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(U) Magnetic Resonance Elucidation of Molecule/Macromolecule Interaction Stereochemistry

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None.

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

We continue to make significant progress in the development of software for: a) simulating accurate theoretical NOESY spectra based on model structures, motional assumptions, and experimental parameters; b) the statistical comparison of model-dependent calculated spectra and experimental data; and c) the automated extraction of experimental constraints from raw NOESY data. The latter program (DISCON) has been tested using data for a rigid steroid model (in both the small molecule and slow tumbling regime) and simulated data for both a B-DNA and peptide structure. It is currently being used to refine the structures of: GnRH analogs, several peptide macrolide antibiotics, yeast α-factor and small neuropeptides in micelle-associated states, and endothelin (a 21mer bisdisulfide vasoconstrictor produced in the endothelium of the vasculature)

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PRINCIPAL INVESTIGATOR: Niels H. Andersen, Professor of Chemistry

CONTRACTOR: University of Washington

CONTRACT TITLE: **Magnetic Resonance Elucidation of Molecule/Macromolecule Interaction Stereochemistry**

CONTRACT PERIOD: 88FEB01 to 91JAN31

RESEARCH OBJECTIVES: To develop and experimentally verify data collection and reduction protocols suitable for the definition of molecule structure in: 1) the free solution state, 2) the membrane-lipid associated state, and 3) protein-bound states that represent or mimic the steps of molecular recognition and receptor interaction. The specific emphasis will be on computer-aided conformation elucidation.

The anticipated products of this effort will be computer programs that facilitate NOESY-based conformation elucidation and are applicable to data collected for small molecules in various states including dynamic equilibria that include "receptor-bound" states. Application of these programs will be illustrated with rigid models systems, more flexible receptor probes and peptides and, if available, small protein and DNA systems.

PROGRESS (Year 02 + 3/90):

Program NOESYSIM has been revised to include the more theoretically correct Tropp and Woessner[1] treatments of methyl group cross-relaxation and internal relaxation respectively. The final bugs should be removed in all software in use or distributed after 5/90.

DISCON, our program for extracting distances (fully corrected for spin diffusion artefacts) was announced at the 30th ENC in April 1989.[2] In that presentation we used an experimental data set for fluoromethalone (in viscous media such that $\tau_c \cong 1$ vis) as a test. We have now compared four different data sets corresponding to this motional regime. Distances out to 4.0Å were measurable in our experiments. The rms error of distance estimation for individual experiments ranged for 0.33 - 0.49 Å (distances involving methyl groups were not used for this comparison due to the additional geometry factors that confluence $\sigma_{Me,H}$). If the "distances" from each experiment are viewed as independent obervations, precision only slightly exceeds accuracy. In fact, the averages of experimental distances from these four NOESY data sets display much greater accuracy: rms error of averages, 0.23Å (→ 3.4Å), 0.30Å (→ 4.0Å). We have concluded that much of the error is associated with the peak volume integration routine we used to extract the signal intensities.

Recently we collected one additional data set for fluoromethalone paying particular attention to peak quantitation techniques. The data was for a non-viscous solvent in which this steroid displayed τ_c = 210ps. As usual for small molecules, the detection limit corresponded to 3.4Å rather than 4Å. However the rms error was much improved (0.13Å).

Four NOESY data sets for lysobactin, a rigid backbone undecapeptide macrolide, have been subjected to DISCON analysis to derive a new set of distance constraints. (We had previously derived constraints from one of the data sets using a modified ISPA[3]). A total of 66 constraints for refinement (using CHARMm dynamics and minimization) were selected from the DISCON output. These included 30 intraresidue, 23 sequential, and 13 long-range constraints with the following bounds assigned from the DISCON error analysis.

distance range	# of constraints	ave bounds range (Å)
2.0 - 2.7 Å	21	0.23
2.71- 3.1 Å	23	0.29
3.15- 4.4 Å	22	0.54

The best fitting structures from the refinement displayed only two bound violations exceeding 0.4 Å. The typical values of Σ (violations) was 1.1-1.6 Å, quite remarkable for a set of 66 constraints with an average experimental error of only ±0.18 Å. The resulting "structure" also is of high precision; the backbone rmsd for the 3 best fitting structures in the set was 0.29 Å.

We believe these examples fully substantiate our claim that DISCON will provide experimental distances of sufficient accuracy for high precision conformational analysis.

One further test of DISCON deserves mention. It is based on simulated data and is our first foray into the area of DNA structural analysis. Brian Reid and K. Banks of this department had previously used a regularized B-DNA model of a self-complementary dodecamer $[d(CGCGAATTCGCG)]_2$ to generate simulated spectra* for a test of a refinement strategy[4] that can be outlined as: NOESYs (at several τ_m) → initial bounds (from a linear growth ISPA analysis) $\left[\xrightarrow{\text{DSPACE}}\right.$ model #n $\xrightarrow{\text{BKCALC}}$ compare simulated NOESY to "experiment" and adjust bounds$\left.\right]_4$ $\xrightarrow{\text{DSPACE}}$ Refined Model. The Reid/Banks procedure used τ_m = 30, 60, 90, 150 ms simulated data sets to estimate observable NOE build-up rates. We applied model-free DISCON analysis to the 60 and 150 ms data (with no noise) and a 100 ms τ_m simulation which included resonable levels of noise. The rms deviations (relative to B-Model, the coordinates that generated the simulated data) of distance constraints by these methods are summarized below.

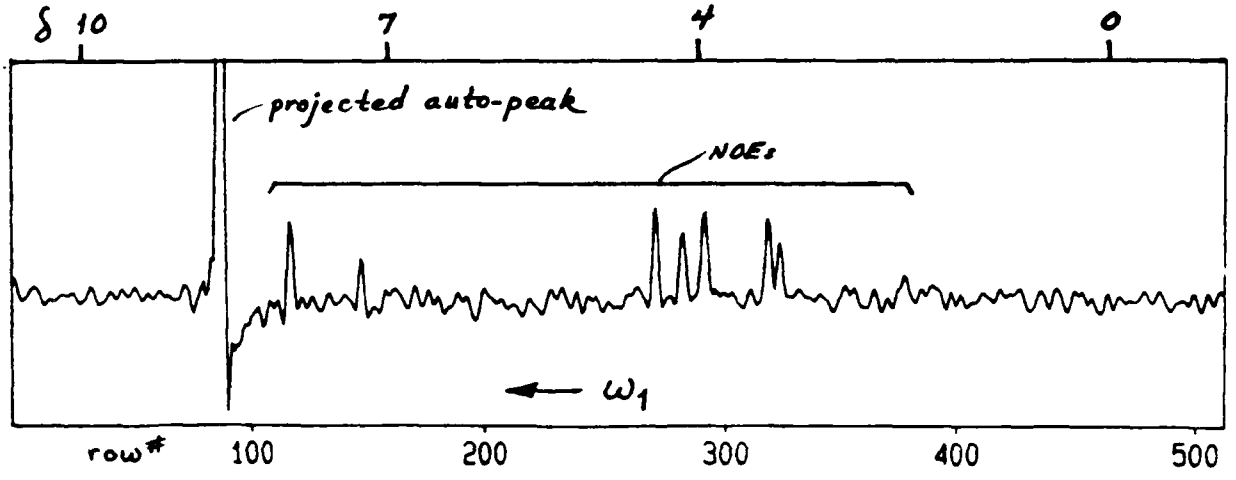
	r.m.s. deviation (Å)				
	B-MODEL		DISCON		
r_{ij} range (Å)	initial rate ISPA bounds	final bounds 4 BKCALC cycles	60 ms	150 ms	100 ms noise
1.91-2.3	0.09	0.12	0.01	0.04	0.02
2.31-3.1	0.11	0.09	0.02	0.08	0.06
3.11-3.8	0.33	0.07	0.03	0.17	0.20
3.81-4.7	0.77	0.22	0.17	0.21	0.41

* NOESYSIM reproduces the semi-empirical BKCALC simulation to better than ±3% (relative) when we use τ_c = 3.5 ns, ρ^* = 0.19 s^{-1}.

Experimental Results.

Further studies of the binding of Trp and 6-F-Trp enantiomers to albumin have confirmed that the off-rates are such as to complicate the derivation of a specific-receptor bound conformation. Additional studies are proposed.

We recently worked out procedures for obtaining NOESY data (including backbone NH connectivities) for yeast α-factor (a 13mer pheromone) and [Arg^8]-α-neoendorphin(1-8), a neuropeptide, in micelle-associated states. Other workers have had to relie on spectra in D_2O which thus lack the structure diagnostic NH interactions. A cross-section of the NOESY of the latter neuropeptide, showing the cross-relaxation spectrum of one of its backbone Gly-NH resonances, appears below.



Clearly we will be able to obtain the required matrix of data necessary for structure elucidation. The yeast α-factor data shows even greater S/N and resolution.

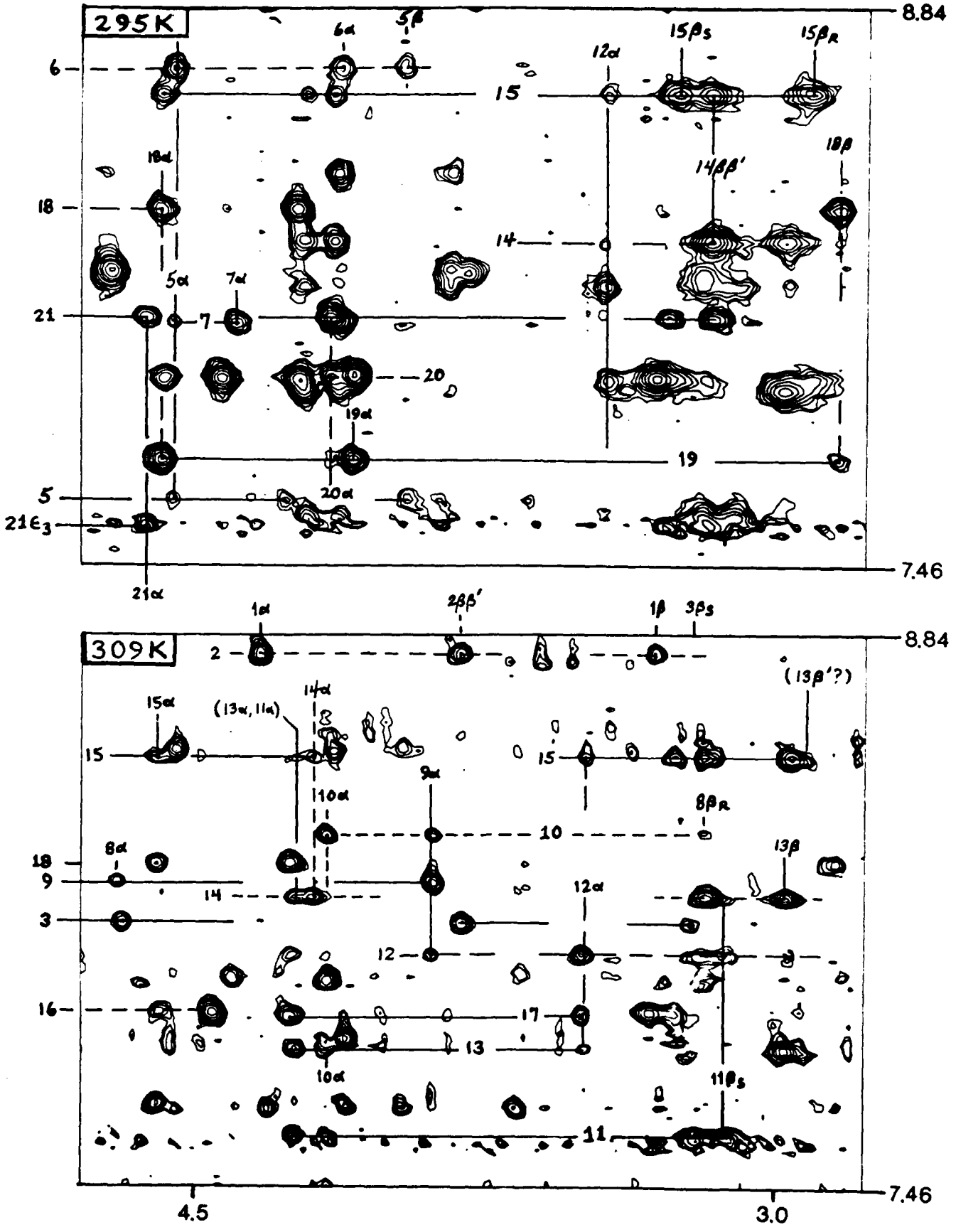
Endothelins, members of a new class of peptide vasoconstrictors, have been the other major arena for data collection. Three representative structures are shown below

							6		8								17	18				
ET-1	C	S	C	S	S	L	M	D	K	E	C	V	Y	F	C	H	L	D	I	I	W	
	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
ET-2	:	:	:	:	:	W	L	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
ET-3	:	T	:	F	T	Y	K	:	:	:	:	:	:	Y	:	:	:	:	:	:	:	:

ET-2 is perhaps the most potent endogenous vasoconstrictor known. As 21-residue bisdisulfides, they should be ammenable to conformation elucidation by 2D NMR using DISCON and restrained dynamics annealing.

We have now collected NOESY data for native ET-1 endothelin in a variety of water/glycol mixtures at 295 and 309° K for both the protonated and neutral His^{16} forms in both protonic and deuteronic media. The spectra have been completely assigned at both pHs and at glycol concentrations from 20-80% (v/v). At 60% glycol the temperature gradients for backbone amide-NHs span a wide range (-11.4 → +2.7 ppt/°C) and thus NOESY spectra at different temperatures provide different unambiguous cross-peak intensi- ties. **Figure S1** illustrates this by partially annotated NH/α, /β-H region NOESY for the acid form of ET-1.

Figure S1. The crowded NH/αH βH region of the NOESY spectra of 4 mM ET-1 in acidic 4:6 H_2O/d_6-glycol at 309°C (τ_m= 100 ms) and 295°C (τ_m = 160 ms).



Quantitative analysis and refinement of endothelin data sets is in progress, but a number of structural features are obvious from a qualitative analysis: 1) residues 9 → 15 form a distorted helix, 2) segmental motion occurs in the backbone for at least a portion of the 1 → 4 region and also probably at 7, 8, 3) a relatively persistent turn occurs in the vicinity of Ser^5, and 4) the C-terminal tail is not a random coil as might be expected.

WORK PLAN (year 03):

Software Development. We plan to develop automated routines for converting DISCON output into constraints suitable for direct use in XPLOR,[5] the present gold-standard of restrained dynamics annealing programs for structure refinement based on NOESY data. At present[6] this program is typically used with very wide bounds categories such as: 1.8-2.7, 2.0-3.5, 2.3-5 and 2.8-6Å. We expect DISCON constraint bounds of ±0.2 Å to 3.4Å, ~ ±0.4Å out to 4^+ Å should yield much more precise structures; but the best means for incorporating such high precision constraints into XPLOR is problematic. We will use lysobactin as the primary test system for this effort, but the cyclic hexapeptide somatostatin analogs will also serve in this capacity since their structural variability has been better documented in other laboratories.

Experimental Work and Specific Structure Refinements

We will continue our refinement studies for endothelin and for yeast α-factor and [Arg^8]-α-neoendorphin in micelles. The latter study will be extended to a 13mer neuropeptide, dynorphin. We expect to use the DISCON-XLPOR combination in the final stages of structure refinement in all cases.

As previously noted, we will continue to explore the binding geometry associated with the specific binding site of serum albumin for tryptophan. We will evaluate the displacement kinetics of Ac-G-H-K-W using ^{19}F-NMR with L-6-fluorotryptophan as the specific binding probe. Once we have found conditions with $k_{off} \simeq 50\ s^{-1}$ for the tetrapeptide, we will use exchange transferred NOESY to derive its receptor-bound conformation.

Somatostatin Analogs, rigid systems for testing DISCON

Dr. Freidinger (Merck) has kindly agreed to provide us with two remarkably potent somatostatin analogs (L363301 and L363586, below):

NMe-Ala^6-Tyr^7-D-Trp^8	Pro^6-Phe^7-D-Trp^8	D-Pro^6-Phe^7-Trp^8
Phe^{11}-Val^{10}-Lys^9	Phe^{11}-Thr^{10}-Lys^9	Phe^{11}-Thr^{10}-Lys^9
L363586	*L363301*	***1***

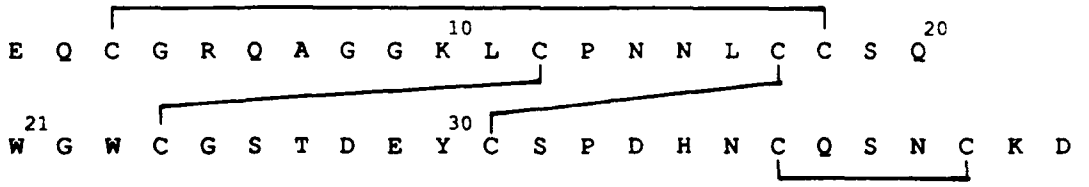
This class of cyclic hexapeptides has been extensivly studied. Kessler *et al.*[7] applied every known 2D NMR method (including E-COSY, HETCORs, and a quantitative ROESY) to analog **1** and derived a detailed solution structure and compared it to the high resolution x-ray structure of the Lys → Phe substitution analog.

Our studies will focus on L363586 and 363301 in DMSO containing 10-40% H_2O. This cryogenic media should allow us to perform NOESY spectra at sufficiently low temperature so that $\omega\tau_c > 3$. These results will be compared to a room temperature ROESY. We will attempt to measure every 3-bond J_{CH} and J_{HH} for the molecule and use amide-NH $\Delta\delta/\Delta T$ gradients to determine which backbone NHs are involved in persistent H-bonds.

Distant constraints derived by ISPA (a linear growth approximation)[3] and DISCON will be compared to those in the final refined structure and to an idealized structure bearing the ϕ/Ψ values observed in the crystal structures of other analogs.

Extension to Proteins

Finally we will explore the application of our methods to a small protein, hevein, which was isolated from a rubber tree species.[8] Its structure is given below



It represents a significant challenge even though it is only a 43mer due to the high frequency of repeated or similar residues. Preliminary control spectra indicate that suitable high resolution data will be obtainable. Dr. Arreguin (Instituto de Quimica, Ciudad Universitaria Coyoacan, Mexico DF) is pursuing an independent x-ray crystallographic structure determination which should serve as a check on the NMR method. We have instructed our Mexican collaborators not to provide as any crystallographic structural information so that the method can be totally independent.

The hevein studies may be included in an upcoming NIH grant application and in the renewal request for this contract.

References and Notes

1. a) J. Tropp, *J. Chem. Phys.* **72**, 6035 (1980); b) D.E. Woessner, *ibid* **36**, (1962); **42**, 1855 (1965).

2. X. Lai, T.M. Marschner, C. Chen and N.H. Andersen, "DISCON, A New Program for Obtaining Distance Constraints Corrected for Spin Diffusion," Poster #W20, 30th E.N.C., Asilomar, CA (4/89).

3. ISPA refers to the "isolated spin pair assumption" used in most NOESY analysis: G.M. Clore and A.M. Gronenborn, *J. Magn. Reson.* **61**, (1985); for critiques of ISP analyses see: b) B.A. Borgias and T.L. James, *J. Magn Reson.* **79**, 493 (1988); c) N.H. Andersen, H.L. Eaton and X. lai, *Magn. Reson. in Chem.* **27**, 515 (1989).

4. K.M. Banks, D.R. Hare and B.R. Reid, *Biochemistry*, **28**, 6996 (1989).

5. A.T. Brünger, G.M. Clore, A,M. Gronenborn and M. Karplus, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3801 (1986); A.T. Brünger, *X-PLOR Manual*, Yale University, New Haven, CT (1988).

6. For example, the recent elucidation of the structure of interleukin-8 by the Clore and Gronenborn group: *Biochemistry*, **29**, 1689 (1990).

7. H. Kessler, J.W. Bats, C. Griesinger, S. Koll, M. Will and K. Wagner, *J. Am. Chem. Soc.* **110**, 1033 (1988).

8. a) A. Rodriguez, B. Arreguin, A. Hernandez and M. Soriano, abstract reprinted in *Biochemistry*, **27**, 3092 (1988); b) A. Rodriguez-Romera, B. Arreguin and A. hernandez-Arana, *Biochimica et Biophysics Acta*, **998**, 21 (1989).

INVENTIONS: None to report. The computer software for structure refinement that will result should be viewed as the result of joint support by ONR and NSF funding. We do not intend to pursue patent protection but rather will promote use in the research community by open literature publication and code distribution (at nominal cost) to potential academic and government laboratory user groups. Distribution to corporate users will be contingent upon contribution of unrestricted funds for support of continuing development of these computational methods and may be handled by a software vendor.

PUBLICATIONS, PRESENTATIONS AND REPORT (cummulative for this contract):

1. H.L. Eaton, N.H. Andersen and X. Lai, Recent Extensions of NOESYSIM, A Program for Rapid Computation of NOESY Intensity Matrices from Atomic Coordinates and Experimental Conditions, Abstract #112, 29th Exper. NMR Conf. (Rochester, N.Y., 4/88).

*2. X. Lai, P.K. Hammen and T.M. Marschner, Computer-Aided Conformational Analysis Based on NOESY Signal Intensities, ACS Nat'l Mtg (Los Angeles, CA, 9/88); in press as a chapter in an ACS Symposium Volume.

*3. X. Lai, T.M. Marschner, C. Chen and N.H. Andersen, "DISCON, A New Program for Obtaining Distance Constraints Corrected for Spin Diffusion," Poster #W20, 30th E.N.C., Asilomer, CA (4/89).

*4. N.H. Andersen, H.L. Eaton and X. Lai, *Magn. Reson. in Chem.* **27**, 515 (1989).

*5. N.H. Andersen, X. Lai, T.M. Marschner, C. Chen, P.K. Hammen and S. Harris, "Programs for Quantitative NOESY Analysis and Application to Biorecognition Phenomena," Molecular Recognition Research Mtg., Charleston, SC (1/90).

*6. P.K. Hammen K.T. Nguyen and N.H. Andersen, "Media-Induced Changes in GnRH Conformation: an NMR Study," *Biopolymers*, submitted.

*7. N.H. Andersen and P.K. Hammen, A Conformation-Preference/Potency Correlation for GnRH Analogs: NMR Evidence," *Tetrahedron Let.*, submitted.

*8. K.M. Banks, B.R. Reid, N.H. Andersen and X. Lai, "A Comparison of Distance Constraints derived by iterative BKCALC bounds and those from application of DISCON to a single NOESY matrix," *TAMU NMR Newsletter*, submitted.

*9. N.H. Andersen, P.K. Hammen, K. Banks, T. Pratum, M.A. Porubcan and A.A. Tymiak, "Two-Dimensional NMR Elucidation of the Structure of Lysobactin, an Unusual Macrocyuclic Peptide," *Int. J. Peptides and Proteins*, ms in preparation.

Manuscripts and preprints (as available) will be provided together with the Progress Report due 90 JUL.

STUDENTS AND TRAINEES SUPPORTED (89/02/01 - 90/01/31):

name	status[a]	man-month salary equiv.	requested data
Thomas Marschner	post-doc	5.64	U.S.A. citizen
Xiaonian Lai[b]	G.S.	1.5(3.0)[c]	P.R.C.; Han Chinese
Bolong Cao[b]	G.S.	1.5(3.0)[c]	P.R.C.; Han Chinese
Philip K. Hammen[b]	G.S.	1.5(2.5)[c]	U.S.A. citizen
Chinpan Chen	G.S.	1.5	Taiwan, Chinese
Scott Harris[b]	G.S.	(1.5)[c]	U.S.A. citizen

a) Status indicated as post-doctoral research associate (post-doc) or a graduate student research assistant (G.S.)

b) Has also worked on contract goals while supported as a teaching assistant.

c) Effort estimate.