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Citrinin Hydrate Inhibits Serotonin *N*-Acetyltransferase Catalyzing the Conversion of Serotonin to *N*-Acetylserotonin

LEE, IN-KYOUNG, BONG-SIK YUN, KYONG-TAI KIM[1], BO-HWA CHOI[1], TAE-JU PARK[1], YOUNG-HO KIM[2], AND ICK-DONG YOO*

The Korean Research Institute of Bioscience and Biotechnology, P.O.Box 115, Yusong, Taejon 305-600, Korea
[1]Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea
[2]College of Pharmacy, Chungnam National University, Taejeon 305-764, Korea

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Abstract In an attempt to search for serotonin *N*-acetyltransferase (arylalkylamine *N*-acetyltransferase, AA-NAT) inhibitors from microbial metabolites, we found the culture broth of *Penicillium* sp. 80722 which showed a strong inhibitory activity against AA-NAT. The active principle has been identified as citrinin hydrate through bioassay-guided fractionation of cultural broth, and structure elucidation derived by spectroscopic analyses. Citrinin hydrate inhibits AA-NAT with an IC_{50} value of 173 μM in a dose-dependent manner. Although citrinin hydrate was previously isolated as human rhinovirus 3C-protease inhibitor, this was recognized as the first AA-NAT inhibitor isolated from natural sources.

Key words: Serotonin *N*-acetyltransferase (AA-NAT) inhibitor, citrinin hydrate, *Penicillium* sp.

Serotonin *N*-acetyltransferase(arylalkylamine *N*-acetyltransferase, AA-NAT, EC 2.3.1.87) catalyses the conversion of serotonin to *N*-acetylserotonin, which is a rate-limiting enzyme in the biosynthetic pathway of melatonin. Melatonin is a pineal hormone which modulates a variety of endocrinological, neurophysiological, and behavioral functions in vertebrates [9]. The synthesis of melatonin occurs mainly in the pinealocytes of the pineal gland, where it reaches its maximum level during the night, and it is also synthesized within the retina [10]. Melatonin receptors have been characterized and several melatonin receptor antagonists and agonists have been described [2, 3]. In order to understand the role of melatonin, availability of melatonin receptor antagonists and AA-NAT inhibitors are essential. For this purpose, several AA-NAT inhibitors are synthesized [11]. On the other hand, a dysregulation in serotonin function has been implicated in the pathophysiology of depression. AA-NAT plays a key role in a number of disorders, such as depression [8] and delayed sleep-phase syndrome [13]. The inhibitors of AA-NAT, which reduces serotonin level, could possibly constitute a very useful antidepressive agent.

A screening process has been adapted for the biologically active substances from natural sources such as the higher plants, microbial metabolites, and mushrooms [7, 14, 15]. As a part of this search to find a new molecule, AA-NAT inhibitors have been searched from microbial metabolites and resulted in the isolation of citrinin hydrate (Fig. 1) from the culture broth of *Penicillium* sp. 80722. In this report, we decribe the isolation, structure determination, and biological activity of citrinin hydrate.

The AA-NAT protein was expressed in *Escherichia coli* BL21(DE3)pLysS culturing in a LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) by induction with isopropyl-β-D-thiogalactopyranoside. The AA-NAT assay was performed according to the method of Chae *et al.* [1]. The AA-NAT was incubated in the presence of 5 μl tryptamine-HCl (10 mM), 1 μl acetyl CoA (0.5 mM), 1 μl [^{3}H]acetyl CoA (3.6 Ci/mmol, 250 μl Ci/ml), 1 μl inhibitor, and 8 μl 50 mM phosphate buffer (pH 6.8). After incubating at 37°C for 30 min, the reaction mixture was stopped by diluting with an additional 180 μl of 50 mM phosphate buffer (pH 6.8) and

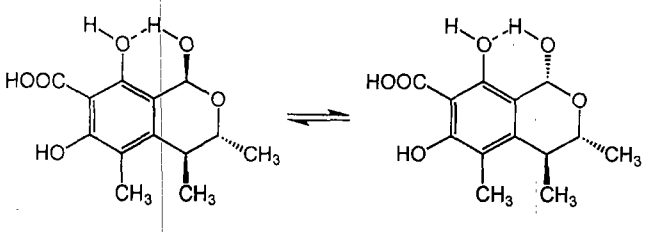

Fig. 1. Structure of citrinin hydrate.

*Corresponding author
Phone: 82-42-860-4330; Fax: 82-42-860-4595;
E-mail: idyoo@mail.kribb.re.kr

then 1.3 ml of Econofluor (1,2,4-trimethylbenzene (99%), 2,5-diphenyloxazole (0.7%, w/v), 1,4-bis(2-methylstyryl) benzene (0.05%, w/v)) and scintillation fluids were added to the reaction tube. After mixing with vortex, the amount of radiolabeled acetyltryptamine was determined in a liquid scintillation counter. Inhibitory activity was calculated as follows; [1−(I-B)/(C−B)]×100 (%), in which I, C, and B are the amount of radiolabeled acetyltryptamine of the inhibitor treatment, control (without an inhibitor), and blank (without enzyme).

The organism which produced a fungal strain *Penicillium* sp. 80722 was isolated from a soil sample. The seed culture was incubated in a medium consisting of 0.4% (w/v) yeast extract, 0.4% (w/v) malt extract, 0.4% (w/v) soytone and 1.0% (w/v) glucose (adjusted to pH 7.0 before sterilization) at 28°C for 4 days, and then transferred to a jar fermentor containing 3.5 l of the same medium. Cultivation was carried out at 28°C for 5 days with aeration of 3 l/min and agitation of 130 rpm.

The active compound was isolated from the fermentation broth (7 l) of *Penicillium* sp. 80722. The broth filtrate was applied to a column of Diaion HP-20. The column was washed with 30% MeOH and eluted with 70% aq. MeOH. The active eluate was concentrated *in vacuo* to eliminate MeOH, and aqueous solution was extracted with ethyl acetate. After concentrating the solvent layer *in vacuo*, the residue was chromatographed on a silica gel column with $CHCl_3$-MeOH (10:1). The concentrated active eluate was applied to a column of Sephadex LH-20 and eluted with 100% MeOH. Active fractions were combined and concentrated to yield 35 mg of the pure compound.

The physicochemical properties of active compound are as follows: Brown powder; UV λ_{max} (MeOH)(ε) 214 (74,000), 253 (24,000), 316 (17,000) nm; ^{1}H NMR (CD_3OD, 500 MHz) major isomer: δ 5.60 (1H, s, H-1), 2.68 (1H, m, H-8), 3.98 (1H, m, H-9), 1.35 (3H, d, *J*=6.6 Hz, H-10), 2.07 (3H, s, H-12), 1.21 (3H, d, *J*=6.6 Hz, H-13), minor isomer: 5.48 (1H, s, H-1), 2.73 (1H, m, H-8), 4.09 (1H, m, H-9), 1.36 (3H, d, *J*=7.2 Hz, H-10), 2.07 (3H, s, H-12), 1.18 (3H, d, *J*=7.2 Hz, H-13); ^{13}C NMR (CD_3OD, 125 MHz) major isomer: δ 96.1 (C-1), 112.8 (C-2), 157.6 (C-3), 101.1 (C-4), 160.9 (C-5), 111.8 (C-6), 144.5 (C-7), 37.8 (C-8), 70.5 (C-9), 20.7 (C-10), 179.4 (C-11), 11.7 (C-12), 19.5 (C-13), minor isomer: 95.8 (C-1), 112.2 (C-2), 158.3 (C-3), 101.6 (C-4), 160.6 (C-5), 109.9 (C-6), 142.9 (C-7), 35.9 (C-8), 74.3 (C-9), 22.1 (C-10), 179.5 (C-11), 9.7 (C-12), 20.7 (C-13); ESI-MS *m/z* 827 $[3M+Na]^+$. The ^{1}H and ^{13}C NMR spectra of purified compound revealed that it was a mixture of isomers in the ratio of 2:1 in CD_3OD. On the basis of the above physicochemical and spectral data, the compound was identified as a citrinin hydrate, and was further confirmed by HMBC spectral data. Citrinin hydrate was previously isolated as a human rhinovirus 3C-protease inhibitor and produced by a *Penicillium* sp. that was isolated from marine invertebrate [5].

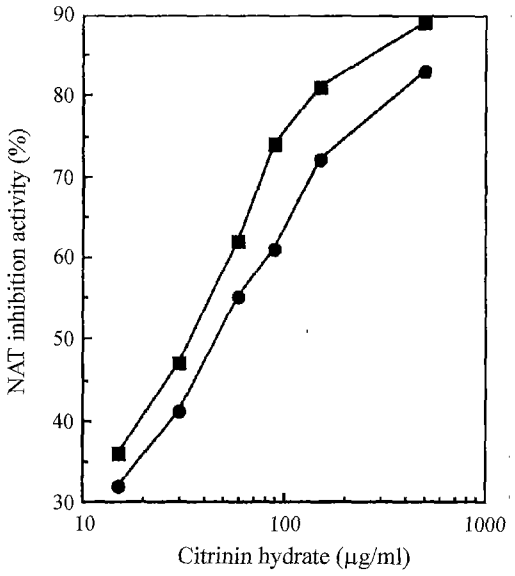

Fig. 2. Inhibitory activity of citrinin hydrate against arylalkylamine-*N*-acetyltransferase(AA-NAT).
AA-NAT inhibition activity of citrinin hydrate was estimated by using AA-NAT in the pineal gland of a rat (■) and in expressed *Escherichia coli* BL21(DE3)pLysS (●).

Citrinin hydrate inhibited NAT in a dose-dependent manner with IC_{50} value of 173 μM, as shown in Fig. 2. In order to confirm the NAT inhibitory activity of citrinin hydrate, AA-NAT in the pineal gland of a rat was used. Rats were sacrificed by decapitation at midnight to harvest pineal glands with high AA-NAT activity. The pineal glands were removed, frozen in a liquid nitrogen, and stored at −70°C until they were used for AA-NAT activity. For *in vitro* organ culture, rat pineal glands were cultured by using a method described previously with some modifications [12]. Pineal glands were placed directly into ice-cold Dulbecco's modified Eagle's medium (DMEM, Gibco Co., Grand Island, NY, U.S.A.) containing 10% bovine calf serum and 1% penicillin/streptomycin. After removing extraneous tissue, the pineal glands were placed on nylon mesh that rested on the DMEM culture medium. The glands were incubated at 37°C in a humidified atmosphere of 5% CO_2. They were preincubated for 3 h before adding isoproterenol, and then further incubated for 4 h after the isoproterenol treatment. The AA-NAT assay was carried out as described by Chae *et al.* [1]. The IC_{50} for AA-NAT inhibition of citrinin hydrate was determined to be 111.5 μM (Fig. 2). These results indicate that citrinin hydrate had some reliable AA-NAT inhibitory activity. On the other hand, serotonin is catabolized by AA-NAT as well as monoamine oxidase (MAO). It is well known that depression is related to a deficit of monoamines such as norepinephrine, epinephrine, dopamine, and serotonin at critical synapses

[4]. Thus, we measured the MAO inhibitory activity of citrinin hydrate.

Monoamine oxidase activity was assayed with kynuramine as the substrate fluorometrically by the Krajl method [6]. Inhibitor (5 μl) dissolved in methanol was added to 0.2 M potassium phosphate buffer (70 μl, pH 7.4) containing 5 μl of MAO suspension and 20 μl of 500 μM kynuramine. After incubating at 37°C for 30 min, the reaction was stopped by addition of 25 μl of 10% $ZnSO_4$ and 5 μl of 1 N NaOH, and then centrifuged at 3,000 ×g for 5 min. Seventy μl of the supernatant were then transferred to a fluoro 96-well plate and 140 μl of 1 N NaOH were added to the plate. The fluorescence intensity of the reaction product, 4-hydroxyquinoline, was measured at 380 nm (emission) with excitation at 315 nm (fluorophotometer: Model F-300, Hitachi, Tokyo, Japan). The blank reaction was carried out by omitting the substrate.

Citrinin hydrate inhibited MAO in a dose-dependent manner, with IC_{50} value of 4.21 μM. The potency of inhibition by citrinin hydrate was comparable to that of a clinically used MAO inhibitor, such as clorgyline (0.71 μM). Thus, it is confirmed that citrinin hydrate had a dual potency of activity that effectively provides serotonin to remain in the pineal gland. This is the first AA-NAT inhibitor isolated from natural sources as a potentially important molecule to treat depression.

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