# Structural Studies of α-Bungarotoxin. 2. $^1$H NMR Assignments via an Improved Relayed Coherence Transfer Nuclear Overhauser Enhancement Experiment†

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ABSTRACT: Complete sequence-specific assignments of the $^1$H NMR spectrum of bungarotoxin were reported in the previous paper [Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry* (first paper of three in this issue)]. The assignment was significantly aided by the use of the homonuclear Hartman–Hahn relayed coherence transfer nuclear Overhauser enhancement spectroscopy experiment (HRNOESY) which we present here, as a modification of relayed coherence transfer nuclear Overhauser enhancement spectroscopy (relayed NOESY) [Wagner, G. (1984) *J. Magn. Reson. 57*, 497]. As shown here, HRNOESY resolves problems of proton resonance overlap especially in extended chain conformations as found in β-sheets.

The sequential assignments of α-bungarotoxin, a 74 amino acid protein from the venom of *Bungarus multicinctus*, were presented in the previous paper (Basus et al., 1988). We have used the assignment procedure developed by Wüthrich and co-workers (Wüthrich et al., 1982; Billeter et al., 1982), which depends on the observation and assignment of nuclear Overhauser enhancements (NOEs) between the α-proton of one residue and the backbone NH proton of the next residue. Coincidence or near coincidence of chemical shifts hinders this assignment procedure. The NH chemical shifts, however, show a temperature dependence, which is, in general, different for each NH proton. This differential temperature dependence can be used, as has been done in the preceding paper, to identify the residue to which the NH chemical shift of a two-dimensional NOE (NOESY) cross-peak belongs, by comparison with two-dimensional *J*-correlated (COSY) or two-dimensional homonuclear Hartmann–Hahn (HOHAHA) spectra. However, the same cannot be done with the α-proton chemical shift or that of any other side-chain resonance since there is, in general, little or no temperature dependence of these shifts. As demonstrated in the preceding paper, the pH dependence of these resonances can be used to sort out the identity of the residue to which the α-proton or other side-chain chemical shift belongs. Although this procedure is able to provide us with more assignments, several of these resonances show little or no pH dependence. For the α-proton chemical shifts, which are essential for the sequential assignment, we have the added difficulty that these resonances are near the $H_2O$ solvent resonance, so that these cross-peaks can only be observed from the modulation of the NH resonances as the evolution time is incremented. Time restrictions on data acquisition limit the digital resolution in this dimension, further hindering the assignment of the NOESY cross-peaks in the α-proton region. Such problems become more severe for larger proteins since not only will there be more resonances in the same chemical shift range but also the resonances will be broader due to longer rotational correlation times.

An interesting experiment which would help in the assignment of such resonances has been developed by Wagner (1984), which he has called relayed NOESY. This experiment relays the magnetization transferred between two protons by NOE to a third proton via *J* coupling. Thus an NH to α-proton NOE can be relayed to the NH proton to which the α-proton is coupled. This should be sufficient to assign the α-proton chemical shift of a NOESY cross-peak, especially since we could now also make use of the differential temperature dependence of the NH chemical shifts. The sensitivity of this experiment is relatively low, however, and we present here a modification which improves its sensitivity. We also demonstrate the use of this experiment for the assignments of NOESY cross-peaks in α-bungarotoxin.

## Materials and Methods

*Sample Preparation.* Purification of bungarotoxin is described in the succeeding paper (Kosen et al., 1988). Samples used here contained 5–7 mM bungarotoxin in 95% $H_2O$–5% $D_2O$ solution and with approximately 1 mM sodium (trimethylsilyl)propionate (TSP) as a chemical shift reference.

*NMR Spectra.* All spectra were obtained with 800–1024 $t_1$ increments and according to the time-proportional phase incrementation procedure (Marion & Wüthrich, 1983), on a General Electric GN-500 NMR spectrometer modified for the HOHAHA experiment as described in the previous paper (Basus et al., 1988). A total of 4096 complex data points were acquired in $t_2$. Zero filling was used in $t_1$, creating a final real matrix of 1024 by 4096 data points in the $t_1$ and $t_2$ dimensions, respectively. Spectral widths of 6024 Hz were used in both dimensions. The carrier frequency was placed on the $H_2O$ resonance, which was irradiated at all times except for the evolution ($t_1$) and acquisition ($t_2$) periods. Base-line correction was used as described in the previous paper (Basus et al., 1988). All contour plots were plotted with a factor of 1.4 between consecutive contour levels.

## Results and Discussion

The relayed NOESY experiment (Wagner, 1984), as shown in sequence A of Figure 1, is composed of a standard NOESY sequence where incoherent magnetization is transferred during

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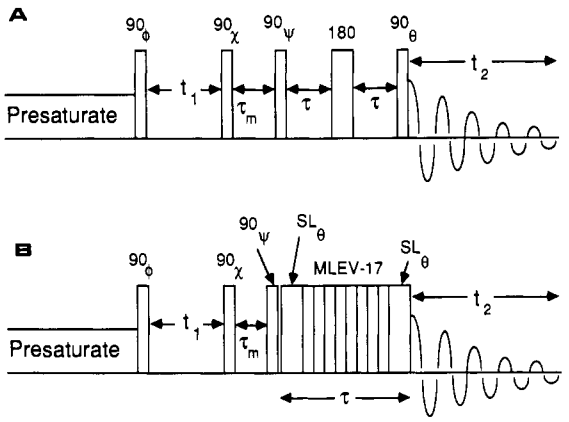


FIGURE 1: Pulse sequences for the relayed NOESY (A) and HRNOESY (B) experiments. The phase cycling is as follows: $\phi = (x,-x,y,-y,-x,x,-y,y)$; $\chi = (x,x,y,y,-x,-x,-y,-y)$; $\psi = x$; $\theta = y$. The signals are alternatively added and subtracted.

$\tau_m$ through the NOE between spin $H_k$ to spin $H_l$, followed by a coherent magnetization transfer step during $2\tau$ from spin $H_l$ to spin $H_m$ through $J$ coupling between $H_l$ and $H_m$. This creates a cross-peak in the relayed NOESY spectrum between protons $H_k$ and $H_m$, thus revealing an NOE between $H_k$ and $H_l$, even if the direct NOESY cross-peak between $H_k$ and $H_l$ is obscured by cross-peak overlap.

The coherent magnetization transfer step in the relayed NOESY experiment is based on the same principle as that of COSY spectra (Aue et al., 1976; Bax & Freeman, 1981). Recently, Bax and Davis (1985a) have developed a new sequence which transfers magnetization through a $J$-coupled network of spins via a homonuclear magnetization transfer based on the Hartmann–Hahn condition (Hartmann & Hahn, 1962) which had been developed for heteronuclear magnetization transfer. This homonuclear experiment was named HOHAHA (Bax & Davis, 1985a). It is equivalent to the TOCSY experiment developed by Braunschweiler and Ernst (1983), however with improved performance. It shows the same cross-peaks as in COSY spectra, with higher sensitivity in many cases (Davis & Bax, 1985), as well as extra peaks as are observable in relayed COSY spectra (Eich et al., 1982). With short mixing times, very few of these relayed cross-peaks will appear. All peaks are in the pure-absorption mode.

Our modification of the relayed NOESY experiment involves the replacement of the coherent magnetization transfer step with a spin-locked magnetization transfer as in the HOHAHA experiment. The sequence is shown in Figure 1. We have used the MLEV-17 version of the HOHAHA experiment (Bax & Davis, 1985b) for best results.

As in the relayed NOESY experiment, our HRNOESY (*H*OHAHA *R*elayed *NOESY*) can be done in two ways: one as shown in sequence B of Figure 1, with the incoherent magnetization transfer first, and another with the spin-locked magnetization transfer first. The resulting spectra are asymmetric, and the two sequences give spectra which are related to each other by a mirror image through the diagonal.

Since the information content in the NH to NH cross-peak region is the same for either sequence, only sequence B was used here. The results for a 7 mM solution of $\alpha$-bungarotoxin in $H_2O$ at 500 MHz, for both the HRNOESY experiment being introduced here and the relayed NOESY experiment, are shown in Figures 2 and 3. The relayed NOESY was obtained with $\tau = 31$ ms, which is optimum for 8-Hz coupling constants (neglecting relaxation effects), since the relayed cross-peak intensity is proportional to $\sin(2\pi J\tau)$ (Wagner, 1984), where $J$ is the coupling constant through which the

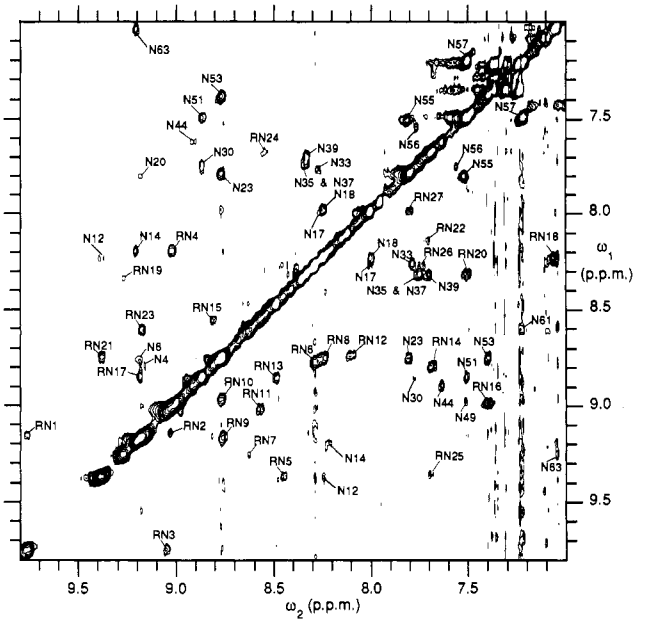


FIGURE 2: Portion of the HRNOESY spectrum showing most of the relayed NOESY cross-peaks (RN$i$) and also several NOESY cross-peaks (N$i$).

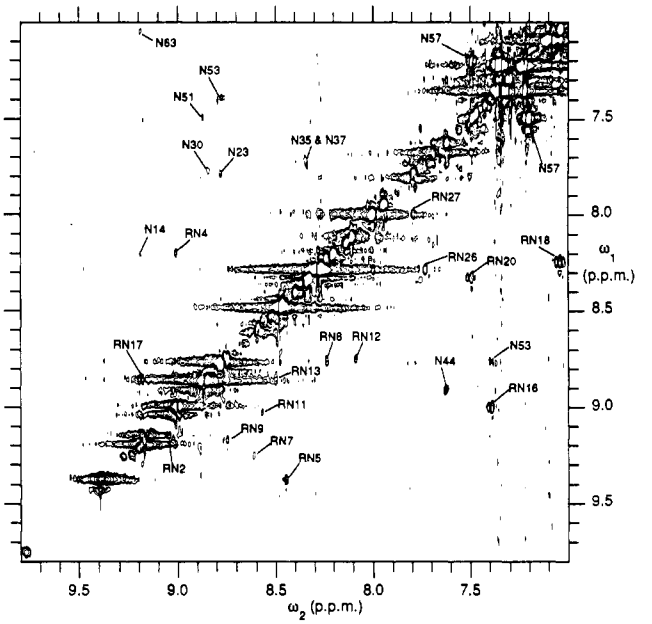


FIGURE 3: Same region as in Figure 2, using the original relayed NOESY pulse sequence (sequence A of Figure 1).

relay proceeds. On the basis of our experience with HOHAHA spectroscopy of this protein, the HRNOESY mixing time $\tau$ was chosen to be 30 ms to maximize peak intensities to the directly coupled protons. With larger mixing times more signal would be lost due to relaxation effects and by transfer of magnetization to other spins via $J$ coupling. Both experiments were run on the same sample in the same amount of time and at the same digital resolution. They were run consecutively, so that all other instrumental parameters such as, for example, static field homogeneity were the same for both experiments. The contour plot in Figure 3, the relayed NOESY experiment, is such that one more contour level (i.e., a factor of 1.4 lower) would be into the noise level of this spectrum. Both spectra were base line corrected in the same manner, as described under Materials and Methods. Close inspection of the cross-peaks far enough from the diagonal to be observable in Figure 3 shows that the intensity of these

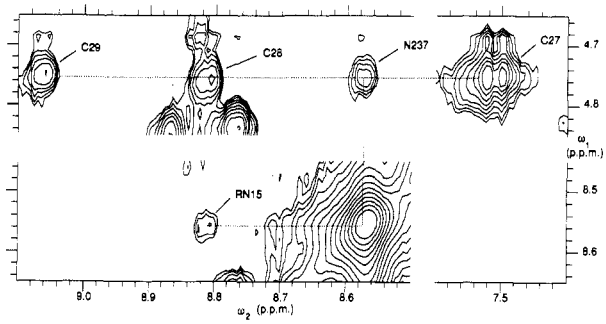


FIGURE 4: Portions of the HRNOESY spectrum of α-bungarotoxin showing the assignment of the α-proton chemical shift of the NOESY cross-peak N237 to the spin system of C28, a COSY (or HOHAHA) cross-peak.

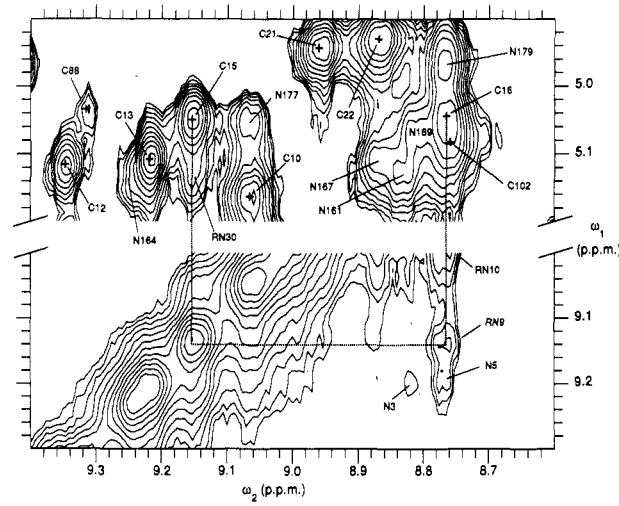


FIGURE 5: Portions of the HRNOESY spectrum showing evidence for the existence of a NOESY cross-peak between the NH of the spin system of C15 and the α-proton of the spin system of C16, at the position of the intraresidue NOESY between the α-proton and NH proton of C15 (COSY position indicated), through HRNOESY RN9.

peaks is at least a factor of 2 lower than that of those in the HRNOESY spectrum of Figure 2. In order to have a more direct comparison of mixing times, the relayed NOESY experiment was run with $\tau$ = 15 ms. The resulting spectra had a more intense diagonal, and many of the cross-peaks that are observable in Figure 3 were lost in the dispersive tails of the diagonal peaks.

The relayed NOE cross-peaks were identified by comparison with the NOESY and COSY or HOHAHA spectra and are labeled RN*i*, where *i* corresponds to an arbitrary numbering scheme. A comparison of the HRNOESY and the relayed NOESY spectra of Figures 2 and 3 shows that 15 of the 27 relayed NOE cross-peaks in the HRNOESY spectrum are seen in the relayed NOESY spectrum. It is possible, as indicated by Wagner (1984), that other relayed NOE cross-peaks may appear with a different mixing time. However, with a single instrument setting the HRNOESY spectrum shows almost twice as many NOE cross-peaks as the relayed NOESY spectrum.

Interpretation of the relayed peaks is illustrated in Figure 4. The HRNOESY spectrum contains direct NOESY (denoted N*i*) and HOHAHA (C*i*) cross-peaks in addition to the relayed (RN*i*) peaks. The NOESY cross-peaks arise from magnetization which is not relayed through *J* coupling during the mixing time $\tau$, and HOHAHA cross-peaks arise from magnetization which was not transferred to a neighboring spin during $\tau_m$. The relayed cross-peak RN15 reveals that the α-proton involved in the NOESY cross-peak N237 is the same as that giving rise to the COSY (or HOHAHA) cross-peak C28. This relayed cross-peak is generated as follows: (1) Magnetization due to the NH component of N237 (an α-proton to NH NOESY cross-peak is also present in this spectrum) precesses with a frequency $\omega_1$ = 8.56 ppm during $t_1$ and is transferred to the α-proton of C28 via NOE during the mixing time $\tau_m$. (2) In this first mixing time, the frequency labeling in the evolution time $t_1$ which had been converted to longitudinal magnetization by the second 90° pulse is being transferred via NOE to the α-proton of C28, remaining as longitudinal magnetization. (3) The third 90° pulse converts this to transverse magnetization and is followed by a spin-locking field modulated with an MLEV-17 sequence, as in the HOHAHA experiment (Bax & Davis, 1985b), during a second mixing period labeled as $\tau$ in Figure 1. In this mixing period, the homonuclear Hartmann–Hahn condition is established, and magnetization transfer occurs via *J* coupling from the α-proton component of N237 to the NH to which it is coupled at a frequency $\omega_2$ = 8.81 ppm. Figure 4 shows that the α-proton component of N237 could have been assigned to the α-proton of the spin systems of C27, C28, or C29. The $\omega_2$ frequency of RN15 corresponds to that of C28, so that at least the major part of N237 is due to an NOE to the α-proton of the spin system of C28. The NH chemical shift of N237 corresponds to that of the spin system of C7, so that RN15 establishes the existence of an NOE between the NH of C7 and the α-proton of C28.

From the complete assignment (Basus et al., 1988), C7 corresponds to Val-57 and C28 to Glu-56. These residues are in the β-sheet shown in Figure 8 of the previous paper. In β-sheets, both the NOE between the NH and α-proton of sequential residues and the coupling between the NH and α-proton of each residue are large. This situation should give rise to the largest relayed cross-peaks in the HRNOESY spectrum. Fifteen sequential α-proton to NH cross-peaks are expected in this β-sheet. Of these only three did not show the corresponding HRNOESY cross-peaks. The missing cross-peak between Tyr-24 and Arg-25 can be explained by the proximity of this cross-peak to the diagonal since the NH chemical shifts are 9.05 and 8.99 ppm, respectively. A similar situation is found between Cys-59 and Cys-60 where the chemical shifts are 9.16 and 9.19 ppm, respectively. The region around Gly-43 is also missing HRNOESY cross-peaks. This can be explained by the weakness of the HOHAHA cross-peaks for this residue, which indicates small α-proton to NH coupling constants. This may be due to a different conformation for this residue than would be expected for a residue in a β-sheet. In the crystal structure, the β-sheet appears to twist starting at this residue which would be consistent with an unusual conformation for this residue.

An unusual overlap occurs between the intraresidue NOESY cross-peak involving the α-proton and NH proton of spin system C15 and the interresidue NOE between the α-proton of spin system C16 and the NH of spin system C15 at pH 4.8. The existence of the interresidue NOE could only be established by the presence of the HRNOESY cross-peak RN9, as shown in Figure 5. Spin systems C16 and C15 were assigned to the sequential residues Cys-3 and His-4, respectively, mostly on the basis of this NOE.

Since HOHAHA spectra show cross-peaks relayed through one or more couplings, as well as cross-peaks between directly coupled spins, care must be taken in the interpretation of HRNOESY spectra. If there is a relatively strong HOHAHA cross-peak from the NH to a β- or γ-proton, then the NOESY spectrum should be checked in the region of the β- or γ-protons

for a relatively strong NOE between these protons and a second NH proton, for which such a situation could also give rise to an HRNOESY cross-peak linking these two NH proton resonances. For none of the HRNOESY cross-peaks of α-bungarotoxin has there been any ambiguity of this nature, although in some cases the β-proton to NH HOHAHA cross-peaks was relatively strong.

In conclusion, the HRNOESY experiment has proven quite useful in resolving assignment ambiguities for NOESY cross-peaks involving α-proton resonances which overlap with other resonances. In particular, for α-bungarotoxin it has made possible the assignment of 13 NOESY cross-peaks not assignable previously due to overlap, even after taking advantage of the NH chemical shift temperature dependence. Only one of these NOESY cross-peaks was due to a nonsequential NOE. The sensitivity improvement and reduction of diagonal peak dispersion components, as compared to the original relayed NOESY experiment, have made this experiment more useful for obtaining resonance assignments in larger proteins, where coincidence of chemical shifts will occur more often. HRNOESY is especially useful for those proteins containing β-sheets where the NH to α-proton NOEs and $J$ couplings are favorable for the regular appearance of relayed cross-peaks.

**Registry No.** α-Bungarotoxin, 11032-79-4.

REFERENCES


Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229.

Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry* (first paper of three in this issue).

Bax, A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542.

Bax, A., & Davis, D. G. (1985a) *J. Magn. Reson. 63*, 207.

Bax, A., & Davis, D. G. (1985b) *J. Magn. Reson. 65*, 355.

Billeter, M., Braun, W., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 321.

Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521.

Davis, D. G., & Bax, A. (1985) *J. Am. Chem. Soc. 107*, 2821.

Eich, G., Bodenhausen, G., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 3732.

Hartmann, S. R., & Hahn, E. L. (1962) *Phys. Rev. 128*, 2042.

Klevit, R. E., & Drobny, G. P. (1986) *Biochemistry 25*, 7770.

Kosen, P. A., Finer-Moore, J., McCarthy, M., & Basus, V. J. (1988) *Biochemistry* (third paper of three in this issue).

Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967.

Wagner, G. (1984) *J. Magn. Reson. 57*, 497.

Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 319.


# Structural Studies of α-Bungarotoxin. 3. Corrections in the Primary Sequence and X-ray Structure and Characterization of an Isotoxic α-Bungarotoxin†


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ABSTRACT: The most plausible set of chemical shift assignments for α-bungarotoxin as deduced from the combined use of two-dimensional $J$-correlated and two-dimensional nuclear Overhauser effect $^1$H nuclear magnetic resonance (NMR) spectroscopy was in conflict with the accepted amino acid sequence between residues 8 and 12 and residues 66 and 70 [Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry* (first paper of three in this issue)]. Furthermore, NMR spectra of α-bungarotoxin, purified by conventional methods, evidenced a second species at the level of approximately 10% total protein. The minor component was separated from α-bungarotoxin by Mono-S (cationic) chromatography. Sequencing of Mono-S-purified α-bungarotoxin and one of its tryptic peptides showed that the correct sequence for α-bungarotoxin is Ser-Pro-Ile at positions 9–11 and Pro-His-Pro at positions 67–69. The electron density map of α-bungarotoxin [Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37] was refined with the new sequence data. Improvements in the structure were found primarily for residues 9–11. Sequence analysis of two overlapping tryptic peptides proved that the minor species differed from α-bungarotoxin by replacement of a valine for an alanine at position 31. This new toxin, α-bungarotoxin$^{(Val\text{-}31)}$, binds to the acetylcholine receptor with an affinity that is comparable to that of α-bungarotoxin.


α-Bungarotoxin is a 74-residue protein belonging to the homologous family of long (snake) neurotoxins [reviewed by Karlsson (1979), Low (1979), Bystrov et al. (1983), and Dufton and Hider (1983)]. Because these neurotoxins are readily available and because they bind tightly to the nicotinic acetylcholine receptor of vertebrate muscle and electric eel organ, α-bungarotoxin and others of this toxin family have been extensively utilized in physiological studies directed at understanding neuromuscular transmission in general and in biophysical studies directed at the structure and mechanism of the acetylcholine receptor [e.g., Changeux et al. (1970), Albuqueruqe et al. (1979), Bystrov et al. (1983), and Surin et al. (1983)]. In the future, a toxin–receptor complex may find use as a direct model system for high-resolution membrane protein–protein structural studies.


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