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Isolation of a novel substrate-competitive tyrosine kinase inhibitor, desmal, from the plant *Desmos chinensis*

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In the course of a screening program for tyrosine kinase inhibitors, the chloroform extract of a tropical plant, *Desmos chinensis*, strongly inhibited the enzyme activity. The active substance was purified by silica gel, gel filtration, and finally crystallized. The structure was elucidated by mass spectrometry and X-ray crystallography to be 8-formyl-2,5,7-trihydroxy-6-methylflavanone, and we named it desmal. Desmal competed with peptide substrate and non-competed with ATP. It inhibited tyrosine kinase in situ in epidermal growth factor (EGF) receptor-overexpressing NIH3T3 (ER12) cells. It also inhibited EGF-induced inositol phosphate formation and morphological changes.

Tyrosine kinase; *Desmos chinensis*; Desmal; Epidermal growth factor

1. INTRODUCTION

A variety of mitogen receptors and oncogene products possess tyrosine kinase activity. Of them, especially, epidermal growth factor (EGF) receptors are often overproduced in tumour cells [1,2], and erb B2 products are often overexpressed in breast carcinomas [3]. The *abl* oncogene is activated in most chronic myelogenous leukemia by translocation, which results in activation of the tyrosine kinase in the *abl* oncogene product [4]. Therefore, tyrosine kinase inhibitors may help to suppress development of these neoplasms.

We have isolated tyrosine kinase inhibitors such as erbstatin [5] and lavendustin A [6] from microorganisms. However, screening of microorganisms has been so popular that it becomes difficult to avoid the rescreening of known compounds. For example, we often isolate genistein and staurosporin from *Streptomyces* as tyrosine kinase inhibitors. So, we began screening for tyrosine kinase inhibitors from tropical plant extracts, and isolated a new flavanone compound, desmal (Fig. 1). We also studied the mechanism of inhibition and biological activity of desmal.

2. MATERIALS AND METHODS

2.1. *Materials*

ER12 cells [7] were kindly supplied by Dr. M. Shibuya, Institute of Medical Science, University of Tokyo. [γ-^{32}P]ATP (10 Ci/mmol) and [^{3}H]inositol (23 Ci/mmol) were purchased from DuPont-New England Nuclear.

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2.2. *Preparation of the chloroform extracts*

Both leaves and stems of *Desmos chinensis* were crushed into pieces. The dried material (50 g) was extracted 3 times by vortexing with 200 ml of chloroform for 8 h each time to give 3.3 g of oily material.

2.3. *Isolation of desmal*

The extract (450 mg) was dissolved in a small amount of hexane, applied to a silica gel column, and eluted with a mixture of hexane-ethyl acetate (8/1). The active fractions were combined and dried to give a yellow powder (160 mg). This residue was dissolved in MeOH and subjected to Toyopearl HW-40 column chromatography. After elution with MeOH, the active fractions were combined and dried to yield a yellow powder (140 mg). Next, it was dissolved in ethyl acetate, and the mixture was washed with 1 N HCl and H_2O. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give a yellow powder. It was crystallized from benzene/toluene/methanol to yield 80 mg of desmal.

2.4. *Physico-chemical properties*

Desmal crystals appeared as pale yellow needles. They were soluble in MeOH, EtOAc, $CHCl_3$, DMSO, Me_2CO, but not in H_2O.

Desmal melted at 164–167°C. The UV spectra showed maxima at 270 nm (ε 630) and 340 nm (ε 85) in MeOH, at 270 nm (ε 660) and 340 nm (ε 85) in 0.1 N HCl, and at 290 nm (ε 430), 340 nm (ε 25), and 390 nm (ε 360) in 0.1 N NaOH.

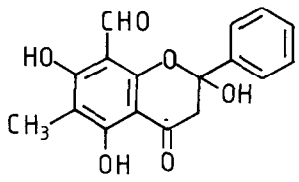

Fig. 1. Desmal.

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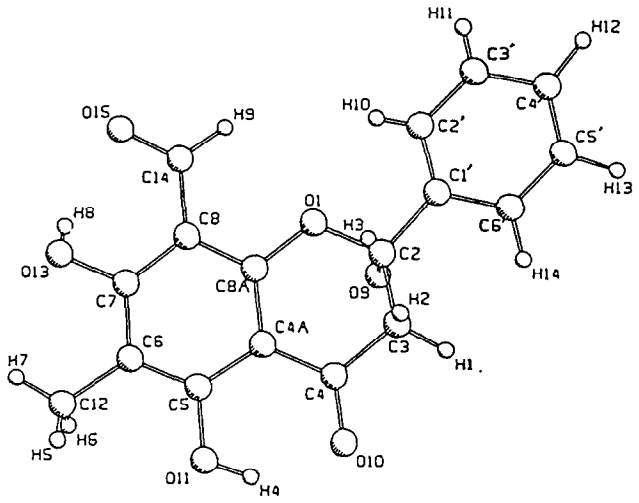

Fig. 2. Molecular structure of desmal drawn by PLUTO. There are two planes in the molecule: one formed by C(1′)–C(6′) atoms and the other involving C(4A), C(5), C(6), C(7), C(8), C(8A), C(4), O(11), C(12), O(13), C(14), C(4) and O(10) atoms. The dihedral angle between the two planes is 25.8°. The oxygen atom O(9) forms an intramolecular hydrogen bond with O(10) [O(9) ··· O(10) = 2.790(5) Å].

The IR spectra of desmal gave peaks at 3,341, 2,928, 1,630, 1,446, 1,329, 1,221, 1,201, 1,141, 1,018, 979, 945, 902, 756, 700 and 619 cm^{-1}.

2.5. *X-ray structure analysis*

Transparent prism crystals of desmal were grown in benzene/toluene/methanol solution. A crystal having approximate dimensions of 0.3 × 0.1 × 0.1 mm was mounted on a goniometer head for the diffraction experiment. All measurements were made with a Rigaku AFC5R diffractometer using β-filtered CuKα radiation. The crystal of desmal was monoclinic and belonged to the space group $P2_1/n$. The cell dimensions were as follows: a = 12.940(1) Å, b = 6.3589(9) Å, c = 18.352(1) Å, β = 105.007(6)°. The Z value, Dx, and μ for CuKα were 4, 1.431 $g \cdot cm^{-3}$, and 8.8 cm^{-1}, respectively. The intensity data were collected by the $\omega - 2\theta$ scan technique to a maximum 2θ value of 123.9°. A total of 1,308 independent reflections ($I > 3\sigma$) was used for the subsequent structure determination. The structure was solved by the direct method [8] and refined by the method of full-matrix least-squares. All hydrogen atoms were located on the difference Fourier map. The final R-value was 0.061. The molecular structure was drawn by PLUTO [9].

Atomic coordinates for this structure have been deposited with the Cambridge Crystallographic Data Centre and can be obtained on request from Dr. Olga Kennard, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EN, UK.

2.6. *EGF receptor-associated tyrosine kinase assay*

EGF receptor-associated tyrosine kinase activity was measured by the method described before [5]. In brief, the reaction mixture contained 1 mM $MnCl_2$, 100 ng of EGF, 40 μg of protein of A431 membrane fraction [10], 75 μg of albumin, 3 μg of histone in 50 μl of HEPES (20 mM, pH 7.4) buffer. The reaction tubes were placed on ice and incubated for 10 min in the presence or absence of inhibitor. The reaction was initiated by the addition of [γ-^{32}P]ATP (10 μl), and the incubation was continued for 30 min at 0°C. Then the reaction was stopped by the addition of cold 10% TCA (200 μl) containing 0.01 M sodium pyrophosphate and kept at 0°C for 15 min. Finally, TCA-insoluble material was collected on glass filter papers by use of a cell harvester and counted in a liquid scintillation counter. The kinetic study was carried out as described before [6].

2.7. *Intracellular tyrosine phosphorylation*

ER12 cells (2×10^5) were plated in 35-mm plastic dishes and cultured in 2 ml of DMEM containing 5% calf serum for 60 h. During this period the cells became quiescent by density-dependent inhibition. The cells were first preincubated with desmal (25 or 50 μg/ml) for 3 h, and then washed twice with 0.5 ml of serum-free DMEM and given 1 ml of serum-free DMEM containing 100 μM orthovanadate and desmal (25 or 50 μg/ml). After incubation for 1 h, 100 ng/ml of EGF was added. After incubation for 5 min, the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), scraped into PBS, and collected by centrifugation at 15,000 × g for 5 min at 4°C. The precipitate was then dissolved in 50 μl of 10 mM HEPES buffer (pH 7.4, containing 2 mM Na_3VO_4, 10 mM NaF, 10 mM pyrophosphate, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and vigorously stirred. Next, 50 μl of electrophoresis loading buffer (42 mM Tris, pH 6.8,

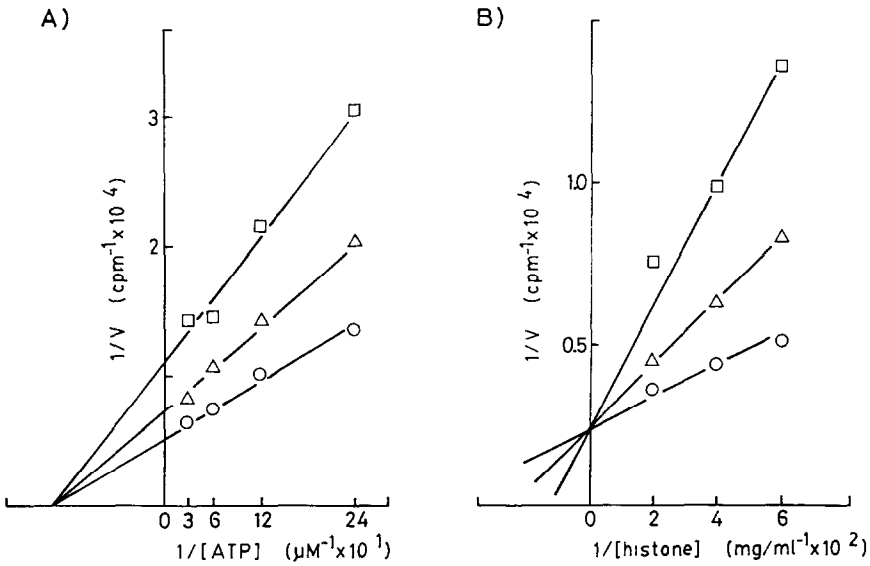

Fig. 3. Lineweaver–Burk plot of tyrosine kinase reaction with desmal. (A) A Lineweaver–Burk plot showing non-competitive inhibition by desmal against ATP. Inhibitor concentrations are 0 (○), 2.5 (△), and 5 (□) μg/ml, respectively. (B) A Lineweaver–Burk plot showing competitive inhibition by desmal against histone. Inhibitor concentrations were 0 (○), 1.0 (△), and 5 (□) μg/ml, respectively.

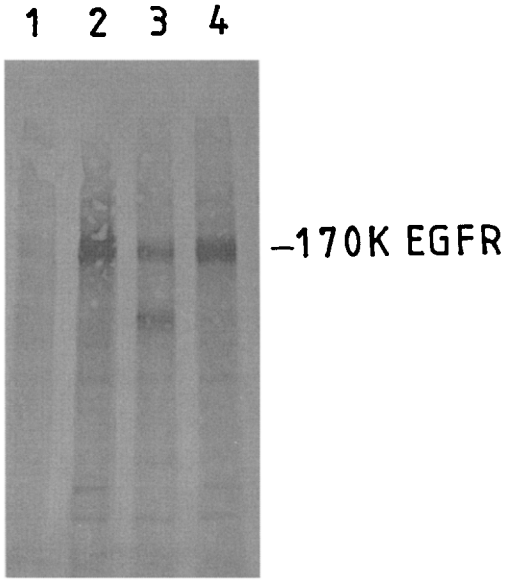

Fig. 4. Inhibition by desmal of intracellular tyrosine phosphorylation in ER12 cells. ER12 cells were incubated without (lane 1 and 2) or with desmal (lane 3, 50 μg/ml, lane 4, 25 μg/ml) for 4 h and then incubated with EGF (100 ng/ml, lanes 2–4) for 5 min. Then, the cells were solubilized, electrophoresed, and reacted with anti-phosphotyrosine antibody as described in Section 2.

containing 10% 2-mercaptoethanol, 10% glycerol, 8% SDS, and 0.002% bromophenol blue) was added to the mixture, which was then boiled for 5 min and centrifuged at 12,000 rpm for 20 min. The supernatant was electrophoresed on an SDS-polyacrylamide gel, after which the gel was reacted with anti-phosphotyrosine antibody (PY20, Seikagaku Kogyo Co., Ltd.).

2.8. *Inositol phosphate formation*

ER12 cells (3×10^5) were plated in 24-well plates and cultured in 1 ml of DMEM containing 5% calf serum for 48 h, during which period the cells became quiescent. They were then prelabeled with [^{3}H]inositol (1 μCi/ml) in inositol-free DMEM for 18 h, washed twice with 0.5 ml of DMEM, and given 0.5 ml of DMEM containing 30 mM LiCl and desmal (0–100 μg/ml). After incubation for 30 min, EGF (100 ng/ml) was added and the cells were further incubated for 5 min. The reaction was terminated by the addition of ice-cold 10% perchloric acid, and then the mixture was neutralized by 1.53 M KOH in 75 mM HEPES. The solution was kept on ice for 15 min, after which it was centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was applied onto an Amprep SAX column, which was then washed with water and eluted with 0.17 mM $KHCO_3$. The obtained eluate was counted with a liquid scintillation counter.

2.9. *Fluorescent staining of filamentous actin*

ER12 cells (1×10^5) were plated in 12-well plates and cultured in 1 ml of DMEM containing 1% calf serum for 16 h without or with desmal (50 μg/ml) and EGF (100 ng/ml). Then, the cells were fixed with 3.5% paraformaldehyde in Ca^{2+}/Mg^{2+}-free PBS for 20 min at room temperature. After a brief rinse in the Ca^{2+}/Mg^{2+}-free PBS, the fixed cells were exposed to acetone for 5 min at −20°C. The cells were washed again with Ca^{2+}/Mg^{2+}-free PBS and incubated with rhodamine-conjugated phalloidin for 20 min at 37°C. They were washed with Ca^{2+}/Mg^{2+}-free PBS a final time and sealed in glycerin buffer. The cells were examined by fluorescence microscopy.

3. RESULTS

The chloroform extract of *Desmos chinensis* leaves showed potent inhibitory activity toward EGF receptor-associated tyrosine kinase activity. The active fraction was purified by chromatography and crystallized. The molecular formula of the active substance was determined to be $C_{17}H_{14}O_6$ based on FDMS spectral data (FDMS m/z 314) and elemental analysis (Found: C,

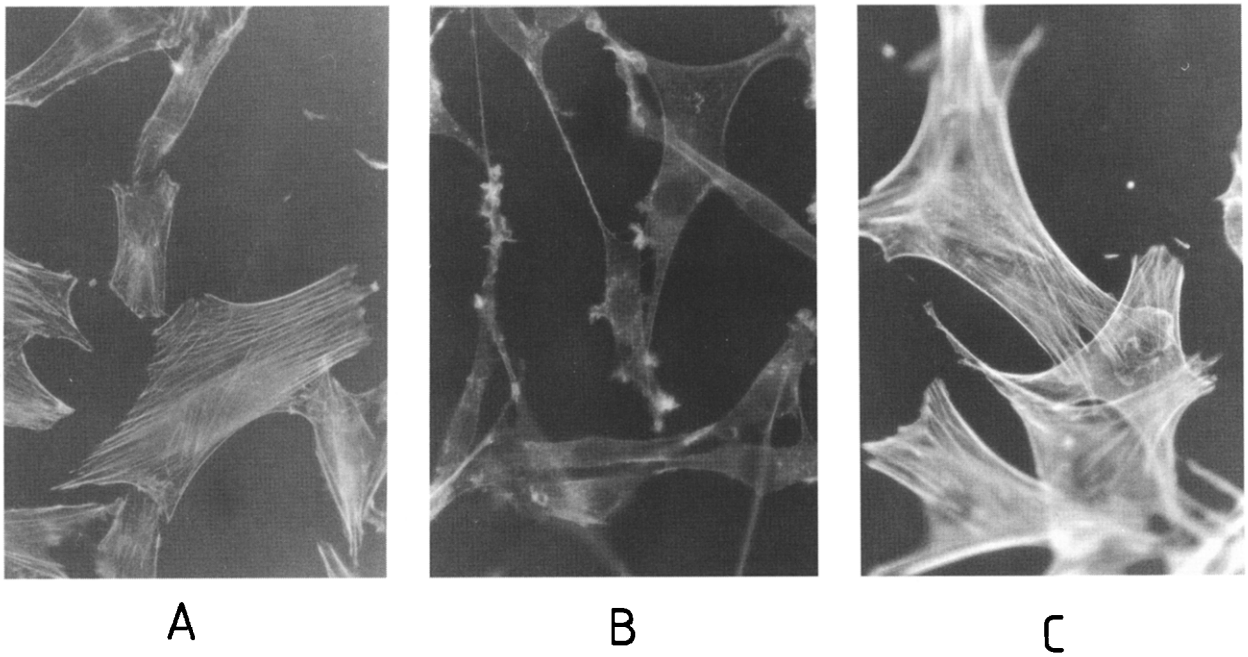

Fig. 5. Inhibition by desmal of EGF-induced cytoskeletal changes in ER12 cells. ER12 cells were incubated with no addition (A), 100 ng/ml of EGF (B), or EGF and 50 μg/ml of desmal (C) in DMEM containing 1% calf serum for 16 h at 37°C. Then, the cells were stained with rhodamine-phalloidin as described in Section 2.

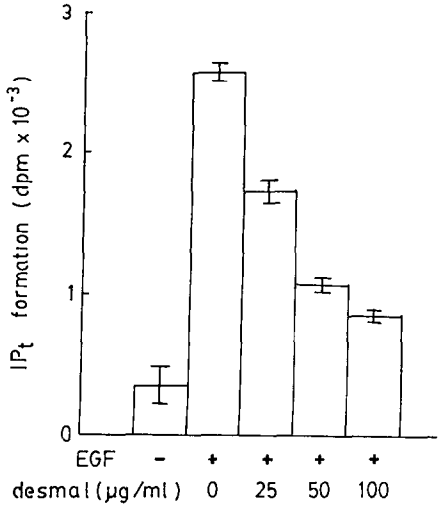

Fig. 6. Inhibition by desmal of inositol phosphate production in ER12 cells. After treatment of quiescent ER12 cells with various concentrations of desmal for 30 min at 37°C, EGF (100 ng/ml) was added to the cells and incubation continued for 5 min at 37°C. Total inositol phosphates were measured as described in Section 2. The results are mean ± S.D. of triplicate determinations.

65.29; H, 4.62%; Calculated: C, 64.96; H, 4.49%). The structure was postulated by proton and carbon-13 spectroscopy, and determined by X-ray crystallographic analysis to be 8-formyl-2,5,7-trihydroxy-6-methylflavanone, as shown in Fig. 2. As it had not been isolated previously, we named it desmal.

Desmal inhibited EGF receptor-associated tyrosine kinase with an IC_{50} of 2.5 μg/ml. Lineweaver–Burk plotting with histone as a substrate showed that desmal competed with the protein but not with ATP, as shown in Fig. 3.

Desmal inhibited intracellular tyrosine phosphorylation in EGF receptor-overexpressing NIH3T3 (ER12) cells at 25–50 μg/ml, as shown in Fig. 4. EGF induces cytoskeletal changes in ER12 cells in 16 h [11], and desmal inhibited such changes at 50 μg/ml, as shown in Fig. 5. We found earlier that EGF induces inositol phosphate formation in ER12 cells as in A431 cells [12]. The EGF-induced activation of inositol phosphate formation was inhibited by desmal at 25–100 μg/ml, as shown in Fig. 6.

4. DISCUSSION

The structure of desmal was predicted by NMR as one of the possible forms of a plant product from *Unona lawii*, 3-formyl-2,6-dihydroxy-4-methoxy-5-methyldibenzoylmethane, in acetone-d_6 solvent [13], but it was never isolated. We isolated desmal from a different plant and determined its structure by X-ray crystallography.

As a tyrosine kinase inhibitor desmal competed with the peptide substrate. It is rather unexpected since all isoflavonoids such as genistein and orobol inhibit tyrosine kinase by competing with ATP. A substrate-competitive inhibitor would be advantageous for specific inhibition of tyrosine kinase. Desmal did not inhibit protein kinase C or phosphatidylinositol kinase at 100 μg/ml.

Desmal inhibited the growth of A431 cells, ER12 cells, and temperature-sensitive Rous sarcoma virus-transformed NRK (RSV^{ts}-NRK) cells at 33°C and 39°C with IC_{50}s of 26, 31, 14, and 13 μg/ml, respectively. In EGF-overexpressing fibroblasts desmal modified the EGF-induced phenotypic change. Therefore, it may be a tool to study the role of tyrosine kinase in various cellular events.

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