

SYNTHESIS OF ADENYLYL IMIDODIPHOSPHATE- $\beta, \gamma$ - $^{15}\text{N}$ 

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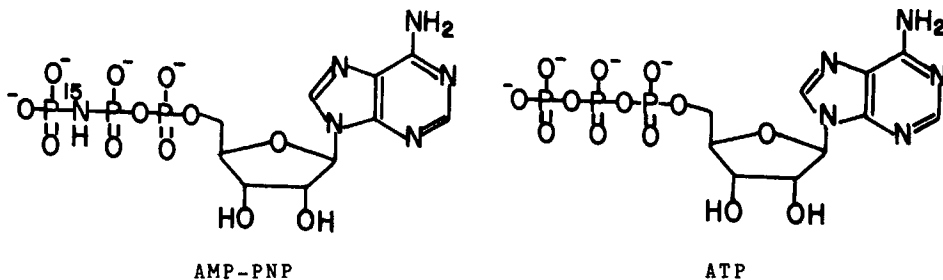
SUMMARY

Adenylyl imidodiphosphate, specifically  $^{15}\text{N}$  labelled in the  $\beta, \gamma$ -bridging nitrogen position, was synthesized in 5% overall yield from  $^{15}\text{NH}_3$ .

Key Words: Adenylyl imidodiphosphate- $\beta, \gamma$ - $^{15}\text{N}$ ;  $^{15}\text{N}$ -NMR

## INTRODUCTION

In 1971, Yount *et al.* (1) reported the synthesis of adenylyl imidodiphosphate (AMP-PNP), which is a close structural analog of adenosine 5'-triphosphate (ATP). Since the P-N bond is relatively stable in basic solutions, this compound has proved to be a widely applicable competitive inhibitor of enzymes that normally catalyze



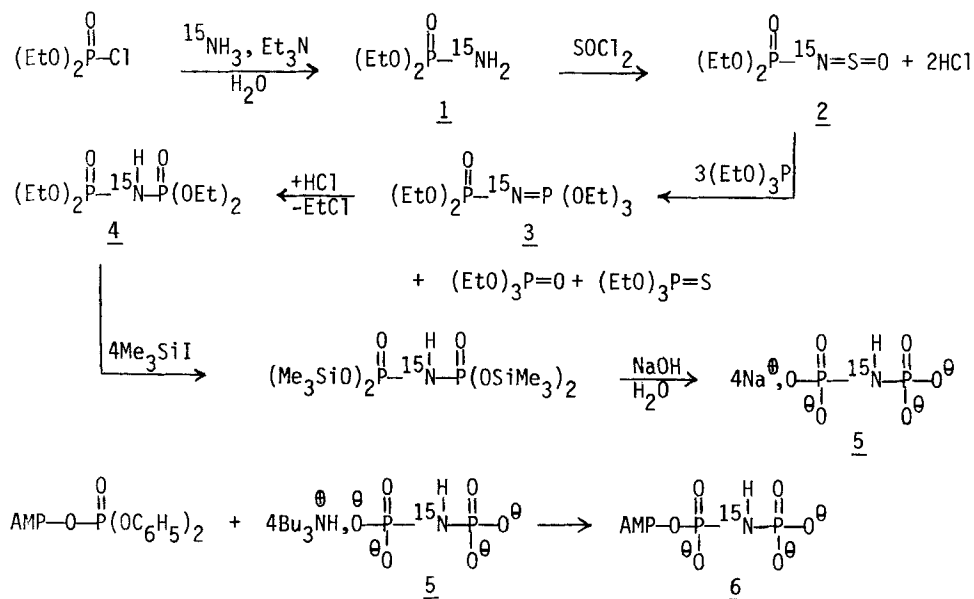
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either cleavage or transfer of the  $\gamma$ -phosphoryl group of ATP (e.g., adenosine triphosphatases and kinases). More recently,  $^{31}\text{P}$ -NMR studies (2) on AMP-PNP as a function of both pH and divalent metal ion concentration (e.g.,  $\text{Mg}^{2+}$ ) have revealed somewhat anomalous behavior relative to the corresponding behavior of ATP itself (3). Namely, the  $\beta$ -phosphorus experiences a greater change in chemical shift upon titration in the 4-10 pH range than does the  $\gamma$ -phosphorus, whereas with ATP the reverse is true. These studies have prompted us to synthesize AMP-PNP that has a specific  $^{15}\text{N}$  label in the  $\beta, \gamma$ -nitrogen position (compound 6 in Scheme I). This compound should be useful in  $^{15}\text{N}$ -NMR studies of AMP-PNP as a function of both pH and divalent metal ion concentration.

## DISCUSSION

Adenylyl imidodiphosphate (6), containing 99%  $^{15}\text{N}$  in the  $\beta, \gamma$ -nitrogen position, has been prepared according to Scheme I.

## Scheme I



where AMP is the adenosine-5'-monophosphate moiety

The method of Goehring and Niedenzu (4) was modified for the synthesis of the diethyl phosphoramidate- $^{15}\text{N}$  (1). The rest of the scheme up to compound 4 was adapted from the synthesis of Wieczorowski (5, 6) as this scheme does not depend on a step where excess ammonia- $^{15}\text{N}$  would be required. Removal of the ethyl ester groups from 4 using trimethylsilyl iodide was an adaptation of the method of Chojnowski *et al.* (7), who used this reagent to prepare tetrasodium thiopyrophosphate from its tetraethyl ester. Although the overall yield is not great, 2.4 g of tetrasodium imidodiphosphate- $^{15}\text{N}$  (5) can be obtained per 1.0 g of enriched ammonia. The procedure of Yount *et al.* (1, 8), an adaptation of the Michelson synthesis of ATP itself (9), was used to convert 5 to the desired nucleotide product 6.

#### EXPERIMENTAL

Ammonia- $^{15}\text{N}$  (99%  $^{15}\text{N}$ ) was obtained from KOR Isotopes, Inc. Triethyl phosphite, triphenyl phosphite, diphenyl chlorophosphate, diethyl chlorophosphate, and trimethylsilyl iodide were all from Aldrich Chemical Co. Adenosine-5'-monophosphate was the product of Sigma Chemical Co. Unlabeled AMP-PNP was purchased from Boehringer-Mannheim Co. All other chemicals were reagent grade.

Melting points and boiling points are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory, University of California, Berkeley.  $^1\text{H}$ -NMR spectra were taken on a Varian Model FT-80 spectrometer, and  $\delta$  values are reported relative to tetramethylsilane as an internal standard.  $^{31}\text{P}$ -NMR spectra were obtained using a Varian Model XL-100 spectrometer at 40.5 MHz, and  $\delta$  values are reported relative to 85%  $\text{H}_3\text{PO}_4$  as an external standard.  $^{15}\text{N}$ -NMR spectra were recorded on the same instrument operat-

ing at 10.14 MHz with  $\delta$  values reported relative to ammonium- $^{15}\text{N}$  chloride as an external standard.

Diethyl Phosphoramidate- $^{15}\text{N}$  (1). Ammonia- $^{15}\text{N}$  (2.0 l., 89 mmol) was dissolved in 8.92 ml of distilled, deionized water in a sealed vessel at  $0^\circ\text{C}$  to make a 10 M solution. This solution was transferred to a 50-ml flask, and freshly distilled triethylamine (8.21 g, 81 mmol) was added. Diethyl chlorophosphate (14.00 g, 81 mmol) was then added dropwise to the stirred solution at  $0^\circ\text{C}$ . After addition was complete, the solution was stirred for 15 min at room temperature. More water (5 ml) was then added, and the product was extracted with two successive additions of 200-ml portions of  $\text{CH}_2\text{Cl}_2$ . The  $\text{CH}_2\text{Cl}_2$  layer was dried over  $\text{MgSO}_4$ . After removal of solvent at reduced pressure, 15 ml of benzene was added and similarly removed to rid the product of traces of water. A flaky, white solid was obtained which melted between  $48\text{--}51^\circ\text{C}$ . [literature m.p.  $52^\circ\text{C}$  (4)]. The product weighed 8.0 g (64% of the theoretical yield).

Diethyl Sulfinylphosphoramidate- $^{15}\text{N}$  (2). Thionyl chloride was heated at reflux with triphenyl phosphite (20 ml per 200 ml  $\text{SOCl}_2$ ) and then freshly distilled (b.p.  $76^\circ\text{C}$ ). Benzene was dried over molecular sieves for at least 3 days. Compound 1 (8.0 g, 52.5 mmol) was added to 40 ml of dry benzene in a flask equipped with a reflux condenser and  $\text{CaCl}_2$  drying tube.  $\text{SOCl}_2$  (6.22 g, 52.5 mmol) was then added, and the apparatus was purged with dry  $\text{N}_2$ . Using an oil bath, the temperature was raised to  $80^\circ\text{C}$ .  $\text{HCl}$  gas began to evolve above  $45^\circ\text{C}$ . Heating was continued until  $\text{HCl}$  gas evolution ceased (ca. 45 min). The cooled mixture was filtered through activated carbon, and the benzene was removed under reduced pressure to give 9.24 g of crude product as a pale yellow liquid. This crude product was purified by vacuum distillation at  $72\text{--}74^\circ\text{C}$  (0.3

torr) [literature b.p.  $77^\circ\text{C}$  at 1 torr (5)]. This fraction weighed 3.81 g (37% of the theoretical amount).

Diethylphosphoryl Triethoxyiminophosphorane- $^{15}\text{N}$  (3).

Triethylphosphite (9.54 g, 57.4 mmol) was dissolved in 8 ml of dry benzene, and 3.81 g of 2 in 6 ml dry benzene at  $0^\circ\text{C}$  was added dropwise with stirring under a purge of dry  $\text{N}_2$ . Stirring was continued for 15 min at room temperature following complete addition, and the benzene was removed at reduced pressure. The side products, triethyl phosphate and triethyl phosphorothioate, distilled together at  $50\text{--}53^\circ\text{C}$  (0.20 torr) [literature b.p.  $42^\circ\text{C}$ . (0.2 torr) (6)] and weighed 6.36 g. The desired product (3) distilled at  $95^\circ\text{C}$  (0.005 torr, oil diffusion pump) and weighed 5.57 g (91.5% of the theoretical yield).  $^1\text{H-NMR}$  (neat):  $\delta$  1.29 (6H, t,  $J_{\text{HH}} = 7.1$  Hz), 1.33 (9H, t,  $J_{\text{HH}} = 7.1$  Hz), 4.09 (10 H, m).

Anal. Calcd. for  $\text{C}_{10}\text{H}_{25}\text{NO}_6\text{P}_2$ : C, 37.74; H, 7.92;  $^{15}\text{N}$ , 4.71; P, 19.46. Found: C, 37.52; H, 7.85;  $^{15}\text{N}$ , 4.70; P, 19.40.

Diethylphosphoryl Diethylphosphoramidate- $^{15}\text{N}$  (4). Compound 3

(5.35 g, 16.8 mmol) was dissolved in 15 ml of ice-cold, dry benzene, and dry HCl (passed through conc.  $\text{H}_2\text{SO}_4$ ) was introduced over a period of 15 min. The benzene was removed at reduced pressure to give 5.0 g of crude product. This product was distilled at  $112\text{--}117^\circ\text{C}$  (0.003 torr), and weighed 3.26 g (66.8% of the theoretical yield).  $^1\text{H-NMR}$ :  $\delta$  1.32 (12 H, t,  $J_{\text{HH}} = 7.1$  Hz), 4.10 (8 H, apparent quintet).  $^{31}\text{P-NMR}$  (proton decoupled):  $\delta = -2.816$  (d,  $J_{\text{PN}} = 33.6$  Hz).  $^{15}\text{N-NMR}$  (proton decoupled):  $\delta = 44.49$  (t,  $J_{\text{PN}} = 33.6$  Hz).

Anal. Calcd. for  $\text{C}_8\text{H}_{21}\text{NO}_6\text{P}_2$ : C, 33.11; H, 7.29;  $^{15}\text{N}$ , 5.17; P, 21.35. Found: C, 33.83; H, 7.27;  $^{15}\text{N}$ , 4.94; P, 20.90.

Tetrasodium Imidodiphosphate-<sup>15</sup>N (5). Compound 4 (1.00 g, 3.45 mmol) was dissolved in 1.0 ml of dry CH<sub>2</sub>Cl<sub>2</sub>, and the resulting solution was cooled to ca. -40°C using a Dry Ice-acetone bath. Trimethylsilyl iodide (2.76 g, 13.8 mmol) was added dropwise with stirring over the course of ca. 15 min, and the cooled mixture was stirred for an additional 45 min. Stirring was continued at room temperature overnight. Water (10 ml) was then added, and the mixture was stirred at room temperature for 30 min. Next, a 50% aqueous NaOH solution (2.21 g, 27.6 mmol NaOH) was added. The aqueous phase was then washed with 5 ml CH<sub>2</sub>Cl<sub>2</sub> and diluted with an additional 10 ml H<sub>2</sub>O. The product (white needles) was precipitated by dropwise addition of ethanol at 0°C. After one recrystallization from aq. EtOH, the needles weighed 1.2 g (78.1% yield).

Anal. Calcd. for Na<sub>4</sub>H<sup>15</sup>NO<sub>6</sub>P<sub>2</sub>·10 H<sub>2</sub>O: H, 4.74; <sup>15</sup>N, 3.36; P, 13.89; Na, 20.62. Found: H, 4.60; <sup>15</sup>N, 2.81; P, 13.90; Na, 21.00.

Adenylyl Imidodiphosphate β,γ-<sup>15</sup>N (6). Compound 5 was transformed into its tributylammonium salt and converted to 6 exactly as described by Yount and co-workers (1, 8). Essentially the same yield that they report was obtained (~40%). The product had an identical R<sub>f</sub> value (0.16) when compared to authentic unlabeled AMP-PNP using polyethylenimine cellulose thin-layer chromatography with 1.2 N NaCl as eluent (10). The spot, as expected, could be visualized with ultraviolet light. <sup>15</sup>N-NMR: δ = 53.43 (pH = 7.3).

#### ACKNOWLEDGMENTS

This work was supported by USPHS Grants AM 17323 (G.L.K.) and GM 22982 (N.J.O.). G.L.K. is an NIH Research Career Development Awardee, AM 00014, 1975-1980. N.J.O. is also an NIH Research Career Development Awardee, CA 00587, 1979-1984.

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