Supporting Information.

Hyperdimensional NMR spectroscopy.

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Comparison with 3D HN(CA)CO spectrum. Figures S1 and S2 compare the F1F3 projection of the conventional 3D HN(CA)CO spectrum of agitoxin with the corresponding projection, F6F9 of a hypothetical ten-dimensional spectrum of agitoxin. Note that due to the low sensitivity of the conventional 3D HN(CA)CO experiment several peaks that are observed in the F6F9 reconstruction are missing from the F1F3 projection of the HN(CA)CO spectrum. Note also the lower overall resolution of the F1F3 projection of the conventional HN(CA)CO spectrum. The resolution in this spectrum is determined by the total duration of the experiment which was limited to 24 hours. The reconstructed spectrum lacks responses from the residues adjacent to prolines (which have no NH protons thus precluding indirect measurements).

Figure S1. The F1F3 (NH, CO) projection from the conventional 3D HN(CA)CO spectrum recorded on a Varian 800 MHz instrument in ca 24 hours. The spectral width in F1 was 3200 Hz and 12500 Hz in F3, 64 and 32 complex points were recorded in F1 and F2 respectively. The number of complex points was doubled by linear prediction before applying a sine-squared window function and zero filling to 512 x 256 x 1024 matrix size.

Figure S2. The reconstructed F6F9 projection of a hypothetical ten-dimensional spectrum of agitoxin (see text for the details). The spectrum corresponds to the F1F3 projection of the conventional 3D HN(CA)CO experiment shown in Figure S1.

Resolving Overlap and Step-by-Step Reconstruction. Below the problem of overlap is further considered and reconstruction of the F1F6 projection is demonstrated in detail. Figure S3 shows the F1F3 (CA, NH) plane of the conventional 3D HNCA spectrum. There is a strong overlap in the NH domain (at 8.26 ppm) between two pairs of cross-peaks as shown by the dotted line. The corresponding peaks in the F2F3 (NH) plane are shown in figure S4. First the 3D HNCA spectrum is reconstructed from two orthogonal projections, F1F3 (Fig. S3) and F2F3 (Fig. S4) and a pair of planes tilted at $+60^\circ$ and -60° (not shown here). An example of the reconstructed F1F2 plane taken at $f_3 = 8.26$ ppm (H-1) is shown in Fig. S5. This solves the overlap problem in the NH domain and once the overlap has been dealt with, the NH(i)-NH(i-1) correlation map can be constructed (see Fig. S6) in analogy with covariance spectroscopy (see reference 13 in the

Figure S3. The F1F3 (CA, NH) plane of the conventional 3D HNCA spectrum of agitoxin. The dotted line shows the position of the reconstructed F1F2 plane in Figure S5. Four peaks that overlap in F3 are color-coded.

Figure S5. The reconstructed F1F2 projection of the conventional 3D HNCA spectrum of agitoxin at position shown in Figures S3 and S4 ($f_3 = 8.26$ ppm). The color coding corresponds to that in Fig. S3.

Figure S4. The F2F3 (NH) plane of the conventional 3D HNCA spectrum of agitoxin. The dotted line shows the position of the reconstructed F1F2 plane in Figure S5. The color coding corresponds to that in Figure S3.

main text). In cases where simultaneous overlap of peaks occurs in two domains (e.g. NH and CA) more direct correlations may need to be recorded before the indirect correlations can be established.

Experimental Details. All spectra were recorded at 30° C on a Varian INOVA spectrometer operating at 800 MHz H-1 frequency. The sample was 0.3 mM protein agitoxin (39 residues) in H₂O/D₂O (9:1). The spectral width of 12500 Hz in the observe dimension (H-1) was digitized using 1024 complex data points.

Figure S6. The reconstructed F1F6 projection of the hyperdimensional 10D spectrum of agitoxin. The color coding corresponds to that in Figures S3 to S5.

The number of complex data points in the indirectly detected dimension was 256 (N-15 HSQC, C-13 HSQC, HNCA and HNCOCa), 128 (HNCA), 100 (*PR*-HNCO) 200 (*PR*-HNCA, *PR*-HNCOCa). The spectral width in the indirect dimension was 3200 Hz (N-15 and CO) and 7200 Hz (CA). The experimental times were 23 minutes for N-15 HSQC, C-13 HSQC, and *PR*-HNCO (plane tilted at 60 degrees), 24 min for HN(CO)CA (HN-CA plane), 45 minutes (HNCA, HN-CA plane), 12 minutes for HNCO (HN-CO plane), 38 minutes for HN(CO)CA (plane tilted at 60 degrees) and 46 minutes for *PR*-HNCA (plane tilted at 60

degrees). The total experiment time was approximately 4 hours. All spectra were processed using zero filling to 2048 complex points in the direct dimension and 1024 complex points in the indirect dimension. Linear prediction was used to double the number of complex data points in the indirect dimension before sine-squared window function and zero filling were applied in both dimensions.