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The first chemical synthesis of pyrazofurin 5′-triphosphate

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ABSTRACT

As an archetype *C*-nucleoside, pyrazofurin possesses broad-spectrum antiviral and antitumor activities. However, the presence of the acidic enol in the nucleobase of pyrazofurin poses a huge challenge to the conventional NTP synthetic methods. On the basis of a selective phosphoylation method and the P(V)-N activation strategy, the first chemical synthesis of pyrazofurin 5′-triphosphate (PTP) was accomplished, which will greatly facilitate the investigation on the interactions of viral RNA polymerases and PTP, an important cellular metabolite of pyrazofurin.

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Pyrazofurin, a naturally occurring *C*-nucleoside first isolated from the fermentation of a strain of *Streptomyces candidus* in 1969,[1] is a close structural analog of a natural imidazole nucleoside, bredinin,[2] and a synthetic triazole nucleoside drug, ribavirin (Figure 1).[3] As a microbial metabolite, pyrazofurin possesses broad-spectrum antiviral activities against both RNA and DNA viruses[4] and antitumor properties.[5]

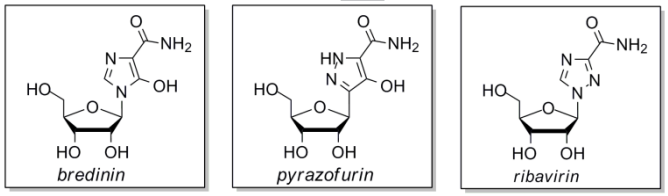
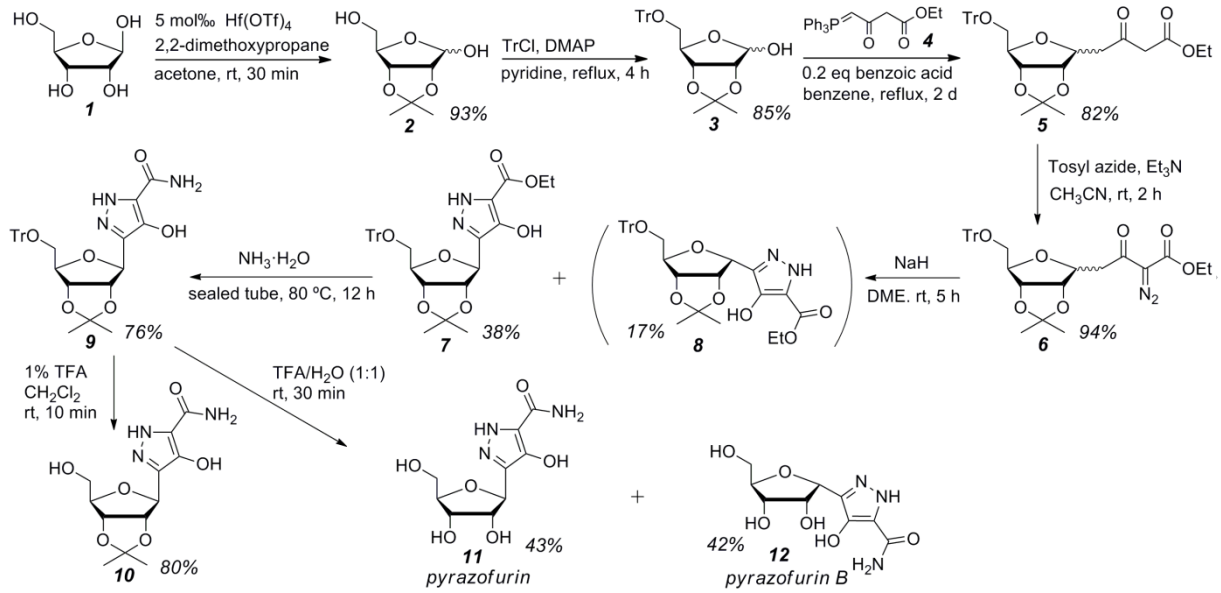


Figure 1. Structures of pyrazofurin and its analogs

In cellular metabolism, both pyrazofurin and ribavirin are converted to their monophosphates and triphosphates.[6-7] Early research on the mechanism of action of the two antiviral agents showed that pyrazofurin 5′-monophosphate (PMP) and ribavirin 5′-monophosphate (RMP) inhibit the orotidine 5′-monophosphate (OMP) decarboxylase[8] and inosine 5′-monophosphate (IMP) dehydrogenase,[9] respectively, and thus block the *de novo* pyrimidine and purine biosynthesis. In 2000, with the assistance of synthetic ribavirin 5′-triphosphate (RTP), Cameron and his coworker were able to investigate the interactions of RTP with viral RNA polymerases on the basis of a novel primer-extension assay and found that non-specific incorporation of ribavirin into viral genome leads to lethal mutagenesis of virus population.[10-12] Similarly, in the mechanistic investigation of T-705, a pyrazine nucleoside drug (favipiravir), synthetic T-705 5′-triphosphate (T-705TP) helped Furuta and coworkers reveal that the drug's antiviral activity comes from the inhibition of influenza virus RNA polymerase by T-705TP.[13] In contrast, due to the unique enol-containing nucleobase in pyrazofurin, the chemical synthesis of pyrazofurin 5′-triphosphate (PTP) has always been an unsolved problem for phosphorus chemists. But it can be envisioned that the availability of synthetic PTP will help virologists and medicinal chemists elucidate the potential roles of PTP in halting virus replication and develop effective antiviral strategies.

In recent years, our research group reported the P(V)-N activation strategy for the synthesis of nucleoside polyphosphates,[14-15] nucleoside diphosphate sugars,[16] and dinucleoside polyphosphates.[17] The high coupling efficacy and tolerance of diverse functional groups on nucleobases offer an ideal approach to PTP. We report herein the first chemical synthesis of PTP from the corresponding pyrazofurin 5′-monophosphate, which was efficiently prepared from isopropylidene-protected pyrazofurin via selective phosphorylation without enol protection.

Due to the poor commercial availability of pyrazofurin, we first synthesized the isopropylidene-protected pyrazofurin (**10**) according to a known synthetic route.[18-20] The detailed synthetic methods of certain steps were optimized to improve the practicability (Scheme 1). First, D-ribose (**1**) was treated with catalytic amount of *p*-TsOH in 2,2-dimethoxypropane and acetone. But the yield of 2,3-*O*-isopropylidene-D-ribose (**2**) was only around 70%.[21] Instead, the application of 5 mol‰ $Hf(OTf)_4$ as the catalyst afforded **2** in 93% yield within 30 min.[22] Direct tritylation of the 5-OH of **2** with TrCl in refluxing pyridine

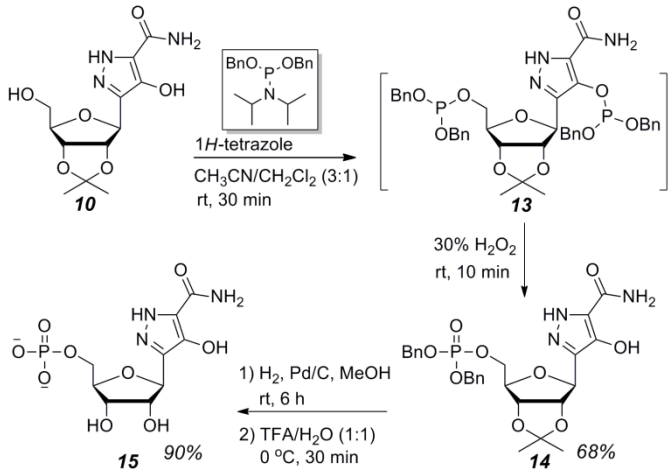


Scheme 1. Optimized synthesis of isopropylidene-protected pyrazofurin (**10**)

was sluggish (45% yield, 24 h).[21] We found that addition of 4 equiv of DMAP significantly shortened the reaction time to 4 h and increase the yield of **3** to 85%.[23] In the Wittig reaction, **3** and phosphorane **4** were initially refluxed in acetonitrile as described in literature.[18] However, the reaction was extremely slow and *C*-nucleoside **5** was obtained in only 60% yield after 7 days. When the reaction of **3** and **4** was conducted in benzene,[19] catalytic amount of benzoic acid (5 mol%) resulted in moderate improvement (66% yield, 5 d). Therefore, we gradually increased the amount of benzoic acid from 5 mol% to 50 mol%, and found that 20 mol% acid catalyst resulted in the best outcome (82% yield, 2 d). The α/β ratio was determined as 1.4:1. Then, the diazotization of **5** with tosyl azide/Et_3N and subsequent cyclization of **6** with NaH in DME were conducted strictly according to literature procedures which afforded the diazo compound **6** and anomeric mixtures of pyrazole **7** and **8** in reproducible yields. **7** and **8** were separated by column chromatography and obtained in 38% and 17% yields, respectively. In order to reduce the risk of potential explosion resulting from the aminolysis of **7** with NH_3/MeOH in a sealed tube at 90–95 ºC, concentrated ammonia was used instead in our method. The aminolysis was performed at 80 ºC for 12 h and gave the amide **9** in comparable yield. Finally, the trityl was cleaved with 1% TFA in CH_2Cl_2 to afford **10** in 80% yield. Alternatively, treatment of **9** with TFA/H_2O (1:1) at room temperature efficiently yielded equal amounts of pyrazofurin (**11**) and its epimer, pyrazofurin B (**12**). This result was in agreement with previous reports that pyrazofurin is prone to epimerize in acidic aqueous solution.[1,24]

To apply the P(V)-N activation strategy to the synthesis of PTP, efficient preparation of PMP is quite essential. In a previous report, Revankar *et al.* prepared PMP from 4-*O*-benzyl pyrazofurin with $POCl_3$. While the benzylation/ debenzylation of enol took extra steps, phosphorylation with $POCl_3$ was low-yielding. In addition, a pressurized hydrogenator was required for debenzylation of the 4-*O*-benzyl ether.[25] Though direct phosphorylation of pyrazofurin with $POCl_3$ has been reported, however, the very low yield and tedious purification procedures prohibited its application in our synthesis.[26] Therefore, on the basis of the different reactivity of 5′-OH and enol, we attempted to selectively phosphorylate 5′-OH of **10** without enol protection. A series of P(V) and P(III) reagents were tested. Our first trial with $POCl_3$ as the phosphorylating reagent yielded the desired isopropylidene-protected PMP in very low yield (<10%), and the contaminated inorganic phosphate was hard to separate. The less reactive dibenzylphosphoryl chloride[27] was highly prone to hydrolysis and failed to generated any phosphorylated product in this case.

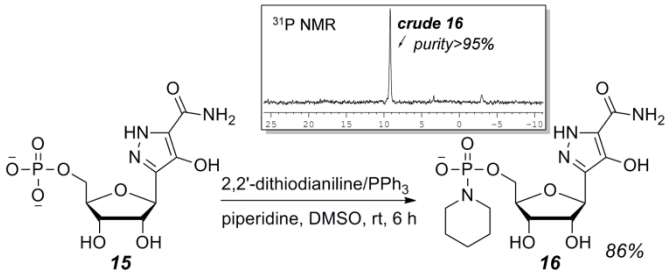
Then, we switched to more reactive phosphoramidite reagents. It was anticipated that if excess P(III) reagent was used, both 5′-OH and enol should be phosphitylated. However, the phosphite triester on enol should be much less stable than the one at 5′-*O* position. Therefore, upon *in situ* oxidation with aqueous H_2O_2, the phosphite triester might be cleaved by hydrolysis to afford 5′-OH selectively phosphorylated product. In our experiments, the hindered di-*tert*-butyl *N,N*-diisopropylphosphoramidite[28] was not reactive at all. In contrast, excess dibenzyl *N,N*-diisopropylphosphoramidite[29-30] reacted with both 5′-OH and enol as we speculated. In the ^{31}P NMR spectrum of the diphosphite intermediate (**13**), two peaks of equal intensity showed up at 139.4 ppm and 137.5 ppm corresponding to the two phosphite triesters at 5′-*O* and 4-*O* positions. After H_2O_2 was added, the major product was isolated in 68% yield and characterized as the 5′-*O*-dibenzyl phosphate (**14**), indicating that the phosphite triester on enol was hydrolytically removed upon addition of H_2O_2. Sequential removal of benzyl esters and isopropylidene afforded the desired PMP (**15**) in excellent yield (Scheme 2). To our surprise, when the phosphate



Scheme 2. A selective phosphorylation method of **10** for synthesis of PMP (**15**).

intermediate was treated with TFA/H_2O (1:1) at room temperature, only ~10% of **15** epimerized to α-anomer as seen on ^{1}H NMR. Further decreasing the deprotection temperature to 0 ºC effectively inhibited the epimerization of **15**.

In the following research, **15** was converted to the corresponding phosphoropiperidate (**16**) according to the redox condensation method described in a previous paper[15] (Scheme 3). The treatment of **15** with 2,2′-dithiodi-aniline/PPh_3 and piperidine in DMSO afforded **16** smoothly over 6 h. Addition of acetone solution of NaI precipitated **16** as sodium salt. Our attempt to purify **16** with LH-20 size-exclusion chromatography caused partial decomposition. Therefore, **16** was directly transformed into triethyl-ammonium salt form by passing through ion exchange resin and used in the next step without further purification (86% yield, purity >95%).



Scheme 3. The redox condensation method for the synthesis of pyrazofurin 5′-phosphoropiperidate (**16**).

According to the P(V)-N activation method,[14] **16** was treated with 2 equiv of pyrophosphate in the presence of 6 equiv of 4,5-dicyanoimidazole (DCI) as the activator. ^{31}P NMR tracing of the reaction showed that the coupling of pyrophosphate with **16** proceeded efficiently and was not affected by the enol on the nucleobase (Figure 2). Upon completion, ^{31}P NMR spectrum of the precipitated crude triphosphate as sodium salt form (Figure 2 inset) indicated that conversion ratio from **16** to triphosphate **17** was over 90%.

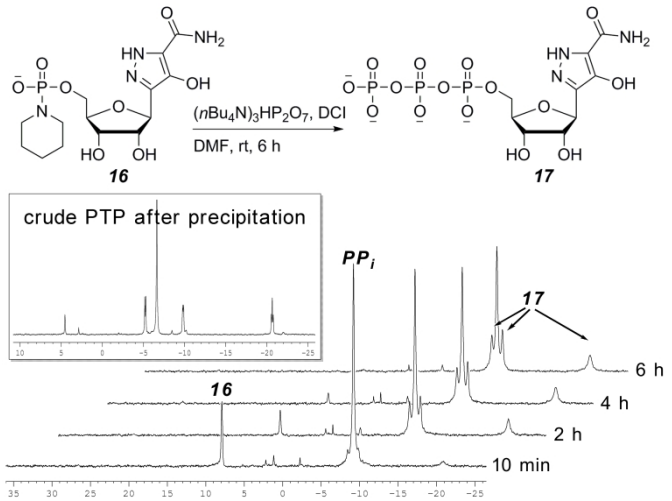


Figure 2. ^{31}P NMR tracing of the P(V)-N activation strategy for the synthesis of PTP (**17**).

The crude product was purified by ion exchange chromatography. Passage through an ion exchange resin (Na^+ form) and lyopholization afforded the target PTP **17** in 65% yield as a white solid. The HPLC analysis of **17** (for detailed conditions, see SI) determined that the triphosphate prepared by this method was of high purity (>98%). On the high resolution mass spectrum of **17** (ESI–), both the molecular ion peak of **17** and fragment peaks corresponding to pyrazofurin 5′-diphosphate and 5′-monophosphate were observed. It is worth to mention that upon extended stay in aqueous solution for several days, ~10% epimerized pyrazofurin B 5′-triphosphate was observed by ^{1}H NMR.

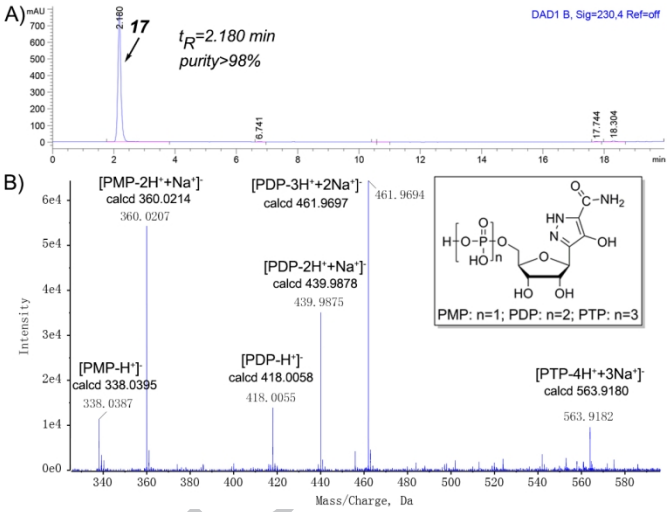


Figure 3. HPLC (A) and HRMS (B) analysis of synthetic PTP (**17**).

In summary, for the first time, pyrazofurin 5′-triphosphate (**17**) was synthesized via the P(V)-N activation strategy, which exhibited excellent coupling efficacy and tolerance of acidic enol functional group on nucleobase. Moreover, certain procedures for the synthesis of isoproylidene-protected pyrazofurin (**10**) in the Wittig reaction-based route were optimized and a selective phosphorylation method for the preparation of pyrazofurin 5′-monophosphate (**15**) was also developed. The current research will be useful for virological and medicinal investigations of the role of pyrazofurin 5′-triphosphate (**17**), an important cellular metabolite of pyrazofurin, for the marked antiviral activity of pyrazofurin, which may be helpful for the discovery of novel antiviral strategies.

Acknowledgments

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Supplementary Material

Supplementary data (experimental procedures and characterization data) associated with this article can be found at

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- Pyrazofurin triphosphate was first synthesized via P(V)-N activation strategy.
- A selective phosphorylation method was developed for pyrazofurin 5′-phosphate.
- Certain synthetic procedures for isoproylidene-protected pyrazofurin were modified.

Graphical Abstract

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