

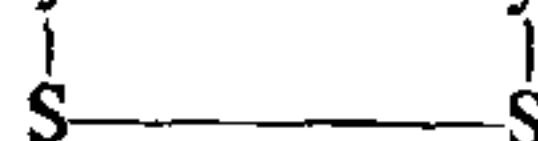
## APPLICATION OF TWO-DIMENSIONAL CORRELATION SPECTROSCOPY FOR ASSIGNMENT OF NMR SPECTRA OF PEPTIDES

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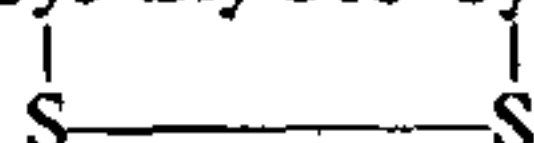
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### ABSTRACT

Two-dimensional correlated NMR experiments have been utilized for resonance assignments of two cyclic cystine peptides (I) Boc-Cys-Pro-Leu-Cys-NHMe and



(II) Boc-Cys-Gly-Pro-Cys-NHMe and a linear hexapeptide



(III) Boc-Asp(Bzl)-Leu-Thr-Gly-Gly-Val-OBzl.

Complete resonance assignments have been obtained, by a single two-dimensional correlated experiment in each case. The details of resonance assignment are outlined.

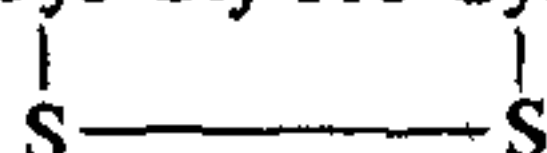
### INTRODUCTION

NUCLEAR magnetic resonance (NMR) plays a pivotal role in the study of structure and dynamics of biological molecules in solution. A central problem in the application of NMR to biological systems is the assignment of spectral lines to the various protons of the polymeric chain. Using chemical shift information, which provides the first clue, and spin-spin couplings, several conventional double-resonance techniques have been evolved to identify resonances that are spin-coupled<sup>1</sup>. However, one needs to perform a large number of selective decoupling experiments, with varying degrees of success, to obtain such information. The development of two-dimensional correlated spectroscopy (COSY) has provided a great impetus in this direction<sup>2-4</sup>. A single COSY experiment yields a totality of information on the coupling networks of a biological system and allows the assignments of all the spin-coupled resonances<sup>4-11</sup>. We have utilized this technique for assignment of resonances in several oligopeptides and in this paper, its application to two cystine-bridged cyclic tetrapeptides

(I) Boc-Cys-Pro-Leu-Cys-NHMe



(II) Boc-Cys-Gly-Pro-Cys-NHMe



and (III) Boc-Asp(Bzl)-Leu-Thr-Gly-Gly-Val-OBzl, is described. Peptides I and II are conformationally constrained disulphides used to model the active sites

of redox proteins like thioredoxin and glutaredoxin<sup>12</sup>. Peptide III is a synthetic fragment of an analog of sperm-activating peptide<sup>13</sup>. Assignment of <sup>1</sup>H resonances is a pre-requisite to the conformational analysis of these peptides by NMR. Using a single COSY experiment, a complete resonance assignment has been achieved in each case.

### METHOD

The COSY experiment consists of application of two 90° pulses, spaced at a time interval  $t_1$ , to a spin system in equilibrium and collection of signal as a function of time  $t_2$  immediately following the second pulse<sup>2</sup>. The experiment is repeated for a set of  $t_1$  values and the two-dimensional (2D) time domain data  $s(t_1, t_2)$  yields on 2D Fourier transformation, the 2D frequency domain spectrum  $S(\omega_1, \omega_2)$ . The 2D spectrum consists of peaks along the diagonal which represent the normal one-dimensional (1D) spectrum and off-diagonal peaks (cross-peaks) indicating that resonances at the two-frequency coordinates of the cross-peak are spin coupled. A single COSY experiment provides this information for the entire spectrum. Since the cross-peaks do not contain a net transfer but only a differential transfer of magnetization from one proton to another, an overlap of various lines of the multiplets of a given proton tends to cancel their intensities<sup>14</sup>. As a result, cross-peaks are observed only for large proton-proton couplings, such as two-bond and three-bond couplings. This is especially so, if the

data size of the 2D spectrum is severely limited, as is the case, in general for 2D spectroscopy.

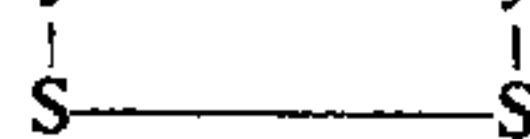
The 2D and the 1D spectra of peptides I-III are shown in figures. 1-5. The spectra have been recorded on a Bruker WH-270 FT NMR spectrometer of the Sophisticated Instruments Facility, Bangalore, with a home-written 2D software for the BNC-12 computer.

### MATERIALS AND RESULTS

The synthesis of I and II have been described<sup>15, 16</sup>, while III was prepared by classical solution phase procedure which will be detailed elsewhere. Approximately 10 mM solutions were prepared in CDCl<sub>3</sub> in each case and spectra recorded at room temperature.

#### A. Assignment of resonances of (I)

Boc-Cys-Pro-Leu-Cys-NHMe



It can be seen from figure 1, that the resonances at 6.6 and 2.8 ppm are mutually connected through two cross-peaks. These two peaks are unequivocally assigned respectively to the amide and the methyl protons of the terminal methylamide group. The high field amide resonance at 5.5 ppm is assigned to Cys (1), as it is adjacent to the urethane group<sup>17</sup> (figure 2). A cross-peak to this resonance leads to the assignment of the C<sup>α</sup>H proton at 4.8 ppm. Finally, the cross-peak at the chemical shift coordinates (4.8, 3.3) identifies the two C<sup>β</sup>H<sub>2</sub> protons of Cys (1) at 3.3 ppm. Out of the remaining two unassigned amide resonances, the one

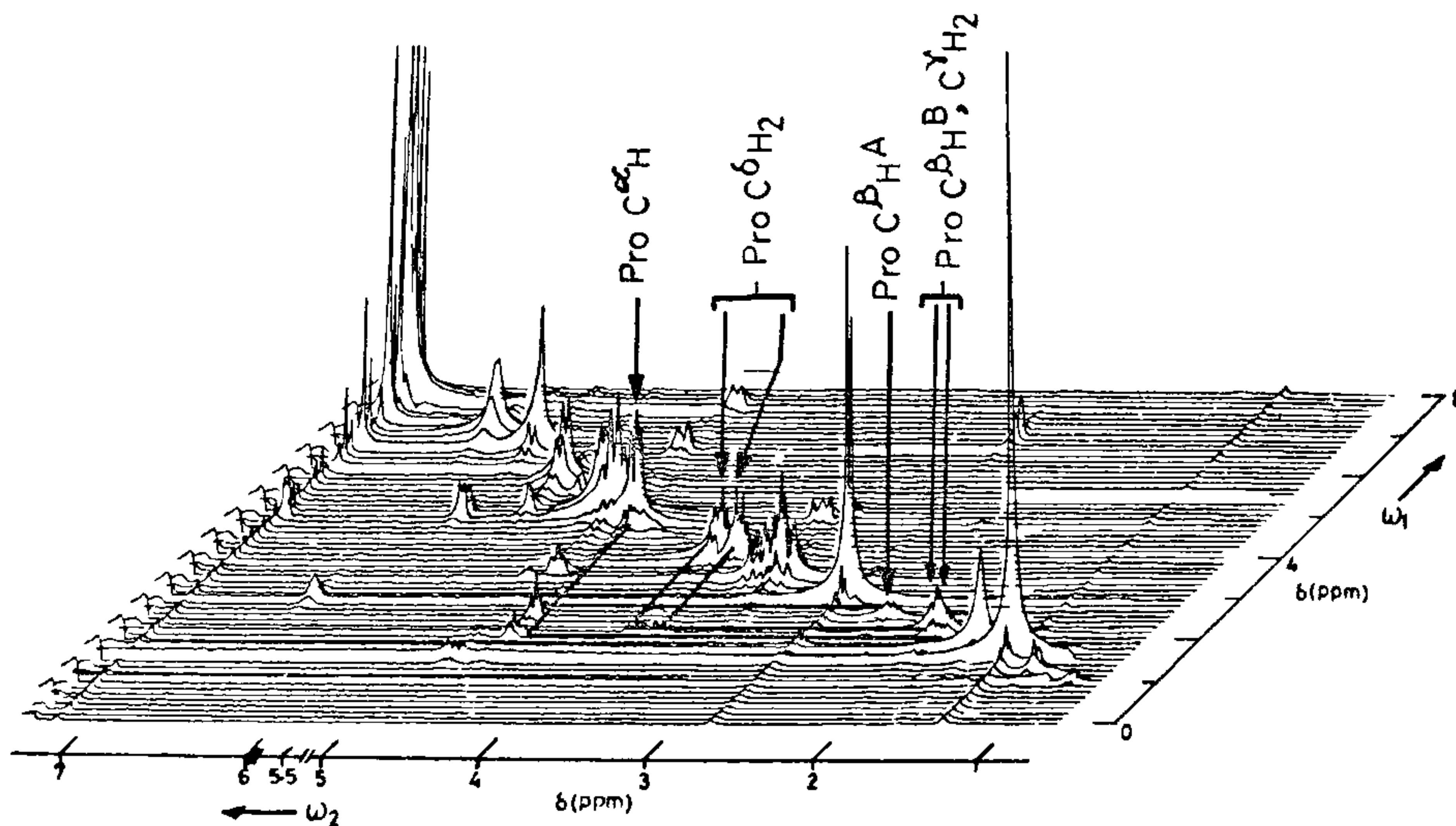


Figure 1. 270 MHz two-dimensional COSY spectrum of (I) Boc-Cys-Pro-Leu-Cys-NHMe. The algorithm



used for the 2D experiment is as follows. The  $t_2$  time domain data, was collected in the memory of the computer using 4K data points. Single-channel detection was used with the carrier placed at the left hand end of the spectrum. This data was Fourier-transformed and about 700 relevant data points of the frequency domain were stored in the disc. The experiment was repeated for 128  $t_1$  values at intervals of 0.25 msec, covering the full frequency range of  $\omega_1$  domain. The  $\omega_2$  axis is therefore broken into three blocks while the  $\omega_1$  axis is continuous. The diagonal of the spectrum runs from left hand top corner to the right hand bottom corner. The digital resolution in the  $\omega_2$  domain was 1 Hz/point and in the  $\omega_1$  domain was 34 Hz/point. The same representation is used in all the COSY spectra of this paper. The assignment of the Pro residue is indicated by thick lines.

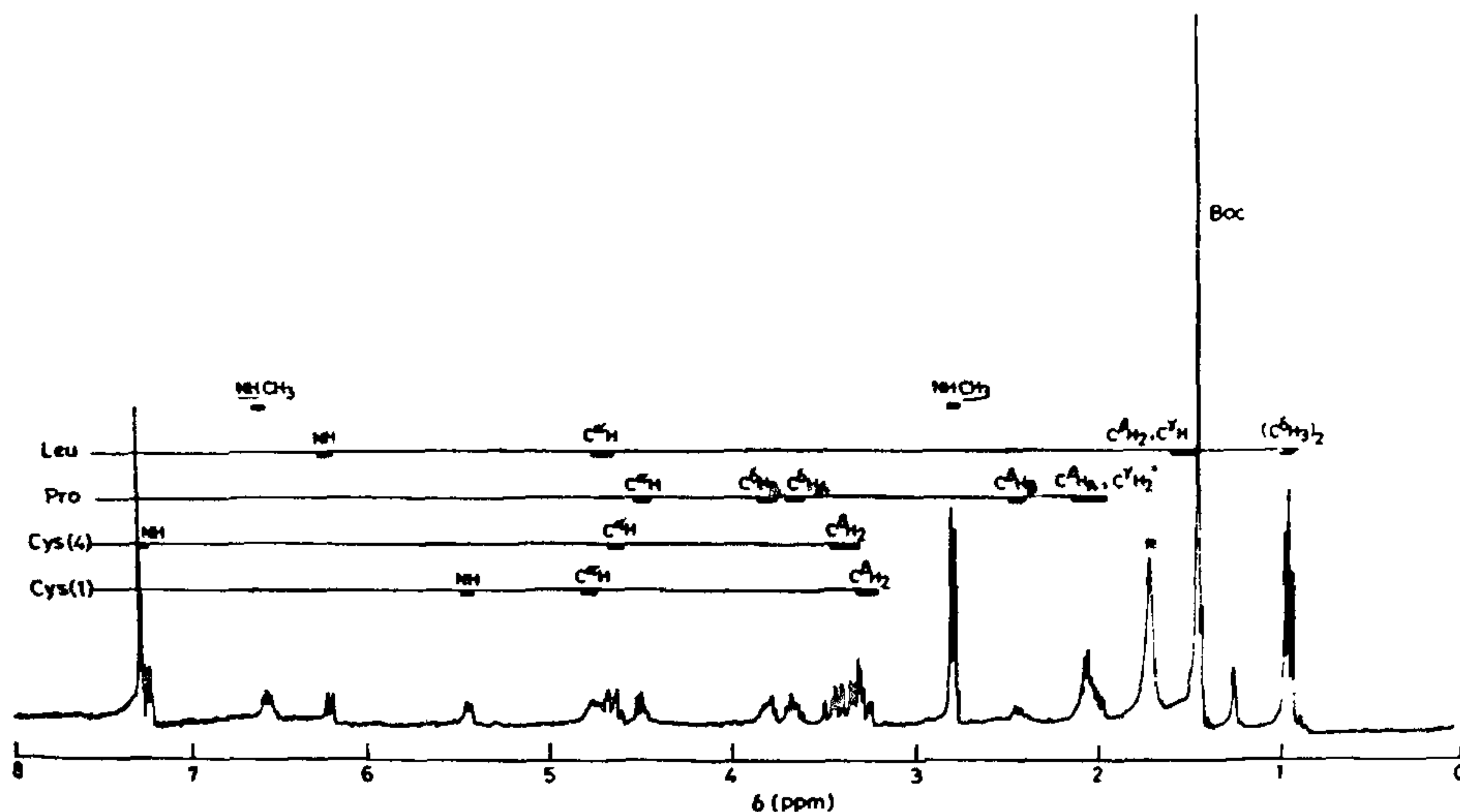


Figure 2. 270 MHz NMR spectrum of (I) Boc-Cys-Pro-Leu-Cys-NHMe with the final assignments obtained from the 2D COSY spectrum of figure 1.

at 6.2 ppm has a cross-peak to a C<sup>α</sup>H proton at 4.7 ppm which in turn has a cross-peak to resonance at 1.5 ppm. Finally, this resonance has a cross-peak leading to the diagonal peak at 1 ppm\*, a characteristic value for a methyl resonance. These resonances are identified as belonging to leucine, the only residue having a methyl group in the sidechain, with the C<sup>β</sup>H<sub>2</sub> and C<sup>γ</sup>H resonances overlapping at 1.5 ppm. Since the C<sup>β</sup>H<sub>2</sub> and C<sup>γ</sup>H resonances of leucine are overlapped, their connectivity cannot be established by the COSY spectrum. This was independently verified by 1D decoupling experiments<sup>16</sup>. Two more coupling networks can be identified in the COSY spectrum. One having resonances at 7.3, 4.6 and 3.4 ppm and the other at 4.5, 3.8, 3.7, 2.5 and 2 ppm. The former group has a resonance in the amide region and among the two remaining amino acid residues only Cys (4) has an amide proton. The resonances of the former group are therefore assigned respectively to the amide, C<sup>α</sup>H and

C<sup>β</sup>H<sub>2</sub> protons of Cys (4). The resonances of the Pro protons follow an interesting pattern and are shown in figure 1. The C<sup>α</sup>H proton at 4.5 ppm has two cross-peaks at 2.5 and 2 ppm identifying the two C<sup>β</sup>H<sub>2</sub> protons. The 2.5 ppm resonance has no clear-cut cross-peak, except a weak cross-peak in 2.1 ppm region indicating that both C<sup>β</sup>H<sub>2</sub> resonances are in the 2.1 ppm region. Two clear-cut cross-peaks from the 2.1 ppm region to 3.8 and 3.7 ppm, then identify the two C<sup>β</sup>H<sub>2</sub> resonances.

#### B. Assignment of resonances for (II)

Boc-Cys-Gly-Pro-Cys-NHMe



Two cross-peaks at 4.2 ppm and 3.6 ppm to the same amide resonances at 7.6 ppm, clearly seen in the COSY spectrum of this compound, point to the glycyl residue (figure 3). The Pro has C<sup>α</sup>H at 4.4 ppm, C<sup>β</sup>H<sub>2</sub> at 2.2 ppm overlapped with C<sup>γ</sup>H<sub>2</sub> resonances, which in turn has two clear-cut cross-peaks at 4.2 and 3.6 ppm, identifying the two C<sup>β</sup>H<sub>2</sub> protons. Such large chemical shift non-equivalence of the two C<sup>β</sup>H<sub>2</sub> protons of the Pro residue is a point of interest in this peptide, pointing to some rather unusual structural feature.

\* The diagonal peak at 1 ppm appeared with an unexpectedly low intensity. This is possibly due to limited digital resolution along the  $\omega_1$  direction in the spectrum, with the sharp methyl peak accidentally falling between two tracks, attenuating its intensity drastically.

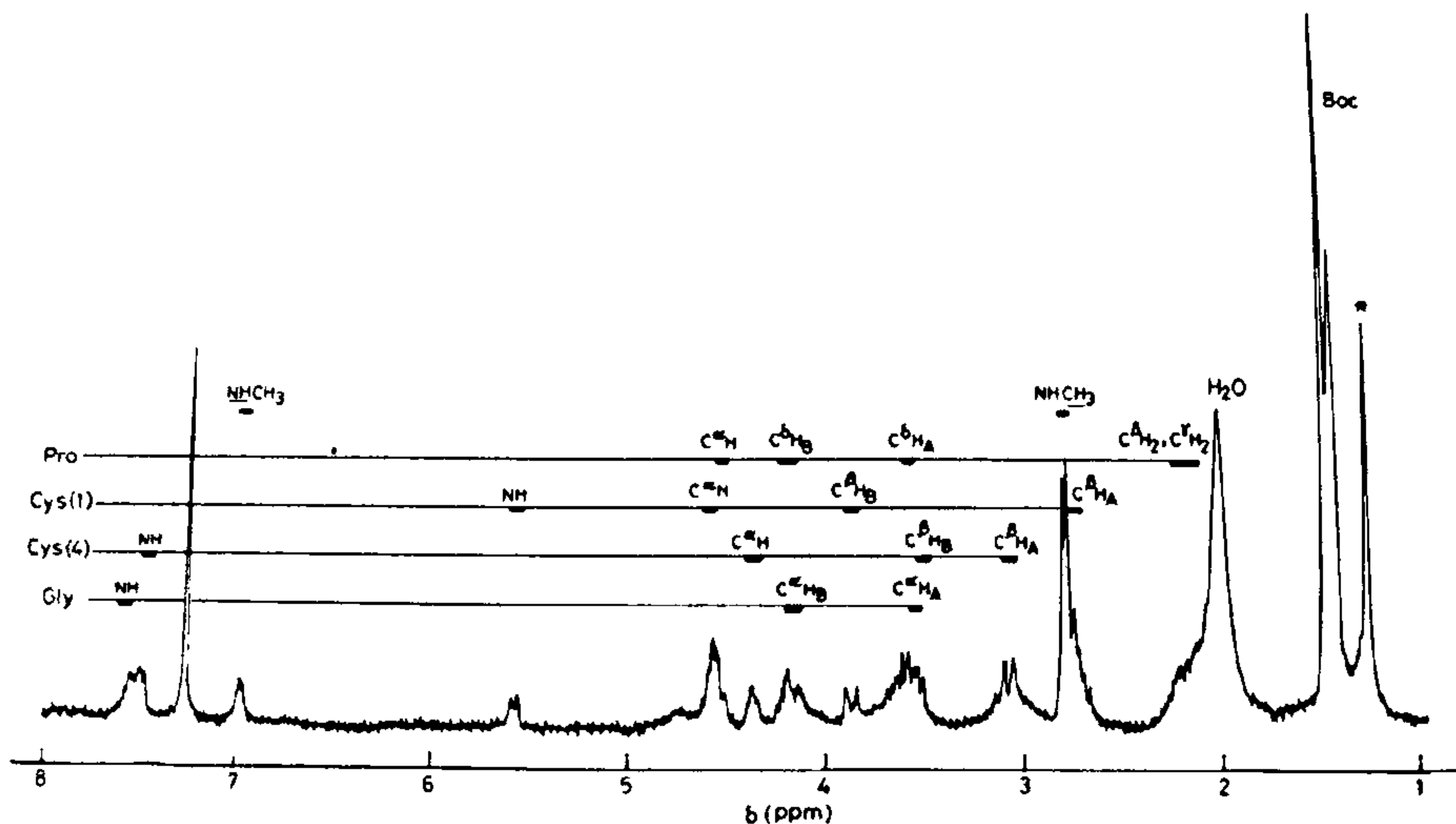


Figure 3. 270 MHz NMR spectrum of (II) Boc-Cys-Gly-Pro-Cys-NHMe with final assignments obtained from the 2D COSY spectrum.

The remaining assignments of the two Cys residues and the terminal methyl amide group are similar to those of (I) and are given in figure 3. It may be mentioned that the assignments of (I) and (II) obtained with the COSY spectra are consistent with those obtained with conventional 1D decoupling experiments<sup>15,16</sup>.

### C. Assignment of resonances for (III)

#### Boc-Asp(Bzl)-Leu-Thr-Gly-Gly-Val-OBzl

Double cross-peaks to the same amide resonance, characteristic of a glycylic residue, identify the two glycylics having amide resonances at 7.4 and 7.6 ppm and C<sup>α</sup>H resonances at 3.6\* and 4.1 ppm and 3.7 and 4.05 ppm respectively (figure 4). The high field amide resonance at 5.8 ppm is assigned to Asp, which is blocked with a urethane protecting group<sup>17</sup>. A cross-

peak to this amide identifies the C<sup>α</sup>H at 4.5 ppm. The multiplet at 2.9 ppm is characteristic of Asp C<sup>β</sup>H<sub>2</sub> protons, which also shows cross-peak to the C<sup>α</sup>H at 4.5 ppm, completing the Asp assignment. The Leu assignment follows a pattern similar to (I) and the resonances at 7, 4.2, 1.7 and 0.9 ppm are identified as belonging to NH, C<sup>α</sup>H, C<sup>β</sup>H<sub>2</sub> and (C<sup>β</sup>H<sub>3</sub>)<sub>2</sub> protons respectively, with the C<sup>γ</sup>H resonances overlapped with C<sup>β</sup>H<sub>2</sub> resonances. The multiplet at 2.2 ppm is unequivocally assignable to Val C<sup>β</sup>H, which has clear cross-peak at 1 ppm, identifying its methyl groups and at 4.5 ppm, identifying its C<sup>α</sup>H multiplet (figures 4 and 5). The downfield methyl doublet at 1.2 ppm is assigned to threonine, as there is an OH group attached to the C<sup>β</sup> carbon in threonine. The cross-peak to this resonance at 4.6 ppm identifies its C<sup>β</sup>H resonance. There are two amide doublets at 6.9 and 7.3 ppm, both having cross-peaks in the C<sup>α</sup>H region at 4.5 ppm, the former of these is tentatively assigned to the amide of Thr and the latter to Val. The threonine C<sup>α</sup>H at 4.5 ppm and C<sup>β</sup>H at 4.5 ppm lie too close to yield resolvable cross-peaks. The complete assignments are given in figure 5.

\* Cross peak seen clearly in a replot of the same data set with a higher gain.

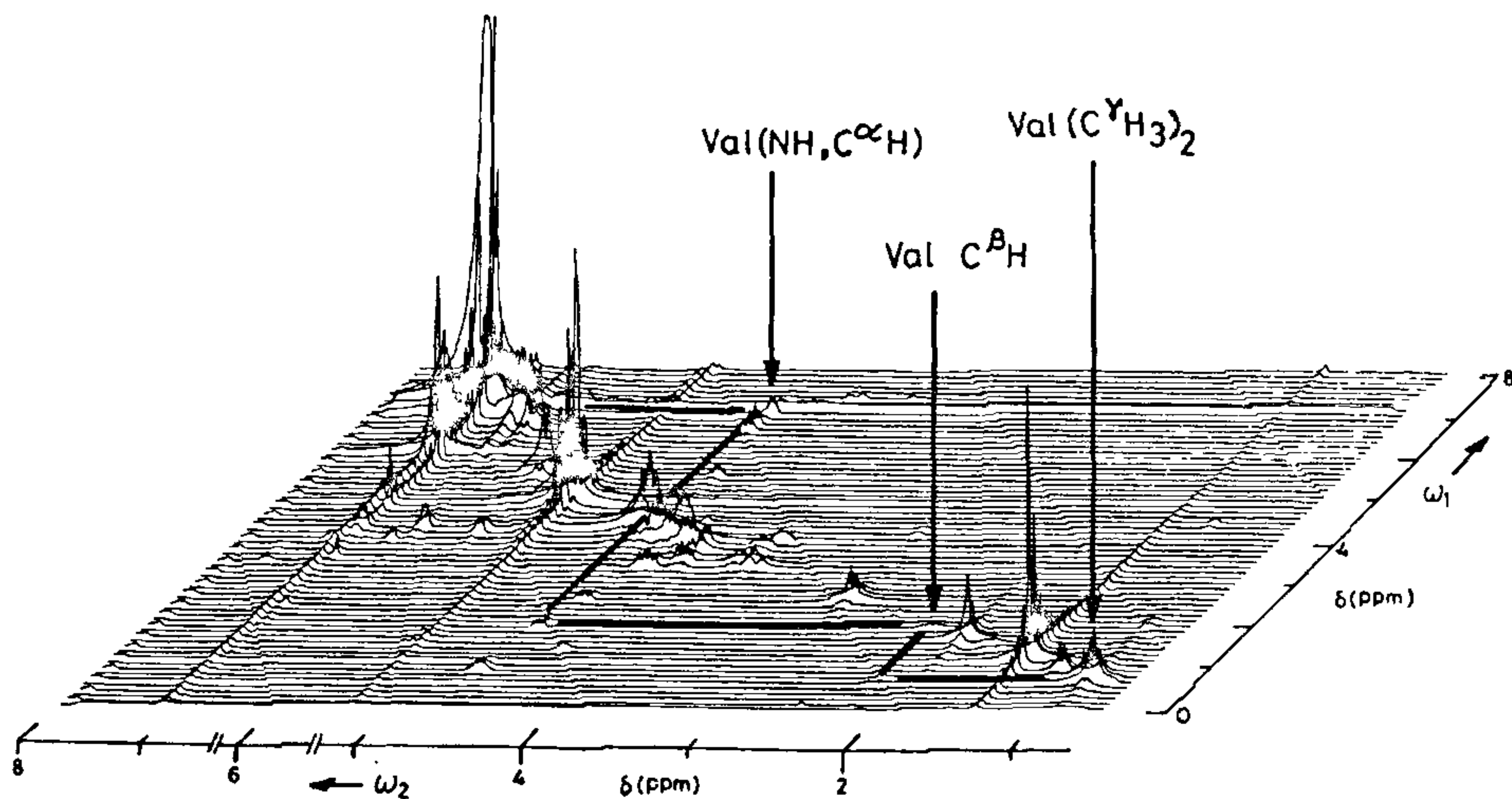


Figure 4. 270 MHz 2D COSY spectrum of (III) Boc-Asp(Bzl)-Leu-Thr-Gly-Gly-Val-OBzl. The assignment of Val residue is indicated by thick lines. For other details see caption for figure 1.

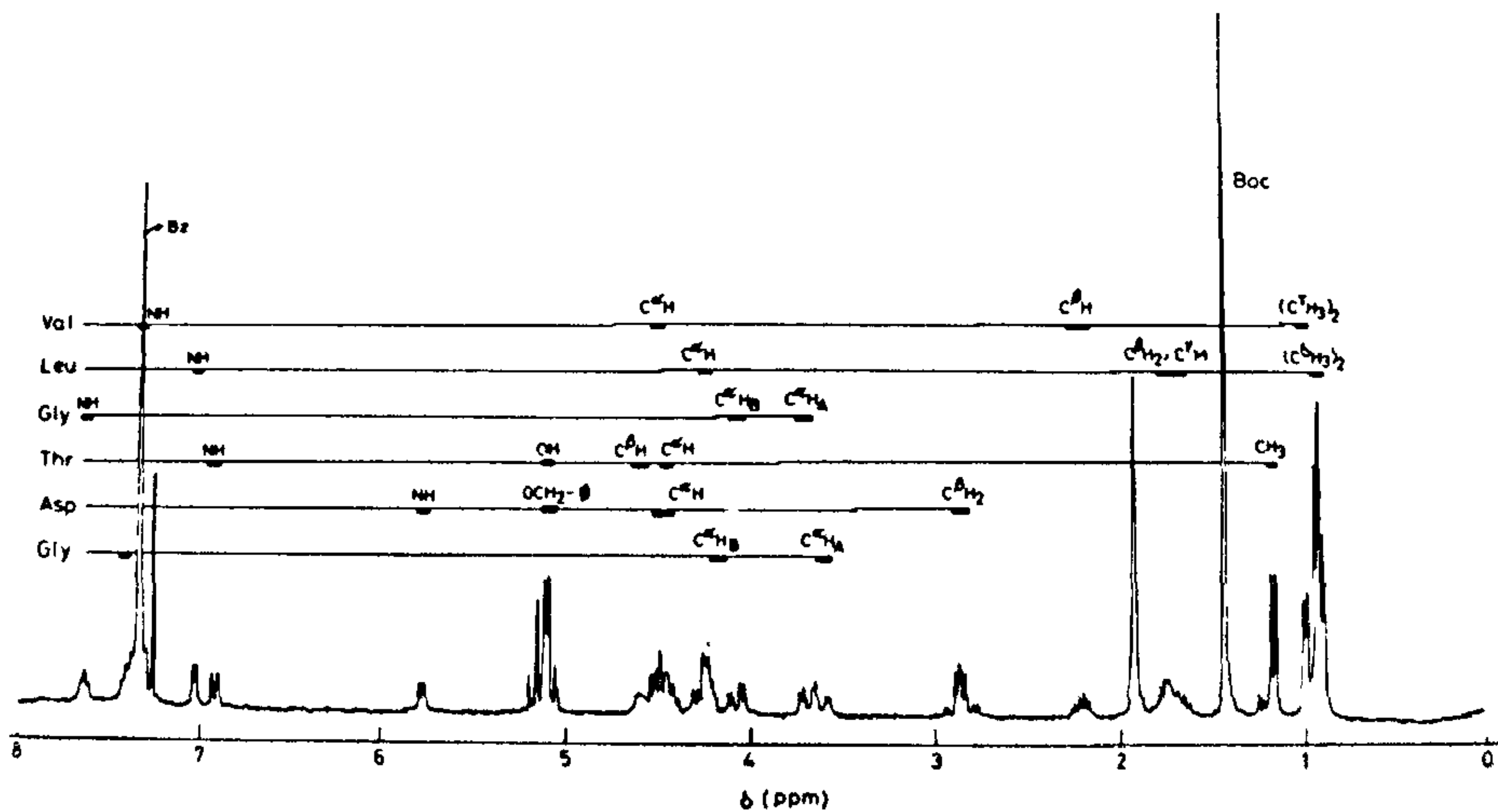


Figure 5. 270 MHz NMR spectrum of (III) Boc-Asp(Bzl)-Leu-Thr-Gly-Gly-Val-OBzl, with final assignments obtained from the 2D COSY spectrum of figure 4.

## CONCLUSIONS

Complete resonance assignments have been obtained for the three peptides by a single COSY experiment in each case. Such assignments using conventional decoupling experiments are considerably more time-consuming, especially as the number of amino acid residues increases in a polypeptide chain, as is the situation in III. These assignments have been obtained despite a rather limited data set, resulting in poor cross-peak intensities. The situation improves dramatically for a larger data set as well as by utilization of contour plots<sup>3-11, 14</sup>.

## ACKNOWLEDGEMENTS

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## ANNOUNCEMENT

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### 29TH GERMAN TIN DAY

The 29th German Tin Day will be held on 30th May, 1984 in Dusseldorf, on the theme "Organotin Chemistry", People interested in attending can obtain

further details from: Zinn-Informationsburo GmbH, Kasemenstrasse 13, 4000 Dusseldorf 1, Federal Republic of Germany.

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