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3D HCCH-COSY-TOCSY experiment for the assignment of ribose and amino acid side chains in ^{13}C labeled RNA and protein

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Abstract

A new 3D HCCH-COSY-TOCSY experiment is presented for the assignment of RNA sugar and protein side chains. The experiment, which combines COSY and TOCSY units, is more powerful than the sum of individual HCCH-COSY and HCCH-TOCSY pulse sequences. The experiment was applied to a ^{13}C, ^{15}N-labeled 26 mer RNA complexed with the antibiotic tobramycin, and a 12 kDa ^{13}C, ^{15}N-labeled FKBP12 protein sample. The power of HCCH-COSY-TOCSY is demonstrated through complete spin system assignments of sugars in the 26 mer RNA sample, which could not be assigned using a combination of HCCH-COSY, HCCH-TOCSY and ^{13}C-edited NOESY experiments.

RNA sugar or protein side chain assignment plays an important role in deriving high resolution RNA or protein structures. There are two major types of heteronuclear experiments used for this purpose. The TOCSY type experiments include: HCCH-TOCSY, (H)CCH-TOCSY (Fesik et al., 1990; Bax et al., 1990a; Olejniczak et al., 1992) and methylene-selected HCCH-TOCSY (Pardi and Nikonowicz, 1992). The COSY type experiments include: HCCH-COSY (Bax et al., 1990b; Kay et al., 1990; Ikura et al., 1991) and HCCH-RELAY (Pardi and Nikonowicz, 1992). In the HCCH-TOCSY type experiment, all the ^{1}H or ^{13}C in a spin system are correlated through a ^{13}C mixing scheme. The disadvantage of this experiment is that spin system identification is not straightforward from the chemical shift values. In the HCCH-COSY type experiment, two neighboring ^{1}H, ^{1}H or ^{13}C, ^{13}C are correlated. The spin types can be identified starting from C1′/H1′ in RNA and C_α/H_α in protein. However, it is difficult to go beyond C2′/H2′ in the sugar ring or C_β/H_β in the amino acid side chain, because chemical shifts of both carbon and proton become crowded. Since both types of the experiments rely heavily on the dispersion of C1′/H1′ in RNA and C_α/H_α in protein, overlaps in these regions make the assignment time consuming and difficult, and sometimes, impossible.

In this report, we propose a 3D HCCH-COSY-TOCSY experiment to facilitate RNA sugar and protein side chain assignments. The experiment is composed of a COSY and a TOCSY step. The COSY, which correlates a proton to its attached carbon as well as the carbon separated by two bonds, makes use of chemical shift dispersion in both proton and carbon nuclei to increase resolution. The TOCSY step transfers the entire ^{1}H-^{13}C COSY correlation within a spin system to a set of well resolved protons, such as H1′ in RNA or H_α in protein. Since the entire ^{1}H-^{13}C COSY spin connectivity is now displayed in a 2D plane, instead of an 1D skewer in TOCSY experiment, overlaps in C1′/H1′ or C_α/H_α do not limit the separation of different spin systems as long as dispersion exists in proton and/or carbon chemical shifts among the spin systems being studied. At the same time, the COSY connectivities make spin system identification straightforward. A similar experiment, HCC-TOCSY-CCH-E.COSY, was published recently (Schwalbe et al., 1995). Since it is specifically designed for measuring $^{3}J_{(H,H)}$ coupling constants of RNA ribose, it may not be suitable for RNA sugar or protein side chain assignment.

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The proposed pulse schemes of HCCH-COSY-TOCSY experiment for RNA and protein samples are displayed in Figures 1A and 1B. The detectable terms at point (a) in Figure 1A are

$$C'_X \cos(\omega_H t_1) \cos^n(2\Delta_3 \pi J_{C-C}) \cos(\omega_{C'} t_2) \cos^n(2T_C \pi J_{C-C}) \quad (1)$$

$$C''_X \cos(\omega_H t_1) x \cos^{n-1}(2\Delta_3 \pi J_{C-C}) \sin(2\Delta_3 \pi J_{C-C}) \cos(\omega_{C''} t_2) x \cos^{m-1}(2T_C \pi J_{C-C}) \sin(2T_C \pi J_{C-C}) \quad (2)$$

deduced from product operator formalism (Sørensen et al., 1983), where C′ is the carbon directly attached to the proton, ω_H is the proton chemical shift, n is the total number of carbons connected to the C′ carbon, C″ is the carbon connected to C′, $2\Delta_3$ ($\Delta_3 = \Delta_1 + \Delta_2$) and $2T_C$ are the duration of COSY and constant time period respectively, ${}^1J_{C-C}$ is the carbon-carbon one bond coupling constant, $\omega_{C'}$ and $\omega_{C''}$ are the chemical shifts of C′ and C″, and m is the total number of carbons connected to C″. It can be seen from term (1) and (2) above that each proton is correlated to its directly attached carbon (C′) and the carbon two bonds away (C″). These correlations are then transferred to all the carbons within the spin system by a DIPSI-3 mixing scheme (Shaka et al., 1988). The reverse INEPT sequence after the mixing returns the magnetization to proton, where signal is detected. When the 3D HCCH-COSY-TOCSY is viewed along the highly resolved direct ^{1}H dimension, the ^{1}H-^{13}C COSY pattern of the entire spin system is laid out in an F1-F2 2D plane (see Figures 2 and 3).

The two gradient Z filters (g4 in Figure 1) that flank the mixing period, especially the one before the mixing destroy the carbon antiphase terms that evolve during the constant time $2T_C$. These terms could generate directed TOCSY transfer (Glaser et al., 1996), in which one set of the cross peaks, Hi′/Ci+1′ (i = 1, 2, 3 ,4 for RNA sugar), will be suppressed in an extent depending on the relative duration of the constant time $2T_C$ and the mixing. The missing COSY peaks from one direction along the side chain makes assignment ambiguous.

Compared to the HCCH-TOCSY experiment, the HCCH-COSY-TOCSY experiment has an extra COSY step which reduces the sensitivity of HCCH-COSY-TOCSY by a factor of $\cos^n(2\Delta_3 \pi J_{C-C})$ and $\cos^{n-1}(2\Delta_3 \pi J_{C-C}) \sin(2\Delta_3 \pi J_{C-C})$ for carbon C′ and C″ without considering relaxation effect during the COSY step. The duration of COSY step is $1/4^1 J_{C-C}$, and the reduction factors are thus the same for C′ and C″, 0.71, 0.50 and 0.36 for n = 1, 2 and 3 respectively. The trigonometry factors during the constant time period ($2T_C$) are not considered because there is also an intensity loss during the ^{13}C chemical shift labeling period in the HCCH-TOCSY experiment due to broader ^{13}C line width caused by the J_{C-C} modulation. To compare the sensitivity of these two experiments, 2D ^{13}C-^{1}H correlated spectra have been obtained using HCCH-TOCSY and HCCH-COSY-TOCSY pulse sequences under the same conditions. The signal-to-noise ratios (S/N) of 35 well resolved cross peaks are compared. S/N ratios of 6 peaks from HCCH-COSY-TOCSY are up by 2∼60% compared to HCCH-TOCSY, 9 peaks are down by <40%, and 20 peaks are down by 40∼67%. The possible reason that a few peaks from HCCH-COSY-TOCSY have better S/N ratios is that these peaks must be extreme mobile so that the relaxation does not reduce the sensitivity much during the COSY and constant time periods, on the other hand, the large J_{C-C} modulation broadens the ^{13}C line width in the HCCH-TOCSY.

The pulse schemes shown in Figures 1A and 1B were tested on ^{13}C and ^{15}N uniformly labeled RNA and protein samples. The RNA sample is a 26-mer X1 RNA (GGGACUUGGUUUAGGUAAUGAGUCCC) stem loop (Wang and Rando, 1995; Jiang et al., unpublished results) complexed with antibiotic tobramycin. The protein is a 12 kDa FK506 Binding Protein (FKBP12), whose NMR chemical shifts are available (Rosen et al., 1991; Xu et al., 1993; Kakalis and Rosen, unpublished results). Concentrations of X1 RNA and FKBP12 are 3.0 and 3.1mM in D_2O. All experiments were carried out on a Varian INOVA 500 equipped with actively shielded Z-gradients performa II at 25 °C. The experiments were processed and analyzed using NMRPipe (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994) on SGI Power Challenge and SGI O2 workstations.

Despite being a 26 mer, the complete sugar assignment of the X1 RNA stem loop complexed with tobramycin is not available from combination of HCCH-TOCSY, HCCH-COSY, and ^{13}C edited HMQC-NOESY due to severe degeneracy of proton and carbon chemical shifts, especially H1′ and C1′, among some sugars. Specifically, G8 and G14 assignments are ambiguous from HCCH-TOCSY and HCCH-COSY experiments, and the complete assignments of U6, U10 and U23 are not available using HCCH-TOCSY, HCCH-COSY and ^{13}C edited HMQC-NOESY experiments. The complete spin system assignments of these five sugars are obtained using single HCCH-COSY-TOCSY experiment, and

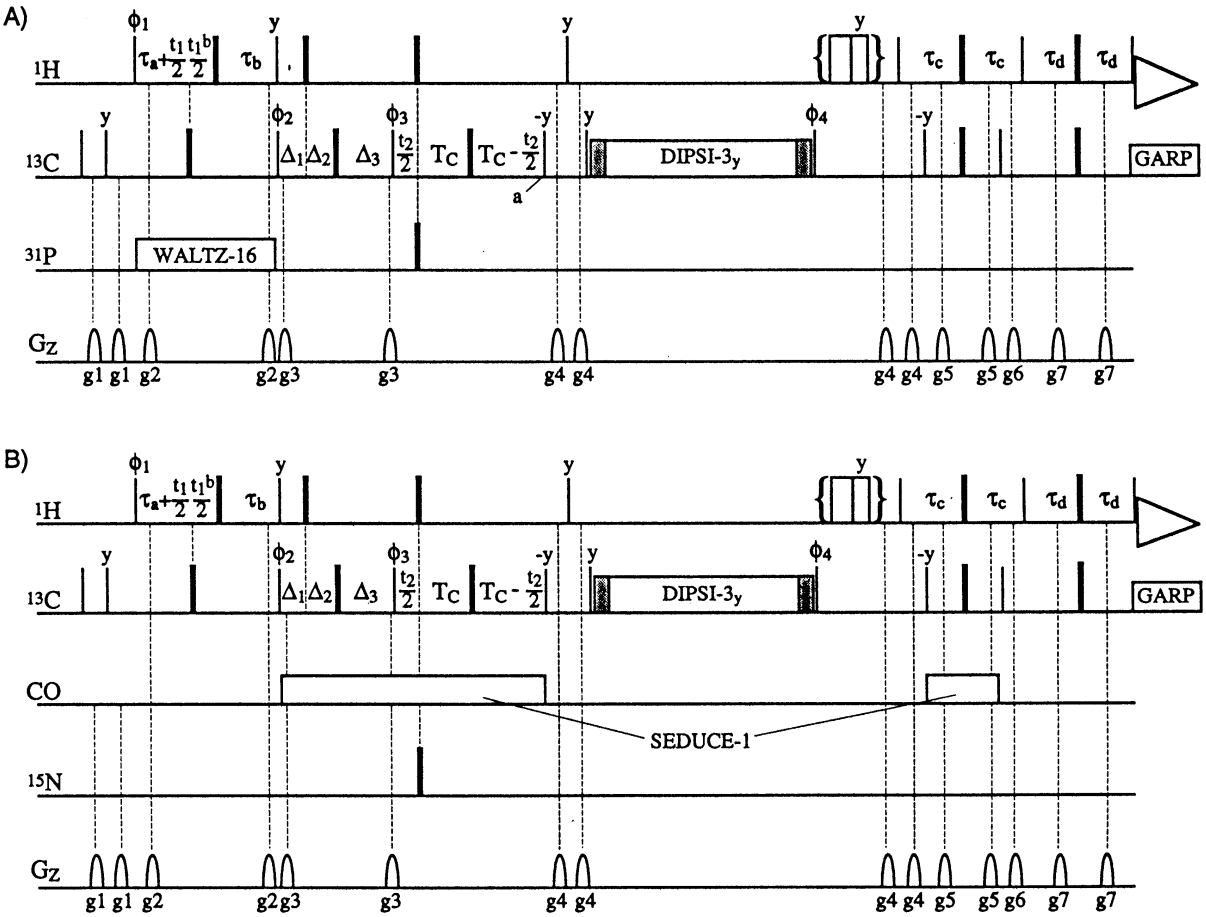

Figure 1. Pulse sequences of 3D HCCH-COSY-TOCSY experiment for ^{13}C-labeled RNA (A), and ^{13}C-labeled protein (B). Narrow and wide bars correspond to 90° and 180° pulses, respectively. The pulses in braces are used to suppress water for samples dissolved in H_2O. Trim pulses are shown as shaded pulses flanking DIPSI-3 sequence (Shaka et al., 1988). The phases of all pulses are x axis unless otherwise indicated. The ^{1}H, ^{13}C and ^{31}P carrier frequencies were 5.1, 79 and −3.5 ppm respectively in (A). The ^{1}H, ^{13}C and ^{15}N carrier frequencies were 3.2, 46 and 120ppm respectively in (B). Field strengths of the ^{1}H pulse, ^{13}C high power pulse, trim pulses, DIPSI-3 mixing, GARP decoupling on ^{13}C (Shaka et al., 1985), WALTZ-16 decoupling (Shaka et al., 1983) on ^{31}P and ^{31}P hard pulse were 28.7, 19.2, 7.4, 7.4, 2.5, 0.98 and 4.4 kHz respectively in sequence (A). Field strengths of ^{1}H pulse, ^{13}C hard pulse, trim pulses, DIPSI-3 mixing, GARP decoupling on ^{13}C, SEDUCE-1 (McCoy and Mueller, 1992) decoupling on carbonyl carbon, and ^{15}N hard pulses were 28.1, 18.5, 7.8, 7.8, 2.5, 0.76 and 5.6 kHz respectively in sequence (B). All ^{13}C pulses were applied on resonance with the exception of the carrier of the carbonyl decoupling which was shifted to 175 ppm through phase modulation. Strengths and duration of gradients were: g1 = (8 G/cm, 0.5 ms), g2 = (8 G/cm, 0.5 ms), g3 = (8 G/cm, 0.25 ms), g4 = (20 G/cm, 1.2 ms), g5 = (20 G/cm, 1.8 ms), g6 = (2 G/cm, 0.5 ms), g7 = (−15 G/cm, 1.5 ms), g8=(3 G/cm, 0.5 ms). ^{13}C-TOCSY mixing times were 14.8 and 13.9 ms for (A) and (B) respectively. The delay $\Delta_1 = 1/8J_{C-H}$, where J_{C-H} is the one bond J coupling constant of ^{1}H-^{13}C. Values of J_{C-H} used in (A) and (B) are 160 and 130 Hz respectively. The delay $\Delta_2 = \Delta_3 - \Delta_1$. Additional delays $\tau_a = 1.5$ ms, $\Delta_3 = 3.2$ ms, $T_C = 3.3$ ms, $\tau_c = 1.5$ ms and $\tau_d = 1.5$ m in scheme (A), and $\tau_a = 1.6$ ms, $\Delta_3 = 3.4$ ms, $T_C = 3.6$ ms, $\tau_c = 1.0$ ms and $\tau_d = 1.6$ ms in scheme (B). Delay $\tau_b = \tau_a - n\delta$, $t_1{}^b/2 = t_1/2 - n\delta$, where τ_a is set to $\sim 1/4J_{C-H}$, n is the incremental point of proton dimension increasing from 0 to (N–1), N is the total complex points of proton dimension, δ, a portion of delay τ_a, is set to $\tau_a/(N-1)$ (Grzesiek and Bax, 1993; Logan et al., 1993). Phase cycling for (A) and (B) was ϕ_1 = x, -x; ϕ_2 = x, x, −x, −x; ϕ_3 = 4(x), 4(−x); ϕ_4 = 4(y), 4(−y) and Acq. = x, −x, −x, x, −x, x, x, −x. Quadrature detection during t_1 was achieved via States-TPPI (Marion et al., 1989) on ϕ_1, and on ϕ_2 and ϕ_3 during t_2.

Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of five RNA sugars in the tobramycin-RNA aptamer complex

	H1′ C1′	H2′ C2′	H3′ C3′	H4′ C4′	H5′/H5″ C5′
U6	5.63	4.75	4.46	4.44	4.11/4.56
	94.0	75.8	72.7	82.5	64.8
G8	5.84	4.95	4.75	4.50	4.11/4.64
	93.6	75.6	72.4	83.0	64.2
U10	5.62	4.38	4.23	4.46	4.07/4.53
	93.8	75.7	71.7	82.3	63.9
G14	5.85	4.01	4.88	4.23	4.31/3.98
	93.5	77.0	71.9	83.6	64.5
U23	5.62	4.53	4.53	4.46	4.09/4.57
	94.0	75.6	72.6	82.5	64.6

the results are listed in Table 1. The sequential assignment of sugars was obtained from HMQC-NOESY experiment (Jiang et al., unpublished results).

Shown in Figure 2A are the COSY connectivities for the G8 and G14 ribose rings in a F1-F2 slice at the H1′ chemical shift of sugars. In the Figure 2A, Ci′/Hi′ diagonal peaks of G8 and G14 are enclosed in parenthesis and bracket respectively. Starting from C1′ and H1′ at the left bottom corner, the diagonal peaks are connected by the cross peaks of Hi′/Ci+1′ and Hi+1′/Ci′. Although the C5′/H5′ and C5′/H5″ diagonal peaks are relatively weak compared to others, H5′/H5″ chemical shift values can also be read out from the skewer along C4′ chemical shift values of G8 and G14, i.e., the C4′/H5′ and C4′/H5″ two cross peaks. In this way, the sugar spin systems of G8 and G14 have been determined unambiguously although their C1′ and H1′ are superimposed.

A big challenge for the HCCH-COSY-TOCSY experiment is the spin system assignment of U6, U10 and U23 sugars. The riboses of these three nucleotides have not only the same C1′ and H1′ chemical shifts, but also some other identical ^{13}C and ^{1}H chemical shifts (see Table 1). Thus, their COSY patterns are indistinguishable in the F1-F2 plane sliced at their H1′ chemical shift. In order to identify each spin system, a F1-F3 slice at C1′ chemical shift of three sugars is examined in Figure 2B. The bottom skewer labeled with 'TOCSY' and 'U6, U10, U23' contains all ^{1}H chemical shift values from three sugars except for H5′/H5″ because the read out delay (τ_C) in reverse INEPT is optimized for CH group. This is the skewer one usually observes in normal HCCH-TOCSY experiment. There is no way to find out which chemical shift values belong to which ribose ring from this over crowded skewer. Nevertheless, the COSY part of HCCH-COSY-TOCSY experiment generates three sub-TOCSY skewers anchored at C1′/H2′, and these sub-TOCSY skewers are labeled as 'U6', 'U10' and 'U23' in the spectrum. The H2′, the possible H3′ and H4′ chemical shift values for the three sugars are thus obtained from the sub-TOCSY skewers. There are three distinguishable ^{1}H values for U10, and two for U6 and U23. These suggest that two protons of H2′, H3′ and H4′ in U6 and U23 are degenerate. In the following discussion, U23 will be used as an example to demonstrate how to obtain a complete assignment for these three sugars.

From the sub-TOCSY of U23 in Figure 2B, the possible H3′ and H4′ values are 4.53 ppm and 4.46 ppm, while that of H2′ is 4.53 ppm. Thus the ^{1}H-^{13}C COSY connectivity of U23 can be examined at either F3 = 4.53 ppm or F3 = 4.46 ppm depending upon which value locates at less crowded region. Shown in Figure 2C is the F1-F2 slice at F3 = 4.53 ppm. Starting from C2′/H2′, C1′/H1′ can be easily identified, and this connection assures that the correct spin system is being examined. For the assignment of C3′/H3′, if H3′ resonates at 4.46 ppm, there should be a C2′/H3′ cross peak at (75.6 ppm, 4.46 ppm). The absence of cross peak suggests that H3′ does not resonate at 4.46 ppm. On the other hand, there is a peak at (72.6 ppm, 4.53 ppm), which connects to a peak at (72.6 ppm, 4.46 ppm). Therefore, the peak at (72.6 ppm, 4.53 ppm) corresponds to both the C3′/H3′ diagonal peak and the C3′/H2′ cross peak due to the same chemical shifts of H2′ and H3′. The peak at (72.6 ppm, 4.46 ppm) corresponds to the C3′/H4′ cross peak. From this cross peak, the C4′/H4′ and C5′/H4′ are thus identified, leading to the C5′-H5′ and C5′/H5″ diagonal peaks. Similarly, the complete spin system assignments of U6 and U10 have been obtained from the F1-F2 slices at F3 = 4.73 ppm (H2′ of U6) and F3 = 4.22 ppm (H3′ of U10).

The protein version of the HCCH-COSY-TOCSY pulse scheme is illustrated in Figure 1B. Compared to the HCCH-COSY and HCCH-TOCSY experiments, side chain assignment of protein by HCCH-COSY-TOCSY is more straightforward because the assignment is based on the COSY connectivities instead of chemical shift values of ^{1}H and ^{13}C. Since the entire ^{1}H-^{13}C COSY connectivities of amino acid residues are displayed in a 2D plane, the overlaps in C_α/H_α are less likely to generate the ambiguous as-

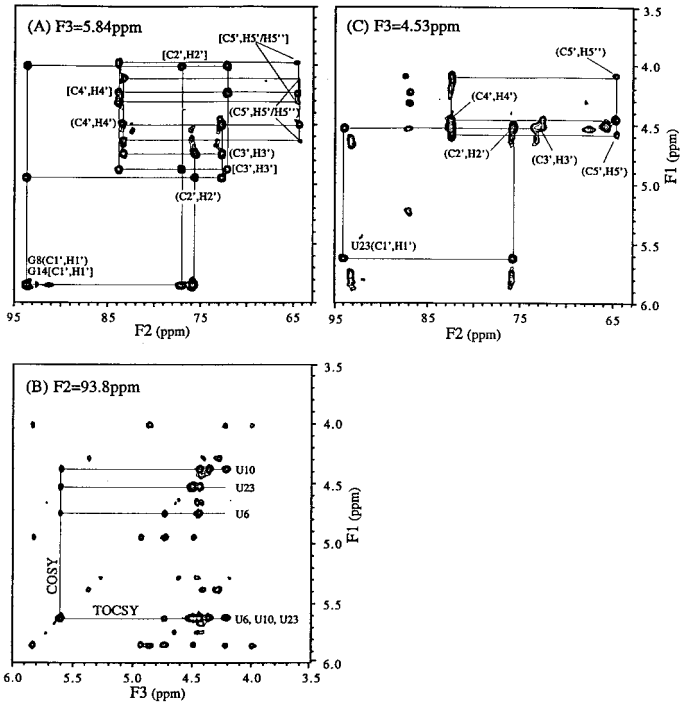

Figure 2. 2D slices of the 3D HCCH-COSY-TOCSY experiment of some sugars from ^{13}C and ^{15}N uniformly labeled 26 mer of the X1 RNA stem loop. The spectral widths(Hz)/complex points along F1, F2 and F3 dimensions were 1800/128, 4300/28 and 2000/256, respectively with 8 transients per FID. The total experimental time was 40 h. For more details, see the legend to Figure 1 and the text. The F2 resolution was enhanced through mirror-image linear prediction (Zhu and Bax, 1990) resulting in a final matrix size 256×128×512 real points. (A) The F1-F2 slice at F3 = 5.84 ppm, the H1′ chemical shift of G8. The H1′ chemical shift of G14 is 5.85 ppm. The slice shows the complete assignment of ^{1}H and ^{13}C of G8 and G14 sugars (see text). (B) F1-F3 sliced at F2 = 93.8 ppm. The crowded normal TOCSY skewer labeled as 'U6, U10, U23' at F1 = 5.62 ppm is spread into three sub-TOCSY skewers due to the COSY transfer. These three sub-TOCSY skewers anchor at H2′ chemical shift values of the three sugars along the F1 dimension. (C) The complete assignment of U23 sugar in F1-F2 slice at F3 = 4.53 ppm, the H2′ chemical shift of U23.

signment as long as the entire ^{1}H-^{13}C COSY patterns are different among the residues being studied. The utility of HCCH-COSY-TOCSY is demonstrated on five FKBP12 residues. Shown in Figure 3A are the complete ^{1}H-^{13}C COSY connectivities of five residues in the F1-F2 plane at F3 = 5.34 ppm. Figure 3B is an expansion of the boxed region in (A). For simplicity, chirality of proton or carbon is not labeled in these two figures.

Similarly to the assignment of RNA sugar, the assignment for these five residues starts from their C_α/H_α diagonal peak. Locations of the corresponding C_β/H_β can be found through COSY cross peaks, i.e., the H_α/C_β and H_β/C_α. Thus, the C_β/H_β diagonal peaks of F15 and L106 can be easily identified. Although the C_β/H_β of V98 is merged with one of the two C_β/H_β diagonal peaks of K73, the C_β/H_β diagonal peak of V98 can be assigned from the two cross peaks as described above for F15 and L106. The two H_β values (1.97 and 2.03ppm) of M29 which are not distinguishable in the F1 dimension are well resolved in the F3 dimension in an F1-F3 plane viewed at F2 = 54.4 ppm (results not shown). The complete assignment scheme from C_β/H_β to the rest of the side chain of M29, K73, L106 and V98 are shown in Figure 3B. The assignment of K73 will be used as an example in the following discussion. The two C_β/H_β diagonal peaks of K73 are connected to two C_γ/H_γ diagonal peaks through two pairs of cross peaks: the two H_β/C_γ, and the two H_γ/C_β transfers. The single C_δ/H_δ diagonal peak is connected to two C_γ/H_γ peaks by a pair of H_γ/C_δ peaks, and a single H_δ/C_γ peak, which is superimposed with one of the two H_β/C_γ cross peaks. Finally, the C_ϵ/H_ϵ diagonal peak is connected to C_δ/H_δ by H_δ/C_ϵ and H_ϵ/C_δ cross peaks.

In summary, a new HCCH-COSY-TOCSY experiment which combines the advantages of COSY and TOCSY experiments is presented in this communi-

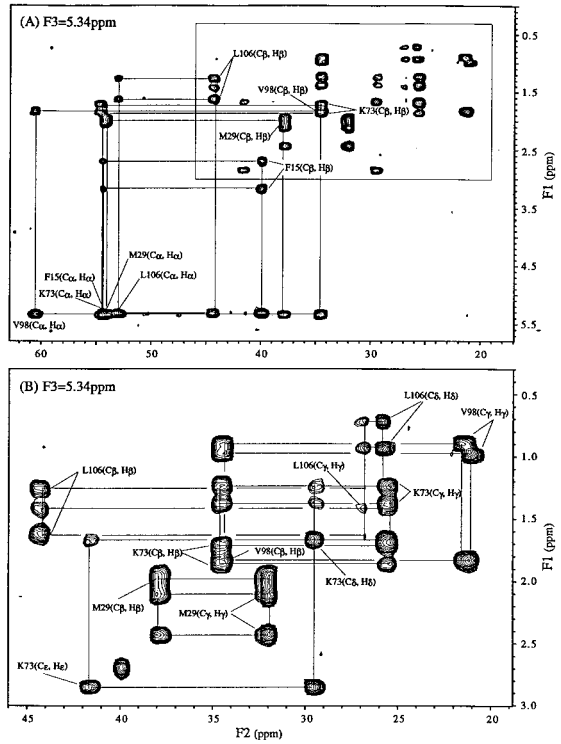

Figure 3. F1-F2 slices of 3D HCCH-COSY-TOCSY experiment at F3 = 5.34 ppm for five amino acids from ^{13}C and ^{15}N uniformly labeled FKBP12. The spectral widths(Hz)/complex points along F1, F2 and F3 were 3500/100, 7500/52 and 4000/256 respectively with 8 transients per FID. The total experimental time was 50 h. For more details, see the legend to Figure 1 and the text. The F2 resolution was enhanced through mirror-image linear prediction (Zhu and Bax, 1990) resulting in a final matrix size of 256×128×512 real points. Shown in (A) are the complete ^{1}H-^{13}C COSY connectivities for the five residues (F15, M29, K73, V98 and L106) of FKBP12. The $^{1}H_{\alpha}/^{13}C_{\alpha}$ chemical shifts of these five residues are F15 (5.34/54.4 ppm), M29 (5.35/54.0 ppm), K73 (5.35/54.6 ppm), V98 (5.34/60.5 ppm) and L106 (5.32/53.0 ppm). The $^{13}C_{\alpha}/^{1}H_{\alpha}$ and $^{13}C_{\beta}/^{1}H_{\beta}$ diagonal peaks of the five residues are connected with fine lines through the cross peaks. (B) Shows an expansion of the boxed region in (A). Assignments of the side chains of M29, K73, V98 and L106 starting from $^{13}C_{\beta}/^{1}H_{\beta}$ diagonal peaks are demonstrated.

cation. By combining COSY and TOCSY transfer schemes into one experiment, HCCH-COSY-TOCSY is more powerful than the sum of two individual HCCH-COSY and HCCH-TOCSY experiments in RNA ribose and protein side chain assignments. The experiment, which resolves spin systems by recognition of whole ^{1}H-^{13}C COSY patterns at a plane sliced at well resolved ^{1}H locations, makes it excellent for assignment of both poorly dispersed RNA ribose and amino acid side chains of proteins. Spectral analysis of this experiment is straightforward. The explicit COSY connectivities make it unambiguous in identifying both ^{1}H and ^{13}C spin types in a spin system, which is very important in deriving structure from NOESY data.

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