

The carbonyl adduct **3** is extremely labile, a feature which has made complete characterization difficult. Thus briefly flushing a solution of **3** with nitrogen rapidly causes a color change to orange,¹⁵ and the ¹H and ³¹P NMR spectra of this solution are identical with those of **1**.¹⁶ This ready recovery of **1** from **3** strongly suggests that the latter remains trinuclear and its infrared and NMR spectra¹⁷ indicate a symmetrical structure. The addition of 3 equiv of *tert*-butylisocyanide to **1** produces a single species, **4**, as indicated by the ¹H and ³¹P NMR spectra¹⁸ which are invariant over the temperature range 298–224 K. Given the stoichiometry of the reaction and the lack of evidence in the ³¹P NMR spectrum for unsymmetrical substitution or the presence of more than one species (i.e., fragmentation of the trimer) we believe that **4** is also a symmetrical adduct of **1**. Although fluxionality in **4** cannot be conclusively ruled out, the invariance of the NMR spectra with temperature would require a high degree of fluxionality which does not seem likely based on previous experience with isocyanide complexes of the "A-frame" type.¹⁹ Unfortunately, attempts to determine if *intermolecular* exchange of isocyanide ligands occurs were frustrated by further reaction of **4** with the added isocyanide to ultimately form $[\text{Rh}(\text{CNBu}^t)_4]^+$.

The ready recovery of **1** from **3** and the apparent formation of a single species from **1** and 3 equiv *tert*-butylisocyanide suggests that **3** and **4** be formulated as $[\text{Rh}_3\text{Cl}_3\text{L}_3(\text{MeN}(\text{PF}_2)_2)_3]$ (L = CO, CNBu^t). In the absence of structural data we cannot say whether these ligands have simply added to the metal atoms or whether cleavage of the chloride bridges has also occurred. The high value of ν_{CO} in **3** tentatively suggests the latter.

The results obtained here underscore the unpredictable complexing tendencies of $\text{RN}(\text{PR}'_2)_2$ (R = Me, Et; R' = F, OMe, OPr^t, OCH₂-) ligands. Thus with rhodium alone it is possible to obtain monomers,²⁰ a variety of symmetrical^{20–22} and unsymmetrical^{4,21} dimers, and even trimers depending on the nature of the substituents on both nitrogen and phosphorus. We are continuing to explore these interesting systems and will report further details in the future.

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Supplementary Material Available: Tables of positional parameters, bond lengths, interbond angles, anisotropic thermal parameters, and calculated hydrogen atom positions (8 pages). Ordering information is given on any current masthead page.

(14) ¹H NMR ((CD₃)₂CO) δ 3.19 (3 H, t ($J = 7.3$ Hz)), 3.02 (6 H, m). The complex was insufficiently soluble to obtain a satisfactory ³¹P NMR spectrum.

(15) Evaporation of the solution of **3** with a CO stream yields a dark greenish blue solid which rapidly becomes orange in vacuo. Adduct **3** is thus quite labile even in the solid state.

(16) ¹H NMR (CDCl₃) δ 2.99 (t, $J_{\text{P-H}} = 7.4$ Hz); ³¹P NMR (CDCl₃/CH₂Cl₂) δ 135 (AA'X₂X'₂M).

(17) IR spectrum $\nu_{\text{CO}} = 2056$ cm⁻¹ (Nujol), 2064 cm⁻¹ (CH₂Cl₂ solution); ¹H NMR (CDCl₃, 224 K) δ 3.04 (t, $J_{\text{P-H}} = 7.0$ Hz); ³¹P NMR (CDCl₃, 224 K) δ 135 (m). Although not well-resolved, the ³¹P NMR spectrum appears as a symmetrical multiplet which is clearly different from that of **1**. The symmetrical appearance together with the single terminal carbonyl stretching frequency indicates the presence of a single species with a symmetrical disposition of carbonyl ligands. On warming the ³¹P resonance broadens presumably because of CO exchange and on flushing with nitrogen becomes that observed for authentic **1**.

(18) IR spectrum $\nu_{\text{CN}} = 2210, 2171$ cm⁻¹ (toluene); ¹H NMR (CDCl₃, 298 K) δ 2.94 (9 H, t ($J_{\text{P-H}} = 7.1$ Hz)), 1.49 (27 H, s); ³¹P NMR (CDCl₃, 298 K) δ 137 (m). The ¹H and ³¹P NMR spectra are invariant down to 224 K, and the latter appears as a symmetrical complex multiplet. Because of the large number of spins involved, a simulation was not feasible, but the symmetrical appearance strongly argues for chemical equivalence of all phosphorus atoms, a conclusion consistent with the single chemical shifts observed for the ligand methyl groups and for the *tert*-butyl groups.

(19) Loss of isocyanide occurred on drying the apparently crystalline samples of **4** obtained from these solutions which prevented obtaining reliable analyses. This loss was evident from ³¹P NMR spectra of the dried solid which showed a mixture of **1** and **4** to be present.

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An Approach for Studying the Active Site of Enzyme/Inhibitor Complexes Using Deuterated Ligands and 2D NOE Difference Spectroscopy

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Obtaining detailed structural information on enzyme/inhibitor complexes by NMR spectroscopy is a formidable problem due to the difficulties in analyzing the large number of broad, overlapping NMR signals. In order to simplify the proton NMR spectra of ligand/macromolecule spectra, several experimental approaches have been proposed.^{1–5} Recently, we have described^{4,5} a method for studying enzyme/inhibitor complexes with use of isotope-editing techniques⁶ in which only those protons attached to the isotopically labeled nuclei (¹³C, ¹⁵N) of the ligand are detected. By using these techniques, we were able to determine the conformation of a tightly bound inhibitor of porcine pepsin and help define its active-site environment.⁵

In this communication, we present a simple, alternative method for providing the same type of structural information on large, enzyme/inhibitor complexes that has several practical advantages over previously proposed techniques. The method involves the subtraction of two-dimensional NOE spectra of two enzyme/inhibitor complexes prepared with either a protonated or a deuterated inhibitor. At short mixing times, only NOEs involving ligand protons that have been replaced by deuterium are observed in the 2D NOE difference spectrum.

The technique is illustrated by using the same pepsin/inhibitor (Figure 1) complex (MW = 35 kD) that has been previously studied by isotope-editing procedures.⁵ This system was chosen to be able to evaluate the reliability of the method. Figure 2A depicts a contour map of a 2D NOE spectrum of the protonated inhibitor (Figure 1) complexed to pepsin minus a 2D NOE spectrum of pepsin bound to the inhibitor perdeuterated at P₃. The 2D NOE difference spectrum is markedly simplified compared to the individual 2D NOE data sets (not shown), making it possible to interpret the data. NOEs between ligand protons (e.g., P₃H ^{α} /P₃H ^{β} , P₃H ^{α} /P₃H ^{δ}) help define the P₃ side-chain conformation of the bound inhibitor, and NOEs between the ligand and enzyme (boxed NOEs) provide structural information on the active site. For example, the NOEs observed between the P₃ methyl groups of the ligand and enzyme indicate that the P₃ side

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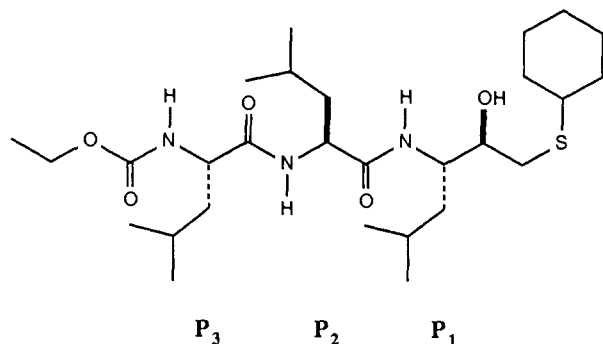


Figure 1. Structure of the pepsin inhibitor.

chain of the inhibitor is close to aromatic protons (Ha, Hb), and other protons of the enzyme that resonate at 2.65 ppm (Hd) and 0.56 ppm (Hg).

In Figure 2B a 2D NOE difference spectrum is shown of the protonated inhibitor (Figure 1) bound to pepsin minus a complex prepared with the inhibitor perdeuterated at P₂. NOEs are observed between the P₂ protons of the ligand (e.g., P₂H^α/P₂H^{β3}, P₂H^α/P₂H^γ) and between the protons of the inhibitor and enzyme (H_c, H_e, H_f). In contrast to the NOEs obtained for the P₃ side chain, no NOEs were observed between P₂ and any aromatic protons of the enzyme.

The NOEs that were obtained from the 2D NOE difference spectra are the same as those observed in the isotope-edited 2D NOE spectra of the pepsin/inhibitor complex prepared with ligands ¹³C-labeled at P₂ or P₃.⁵ Furthermore, the NOE data are consistent with a model of this pepsin/inhibitor complex⁵ built from a partially refined X-ray crystal structure of porcine pepsin complexed with a similar inhibitor.⁷ Thus, this method is reliable and can be used to obtain NOEs which are important for defining the active site structure of enzyme/inhibitor complexes. In addition, a high signal to noise is obtained in this experiment as illustrated by the ω₂ traces extracted at the frequencies of H_b (Figure 2A) and P₂H^α (Figure 2B).

In order to obtain 2D NOE difference spectra such as those depicted in Figure 2 which are relatively free from artifacts, the NMR samples of the enzyme/inhibitor complexes had to be prepared in an identical manner from the same stock solution of enzyme, and the experimental conditions to acquire the NMR data (see legend of Figure 2) needed to be kept constant. In addition, to avoid base line distortions, the receiver phase of the first 2D NOE slice of each data set was adjusted for pure absorption (0°) prior to the start of the experiment,⁸ and, to improve the dynamic range, the data were oversampled⁹ in t₂ by using a spectral width of 23 810 Hz over 2048 complex points.

It was also found to be important to process the NMR data in a particular manner. For example, a base line correction in ω₁ (fifth-order polynomial) was required before subtracting the 2D NOE data sets. It was also necessary to multiply each 2D NOE data set by a suitable constant before subtracting the data to minimize the residual signals in the 2D NOE difference spectra. Further improvements in the data set were obtained by applying a base line correction to the subtracted data sets in ω₂ and ω₁ using a fifth-order polynomial followed by symmetrization of the data. No artifacts were observed in the NOE difference spectra due to the narrowing of the proton signals by nearby deuterons, nor were difference NOEs observed at a mixing time of 50 ms between protons of the protein that might arise from three spin effects altered by the presence of deuterium. With use of these methods, the only remaining artifacts in the 2D NOE difference spectra were due to the imperfections associated with the subtraction of

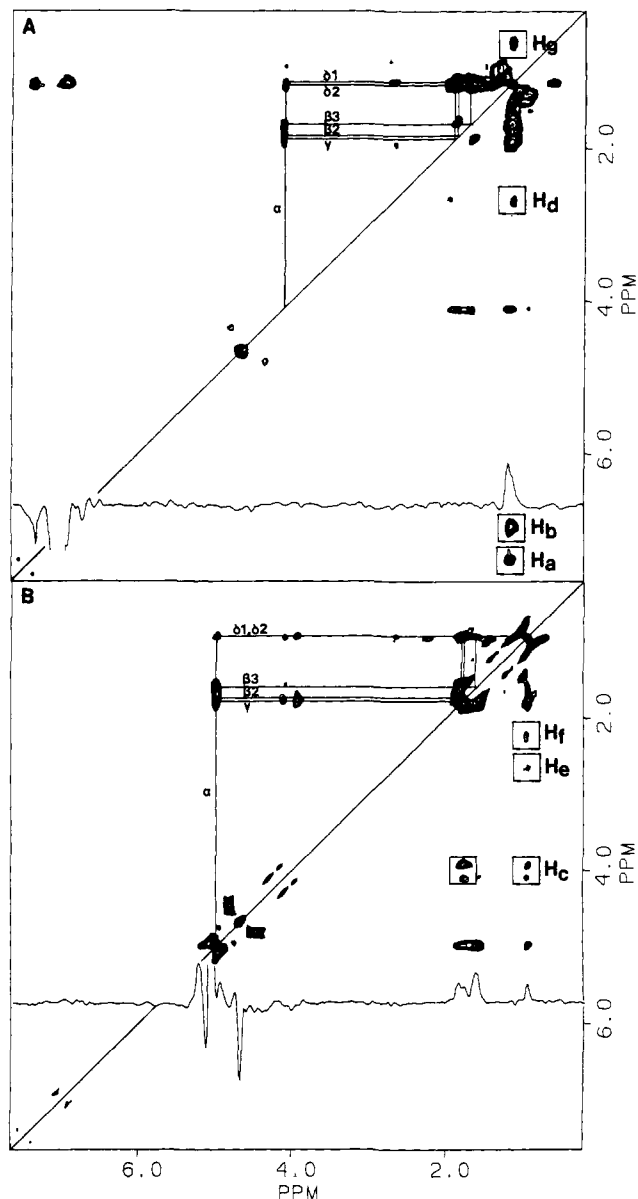


Figure 2. Two-dimensional NOE difference spectra calculated from a 2D NOE spectrum of a protonated inhibitor complexed to pepsin minus a 2D NOE spectrum of pepsin bound to an inhibitor perdeuterated at (A) P₃ or (B) P₂. Before subtraction, the 2D NOE data of the pepsin/inhibitor complex obtained with the inhibitor perdeuterated at P₃ and P₂ were multiplied by a constant of 0.75 and 0.87, respectively. The individual 2D NOE data sets were acquired at 40 °C with identical parameters on a Bruker AM 500 NMR spectrometer. A conventional 2D NOE pulse sequence was employed by using a mixing time of 50 ms, an acquisition time of 86 ms, and a 1.3-s delay between scans. (T₁ = 0.7–1.1 s for pepsin protons.) The residual solvent signal was suppressed by applying rf irradiation at very low power (γB₂ = 10 Hz) during the mixing time and delay between scans. A total of 256 scans were acquired for 2 × 110 t₁ increments. The NMR data were processed on a Vax 8350 with a slave CSPI array processor by using the FT NMR program of Dr. Dennis Hare and software written at Abbott Laboratories. A Gaussian multiplication was applied in both dimensions before Fourier transformation. NOEs between ligand protons are connected by solid lines, and inhibitor/pepsin NOEs are shown in boxes and labeled as previously described.⁵ Traces along ω₂ were extracted from the 2D NOE difference spectra at the frequencies (ω₁) of (A) H_b and (B) P₂H^α.

(7) This crystal structure was solved by using the inhibitor (2*S*,3*R*,4*S*)-2-(EtOC-p-I-Phe-Leu-amino)-1-cyclohexyl-3,4-dihydroxy-6-methylheptane complexed with porcine pepsin. A preliminary account of this work was presented by Erickson, J.; Abad-Zapatero, C.; Rydel, T. J.; Luly, J. at the 14th International Congress of Crystallography, Perth, Australia, 1987.

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the large diagonal peaks (see traces, Figure 2). Unfortunately, these artifacts limit the utility of this experiment for unambiguously identifying NOEs close to the diagonal. Another potential limitation may be the difficulties in obtaining good NOE difference spectra in H₂O, hindering the application of this approach for studying the exchangeable amide protons.

In summary, complicated proton NMR spectra of enzyme/inhibitor complexes can be simplified and made interpretable by using 2D NOE difference spectroscopy and deuterium labeled ligands. Analogous to the structural information obtained by isotope-editing procedures,⁶ these techniques allow the conformations of bound ligands and their active site environments to be defined. Unlike the isotope-editing techniques, however, this method does not require additional hardware for x-nucleus decoupling, fixed delays in the experiment which decrease the sensitivity, or carbon-13 labeling of ligands which is typically more difficult and expensive than deuterium labeling.

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Isolation and Structure of the Cytostatic Depsipeptide Dolastatin 13 from the Sea Hare *Dolabella auricularia*¹

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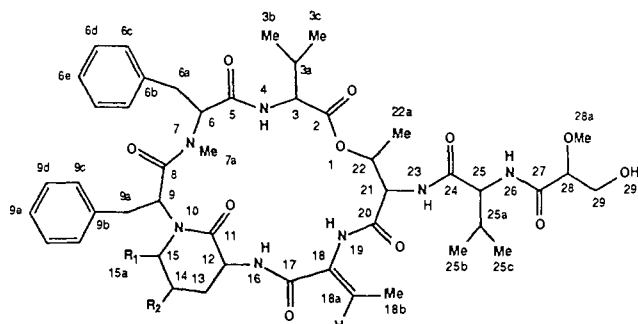
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The Indian Ocean shell-less mollusc *Dolabella auricularia* superficially appears to lack predator defenses. Such seemingly defenseless animals are only attacked by certain carnivorous^{2a} members of this gastropod subclass. Evidence is now accumulating that Opisthobranchia species have developed very powerful chemical defenses by careful selection and/or biosynthetic manipulation of various dietary sources such as blue-green algae^{1a} and sponges^{2b,3} (employed by nudibranchs, including possible protection of egg masses⁴). Our early (1968-1972)⁵ assumptions that certain species of shell-less molluscs of, e.g., the *Aplysia*⁵ and *Dolabella*⁶ genera contain potentially useful defensive constituents of the cell growth inhibitory type has been amply realized by isolation of the exceptionally potent antimelanoma pentapeptide dolastatin 10 from *D. auricularia*.⁶ We now report the isolation and structural elucidation of a new cell growth inhibitory (P388 lymphocytic leukemia, PS system⁷) constituent of this animal that

represents a hitherto unknown type of cyclodepsipeptide.

A small (1.72 g), albeit PS active, fraction prepared as previously summarized¹ from 1600 kg (wet wt) of *D. auricularia* collected (1982) in the Indian Ocean (East Africa) was further separated (PS bioassay) by gradient HPLC (RP8 silica gel, 1:1 methanol-water 100% methanol as mobile phase) to afford dolastatin 13 (1) as crystals (from methylene chloride-hexane, 10.6



1: R₁=OH; R₂=H
2: R₁=R₂=Δ^{14,15}

mg, 6 × 10⁻⁸% yield): mp 286-289 °C; [α]_D +94° (c 0.01, CH₃OH); R_f 0.56 in 90:10:0.8 CH₂Cl₂-CH₃OH-H₂O; see ref 13 for mass spectroscopy; UV (CH₃OH) λ_{max} (log ε), 220 (3.04) nm; and IR (NaCl plate), and ν_{max} 3384, 3315, 2960, 2930, 1733, 1677, 1653, 1529, 1205, 750, and 700 cm⁻¹. Dehydrodolastatin 13 (2) was obtained as a minor component together with depsipeptide 1 from the same fraction; crystals from methylene chloride-hexane (0.74 mg, 4 × 10⁻⁹% yield); mp 127-132 °C; [α]_D +38° (c 0.005, CH₃OH); R_f 0.64 (in preceding solvent system); HR FAB MS[M + H]⁺ obsd 888.4492, calcd 888.4508 for C₄₆H₆₃N₇O₁₁; UV_{max} (CH₃OH) λ_{max} (log ε), 220 (3.11) nm; and IR (NaCl plate), λ_{max} 3382, 3311, 2960, 2930, 1732, 1678, 1653, 1530, 1467, 1202, 750, and 700 cm⁻¹.

On the basis of results of detailed high field (400 MHz) ¹H and ¹³C NMR and high resolution FAB MS peak matching experiments, a molecular formula of C₄₆H₆₃N₇O₁₂ was deduced for dolastatin 13 (1). A combination of ¹H, ¹H COSY, ¹H, ¹³C COSY, and ¹H, ¹H relayed COSY⁸ experiments indicated eight discreet spin coupled systems of which four corresponded to the well-known amino acids threonine (Thr), *N*-methylphenylalanine (MePhe), and valine (Val, two units). Threonine and the two valine units were also detected by amino acid analyses of the products from acid-catalyzed (6 N HCl, 110 °C, 24 h) hydrolysis. Assignment of the fifth and sixth units as an *N,N*-disubstituted phenylalanine derivative was realized by NMR interpretations. From a series of double and triple relayed coherence transfer experiments (homonuclear relay) and sensitivity enhanced heteronuclear multiple bond correlation experiments (HMBC)⁹ the sixth unit was found to be the new cyclic hemiacetal (Ahp, for 3-amino-6-hydroxy-2-piperidone, cf. 1) presumably derived from a Phe-Glu dipeptide precursor (Glu-γ-carboxyl-γ-aldehyde).¹⁰

Continuation of the NMR experiments led to assignment of the seventh unit as the rare¹¹ dehydro amino acid α,β-dehydro-2-aminobutanoic acid (*cis*-Abu),¹² presumably from dehydration of Thr, and the eighth, as 2-*O*-methylglyceric acid (MeGlc). Because of some ambiguity, the Δ-Abu olefin was only tentatively

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