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SYNTHESIS AND HETERONUCLEAR NMR ANALYSIS OF DEUTERIUM- AND TRITIUM-LABELLED METHYLENEBISPHOSPHONIC ACID

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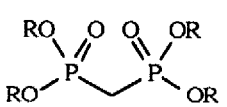
Abstract. Deuterium has been substituted into the methylene group of methylenebisphosphonic acid, MDP. Analysis of the phosphorus NMR spectrum of the product shows a linear high-frequency shift on sequential mono- and di-deuteriation. The corresponding tritium labelling of MDP achieved a specific activity of 30 mCi/mmol.

Methylenebisphosphonic acids possess unique physico-chemical affinities for the alkali metals (notably calcium and magnesium) and certain heavy metal cations (*e.g.* tin and iron) that have led to many applications in industry and medicine.[1] Industrially, their chief use is in the prevention of calcium and magnesium scale formation in boilers and pipes.[1,2] Medically, compounds of this class are used as drugs for prophylaxis and therapy of abnormal calcium phosphate metabolism *e.g.* Paget's disease, and for diagnosis of bone pathologies.[1,3,4]

The parent compound, methylenebisphosphonic acid (**1a**) is a stable analogue of pyrophosphoric acid, PP_i, and is widely used in biochemical investigations. For example, the complete series of phosphonate analogues of the nucleotides ADP, ATP, GDP, and GTP having α,β- or β,γ-methylene groups has been synthesised.[5] A phosphonic acid analogue of thiamine diphosphate has also been prepared.[6] In general, (**1a**) can mimic PP_i in many differing metabolic processes, as for example in the pyrophosphorolysis reaction catalysed by the *E.coli* RNA polymerase.[7] In addition, (**1a**) efficiently inhibits the growth of the slime mould, *Dictyostelium discoideum*, presumably as a result of the *in vivo* formation of nonhydrolysable analogues of ATP and of Ap_4A.[8]

Notwithstanding the general stability of the P-C bond to enzymatic cleavage, some microorganisms exist which are capable of utilising (**1a**) as their sole source of phosphorus for growth.[9,10] In particular, the uptake of (**1a**) by *Pseudomonas* PG 2982 appears to give rise to the formation of methane.[9] To enable us further to explore the application of (**1a**) for research in both *in vitro* and *in vivo* biological studies, we required a route for the tritium labelling of (**1a**) in good yield and with a specific activity in the region of 30 mCi/mmol.



(**1a**) R = H
(**1b**) R = Et
(**1c**) R = Me_3Si

We chose to pilot this preparation by investigation of the pattern and extent of deuteriation of tetraethyl

methylenebisphosphonate (**1b**), basing our approach on the known reactions of tetraethyl sodiomethylenebisphosphonate with halides, with halogens, and with oxygen.[11]

Our attempts to introduce the isotopic label by quenching a solution of tetraethyl lithiomethylenebisphosphonate in THF at -40°C with deuteriated water led to the partial hydrolysis of phosphonate esters to give water-soluble products only. Accordingly, we chose to quench the corresponding sodio-carbanion with deuteriated trifluoroacetic acid to circumvent alkaline hydrolysis. Tetraethyl methylenebisphosphonate (**1b**) (6 mmol) (Lancaster Synthesis) was metallated with sodium hydride (6.6 mmol) in toluene (2.5 ml) under nitrogen at 0°C. After stirring 18 h at 10°C, D_2O (4.8 mmol) premixed with trifluoroacetic anhydride (4.2 mmol) in THF (1.5 ml) and incubated 5 min at 5°C was added to the reaction mixture at -40°C under nitrogen. After 1 h, the reaction was quenched with 2 M Na_2SO_4 in 1 M sodium citrate (pH 5.0) and extracted with ethyl acetate to give deuteriated (**1a**) in 92% yield. De-esterification was carried out using bromotrimethylsilane by standard procedures,[12] and the *tetrakis*-trimethylsilyl ester (**1c**) taken up in methanol and brought to pH 7.5 by addition of sodium hydroxide (3 equiv) to give the trisodium salt of (**1a**) in quantitative yield, M/z 290 ([M+H]$^+$ 100%). Its ^1H, ^{13}C, and ^{31}P NMR characteristics are provided in the Table.

Table. Heteronuclear chemical shifts (CS),[a] spin coupling constants (SCC),[a] and isotope chemical shift resulting from deuteriation (ICS)[b] for tris-sodium methylenebisphosphonate.

SCC Hz					
$^1J_{(^{13}C,^1H)}$	$^1J_{(^{13}C,^2H)}$	$^1J_{(^{13}C,^{31}P)}$	$^2J_{(^3H,^1H)}$	$^2J_{(^1H,^{31}P)}$	$^2J_{(^3H,^{31}P)}$
121.5	18.7	121.5	-16.5	19.8	20.2
CS ppm			ICS ppm		
PCH_2P	PCH_2P	PCH_2P	$^1\Delta^{13}C$ (D)	$^2\Delta^1H$ (D)	$^2\Delta^{31}P$ (D)
δ_H 2.15, t	δ_C 29.2, t	δ_P 16.0, t	+0.290	+0.016	-0.035[c]

[a] Error ±0.3Hz; [b] error ± 1 ppb; [c] low frequency shift is positive. Spectra recorded at 250.13 MHz for ^1H (DSS internal reference, 62.9 MHz for ^{13}C with CD_3OD internal reference, 101.26 MHz for ^{31}P with $(MeO)_3PO$ internal reference, and 266.8 MHz for ^3H in D_2O solutions using a Bruker AC250 instrument.

Analysis of the proton-coupled ^{31}P NMR spectrum (Figure 1A) showed the presence of three isotopomers: a triplet corresponding to undeuteriated (**1a**), (42%), a broad doublet, corresponding to monodeuteriated (**1a**) (42%), and a very broad singlet, corresponding to dideuteriated (**1a**), 16%. Unexpectedly, the chemical shift for phosphorus is

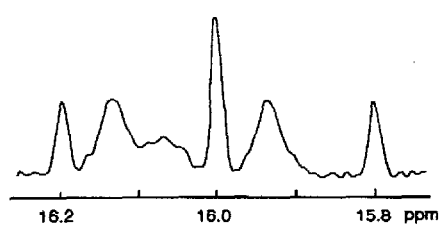


Figure1A ^{31}P NMR spectrum of (**1a**) at 101.26 MHz

moved -0.035 ppm to*high frequency* by both of the successive deuterium substitutions as a result of the two-bond deuterium isotope effect. Only one unambiguous example of a comparable linear high-frequency shift has been reported hitherto, that being for dialkyl benzylphosphonates.[13]

The proton (Figure 1B) and carbon-13 (Figure 1C) spectra were observed for the first two isotopomers and showed typical low-frequency chemical shifts for deuterium substitution and with regular values for spin-spin couping constants (Table) though the geminal deuterium isotope shift for the proton signal of (**1a**) was rather higher (16 ppb) than usual.[14] We observed coincidence of the magnitude of carbon-13 coupling to proton and to phosphorus, leading to a near-perfect quintet spectrum (Figure 1C).

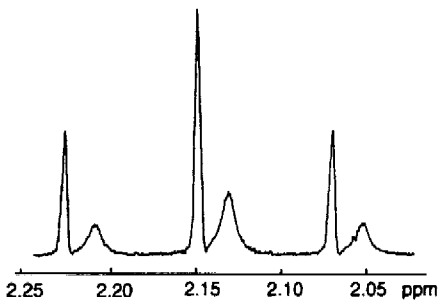


Figure 1B ^{1}H NMR spectrum of (**1a**) at 250.13 MHz

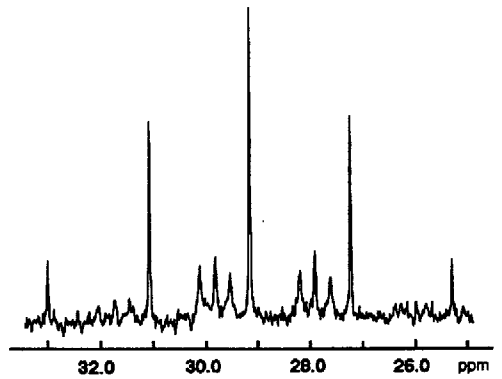


Figure 1C ^{13}C NMR spectrum of (**1a**) at 62.9 MHz

The tritium-labelling of (**1a**) was carried out exactly as for the deuteriation experiment but using tritiated water (Amersham International plc, 0.1 ml, 90 Ci/mole) in place of D_2O. The dry tris-sodium salt product contained 197.3 mCi, giving a specific activity of 30±1 mCi/mmole. Compared to the theoretical optimum specific activity of 45 mCi/mmol, this result indicates that there is at most only a small primary kinetic isotope effect operating in the protonation of the carbanion of (**1b**). Moreover, the radiochemical yield of close to 40 is very satisfactory. Proof of structure was established by tlc comparison with MDP, R_f 0.40 (Cellulose F 1440, AVICEL [Schleicher and Schüll]; water:25% NH_4OH:10% TCA:MeOH; 6:3:1:10) with coincidental visualisation both by direct tlc radioscanning and using the standard ammonium molybdate spray reagent for phosphorus. The isotope appears not to exchange to any detectable extent out of (**1a**) after one week at room temperature in solution in water at pH 7-8. The tritium NMR spectrum[15] (at 266.8 MHz) shows a broad triplet in the proton-decoupled mode and a double triplet when proton-coupled. Relevant NMR parameters are provided in the Table.

In conclusion, the method here described permits the simple preparation of tritiated MDP at specific activities comparable to those of available tritiated water and in ample quantity both for further synthetic incorporation into nucleotides and for metabolic studies. The methodology developed is of general applicability for the hydrogen-isotope labelling of alkanephosphonic acids.

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