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Heteronuclear NMR of DNA with the heteronucleus in natural abundance: facilitated assignment and extraction of coupling constants

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ABSTRACT

Two heteronuclear proton-carbon NMR experiments are applied to the DNA-octamer d(TTGGCCAA)$_2$ with carbon in natural abundance. They lead to a complete assignment of the carbon resonances of the sugars and bases. In addition, several heteronuclear coupling constants, proton-carbon as well as proton-phosphorous and phosphorous-carbon, were determined. The information can be obtained in a resonable measuring time and offers valuable information for a detailed picture of DNA structure.

INTRODUCTION

Heteronuclear experiments can provide useful information during a structure determination by NMR (1-5). The higher dispersion of the resonances and the smaller linewidth of the heteronucleus compared to protons facilitate the spectral assignment considerably. In addition, heteronuclear coupling constants offer a wealth of structural information. They can be used for stereospecific assignment of diastereotopic groups (6) and can be related to the dihedral angles via a Karplus equation (7,8) in a similar manner as homonuclear coupling constants (9,10). Thus they provide information on these angles and can help to remove ambiguities left from the exclusive use of homonuclear coupling constants.

Heteronuclear NMR experiments have been applied extensively to proteins, were a complete set of new heteronuclear techniques for spectral assignment have been developed in recent years (11–14) and nearly all homonuclear and heteronuclear coupling constants can be determined (15–20). In the case of proteins, this has been facilitated by the fact that complete enrichment with carbon-13 and/or nitrogen-15 is relatively easy and inexpensive (21,22). Also in the case of RNA examples of the application of heteronuclear techniques to completely labeled material have been given (23,24). However, an enrichment of DNA or RNA is usually too tedious and expensive to be generally applicable and samples with the heteronucleus in natural abundance have to be used. The problem of sensitivity connected with the low natural abundance of ^{13}C and ^{15}N as the heteronucleus can be overcome with the application of techniques with proton detection (25–27), as has recently been demonstrated with DNA and RNA samples by several groups (28–36). However, a complete assignment, especially of the sugar resonances, has only been possible in a few select cases and the only coupling constants that have been extracted quantitatively have been one-bond couplings.

Here, we demonstrate the application of two heteronuclear techniques with carbon-13 as the heteronucleus to a sample of the DNA-octamer d(TTGGCCAA)$_2$ leading to a complete assignment of the carbon resonances and an extraction of proton-carbon, proton-phosphorous and carbon-phosphorous coupling constants. The chemical shifts and proton-carbon coupling constants can provide information on the sugar pucker (37,38) and the structure of the bases. In addition, all coupling constants provide information on several dihedral angles in DNA (39). Of especial importance is the determination of the carbon-phosphorous coupling constant, which can only be determined with this accuracy using the methods described here.

MATERIALS AND METHODS

The DNA-octamer d(TTGGCCAA)$_2$ was synthesized via a solid phase approach of an improved phosphotriester method (40,41). After purification the compound was treated with a DOWEX cation-exchange resin (Na^+) to yield the sodium salt.

The sample had a single strand concentration of 6 mM (0.02 mM EDTA was added to neutralize paramagnetic contaminations, the pH was adjusted to 6.9, 5 mM phosphate buffer,) and was dissolved after three lyophilization steps in 0.325 ml D_2O (99.95%, Aldrich), Tetramethylammoniumchloride (TMA) was added as an internal standard. The sample was degassed and sealed.

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All 2D-spectra have been recorded on a Bruker AMX-600 at 298 K using an inverse probe. The sample was not spun. The ^{13}C-1D-spectrum was recorded with continuous proton decoupling on a Bruker AMX-500 at 298 K using a broadband probe. TSP (3-trimethylsilyl-1-propionate) was used as an external standard. The spectra were processed on a Bruker X32 data station, coupling constants were extracted by means of 2D curve-fitting (HSQC) or curve-fitting of 1D-slices (HMBC) with the NMRi program, running on a Silicon Graphics IRIS 4D25G workstation. The proton assignment was carried out utilizing standard procedures (42). The pulse sequences are shown in Figure 1.

The HMQC (25–27) was recorded with 1024 experiments of 2048 points and 36 scans each. The carrier was positioned at 1.39 ppm for protons and 85.09 ppm for carbon. The spectral width was 5555 Hz (9.3 ppm) in the proton dimension and 25000 Hz (165.7 ppm) in the carbon dimension. Heteronuclear decoupling was acheived with the GARP-1 sequence (42). The total measuring time was 13 hours.

The HSQC ('Overbodenhausen') (44,45) was recorded with 1890 experiments of 2048 points and 32 scans each. The GARP-1 sequence (42) was used for heteronuclear decoupling. The carrier was positioned at 1.39 ppm for protons and 84.66 ppm for carbon. The spectral width was 5555 Hz (9.3 ppm) in the proton-dimension and 3012 Hz (19.9 ppm) in the carbon dimension leading to extensive folding. The first-order phase-correction parameter was set to 180° by proper adjustment of the length of the first value for the evolution time as described elsewhere (46,47). The total measuring time was 25 hours.

The HMBC (48) was recorded with 1006 experiments of 4096 points and 144 scans each. Presaturation of the HDO signal was applied during the relaxation time to optimize the digitization. The carrier was positioned at 1.60 ppm for protons and 90.12 ppm for carbon. The spectral width was 5555 Hz (9.3 ppm) in the proton dimension and 26316 Hz (174.4 ppm) in the carbon dimension. The total measuring times was 60 hours.

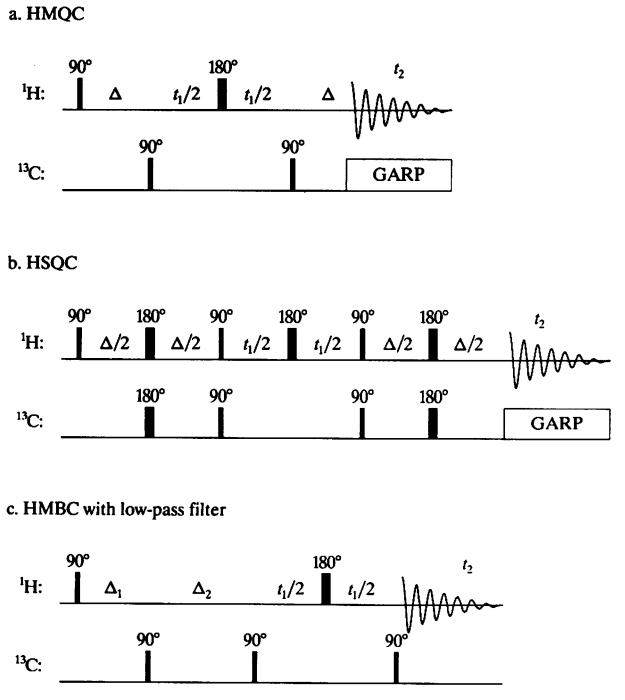

Figure 1. Pulse sequences of the experiments used in this study. (a) HMQC, the delay Δ is set to $(2^1J_{CH})^{-1}$, (b) HSQC, the delay Δ is set to $(2^1J_{CH})^{-1}$, (c) HMBC with low-pass-filter, Δ_1 is set to $(2^1J_{CH})^{-1}$, Δ_2 is set to 50 msec.

RESULTS AND DISCUSSION

Assignments

Figure 2 shows a comparison between a conventional HMQC and the HSQC of d(TTGGCCAA)$_2$. Only the region containing the sugar resonances is shown. An increased separation of the signals compared to homonuclear spectra is already visible in the HMQC (Figure. 2(a)) and with the proton assignment available, the assignment of the C1′, C3′ and C4′ (and of A-C2/8, G-C8, C-C5/6, T-C6 and methyl groups of T (not shown)) is straightforward. Ambiguities in the assignments from homonuclear spectra can be resolved at this point. The complete assignment of the C2′ and C5′ carbons, however, is usually not possible because of spectral overlap, even if the proton assignment is known. Both are methylene groups, exhibiting two signals and distortions from homonuclear couplings. Increasing the resolution by recording more experiments does not improve the situation, since the distortions due to homonuclear couplings get worse.

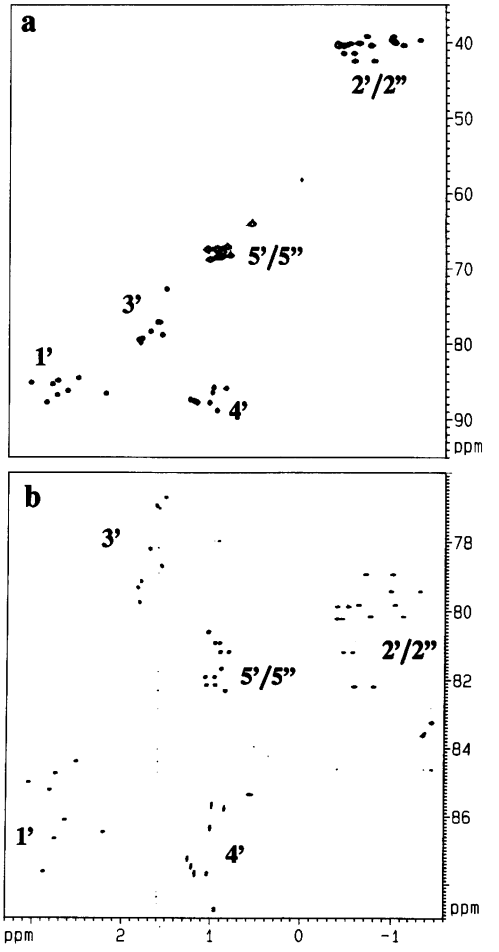

Figure 2. Comparison of an HMQC (a) and an HSQC (b) with extensive folding of d(TTCCGGAA)$_2$, illustrating the increased resolution in the HSQC. The signals of the 3′, 4′ and 5′ protons exhibit the typical 'E.COSY' patterns.

In the HSQC no homonuclear couplings are present during the evolution time and thus no distortions occur. To increase the resolution an extensive folding is advantageous. The optimal folding can be achieved from a low resolution HMQC, that can be obtained in a short period of time (e.g. 1 hour). All overlap still present in the HMQC is then completely resolved (Figure 2(b)). The digital resolution in the carbon-dimension is comparable to that of the 1D spectrum (1.5 Hz/point vs. 0.8 Hz/point), in fact some signals that are too close to be clearly separated in the 1D can be separated in the 2D because of a difference in the proton shifts. All sugar resonances can now be assigned based on the available proton assignment. In addition, there is still space for more signals in the HSQC, making the assignment of larger DNA-fragments feasible as well.

Since both spectra shown in Figure 2 rely on the use of the large proton-carbon one-bond coupling (usually between 130 and 220 Hz), signals from carbons not directly bound to a proton are not visible in either spectrum. Those quaternary carbons occur in the aromatic ring of the bases and can be assigned from the HMBC-experiment, which utilizes proton-carbon two-bond and three-bond coupling constants. Figure 3 shows the region of the correlations from H1′ and H5 to the carbons of the bases.

The resonances of C2 and C6 in Guanine, however, can not be assigned, since there is no proton-carbon coupling of sufficient size to yield a correlation in the HMBC. These resonances are found in the 1D spectrum but a sequence specific assignment is still not possible with the techniques used here. A list of the complete carbon assignment is given in Table I together with the proton assignment.

Table I(a). Protons chemical shifts of d(TTGGCCAA)$_2$ at 298 K. Reference is TMA (0.0 ppm) (Proton chemical shifts can be converted to the TSP scale by addition of 3.144 ppm to the proton chemical shifts given here).

	T(1)	T(2)	G(3)	G(4)	C(5)	C(6)	A(7)	A(8)
H1′	2.870	2.626	2.510	2.738	2.744	2.181	2.802	3.043
H2′	−1.037	−0.981	−0.456	−0.572	−1.149	−1.327	−0.516	−0.597
H2″	−0.635	−0.716	−0.402	−0.468	−0.769	−0.997	−0.398	−0.815
H3′	1.556	1.695	1.836	1.795	1.609	1.584	1.815	1.503
H4′	0.943	0.991	1.198	1.243	0.972	0.835	1.170	1.027
H5′			0.898		1.041		0.767	1.067
	0.558[a]	0.883[a]		1.026[a]		0.835[a]		
H5″			0.951		0.940		0.894	0.956
H2/H5	−1.459	−1.314	–	–	2.133	2.454	4.581	4.485
H6/H8	4.387	4.321	4.757	4.619	4.193	4.250	4.962	4.941

[a] isochronous H5′ and H5″ protons

Table I(b). Carbon chemical shifts of d(TTGGCCAA)$_2$ at 298 K. Reference is TSP (0.0 ppm).

	T(1)	T(2)	G(3)	G(4)	C(5)	C(6)	A(7)	A(8)
C1′	87.62	86.12	84.40	84.74	86.66	86.47	85.23	85.01
C2′	39.89	39.03	40.24	41.26	40.23	39.51	39.90	42.28
C3′	78.69	78.23	79.29	79.14	76.98	76.99	79.75	72.60
C4′	88.79	86.35	87.48	87.26	85.68	85.77	87.63	87.63
C5′	63.94	67.63	68.41	68.71	67.17	66.96	68.12	67.40
C2	153.88	153.71	a	a	158.93	159.09	154.99	154.98
C4	169.52	169.76	154.02	153.39	167.94	168.32	150.54	150.61
C5	114.06	113.80	117.64	117.65	98.56	98.96	120.05	120.05
C6	139.49	139.84	a	a	142.61	143.35	157.54	157.88
C8	–	–	138.54	137.57	–	–	141.89	141.88
CH_3	14.28	14.58	–	–	–	–	–	–

a) no peak in the HMQC or HMBC, no sequence specific assignments, the chemical shifts of the G−C6 are 161.97 and 161.37 ppm, of the G−C2 157.29 and 156.71 ppm, respectively.

Determination of coupling constants

A general problem with the determination of coupling constants from NMR spectra is that the apparent splitting of the signals is, even in first order spectra, not a true representation of the coupling constants but is also dependent on the linewidth. To obtain accurate information one has to apply linewidth-independent methods.

One method to achieve this goal is the creation of 'E.COSY'-type patterns (49). It has been extensively used in peptides and proteins for many different coupling constants (15-20). Here we show that the extraction of proton-phosphorous and carbon-phosphorous coupling-constants is straightforward from an HSQC with extensive folding. Since the phosphorous nucleus has a natural abundance of 100%, the coupling to both carbons and protons is always present. However, since no phosphorous pulses are applied, only connected transitions can appear in the spectrum, yielding the typical 'E.COSY'-patterns shown in Figure 4. This pattern is also present in the HMQC. However, due to the lower resolution and distortions from homonuclear couplings it is usually not visible.

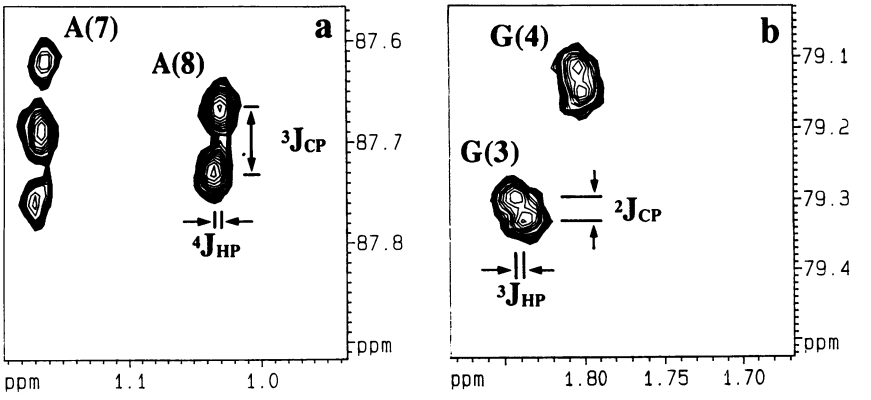

Figure 3. Region from the HMBC of d(TTCCGGAA)$_2$ containig the corelations from H1′ and C-H5 protons to the base carbons C2, C4, C6 and C8. Quaternary carbons can be sequence specifically assigned, 1D-slices from this spectrum can be used for a determination of coupling constants in a curve-fitting procedure.

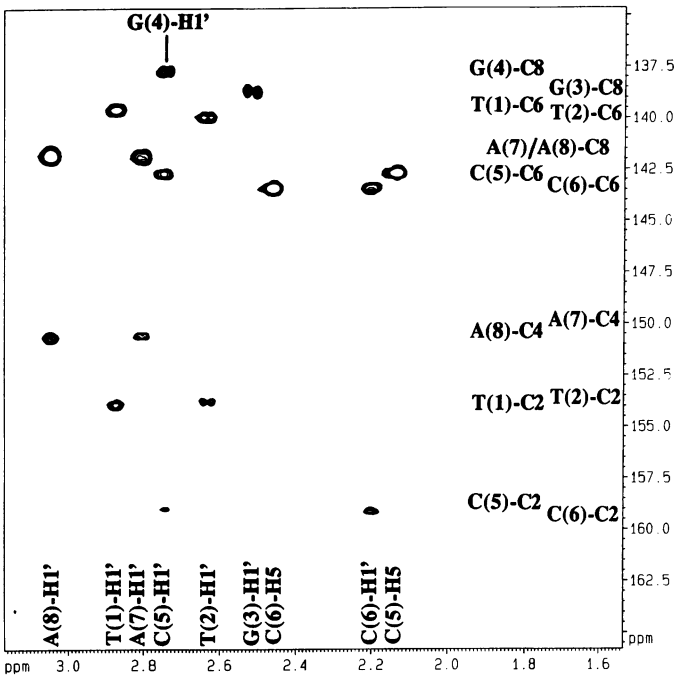

Figure 4. Regions from the HSQC with extensive folding. (a) region of the Adenosine H4′/C4″ correlation, (b) region of the Guanine H3′/C3″ correlation. The 'E.COSY' patterns are clearly visible, coupling constants can be measured as the displacement of multiplet components (see text for details) and the relative sign of the coupling constants can be determined from the tilt in the multiplets.

The splitting in the carbon dimension results from the carbon-phosphorous coupling, the splitting in the proton dimension from the proton-phosphorous coupling. The coupling constants can then be extracted from the displacement of the components of the multiplet. Since the coupling constants are small in both dimensions, a high digital resolution is necessary, that is provided conveniently from the HSQC with extensive folding. Figure 4(a) shows peaks correlating H4′ and C4′ of A(7) and A(8), the couplings constants creating the 'E.COSY'-pattern are $^3J_{CP}$ and $^4J_{HP}$. In the case of A(7) there are two phosphorous atoms as coupling partners, resulting in four multiplet components instead of two, whereas in A(8), lacking the 3′-terminus phosphate, only one phosphorous is present leading to a doublet. Figure 4(b) shows signals correlating H3′ and C3′ of G(3) and G(4), here the coupling constants are $^2J_{CP}$ and $^3J_{HP}$ to the phosphorous at the 3′ end. Furthermore, the relative sign of the coupling constants, information that can usually not be obtained by other methods is extracted conveniently from the tilt of the E.COSY-patterns. Another advantage compared to other methods for the extraction of proton-phosphorous coupling constants is the fact, that overlap of the H4′ and H5′/H5″ protons is not a problem here. The method for the determination of carbon-phosphorous coupling constants is, to the best of our knowledge, the most sensitive and accurate method presented so far.

Other methods for the elimination of the linewidth for the determination of coupling constants involve fitting routines to simulate peak patterns from the HMBC, yielding proton-carbon long-range coupling constants. If the passive, homonuclear coupling constants are known, curve-fitting is possible with the multiplets from the HMBC alone. Otherwise a more elaborate method has to be used, e.g. the method recently developed in the group of J. Keeler (50). In the case of the molecule studied here, the homonuclear proton coupling constants have been determined and curve fitting using these values yielded the desired heteronuclear coupling constants. Resonances in the region of the HMBC shown in Figure 3, exhibiting the signals from H1′ to C6/C8 and C2/C4 carbons, are suitable for that procedure. If the signal is too weak or spectral overlap prevents curve fitting, only a qualitative determination is possible. A list of the quantitatively determined coupling constants is given in Table II.

Here, only one example of the use of the heteronuclear coupling constants is shown: the coupling constants from the H1′ to the C8 and C4 carbons in purine bases and the C6 and C2 carbons in pyrimidine bases give direct evidence for the syn-anti equilibrium at the glycosidic bond. The coupling constants from the H1′ to the C6/C8 carbons have been determined (Table II) and are larger than those to the C2/C4 carbons, which could only be obtained qualitatively by inspection of the HMBC cross-peak intensity in Figure 3. This establishes the preferred anti-conformation. A more detailed discussion of the use of the coupling constants will be presented elsewhere in connection with a full description of the structure of the DNA-octamer.

Table II(a). Carbon-phosphorous and proton-phosphorous coupling constants of d(TTGGCCAA)$_2$, the accuracy is 0.3 Hz.

	T(1)	T(2)	G(3)	G(4)	C(5)	C(6)	A(7)	A(8)
C2′P	<2	<2	<2	<2	<2	<2	<2	b
C3′P	−5.7	−5.6	−5.1	−5.7	−5.9	a	−5.0	b
H3′P	5.6	4.4	4.4	2.7	4.0	a	3.3	b
C4′P	9.2	18.6[c]	18.5[c]	19.9[c]	21.7[c]	19.3[c]	20.4[c]	9.4[c]
H4′P	0.0[d]	2.8[e]	3.5[e]	4.0[e]	3.0[e]	3.0[e]	3.0[e]	2.7[e]
C5′P	b	−5.4	a	−5.6	−5.4	−5.3	−5.7	−5.4
H5′P	b		4.0		2.8		4.5	3.7
		4.0[f]		3.8[f]		2.7[f]		
H5″P	b		<2.5		<2.5		1.7	2.2

a) could not be determined
b) no phosphate at 5′ and 3′ terminus
c) Sum of coupling constants C4′(n)−P(n) and C4′(n)−P(n+1). Line width for the C4′−H4′ triplet crosspeak patterns suggest equal values for both coupling constants for residues G(4), C(5) and C(6), but unequal values (within 2−3 Hz) for residues T(2), G(3) and A(7)
d) H4′(n)−P(n+1) coupling constant
e) Sum of coupling constants H4′(n)−P(n) and H4′(n)−P(n+1), but the H4′(n)−P(n+1) coupling constant usually is around 0.0 Hz
f) isochronous H5′ and H5″ resonances; sum of both coupling constants

Table II(b). Proton-carbon coupling constants of d(TTGGCCAA)$_2$ that could be determined quantitatively. The accuracy is 0.5 Hz, the coupling constants involving methyl groups in Thymidine are: ^2J(Me-C5) = −6.7 and −6.6, ^2J(H6-C7) = −3.9 (both), ^3J(Me-C5) = 6.4 and 6.3, ^3J(Me-C4) = 2.9 and 3.0 for residues T(1) and T(2), respectively.

	T(1)	T(2)	G(3)	G(4)	C(5)	C(6)	A(7)	A(8)
H8-C4			5.3	4.7			5[a]	5[a]
H8-C5			10.8	10.9			9.8	9.4
H6-C2	7.9	8.1			6.4	6.5		
H6-C4	9.7	9.7			9.0	9.0		
H6-C5	−4.0	−3.9			−4.8	−4.4		
H5-C6					−4.4	−4.5		
H5-C4					−2.5	−2.6		
H2-C4							12.0	11.9
H2-C6							10.8	10.7
H6-C1′	3.6	3.7			3.4	3.6		
H1′-C6	5.1	5.1			4.4	5.1		
H1′-C8			5.5	5.8			5.0	4.9

a) accuracy 1 Hz

It should be noted that, though the sensitivity of the methods described here is rather high, their application might be difficult if the concentration of the sample decreases and the size of the molecules increases. This is especially true for the HMBC experiment. Here, the extraction of quantitative data will first become difficult, then even the extraction of qualitative data might fail. However, the HSQC experiment is certainly much more robust, since it relies only on direct proton-carbon coupling constants and will thus work also when the linewidth increases. As mentioned above overlap is not a problem in this experiment and it should be applicable with concentrations that are reasonable for NMR-measurements in an acceptable measuring time. The measurement of protein-DNA-complexes, however, will not be possible with the method described here, since the folding procedure can not be applied in that case.

In summary, we have demonstrated that a complete assignment and an extraction of several heteronuclear coupling constants is feasible from only two heteronuclear experiments recorded in a rather short period of time using a DNA-sample of 6 mM concentration. The assignment can help to utilize information from NOESY spectra and produce structural details of DNA. The coupling constants can be related to important dihedral angles in DNA molecules (39).

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