Screening of Microbial Extracts for Tyrosine Kinase Inhibitors

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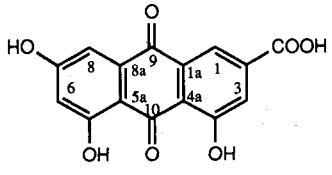
Protein tyrosine kinase activity (PTK) is associated with the activity of many cellular and viral oncogene products[1,2] as well as signal transduction events for several growth factor receptors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin[3,4]. Activation of specific PTK mediated processes have been associated with proliferative diseases such as cancer[5], artherosclerosis[6] and probably psoriasis[6,7]. This has led to the general concept that PTK inhibition may combat hyperproliferative conditions which result from enhanced activity of PTKs[8,9]. Several synthetic inhibitors of PTKs have been characterized along with a number of natural products of microbial, plant and marine origin. Among these are tyrphostins[9], erbstatin[10], genistein[11], and halenaquinone[12].

We conducted a high throughput screen aimed at obtaining novel inhibitors of $p56^{lck}$ tyrosine kinase of microbial origin and found that emodic acid (**1**), chartreusin (**2**), 3,5-dimethoxybenzoic acid (**3**), 3,5-dihydroxybenzoic acid (**4**) and 3,4-dihydroxy-6-aminobenzoic acid (**5**) strongly inhibit the enzyme.

The $p56^{lck}$ and $p59^{fyn}$ enzymes were purchased from Upstate Biotechnology. A cDNA encoding the kinase domains of the mouse EGF receptor was isolated using RT/PCR and subcloned into a baculovirus expression vector, pBlueBacHis C (Invitrogen) for expression in Sf9 cells. Tyrosine kinase activity was assayed by monitoring the phosphorylation of the substrate poly(Glu-Na, Tyr) 4:1 bound to an ELISA plate. Successive layerings of anti-phosphotyrosine antibodies, biotin-linked goat anti-mouse IgG and streptavidin-linked β-galactosidase conjugate allowed fluorescent detection using fluorescein-di-β-galactopyranoside which was hydrolyzed to fluorescein and detected by a Cytofluor 300 fluorescence plate reader (Millipore).

After screening for PTK inhibitors from thousands of microbial extracts (both fungal and actinomycete), we have identified three fungal extracts which showed potent inhibitor activity in the PTK assay. Subsequent bioassay directed fractionation afforded emodic acid (**1**), chartreusin (**2**), 3,5-dimethoxybenzoic acid (**3**), 3,5-dihydroxybenzoic acid (**4**) and 3,4-dihydroxy-6-aminobenzoic acid (**5**) from these extracts. For isolation of emodic acid, a fungal strain AM14130 (*Penicillium* sp.) was cultured in media containing glycerol 30.0 g/liter, pharmamedia 20.0 g/liter, dry yeast 20.0 g/liter, potassium phosphate, monobasic 21.8 g/liter, sodium phosphate, dibasic 5.6 g/liter, magnesium chloride, hydrated 5.0 g/liter. Chartreusin was isolated from the fungus AM15511 (*Nocardioides* sp.) and phloroglucinol, 3,4-dihydroxy-6-aminobenzoic acid and 3,5-dihydroxybenzoic acid were isolated from the fungus AM13971 (*Botrytis* sp.). Both fungi were cultured in the medium containing glycerol 20.0 g/liter, potato starch 20.0 g/liter, corn steep liquor 2.0 g/liter, Nutrisoy grits 10.0 g/liter, beef extract 5.0 g/liter, yeast extract 3.0 g/liter calcium carbonate 3.0 g/liter, magnesium phosphate, hydrated 10.0 g/liter. In all cases, the fermentation broth (3 liters) was homogenized, extracted with ethyl acetate and



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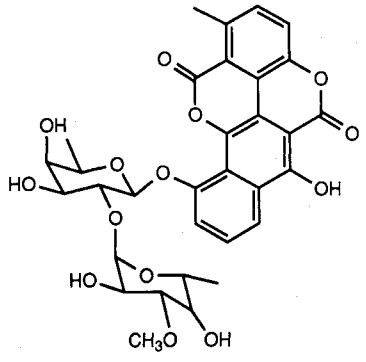

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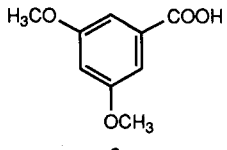

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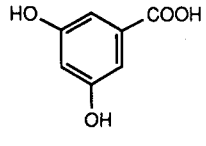

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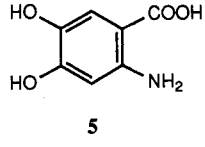

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Table 1. NMR data of emodic acid.

Carbon number	δ ^{1}H (multiplicity and coupling constant)	δ ^{13}C
1	7.95 (d, J=1.5 Hz)	134.8
1a		118.8
2		137.4
3	7.60 (d, J=1.5 Hz)	124.1
4		161.0
4-OH	11.80 (s)	
4a		118.1
5		164.7
5-OH	11.96 (s)	
5a		109.0
6	6.50 (d, J=2.3 Hz)	108.0
7		166.0
8	7.01 (d, J=2.3 Hz)	109.1
8a		133.3
9		180.5
10		189.1
*C*OOH		165.3
COO*H*	11.52 (s)	

fractionated by the dual mode HSCCC[13]. The active HSCCC fractions from each individual extract were combined and fractionated further by the semi-preparative HPLC to yield pure emodic acid (**1**), chartreusin (**2**), 3,5-dimethoxybenzoic acid (**3**), 3,5-dihydroxybenzoic acid (**4**) and 3,4-dihydroxy-6-aminobenzoic acid (**5**). Charteusin was identified by direct comparision of its spectroscopic properties with those reported in the literature[14] while 3,5-dimethoxybenzoic acid, 3,5-dihydroxybenzoic acid and 3,4-dihydroxy-6-aminobenzoic acid were identified by analysis of ^{1}H, ^{13}C NMR and mass data.

The compound isolated from the extract of the fungal strain AM14130 was tentatively identified as emodic acid (**1**) by comparing UV and mass data with those reported in the literature for emodic acid[15]. However, in spite of this relationship (UV and mass data), our structure elucidation work was prolonged because the NMR data of emodic acid was not reported in the literature, and the specific details of its structure elucidation have never been published. Therefore, all the assignments and overall structure was ascertained by extensive 2D NMR (HMQC and HMBC) spectroscopy.

Emodic acid (**1**) was isolated as a red solid. It exhibited characteristic UV spectra, with maxima at λ_{max} 220, 255, 270 and 443. The ion-spray mass spectrum showed the molecular weight to be 300 and the HREI mass measurements revealed the molecular formula to be $C_{15}H_8O_7$. The ^{13}C NMR of **1** (Table 1) showed signals for four aromatic methines and **11** aromatic quarternary carbons, three of which are carbonyl carbons. ^{1}H NMR (Table 1) exhibited two sets of meta coupled proton signals at δ 6.50 (d, J=2.3 Hz) and 7.01 (d, J=2.3 Hz), 7.98 (d, J=1.5 Hz) and 7.60 (d, J=1.5 Hz). In addition, the ^{1}H NMR spectrum showed three signals at δ 11.8, 11.96 and 11.52 due to phenolic protons and a carboxylic proton, respectively.

Table 2. Inhibition of p56^{lck} tyrosine kinase activity

Compounds name	p56^{lck} Tyrosine kinase IC_{50} (μg/ml)
Emodic acid	1.07
Chartreusin	0.002
3,5-Dimethoxybenzoic acid	10.7
3,5-Dihydroxybenzoic acid	0.43
3,5-Dihydroxyaminobenzoic acid	0.42

All five compounds (emodic acid, chartreusin, 3,5-dimethoxybenzoic acid, 3,5-dihydroxybenzoic acid and 3,4-dihydroxy-6-aminobenzoic acid) elicited potent dose-dependent inhibition of p56^{lck} tyrosine kinase (Table 2). Emodic acid also inhibited the EGF receptor and p59^{fyn} PTK with an IC_{50} of 0.078 and 0.080 μg/ml, respectively. Furthermore, it is noteworthy that emodic acid did not inhibit the proliferation of human foreskin fibroblast cells and also did not produce any cytotoxic effects on these cells, as determined by ^{3}H thymidine incorporation and alamar blue cytotoxicity analysis (Data not shown).

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