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^1H AND ^{13}C NMR STUDY OF PHOSPHOPEPTIDES

I—ACETYLPHOSPHOSERINE AND ACETYLPHOSPHOTHREONINE

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Abstract—The ^1H and ^{13}C NMR spectra of acetylserine, acetylphosphoserine and acetylphosphothreonine have been measured and completely analysed as a function of pD. For pD > 4 the NMR parameters are only slightly pD dependent. The second titration step of the phosphate group ($pK_2 = 6.5$) is much more difficult to detect in AcPSer (AcPTher) than in PSer (PThr). The ionization of the phosphate is of minor direct influence on the NMR parameters, their comparatively much larger changes in PSer (PThr) are due to conformational changes caused by the electrostatic interaction between the amino and phosphate group. The conformation in which the atoms $H\alpha$–$C\alpha$–$C\beta$–O–P form a planar W-type arrangement predominates at all pD values in the studied region from 4 to 14 in contrast to the non-cetylated phosphoamino acids in which its percentage passes a minimum at pD 8.

In preceding articles we have reported H and ^{13}C NMR results for phosphoserine and phosphothreonine[1–3] the biological importance of which has recently been discussed.[4–10] In this paper we present an NMR study of acetylphosphoserine and acetylphosphothreonine (AcPSer and AcPThr) which first are simple model compounds for phosphopeptides and, secondly, constitute one of the terminal units in Histones H2A and H4 (AcPSer). Acetylserine (AcSer) has been investigated in order to study the changes in NMR parameters upon phosphorylation. The measurements are conducted with particular attention to the physiological pD region (4–8) where appreciable amounts of both the mono- and dianion of the phospho-group are present and where Ser-phosphorylated proteins are reported to exhibit pronounced biological activity.[11,12] Our results will aid the assignment of NMR lines in more complex phosphopeptides and may eventually be used to determine the position of the phospho-protein bond.[13,14]

EXPERIMENTAL

N-acetyl-DL-serine was obtained from Sigma Chemical Company. N-acetyl-O-phospho-L-serine and N-acetyl-O-phospho-DL-threonine were synthetized from commercial O-phospho-L-serine and O-phospho-DL-threonine following the acetylation method of Refs 15, 16. Solutions were 1 M for AcSer, 0.2 M for AcPSer, and 0.15 M for AcPThr in D_2O. Conc. DCl and NaOD were added to achieve different pD values. ^{13}C and ^1H NMR spectra were recorded as in Ref. 1 and Refs 2, 3. 100, 1600 and 3000 scans (AcSer, AcPSer and AcPThr) were accumulated using 8K data points. One spectrum (32 K scans for AcSer at pD = 4) was taken without proton noise decoupling in order to check the assignment of the ^{13}C resonances. ^{13}C NMR spectra of AcPSer in extreme basic solutions were not measured since at the elevated temps caused by the decoupling rf field, considerable sample degradation occured during the long accumulation time. AcPThr appeared to be more resistent to basic decomposition. Although it seems very improbable, sample restructuration cannot completely be excluded to that the respective NMR parameters are to be used with care. Theoretical spectra were calculated and plotted as in Ref. 2, the full half-width being 0.8 Hz for AcSer and 1.2 Hz for AcPSer.

RESULTS

The ^1H spectra of AcSer and AcPSer can be analysed according to an AMN and AMNX type spectra ($H\alpha H\beta_1 H\beta_2$ and $H\alpha H\beta_1 H\beta_2 P$ respectively). The resultant spectral parameters are collected in Tables 1 and 2. Assignments are based on previous considerations.[2] Theoretical and experimental lines deviate by no more than 0.1 for AcSer and 0.2 Hz for AcPSer. As a typical example Fig. 1 shows the experimental and calculated ^1H spectra of AcSer and AcPSer at pD = 10. At pD = 0 only approximate values have been obtained for AcPser due to a low signal/noise ratio and the overlap of the $H\alpha$ signal with the strong HDO solvent peak. The NMR parameters of AcPThr follow directly from a first order analysis (see Ref. 3). In acidic solutions the $H\beta$ resonance lines overlap with the HDO solvent peak so that only the approximate value for $\delta(H\beta)$ and $J(\beta P)$ can be given. The larger uncertainty for $J(\alpha\beta)$ at pD = 4 in comparison to higher pD is due to line broadening caused by unresolved long-range coupling of $H\alpha$ to $H\gamma$ and P which increases in acidic solutions. The respective ^{13}C NMR parameters are also collected in Tables 1–3. The undecoupled AcSer spectrum at pd = 4 was used to confirm the assignment. Only approximate values for the small $J(PC\gamma)$ in AcPThr are given in Table 3 which is due to the experimental line-width and the rather low signal/noise ratio attainable in the given measuring time.

DISCUSSION

Only one signal is observed for the various ^1H and ^{13}C nuclei so that, based on both NMR and IR studies on small model peptides,[17,18] we assume that the presented NMR parameters apply for the *trans* isomer of the *cis–trans* isomerism about the peptide bond. As other

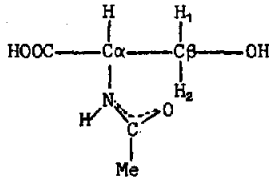
Table 1. Chemical shifts and coupling constants of AcSer

pD	^{1}H n.m.r. (270 MHz) δ[a)] α	β1	β2	Me(Ac)	J[b)] β1β2	αβ1	αβ2
4.0	4.314	3.862	3.841	2.061	-11.5	3.4	6.4
7.5	4.275	3.857	3.817	2.059	-11.5	3.8	6.2
10.0	4.277	3.860	3.821	2.058	-11.5	3.7	6.3

	^{13}C n.m.r. (22.63 MHz) δ[c)] C_o(Ac)	C_o	Cβ	Cα	Me(Ac)	J[d)] CαHα	CβHβ	CH(Ac)
4.0	179.02	176.85	65.04	59.82	25.07	141.2	144.0	128.7
7.5	179.45	176.80	65.17	60.17	25.12			
10.2	179.42	176.78	65.17	60.15	25.10			

a) Given in ppm with reference to internal DSS, ±0.004 ppm relative to DSS.
b) in Hz, ±0.08 Hz.
c) Given in ppm withreference to ext. TMS, ±0.044 ppm.
d) in Hz, ±1.5 Hz.

AcSer :



amino-acids,[13] acetylated Ser and Thr are not greatly affected by pD changes and it is noteworthy that the introduction of the phosphate group does not remove this insensitivity to pD changes.

Chemical shifts. In the studied pD region from 4 to 14, ^{1}H and ^{13}C titration shifts are to be expected only from the phosphate second deprotonation step (pK_2). Chemical shifts in AcPSer, however, do not show a sigmoidal dependence on pD, contrary to PSer ($pK_2 = 6.5$).[4,5] From the data in Table 2 one can estimate that the upper limit of the pK_2 titration shifts for Hα, $H\beta_1$, $H\beta_2$ and Cβ are respectively 0.04, 0.05, 0.06 and −0.2 ppm. This little influence of the phosphate ionization state is in accordance with earlier findings on sp^3 hybridized carbons (or attached protons) in peptide fragments.[19]

The comparatively large shift changes in the pD region at 4–5 for AcSer and AcPSer are presumably caused by the interaction of protons with the peptide bond. This seems to be a particular feature of these dipeptide models since the chemical shifts of the respective nuclei of the peptide Ser residue in the tripeptide $(Ser)_3$ are almost pD independent.[21]

Coupling constants and rotational isomerism. The measured coupling constants are for the greater part almost pD independent, changes are most pronounced on going from pD 4 to 5. This again indicates that the charge of the phosphate group has minor influence upon the NMR parameters. In the following we shall use the measured vicinal coupling constant ^3J to calculate the fractional populations p_i of the three rotamers for both the Cα–Cβ and Cβ–O bonds. Although ^3J change little with pD, we have chosen to evaluate ^3J at pD = 7 and 13(14) since some of the ^3J's appear to be notably different.

Cα–Cβ Bond. The observed $^3J(\alpha\beta 1,2)$ are the weighted average of the various *trans* and *gauche* coupling constants $^3J_i^{t,g}$ (i = rotamer index, see Fig. 3):

$$p_a J_a^t + p_b J_b^g + p_c J_c'^g = J(\alpha\beta 2)\text{obs.}$$

$$p_a J_a^g + p_b J_b^t + p_c J_c^g = J(\alpha\beta 1)\text{obs.}$$

$$p_a + p_b + p_c = 1.$$

In the Pachler approach[22] (*cf.* also recently Refs. 23, 24) all J_i^g are assumed to be equal ($J^g = 2.6$ Hz, $J^t = 13.6$ Hz). Recently Abraham *et al.*[25] proposed a scheme to calculate J_i^g from increments which have been tabulated for the various substitutions of the Cα–Cβ bond. It yields for AcSer and AcPSer:

$^3J(\alpha\beta 1)$: $J_a^g = 3.7$ $J_b^t = 11.2$ $J_c'^g = 2.1$ Hz

$^3J(\alpha\beta 2)$: $J_a^t = 11.2$ $J_b^g = 4.8$ $J_c^g = 1.0$ Hz.

We have used both methods and the resulting p_i are collected in Table 4, including the interpolated values of $^3J(\alpha\beta 1,2)$ at given pD.

The populations p_i differ for the two methods but the qualitative behaviour is similar. For instance, in the whole pD range, p_b is small (about 5%) and p_c is greater than p_a. The projection formula for rotamer b is suggestive for a maximal Coulomb repulsion between the negatively charged phosphate and carboxyl group. From the molecular model it can, however, be seen that due to the rotation around the C–O and O–P bonds this repulsion is about the same for rotamers a and c. Furthermore, the model suggests a maximal Coulomb energy for a dihedral angle Hα–Hβ1(Hα–Hβ2) of approximately 30° (90°) which corresponds to $p_c \approx 0.75$ and $p_a \approx 0.25$ if one neglects p_b. This compares quite well with the experimental values (Table 4). When the charged phosphate group in AcPSer is removed, p_c decrease by about

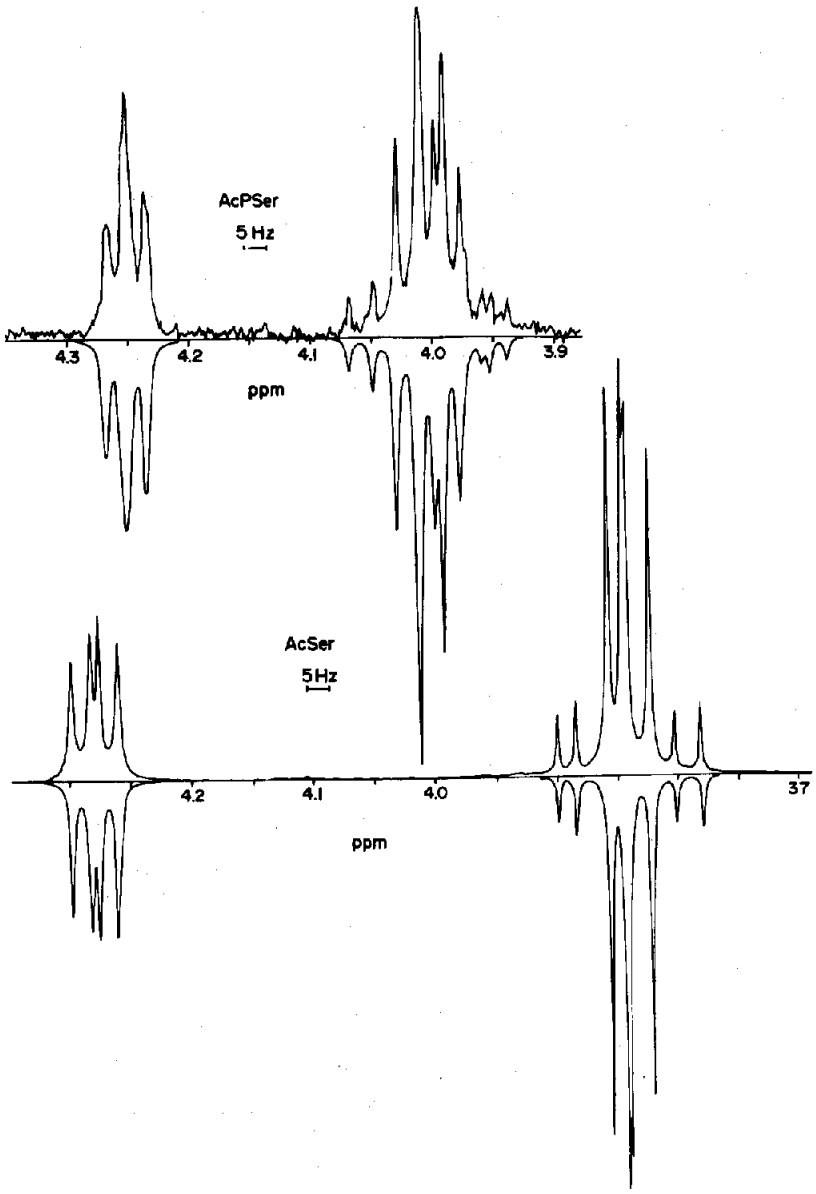


Fig. 1. Experimental and calculated 270 MHz ^{1}H NMR spectra of AcPSer and AcSer in D_2O at pD 10.

20% whereas p_a and p_b increase, which indicates that not only the phosphate group is responsible for the dominance of the rotamer c.

Cβ–O Bond. The evaluation of ^{3}J (PH, PC) relies upon the assumption made by several authors (*cf.* Refs. in Table 5) that ^{3}J follows a Karplus-like relationship of the form

$$^3J(\text{PH or PC}) = J_A \cdot \cos^2\theta - J_B \cdot \cos\theta$$

where θ is the dihedral angle for P–O–C–H and P–O–C–C, respectively. The proposed values for $J_{A,B}$ and the calculated $^3J^{4-6}$ are collected in Table 5. As in our previous work[4-6] we have used the set 2 in Table 5. In AcPSer as well as AcPThr three values for ^{3}J are available thus two independent evaluations (one of which gives the sum $p_i + p_k$) are possible for p_i (i, k = a', b', c'). The resulting populations are given in Table 4. The two sets agree reasonably well. Furthermore, the populations are not sensitive to temperature since ^{3}J(PH) and ^{3}J(PC) have been measured at different temperature (28 and 35°, respectively). The rotamer populations of the Cα–Cβ bond in serine behave similarly. The spectra of the anion and zwitterion were found[26] to change only slightly on raising the temperature from 28 to 74°. The spectra of the cation showed a more pronounced temperature dependence in the range of 28–65° but changes are less than 10% on going from 28 to 37°. In leucine,[24] Cα–Cβ rotamer populations at 45° differ by less than 5% from those at 25°.

The percentage of the most favoured rotamer c' stays constant for pD = 5 whereas in PSer[1,2] and PThr[3] p_c, passes a distinct minimum in the intermediary pD region (amino group with charge +1). Here again we encounter the difference in electrostatic interaction between acetylated and non-acetylated phosphoamino acid

Table 2. Chemical shifts and coupling constants of AcPSer

	^{1}H n.m.r. (270 MHz)									
	δ[a]				J[b]					
pD	α	β2	β1	Me(Ac)	β1β2	αβ1	αβ2	Pβ1	Pβ2	Pα
4.4	4.375	4.156	4.090	2.077	-10.9	3.2	4.9	5.2	5.4	1.2
5.0	4.328	4.108	4.053	2.070	-10.6	2.7	5.0	5.2	5.2	0.9
6.3	4.272	4.045	4.004	2.066	-10.5	2.9	5.2	5.4	5.2	0.8
7.5	4.250	4.016	3.978	2.062	-10.5	3.1	5.4	5.8	5.4	0.7
8.7	4.248	4.015	3.962	2.061	-10.5	3.1	5.4	5.8	5.4	0.7
10.3	4.251	4.022	3.984	2.062	-10.5	3.3	5.6	5.6	5.2	0.8
14	4.250	4.020	3.982	2.061	-10.5	3.3	5.6	5.6	5.2	0.8

	^{13}C n.m.r. (22.63 MHz)						
	δ[c]					J[d]	
	C_0(Ac)	C_0	Cβ	Cα	Me(Ac)	PCα	PCβ
4.1	179.24	177.14	68.76	58.80	25.50	8.2	4.8
5.1	179.17	176.71	68.03	58.67	25.16	7.4	4.8
6.1	179.90	176.94	67.62	59.35	25.32	7.2	4.7
7.5	180.02	176.91	67.37	59.39	25.34	7.2	4.6
8.7	179.89	176.76	67.26	59.33	25.24	7.2	4.7
10.4	180.31	177.22	67.67	59.77	25.58	7.4	4.7

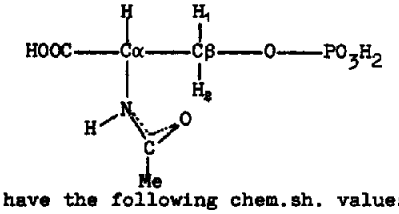
a) Given in ppm with reference to int.DSS, ±0.007 ppm relative to DSS

b) in Hz, ±0.15 Hz.

c) Given in ppm with reference to ext.TMS, ±0.09 ppm.

d) in Hz, ±0.2 Hz.

AcPSer :



e) For pD ≅ 0 we have the following chem.sh. values

δ Hα≅4.652 δ Hβ2≅4.288, δ Hβ1≅4.144, δ Me(Ac)=2.056.

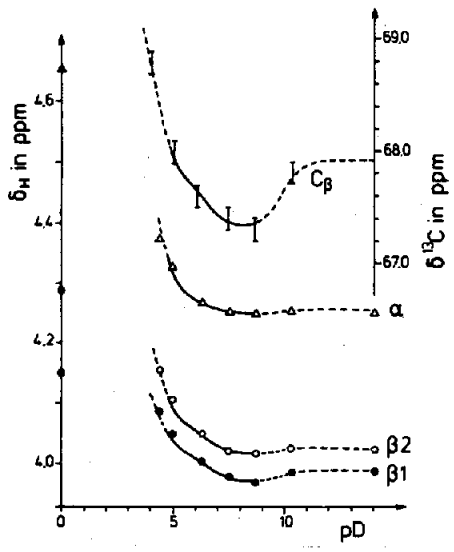


Fig. 2. pD dependence of Hα, Hβ2, Hβ1 and ^{13}Cβ chemical shifts of AcPSer in D_2O solution. Full lines indicate the intermediary pD region with the second deprotonation step of the phosphate group -O-PO_3H_2 at pD ≈ 6.5.

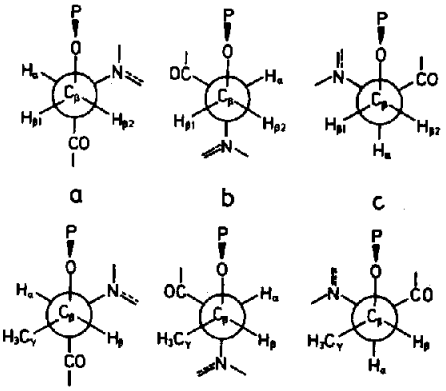


Fig. 3. Cα–Cβ rotamer notation for AcPSer (top) and AcPThr P has to be replaced by H in AcPSer to obtain AcSer.

which has already been discussed in the preceding paragraph.

Hα–Cα–Cβ–O–P *Fragment.* It has been reported[27] that the long-range coupling constant ^{4}J (PH) provides a measure for the preferential total conformation such that for ^{4}J = 1 Hz the four bonds have a near planar W-type

Table 3. Chemical shifts and coupling constants of AcPThr

PD	^{1}H n.m.r. (270 MHz) δ[a)] β	α	γ	Me(Ac)	J[b)] αβ	Pβ	βγ	Pα
4.0	4.690$^+$	4.244	1.307	2.117	5.0$^+$	6.5$^+$	6.4	1.0
7.9	4.404	3.971	1.270	2.073	5.4	6.5	6.3	0.7
14	4.425	3.991	1.293	2.098	5.4	6.5	6.3	0.6

	^{13}C n.m.r. (22.63 MHz) δ[c)] C_o(Ac)	C_o	Cβ	Cα	C_γ	Me(Ac)	J[d)] PCα	PCγ$^+$	PCβ
4.0	179.9	177.7	76.3	63.3	22.2	24.7	7.0	1.	5.1
7.5	181.1	177.5	74.2	65.4	22.3	24.6	6.1	2.	5.0
14	181.3	177.6	74.3	65.6	22.7	24.7	6.0	2.	4.9

a) Given in ppm with reference to DSS, ±0.007 ppm.
b) in Hz, ±0.2 Hz.
c) Given in ppm with reference to ext. TMS, ±0.10 ppm.
d) in Hz, ±0.2 Hz
+) Approximated values, see text.

AcPThr :

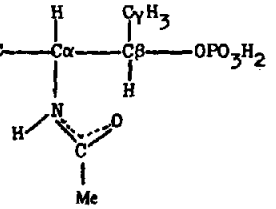


Table 4. Measured vicinal coupling constants and fractional populations p_i for the Cα–Cβ and Cβ–O bond rotamers

pD	AcPSer $^3J(\alpha\beta 1)$	$^3J(\alpha\beta 2)$	p_a	p_b	p_c	$^3J(P\beta 1)$	$^3J(P\beta 2)$	p_a'	p_b'	p_c'	$^3J(PC\alpha)$	p_c'	$p_a'+p_b'$
4	3.3	4.9	.21 (.36)	.06 (.07)	.73 (.57)	5.1	5.5	.12	.10	.78	8.4	.82	.18
8	3.1	5.4	.25 (.42)	.05 (.04)	.70 (.54)	5.8	5.4	.11	.13	.76	7.2	.69	.31
13	3.3	5.6	.27 (.43)	.06 (.06)	.67 (.51)	5.6	5.2	.11	.12	.77	7.5	.72	.28
	AcSer												
7.5	3.8	6.2	.33 (.47)	.11 (.10)	.56 (.43)								
	AcPThr												
	$^3J(\alpha\beta)$		p_a	p_b+p_c		$^3J(P\beta)$		p_a'	$p_b'+p_c'$		$^3J(PC\alpha)$	$^3J(PC\gamma)$	p_a' p_b' p_c'
4	5.0		.22 (.38)	.78 (.62)		6.5		.16	.84		7.0	1.	.3 .0 .7
7.	5.4		.25 (.42)	.75 (.58)		6.5		.16	.84		6.1	2.	.3 .1 .6
14	5.4		.25 (.43)	.75 (.57)		6.5		.16	.84		6.0	2.	.3 .1 .6

Table 5. Phosphorus vicinal coupling constants[a]

	J(PH)/Hz					J(PC)/Hz				
	J_A	J_B	J^t	J^g	Refs.	J_A	J_B	J^t	J^g	Refs.
1)	16.3	4.6	20.9	1.8	(26)	8	0	8	2	(29)
2)	21.9	6	27.9	2.5	(30)	8	2	10	1	(31)
3)	20.7	7.3	28	1.5	(32)	9.5	0.6	10.1	2.1	(33)
4)	17.3	6.7	24	1	(34)					
5)	18.1	4.8	22.9	2.1	(35)					

a) $^3J^{t,g} = J_A\cos^2\theta^{t,g} - J_B\cdot\cos\theta^{t,g}$

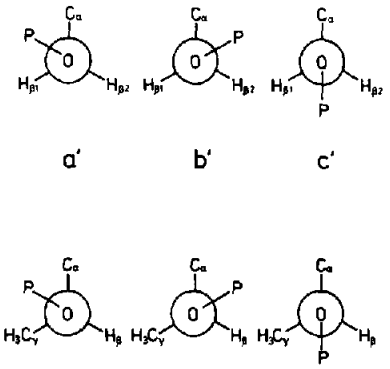


Fig. 4. Cβ–O rotamer notation for AcPSer (top) and AcPThr.

arrangement. If we accept that for the planar "W" conformation ^4J is equal to 1.7 Hz,[28] the "W"-rotamer c–c' contributes with $1.7 \cdot p_c \cdot p_{c'}$ Hz to the observed average value of ^4J(PH). For pD 4, 8, 13 we obtain 1.0 (0.8), 0.9 (0.7) and 0.9 (0.7) Hz (values in parenthesis correspond to p_i in parenthesis, Table 4) as compared with the experimental values 1.2, 0.7 and 0.8 Hz. Accordingly, the contributions from the remaining eight rotamers i–k' (i,k = a, b, c except c–c') are relatively small as is the case when ^4J(PH) gets small for non-planar conformations. The various data sources for ^3J and ^4J thus seem to result a rather consistent picture for the conformations in the acetylated phosphoamino acids.

CONCLUSIONS

The NMR study of acetylated phosphoamino acids provides a simple and fully analyzable model system for the investigations of structure and function of phosphorylated sidechain residues in phospho-peptides and phospho-proteins. ^1H and ^{13}C spectra are only slightly pD dependent. The biologically important second titration of the phosphate group is difficult to detect by H and ^{13}C NMR. The preferred conformation of the phospho-sidechain unit is the c–c' one in which Hα–Cα–Cβ–O–P form a planar W-type arrangement. The rotamer c concentration is increased upon the phosphorylation of the acetylated amino acid.

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