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Enzyme-assisted synthesis of the glucuronide conjugate of psilocin, an hallucinogenic component of magic mushrooms

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An enzyme-assisted synthesis of psilocin glucuronide (PCG), a metabolite excreted in the urine of magic mushroom (MM) users, is described. In the presence of Aroclor 1254 pretreated rat liver microsomes, psilocin and the cofactor UDPGA were incubated for 20 h. Purification by HPLC gave PCG in 19% yield (3.6 mg). The compound structure was characterized by MS and NMR. The milligram amounts of PCG produced by this method will allow the direct identification and quantification of PCG in the urine of MM users.

Keywords: psilocin; glucuronide; enzyme-assisted synthesis; uridine 5′-diphospho-glucuronosyltransferase

Introduction

Psilocybin (PB) and psilocin (PC) are hallucinogenic compounds found in *Psilocybe* mushrooms, also called 'magic mushrooms' (MM). The structure of these compounds is similar to the neurotransmitter serotonin. The hallucinogenic effect of PB and PC is attributed to their agonist activities to serotonin 5-HT_{2A} receptors.[1–3] In Japan, the possession, cultivation, and intake of MM containing PB and PC have been prohibited by the Narcotics and Psychotropic Control Law since 2002.

Several studies on the metabolism of PB and PC have been reported. The metabolic pathway for PB and PC is shown in Figure 1. After ingestion of MM, PB is rapidly dephosphorylated to PC by an intrinsic phosphatase.[4] PC is metabolized by conjugation with glucuronic acid to PC glucuronide (PCG).[5] Direct analysis by liquid chromatography-mass spectrometry (LC-MS) of urine from MM users has determined that psilocin is mostly excreted as PCG.[6] Thus, the detection of PCG in urine is useful for unequivocal proof of MM use. However, PCG reference material is not currently available. In addition, the preparation of PCG has not been described in the literature. The development of a synthetic method for the preparation of PCG is important for forensic analysis.

Enzyme-assisted synthesis offers an effective way of preparing glucuronides. The advantage of biocatalyzed reactions is that the formation of solely the biologically relevant β-anomer conjugate can be expected. Usually, enzyme-assisted synthesis uses liver microsomes as a general source of glucuronidation enzymes, and this has made it possible to synthesize glucuronides of several types of substrates, for example, COMT inhibitors,[7] silybin,[8] hydroxypyrene,[9] propofol,[10] buprenorphine,[10] anthraflavic acid,[10] octylgallate,[10] anabolic-androgenic steroids,[11,12] dobutamine,[13] losartan,[14] and benzodiazepines.[15] Recently, we reported the enzyme-assisted synthesis of the glucuronide of 4-hydroxy-3-methoxymethamphetamine, one of the metabolites of methylenedioxymethamphetamine (MDMA).[16]

This paper describes the synthesis of PCG by an enzyme-assisted synthesis. The synthesized product was characterized by NMR and MS. PCG was readily prepared in milligram amounts, quantities sufficient for *in vitro* enzyme kinetic studies or reference standards required for *in vivo* pharmacokinetics and metabolism studies, as well as for forensic analysis.

Experimental

General information

UDPGA and alamethicin were purchased from Sigma-Aldrich (St Louis, MO, USA). Psilocin was synthesized according to literature procedures.[17] All other reagents and solvents were purchased from Wako Pure Chemical (Osaka, Japan), Tokyo Kasei Kogyo (Tokyo, Japan), and Kanto Chemical (Tokyo, Japan) and were used without purification. Aroclor 1254-induced male Sprague-Dawley rat liver microsomes (Lot No. ADM) were purchased from Charles River Laboratories (Wilmington, MA, USA).

Analytical HPLC was performed using a CBM-20A system controller, LC-20A pump, SPD-M20A UV/Vis photodiode array detector, and CTO-10AC column oven (Shimadzu, Kyoto, Japan) equipped with a CAPCELL PAK C18 MGII 5 µm, 4.6 × 250 mm column (Shiseido, Tokyo, Japan). The mobile phases were A: 0.1% trifluoroacetic acid (TFA)/H_2O and B: 0.1% TFA/CH_3CN. The gradient was programmed to begin the analysis at 5 : 95 A/B and linearly increase to 30 : 70 A/B over 15 min at a flow rate of 1 ml/min. Preparative HPLC was performed using an SSC-6600 gradient controller, SSC-3465 pump, SSC-5410 UV/Vis detector and SSC-3465 column oven equipped with a SenshuPak PEGASIL ODS column 5 µm, 20 × 250 mm (Senshu Kagaku, Tokyo, Japan). The mobile phases were A: 0.1% TFA/H_2O and B: 0.1% TFA/CH_3CN. The gradient was programmed to begin the analysis at 5 : 95 A/B and linearly increase to 30 : 70 A/B over 20 min at a flow rate of 10 ml/min. The gradient condition was LC-MS was performed using a Dual λ Absorbance Detector 2487

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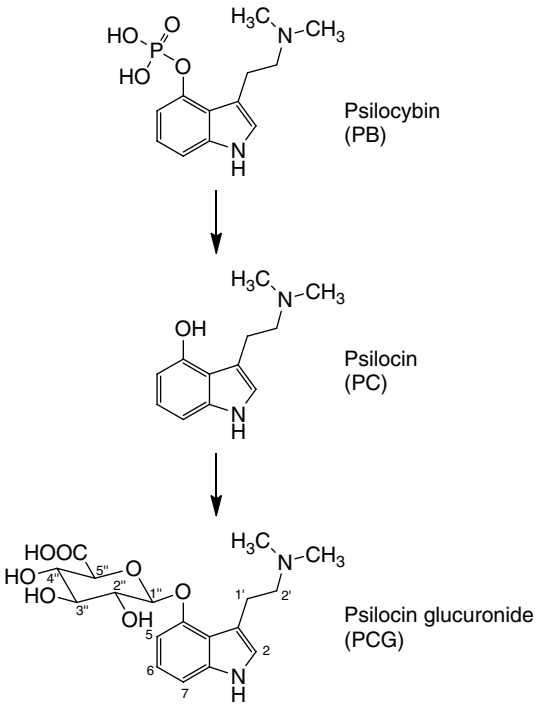

Figure 1. Metabolic pathway for psilocybin and psilocin.

(Waters, Milford, MA, USA), micromass ZQ (Waters) and an Alliance model 2695 (Waters) equipped with a CAPCELL PAK C18 MGII 5 μm, 4.6 × 250 mm column (Shiseido, Tokyo, Japan). The mobile phases were composed of A: 0.1% $HCOOH/H_2O$ and B: CH_3CN. The gradient was programmed to begin the analysis at 5 : 95 A/B and linearly increase to 30 : 70 A/B over 15 min at a flow rate of 1.0 ml/min. The NMR spectra were recorded on a Varian AS 400 Mercury spectrometer and a JEOL ECA-600. Assignments were made *via* ^1H NMR, ^{13}C NMR, ^1H-^1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond connectivity (HMBC), and rotating frame nuclear overhauser effect (ROE) spectra. Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-propionate-2,2,3,3,-d_4 (δ scale). High-resolution mass spectra were obtained on an LTQ Orbitrap equipped with an electrospray ionization source.

Enzyme-assisted synthesis of PCG

A mixture of 10 ml of buffer solution containing 50 mM of Tris-HCl (pH 7.5), 8 mM of $MgCl_2$, 25 μg/ml of alamethicin, 5 mM of UDPGA, and 5 mM of PC was stirred in a 37 °C water bath. The reaction was started by the addition of 200 μl of Aroclor 1254-induced rat liver microsomes (24 mg protein/ml) and was gently stirred for 20 h under protection from light. The reaction was stopped with 5 ml of 10% $HClO_4$. The precipitated proteins were removed by centrifugation (3000 rpm, 10 min, 4 °C), and the supernatant was filtered. The filtrate was purified by preparative HPLC, and after evaporation of the fraction containing the product, PCG was obtained as a white solid (3.6 mg, yield 19%).

Results and discussion

The enzyme-assisted synthesis of PCG was carried out using a 10-ml reaction volume. The incubation condition was based on our recent report.[16] The reaction mixture contained PC (5 mM), Uridine 5′-diphosphoglucuronic acid (UDPGA, 5 mM), alamethicin (25 μg/ml) and Aroclor 1254-induced rat liver microsomes (0.48 mg protein/ml). Alamethicin, a pore-forming peptide, was added to the reaction system to activate UGT activity in the liver microsomes.[18]

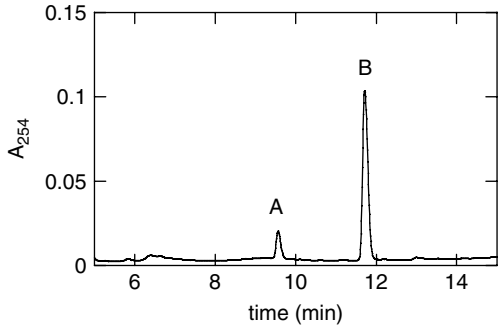

Figure 2. HPLC chromatogram after enzyme-assisted synthesis of PCG. A: PCG; B: PC.

Treatment of rats with Aroclor 1254, a mixture of polychlorinated biphenyls, increased the UGT activity of the rat liver microsomes.[19] The reaction mixture was incubated for 20 h at 37 °C with gentle shaking under protection from light. After protein precipitation and centrifugation, the supernatant was analyzed by HPLC. A representative chromatogram of the reaction mixture is shown in Figure 2. The new peak at retention time 9.6 min (peak A) was found together with the PC peak at 11.6 min (peak B). The purification of peak A was carried out by preparative HPLC. After removal of the eluent, 3.6 mg of white solid was obtained from the initial 10.2 mg of PC.

The most abundant protonated $[M+H]^+$ (*m/z* 381) and deprotonated $[M-H]^-$ (*m/z* 379) molecules recorded in the positive and negative electrospray ionization mass spectra, respectively, indicated the correct molecular weights for PCG, and the observed mass obtained by high-resolution MS (*m/z* 381.1661) corresponded well with the calculated mass for $[M+H]^+$ of PCG (*m/z* 381.1656). ^1H and ^{13}C NMR spectra in D_2O were fully assigned on the basis of ^1H-^1H COSY, HMQC, and HMBC spectra (Table 1). One-dimensional ^1H NMR was used to determine the anomer composition of the glucuronic acid moiety in the PCG. The anomeric proton H1″ was identified by its characteristic chemical shift at 5.28 ppm, and the coupling constant between H1″ and H2″ was 7.6 Hz, which is consistent with a β-configuration. In addition, the irradiation of the H1″ proton at 5.28 ppm resulted in ROE on H5 of the indole proton at 6.70 ppm. This signal indicated that adduct formation could only have occurred at the indole hydroxyl group. Thus, the MS and NMR spectral data confirm the structure of this compound to be PCG.

We also examined the chemical synthesis of PCG by typical procedures for preparing glucuronides, such as the Koenigs-Knorr and the Williamson ether syntheses. However, all attempts with these procedures failed to produce PCG. To date, the enzyme-assisted synthesis is the only successful method for preparing the glucuronide of PC. This method allows the synthesis of the stereochemically pure β-anomer of PCG in milligram amounts (3.6 mg, yield 19%), which is sufficient for use as reference material in forensic analysis.

It has been reported that PC was decomposed during incubation in the presence of microsomes, presumably due to nonenzymatic oxidation.[20] The enzyme-assisted synthesis in this study was

Table 1. ^{1}H and ^{13}C chemical shift assignments of psilocin glucuronide in D_2O

Position	$^{1}H^{a}$ δ (*m*, *J* in Hz)	$^{13}C^{b}$ δ
NCH_3	2.74 (3H, s), 2.76 (3H, s)	41.6
1′	3.15-3.28 (2H, m)	24.7
2′	3.36-3.46 (2H, m)	61.8
2	7.12 (1H, s)	127.2
3		110.9
3a		119.7
4		153.5
5	6.70 (1H, d, 7.6)	106.3
6	7.05 (1H, dd, 7.6, 8.4)	125.9
7	7.10 (1H, d, 8.4)	110.1
7a		141.5
1″	5.28 (1H, d, 7.6)	102.7
2″, 3″, 4″	3.49-3.59 (3H, m)	74.6, 75.9, 78.7
5″	3.90 (1H, d, 7.6)	79.2
6″		178.2

[a] 400 MHz.
[b] 125 MHz.

carried out under protection from light, resulting in the formation of glucuronide as the sole product without any degradation products which were not detected in HPLC chromatogram as shown in Figure 2. Protecting the reaction mixture from light is enough to synthesize the glucuronide without accompanying the degradation of PC.

The lower yield of PCG than that of 4-hydroxy-3-methoxymethamphetamine glucuronide[16] might be attributed to low capacities of the microsomes to catalyze the glucuronidation of PC. Our preliminary data indicate that the yield of PCG using β-Naphthoflavone-induced rat liver microsomes as a biocatalyst is 1.3 times higher than that using Aroclor 1254-induced rat liver microsomes (data not shown). Therefore, the optimization of the incubation conditions for large-scale synthesis is under consideration.

In conclusion, we have demonstrated an efficient method for the preparation of PCG by an enzyme-assisted synthesis. The PCG synthesized by this method makes it possible to directly identify and quantify PCG in the urine of MM users. Further studies for the identification of MM intake using PCG as a reference material are now in progress.

Acknowledgement

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