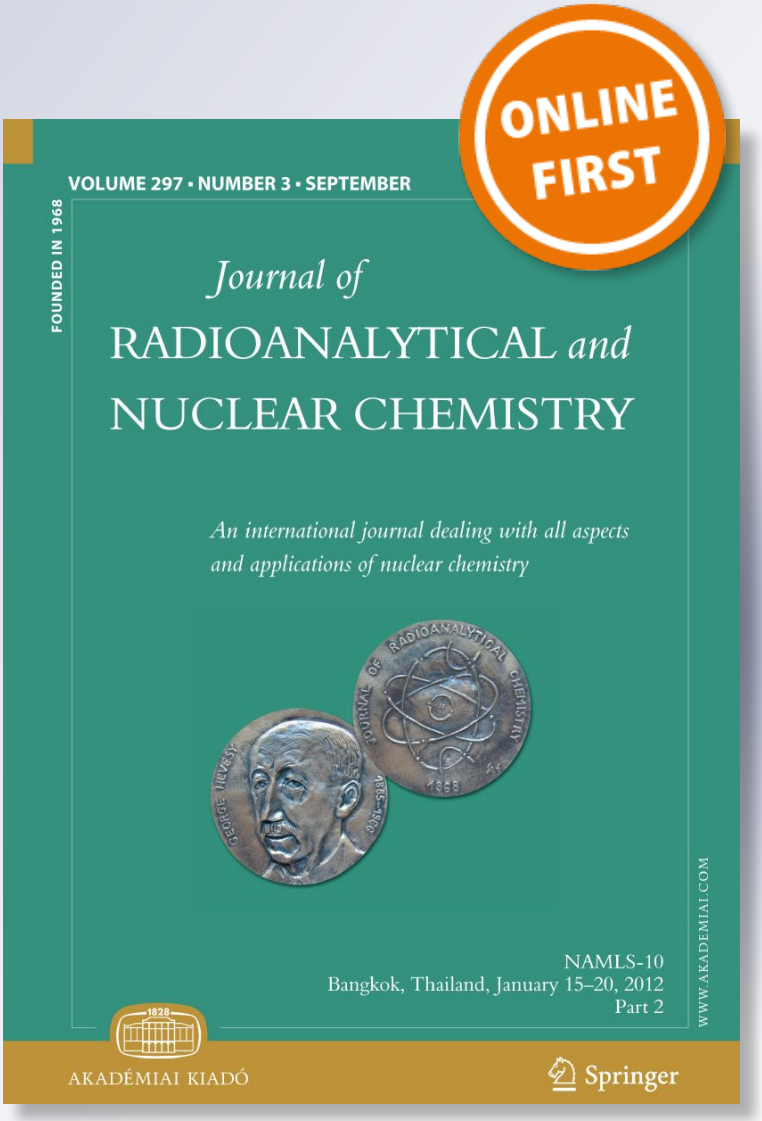
# *Synthesis of high molar activity $^{33}$P-labeled phosphorous acid*

**Benjamin A. S. Van Mooy**

**Journal of Radioanalytical and Nuclear Chemistry**
An International Journal Dealing with All Aspects and Applications of Nuclear Chemistry

ISSN 0236-5731

J Radioanal Nucl Chem
DOI 10.1007/s10967-019-06548-z



Journal of Radioanalytical and Nuclear Chemistry
https://doi.org/10.1007/s10967-019-06548-z

# Synthesis of high molar activity $^{33}$P-labeled phosphorous acid

**Benjamin A. S. Van Mooy**[1]

Received: 7 March 2019


**Abstract**
Studies of phosphorus cycling in the ocean have been greatly facilitated by the use of high molar activity $^{32}$P- and $^{33}$P-labeled phosphate (phosphoric acid) in biological incubation assays. Recently, phosphite (phosphorous acid) has been shown to play an important role in the ocean. Here I report the microscale (100 µmol) synthesis of high molar activity $^{33}$P-labeled phosphorous acid. The scheme incorporates a new combination of known synthetic routes, which requires 20 times less radioactivity than existing methods. The economical production of $^{33}$P-phosphorous acid with molar activity > 37 GBq mol$^{-1}$ for use in assays is readily achievable with this scheme.

**Keywords** Phosphorus · Phosphorous acid · Phosphite · $^{33}$P · $^{32}$P · Synthesis

## Introduction

Phosphorus is an essential element for the growth of all organisms, including the approximately one billion microorganisms that reside in a liter of typical seawater from the surface ocean [1]. Yet in most of the surface ocean dissolved phosphorous concentrations are in the nanomolar range, raising a fundamental question in chemical and biological oceanography: how do microorganisms, which form the base of open-ocean food webs, thrive despite the lack of phosphorus [2]? For decades, phosphate (phosphoric acid) was thought to be the sole form of dissolved inorganic phosphorus in ocean, but recent work has called this into question. Pasek et al. [3] showed that phosphite (phosphorous acid, a.k.a. phosphonic acid) is present in rivers and estuaries, raising the possibility of substantial delivery of phosphite to the ocean. Some of the most abundant genera of marine microorganisms appear to be able to grow on phosphite as their sole source of phosphorus [4–6]. My colleagues and I found that microbial cycling of phosphorus between P(V) and P(III) oxidation states, such as the shuttling of phosphorus between phosphate and phosphite, could drive an internal redox flux of phosphorus that is many times greater than the delivery of phosphorus to the ocean from rivers [7, 8].

The use of commercially available $^{32}$P- and $^{33}$P-labeled phosphoric acid in biological incubation assays has been a fundamental tool for oceanographers for decades [9–11]. More recently, my colleagues and I used $^{33}$P-labeled phosphoric acid to make the first estimates of rates of P(V) → P(III) reactions by microorganisms in the ocean [7, 12]. These assays were simple in design: seawater was collected in bottles, $^{33}$P-labeled phosphoric acid was added to the bottles, the bottles were incubated, microbial biomass was filtered from the seawater, and P(III) compounds were isolated and their radioactivity determined. However, studies of the reverse redox reaction, P(III) → P(V), are hindered by a lack of commercially available $^{32}$P- or $^{33}$P-labeled phosphorous acid for use in these simple assays. The synthetic reduction of phosphoric acid to phosphorous acid is challenging due to the large reduction potential of the reaction and the reactivity of intermediates with water. These obstacles were first overcome by Zhang and Casida [13], but the scale of the scheme they described is cost prohibitive and yields $^{33}$P-phosphorous acid with a molar activity (Bq mol$^{-1}$) that is too low for use in oceanographic studies. Since half-saturation constants for the uptake of phosphite by marine microorganisms are thought to be less than 100 nmol L$^{-1}$ [14], additions of phosphite in the range of 10 nmol L$^{-1}$ are prescribed. Yet 100 Bq L$^{-1}$ of $^{32}$P or $^{33}$P is required in the assays for reliable and repeatable

✉ Benjamin A. S. Van Mooy
bvanmooy@whoi.edu

[1] Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, 266 Woods Hole Rd., Woods Hole, MA 02543, USA

Published online: 11 May 2019

quantification of radioactivity by liquid scintillation counting at the very least. Thus, a scheme for the production of $^{33}$P-phosphorous acid with a molar activity substantially greater than 10 GBq mol$^{-1}$ is required.

## Experimental

A number of overarching constraints drove the design of the synthesis scheme presented here. First, since the goal was to produce labeled phosphorous acid for use on oceanographic research cruises, which are often months in duration originating from ports over the world, I chose to use longer-lived $^{33}$P-labeled phosphoric acid. This stated, there is no a priori reason why the scheme could not also be used with $^{32}$P-labeled phosphoric acid. Second, since $^{33}$P is considerably more expensive than $^{32}$P, the scale of each synthesis was limited to approximately 3.7 MBq $^{33}$P. Third, phosphate binds to glass, which may attenuate reaction yield. Thus, glass was avoided where possible. When glass was necessary it was treated with a solution of 3 mol L$^{-1}$ hydrofluoric acid and 2 mol L$^{-1}$ hydrochloric acid as described [15], and then baked overnight at 450 °C. Fourth, since water reacts with intermediates in the synthesis scheme, practical steps were taken to reduce introduction of water to reactions. All reagents used were of the highest available purity from Sigma-Aldrich in a further effort to eliminate water. Finally, since the immediate purpose of developing a micro-scale synthetic route for $^{33}$P-phosphorous acid is to benefit oceanographers, the scheme utilizes common laboratory equipment and simple techniques wherever possible.

The first step in the scheme (Fig. 1a) is to convert phosphoric acid ([$^{33}$P]$H_3PO_4$) to phosphorus oxychloride ([$^{33}$P]$POCl_3$). Zhang and Casida [13] utilized a reaction involving a treatment of $^{33}$P-$H_3PO_4$ with phosphorus pentachloride, but I found that this reaction was not amenable to miniaturization. Instead, [$^{33}$P]$H_3PO_4$ was equilibrated directly with $POCl_3$ as described by Keenan et al. [16]. Ten µL of a 37 GBq L$^{-1}$ solution of carrier-free [$^{33}$P]$H_3PO_4$ (Perkin Elmer), equivalent to 370 kBq or 2 pmol of phosphorus, was carefully added to the bottom of a 100 µL conical reaction vial (Wheaton V-Vial) using an adjustable pipette with a plastic tip. The water was then evaporated from the vial in a vacuum centrifuge (Eppendorf Vacufuge) at 60 °C

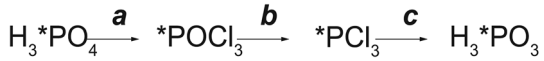



**Fig. 1** Reaction scheme for production of [$^{33}$P]$H_3PO_3$ from [$^{33}$P]$H_3PO_4$ using the following reagents and conditions: **a** equilibration with $POCl_3$, reflux 110 °C, [16]; **b** reduction of [$^{33}$P]$POCl_3$ with $PPh_3$, reflux in toluene at 110 °C, [13]; **c** reaction of [$^{33}$P]$PCl_3$ with water, − 78 °C [13]

for 30 min. This step was repeated 10 more times, yielding 3.7 MBq in the vial. The vial was then lyophilized in a vacuum desiccator for 3 days (Best Value Vacs), after which a barely visible smudge of [$^{33}$P]$H_3PO_4$ was observed at the bottom of the vial. Next, 10 µL of $POCl_3$ (≈ 100 µmol) was added to the bottom of the vial with a 100 µL syringe, and the vial was flushed with nitrogen and tightly sealed with a Teflon-lined screwcap. The vial was partially immersed (2 mm) in an oil bath set to 110 °C, which effected the reflux of $POCl_3$. Approximately every hour, the vial was removed from the oil bath and tapped on the bench to bring any condensed [$^{33}$P]$POCl_3$ back to bottom of the vial. This was repeated for 24 h, after which the vial was removed from the bath, allowed to cool, and centrifuged briefly to be sure all the [$^{33}$P]$POCl_3$ was at the bottom of the vial.

The next step in the scheme (Fig. 1b) is to reduce the [$^{33}$P]$POCl_3$ to phosphorus trichloride ([$^{33}$P]$PCl_3$) using triphenylphosphine ($PPh_3$). This step was done by first placing 39 mg (≈ 150 µmol) of $PPh_3$ in a 1 mL Teflon bottle (Cole Parmer), to which a micro stir bar was added. Next, 100 µL of anhydrous toluene was added to the vial containing the [$^{33}$P]$POCl_3$ from the previous step, and the solution of [$^{33}$P]$POCl_3$ in toluene was transferred to the bottle. The vial was then rinsed 4 times with 100 µL anhydrous toluene, with each rinse being added to the Teflon bottle. The bottle was then flushed with nitrogen and capped. The bottle was partially immersed (5 mm) in a 110 °C oil bath and the stirrer was set to 200 rpm. After 18 h, the bottle was removed from the bath and vigorously shaken to bring any [$^{33}$P]$PCl_3$ that may have condensed on the cap or sides of the bottle back down into the solution. The bottle was then partially immersed (5 mm) in a dry-ice/acetone bath in preparation for the next step.

The final step (Fig. 1c) is the reaction of [$^{33}$P]$PCl_3$ with water to form $^{33}$P-labeled phosphorous acid ([$^{33}$P]$H_3PO_3$). To a new 1 mL Teflon bottle, 500 µL of water was added. This bottle was then partially immersed (5 mm) in dry-ice/acetone bath for 5 min to completely freeze the water. Working very quickly, the two bottles were removed from the dry-ice/acetone bath, and the 500 µL solution of [$^{33}$P]$PCl_3$ in toluene was added to the bottle containing the frozen water. The bottle was then partially immersed in the dry-ice/acetone bath. The vial remained there for several hours; as the dry ice sublimed, the level of the bath gradually descended, thereby slowly removing the vial from the bath and allowing the frozen water to very gradually melt.

The combined 1 mL mixture in the Teflon bottle was transferred to a 10 mL glass centrifuge tube, and the bottom aqueous layer containing the [$^{33}$P]$H_3PO_3$ was transferred to a 1.5 mL plastic microcentrifuge tube. The remaining organic phase was washed twice with 500 µL of ice-cold water, and the aqueous wash phases were combined in the microcentrifuge tube. The microcentrifuge tube containing the [$^{33}$P]

$H_3PO_3$ was then dried overnight in the vacuum centrifuge to remove residual toluene. After dissolving the $[^{33}P]H_3PO_3$ in 1 mL water, aliquots were taken for purity assessment by $^{31}P$-NMR and molar activity analysis by preparative ion exchange chromatography.

To access purity by $^{31}P$-NMR analysis, 200 µL of 0.6 mol $L^{-1}$ deuterium chloride in $D_2O$ was added to 800 µL of $[^{33}P]H_3PO_3$ solution. The solution was analyzed by the Woods Hole Oceanographic Institution Organic Spectroscopy Facility on a Bruker AVANCE 400 NMR spectrometer using phosphate as the reference ($\delta \approx 0$ ppm). The frequency was 162 mHz, temperature was 295 K, and the recycle time was 3 s. Peak areas of the spectrum were integrated and compared to standard solutions of $H_3PO_3$ and $H_3PO_4$ to provide an estimate of the purity of the $H_3PO_3$ product.

For ion exchange chromatography, a 10 µL aliquot was diluted with 990 µL water and 100 µL was injected onto an ion chromatograph (Thermo Dionex ICS-2100). The $[^{33}P]H_3PO_3$ was quantified by conductivity against a pure phosphorous acid standard, and the eluting fraction corresponding to phosphorous acid was collected and analyzed by liquid scintillation counting to determine $^{33}P$ activity. A detailed description of this method was published previously [7].

## Results and discussion

The synthesis scheme yielded relatively pure $H_3PO_3$ (Fig. 2). The $^{31}P$-NMR showed the expected single hydride doublet with two major peaks of identical intensity at 2.91 and 7.08 ppm ($JP$-H = 676 Hz). These peaks were identical in relative intensity and chemical shift to standard solutions of $H_3PO_3$. The $H_3PO_3$ doublet represented approximately 93% of the phosphorus, indicating that $H_3PO_3$ was the overwhelmingly dominant product. The major impurity was $H_3PO_4$.

The $H_3PO_3$ was also of sufficient molar activity for use in biological incubation assays at sea. The $^{33}P$ activity of the $H_3PO_3$ purified by ion exchange chromatography varied between 0.7 and 2.8 MBq representing a reaction efficiency between 21 and 75%. Regardless of the overall yield, the molar activity of the product ranged between 41 and 59 GBq $mol^{-1}$. This result is somewhat higher than the expected molar activity of 37 GBq $mol^{-1}$, which is defined by the ratio of $^{33}P$ radioactivity from $[^{33}P]H_3PO_4$ and the moles of $POCl_3$. I attribute this discrepancy to error in dispensing the $POCl_3$, which is highly viscous at room temperature and difficult to quantitatively draw into and eject from a syringe. Regardless, this result affirms the observation of Keenan et al. [16] that $[^{33}P]H_3PO_4$ completely equilibrates with $POCl_3$ at high temperature. Interestingly, radioanalytical analysis of the synthesis product by preparative ion exchange chromatography showed that $H_3PO_3$ was effectively the sole $^{33}P$-labeled product (Fig. 3). This indicated that the $H_3PO_4$ identified by NMR did not also include $[^{33}P]H_3PO_4$, and, thus, must not have been introduced as a contaminant in the first step of synthesis. The aforementioned preparative ion exchange chromatography clearly separated $H_3PO_3$ from $H_3PO_4$, allowing isolation of very high purity $[^{33}P]H_3PO_3$ final product.

A number of potential pitfalls were identified during the course of the development of this scheme for $[^{33}P]H_3PO_3$ synthesis. First and foremost, water in the reaction appeared to have a number of deleterious effects, as expected. During method development it appeared that traces of moisture

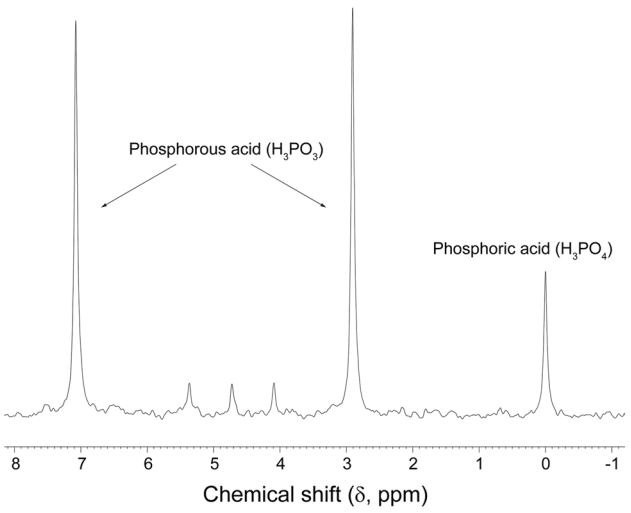



**Fig. 2** $^{31}P$ NMR spectrum of final synthesis product prior to further purification of $[^{33}P]H_3PO_3$ by preparative ion exchange chromatography

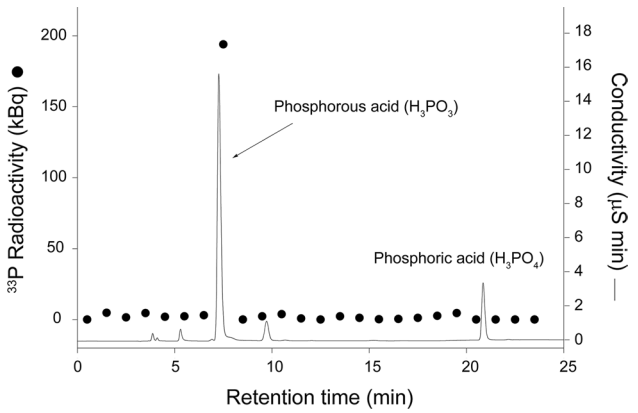



**Fig. 3** Preparative ion exchange chromatograph of final synthesis product shown in Fig. 2. The black circles indicate the $^{33}P$ radioactivity of fractions collected at one-minute intervals. The solid line is a conductivity trace. Retention times of phosphorous acid and phosphoric acid were verified with pure standards

present in the first step (Fig. 1a) reacted with $POCl_3$, yielding $H_3PO_4$ that diluted the $[^{33}P]H_3PO_4$ and reduced the molar activity of the end product. Complete lyophilization of the $[^{33}P]H_3PO_4$ at the outset of the synthesis appeared very important for the success of the first step, as was baking the glassware. If water was present in the second step (Fig. 1b), for example as a contaminant in a previously-opened bottle of toluene, then any newly synthesized $[^{33}P]PCl_3$ reacted with it to make $[^{33}P]H_3PO_4$. Using anhydrous toluene is a simple and inexpensive precaution. A related pitfall is the controlled reaction of $[^{33}P]PCl_3$ with water in the third step (Fig. 1c). It appeared to be insufficient to allow the reaction between $PCl_3$ and ice to proceed on the bench: the reaction must be further slowed by initiating the reaction on dry ice (−78 °C) and allowing the dry ice to sublime over the course of a few hours. Finally, tight-fitting caps are essential, since 100 µmol of $POCl_3$ (bp 106 °C) or $PCl_3$ (bp 76 °C) vapor can rapidly escape a reaction at 110 °C and condense on nearby surfaces.

## Conclusions

The $[^{33}P]H_3PO_3$ synthesis method described here yields $[^{33}P]H_3PO_3$ with a molar activity nearly 3 times greater than described by Zhang and Casida (15.4 GBq $mol^{-1}$ vs. 48 GBq $mol^{-1}$) while starting with 5% of initial radioactivity (74 MBq vs. 3.7 mBq). This new synthesis scheme accomplishes the goals of producing a substrate that is amenable to experimentation at sea while minimizing the initial quantity of $^{33}P$ and associated costs.

**Acknowledgements** I gratefully acknowledge Carl Johnson (Woods Hole Oceanographic Institution) for conducting the $^{31}P$ NMR analyses. This work was supported by a grant from the Simons Foundation (329108 to B.A.S.V.M.), and is a contribution of SCOPE. This work was also supported by the National Science Foundation (OCE-1536346 to B.A.S.V.M.).

## References

1. Karl DM (2014) Microbially mediated transformations of phosphorus in the sea: new views of an old cycle. Annu Rev Mar Sci 6:279–337
2. Dyhrman ST, Ammerman JW, Van Mooy BAS (2007) Microbes and the marine phosphorus cycle. Oceanography 20:110–116
3. Pasek MA, Sampson JM, Atlas Z (2014) Redox chemistry in the phosphorus biogeochemical cycle. Proc Natl Acad Sci USA 111:15468–15473
4. Polyviou D, Hitchcock A, Baylay AJ, Moore CM, Bibby TS (2015) Phosphite utilization by the globally important marine diazotroph *Trichodesmium*. Environ Microbiol Rep 7:824–830
5. Feingersch R, Philosof A, Mejuch T, Glaser F, Alalouf O, Shoham Y, Beja O (2012) Potential for phosphite and phosphonate utilization by *Prochlorococcus*. ISME J 6:827–834
6. Martínez A, Osburne MS, Sharma AK, DeLong EF, Chisholm SW (2012) Phosphite utilization by the marine picocyanobacterium *Prochlorococcus* MIT9301. Environ Microbiol 14:1363–1377
7. Van Mooy BAS, Krupke A, Dyhrman ST, Fredricks HF, Frischkorn KR, Ossolinski JE, Repeta DJ, Rouco-Molina M, Seewald JS, Sylva SP (2015) Major role of planktonic phosphate reduction in the marine phosphorus redox cycle. Science 348:783–785
8. Repeta DJ, Ferron S, Sosa OA, Johnson CG, Repeta LD, Acker M, DeLong EF, Karl DM (2016) Marine methane paradox explained by bacterial degradation of dissolved organic matter. Nat Geosci 9:884–887
9. Björkman K, Karl DM (1994) Bioavailibility of inorganic and organic P compounds to natural assemblages of microorganisms in Hawaiian coastal waters. Mar Ecol Prog Ser 111:265–273
10. Perry MJ, Eppley RW (1981) Phosphate uptake by phytoplankton in the central North Pacific. Deep Sea Res 28:39–49
11. Van Mooy BAS, Fredricks HF, Pedler BE, Dyhrman ST, Karl DM, Lomas MW, Mincer T, Moore LR, Moutin T, Rappé MS, Webb EA (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458:69–72
12. Frischkorn KR, Krupke A, Rouco M, Salazar Estrada AE, Van Mooy BAS, Dyhrman ST (2018) *Trichodesmium* physiological ecology and phosphate reduction in the western Tropical South Pacific. Biogeosciences 15:5761–5788
13. Zhang N, Casida JE (2001) Novel synthesis of $[^{33}P]$-(2-chloroethyl)phosphonic acid. J Org Chem 66:327–329
14. Bisson C, Adams NBP, Stevenson B, Brindley AA, Polyviou D, Bibby TS, Baker PJ, Hunter CN, Hitchcock A (2017) The molecular basis of phosphite and hypophosphite recognition by ABC-transporters. Nat Commun 8:1746
15. Hassenteufel W, Jagitsch R, Koczy F (1963) Impregnation of glass surface against sorption of phosphate traces. Limnol Oceanogr 8:152–156
16. Keenan RW, Martinez RA, Williams RF (1982) Synthesis of $[^{32}P]$ dolichyl phosphate, utilizing a general procedure for $[^{32}P]$phosphorus oxychloride preparation. J Biol Chem 257:14817–14820