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Synthesis of tritium-labeled cyadox, a promising antimicrobial growth-promoting agent with high specific activity

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HIGHLIGHTS

- An efficient synthesis of tritium-labeled growth-promoting agent cyadox is presented.
- A high-specific activity product was obtained by radiosynthesis at a micromolar scale.
- In all the reaction steps, high radiochemical yields of 36.16–94.75% were achieved.
- Specific activity and radiochemical purity of the products were more than 99%.
- 4-[^{3}H]-ONA can be used as the starting material to produce tritium-labeled quinoxaline-N,N-dioxides and quinoxaline derivatives.

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ABSTRACT

Cyadox is a new antimicrobial growth-promoting agent for food-producing animals. Studies on radiolabeled compounds enable the use of sensitive radiometric analytical methods and help in the elucidation of metabolic and elimination pathways. In the present study, 6-[^{3}H]-cyadox with a high specific activity of 2.08 Ci/mmol was prepared by the catalytic bromine–tritium exchange of 4-bromo-2-nitroaniline followed by a three-step microscale synthesis, giving a high yield between 36.16% and 94.75%.

1. Introduction

Cyadox (CYA, Scheme 1), 2-formylquinoxaline-N_1, N_4-dioxide cyanoacetylhydrazone, a derivative of quinoxaline-*N*,*N*-dioxides, is an antimicrobial growth-promoting agent of quinoxalines. It has been proven to be effective against most of the pathogenic bacteria in food-producing animals (Fan et al., 2000; Huang et al., 2003), promote the average daily gain and feed conversion ratio, and prevent *Escherichia coli* infection in pigs and chickens (Wang et al., 2005; Ding et al., 2006a, 2006b; Huang et al., 2002). CYX did not show any adverse effects in carcinogenicity tests with rats and long-term toxicity tests with rats or pigs (Ševčík, 1986). Subchronic oral toxicity study suggested that CYX possesses significantly lower toxicity than olaquindox (Fang et al., 2006), which is another derivative of quinoxaline-N_1, N_4-dioxide. The phototoxicity of CYX was mild and reversible, which demonstrated a good safety profile of CYX in terms of phototoxicity (He et al., 2006). However, the widespread use of CYX, as a new member of the quinoxaline-N_1, N_4-dioxides, in food-producing animals may produce residues that remain in edible tissues after slaughter. Sponsors of drugs used in food animals must demonstrate that the residue that remains in edible tissues of treated animals are safe to consumers (FDA, 2005), and the presence of drug residue in tissues of food-producing animals is undesirable from a public safety standpoint. Radiolabeled drugs are often used to identify and elucidate the metabolites formed, as well as to investigate the extent of absorption and excretion, tissue distribution, and metabolism (Marathe et al., 2004). In another study, we

Abbreviations: T, Tritium; D, Deuterium; RP HPLC, Reverse-phase high pressure liquid chromatography; UV, Ultraviolet spectrophotometer; LSC, Liquid scintillation counter; TLC, Thin layer chromatography; CYX, Cyadox; ONA, O-nitroaniline; 4-Br-ONA, 4-bromo-2-nitroaniline; BFO, Benzofuroxan; FQDDA, 2-formylquinoxaline-1,4-dioxide dimethylacetal; DMSO, Dimethylsulfoxide; DMF, *N,N*- dimethylformamide; NaClO, Sodium hypochlorite solution; PDA, Pyruvaldehyde dimethylacetal

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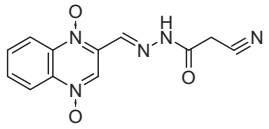

Scheme 1. Structure of CYX.

investigated the mass balance, metabolism, tissue distribution, and depletion of CYA in pigs, chickens, carp, and rats after an oral administration of 6-[^{3}H]-CYA (Huang et al., 2015) synthesized in this study.

The purpose of this study was to synthesize a high-specific activity tritium-labeled CYX. Synthesis of tritium-labeled CYX was accomplished by the microscale synthesis method. Tritium atom was labeled at the sixth position of aromatic ring, which likely to be portion of most toxicological concern for the CYX, and its potential metabolites in animal tissues and excreta after metabolism.

2. Experimental

2.1. Chemicals

Analytical-grade starting material 4-Br-ONA and 10% Pd/C catalyst were purchased from Sigma-Aldrich. Tritium gas was provided by Shanghai Institute of Applied Physics Chinese Academy of Sciences. Standard and reference compounds CYX, benzofuroxan (BFO), 2-formylquinoxaline-1, 4-dioxide dimethylacetal (FQDDA), and cyanoacetylhydrazine were provided by the Institute of Veterinary Pharmaceutics (Huazhong Agricultural University, Wuhan, Hubei, China). HPLC solvents were filtered through a 0.22-μm durapore membrane (Millipore). Deionized water was obtained from a Milli-Q System (Millipore, Bedford, MA, USA). Monophase-S was purchased from PerkinElmer Life and Analytical Sciences. All other chemicals and solvents were of analytical grade or higher and used without purification.

2.2. Apparatus

Tritiation was carried out on a tritium manifold system (RC TRITEC AG in Switzerland). Evaporations were carried out on a ZHENJIE rotary evaporator (Model RE-52AA). Solution radioassays were conducted with a PerkinElmer® TriCarb® 2900 instrument. Radiochromatogram scan was performed on a Bioscan's AR-2000 radio-TLC scanner.

Analytical and preparative HPLC were performed on a comprising Waters 600 controller, 717plus Autosampler, and 2996 Photodiode Array detector. Separation and purification of 4-[^{3}H]-ONA were performed using a venusil XBP-C18 (21.5 mm × 150 mm, 10 μm, 100 Å) (Agela Technologies Inc., USA) semipreparative HPLC column. The temperature of the HPLC column was maintained at 30 °C. The mobile phase consisted of methanol and water (75:25, V/V), with a flow rate of 5.0 mL/min. The sample volume injected was 500 μL. The detection wavelength was 231 nm. The analysis of 4-[^{3}H]-ONA was accomplished on a venusil XBP C18 column (4.6 mm × 250 mm, 5 μm) (Agela Technologies Inc., USA), and the mobile phase consisted of methanol and water (60:40, V/V). The flow rate was 1 mL/min^{-1}. The sample volume injected was 20 μL.

The analysis of 6-[^{3}H]-CYX was accomplished on a venusil XBP C18 column (4.6 mm × 250 mm, 5 μm) (Agela Technologies Inc., USA). The mobile phase consisted of acetonitrile and water (20:80). The flow rate was 1 mL/min. The UV detector was set at a wavelength of 306 nm. The sample volume injected was 20 μL.

4-[^{2}H]-ONA and 6-[^{2}H]-CYX were confirmed using SHIMADZU LC/MS-IT-TOF (Shimadzu Corp., Kyoto, Japan) instrument with direct injection. The IT-TOF-MS was equipped with an electrospray ionization (ESI) source operated in the positive ionization mode. Mass spectroscopic analyses were carried out on a full-scan mass spectrometer with a mass range of 100–500 Da. High-purity nitrogen was used as nebulizing gas at a flow rate of 1.5 L/min. The interface and detector voltages were set at 4.5 and 1.6 kV, respectively. The curved desorption line (CDL) and heat block temperatures were both 200 °C. The MS^2 spectra were produced by the collision-induced dissociation (CID) of the selected precursor ions with Ar as the collision gas. The ion accumulation time and relative collision energy were set at 50 ms and 50%, respectively. Data acquisition and processing were carried out using the software LCMS solution version 3.41 supplied with the instrument.

2.3. Preparation of 4-[^{3}H]-ONA

A solution of 20 mg of 4-Br-ONA in ethanol with 5 mg of 10% Pd/C catalyst, 4 mg of NaOH, and a teflon magnetic stirrer were introduced into a 5-mL reaction flask. The reaction flask was introduced into the tritium manifold system (RC TRITEC AG in Switzerland) and vigorously stirred with tritium gas at a pressure of 150 mmHg. The reaction temperature was 30 °C. The optimal yield of product formation was achieved at 45 min. After this time, unreacted tritium was recovered to a tritium storage tank, and the catalyst was removed from the product by filtration. Labile tritium was removed by several evaporations of ethanol, and the reaction mixture was dissolved in 1 mL of ethanol and preserved in a sealed tube under nitrogen at − 20 °C. The total radioactivity of the reaction mixture was determined to be 589 mCi using a liquid scintillation counter (LSC). Radiochromatogram scan of crude reaction mixture was performed on a Radio-TLC system, and the TLC plate was coated with silica gel. The developed solvent consisted of n-hexane, ethyl acetate, and methylbenzene (10:2:1), and it was ≥ 99% radiochemically pure (Fig. 1).

The reaction mixture was dissolved in 3 mL of ethanol and injected in RP HPLC. 4-[^{3}H]-ONA fraction was pooled, and evaporation of the methanol of the mobile phase was carried out on a rotary evaporator at a bath temperature below 40 °C. 4-[^{3}H]-ONA was extracted 3 times with methylene dichloride from water phase. The extracts were pooled and evaporated to dryness on a rotary evaporator at a bath temperature below 35 °C. 4-[^{3}H]-ONA was removed by 5 mL of ethanol and diluted

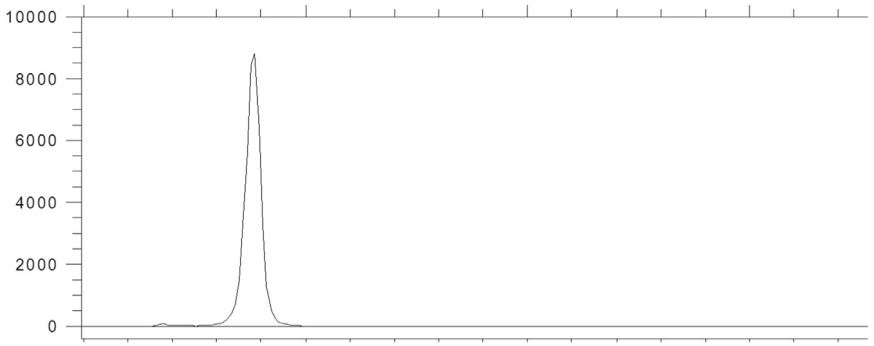

Fig. 1. Radiochromatogram scan of the tritiated ONA reaction mixture (Y-axis: cpm; X-axis: cm; without purification).