

Assignment of Overlapping ^1H NMR Signals in Carp Seminal Plasma by Proton-Detected 2D C,H Correlation Spectroscopy

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The ^1H NMR spectrum of the perchloric acid extract of carp seminal plasma was heavily congested. It is demonstrated that proton-detected C,H chemical shift correlation spectroscopy (HSQC, HSQC-TOCSY) allows an unequivocal identification of proline, glutamate, taurine, and methionine sulfoxide, although several key proton signals were strongly overlapped.

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The assignment of resonances in the ^1H NMR spectra of biological fluids is generally very difficult because of the presence of a large number of low-molecular-weight compounds which lead to severe signal overlap even at the highest magnetic fields (1, 2). Therefore, the application of conventional 2D NMR methods, for example, COSY, TOCSY, or NOESY, to establish proton–proton coupling pathways, often fails. In the case of a perchloric acid (PCA) extract of carp seminal plasma (3) the latter spectra from homonuclear transfer steps turned out to be very complex and difficult to analyze. As an alternative procedure for the assignment in molecular mixtures, we present here the results from a heteronuclear single quantum correlation (HSQC) experiment (4) in combination with HSQC-TOCSY experiments (5) at variable spin-lock mixing times. In addition, a heteronuclear multiple bond experiment (HMBC) (6) was recorded to examine the long-range coupling relations to distant functional groups. All experiments were performed with the aid of magnetic field gradients to improve the suppression of artifacts (7).

MATERIALS AND METHODS

Seminal plasma of carp was deproteinized with cold PCA solution. The resulting suspension was centrifuged at $30,000 \times g$ for 15 min at

4°C, the supernatant decanted and KOH solution added. The supernatant was again decanted, frozen and lyophilized. Immediately prior to NMR spectroscopy 0.5 ml of D_2O was added. The final pH of the solution was 7.1 (meter reading).

NMR spectra were recorded on a Varian UNITY plus 600 spectrometer equipped with a 5 mm $^1\text{H}\{^{15}\text{N}-^{31}\text{P}\}$ pulsed field gradient indirect probe operating at 600 and 150.8 MHz for ^1H and ^{13}C , respectively. The z-field gradients had a maximum strength of 30 Gauss/cm.

The gradient-selected HSQC (4) and HMBC (6) experiments and their conditions were described in a previous paper on a similar investigation on seminal plasma of boar (8). The HSQC-TOCSY experiments were performed gradient-selected according to the pulse sequence of Willker *et al.* (5). Spectral width in f_2 5 kHz and in f_1 26 kHz, 256 increments with 2K data in f_2 , zero-filling in f_1 to 2K. Homospoil pulse during the relaxation delay (1 s). GARP decoupling of ^{13}C nuclei. p-Type spectra by pulse field gradients in the ratio 4:–1 with gradient lengths of 2 and 0.5 ms, filtering by Gauss functions in both dimensions.

RESULTS AND DISCUSSION

Figure 1 shows a portion of the gradient-selected HSQC spectrum of the PCA extract of carp seminal plasma. The correlation was made via the one-bond C,H couplings with multiplicity editing. In contrast to the overlapping resonances in the ^1H NMR spectrum of this complex mixture of low-molecular-weight compounds (Fig. 1, on the left), the resonances of the ^1H broad-band decoupled ^{13}C NMR spectrum (Fig. 1 on the top) were rather well resolved. Obviously, several of the ^{13}C resonances between 55 and 65 ppm were indicative of 2C carbons from amino acids, and in particular those at 64.3 and 57.7 ppm which correlated with the 2-CH protons at 4.20 and 3.82 ppm, respectively, could be tentatively assigned to proline and glutamate (8). However, the latter assignments were only from chemical shift arguments and therefore not very satisfactory. In order to obtain an unequivocal assignment of Pro and Glu, the analysis of the full H,H coupling pattern of both spin systems was necessary. Yet, the discrimination of the overlapping resonances in the chemical shift range between 2 and 2.5 ppm, where the

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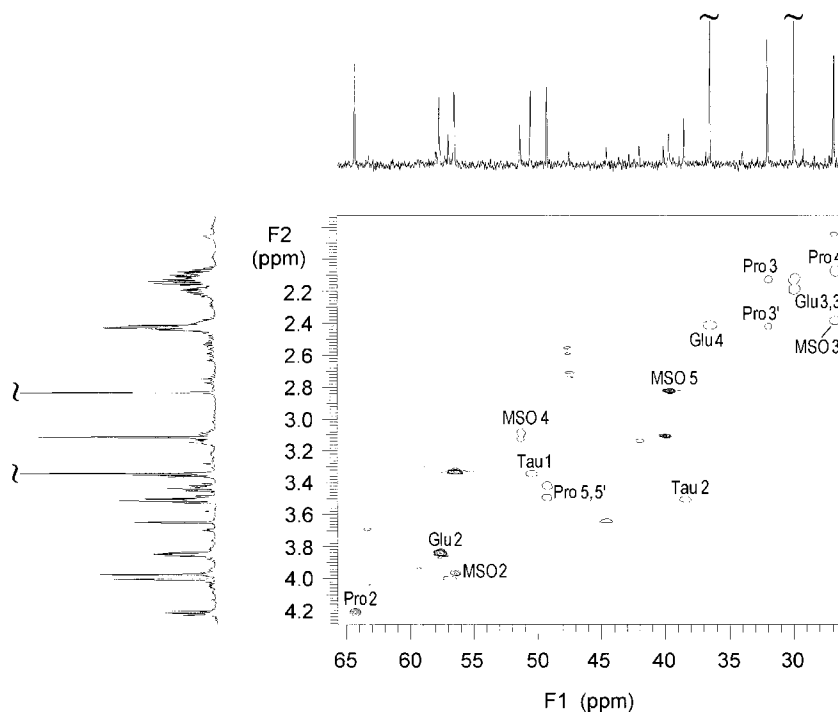


FIG. 1. HSQC spectrum of the extract of carp seminal plasma recorded at pH 7.1 in D_2O ; $T = 30^\circ C$. Gradient selection and editing, with CH_2 peaks negative (shown with one contour line) and CH/CH_3 peaks positive. Optimization for $^1J(C,H) \sim 140$ Hz. 256 increments, experimental time ~ 6 h.

$3-CH_2$ and $4-CH_2$ protons of both Pro and Glu were expected (8) seemed impossible, especially since resonances from other compounds also contributed to this part of the spectrum. Similarly, the region around 3.4 ppm, where the $5-CH_2$ resonances of Pro were expected, was overcrowded by resonances of the other compounds leading to a complex, if not failing analysis when homonuclear correlation techniques were applied (COSY and TOCSY spectra not shown).

HSQC-TOCSY experiments (5) give spectra which essentially contain the same connectivity information as the homonuclear COSY and TOCSY versions, with the difference that the signals are labelled with their ^{13}C chemical shifts in f_1 instead of the 1H chemical shifts. Figure 2 shows a HSQC-TOCSY spectrum where the mixing time was chosen short enough to transfer magnetization only to the neighbouring vicinally coupling protons, and intense correlation responses were caused only by COSY-type transfers. For the case of Pro it can be seen (Fig. 2) that the 2-CH proton correlates with the diastereotopic $3-CH_2$ protons at 2.13 and 2.42 ppm, and the $3-CH_2$ protons, additionally, correlate with the $4-CH_2$ protons at 2.06 ppm. Finally, the latter correlate with the $5-CH_2$ protons at 3.45 and 3.40 ppm. Clearly, the HSQC-TOCSY, performed in this way, not only gave the information of the coupling protons but also the chemical shifts of the carbon atoms bound to the respective protons. The separation of the overlapping resonances of $3-CH_2$ and $4-CH_2$ of Pro,

as well as that of the corresponding resonances of Glu (see below), was thus possible in spite of the nearly identical proton chemical shifts.

When the mixing time in the HSQC-TOCSY experiment was increased (Fig. 3) long-range transfers became visible, and as in a TOCSY-type spectrum, the full proton coupling pattern could be observed at the chemical shift of a carbon contained in the skeleton of this molecular fragment. Thus, all proton multiplets of proline corresponding to a $A_2(T_2)MPX$ spin system (9) were expected at either one of the chemical shift positions of C2 to C5 although they could be best observed at $\delta^{13}C = 32.0$ ppm and 49.2 ppm (Fig. 3). Performing the experiment under these conditions allowed a clear control of the proton spin systems assigned before to an individual compound in the seminal plasma.

The correlations of the vicinally coupling protons of glutamate could also be well observed in the HSQC-TOCSY spectrum of Fig. 2, and the fully coupling pattern corresponding to an $AM(PT)X$ spin system (9), was clearly visible in the HSQC-TOCSY spectrum with long mixing time (Fig. 3), at best at $\delta^{13}C = 36.5$ and 57.7 ppm, where the resonances of 2-CH at 3.82 ppm, those of $3-CH_2$ at 2.10 and 2.17 ppm, as well as those of $4-CH_2$ at approx. 2.4 ppm, were clearly resolved. The HMBC spectrum (Fig. 4) revealed the long-range coupling correlations from the methylene and methine protons to the carboxyl groups at 177.6 ppm (C-1) and 184.3 ppm (C-5), respectively. Glutamine could be ex-

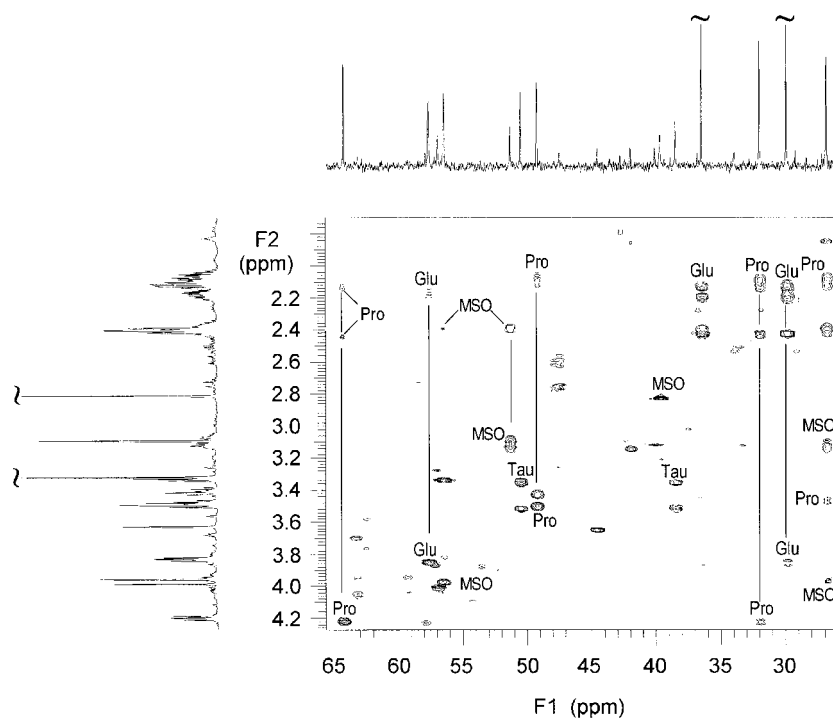


FIG. 2. Gradient selected HSQC-TOCSY spectrum of the extract of carp seminal plasma. Optimization for $^1\text{J}(\text{C},\text{H}) \sim 140$ Hz. The mixing times was 20 ms. The spectrum was recorded with 256 increments, experimental time ~ 6 h.

cluded as an alternative since the C-5 chemical shift position of this compound was expected at much lower frequency ($\delta^{13}\text{C} = 180.7$ ppm) (8).

The HSQC-TOCSY spectrum of Fig. 3 revealed a correlation between two multiplets at 3.32 and 3.48 ppm. In the 1D ^1H -NMR spectrum the latter were

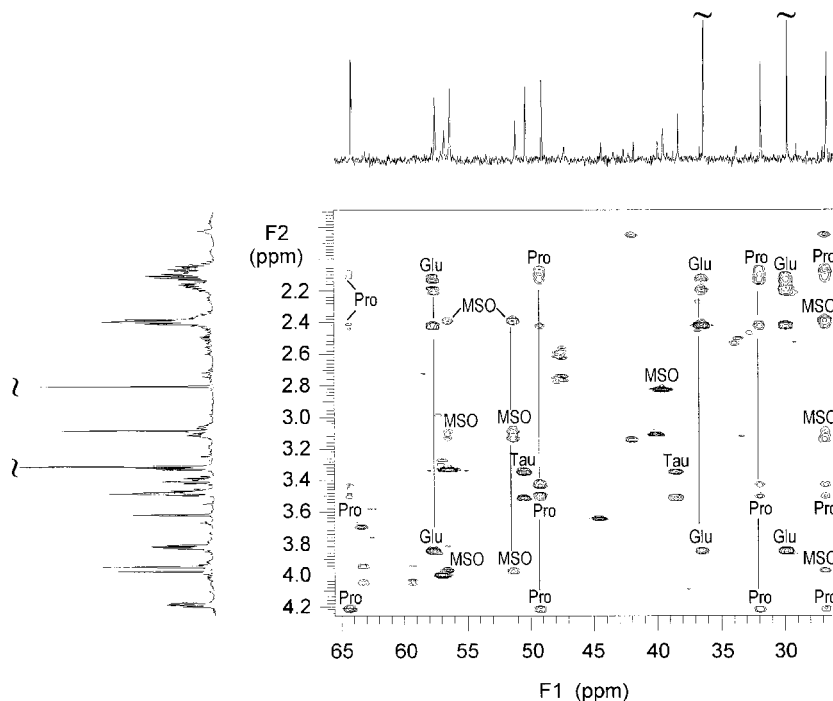


FIG. 3. The same experiment as in Fig. 2, however, performed with a mixing time of 80 ms.

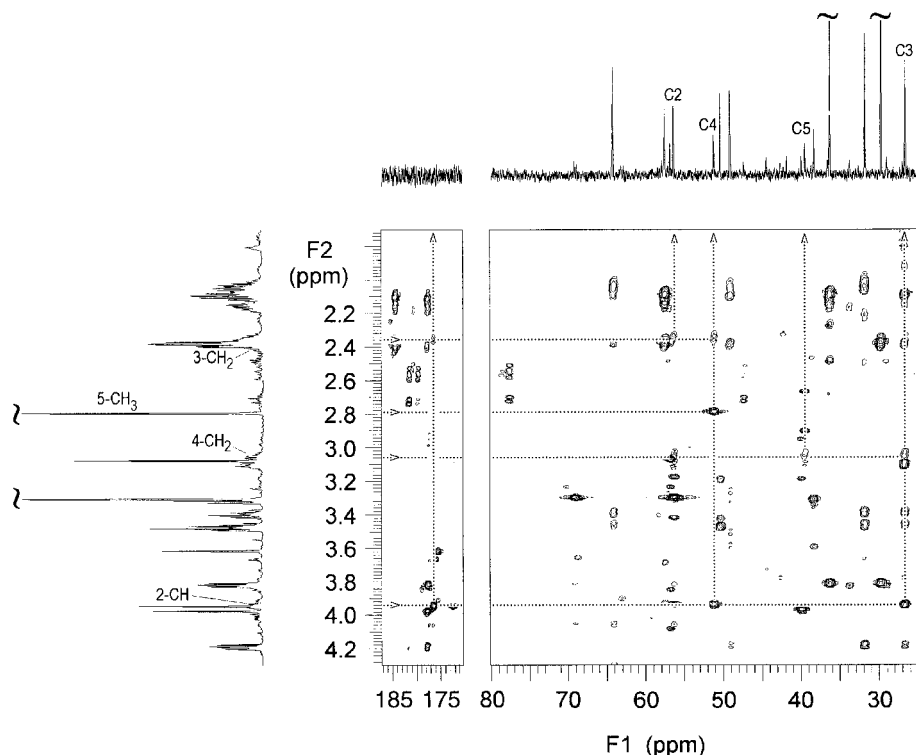


FIG. 4. Gradient-selected HMBC spectrum of the extract of carp seminal plasma. Optimization for $^{23}\text{J}(\text{C},\text{H}) \sim 8$ Hz. The correlation lines indicated are from couplings within methionine sulfoxide.

strongly overlapped by signals of Pro and betaine (10). From the HSQC-ed spectrum (Fig. 1) they could be identified to signals from two methylene groups. Thus, the coupling pattern corresponded to a compound of the type $\text{XCH}_2\text{CH}_2\text{Y}$. Both proton correlation peaks were visible at the ^{13}C chemical shift positions of 38.5 and 50.5 ppm. The ^1H and ^{13}C chemical shifts of the methylene groups (11) and the observed “cross-over” effect (12) (Table 1) assigned this compound to taurine.

Finally we describe the identification of a compound in the seminal plasma of carp which, to our knowledge, has so far not been detected in animal or human sperms. The ^1H NMR spectrum showed a singlet at 2.82 ppm which from the HSQC-ed spectrum (Fig. 1) could be attributed to a methyl signal ($\delta^{13}\text{C} = 39.7$ ppm). In the HMBC spectrum (Fig. 4) these methyl protons revealed a long-range coupling to a carbon atom at 51.3 ppm which was part of a methylene group with the protons appearing at approx. 3.1 ppm (HSQC-ed) (Fig. 1), being overlapped by resonances of creatine and putrescine (10). The same methylene protons, besides a retro-correlation to the methyl carbon at 39.7 ppm, showed a correlation to a carbon resonance at 26.8 ppm (HMBC) (Fig. 4). The full proton coupling pattern was again determined with the help of HSQC-TOCSY experiments. In Fig. 2, the methylene protons at 3.1 ppm showed, at the ^{13}C chemical shift position of 51.3 ppm, a correlation with another methylene group at approx. 2.4 ppm. This experiment

(Fig. 2) was an obvious help in avoiding the strong overlap by the (more intense) proton multiplets of Pro and Glu in this spectral region. However, the ^{13}C chemical shift position of this second methylene group could be only tentatively fixed at 26.8 ppm. Indeed, the ^{13}C signal at 26.8 ppm was composed of the resonance of Pro C-4 (see above and Table 1) and of that of putrescine (10), in addition to that of the searched compound. In order to decide if further protons were part of the spin system, the best way was to perform a HSQC-TOCSY experiment with a long mixing time (Fig. 3) and to go back to the ^{13}C chemical shift position at 51.3 ppm which was free of resonances from other molecules. Figure 3 shows clearly that a third response at 3.95 ppm completed the spin system of the two methylene groups. The HSQC-ed spectrum informed that the multiplet at 3.95 ppm corresponded to a methine group with $\delta^{13}\text{C} = 56.5$ ppm. Obviously, the searched compound contained a fragment of the type $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{Y}-\text{CH}_3$. Since the methine proton at 3.95 ppm showed a long-range coupling to a carboxyl resonance at 176.3 ppm (HMBC) (Fig. 4) an amino acid could be anticipated. Methionine, the only natural amino acid with a $\text{AM}(\text{PT})\text{X} + \text{A}_3$ spin system (9) was the strongest candidate. However, methionine could be excluded because the chemical shift values, in particular those reported for the methyl resonance ($\delta^1\text{H} = 2.13$ ppm, $\delta^{13}\text{C} = 15.4$ ppm) (8) were clearly different from those observed (Table 1). To obtain additional information on

TABLE 1

¹H and ¹³C NMR Chemical Shifts of Compounds Identified in Carp Seminal Plasma^a

Compound	Structure		δ (ppm) ^b	
			¹³ C	¹ H
Proline (Pro)		1C	177.7	
		2CH	64.3	4.20
		3CH ₂	32.0	2.42
		4CH ₂		2.13
		5CH ₂	26.8	~2.06
			49.2	3.45 3.40
Glutamate (Glu)		1C	177.6	
		2CH	57.7	3.82
		3CH ₂	29.9	2.17
				2.10
		4CH ₂	36.5	2.40
		5C	184.3	
Taurine (Tau)		1CH ₂	50.5	3.32
		2CH ₂	38.5	3.48
Methionine-sulfoxide (MSO)		1C	176.3	
		2CH	56.5	3.95
		3CH ₂	26.8	~2.4
		4CH ₂	51.3	~3.1
		5CH ₃	39.7	2.80

^a Lyophilisate, pH 7.1.^b Relative to internal TSP. For comparison with literature values, take notice of δ_H(TSP) = δ_H(TMS), but δ_C(TSP) ≡ δ_C(TMS) + 1.7 ppm.

the functional group Y at which the methyl group was attached, the methyl one-bond C,H coupling constant was determined. Both the chemical shift data (Table 1) and the value of ¹J = 139 Hz indicated the presence of either an amine or a sulfoxide group for Y. In conclusion, two compounds appeared favourable for attribution: (a) 5-azanolvaline, to our knowledge, however, this compound was never identified as natural and no chemical shift data were available in the literature. (b) Methionine sulfoxide (MSO), a natural amino acid, occurring as the oxidation product of methionine (13). Indeed, the observed ¹H chemical shift values were in very good agreement with data reported for MSO (14). Finally, we performed a "spiking" experiment (2, 7) which turned out as a clear proof for MSO. We want to mention that MSO occurred only in the seminal plasma of carp but not in the sperm cells (15).

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REFERENCES

- Nicholson, J. K., and Wilson, I. D. (1989) *Progr. NMR Spectrosc.* **21**, 449–501.
- Lynch, M. J., Masters, J., Pryor, J. P., Lindon, J. C., Spraul, M., Foxall, P. J. D., and Nicholson, J. K. (1994) *J. Pharm. Biomed. Anal.* **12**, 5–19.
- Kamp, G., Büsselmann, G., and Lauterwein, J. (1996) *Experientia* **52**, 487–494.
- Davis, D. G. (1991) *J. Magn. Reson.* **91**, 665–672.
- Willker, W., Leibfritz, D., Kerssebaum, R., and Bermel, W. (1993) *Magn. Reson. Chem.* **31**, 287–292.
- Bax, A., and Summers, M. F. (1986) *J. Am. Chem. Soc.* **108**, 2093–2098.
- Hurd, R. E., and John, B. K. (1991) *J. Magn. Reson.* **91**, 648–653.
- Wüthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland, Amsterdam.
- Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
- Kalic, M., Lauterwein, J., Büsselmann, G., and Kamp, G. (2000) Submitted.
- Sherry, A. D., Malloy, C. R., Jeffrey, F. M. H., Chavez, F., and Srere, H. K. (1990) *J. Magn. Reson.* **89**, 391–398.
- Kalic, M., Büsselmann, G., Lauterwein, J., and Kamp, G. (1997) *Biochim. Biophys. Acta* **1320**, 208–216.
- Ripp, S. L., Itagaki, K., Philpot, R. M., and Elfarra, A. A. (1999) *Arch. Biochem. Biophys.* **367**, 322–332.
- Billington, D. C., and Golding, B. T. (1982) *J. Chem. Soc. Perkin Trans. I*, 1283–1290.
- Kalic, M., Büsselmann, G., Kamp, G., and Lauterwein, J. (2000) Manuscript in preparation.