm, s) to C-2 (112.6 ppm) and C-1″ methylene (35.8 ppm), and from 1″-H benzylic protons (2.423 ppm, t) to C-4, C-6 (two equivalent signals, 108.0 ppm) and C-5 (142.2 ppm) proved the presence of a 2,5-dialkylresorcinol moiety (Fig. 2A).

Since two equivalent methyl protons at 0.854 ppm (10′-H and 11′-H, d) were coupled with the C-8′ methylene carbon (39.0 ppm) and other two equivalent methyl protons (0.866 ppm, 5″-H and 6″-H, d) were coupled with the C-3″ methylene carbon 38.7 ppm), the two alkyl side chains were elucidated to be of iso-type (Fig. 2B). In addition, C-3″ methylene carbon (38.7 ppm) was long-range coupled to the benzylic methylene protons (2.423 ppm, 1″-H, t), which were coupled to the 2″-H methylene protons (1.55 ppm, 2H) as revealed by ^{1}H-^{1}H COSY experiments. These relationships proved that one of the side-chain located at the resorcinol nucleus was an isohexyl residue (Fig. 2C). Consequently, the other side chain was determinated to be an isoundecyl group by elimination. This structure was corroborated by EI-MS experiments.

In the EI-MS spectrum, two fragment ions at *m/z* 207 and 278 were interpreted as caused by cleavage

Fig. 3. Structure and EI-MS fragmentations of resorcinin.

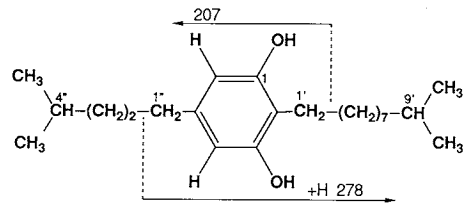


Table 1. ^{1}H and ^{13}C NMR assignments of resorcinin (500 MHz and 125 MHz, $CDCl_3$).

	δ_H	δ_C
1, 3	—	154.4
2	—	112.6
4, 6	6.220 (s)	108.0
5	—	142.2
1′	2.569 (t)	23.1
2′	*ca.* 1.54	29.3
8′	*ca.* 1.14	39.0
9′	*ca.* 1.51	28.0
10′, 11′	0.854 (d)	22.65[a]
1″	2.423 (t)	35.8
2″	*ca.* 1.55 (m)	28.9
3″	*ca.* 1.19 (m)	38.7
4″	*ca.* 1.53 (m)	27.9
5″, 6″	0.866 (d)	22.57[a]

The hydroxy protons at C-1 and C-3 were observed at 4.700 ppm as singlets.

[a] Assignments may be exchangeable. The ^{1}H signals from 3′ to 7′ were observed as envelopes from 1.16 ppm to 1.42 ppm (total 10H). The ^{13}C signals from 3′ to 7′ were observed as the following five signals: 29.9, 29.8, 29.7, 29.6 and 27.4 ppm.

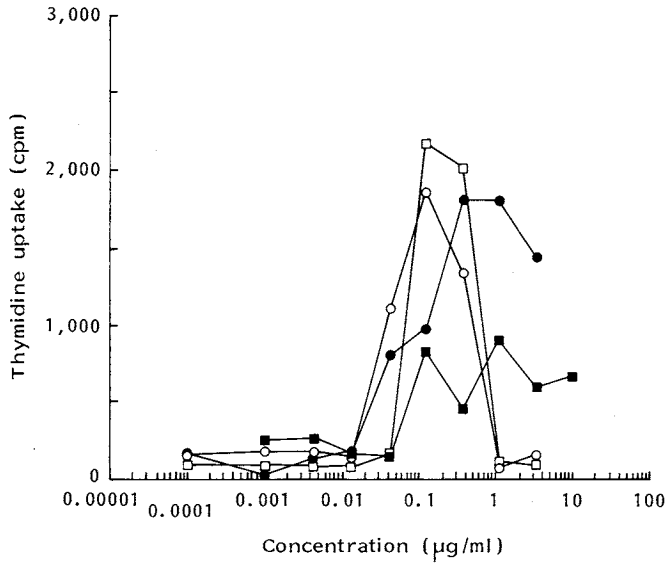
at benzylic positions[5] as shown in Fig. 3. The fragment ion at *m/z* 207 confirmed the presence of the isohexyl side-chain and the fragment ion at *m/z* 278 revealed that the other side chain is of the isoundecyl type.

By these studies the structure of resorcinin was elucidated to be 2-isoundecyl-5-isohexylresorcinol as shown in Fig. 3. The partial ^{1}H and ^{13}C NMR assignments are summarized in Table 1.

Resorcinin proliferated NIH 3T3 cells in the presence of 1% FCS at low dose. The growth-stimulative activity was measured as follows: NIH 3T3 cells were cultured in DULLBECCO's Modified Eagle Medium containing 10% FCS until reaching before the semiconfluent stage. The cells were trypsinised and re-suspended in 1% FCS containing DULLBECCO's Modified Eagle Medium (96-well micro-plates, each well containing 100 μl). Two

Fig. 4. Growth stimulative activities of resorcinin and its related compounds against NIH 3T3 cells.

□ Resorcinin, ○ 3-*n*-pentadecylphenol, ● 5-methylresorcinol, ■ resorcinol.



hours later, test samples were added as methanol solutions. The activities were measured by cell-number counting or [^{3}H]thymidine uptake (pulsing for 1~2 hours) after 24~48 hours later.

The stimulative activities of resorcinin and its structural analogs against NIH 3T3 cells in 1% FCS as measured by [^{3}H]thymidine uptake is shown in Fig. 4. Resorcinin proliferates the growth of NIH 3T3 cells at the concentration from 0.2 to 2 μg/ml. The [^{3}H]thymidine uptake level increased from 80 cpm to over 2,000 cpm by the addition of 0.2 μg/ml of resorcinin. This activity is slightly higher than that of 3-*n*-pentadecylphenol, a compound with a long alkyl side chain. On the other hand, resorcinol derivatives with a short alkyl chain or without a substituent are apparently less active than resorcinin. This result implies that the presence of at least one long alkyl side chain is important for the cell growth promoting activity for phenol derivatives. The toxicity of resorcinin for mammalian cells is low with IC_{50} against P388 murine leukemia being 9.8 μg/ml. Although resorcinin had been synthesized by ACHENBACH *et al.*[7], no biological activity was reported.

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