CITRININ HYDRATE AND RADICININ: HUMAN RHINOVIRUS 3C-PROTEASE INHIBITORS DISCOVERED IN A TARGET-DIRECTED MICROBIAL SCREEN

SUNIL KADAM, JENNIFER PODDIG, PAT HUMPHREY, JAMES KARWOWSKI, MARIANNA JACKSON, SHAUN TENNENT, LUCY FUNG, JILL HOCHLOWSKI, RONALD RASMUSSEN and JAMES MCALPINE

New Lead Discovery, Abbott Laboratories, Abbott Park, IL 60064, U.S.A.

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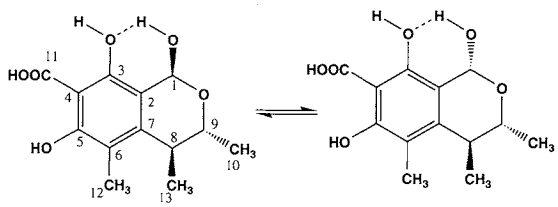
Human Rhinoviruses belong to one of the largest family of positive sense RNA viruses; the *Picornaviridae*, which includes human pathogens such as enteroviruses (poliovirus, coxsackeivirus, echovirus and hepatitis A virus), cardioviruses (encephalomyocarditis and mengo virus), and apthoviruses (foot and mouth disease virus). They are responsible for 50% of common colds[1,2]. A common feature of rhinoviruses is the direct translation of the positive strand RNA to produce a single polyprotein that must be processed, by proteolysis, to generate several structural and functional viral gene products[2,3]. This proteolytic cleavage is dependent upon two virally coded proteases (2A and 3C), for which no known host enzyme can substitute[2,4]. The virally coded proteases therefore provide an attractive target for inhibition of viral replication and therapeutic intervention against common colds. Proteolytic cleavage is very specific, between a glutamine and a glycine (Q-G) for 3C and a tyrosine and a glycine (Y-G) for 2A[3].

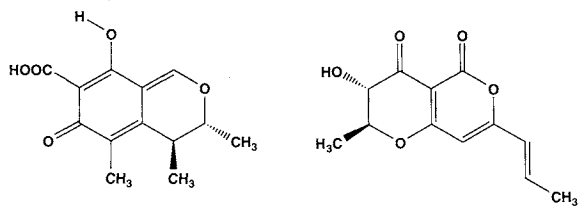
In the course of screening microbial extracts, we identified citrinin hydrate and radicinin. These compounds were identified with a, target-directed, "dual-plasmid", microbial assay. The screening construct consisted of a recombinant *Escherichia coli*, containing a plasmid carrying the rhinovirus type 14 protease 3C gene under the control of an arabinose inducible promoter and, a second plasmid in which the gene coding for the tetracycline resistance protein was modified to contain a nine amino acid coding sequence which generated the Q-G protease 3C cleavage site. Induction of protease 3C, upon the addition of arabinose, to growing cultures carrying the enzyme and substrate coding plasmids, resulted in expression of the enzyme, cleavage of the tetracycline resistance protein and progressive sensitivity of the host cell to tetracycline. Crude extracts obtained from cultures producing citrinin hydrate and radicinin (Fig. 1) were identified by their ability to support the growth of the screening construct in the presence of arabinose (0.2%) and tetracycline (5 μg/ml) as shown in Fig. 2.

Citrinin hydrate was produced by a *Penicillium* sp. strain AB 2089ZZD-62 (NRRL 22560) and radicinin was isolated from the mycelium of a *Curvularia* sp. strain AB 2090A-11 (NRRL 22559). Both fungi were isolated from marine invertebrates, collected off the coast of New Britain. To isolate the active compounds, fermentations of cultures producing citrinin hydrate and radicinin were performed

Fig. 1. Structures.



1a Citrinin hydrate **1b**



2 Citrinin **3** Radicinin

in media containing synthetic sea salts (Aquarium Systems). Inoculum was grown in potato dextrose broth (Difco) containing 36 g/liter of synthetic sea salts. Seed flasks were incubated at 28°C for 72 hours on a rotary shaker operated at 225 rpm. Fermentations were performed in 22-liter fermentors. The citrinin hydrate producer was fermented in a medium consisting of 0.4% glucose monohydrate, 1% malt extract (Difco), 0.4% yeast extract (Difco) and 3.6% synthetic sea salts. The radicinin producer was grown in a medium consisting of 5% glucose monohydrate, 0.5% soy flour, 2% malt extract, 0.1% yeast extract, 0.1% NaCl, 0.04% KH_2PO_4, 0.008% K_2HPO_4 and 3.6% synthetic sea salts. Both fermentations were inoculated at 5% with the 72-hour growth seed. The fermentors were operated at 22°C, with an agitation rate of 250 rpm and air flow at 0.7 v/v/minute. The citrinin hydrate producing fermentor was harvested after 11 days and the radicinin producing fermentor was harvested on the 9th day. Radicinin (**3**) was isolated from the fungal mycelia sequentially with acetone and MeOH. Extracts were combined, concentrated and the residue was partitioned in a mixture of $CHCl_3$-MeOH-water (2:2:1). The lower layer was concentrated to a semisolid mass which was separated by silica gel chromatography. The product was eluted with MeOH-$CHCl_3$ (1:9). Its structure, (Fig. 1), was identified by NMR, UV and mass spectroscopy and the stereochemistry was confirmed by X-ray analysis[5]. The isolation of citrinin hydrate (Fig. 1) was accomplished as follows. Upon completion of fermentation, 1.7 liters of XAD-16 was added to 11 liters of whole broth and the mixture was stirred overnight at 4°C. The resin and fungal mass was removed by centrifugation and filtration and the organics were eluted from this solid with 4 liters of MeOH which was concentrated to leave an oily residue. This residue was triturated sequentially with 1 liter each of; hexane, EtOAc, acetone, EtOH, MeOH and water. The EtOH and MeOH triturates were combined, concentrated to dryness and partitioned between EtOAc-EtOH-water (600 ml, 200 ml, 400 ml, respectively). The lower layer from this partition was concentrated to an oil which was chromatographed over a Sephadex LH-20 column developed with MeOH. Active fractions from this column were combined and concentrated to yield 18 mg of pure citrinin hydrate (**1**). The ^1H and ^{13}C NMR spectra (See Tables 1 and 2) of (**1**) revealed that the active component was a mixture of isomers as the ratio of these varied with solvent, it was deduced that the isomers were an equilibrium mixture (such mixture in the ratio of 70:30 in CD_3OD). The signals in the ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2. These assignments were made by analyses of the COSY, HMQC[3], HMBC[5] experiments. A 2D NOE experiment[8] reveals the stereochemistry of the major anomer to be (**1a**) as evidenced by an NOE between the C-1 proton signal at δ 5.55 and the C-10 methyl protons signal at δ 1.31 and that of the minor anomer to be (**1b**). (An NOE is seen between the C-1 proton at δ 5.43 and the C-13 methyl proton signal at δ 1.15). Presumably the hydrogen bonding between the anomeric hydroxyl at the C-3 hydroxyls causes each anomer to assume the twist envelope conformation in which the anomeric hydroxyl is pseudo-equatorial. The assigned structure represents a formal addition of water to the previously described antibiotic, citrinin[9], and hence we have named compound **1**, citrinin hydrate.

The characteristics of citrinin hydrate were as

Fig. 2. Protease 3C inhibition and cell-growth on agar plates in the vicinity of 5 μl spots containing citrinin hydrate (1), radicinin (2) and *N*-Tosyl-L-phenylalanine chloromethyl ketone, a non-specific thiol protease inhibitor (3).

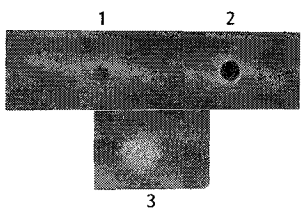


Table 1. ^1H NMR assignments in CD_3OD for citrinin hydrate.

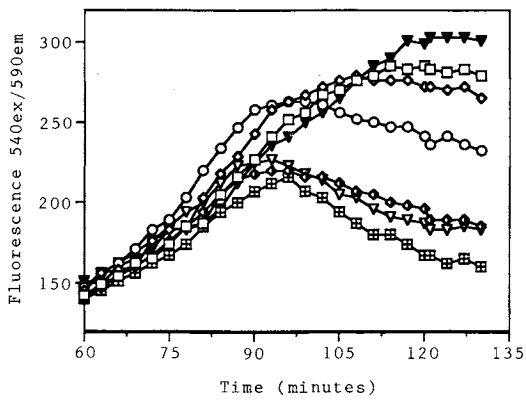
H on C number	Major isomer (**1a**)	Minor isomer (**1b**)
1	5.55 (s, 1H)	5.43 (s, 1H)
8	2.65 (qd, 1H, J=6.8, 6.6 Hz)	2.72 (qd, 1H, J=7.0, 0.9 Hz)
9	3.94 (dq, 1H, J=6.6, 6.2 Hz)	4.05 (qd, 1H, J=7.0, 0.9 Hz)
10	1.31 (d, 3H, J=6.2 Hz)	1.33 (d, 3H, J=7.0 Hz)
12	2.03 (s, 3H)	2.03 (s, 3H)
13	1.19 (d, 3H, J=6.8 Hz)	1.15 (d, 3H, J=7.0 Hz)

Table 2. ^{13}C NMR assignments in CD_3OD for citrinin hydrate.

Carbon number	Major isomer (1a)	Minor isomer (1b)
1	96.6 (CH)	96.3 (CH)
2	112.7 (Q)	112.1 (Q)
3	158.1 (Q)	158.8 (Q)
4	102.2 (Q)	102.8 (Q)
5	161.4 (Q)	161.2 (Q)
6	111.9 (Q)	110.0 (Q)
7	143.9 (Q)	142.4 (Q)
8	38.2 (CH)	36.3 (CH)
9	71.1 (CH)	74.8 (CH)
10	20.8 (CH_3)	22.2 (CH_3)
11	178.2 (Q)	178.3 (Q)
12	11.6 (CH_3)	9.8 (CH_3)
13	19.8 (CH_3)	20.9 (CH_3)

Fig. 3. Measurement of growth in the presence of various concentrations of radicinin.

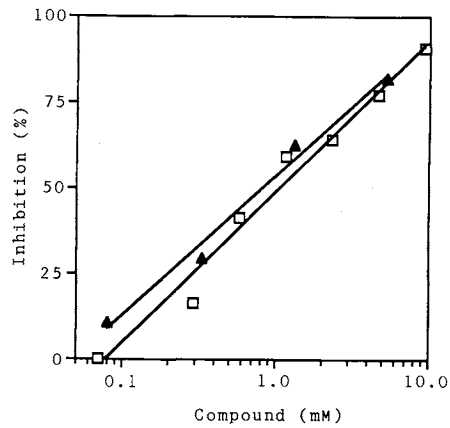
(□) 10 mM, (◇) 5.2 mM, (○) 2.5 mM, (◆) 1.3 mM, (▽) 0.3 mM and (⊞) 0 mM. Growth in the presence of tetracycline without protease 3C induction is represented by (▼).



follows: Citrinin hydrate, $C_{13}H_{16}O_6$, was an orange oily solid with an Rf of 0.8 in MeOH, an Rf of 0.1 in EtOAc and an Rf of 0.7 in CH_2Cl_2 - MeOH (1 : 1). (Rf values were acquired on Merck silica gel TLC plates). UV spectrum (MeOH) neutral and basic λ_{max} 214 nm (ε 74,000), 253 (24,000) and 316 (17,000), acidic λ_{max} 224 nm (ε 44,000), 251 (26,000) and 322 (26,000).

The purified compounds were tested for inhibition of protease 3C using the "dualplasmid" construct. Cell growth in the presence of tetracycline was measured 1 hour after induction of protease 3C with arabinose. Increasing concentrations of radicinin were added and resistance to tetracycline was determined by measuring cell growth as show in Fig. 3. Inhibition of protease and restoration of antibiotic resistance corresponded with the addition of increasing concentrations of radicinin. Based on this assay, the IC_{50} for protease inhibition for radicinin and citrinin hydrate was determined to be 0.7 and 1.0 mM, respectively (Fig. 4).

Fig. 4. IC_{50} determination for citrinin hydrate (□) and radicinin (▲).



In this report we have identified two compounds that inhibit the protease 3C of human rhinovirus. The compounds were discovered in a novel, whole-cell screen and their potency was determined by their ability to inhibit the cleavage of a modified target which provided an antibiotic resistance phenotype. We have described an assay which can be used as a general screen for inhibitors of other targets where the appropriate enzyme and the substrate cleavage site is known. Although inhibitors of the rhinovirus protease 3C, have been reported from microbial sources[10], this is the first report of agents identified in such a "dual-plasmid" mechanisms-based screen.

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