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Synthesis and Biological Activity of Fungal Metabolite, 4-Hydroxy-3-(3'-Methyl-2'-Butenyl)-Benzoic Acid

KIM, HYE JIN AND HO JEONG KWON*

Chemical Genomics Laboratory, Department of Biotechnology, College of Engineering, Yonsei University, Seoul 120-749, Korea

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Abstract 4-Hydroxy-3-(3'-methyl-2'-butenyl)-benzoic acid (HMBA) was previously isolated from *Curvularia* sp. KF119 as a cell-cycle inhibitor. However, the present study used a novel and practical synthetic method to prepare a large quantity of HMBA. The synthetic HMBA was found to inhibit the cell-cycle progression of HeLa cells with a comparable potency to the natural fungal metabolite. The inhibition of the cell-cycle progression by the synthetic HMBA involved both the activation of $p21^{WAF1}$ and the inhibition of cyclin D1 expression in the cells. Consequently, this new synthetic procedure provides an easy and convenient way to produce or manipulate the original fungal metabolite.

Keywords: Fungal metabolite, HMBA, cell-cycle inhibition, synthesis of natural product

As the cell cycle of eukaryotes is orchestrated by the products of oncogenes, as well as tumor suppressor genes [15], deregulation of the eukaryotic cell cycle leads to abnormal cellular proliferation, a characteristic and major factor in the development of many diseases, including cancer [17]. Therefore, small molecules that can correct an aberrant progression of the eukaryotic cell cycle have attracted attention as potential antitumor agents [5, 6, 13].

Fungi are valuable natural sources for a number of important clinical drugs. Indeed, many small molecules from fungi are known to inhibit tumor growth and control cell functions [1, 7, 9, 11]. For example, 4-hydroxy-3-(3'-methyl-2'-butenyl)-benzoic acid (HMBA, **compound 1**), isolated from *Curvularia* sp. KF119, is a novel cell-cycle inhibitor (Fig. 1) [9] that induces $p21^{WAF1}$ expression and inhibits the expression of the positive cell-cycle regulator, cyclin D1. However, its low yield from the fungus and poor structural diversity have made the compound's action mechanism difficult to explore in detail, including the identification of its cellular target protein. Accordingly, this study presents a new and practical synthetic method for producing HMBA, and then the cell-cycle inhibitory activity of the synthetic HMBA is investigated to validate its biological activity.

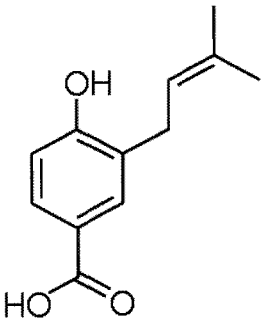

Fig. 1. Chemical structure of 4-hydroxy-3-(3'-methyl-2'-butenyl)-benzoic acid (HMBA, **compound 1**).

The synthesis of HMBA is outlined in Fig. 2. A mixture of 7 g of sodium cut into small pieces, 300 ml of dry toluene, and 50 g of ethyl p-hydroxy benzoate (**compound 2**) was heated under reflux with stirring for 5 h. The mixture was then cooled in an ice-bath and stirred, while 50 g of 1-bromo-3-methyl-2-butene was added dropwise during a period of two hours. Thereafter, the reaction mixture was stirred for a further 15 h at room temperature, and then warmed to 50°C. After filtration, the sodium bromide was removed and the toluene solution concentrated under reduced pressure. The heavy oil was then extracted with ether and dried over magnesium sulfate. The resulting cyclohexane layer was dried over magnesium sulfate and concentrated *in vacuo*. The residue was then dissolved in a boiling

*Corresponding author
Phone: 82-2123-5883; Fax: 82-2-362-7265;
E-mail: kwonhj@yonsei.ac.kr

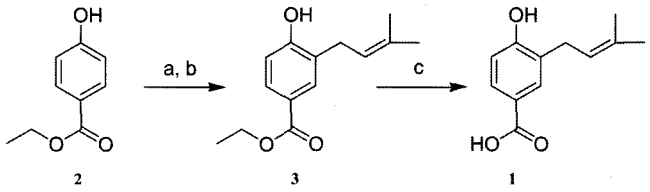

Fig. 2. Reagents and synthetic conditions for HMBA: (**a**) Na, dry toluene, reflux, 5 h; (**b**) 1-bromo-3-methyl-2-butene, rt, 15 h, then 50°C, 0.5 h; (**c**) 4 N NaOH, 40°C, 4 h.

mixture of equal parts of cyclohexane and petroleum ether. After cooling at room temperature for a few hours and at room temperature overnight, the solid was collected. A solution of 7 g of ethyl-4-hydroxy-3-(3-methyl-2-butenyl)-benzoate (**compound 3**) in 30 ml of 4 N NaOH was heated on the steam-cone for 4 h. After cooling in an ice-bath, the solution was acidified with dilute hydrochloric acid and extracted with ether. The combined ether extracts were washed with water, dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The residue was then dissolved in hot benzene and crystallized by the slow addition of cyclohexane. Finally, the crude solid product was washed by n-hexane to produce synthetic HMBA as a white solid with a 62% yield. The synthetic compound was identified as HMBA on the basis of FAB-MS, and ^{1}H and ^{13}C NMR spectral data, and these data were confirmed by comparison with published data (data not shown) [9].

Following the synthesis, the effect of the synthetic HMBA on the proliferation of HeLa cells was investigated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [2, 3, 8]. The HeLa cells were seeded at a density of 5×10^3 cells per well in a 96-well culture plate. The synthetic HMBA (100–300 μg/ml) was then added to each well and the plate incubated for up to 72 h. After 72 h, 50 μl of MTT (2 mg/ml) was added to each well, and the cells incubated for a further 4 h at 37°C. The supernatant was then removed, and 150 μl of dimethylsulfoxide (DMSO) added to dissolve the formazan products. The absorbance was determined spectrophotometrically at 570 nm using an ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.).

The synthetic HMBA was found to inhibit the HeLa cell proliferation with an IC_{50} value of 150 μg/ml. No cytotoxicity was observed up to 300 μg/ml with the synthetic HMBA, as shown by the trypan blue staining (Table 1), implying that the cell-cycle inhibitory activity of the synthetic compound was not merely derived from the cytotoxic effect of the compound on the cells [11]. Natural HMBA from fungal extracts also exhibited similar biological activities to the synthetic HMBA.

Table 1. Inhibitory activity of synthetic HMBA on HeLa cell growth.

Compounds	Growth inhibition IC_{50}, μg/ml	Cytotoxicity IC_{50}, μg/ml
Synthetic HMBA	150	>300
HMBA isolated from *Curvularia* sp. KF119	150	>300

Table 2. Effect of synthetic HMBA on cell-cycle progression of HeLa cells.

Treatment	G_0-G_1	S	G_2-M
Control	43.3	46.11	10.58
Compound 1 (200 μg/ml)	53.82	29.07	17.11
TSA (0.2 μg/ml)	51.48	32.51	16.01

Next, when investigating the effect of the synthetic HMBA on the cell-cycle progression of HeLa cells using flow cytometry, the synthetic HMBA was found to induce an increased proportion of HeLa cells in the G_0-G_1 phase, while inhibiting the cell cycle at the G_1/S transition of the cells (Table 2). The histone deacetylase inhibitor, trichostatin A (TSA), a positive control compound for cell-cycle inhibitors, also induced a G_0-G_1 cell-cycle arrest of HeLa cells [2, 4, 12, 14].

Finally, to validate the molecular mechanisms of HMBA as regards its cell-cycle inhibitory activity, the effect of the compound on the expression of two representative cell-cycle regulators was examined [10, 16]. As shown in Fig. 3, treatment with the synthetic HMBA dose-dependently increased the expression of the cell-cycle inhibitor, $p21^{WAF1}$. In contrast, the expression of the cell-cycle activator, cyclin D1, was dose-dependently suppressed by the synthetic HMBA. Therefore, these data demonstrate that the synthetic HMBA induced G_0-G_1 cell-cycle arrest via modulating the gene expression of the cell-cycle regulators.

In conclusion, HMBA, a fungal metabolite exhibiting cell-cycle inhibitory activity, was prepared in a practical

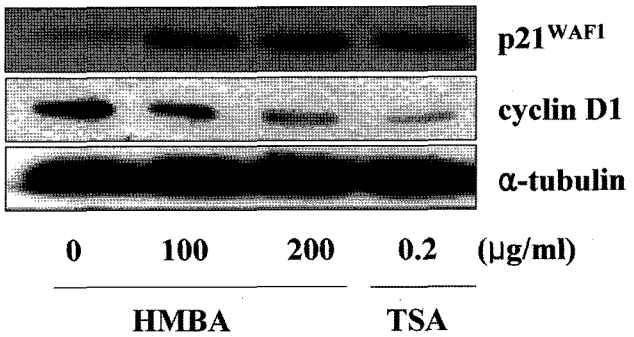

Fig. 3. Effect of synthetic HMBA on expression of $p21^{WAF1}$ and cyclin D1 in HeLa cells.
The effect of the synthetic HMBA on the two cell-cycle modulators was confirmed using Western blot analysis. Tubulin was used as the loading control, and TSA, a well-known histone deacetylase inhibitor, was treated as the positive control for a cell-cycle inhibitor.

synthetic way to provide a simple and easy way for supplying a large quantity of the compound. The biological activity of the synthetic HMBA was also validated, as it exhibited the same activity as the natural compound. Ongoing studies are focused on identifying the cellular target of the compound by developing HMBA derivatives from the established synthetic scheme of the compound.

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