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INHIBITION OF GIBBERELLIN BIOSYNTHESIS IN *GIBBERELLA FUJIKUROI* AND GERMINATION OF *ORYZA SATIVA* BY MEVINOLIN[1,2]

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Abstract

A modified procedure was established for the purification of mevinolin, a hypocholesterolemic natural product and inhibitor of mammalian HMG-CoA reductase, from *Aspergillus terreus*. Purity of mevinolin before inhibitory study was analyzed and confirmed by HPLC method.

Mevinolin strongly inhibits the biosynthesis of gibberellin in *Gibberella fujikuroi*. Identification of GA_3 in the test was carried out by column chromatography and mass spectrometry. Germination of *Oryza sativa* is also blocked by mevinolin. Contents of gibberellins in the trials were greatly reduced and thus too small for quantitative determination. Mevinolin has turned out to be an excellent probe for the study of gibberellin biosynthesis in plants and microorganisms.

Introduction

Compounds biogenetically derived from the condensation of isoprene building blocks are found in all kinds of living organisms and greatly involved in various biological activities. The isoprenoid biosynthetic pathway is responsible for the formation of many plant growth regulators such as gibberellins, abscisic acid and the side chains of some cytokinins (MacMillan, 1980). In mammalian systems, the pathway has contributed to the *de novo* synthesis of cholesterol, ubiquinones, and dolicols. Natural products affecting isoprenoed pathway are therefore excellent tools to elucidate the regulation of isoprenoid formation. Specific inhibitors of isoprenoid biosynthesis can be useful for the study of plant physiology (Goodwin, 1978). Their antimetabolic activities also have great potential in pharmacology and agriculture (Endo *et al.*, 1979; Endo, 1981).

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[2] Part of the results has been presented at the Chinese Biochemical Society Annual Meeting held on October 16, 1982, in Taipei, Taiwan, Republic of China.

Accumulating reports have demonstrated that the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid, an obligatory intermediate catalyzed by HMG-CoA reductase, is the rate determining step of cholesterol biosynthesis in animals (Schroepfer, 1981; Siperstein *et al.*, 1966). As to plants, HMG-CoA reductase activity was reported in sweet potato tubers (Suzuki *et al.*, 1974). Incorporation of HMG-CoA into carotenoids by an extract of maize seedlings was also demonstrated (Berry *et al.*, 1972). Only very recently the regulation of HMG-CoA reductase activity by phytochromes was confirmed in pea seedlings (Wong *et al.*, 1982). Enzymatic study in *Hevea brasiliensis*, trees producing natural rubber which are *cis*-polyisoprenoid compounds was also reported (Sipat, 1982).

Due to the great difficulty in purification and lack of good probe, the regulatory mission of HMG-CoA reductase to the biosynthesis of gibberellin in plants and microorganisms is speculated but never well characterized. We like to report that mevinolin (Fig. 1), a secondary metabolite from *Aspergillus terreus* (Alberts *et al.*, 1980), is a potent inhibitor of gibberellin biosynthesis. It blocks gibberellic acid formation in *Gibberella fujikuroi* and inhibits the germination of *Oryza sativa* (Ogawa, 1963; Kurogochi *et al.*, 1978).

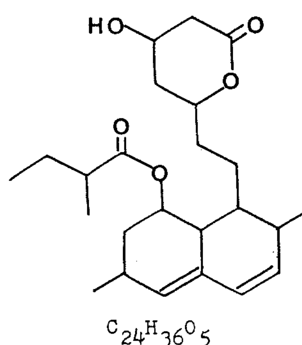

Fig. 1. Chemical structure of mevinolin.

Materials and Methods

Materials

Strains of *Aspergillus terreus* (ATCC 20542, NRRL 265) and *Gibberella fujikuroi* (ATCC 12616) were obtained from American Type Culture Collection (Rockville, Maryland, USA) and Northern Utilization Research and Development Division, U. S. Department of Agriculture (Peoria, Illinois, USA) and maintained on Potato-Dextrose-Agar slants. *Aspergillus* K99 was obtained from the Microbiology Laboratory, Provincial Intustrial Research Institute and kept by Dr. T. C. Tseng at the

Institute of Botany, Academia Sinica. Seeds of *Oryza sativa* cv. Tainan 5 were kindly supplied by Mr. C. T. Lo of this institute. Nutrients for the growth of microorganisms were purchased from Difco Co. (Detroit, Michigan, USA) and E. Merck Co. (Darmstadt, West Germany). Organic solvents for extraction and HPLC were obtained from Alps Co. (Taipei, ROC) and were ultrapure and LC grades respectively. Gibberellic acid (GA_3, 90% purity) and *dl*-mevalonolactone were purchased from Sigma Co. (Saint Louis, Missouri, USA) and were directly used without purification.

Methods

Microorganisms were cultured aerobically in a New Brunswick G-53 shaker or an incubator shaker manufactured by a local company.

Preparative layer chromatography (PLC) was carried out on laboratory prepared 20×20 cm plates, 2 mm thickness, of Merck silica gel PF-254 buffered to pH 7.0. Analytical TLC on Merck precoated silica gel F-254 plates, 0.25 mm thickness, were also routinely utilized.

Proton and ^{13}C-NMR spectra were taken on a Jeol FX-100 spectrometer. All NMR spectra were recorded in $CDCl_3$ and reported as part per million downfield from TMS at ambient temperature. Infrared spectra were taken by a Perkin-Elmer model 283B spectrophotometer with $CHCl_3$ as solvent. Mass spectra were recorded by a Jeol TMS D-100 mass spectrometer at 12 and 75 ev ionization potential and reported as m/e. Analytical HPLC was carried out by using a Waters model 6000A pump equipped with a model 440 254 nm fixed wavelength UV detector and model 401 differential refractometer detector. Samples were introduced by a U6K injector into a μ-Bondapak C-18 column, (0.39×30 cm). Conditions for the separation of mevinolin and gibberellic acid were reported in the text.

*Production of Mevinolin by **Aspergillus terreus***

Aspergillus terreus (ATCC 20542, NRRL 265, K99) were cultured in nutrient solution consisting of 20 g dextrose, 50 g fresh corn extract, 10 g oat flour, 40 g tomato paste, and 10 ml trace element solution per liter of distilled water for 5 days to initiate cell proliferation. Corn extract was prepared by grinding and soaking fresh corn (50 g) in distilled water (150 ml) for 24 h at 4°C. The aqueous extract was collected by filtration through six layers of cheese cloth and added to prepare 1 liter of medium before sterilization. Trace element solution was prepared according to the procedure of Alberts (Alberts *et al.*, 1980). For each 300 ml conical flask, 100 ml of nutrient broth was routinely added. After 5 days growth at 28°C, a 10% by volume of inoculum was subsequently transferred to mevinolin producing medium which was consisted of 60 g dextrose, 20 g skim milk, 4 g peptone, 2.5 g yeast extract per liter of distilled water at pH 7.4. Maximal production of

mevinolin can be achieved after 6 days incubation at 28°C aerobically.

The contents of culture flasks were combined. After removal of mycelia by filtration through cheese cloth, the filtrate was adjusted to pH 3.0 with 6 N HCl and added with equal volume of ethyl acetate. The two-phase mixture was stirred at room temperature for 3 h. After collection of organic phase, the aqueous layer was re-extracted with fresh ethyl acetate (2×1 v). Combined organic extract was dried over anhydrous sodium sulfate and concentrated to dryness by a rotary evaporator. The dark brown concentrate was partially purified by a silica gel column (3.1×42 cm, solvent: EtOAc). Fractions containing mevinolin were pooled. Further separation by PLC (20×20 cm, 2 mm, silica gel, $CHCl_3$: EtOAc=1:1, v/v, Rf=0.31) gave pure mevinolin. Recrystallization was carried out with $CHCl_3$-Petrolium ether. Purity of completely lactonized mevinolin was checked by spectroscopic and HPLC methods before inhibitory study.

*Growth of **Gibberella fujikuroi** and Inhibition of Gibberellic Acid Formation by Mevinolin*

Gibberella fujikuroi (ATCC 12616) was cultured to produce gibberellins (Dockerill *et al.*, 1978). Suitable amount of mevinolin in methanol (2 mg/ml) was pre-socked on filter paper (Whatman No. 1) and added to the three-day-old liquid culture of *G. fujikuroi* (100 ml of broth in each fifteen 300 ml flasks) to a final concentration of 5 μM. After 9 days' growth at 27±1.5°C, the contents of the flasks were pooled and mycelia were removed by filtration. The media were combined and acidified to pH 3.0 with 6N HCl and extracted with equal volume of ethyl acetate three times. The organic layer was dried over sodium sulfate powder and solvent evaporated by a rotary evaporator. The brown preparation was passed through a silica gel column (3.1×44 cm) by eluting with a mixture of EtOAc : MeOH : HOAc (14 : 2 : 1, v/v). Fractions containing GA_3 were pooled and volume of solvent reduced by evaporation. Acetic acid was finally removed by partition between ethyl acetate and water. GA_3 in ethyl acetate layer was subsequently purified by silica gel PLC (solvent, EtOAc : MeOH : HOAc =14 : 2 : 1, v/v, Rf=0.67) or analyzed by HPLC. Identification of GA_3 was carried out by TLC of various solvent systems in conjunction with spraying reagents as well as mass spectroscopic method. Conditions for the analysis of GA_3 by HPLC are described in the result section.

*Inhibition of Germination of **Oryza sativa** by Mevinolin*

Sterilized seeds of *O. sativa* cv. Tainan 5 were soaked in distilled water containing various amounts of mevinolin at 25°C. After 3 days, seeds were removed to containers for germination for another 6 days at the same temperature in the darkness. Degree of rice seedlings was expressed by the percentage retardation

of root and shoot growth in comparison with those of control (Table 1). Attempt was also made to measure the gibberellin contents in 50 g of fresh samples.

Table 1. *Inhibitory effect of mevinolin on the germination of* ***Oryza sativa***

Experiments were carried out at 25°C in the dark for 9 days. Data are mean values of three determinations. For every test group, 30 seeds were used.

Mevinolin (μM)	Length of Shoot		Length of Root	
	mm	% Inhibition	mm	% Inhibition
0	23	—	36	—
5	22	4.3	14	61
12.5	23	0	9	75
25	18	22	8	78

Results

Production and Characterization of Mevinolin

Productivities of mevinolin by three strains of *Aspergillus* were analyzed by HPLC method. Routinely about 40 mg of mevinolin can be obtained from *A. terreus* (ATCC 20542) in one liter of production media. *A. terreus* (NRRL 265) and *Aspergillus* K99 did not produce detectable amount of mevinolin. Presence of mevinolin in the crude preparation after silica gel column and purity of samples before the inhibitory study were analyzed in the same way. The chromatograms are shown in Fig. 2. NMR and IR data shown that mevinolin is completely lactonized in this preparation method. Spectroscopic information is in agreement with structure presented in Fig. 1. The spectroscopic characterization of mevinolin is as follows:

^{1}H-NMR ($CDCl_3$) : δ 0.86 (3H, t, J=7Hz), 0.91 (3H, d, J=7Hz), 0.96 (3H, d, J=7Hz), 1.10 (3H, d, J=7Hz), 1.2-2.1 (11H, m, CH_2, CH), 2.35 (4H, m), 2.64 (2H, d, J=4Hz), 3.84 (1H, bs), 4.3 (1H, bs), 4.6 (1H, bs), 5.3 (1H, bs), 5.5 (1H, bs), 5.75 (1H, dd, J=10, 5 Hz), 5.96 (1H, d, J=10 Hz)

^{13}C-NMR ($CDCl_3$) : δ 11.7, 13.9, 16.2, 22.8, 24.2, 26.8, 27.4, 30.6, 32.6, 32.9, 35.9, 36.5, 37.2, 38.5, 41.4, 62.2, 68.0, 76.6, 128.2, 129.4, 131.5, 132.9, 171.1, 176.9

IR, Vmax ($CHCl_3$): 3610, 3450, 1720, 1460, 1380, 1190, 865 cm^{-1}

Mass, m/e: 404, 302, 284, 224, 198, 172, 159, 157, 143, 126

Inhibition of Gibberellin Biosynthesis in ***Gibberella fujikuroi*** *by Mevinolin*

In the presence of 5 μM mevinolin, gibberellin formation in *G. fujikuroi* was

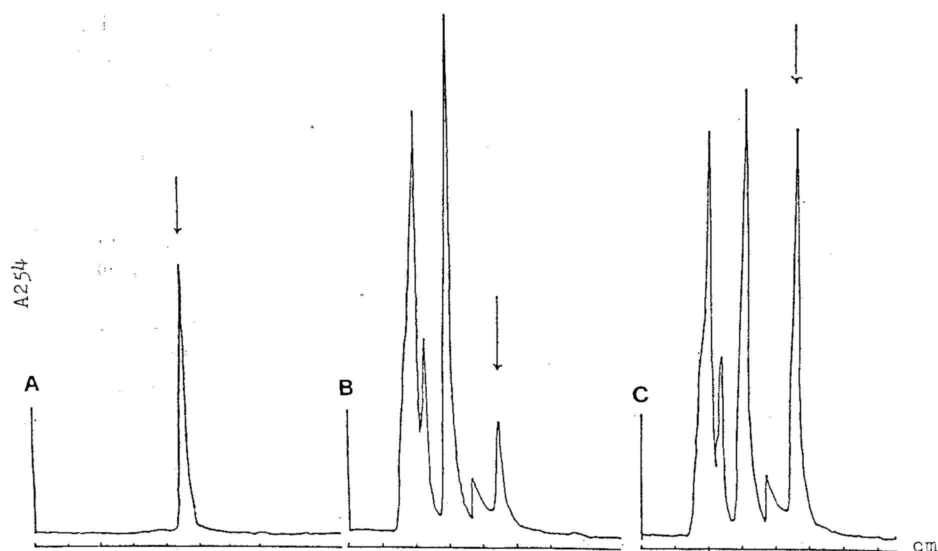

Fig. 2. Chromatograms of highly purified mevinolin and crude preparation of *A. terreus* showing the presence of mevinolin. A: Purified mevinolin, B: Crude preparation of *A. terreus* (Tube No. 25 from silica gel column chromatography), C: A+B, Column: μ-Bondapak C-18, Solvent: MeOH : H_2O=80 : 20, V/V, Flow rate: 1 ml/min, Detector: UV 254 nm, 0.1 x, Chart speed: 1 cm/min, Injection size: 2.5 μl. Peak A is equivalent to 2.5 μg of mevinolin.

greatly inhibited. The amount of GA_3 produced was less than 10% of the control value. The degree of inhibition and identification of GA_3 by HPLC are shown in Fig. 3. Presence of GA_3 was confirmed by a mass spectrometer showing characteristic peaks of m/e at 346, 328, 310, 300, 284, 237, 136. Under the treatment of 5 μM mevinolin, the biomass of *G. fujikuroi* was not significantly changed (5.7 g per liter of broth in the test to 5.6 g per liter of control).

Inhibition of Germination of ***Oryza sativa*** *by Mevinolin*

The inhibitory effects of mevinolin on the seed germination and growth of *O. sativa* are shown in Table 1. About 60% of inhibition of root length was observed in 5 μM mevinolin treatment. The degree of inhibition of root growth was not linearly correlated to the concentration of mevinolin and the growth of shoot was less affected. The amounts of gibberellins in the test samples were extremely low that quantitative determinations by HPLC method have turned out to be unsuccessful.

Discussion

A modified procedure has been established for the isolation and purification of

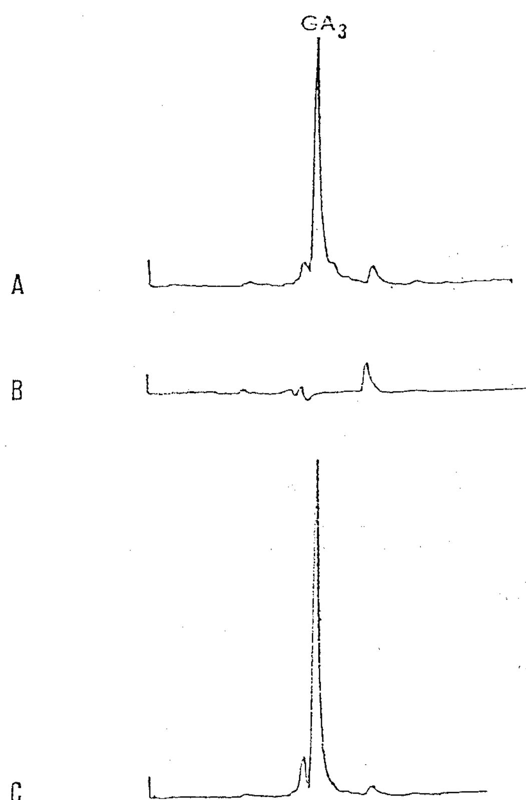

Fig. 3. Mevinolin (5 μM) inhibited GA_3 accumulation in ***G. fujikuroi*** culture as demonstrated in HPLC profiles. A: Control, B: *G. fujikuroi* treated with 5 μM mevinolin, C: B+Authentic GA_3 (50 μg), Column: μ-Bondapak C-18, Solvent: $CH_3CN:H_2O=1:1$, V/V, Flow rate: 1 ml/min, Detector: RI, 32 x, Chart speed: 1 cm/min., Injection size: 5 μl.

mevinolin (Fig. 1). In our scale of preparation, lactonization is complete through prolonged extraction in slightly acidic condition and evaporation of organic phase. This method is simpler and evaporation of high boiling point toluene is not necessary. Purity of mevinolin before the inhibitory study is secured by HPLC analysis. Mevinolin, a hypocholesterolemic natural product and inhibitor of mammalian HMG-CoA reductase, is the first time being tested in plant and microorganism.

In this report, we have demonstrated that gibberellin formation in *Gibberella fujikuroi* was drastically reduced by mevinolin at concentration as low as 5 μM. Due to great reduction of gibberellin contents in the test groups, the predominant GA_3 was the only species analyzed by a mass specyrometer. Since the biomass of *G. fujikuroi* was not significantly changed, the production of gibberellins is probably not obligatory for its growth.

Mevinolin retarded the germination of *Oryza sativa* to various extents. In the experimental scale, gibberellin contents were too low to be quantitatively determined. Since abscisic acid and the side chain of some cytokinins are also biogenetically derived from mevalonic acid, mevinolin might exerts its effect not merely on gibberellin biosynthesis. We also observed that extents of mevinolin inhibition can only be partially reversed by treatment with mevalonic acid in later stages (Shiao, unpublished result). More detailed study using mevinolin in conjunction with tracer techniques is currently underway.

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Mevinolin 抑制 *Gibberella fujikuroi* 徒長素的生物合成與水稻的發芽

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建立簡易的方法，培養麴黴菌，並自其中抽取 mevinolin，此天然物已被證實爲哺乳類酵素 HMG-CoA reductase 的抑制劑，本研究利用高效液相層析法，確定 mevinolin 的產率與純度，並探討其對 *Gibberella fujikuroi* 中徒長素的生物合成與水稻發芽的影響。

在液態的 *G. fujikuroi* 培養液中，加入 mevinolin 則强力抑制了徒長素的生合成，利用層析法配合質譜分析，可知 GA_3 含量大爲下降。臺南5號稻種的發芽亦受到 mevinolin 的抑制，徒長素含量極低，無法準確定量，上述結果顯示 mevinolin 可作爲探測工具，研究徒長素在植物及微生物中的生物合成。