Remarkable Lack of Biological Activity Exhibited by a DNA-reactive and Water-soluble *cis*-Bis(phosphino) Platinum(II) Complex

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The solvolytic behaviour of the complex *cis*-$[Pt(PMe_3)_2Cl_2]$ was investigated in water and dimethyl sulfoxide. Mono- and bis-solvento species have been characterized mainly by ^{31}P NMR spectroscopy. The water-soluble species *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$, thermodynamically stable at physiological pH values and known to be reactive with pyrimidinic nucleobases, is found to be also quite reactive with purified single- and double-strand DNA, as shown by ^{31}P NMR spectroscopy. However, the same hydroxo complex is found to be remarkably inactive towards Eagle's KB cell line and Ehrlich's ascitic tumour cells. This lack of biological activity is maintained also when the complex is administered to Eagle's KB cell line as a suspension of L-α-dipalmitoylphosphatidylcholine liposomes.

The extensive research work dealing with the chemical interaction of *cis*-platinum(II) complexes with DNA-relevant molecules is traditionally motivated by the assumption [1] or the observation [2] that *cis*-$Pt^{II}L_2$ units are capable of producing selective lesions to the DNA of target cells, *in vivo* and *in vitro*. Obviously, understanding in molecular terms the details of this interaction is expected to furnish useful information for the best management of the platinum(II)-based cancer chemotherapy.

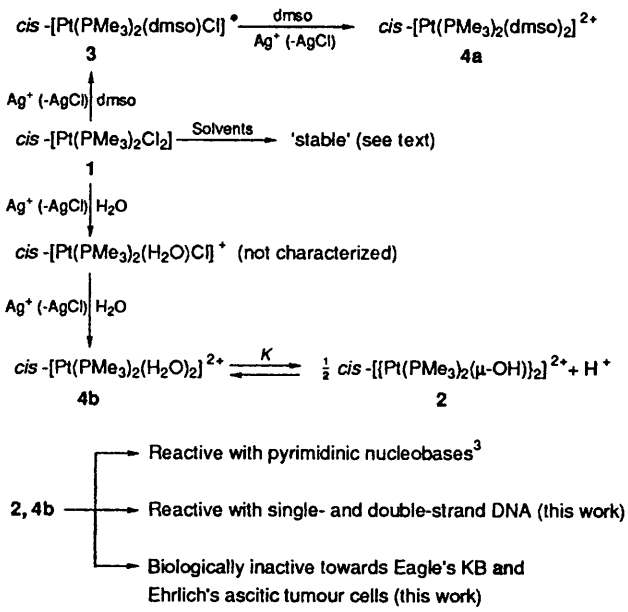
It could be also observed that complexes able to react easily with nucleosides and nucleotides under 'chemical' conditions are expected to be at least plausible candidates for exhibiting a more or less pronounced genotoxic, and therefore potentially cytostatic, activity.

We have recently shown [3] that some water-soluble derivatives of the complex *cis*-$[Pt(PMe_3)_2Cl_2]$ **1** are quite reactive towards pyrimidinic nucleobases and nucleosides. Particularly remarkable is the water solubility and reactivity of the derivative *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ **2**, which is the stable form in the PMe_3–$PtCl_2$–OH^-–H_2O system at physiological pH value. It should be observed that complex **2** is structurally related to *cis*-$[\{Pt(NH_3)_2(\mu\text{-}OH)\}_2]^{2+}$, **4** which is also the known predominant species in the NH_3–$PtCl_2$–OH^-–H_2O system in neutral solutions.[5]

We report here on the solvolytic behaviour of complex **1** in dimethyl sulfoxide (dmso), on the reactivity of complex **2** with either single-strand or double-strand purified DNA and on the remarkable inability of complex **2** to exhibit any biological activity towards Eagle's KB cell line and Ehrlich's ascitic tumour cells.

Results

Solvolytic Behaviour of *cis*-$[Pt(PMe_3)_2Cl_2]$.—The whole of the data herein described together with some taken from the literature are collected in Scheme 1. The complex *cis*-



Scheme 1 Solvolytic behaviour of *cis*-$[Pt(PMe_3)_2Cl_2]$

$[Pt(PMe_3)_2Cl_2]$ **1** is a white microcrystalline thermally-stable compound, which is sparingly soluble in water (*ca.* 10^{-5} mol dm^{-3}) but rather soluble in polar organic solvents, including dmso. In this solvent complex **1** appears to be stable as shown by the invariance of its ^{31}P NMR spectrum with time, even at 80 °C.

This behaviour is in striking contrast with that exhibited by *cis*-$[Pt(NH_3)_2Cl_2]$ which is known to undergo quantitative and relatively fast solvolysis (half-life *ca.* 60 min, at 37 °C) to give

Table 1 Phosphorus-31 NMR data for the phosphine complexes[a]

Compound	$\delta(^{31}P)(^{1}J_{Pt-P}/Hz)$ $[^{2}J_{PP}/Hz]$
1 *cis*-$[Pt(PMe_3)_2Cl_2]$	−22.35 (3469)
3 *cis*-$[Pt(PMe_3)_2(dmso)Cl]^+$	−16.59 (3569)
	−26.75 (3642) [22]
4a *cis*-$[Pt(PMe_3)_2(dmso)_2]^{2+}$	−24.9 (3738)
4b *cis*-$[Pt(PMe_3)_2(H_2O)_2]^{2+}$	−25.25 (3742)[b]

[a] Data at 27 °C, in $(CD_3)_2SO$ unless otherwise stated. [b] Recorded in D_2O.

cis-$[Pt(NH_3)_2(dmso)Cl]^+$.[6] However its phosphine analogue, *cis*-$[Pt(PMe_3)_2(dmso)Cl]^+$ **3**, can be easily obtained upon reacting **1** with a stoichiometric quantity of Ag^+ in dmso at ambient conditions. The reaction of **1** with two equivalents of Ag^+ leads to the instantaneous and quantitative formation of the complex *cis*-$[Pt(PMe_3)_2(dmso)_2]^{2+}$ **4a**. Both complexes **3** and **4** have been characterized by ^{31}P NMR spectroscopy (Table 1). All cationic trimethylphosphine complexes are water-soluble species.

The ^{31}P NMR spectrum of complex **1** exhibits the expected single signal flanked by ^{195}Pt satellites and no other ^{31}P resonances can be detected in *ca.* 0.1 mol dm^{-3} solutions. The spectral pattern does not change after heating at 80 °C for 4 h.

The spectrum of complex **3** displays an AB multiplet with the related ^{195}Pt satellites in agreement with the presence of two different ligands *trans* to PMe_3. Moreover, the spectrum does not show any evidence of further solvolysis after several days at room temperature. The ^{31}P NMR resonance at higher field, characterized by the larger Pt–P coupling constant, is attributable to the phosphine *trans* to the more weakly bonded ligand, *i.e.* dmso.[7]

The quantitative removal of the second chloride ligand from complex **1** in dmso gives rise to complex **4a**, which is characterized by a singlet in agreement with the quantitative formation of the bis(solvento) derivative.

When the chloride removal is carried out in water, the appearance of the bis(aqua) complex **4b** is accompanied by that of a species characterized by a weak singlet at δ −25.58, *i.e.* the dinuclear complex *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ **2**. In fact, as we have already shown, complex **4b** is a fairly acidic species, its pK_a value being in the range 3–4.[3a,8] Careful addition of one equivalent of Ag^+ to complex **1** leads to the quantitative removal of the platinum(II)-co-ordinated chloride ion and to the appearance of a relatively complex ^{31}P NMR pattern. Thus, a broad major signal centred at *ca.* δ −25.6 (flanked by ^{195}Pt satellites, *ca.* 3738 Hz) is accompanied by the spectral pattern of complex **2**. We attribute the broad resonance to a mixture of *cis*-$[Pt(PMe_3)_2(H_2O)Cl]^+$ and *cis*-$[Pt(PMe_3)_2Cl(OH)]$.

It should be concluded that the overall solvolytic pattern of all these phosphine *cis*-platinum(II) complexes as well as their reactivity with nucleobases are similar to those observed for their amino analogues. The major and indeed surprising difference between the chemistry of *cis*-$[Pt(NH_3)_2Cl_2]$ and *cis*-$[Pt(PMe_3)_2Cl_2]$ is the solvolytic stability of **1** in dmso. In fact, as pointed out above, the *cis*-$\{(NH_3)_2\}$ moiety makes the Pt–Cl bond much more prone to solvolysis in dmso than the unit *cis*-$\{(PMe_3)_2\}$, an observation which appears contradictory to the expectation based on the well known higher *trans* effect of PMe_3 compared with NH_3.[9]

Reactivity of cis-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ *with DNA*.—The ^{31}P NMR spectrum of a 53 mmol dm^{-3} (nucleotide) single-strand DNA solution in D_2O (0.5 mol dm^{-3} in $NaNO_3$) exhibits a single rather broad peak at *ca.* δ −1.1. A weak signal due to the terminal phosphate groups at *ca.* δ 2.15 is also detectable [Fig. 1(*a*)]. Gradual additions of aliquots of a D_2O solution of complex **2**, up to a final 1:1 nucleotide:platinum ratio lead to rapid spectral changes [Fig. 1(*b*) and 1(*c*)] which demonstrate a clear reactivity of single-strand DNA towards the platinum(II) complex, with unambiguous metal co-ordination to the DNA nucleotides. In fact, addition of **2** is paralleled by the development of a set of broad signals of nucleotide-co-ordinated *cis*-$\{Pt(PMe_3)_2\}$ units.[3] Moreover, metal co-ordination is clearly indicated by the appearance of the P–Pt satellite peaks, with J_{PtP} *ca.* 3300 Hz, again in close agreement with the persistence of a platinum(II) *cis* co-ordination in the Pt–DNA adduct (see below). When the nucleotide:platinum ratio is brought to 1:0.50 a white precipitate is formed. Interestingly, for a 1.00:0.75 nucleotide:platinum ratio, the very sharp signal at δ −25.56 (J_{PtP} 3398 Hz) due to unreacted complex **2** is clearly apparent in the relevant spectrum (*ca.* 5% with respect to total platinum(II)-bonded phosphorus nuclei present in solution). Further addition of complex **2** up to a 1:1 nucleotide:platinum ratio enhances the signal due to unreacted **2**, without producing major spectral changes. Similar observations are obtained upon reacting double-strand DNA with complex **2** (Fig. 2). Owing to

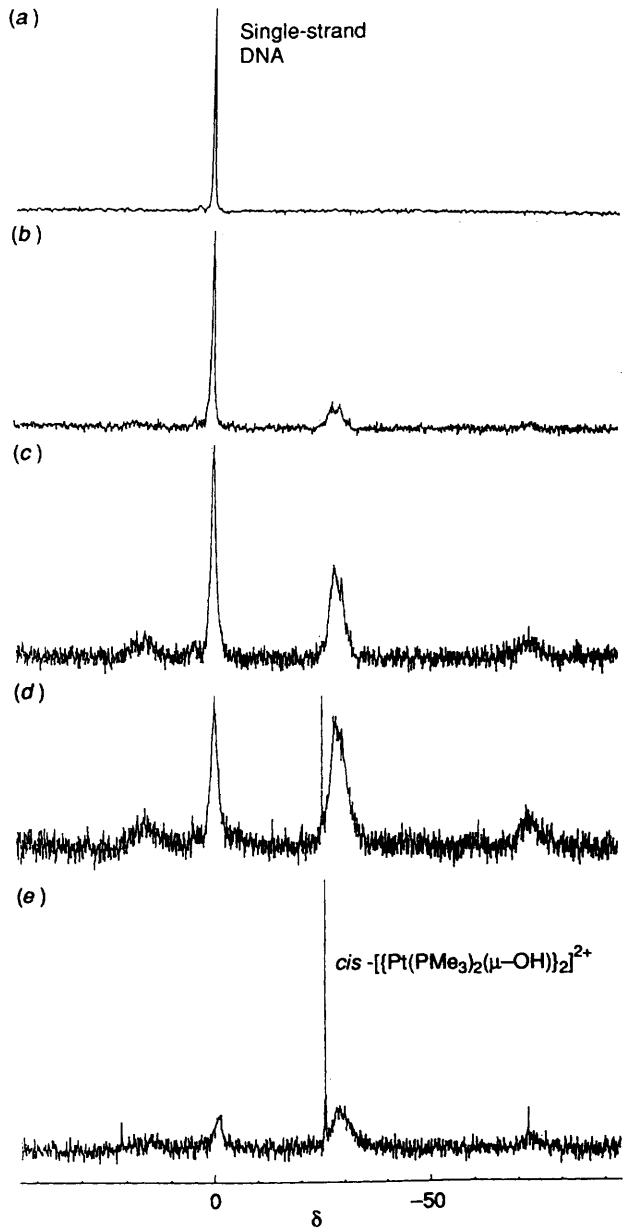


Fig. 1 The $^{31}P-\{^1H\}$ NMR spectra at 36.23 MHz of single-strand DNA solutions (53 mmol dm^{-3} nucleotide in D_2O, 0.5 mol dm^{-3} in $NaNO_3$) containing *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2][NO_3]_2$ at (*a*) 1.00:0, (*b*) 1.00:0.25, (*c*) 1.00:0.50, (*d*) 1.00:0.75 and (*e*) 1.00:1.00 nucleotide:platinum ratios. When nucleotide:platinum > 0.50 a white precipitate is formed

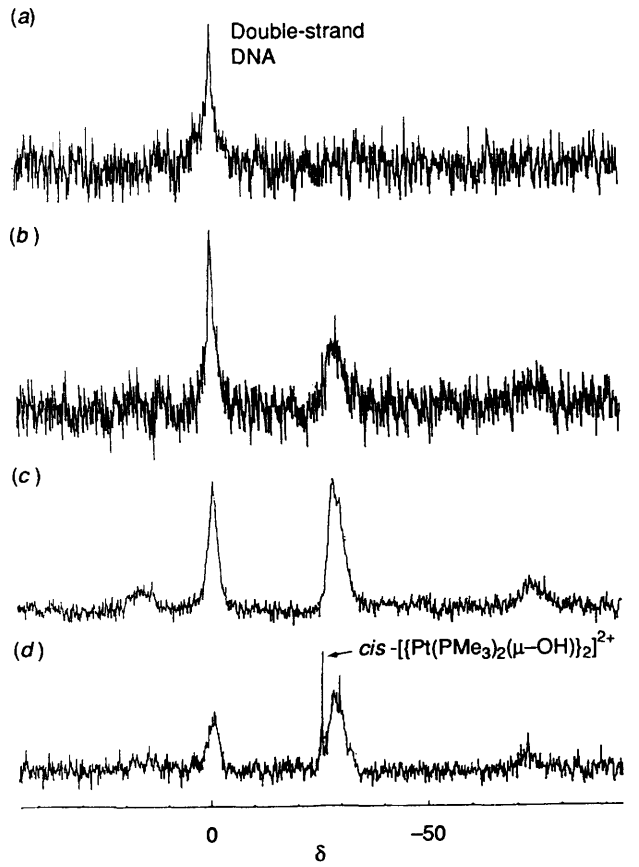


Fig. 2 The ^{31}P-$\{^{1}H\}$ NMR spectra at 36.23 MHz of double-strand DNA solutions (13.6 mmol dm^{-3} nucleotide in D_2O, 0.5 mol dm^{-3} in $NaNO_3$) containing *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2][NO_3]_2$ at (*a*) 1.00:0, (*b*) 1.00:0.25, (*c*) 1.00:0.50 and (*d*) 1.00:0.75 nucleotide:platinum ratios

the lower solubility of double-strand DNA, the initial concentration of the nucleotide solution was 13.6 mmol dm^{-3}. In this case addition of **2** up to a 1.00:0.75 nucleotide:platinum ratio does not induce the formation of a precipitate. Altogether these spectral data leave little doubt as to the ability of **2** to react readily with DNA and the analysis of the platinum(II)-bonded PMe_3 signals suggests that more than one Pt^{II}–DNA species is formed, under these conditions.

Biological Activity of cis-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ *Towards Eagle's KB Cell Line.*—Complex **2** was tested both in water solutions and as a suspension of L-α-dipalmitoylphosphatidylcholine (dppc) liposomes[10] suspensions. Tests in water were carried out with platinum concentrations 1.28, 2.56 and 12.8 μmol dm^{-3}. The first two concentrations gave no inhibition and the third one only a modest effect (*ca.* 10% with respect to the controls). Tests carried out with complex **2** dissolved in the nanometer-sized water pools contained inside the dppc liposomes were performed with two batches at 4.6 μmol dm^{-3} and 18.8 μmol dm^{-3}, respectively. The actual concentrations employed were 0.256, 1.28 and 2.56 μmol dm^{-3} (first batch) and 3.07, 6.40 and 12.8 μmol dm^{-3} (second batch). In this rather extended concentration range, no biological effect was observed.

Biological Activity of cis-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ *Toward Ehrlich's Ascitic Tumour Cells.*—Complex **2** was tested at 20 μg ml^{-1}. No appreciable decrease of thymidine incorporation into the DNA of the platinum(II)-exposed cells was observed, thus ruling out any significant genotoxic activity of **2** under the concentration conditions typically employed for this kind of test.

Discussion

In spite of (*i*) the evident close similarity of the metal solution state of the Pt^{II}–PMe_3–H_2O and Pt^{II}–NH_3–H_2O systems, (*ii*) the water solubility of complex **2** (the predominant species at *ca.* pH 7 in the Pt^{II}–PMe_3–H_2O system), (*iii*) the well documented[3] reactivity of **2** with pyrimidine nucleosides in water and (*iv*) the unambiguous marked reactivity of **2** with both single- and double-strand purified DNA, *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ proves to be completely inactive towards two established platinum(II)-sensitive cell lines.

Arguments dealing with lower lipophilicity of complex **2** appear to be ruled out by the results of our experiments with liposome-carried aqueous solutions of the investigated toxin. In fact, liposomes have been recently shown to be a very effective means for delivering very hydrophilic toxins[10b] and they are well known to improve drug bioavailability.[10a] On the other hand, it is known that the lipophilic complexes *cis*-$[Pt(dppe)Cl_2]$[11] [dppe = 1,2-bis(diphenylphosphino)ethane] and $[PtLCl_2]$(L = 1,1'-bis(diphenylphosphino)ferrocene] exhibit a very low biological activity towards induced P388 leukemia in mice[11] and Eagles KB cell line.[12] Arguments related to unsuitable electronic and/or geometric features of complex **2** appear to be contradicted by (*i*) and (*iii*). A hypothesis that the bulkier *cis*-$\{Pt(PMe_3)_2\}$ moiety is unable to react with the nucleobases when these ligands are constituents of the DNA is apparently belied by (*iv*).

It appears therefore necessary to hypothesize that, in the course of the concentration-gradient-driven motion of *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$ from the periphery of the cell to the nucleus, the metal complex has to undergo a specific and effective scavenging effect by cytosolic ligands or enzymes. This hypothesis may be supported by a possibly peculiar reactivity of the moiety $\{Pt(PMe_3)_2\}$ with cytosolic components (apparently unreactive with the $\{Pt(NH_3)_2\}$ one!) and/or by considering the evident xenobiotical nature of the trimethylphosphino part of the platinum(II) co-ordination sphere, which might be able to trigger a yet undescribed enzymatic defence in the target cells.

Experimental

Complex **1** was prepared according to ref. 13 and purified by dissolution in CH_2Cl_2–1,2-dichloroethane (1:1) followed by treatment with activated carbon. Complex **2**, *cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2][NO_3]_2$, was prepared as in ref. 3(*a*). The resulting colourless solution was concentrated under vacuum to give a microcrystalline white product which was further washed with water and ethanol.

Double-stranded DNA from calf thymus was purchased from Sigma (St. Louis, USA) and purified as reported in ref. 14. Its molecular weight (light scattering) was about 1.8×10^6. Single-stranded DNA from calf lung was a generous gift from CRINOS (Como, Italy). Its double-stranded DNA content was lower than 2%. The molecular weight was between 24 000 and 32 000.

^3H-Labelled thymidine (specific activity 4.77 TBq mM^{-1}) was obtained from Amersham International (UK). Radioactivity measurements were carried out with a toluene-based scintillation fluid [2,5-diphenyloxazole (5 g), 2,2'-(1,4-phenylene)bis-(4-methyl-5-phenyloxazole) (0.25 g) and toluene up to 1l solution]; all determinations were performed with a Packard A 300 CD liquid-scintillation spectrometer.

Preparation of the L-α-*Dipalmitoylphosphatidylcholine* (*dppc*) *Liposomes Containing cis*-$[\{Pt(PMe_3)_2(\mu\text{-}OH)\}_2]^{2+}$.—The compound dppc (Sigma, 0.021 g) was dissolved in absolute ethanol (2 cm^3) at 30–40 °C and complex **2** (1.55 g, 1.82 mmol) dissolved in water (30 cm^3) at room temperature. This latter solution was placed in a 50 cm^3 beaker and thermostatted at 53 ± 2 °C under moderate stirring. The alcoholic solution of dppc was steadily injected in the aqueous solutions, through a 0.15 mm syringe needle, by means of a Gilson Minipuls 2 peristatic pump at a constant 0.1 cm^3 min^{-1} flow rate.

The formation of monocompartmental-monolayer[10] liposomes was revealed by a slight turbidity and by a just appreciable darkening of the so-obtained suspensions.

After cooling down to room temperature, under stirring, the suspension was stored overnight at *ca.* 4 °C and then transferred into a dialysis tube (Visking; internal diameter = 1 cm, cut-off *M* 8000–15 000). The tube was immersed into a 200 cm^3 cylindrical vessel (internal diameter = 4 cm) and dynamic dialytic conditions were ensured with an external turnover flow equal to 280 cm^3 h^{-1}. After 4 d, the suspension was sterilized upon filtration with a Gelman Sciences Sterile Acrodisk (0.2 μm) and stored at 5 °C.

Platinum elemental analyses were carried out on 200 μl aliquots, after transfer into a quartz test tube and mineralization with concentrated nitric acid (Prolabo RP, 1 cm^3) at 95 °C for 1 h. After cooling down to room temperature, the solution was diluted to 100 cm^3. Metal analysis was performed with a Perkin Elmer 3030 spectrometer equipped with a graphite furnace HGA 500.

In Vitro *Evaluation of Cytostatic Activity* (*Eagle's Test*).—The protocol was identical to that described elsewhere.[12]

In Vitro *Evaluation of Cytostatic Activity* (*Ehrlich Cells Test*).—Ehrlich ascites tumour (Lettre' strain from Heidelberg) was routinely transferred by injecting intraperitoneally 2 × 10^6 cells per animal into non-cancer line mice. For the experiments the tumour cells were processed as described in ref. 15, 6–7 d after the transplant. A cell suspension (0.1 cm^3, 2 × 10^7 cells cm^{-3}) in Hank's medium was incubated at 37 °C for 30 min. After the addition of ^{3}H-labelled thymidine (0.4 cm^3) dissolved in the same medium (40 MBq cm^{-3}) the resulting suspension was further incubated at 37 °C for 30 min. The acid-insoluble fraction was subsequently precipitated upon addition of 5% ice-cold trichloroacetic acid and filtered on Whatman GF/C filters (2.5 cm diameter). After several washings with cold 1% trichloroacetic acid, the filters were dried and counted.

The data were expressed as a percentage of the radioactivity incorporated in the DNA of the treated cells with respect to that embodied in the DNA of the control cells (*ca.* 3–6 KBq). Filtration of the biological samples was performed with a sample manifold apparatus (Millipore Corporation, Bedford, USA).

Phosphorus-31 *NMR Experiments*.—The ^{31}P-{^{1}H} NMR spectra were obtained with a JEOL FX90Q spectrometer operating at 36.23 MHz by using a 10 kHz spectral width over 16 K data points with a 8 μs pulse. The delay time between pulses was usually 1 s. Chemical shifts are given on the δ scale and are referenced to external H_3PO_4 (85% w/w).

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