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Synthesis of [^{2}H]- and [^{13}C]-labeled pyronaridine tetraphosphate—an antimalarial drug

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The increasing prevalence of strains of *Plasmodium falciparum* resistant to chloroquine and other antimalarial drugs, necessitates the need for developing novel antimalarial drugs with a potent pharmacological activity. Pyronaridine tetraphosphate (PNDP) is one such drug that is currently undergoing preclinical and clinical trials for use in a chemotherapy treatment of malaria. The present investigation was carried out with the objective of synthesizing carbon-13 [^{13}C]- and deuterium [^{2}H]-labeled PNDP for use in studying the ADME and pharmacokinetics of the drug. Here, we present a methodology to synthesize [^{13}C]- and [^{2}H]-PNDP using a microwave irradiation technique as this method was found to be more advantageous than the classical method. The labeled compounds thus synthesized had a chemical purity of $>$99% as determined by HPLC and were also found to be relatively stable up to 3 months when stored under standard conditions. Further they also revealed satisfactory instrumental analysis data.

Keywords: carbon-13; deuterium; pyronaridine tetraphosphate; microwave irradiation technique; antimalarial drug

Introduction

Malaria, a life-threatening disease caused by the *Plasmodium* parasites transmitted by female anopheles mosquito, is reported to be responsible for about 1 to 2 million deaths each year globally. It is endemic to 90 countries worldwide and affects nearly 40% of the world's population, making it one of the most onerous global health problems.[1] Several antimalarial drugs have been developed over the years and they are being used for treating malaria with varying degree of success. Chloroquine, a 4-aminoquinoline derivative, emerged during the first part of the 20th century as the drug of choice against malaria.[2,3] It proved to be the most successful antimalarial drug on a worldwide scale owing to its wide deployment coinciding with the geographical distribution of *Plasmodium* and also for its high intrinsic antiparasitic efficacy and low toxicity.[4] However, the malarial parasite *P. falciparum* has started to develop a widespread resistance to chloroquine and this has become a major cause for concern. Presently, as the strains of *P. falciparum* have become resistant to chloroquine and their resistance to other alternative antimalarial drugs has also been reported,[5–9] it has become imperative to develop new antimalarial drugs and understand their mechanism of action, an offspring of which is pyronaridine. Pyronaridine, (2-methoxy-7-chloro-10 [3,5-bis(pyrrolidinyl-1-methyl-) 4-hydroxy-phenyl] aminobenzyl-(b)1,5-naphthyridine), is a relatively new antimalarial agent synthesized in the 1970s at the Institute of Parasitic Disease, Chinese Academy of Preventive Medicine.[10–13] It is a benzonaphthyridine derivative that has been in use in China for more than 20 years for the treatment of malaria. The antimalarial activity of pyronaridine has been attributed to both blood schizontocidal and gametocytocidal activities. Pyronaridine was shown in several field studies involving several thousand cases in China, to have a promising therapeutic value in the treatment of a malarial infection. In a clinical trial, pyronaridine was found to be safe and well tolerated by symptomatic children and it is highly efficacious in regions where chloroquine resistance is well established. Pyronaridine is available as a free base and also as pyronaridine tetraphosphate, which is soluble in water.[14] The World Health Organization (WHO) plans to complete preclinical and clinical trials with the aim of replacing chloroquine with pyronaridine as the first line of treatment for malaria, particularly in Africa.

Preclinical studies are usually performed on experimental animals to obtain information regarding the absorption, distribution, metabolism and excretion of a drug in addition to the pharmacological (effective dose) and toxicological (lethal dose) properties of a drug under investigation. The use of a radiolabeled drug is considered to be one of the most effective methods of tracking a drug inside a biological system. It is highly sensitive and is also effective in detecting the metabolites of a drug at desired intervals of time after administering a drug to experimental animals. The present investigation was carried out with the objective of synthesizing carbon-13 [^{13}C]- and deuterium [^{2}H]-labeled PNDP so as to use them in studying the ADME and pharmacokinetics of the drug. Using PNDP-labeled

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with stable isotopes such as [^{13}C] and [^{2}H] is relatively safe when compared with [^{14}C]-, [^{3}H]- and [^{131}I]-labeled PNDP as it does not pose the risk of a radiation exposure. Further, it will also facilitate in an easy detection of its metabolites while performing ADME and pharmacokinetic studies in *in vivo* and *in vitro* systems, due to the advancement in the field of mass spectroscopy (MS). An important point to be considered during a radiolabeling of any drug is the position at which the radioisotope has to be introduced into the chemical structure of a drug. The structure of the expected metabolites of a drug has to be taken into account before selecting the position of a radioisotope introduction. Generally, the position of a radiolabeling is selected such that the labeling is highly stable and is not cleaved from the parent compound during the process of a cellular metabolism when administered to a biological system. This will result in the metabolites also retaining their radioactivity, thus facilitating in a continuous tracking of a drug inside the body. Further, this will enable one to detect not only the metabolites of a drug but will also allow one to detect the end product of a drug undergoing a cellular metabolism. In this study, we present a methodology to synthesize [^{13}C]- and [^{2}H]-labeled PNDP using a microwave irradiation technique. Figure 1 shows the expected metabolites of pyronaridine. The position at which the isotopes [^{13}C] and [^{2}H] were introduced to replace the stable atoms is shown in Figures 2 and 3, respectively, and they were determined by considering the structure of the several expected primary metabolites of pyronaridine.

Results and discussion

The synthesis of pyronaridine tetraphosphate (**3**), [^{13}C]- and [^{2}H]-labeled pyronaridine tetraphosphate starting from PNC (**1**) is outlined in Schemes 1, 2 and 3, respectively.

Synthesis of pyronaridine tetraphosphate (3) by a classical method

Paraformaldehyde (20 eq, 2.862 g), pyrrolidine (20 eq, 7.96 mL) and anhydrous ethanol (10 eq, 16.7 mL) were mixed and refluxed

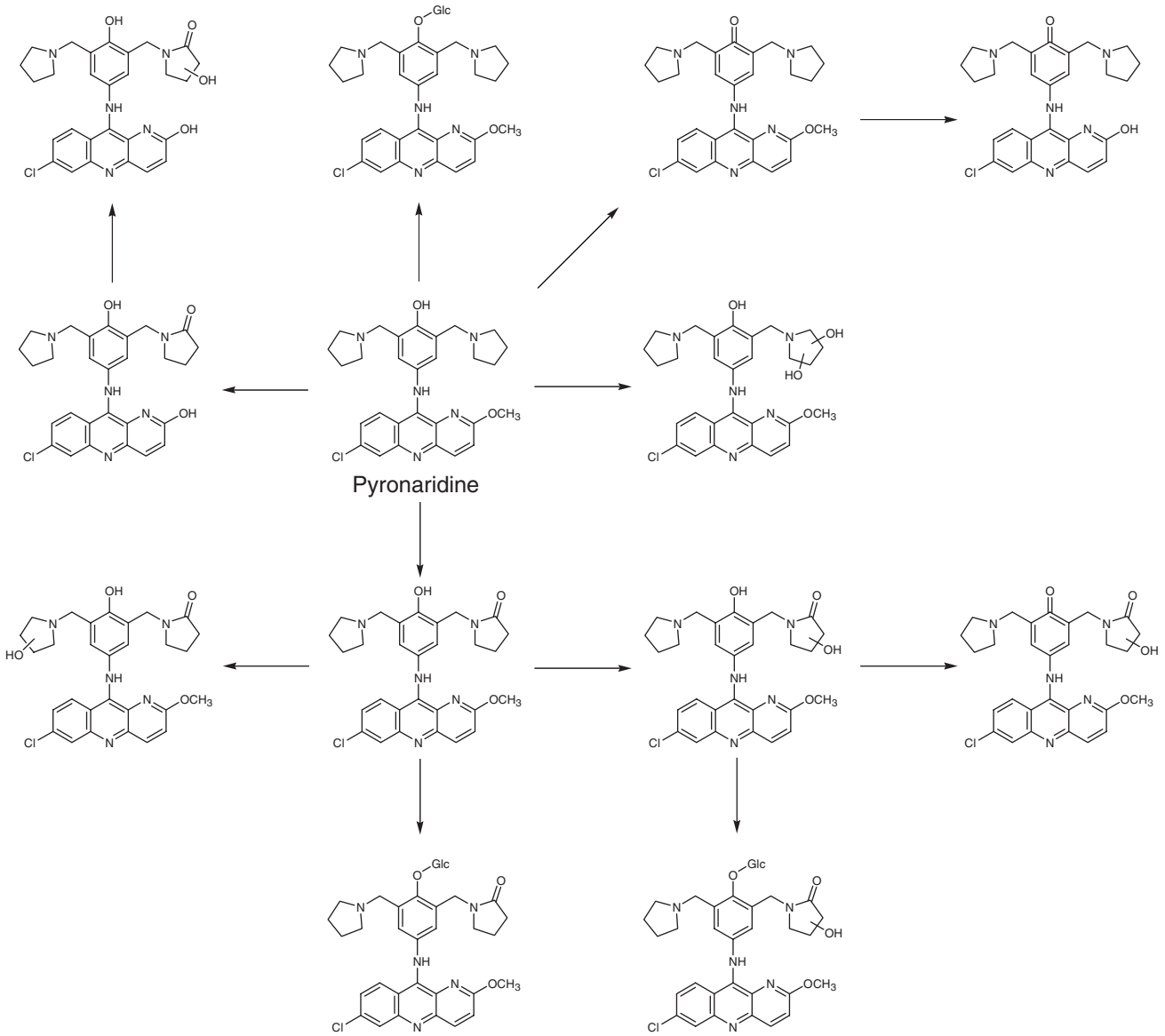

Figure 1. Expected primary metabolites of pyronaridine.

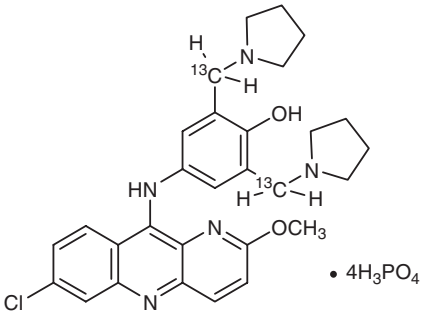

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Figure 2. Structure of [^{13}C]-labeled pyronaridine tetraphosphate showing the position of [^{13}C]-labeling.

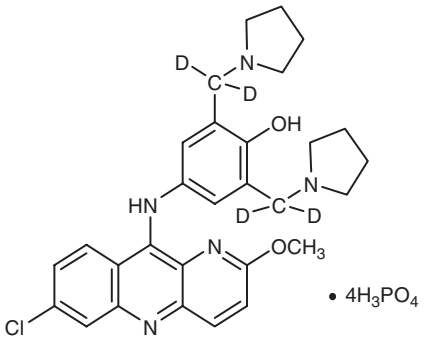

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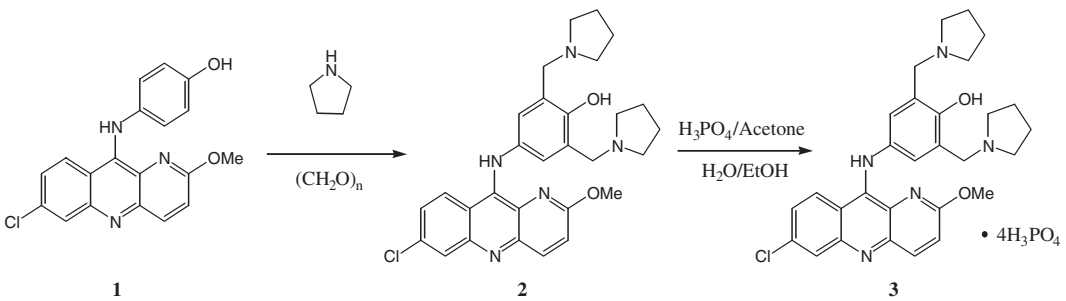
Figure 3. Structure of [^{2}H]-labeled pyronaridine tetraphosphate showing the position of [^{1}H]-labeling.

at 50–60°C for 1 h. Then 1.676 g of PNC (2-2-methoxy-7-chloro-10-(40-hydroxyanilino)-[b]-1,5-naphthyridine, (**1**) was added to the mixture and it was stirred for 14–16 h at 45–50°C. Upon a completion of the reaction, distilled water (10 eq, 16.7 mL) was added to the mixture and stirred at 15–25°C for 1 h and then filtered. The residue was dissolved in anhydrous methanol (10 eq, 16.7 mL) and stirred at 5–10°C for 2 h, then filtered and washed with excessive methanol and finally dried to yield 40% of PND (2-methoxy-7-chloro-10 [3.5-bis(pyrrolidinyl-1-methyl-) 4-hydroxyphenyl] aminobenzyl-(b)-1,5-naphthyridine, (**2**). PND (**2**) thus obtained was mixed with distilled water (10 eq, 16.7 mL) and ortho phosphoric acid (7.5 eq, 3.59 g) and stirred at room temperature for 30 min. The mixture was filtered and washed with distilled water. To the crude product, methanol (3 eq, 5.3 mL) was added and stirred at 5–10°C for 1 h. Then acetone was added to the mixture until the PNDP particles were observed at the bottom, and filtered and washed with acetone again. The solution of the crude product was mixed with 70% ethanol (14 eq, 23.38 mL) and ortho phosphoric acid (2 eq, 0.93 g) and stirred at 60–65°C for 1 h. The mixture was allowed to cool to 10–20°C, filtered and washed with acetone, then dried under a vacuum at 50°C to yield 90% of **3**. ^{1}H-NMR, ^{13}C-NMR, MS, Fourier transform infrared spectroscopy (FT-IR) and elemental analysis (EA) were performed to identify the recovered product. The chemical purity of PNDP (**3**) as determined by a high performance liquid chromatography (HPLC) was found to be >99% as the chromatogram revealed a single peak with a retention time of 26.3 min detected by a UV–V is detector.

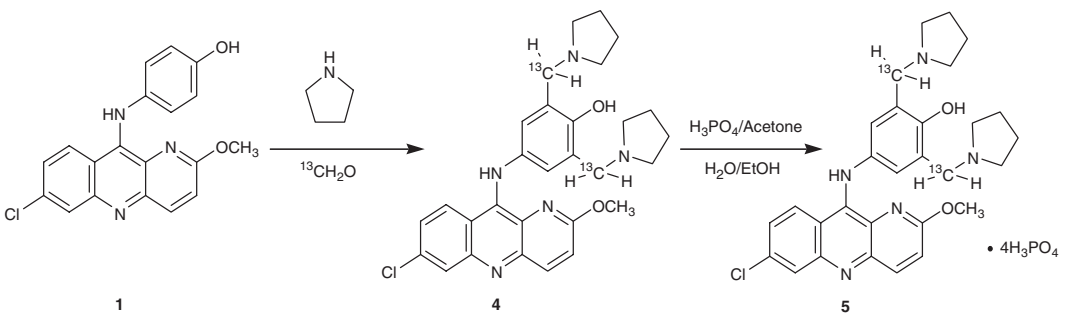
Synthesis of pyronaridine tetraphosphate (3) by a microwave irradiation technique

Microwave has become a useful tool for use in an organic synthesis, in analytical chemical laboratories and in many



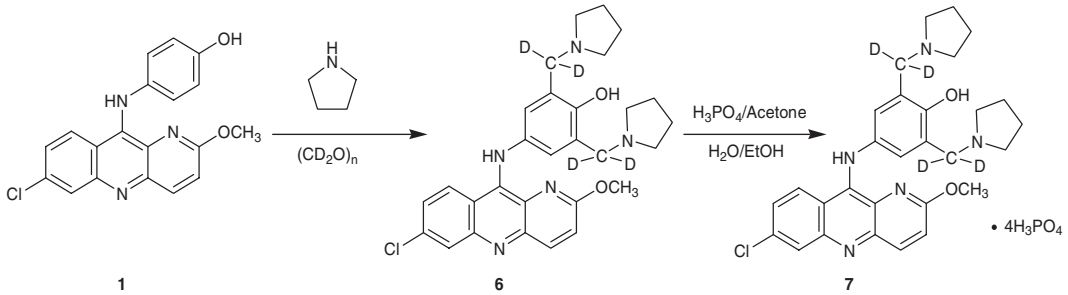
1 **2** **3**

Scheme 1. Synthesis of PND (**2**) and PNDP (**3**) from PNC (**1**).



1 **4** **5**

Scheme 2. Synthesis of [^{13}C]-PND (**4**) and [^{13}C]-PNDP (**5**) from PNC (**1**).



Scheme 3. Synthesis of [^{2}H]-PND (**6**) and [^{2}H]-PNDP (**7**) from PNC (**1**).

chemical manipulations during the process of standardizing procedures. It is becoming evident that rapid microwave protocols can be developed for a large number of chemical transformations requiring heat. The main benefits of performing reactions under microwave irradiation conditions are the significant rate enhancements and the higher product yields that are otherwise not possible by conventional methods. While different hypotheses have been proposed to account for the observed rate enhancements under a microwave irradiation, a generally accepted rationalization remains elusive.[15] Regardless of the origin/existence of a special microwave effect, microwave-enhanced chemistry can be extremely efficient and is applicable to a broad range of practical synthesis.[16,17]

In this paper, a microwave irradiation technique was used in the synthesis of [^{13}C]-labeled PNDP and [^{2}H]-labeled PNDP for a reduction of the chemical reaction time and a high product yield. Purification of **2** and synthesis of **3** were performed essentially in the same manner described for the classical method. In both the classical and microwave irradiation methods, after purification procedures, the products provided satisfactory ^{1}H-NMR, ^{13}C-NMR, MS, IR data and element analysis data.

Paraformaldehyde (5 eq, 0.716 g), pyrrolidine (5 eq, 1.99 mL) and anhydrous ethanol (2.5 eq, 4.175 mL) were mixed and refluxed at 50–60°C for 1 h. Then **1** (0.25 eq, 0.419 g) was added to the mixture and it was irradiated in a microwave reactor for 25 min at 140°C. On completion of the reaction, distilled water (2.5 eq, 4.175 mL) was added to the mixture and stirred at 15–25°C for 1 h, then filtered. The residue was dissolved in anhydrous methanol (2.5 eq, 4.175 mL) and stirred at 5–10°C for 2 h, then filtered and washed with excessive methanol and dried. The residue was dissolved in a mixture of dimethyl sulfoxide (0.25 eq, 1.275 mL) and dichloromethane (1.275 mL). The mixture was filtered, washed using methanol and dried at RT to yield 45% of **2**. After obtaining **2** (0.1 eq, 0.246 g) was mixed with distilled water (2.5 eq, 4.2 mL) and ortho phosphoric acid (1.9 eq, 0.89 g) and stirred at room temperature for 30 min. The mixture was filtered and washed with distilled water. To the crude product, methanol (0.75 eq, 0.325 mL) was added and stirred at 5–10°C for 1 h. Then acetone was added to the mixture and stirred until PNDP particles were observed and filtered, and washed with acetone again. The crude product solution was mixed with 70% ethanol (3.5 eq, 5.84 mL) and ortho phosphoric acid (0.5 eq, 0.23 g) and stirred at 60–65°C for 1 h. The mixture was allowed to cool to 10–20°C, filtered and washed with acetone, then dried under a vacuum at 50°C to yield 95% of **3**.

Synthesis of [^{13}C]-PNDP (5) and [^{2}H]-PNDP (7) by a microwave irradiation technique

On the basis of the optimum condition obtained from the above reactions, [^{13}C]-labeled PNDP (**5**) was synthesized by using formaldehyde-^{13}C by using a microwave irradiation technique. A mixture of formaldehyde-^{13}C (2.5 eq, 0.36 g) and pyrrolidine (5 eq, 1.99 mL) in anhydrous ethanol (2.5 eq, 4.175 mL) was refluxed at 50–60°C for 1 h, then **1** (0.25 eq, 0.419 g) was added and the mixture was irradiated for 25 min so as to reach a temperature of 140°C. The reaction yield 45% of **4**. **4** (0.1 eq, 0.246 g) was mixed with distilled water (2.5 eq, 4.2 mL) and ortho phosphoric acid (1.9 eq, 0.89 g) and stirred at room temperature for 30 min. The mixture was filtered and washed with distilled water. To the crude product **5**, methanol (0.75 eq) was added and stirred at 5–10°C for 1 h. The mixture was filtered and washed with acetone. The solution of crude product **5** was mixed with 70% ethanol (3.5 eq, 5.84 mL) and ortho phosphoric acid (0.5 eq, 0.223 g) and stirred at 60–65°C for 1 h. The mixture was allowed to cool at 10–20°C, filtered and washed with acetone, then dried under a vacuum at 50°C to yield 96% of **5**. In order to investigate the structural change upon a proton, carbon exchange due to a [^{13}C]-labeling, ^{1}H and ^{13}C-NMR analyses were performed and it was foundthat there was no significant structural difference between the labeled and unlabeled compounds. It can be seen from the ^{13}C-NMR spectrum of **5** that owing to a C–^{13}C coupling, the signals of the aromatic carbons occur as a multiplet with spans of 122–123 ppm. ^{13}C-labelling causes the signals in the ^{13}C-NMR spectra of **5** and their corresponding one in **3** and allows for a correct assignment of the chemical shifts to the carbon atoms. The signal of the ^{13}C-labeled atoms in **5** was observed at *m/z* 53.2 and for **3** the signals were found to be too high in the MS analysis. From the ^{1}H-NMR spectrum of **5** it was observed that, owing to strong H-^{13}C coupling, the ^{1}H-NMR signals of **5** were split into two signals at 4.0–4.5 ppm.

[^{2}H]-labeled pyronaridine tetraphosphate (**7**) was synthesized by using paraformaldehyde-d_2 (98%, Aldrich) by a microwave irradiation technique. A mixture of paraformaldehyde-d_2 (2.5 eq, 0.36 g) and pyrrolidine (5 eq, 1.99 mL) in anhydrous ethanol (2.5 eq, 4.175 mL) was refluxed at 50–60°C for 1 h, then **1** (0.25 eq, 0.419 g) was added and the mixture was irradiated for 25 min so as to reach a temperature of 140°C. The product yielded 45% of **6**. After obtaining **6** it was mixed with distilled water (2.5 eq, 4.2 mL) and ortho phosphoric acid (1.9 eq, 0.89 g) and stirred at room temperature for 30 min. The mixture was filtered and washed with distilled water. To the crude product, methanol (0.75 eq, 0.325 mL) was added and stirred at 5–10°C for 1 h. Then acetone was added

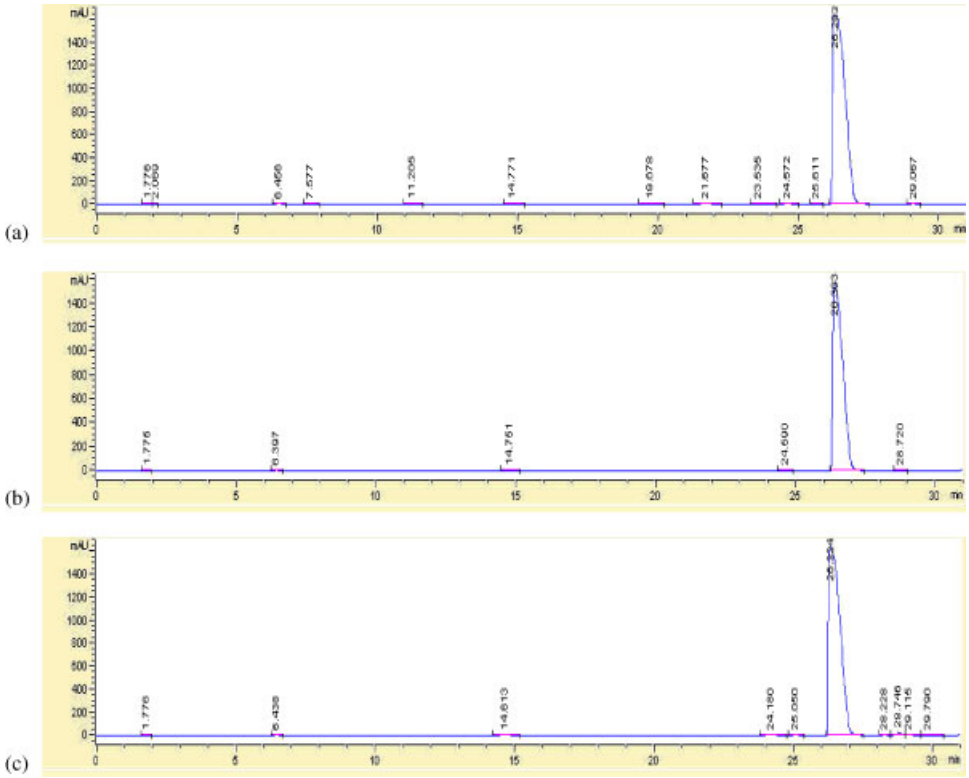


Figure 4. HPLC chromatograms of synthesized compounds: (a) PNDP **3**; (b) [^{13}C]-PNDP **5**; (c) [^{2}H]-PNDP **7**.

to the mixture until PNDP particles were observed and filtered, and washed with acetone again. The crude product solution was mixed with 70% ethanol (3.5 eq, 5.84 mL) and ortho phosphoric acid (0.5 eq, 0.223 g) and stirred at 60–65°C for 1 h. The mixture was allowed to cool at 10–20°C, filtered and washed with acetone, then dried under a vacuum at 50°C to yield 90% of **7**.

In order to investigate the structural change upon a proton, carbon exchange upon a [^{2}H]-labeling, ^{1}H and ^{13}C-NMR analyses were performed and it was found that there was no significant structural difference between the labeled and unlabeled compounds. It was observed from the NMR data of **7** that the signal for the methyl group that disappeared at 4.22 ppm by a [^{2}H]-labeling effect was found to be absent in **3**. The mass spectrum of **3** was found to reveal a peak at *m/z* 518. The peak of the ^{13}C-pyronaridine ion of **5** was found at *m/z* 520. [^{2}H]-labeled pyronaridine ion of **7** was founded at *m/z* 522. These results reveal that the isotope was labeled successfully to pyronaridine and pyronaridine tetraphosphate.

The chemical purity of **5** and **7** were determined by HPLC. The retention times of PNDP (**3**), [^{13}C]-PNDP (**5**) and [^{2}H]-PNDP (**7**) obtained from a UV–V is detector were found to be the same at 26.3 min as can be seen in Figure 4.

Stability test of [^{13}C]-labeled PNDP and [^{2}H]-labeled PNDP

[^{13}C]- and [^{2}H]-labeled PNDP (**5, 7**) were tested for their stability by analyzing their compounds by HPLC at appropriate time intervals, with **5** and **7** being stored at 4°C for up to 3 months. HPLC chromatograms taken before and after storage at 4°C for up to 3 months revealed a single peak of >99% chemical purity with the same retention time indicating **5** and **7** to be highly stable under standard storage conditions.

Experimental

Materials and methods

2-Methoxy-7-chloro-10-(40-hydroxyanilino)-[b]-1, 5-naphthyridine (PNC, **1**) was obtained from Shinpoong Pharmaceutical, Co., Ltd., Seoul, Korea. All other reagents were obtained from Sigma Chemical Co., USA and were used as such without any further purification.

All chromatographic separations were monitored by TLC analyses and performed using glass plates precoated with 0.25 mm 230–400 mesh silica gel impregnated with a fluorescence indicator (254 nm). Solvent removal was accomplished with an aspirator pressure using a rotary evaporator. Microwave assisted organic synthesis was carried out using a microwave reactor (Initatior 2.0, 400 W, Biotage, Sweden). ^{1}H NMR and ^{13}C NMR spectra were recorded with a JNM-ECA500 (JEOL Ltd.) operating at a 500 MHz frequency for ^{1}H-NMR and at a 125 MHz frequency for ^{13}C-NMR. IR spectra were recorded on a VERTEX-70 (BRUKER). MS data were obtained on a JMS-700 Mstation (JEOL Ltd.). EA data were obtained from Flash EA 1112 Series (CE Instruments).

The chemical purities of PNDP (**3**), [^{13}C]- and [^{2}H]-labeled PNDP (**5, 7**) were determined by performing an HPLC (Agilent 1200 Series RRLC, Agilent technologies) using an optimaPAK C_{18} (5 μm; 4.6 × 150 mm) column. HPLC solvents consisted of potassium phosphate buffer: acetonitrile (9:1) (solvent A) and potassium phosphate buffer: acetonitrile (2:8) (solvent B). One liter of the buffer (pH 2) consisted of KH_2PO_4 (1.36 g) and 1-octanesulfonic acid sodium salt hydrate (2.163 g). HPLC gradient: 0–17 min: a linear gradient to 70% A/30% B from 70% A/30% B; 17–25 min: a linear gradient to 60% A/40% B from

70% A/30% B; 25–30 min: a linear gradient to 60% A/40% B from 60% A/40% B; 30–31 min: a linear gradient to 70% A/30% B from 60% A/40% B. The flow rate was maintained at 1 mL/min and the column temperature was maintained at 40°C.

Synthesis of pyronaridine (PND, 2) from 2-methoxy-7-chloro-10-(40-hydroxyanilino)-[b]-1, 5-naphthyridine (PNC, 1) by a classical method

A 250 mL round bottom flask containing a magnetic stir bar was washed with double distilled water (DDW) under an ultrasonication. Then appropriate amounts of paraformaldehyde, pyrrolidine and anhydrous ethanol were added, in that order, and refluxed at 50–60°C for 1 h. **1** (1 eq, 1.676 g) was added to complete the reaction mixture and stirred at 45–55°C for 12–14 h. The final molar composition of any given synthesis solution was: 1.00 PNC: 20 paraformaldehyde: 20 pyrrolidine: 10 ethanol. On completion of the reaction, distilled water (10 eq, 16.7 mL) was added to the mixture and stirred at 15–25°C for 1 h, then filtered. The residue was dissolved in anhydrous methanol (10 eq, 16.7 mL) and stirred at 5–10°C for 2 h, then filtered and washed with excessive methanol. The residue was dissolved in a mixture of dimethyl sulfoxide (3 eq, 5.1 mL) and dichloromethane (3 eq, 5.1 mL). The mixture was filtered, thoroughly washed using methanol and finally dried to yield 40% of **2**. IR (KBr): ν 3307, 2965, 2872, 2806, 1621, 1571, 1519, 1493, 1465, 1403, 1377, 1305, 1271, 1094, 1068, 1000, 927, 867, 834, 808 cm^{-1}; ^1H NMR (CD_3OD):δ 1.81 (t, 8H, CH_2), 2.61 (s, 8H, CH_2), 3.74 (s, 4H, $ArCH_2$), 4.05 (s, 3H, OCH_3), 7.01 (s, 2H, ArH), 7.27 (d, 1H, ArH), 7.64(d, 2H, ArH), 7.87 (s, 2H, ArH), 8.13 (d, 2H, ArH); ^{13}C NMR (CD_3OD): d 23.0, 53.1, 55.3, 119.5, 124.1, 124.7, 126.3, 127.0, 127.9, 135.2, 138.7, 141.9, 148.4, 154.6, 160.3; MS *m/z* 518.24 [M^+]. Calcd for $C_{29}H_{32}ClN_5O_2$: C 67.23, H 6.23, N 13.52. Found: C 66.89, H 6.33, N 13.27.

Synthesis of pyronaridine (PND, 2) from 2-methoxy-7-chloro-10-(40-hydroxyanilino)-[b]-1,5-naphthyridine (PNC, 1) by a microwave irradiation technique

A 250 mL round bottom flask containing a magnetic stir bar was washed with DDW under an ultrasonication. Then appropriate amounts of paraformaldehyde, pyrrolidine and anhydrous ethanol were added, in that order, and refluxed at 50–60°C for 1 h. **1** (0.25 eq, 0.419 g) was added to complete the reaction mixture and it was irradiated in a microwave reactor for 25 min at 140°C. The final molar composition of any given synthesis solution was: 1.00 PNC: 20 paraformaldehyde: 20 pyrrolidine: 10 ethanol. On completion of the reaction, distilled water (2.5 eq, 4.175 mL) was added to the mixture and stirred at 15–25°C for 1 h, then filtered. The residue was dissolved in anhydrous methanol (2.5 eq, 4.175 mL) and stirred at 5–10°C for 2 h, then filtered and washed with excessive methanol. The residue was dissolved in a mixture of dimethyl sulfoxide (0.25 eq, 1.275 mL) and dichloromethane (0.25 eq, 1.275 mL). The mixture was filtered, thoroughly washed using methanol and finally dried to yield 45% of **2**. IR (KBr): ν 3307, 2962, 2780, 1620, 1559, 1519, 1494, 1468, 1402, 1377, 1301, 1273, 1096, 1070, 1000, 929, 867, 836, 808 cm^{-1}; ^1H NMR (CD_3OD):δ 1.81 (t, 8H, CH_2), 2.61 (s, 8H, CH_2), 3.75 (s, 4H, $ArCH_2$), 4.05 (s, 3H, OCH_3), 7.01 (s, 2H, ArH), 7.28 (d, 1H, ArH), 7.65(d, 2H, ArH), 7.87 (s, 2H, ArH), 8.13 (d, 2H, ArH); ^{13}C NMR (CD_3OD): d 23.0, 53.1, 55.3, 119.5, 124.1, 124.7, 126.3, 127.0, 128.1, 135.2, 138.7, 145.9, 148.4; MS *m/z* 518.24 [M^+]. Calcd for $C_{29}H_{32}ClN_5O_2$: C 67.23, H 6.23, N 13.52. Found: C 66.36, H 6.13, N 13.06.

Synthesis of pyronaridine tetraphosphate (PNDP, 3) from pyronaridine (PND, 2)

Appropriate amounts of **2** (0.1 eq, 0.246 g), distilled water and ortho phosphoric acid were mixed and stirred at RT for 30 min. The molar composition of the mixture was: 0.1 PND: 1.9 ortho phosphoric acid: 2.5 H_2O. On completion of the reaction, the mixture was filtered and washed with distilled water. Methanol was (0.75 eq, 0.325 mL) added to the crude product and stirred at 5–10°C for 1 h. Then a sufficient amount of acetone was added to the mixture until PNDP particles were observed, filtered and washed with acetone again. The residue was added to a mixture solution of 70% ethanol (3.5 eq, 5.84 mL) and ortho phosphoric acid (0.5 eq, 0.223 g) and stirred at 60–65°C for 1 h. Then it was filtered and washed with acetone. The solid sample was dried under a vacuum at 50°C to yield 95% of **3**. IR (KBr): ν 3406, 2949, 1631, 1577, 1551, 1526, 1489, 1459, 1389, 1350, 1285, 1091, 991 cm^{-1}; ^1H NMR (D_2O):δ 1.87 (s, 4H, CH_2), 2.00 (s, 4H, CH_2), 3.03 (s, 4H, CH_2), 3.41 (s, 4H, CH_2), 3.88 (s, 3H, OCH_3), 4.32 (s, 4H, $ArCH_2$), 7.10 (d, 1H, ArH), 7.38 (t, 2H, ArH), 7.42 (s, 12H, ArH), 7.73 (s, 1H, ArH), 8.02 (d, 1H, ArH); ^{13}C NMR (D_2O): d 22.4, 53.2, 54.0, 54.5, 111.3, 118.5, 122.1, 123.2, 124.9, 126.1, 127.4, 130.8, 131.3, 132.5, 132.7, 139.7, 140.7, 152.2, 154.5, 160.9; MS *m/z* 518.4 [$M^+ - (H_3PO4)$]. Calcd for $C_{29}H_{32}ClN_5O_2$: C 38.27, H 4.87, N 7.70. Found: C 38.04, H 4.86, N 7.39.

Synthesis of [^{13}C]-labeled pyronaridine tetraphosphate

[^{13}C]-labeled pyronaridine tetraphosphate (**5**) was synthesized by using formaldehyde-^{13}C (20%, Aldrich) by a microwave irradiation technique. A 250 mL round bottom flask containing a magnetic stir bar was washed with DDW under an ultrasonication. Then appropriate amounts of formaldehyde-^{13}C, pyrrolidine and anhydrous ethanol were added, in that order, and refluxed at 50–60°C for 1 h. **1** (0.25 eq, 0.419 g) was added to complete the reaction mixture and it was irradiated in a microwave reactor for 25 min at 140°C. The final molar composition of any given synthesis solution was; 1.00 PNC: 10 [^{13}C]-formaldehyde: 20 pyrrolidin: 10 ethanol. On completion of the reaction, distilled water (2.5 eq, 4.175 mL) was added to the mixture and stirred at 15–25°C for 1 h, then filtered. The residue was dissolved in anhydrous methanol (2.5 eq, 4.175 mL) and stirred at 5–10°C for 2 h, then filtered and washed with excessive methanol. The residue was dissolved in a mixture of dimethyl sulfoxide (0.25 eq, 1.275 mL) and dichloromethane (0.75 eq, 1.275 mL). The mixture was filtered, thoroughly washed using methanol and finally dried to yield 45% of **4**. Appropriate amounts of **4** (0.1 eq, 0.246 g), distilled water and ortho phosphoric acid were mixed and stirred at RT for 30 min. The molar composition of the mixture was: 0.1 PND: 1.9 ortho phosphoric acid: 2.5 H_2O. On completion of the reaction, the mixture was filtered and washed with distilled water. Methanol (0.75 eq, 0.325 mL) was added to the crude product and stirred at 5–10°C for 1 h. Then a sufficient amount of acetone was added to the mixture until PNDP particles were observed, filtered and washed with acetone again. The residue was added to a mixture solution of 70% ethanol (3.5 eq, 5.84 ml) and ortho phosphoric acid (0.5 eq, 0.223 g) and stirred at 60–65°C for 1 h. Then it was filtered and washed with acetone. The solid sample was dried under a vacuum at 50°C to yield 95% of **5**. IR (KBr): ν 3400, 2955, 1635, 1578, 1552, 1528, 1490, 1460, 1389, 1350, 1285, 1093, 991 cm^{-1}; ^1H NMR (D_2O):δ

1.87 (s, 4H, CH_2), 2.01 (s, 4H, CH_2), 3.04 (s, 4H, CH_2), 3.42 (s, 4H, CH_2), 3.88 (s, 3H, OCH_3), 4.18 (s, 4H, $ArCH_2$), 4.47 (s, 4H, $ArCH_2$), 7.11 (d, 1H, ArH), 7.35 (t, 2H, ArH), 7.43 (s, 2H, ArH), 7.74 (s, 1H, ArH), 8.03 (d, 1H, ArH); ^{13}C NMR (D_2O): d 22.4, 53.4, 54.0, 54.5, 111.3, 118.5, 121.9, 122.1, 123.2, 124.9, 126.0, 127.3, 130.7, 131.2, 132.5, 132.5, 139.6, 140.7, 152.2, 154.4, 160.8; MS *m/z* 520.4 [$M^+ - (H_3PO_4)$]. Calcd for $C_{29}H_{32}ClN_5O_2$: C 38.27, H 4.87, N 7.70. Found: C 32.86, H 4.52, N 6.39.

Synthesis of [^{2}H]-labeled pyronaridine tetraphosphate

[^{2}H]-labeled pyronaridine tetraphosphate (**7**) was synthesized by using paraformaldehyde-d_2 (98%) by a microwave irradiation technique. A 250 mL round bottom flask containing a magnetic stir bar was washed with DDW under an ultrasonication. Then appropriate amounts of paraformaldehyde-d_2, pyrrolidine and anhydrous ethanol were added, in that order and refluxed at 50–60°C for 1 h. **1** (0.25 eq, 0.419 g) was added to complete the reaction mixture and it was irradiated in a microwave reactor for 25 min at 140°C. The final molar composition of any given synthesis solution was: 1.00 PNC: 10 [^{2}H]-labeled paraformaldehyde: 20 pyrrolidine: 10 ethanol. On completion of the reaction, distilled water (2.5 eq, 4.175 g) was added to the mixture and stirred at 15–25°C for 10 h, then filtered. The residue was dissolved in anhydrous methanol (2.5 eq, 4.175 g) and stirred at 5–10°C for 2 h, then filtered and washed with excessive methanol. The residue was dissolved in a mixture of dimethyl sulfoxide (0.75 eq, 1.275 ml) and dichloromethane (0.75 eq, 1.275 mL). The mixture was filtered, thoroughly washed using methanol and finally dried to yield 45% of **6**. Appropriate amounts of **6** (0.1 eq, 0.246 g), distilled water and ortho phosphoric acid were mixed and stirred at RT for 30 min. The molar composition of the mixture was: 0.1 [^{2}H]-PND: 1.9 ortho phosphoric acid: 2.5 H_2O. On completion of the reaction, the mixture was filtered and washed with distilled water. Methanol (0.75 eq, 0.325 mL) was added to the crude product and stirred at 5–10°C for 1 h. Then a sufficient amount of acetone was added to the mixture until PNDP particles were observed, filtered and washed with acetone again. The residue was added to a mixture solution of 70% ethanol (3.5 eq, 5.84 mL) and ortho phosphoric acid (0.5 eq, 0.223 g) and stirred at 60–65°C for 1 h. Then it was filtered and washed with acetone. The solid sample was dried under a vacuum at 50°C to yield 95% of **7**. IR (KBr): ν 3394, 3946, 1632, 1577, 1551, 1527, 1488, 1461, 1389, 1350, 1284, 1092, 990 cm^{-1}; ^1H NMR (D_2O):δ 1.87 (s, 4H, CH_2), 2.00 (s, 4H, CH_2), 3.04 (s, 4H, CH_2), 3.42 (s, 4H, CH_2), 3.87 (s, 3H, OCH_3), 4.32 (s, 4H, $ArCH_2$), 7.11 (d, 1H, ArH), 7.38 (t, 2H, ArH), 7.42 (s, 2H, ArH), 7.72 (s, 1H, ArH), 8.04 (d, 1H, ArH); ^{13}C NMR (D_2O): d 22.4, 30.2, 53.9, 54.5, 111.2, 118.5, 122.1, 123.2, 125.0, 126.0, 127.3, 130.7, 131.2, 132.4, 132.7, 139.5, 140.8, 152.1, 154.5, 160.8; MS *m/z* 522.5 [$M^+ - (H_3PO_4)$]. Calcd for $C_{29}H_{32}ClN_5O_2$: C 38.27, H 4.87, N 7.70. Found: C 33.75, H 4.50, N 6.71.

Conclusion

A method for synthesizing stable [^{13}C]- and [^{2}H]-labeled PNDP, an antimalarial drug, using a microwave irradiation, has been described in this study. The use of microwave irradiation facilitated the use of high temperature thereby decreasing the reaction time and also increasing the yield of product formed. The labeled compounds thus synthesized had a chemical purity of >99% as determined by an HPLC and were also found to be relatively stable up to 3 months when stored under standard conditions. They also revealed satisfactory ^{13}C and ^1H-NMR spectrum and the labeled compounds showed different molecular weights when compared with their corresponding unlabeled reveled as determined by a mass spectroscopy. [^{13}C]- and [^{2}H]-labeled PNDP can be used in preclinical studies to determine the absorption, distribution and metabolism, as well as excretion studies and pharmacokinetics of PNDP. As the PNDP is labeled with [^{13}C] and [^{2}H] at a desired position they will be highly stable inside the biological system and will facilitate continuous detection of a drug and its subsequent metabolites. Further, the use of PNDP labeled with stable isotopes, like [^{13}C] and [^{2}H] is relatively safe because unlike unstable isotopes like [^{14}C]-, [^{3}H]- and [^{131}I]-labeled PNDP they do not pose the threat of a radiation exposure. In addition to that, the advancements made in the field of MS will facilitate in an easy detection of their metabolites, while performing ADME and pharmacokinetic studies using *in vivo* and *in vitro* systems.

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References

[1] H. M. Al Arishi, F. E. A. Ahmed, L. A. Al Bishi, *Trans. R. Soc. Trop. Med. Hyg.* **2001**, *95*, 439–440.
[2] K. Yonemitsu, A. Koreeda, K. Kibayashi, P. Ng'walali, M. Mbonde, J. Kitinya, S . Tsunenari, *Leg. Med.* **2005**, *7*, 113–116.
[3] P. G. Bray, S. Deed, E. Fox, M. Kalkanidis, M. Mungthin, L. W. Deady, L. Tilley, *Biochem. Pharmacol.* **2005**, *70*, 1158–1166.
[4] D. Warhurst, *Drug Resistance Updates* **2001**, *4*, 141–144.
[5] C. P. Sanchez, W. D. Stein, M. Lanzer, *Trends Parasitol.* **2007**, *23*, 332–339.
[6] K. Kolaczinski, N. Durrani, S . Rahim, M. Rowland, *Trans. R. Soc. Trop. Med. Hyg.* **2007**, *101*, 1081–1087.
[7] S. Gemma, G. Kukreja, G. Campiani, S. Butini, M. Bernetti, B. P. Joshi, L. Savini, N. Basilico, D. Taramelli, V. Yardley, A. Bertamino, E. Novellino, M. Persico, B. Catalanotti, C. Fattorusso, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3535–3539.
[8] G. D. Li, *Med. Hypotheses* **2007**, *68*, 332–334.
[9] A. Radfar, A. Diez, J. M. Bautista, *Free Radical Biol. Med.* **2008**, *44*, 2034–2042.
[10] E. I. Elueze, S. L. Croft, D. C. Warhurst, *J. Antimicrob. Chemother.* **1996**, *37*, 511–518.
[11] S. D. Jayaraman, S. Ismail, N. K. Nair, V. Navaratnam, *J. Chromatogr. B* **1997**, *690*, 253–257.
[12] C. P. Babalola, G. K. E. Scriba, A. Sowunmi, O. A. Alawode, *J. Chromatogr. B* **2003**, *795*, 265–272.
[13] S. Ramanathan, S. Karupiah, N. K. Nair, P. L. Olliaro, V. Navaratnam, W. H. Wernsdorfer, S. M. Mansor, *J. Chromatogr. B* **2005**, *824*, 45–50.
[14] C. Chang, L. H. Tang, C. Jantanavivat, *Trans. R. Soc. Trop. Med. Hyg.* **1992**, *86*, 7–10.
[15] L. Perreux, A. Loupy, *Tetrahedron* **2001**, *57*, 9199–9223.
[16] P. Lidström, J. Tierney, B. Wathey, J. Westman, *Tetrahedron* **2001**, *57*, 9225–9283.
[17] M. Larhed, A. Hallberg, *Drug Discovery Today* **2001**, *6*, 406–416.