Journal of Radioanalytical and Nuclear Chemistry (2021) 329:1067–1071
https://doi.org/10.1007/s10967-021-07852-3

Tritiation of the botanically derived toxin dihydrorotenone

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Received: 21 April 2021 / Accepted: 13 June 2021 / Published online: 26 June 2021

Abstract
Pests such as insects threaten the efficient production of the world's demanding food requirements. For this reason, insect management has increasingly relied on botanical insecticides as safer alternatives to synthetic agents. Rotenone and related compounds (rotenoids) were among the earliest botanical natural products to be identified as useful insecticides. To elucidate the rotenoid mechanism of action at the insect receptor level and design even more effective insecticides, a tritiated rotenoid analogue was required. As a result, this paper describes the successful synthesis and characterization of [^{3}H] dihydrorotenone at high specific activity. In doing so, it also provides another example of a tritiated dihydro analogue of a parent compound acting as a surrogate for it in biological applications.

Keywords Dihydrorotenone · Rotenone · Tritium

Introduction

With the rapidly growing human population, an especially urgent need is the acceleration of food production to satisfy an increasingly hungry world [1]. While many insects are beneficial for food creation (pollination for instance), others are incredibly harmful pests that destroy crops or food storage and need to be controlled. The global cost of invasive insect infestation is extraordinary and predicted to be exacerbated by ongoing climate change [2]. For many years, synthetic insecticides were exclusively employed for insect management but these could be target indiscriminate and also posed an environmental contamination risk. For this reason, over the past several decades a new strategy of utilizing more environmentally friendly and specific insecticides has emerged. Plants collectively constitute the largest single biomass on Earth [3] and besides supplying food, fiber and fuel, they have been a cornucopia of valuable natural product chemicals for mankind's many needs [4]. It is therefore not surprising that botanical insecticides have been more frequently turned to as a new insect control paradigm [5, 6].

One of the earliest and most selective botanical insecticides to be identified was the structurally complex natural product rotenone (**1,** Fig. 1), isolated from the roots and stems of plant species which are largely members of the genera *Derris* and *Lonchocarpus*. Figure 1 illustrates the conventional rotenone ring lettering designations (A-E) and several key carbon atom numberings. Rotenone is the centerpiece of a group of natural products collectively known as rotenoids, some of which like rotenone are inhibitors of the mitochondrial electron transport chain complex I [7]. After identifying a lead natural product like rotenone, it is important to fully understand its biological activity at the receptor level. This effort is usually best supported by employing a high specific activity radioactive analogue. In this way, structure–activity relationships can be defined, often leading to the discovery of more potent natural product derivatives. For this reason, we needed to obtain a rotenoid labelled with tritium and now describe the synthesis and characterization of [^{3}H] dihydrorotenone.

Experimental

General

Evaporations were carried out on a Buchi® rotary evaporator (model RE 111) at bath temperatures less than 40 °C. Analytical thin layer chromatography (TLC) autoradiography was accomplished on Analtech® 5 × 15 cm silica gel coated glass plates at 0 °C after spraying with 2,5-diphenyloxazole and exposing the plate to X-ray film.

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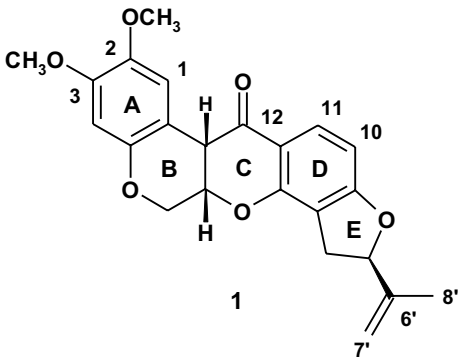

Fig. 1 Structure of rotenone (**1**)

TLC plates were also scanned (~ 3 min) for applied radioactivity (~ 10 uCi) using a Bioscan® AR-2000. Analytical and preparative high-performance liquid chromatography (HPLC) were done on a PerkinElmer® Series 200 instrument with peak detection measured simultaneously by UV and flow scintillation using a PerkinElmer radiomatic 150TR flow scintillation detector. Solution assays were performed with a PerkinElmer Tri-Carb® 3100TR analyzer using PerkinElmer Ultima Gold™ AB as the liquid scintillation cocktail. NMR spectra were obtained on a Bruker® 300 MHz NMR spectrometer and chemical shift values are expressed in parts per million (ppm) downfield from internal tetramethylsilane. UV spectra were done on a PerkinElmer Lambda™ 35 UV/Visible spectrophotometer. All chemicals used were reagent grade.

Dihydrorotenone (2)

A solution of **1** (30 mg, 0.076 mmol, Sigma-Aldrich R8875) in 2 mL of ethyl acetate with 30 mg of 10% Pd/C was stirred under hydrogen at ambient temperature and atmospheric pressure for 3 h. After this time, the catalyst was filtered and the ethyl acetate filtrate was removed by rotary evaporation. The resulting product **2** was homogeneous on silica gel TLC (hexane:ethyl acetate (3:2)) and was also analyzed by proton NMR ($CDCl_3$). The proton NMR spectrum of **2** was in excellent agreement with the structure of **2** and no remaining **1** was observed. In particular, as a result of the hydrogenation, key proton NMR resonances for **1 (**7' position (5.07 and 4.94 ppm), 8' position (1.77 ppm)) were no longer present and were replaced by the corresponding proton NMR resonances for **2** (6' position (1.20 ppm), 7' or 8'positions (0.79 or 0.62 ppm). TLC indicated that **2** was > 95% chemically pure.

[^{3}H] Dihydrorotenone (3)

A solution of **1** (20 mg, 0.05 mmol, Sigma-Aldrich R8875) in 2 mL of ethyl acetate with 20 mg of 10% Pd/C was stirred with 60 Ci of tritium at ambient temperature and atmospheric pressure for 3 h. After this time, the catalyst was filtered and labile tritium was removed by several sequential evaporations of methanol to yield crude product. All of this material was purified by reverse phase HPLC with elution of the solvent system 1% aqueous triethylammonium acetate (pH 4): acetonitrile in a linear gradient of (90:10) to (60:40) at 3 mL/min over the course of 1 h followed by isocratic solvent (50:50) elution for another 20 min with simultaneous UV (220 nm) and radioactive flow detection. Combining of product-containing fractions resulted in 943 mCi. A 255 mCi portion of this material was further purified on two 500 µm silica gel plates (20 × 20 cm) developed with hexane:ethyl acetate (3:2). After development, the plates were briefly air dried and quickly UV visualized. The product-containing silica gel bands were scraped and combined. Ethanol elution of the silica gel bands afforded 156 mCi (a 22.9% extrapolated yield based on **1**) of product **3** which was radiochemically homogeneous on silica gel TLC developed with hexane:ethyl acetate (3:2) as well as > 98% radiochemically pure on reverse phase HPLC (acetonitrile: water (60:40)). Product **3** also co-chromatographed with standard **2** in these chromatographic systems. The UV (ethanol) spectrum of **3** was superimposable on that of **2**. Using the prominent UV band of **3** at 294 nm with ε = 14,280 L/mol cm (and a companion solution radioassay of **3**) gave a specific activity value of 50.5 Ci/mmol for **3**.

Results and discussion

Investigation of the rotenoid family of natural products has been underway for nearly 120 years. Likely the first chemistry efforts in the rotenoid area were those of Japanese researchers and this interesting early history has been summarized by LaForge [8]. During the first part of the twentieth century, gradual progress was made by natural products chemists in identifying the various functional groups (ketone, methoxy, olefin) at the perimeter of the elusive rotenone structure. However, the complete structure of rotenone including details of its ring stereochemistry remained a puzzling conundrum for decades. Remarkably, it was not fully elucidated until 1961 and only after an enormous collaborative effort by George Buchi at MIT and Leslie Crombie at the University of London [9]. What is exceptionally remarkable about this *tour de force* structure determination of rotenone by the Cambridge—London groups is that it was all accomplished by means of logical and methodical rotenone derivatization work before the advent of NMR

spectroscopy. Years later, their proposed structure of rotenone was completely corroborated by unequivocal X-ray crystallography [10]. Since that time, research interest in the rotenoid compound family has accelerated. Currently, the SciFinder® chemistry database lists over 17,000 publications associated with the rotenoid natural product area.

Needing to tritium label a member of the rotenoid compound class at high specific activity for biological applications, consideration was first given to the tritiation of rotenone itself. However, it was soon realized that this would be a daunting task. A general exchange of rotenone with tritium gas or tritiated water by the usual strenuous conditions would certainly be attended by side reactions. A possible (A-ring) halogenation—catalytic tritium dehalogenation strategy was precluded by the complicating presence of the reduction vulnerable rotenone (E-ring) isopropenyl group. Also, concomitant tritiation of the isopropenyl group would likely accompany any attempt at employing Crabtree's catalyst ($[Ir(COD)(Cy_3P)(Py)]PF_6$ [11]) or related iridium catalysts to exchange tritiate the rotenone aromatic 11-position. Another option entertained was demethylating one of the rotenone aromatic methoxy groups and remethylating it with either [^{3}H] methyl iodide or [^{3}H] methyl nosylate. Casida at Berkeley had first explored this demethylation approach (with boron tribromide) as a carbon-14 labelling method for rotenone but encountered epimerization, low yields and other untoward complications [12]. Aware of Casida's earlier results, Crombie later employed an alternative regiospecific demethylating reagent, trimethylsilyl iodide, on rotenone. With this modification, he was able to obtain a demethylated derivative, exclusively at the rotenone aromatic 2-position, in 27% yield [13]. Crombie proposed (text discussion page 782) tritiation of this desmethyl rotenone with [^{3}H] diazomethane to afford [methoxy-^{3}H] rotenone. Although the experimental section of this paper described the use of [^{2}H] diazomethane to prepare [methoxy-^{2}H] rotenone, experimental details for the corresponding tritiation to obtain [methoxy-^{3}H] rotenone were apparently not provided. Based on the current technology to synthesize [^{3}H] diazomethane [14], if this tritiation of rotenone were accomplished, it would likely result in only a modest product specific activity outcome.

Given this technical impasse, a new rotenoid radiolabelling plan was required. It was noted above that the presence of the rotenone E-ring isopropenyl moiety interfered with several possible tritiation methods so we then considered whether this problematic group could instead be leveraged to advantage? We had earlier reviewed the fact that many tritiated dihydro ligands have been successfully synthesized and employed as valuable surrogates for their parent olefin analogues [15]. Likely the earliest illustration of this simple but effective concept from our own laboratories was the synthesis and characterization of [^{3}H] dihydroalprenol and its outstanding performance as a high specific activity proxy radioligand in place of [^{3}H] alprenolol at the beta adrenergic receptor [16]. An even more profound example of this substitution scheme relevant to rotenone was the isopropenyl group-decorated natural product insecticide picrotoxinin (**4,** Fig. 3). For many of the same technical reasons already cited regarding rotenone, we quickly found that picrotoxinin was also very difficult to directly tritium label. Our solution instead was to catalytically tritiate the isopropenyl group of picrotoxinin, giving [^{3}H] dihydropicrotoxinin (**5**) which became a very successful substitute for [^{3}H] picrotoxinin as a GABA receptor radioligand [17]. This compelling result provided encouraging precedent for a similar solution to the rotenone tritiation problem by substitution with the surrogate [^{3}H] dihydrorotenone (**3**, Fig. 3). It was already well known that the beta stereochemistry of the rotenone isopropenyl group (as portrayed in structure **1**) was critical for the successful binding of rotenone to mitochondrial complex I [18]. Our hope was (as in the related case of picrotoxinin) that the isopropenyl group tritium reduction to [^{3}H] dihydrorotenone would not diminish its complex I binding affinity to any great degree.

Our strategy to synthesize [^{3}H] dihydrorotenone was the catalytic tritium reduction of rotenone itself. The corresponding catalytic hydrogenation of rotenone had been studied by several investigators, the earliest description of which appeared to be by Kariyone [19]. Curiously, the literature also contained a single report of a similar plan as described here to obtain [^{3}H] dihydrorotenone [20]. However, useful experimental details were unfortunately lacking in that paper. The authors did not disclose either the final radiochemical yield or HPLC radiochemical purity of the [^{3}H] dihydrorotenone. Furthermore, both the product identity confirmation as well as the specific activity calculation for [^{3}H] dihydrorotenone were ambiguous. This missing technical information prompted our full description here of the synthesis and characterization of [^{3}H] dihydrorotenone.

Since rotenone and related compounds are photochemically unstable [21], precautions were taken during this

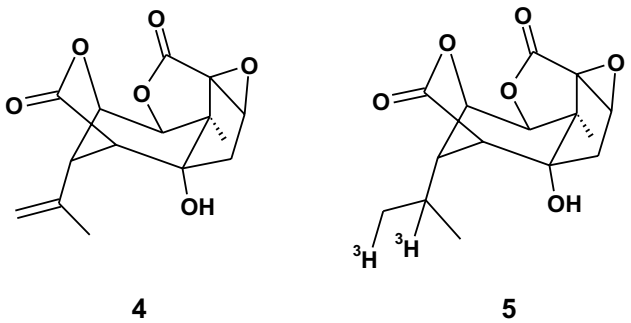

Fig. 2 Structures of picrotoxinin (**4**) and [^{3}H] dihydropicrotoxinin (**5**)

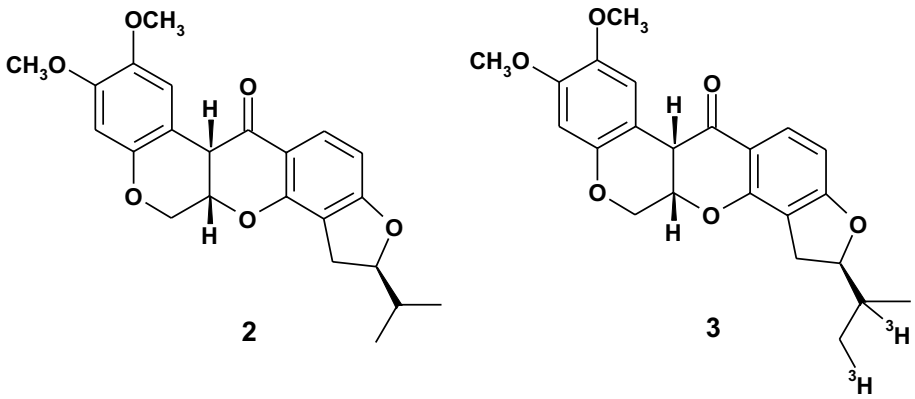

Fig. 3 Structures of dihydrorotenone (**2**) and [^{3}H] dihydrorotenone (**3**)

synthetic campaign to protect reactions and products from light. With past technical precedent as a guide, we first performed a catalytic (10% Pd/C) hydrogenation of rotenone in ethyl acetate at ambient temperature for 3 h. A proton NMR ($CDCl_3$) of the product confirmed that it was indeed dihydrorotenone (**2**), especially evident by loss of the two characteristic peaks for the vinyl protons on the rotenone isopropenyl olefin [22] as well as appearance of several new upfield methyl resonances for the resulting hydrogenated isopropyl group. TLC indicated that the dihydrorotenone was > 95% chemically pure.

Using very similar reduction conditions, we then accomplished a catalytic tritiation of rotenone. Although experience with the earlier catalytic hydrogenation indicated that 3 h for the rotenone tritiation should easily be sufficient, we also decided to employ a purification system which would assure the complete separation of [^{3}H] dihydrorotenone from any remaining rotenone precursor. Literature precedent indicated that reverse phase HPLC using an aqueous acetonitrile solvent system would allow baseline separation of the more lipophilic [^{3}H] dihydrorotenone from any earlier eluting rotenone [23]. We also utilized a gradient HPLC system for an even more effective separation. In the crude tritium reduction, an impurity was observed as a lower Rf (more polar) compound on TLC (silica gel developed with hexane:ethyl acetate (3:2)). Although this side product was not isolated and characterized, its chromatographic behavior was consistent with catalytic tritiation of the rotenone 12-position ketone as observed by the earlier authors [20]. The HPLC purified [^{3}H] dihydrorotenone obtained was > 98% radiochemically pure by both TLC and HPLC and co-chromatographed with dihydrorotenone standard in these chromatographic systems. The UV (ethanol) spectrum of [^{3}H] dihydrorotenone was superimposable on that of dihydrorotenone and (along with a companion solution radioassay of [^{3}H] dihydrorotenone) provided the high specific activity value of 50.5 Ci/mmol for it. The [^{3}H] dihydrorotenone was best stored in ethanol at 1 mCi/mL, −20 °C under nitrogen and free from light. Based on the stability of similar high specific activity radioligands, the HPLC radiochemical purity of [^{3}H] dihydrorotenone would be expected to decrease by about 1% per month.

Conclusions

The successful management of harmful insects has been increasingly accomplished with environmentally friendly agents like botanical insecticides. Rotenone and derivatives of it were among the earliest substances to be identified and evaluated for this purpose. Experimental details have been described here to prepare hundreds of mCi of [^{3}H] dihydrorotenone at high HPLC radiochemical purity and high specific activity. This radioligand has been successfully utilized to label complex I of the mitochondrial electron transport chain [24]. Furthermore, use of [^{3}H] dihydrorotenone *in lieu* of [^{3}H] rotenone has again validated the powerful concept of successfully substituting a more easily tritiated dihydro analogue as a surrogate for a tritiation-challenging parent compound [15].

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