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Research Article

Enzymatic synthesis of [1-^{14}C-*N*-acetyl, P^{18}O$_2$] cytidine monophosphate neuraminic acid

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Summary

A method for the enzymatic synthesis of [1-^{14}C-*N*-acetyl, P^{18}O$_2$] cytidine monophosphate neuraminic acid (CMP-NeuAc) is described. Central to the synthesis of [1-^{14}C-*N*-acetyl, P^{18}O$_2$]CMP-NeuAc was the enzymatic preparation of [γ-P^{18}O$_3$]ATP for use in a reaction with uridine kinase and cytidine to provide 5′-[P^{18}O$_3$]CMP. The [1-^{14}C-*N*-acetyl, P^{18}O$_2$]CMP-NeuAc isotopomer was then synthesized from a reaction involving nucleoside monophosphate kinase, pyruvate kinase and CMP-NeuAc synthetase. The isolated reaction yield was 35%. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: ^{18}O-CMP-NeuAc; uridine kinase [EC 2.7.1.48]; enzymatic synthesis; sialyltransferase

Introduction

Cytidine monophosphate neuraminic acid (CMP-NeuAc) is synthesized in the Golgi apparatus of cells by CMP-NeuAc synthetase and serves as a key intermediate in the sialyltransferase catalyzed biosynthesis of sialylated oligosaccharides. Sialyltransferases are a subset of the glycosyltransferase super family[1] and catalyze the transfer of sialic acid (*N*-acetylneuraminic acid, NeuAc) from an activated CMP-NeuAc donor substrate to non-reducing termini of glycoproteins and glycolipids with inversion of configuration. In nature, these sialylated glycoconjugates function as key recognition molecules for a host of biological processes such as cell–cell regulation, cell adhesion, and biological masking.[2–4]

In recent years, much of the research conducted on sialyltransferases has focused on elucidating the structure and active-site mechanism of this enzyme

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through site-directed mutagenesis and kinetic studies.[5–7] Previous kinetic work conducted in the Horenstein laboratory on the rat recombinant $\alpha(2 \rightarrow 6)$ sialyltransferase, suggested that glycosyl transfer proceeds via a general acid catalyzed mechanism in which a non-bridging phosphate oxygen on CMP-NeuAc may be protonated to facilitate the loss of CMP.[8] We recently synthesized a CMP-NeuAc isotopomer with ^{18}O labels on the non-bridging phosphate oxygens, in order to investigate the mechanism of glycosyl transfer with regard to enzyme interactions at the phosphate leaving group. We were specifically interested in using this CMP-NeuAc isotopomer to conduct ^{18}O kinetic isotope effect (KIE) studies on sialyltransferase. In this paper the enzyme catalyzed synthesis of [1-^{14}C-*N*-acetyl, $P^{18}O_2$]CMP-NeuAc is presented.

Results and discussion

Hydration of PCl_5 with $H_2{}^{18}O$ (95% atom enrichment) followed by addition of 2 M KOH gave $KH_2P^{18}O_4$ **1** in 74% yield. Five phosphate species exist ($P^{18}O_4$, $P^{16}O^{18}O_3$, $P^{16}O_2{}^{18}O_2$, $P^{16}O_3{}^{18}O$, and $P^{16}O_4$) due to the isotopic distribution of the ^{18}O label. ^{31}P NMR and HPLC/(−) ESI-MS analysis of $KH_2P^{18}O_4$ **1** measured the relative isotopic abundance of the five phosphate species to be the following: 80.4% $^{18}O_4$, 16.2% $^{16}O^{18}O_3$, 1.6% $^{16}O_2{}^{18}O_2$, 0.1% $^{16}O_3{}^{18}O$, and 1.7% $^{16}O_4$ (Figures 1 and 2).[9] These results are consistent with the statistical distribution of ^{18}O for the synthesis of $P^{18}O_4$ using 95% atom enriched $H_2^{18}O$.

The enzymatic synthesis with $KH_2P^{18}O_4$ **1** (80% ^{18}O), glyceraldehyde-3-phosphate, (G3P) and cytidine gave $P^{18}O_3$ CMP **2** in 64% isolated yield after purification on HPLC Mono Q. The HPLC/MS analysis of $P^{18}O_3$ CMP **2** showed four peaks at m/z 324, 326, 328 and 330 corresponding to the $P^{16}O_4$ CMP, $P^{16}O_3{}^{18}O$ CMP, $P^{16}O_2{}^{18}O_2$ CMP, $P^{16}O^{18}O_3$ CMP compounds, respectively (Figure 3). A (+) ESI-MS scan measured the relative abundances of the various CMP isotopomers to be: 60% $^{16}O^{18}O_3$, 6.4% $^{16}O_2{}^{18}O_2$, 1.1% $^{16}O_3{}^{18}O$, 32.5% $^{16}O_4$. The results show an approximate 20% dilution of the ^{18}O label from the initial enrichment of $P^{18}O_4$ used in the synthesis. This may be explained by the fact that glyceraldehyde-3-phosphate decomposes at neutral pH to release its phosphate, thus resulting in a dilution of the [$P^{18}O_4^{2-}$]. The decomposition of G3P was minimized in the synthesis by using a shorter incubation time and higher enzyme concentrations. A higher [$P^{18}O_4$] was also used to reduce unlabeled phosphate incorporation.

The [1-^{14}C-*N*-acetyl, $P^{18}O_2$]CMP-NeuAc isotopomer was enzymatically synthesized using $P^{18}O_3$ CMP (60% ^{18}O) and [1-^{14}C-*N*-acetyl]NeuAc. The isolated yield was 35% after purification on HPLC Mono Q. Since we could not obtain ESI-MS spectral data of a radiolabeled compound, a parallel synthesis was conducted to determine approximate isotopic incorporation for

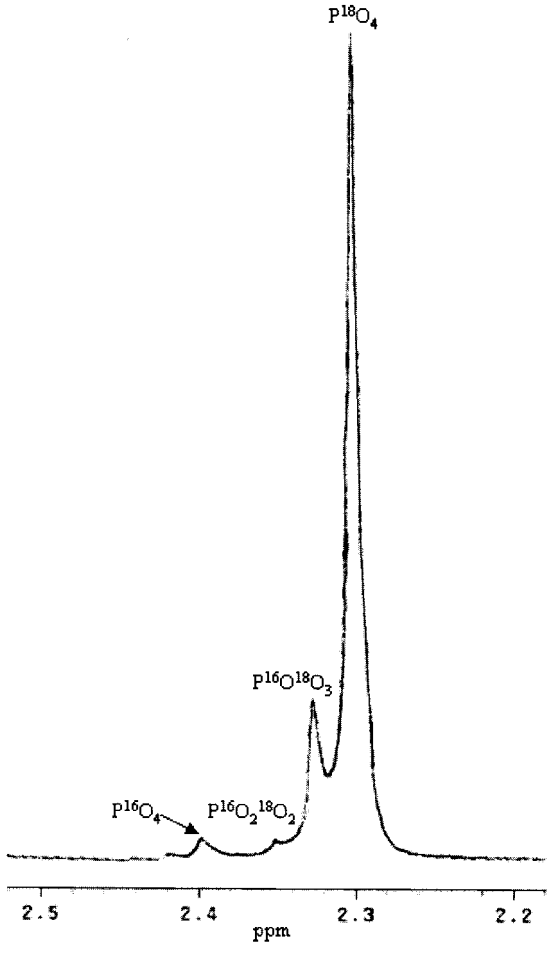


Figure 1. ^{31}P-NMR spectrum of $KH_2P^{18}O_4$ 1(1 M) in D_2O with 4 mM EDTA. Spectrum was obtained after 64 scans

the radiolabeled synthesis. The (+) ESI-MS spectra from the unradiolabeled synthesis of [$P^{18}O_2$]CMP-NeuAc shows the most abundant ions at *m/z* 619 $[M+H]^+$ for [$P^{16}O_2{}^{18}O_2$]CMP-NeuAc and *m/z* 615 $[M+H]^+$ for [$P^{16}O_4$]CMP-NeuAc (Figure 4). The $[M+Na]^+$ adducts of *m/z* 619 and *m/z* 615 were also present at *m/z* 641 and 637, respectively. The *m/z* 619 ion was subjected to MS/MS to yield the labeled *m/z* 328 $[CMP+H]^+$ ion which in a MS/MS/MS scan produced the *m/z* 112 $[Cytidine+H]^+$ ion. Other, less intense ions were detected at *m/z* 310 and 292 which correspond to fragments of the NeuAc moiety. Selected ion monitoring of the ions at *m/z* 619 ([$P^{16}O_2{}^{18}O_2$]CMP-NeuAc), *m/z* 617 ([$P^{16}O_3{}^{18}O$]CMP-NeuAc), and *m/z* 615 ([$P^{16}O_4$]CMP-NeuAc) resulted in a determined isotopic distribution of 56.6, 9.2 and 34.2%, respectively.

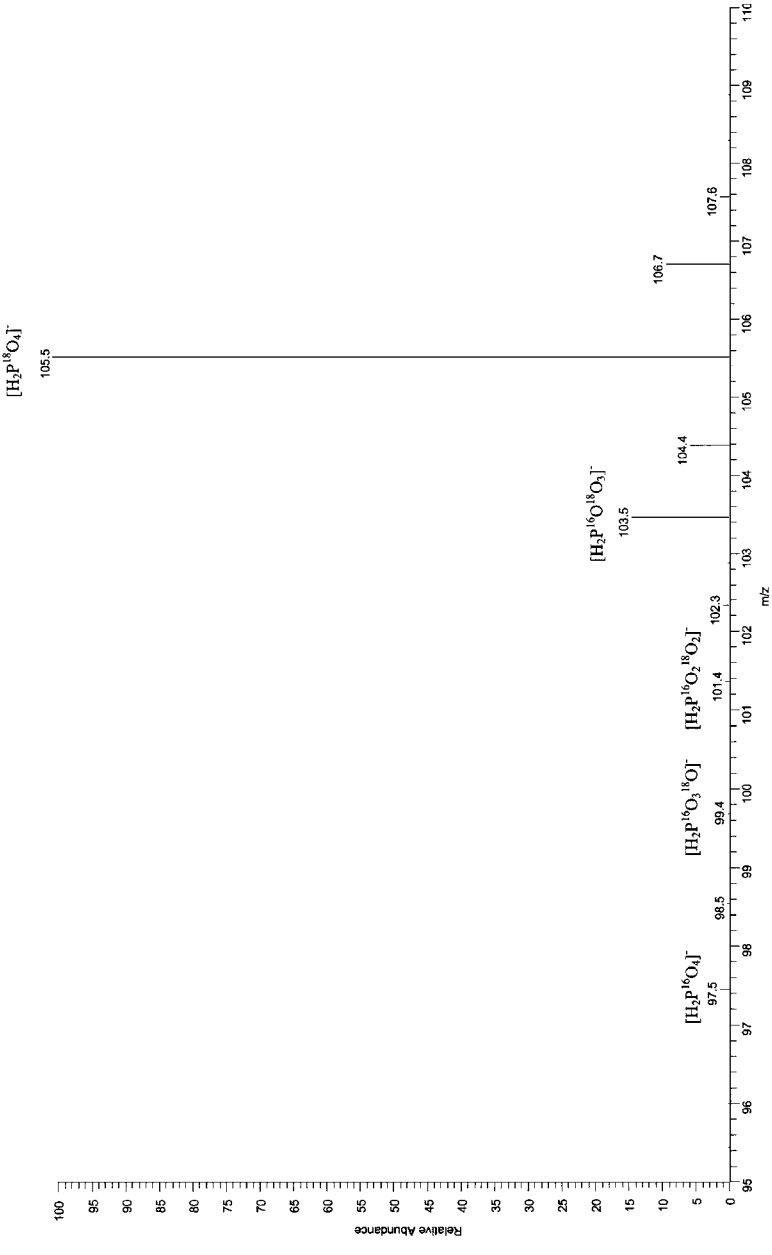


Figure 2. (–) ESI-MS spectrum of $KH_2P^{18}O_4$ 1

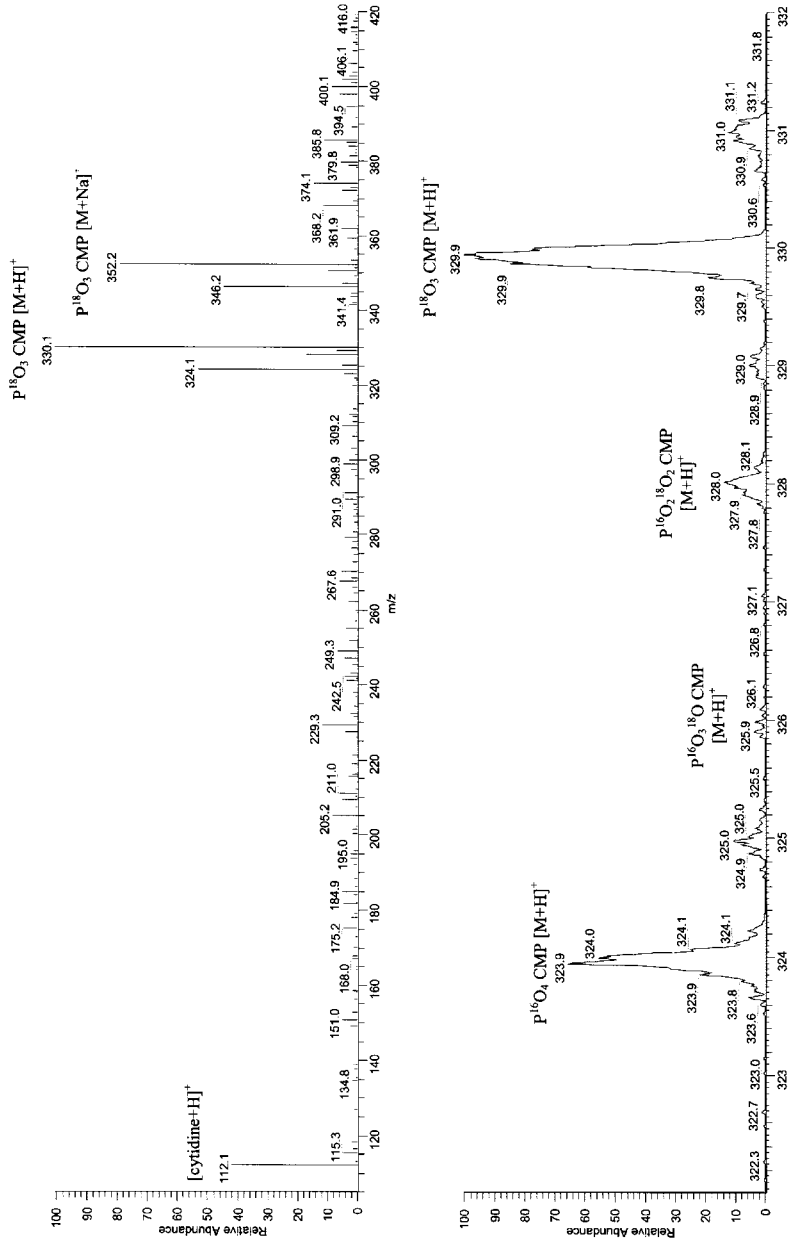


Figure 3. (+) ESI-MS spectrum $P^{18}O_3$ CMP 2 (upper panel) and zoom-MS of the $[M+H]^+$ ions (lower panel)

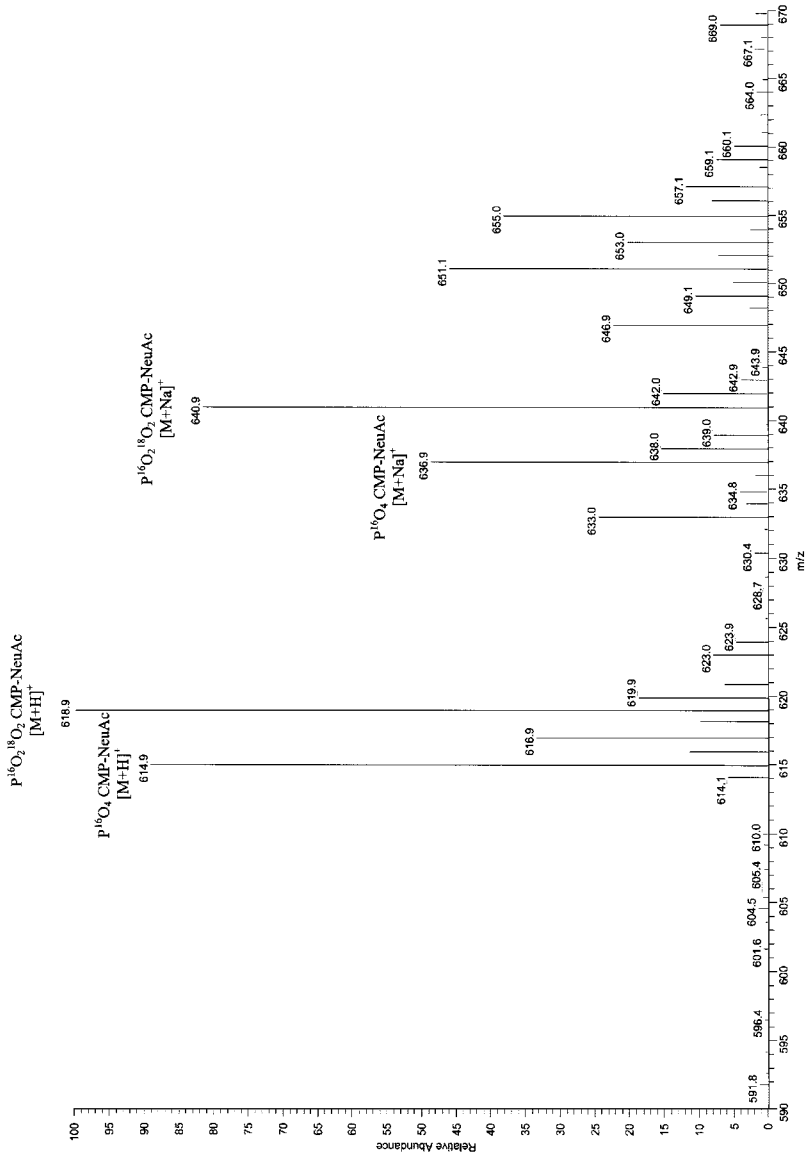


Figure 4. (+) ESI-MS spectrum of $P^{16}O_2^{18}O_2$ CMP-NeuAc

Experimental

General

Reagents and buffers were purchased from Sigma and Fisher and used without further purification. The ^{18}O water (95% atom enrichment) was purchased from Medical Isotopes, Inc. The [1-^{14}C-*N*-acetyl]N-acetyl D-mannosamine isotopomer used in the synthesis of [1-^{14}C-*N*-acetyl] neuraminic acid was purchased from Moravek. Glyceraldehyde-3-phosphate (G3P), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [EC 1.2.1.12] and 3-phosphoglycerate phosphokinase (3-PGK) [EC 2.7.2.3] were purchased from Sigma. Nucleoside monophosphate kinase (NMK) [EC 2.7.4.4] was purchased from Roche Applied Science. The *E. coli* expression plasmid pWV200B haboring the *E. coli* CMP-NeuAc synthetase gene [EC 2.7.2.43) was a generous gift from Dr W. F. Vann at the National Institutes of Health.

Instrumental

A Rainin HPLC system consisting of a HPXL binary pump and a model UV-1 detector was used. HPLC separations were performed on a Mono Q HR 10/10 anion exchange column (Amersham-Pharmacia) monitored at 271 nm. Data collection was achieved on a personal computer using the Star Workstation Version 6.2 software (Varian Inc.). A Rainin-Dynamax fraction collector (model FC-1) was used to collect eluent samples from the HPLC. A Packard 1600 TR instrument was used for liquid scintillation counting. Mass spectrometry (LC-MS) was performed on a ThermoFinnigan (San Jose, CA) LCQ in electrospray ionization (ESI) mode. The system was interfaced with an Agilent (Palo Alto, CA) 1100 binary pump HPLC system consisting of an Applied Biosystems Model 785A programmable absorbance detector set at 254 nm. HPLC separations for LC-MS were performed on a Phenomenex (Torrace, CA) Synergi 4u Hydro-RP 80A Cl8 column (mobile phases = 0.5% HOAc in water/0.5% HOAc in methanol). ^{31}P-NMR spectra were acquired on a 300 MHz Mercury NMR spectrometer.

Cloning, overexpression and purification of uridine kinase [EC 2.7.1.48]

The uridine kinase (UDK) gene[10] was amplified from *E. coli* K12 genomic DNA using the polymerase chain reaction (PCR); (upper primer-5′-ATGACTGACCAGTCTCACCAGCAGTGCG-3′ and lower primer-5′-AAGCTTATTCAAAGAACTGACTTAT-3′). The PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen) and ligated into the pETBlue-1 vector (Novagen). The recombinant plasmid was transformed into NovaBlue (Novagen) competent cells and the plasmid was purified using QIAprep Spin Miniprep Kit (Qiagen). Transformation of Tuner^{TM}(DE3)pLacI competent

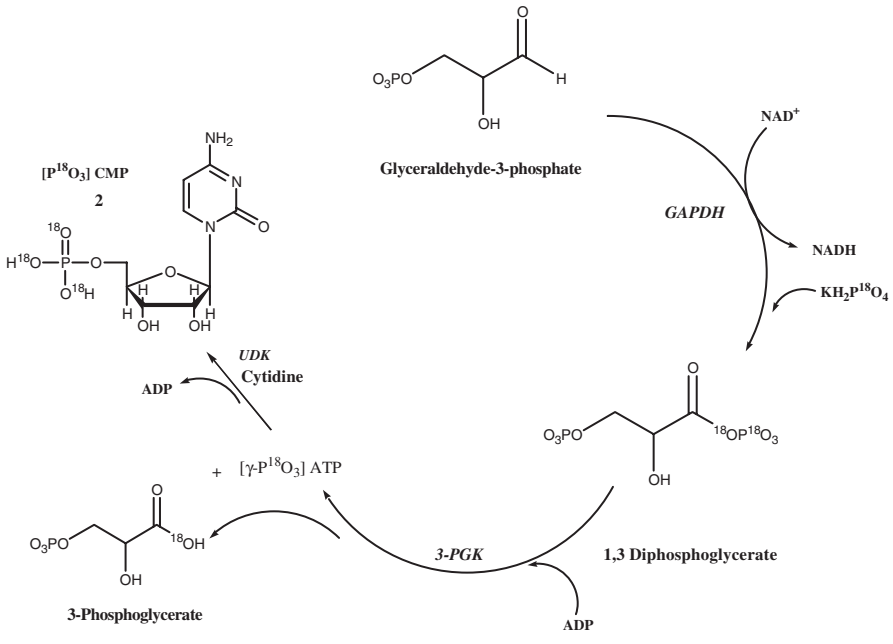
cells with the construct and IPTG-induced overexpression of a 2 l culture yielded the target enzyme. Uridine kinase was purified by Red-A (Millipore) dye affinity column (2.5 × 7 in) using a linear salt gradient from 0–1M KCl. Fractions were analyzed for protein using the Bradford assay method and for UDK activity using the published assay.[11,12] UDK containing fractions were combined and concentrated in an Amicon concentrator to a final volume of 2 ml. The yield was ~150 units and the UDK was 90–95% pure based on SDS-PAGE analysis.

Synthesis of $KH_2P^{18}O_4$

$KH_2P^{18}O_4$ **1** was synthesized using a method similar to that of Risley *et al.*[13] The ^{18}O water (95% atom enrichment) (300 µl, 15 µmol) was added drop wise via syringe to a two-necked flask containing phosphorus pentachloride (428 mg, 2 mmol). The PCl_5 was weighed in a dry box. Once removed, it was immediately placed on a Schlenk line under constant flow of N_2 (g). This was done to reduce $H_2^{16}O$ contamination. The reaction was stirred at 0°C for 1 h under constant flow of dry N_2 (g). The flask was then warmed to room temperature and heated in a water bath at 100°C for 30 min. The remaining reaction solution was cooled to room temperature and ~2 ml of deionized water was added. The solution was titrated to pH 5 with 2 M KOH, and $KH_2P^{18}O_4$ **1** was precipitated from solution by addition of 95% ethanol. The precipitate was collected by concentration under reduced pressure. ^{31}P-NMR (D_2O) δ: 2.303, 2.327, 2.35, 2.39; (−) ESI-MS: *m/z* 105, 103, 101, 99, 97.

Synthesis of $P^{18}O_3$ CMP (Scheme 1)

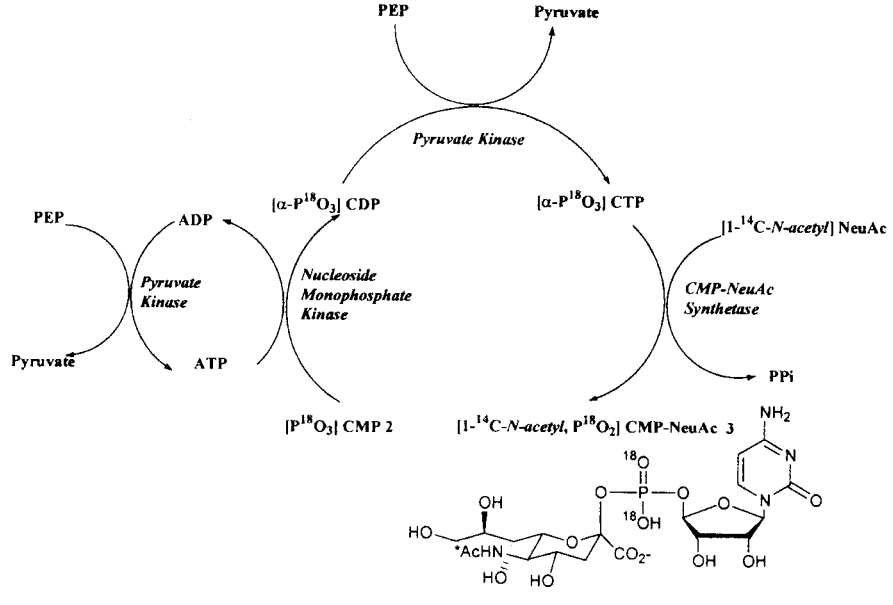
For the synthesis of $P^{18}O_3$ CMP **2** a solution of $KH_2P^{18}O_4$ **1** (1 M, pH 7.0) was made, from which 250–350 µl were mixed with glyceraldehyde-3-phosphate (60 µl, 2 mM), β-NAD (60 µl, 3 mM), ADP (40 µl, 1 mM), $MgSO_4$ (50 µl, 2.8 mM), glycine (50 mM), cytidine (40 µl, 2.5 mM), GAPDH (2 units), 3-PGK (1 unit), and uridine kinase (2 units) in a 1.5 ml microfuge tube and incubated at room temperature for 10 h.[14] Special care was taken to minimize contamination by unlabeled phosphate initially present in some of the reagents used in the reaction. Thus, ADP was freshly prepared and 3-PGK was dialyzed against 0.5 M Tris–HCl, pH 7.5 buffer to remove the orthopyrophosphate storage buffer provided by the manufacturer. The reaction solution was filtered through a microcon filtration unit (Millipore, MWCO 10 kDa) to remove enzymes and $P^{18}O_3$ CMP **2** was purified from the filtrate using isocratic, anion exchange HPLC (75 mM NH_4HCO_3, pH 8.0, 15% methanol, 2 ml/min). The $P^{18}O_3$ CMP **2** containing fractions were pooled and desalted with Amberlite IR 120-H^+ cation-exchange resin. The solution was concentrated to dryness *in vacuo* and resuspended in 300 µl of deionized water. (+) ESI/MS: *m/z* 330, 328, 326, 324.



Scheme 1. Enzymatic synthesis of $[P^{18}O_3]$CMP 2 from $KH_2P^{18}O_4$ 1

Synthesis of [1-^{14}C-N-acetyl, $P^{18}O_2$]CMP-NeuAc (Scheme 2)

The [1-^{14}C-*N*-acetyl, $P^{18}O_2$]CMP-NeuAc **3** was synthesized using a method adapted from that of Ichikawa *et al.*[15] The $P^{18}O_3$ CMP **2** purified from above



Scheme 2. Enzymatic synthesis of [1-^{14}C-*N*-acetyl, $P^{18}O_2$]CMP-NeuAc 3 from $[P^{18}O_3]$CMP 2

was concentrated to dryness *in vacuo* and used in an enzymatic reaction with ATP (5 μmol), PEP monosodium salt (10 μmol), $MnCl_2$ (10 μmol), $MgCl_2$ (10 μmol), NMK (2 units), PK (500 units) and 700 μl of HEPES buffer (0.2 M, pH 7.5). The reaction mixture was incubated for 24 h at 25°C and then filtered through a micron filtration device (Millipore, MWCO 10kDa) to remove enzymes. This step is necessary to prevent the *in situ* recycling of $P^{18}O_3$ CMP **2** and subsequent dilution of ^{18}O labels resulting from the decomposition of CMP-NeuAc in the following synthetic step. [1-^{14}C-*N*-acetyl]NeuAc (10–25 μCi) and CMP-NeuAc synthetase (3 units) were added to the filtrate and the reaction was incubated at 37°C for 6 h. CMP-NeuAc isotopomers were purified using isocratic, anion exchange HPLC (75 mM NH_4HCO_3, 15% methanol, pH 8.0, 2 ml/min). CMP-NeuAc fractions were pooled, desalted with Amberlite IR 120-H^+ cation-exchange resin and concentrated *in vacuo* as previously described.[7,8] (+) ESI/MS: *m*/*z* 619, 617, 615.

Conclusion

The multi enzyme catalyzed synthesis of [1-^{14}C-*N*-acetyl, $P^{18}O_2$]CMP-NeuAc **3** is an effective method to selectively incorporate ^{18}O labels into the non-bridging phosphate oxygens of CMP-NeuAc. This method may be easily adapted for the synthesis of other ^{18}O nucleotide monophosphate esters by substituting in the appropriate enzymes. Alternative radiolabels may also be incorporated into the compound by using other isotopomers of NeuAc, cytidine or inorganic phosphate. Thus, we expect [1-^{14}C-*N*-acetyl, $P^{18}O_2$]CMP-NeuAc to be a useful substrate for *in vivo* and *in vitro* kinetic studies of sialyltransferase.

Acknowledgements

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