Structure and Conformation of a Phenyloxazolone Derivative Used in Studies of Antibodies, Determined Using ^{1}H NMR and Measurement of Long-range (^{13}C, ^{1}H) Coupling Constants

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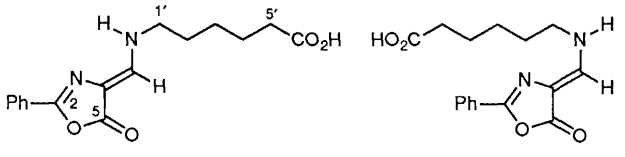
A phenyloxazolone derivative commonly used in studies of antibody–antigen interactions has been shown to exist as a mixture of species in solution, the structures of which were assigned by measuring long range (^{13}C, ^{1}H) *J* couplings using a novel technique; the significance of these results for immunological studies is discussed.

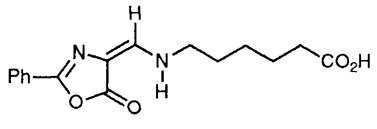
Studies of the physical chemistry of antibody–antigen interactions, and of the evolution of an organism's antibody repertoire, very commonly employ experimental systems based on immunogens prepared by the conjugation of low molecular weight haptens to macromolecular carriers. Both types of study are based on the assumption that a hapten has a unique structure and represents a minimal antigenic determinant. Derivatives of 2-phenyl-5-oxazolone (phOx) have long been used as haptens in such immunochemical studies.[1,2] Here we demonstrate that in low molecular weight amide conjugates, phOx is not a unique structure, but a family of related isomers and slowly exchanging conformers; we presume that the same holds for protein conjugates commonly used in immunisation.

The 300 MHz ^{1}H NMR spectrum of a solution of the oxazolone **1** in [^{2}H$_6$]dimethyl sulphoxide is shown in Fig. 1. It is immediately clear that three species contribute to this spectrum, most obviously from the appearance of three resolved NH signals (NH_A at δ 8.79, NH_B at δ 8.59, and NH_C at δ 8.44). A high resolution, phase-sensitive double quantum filtered (DQF)-COSY spectrum[3] was used to establish the separate networks of coupling connectivities within each side chain starting from these three NH signals. The various species did not show resolvable chemical shift differences between their phenyl signals or between their carboxylic acid proton signals. The assignments are summarised in Table 1. The presence of three sets of signals arises owing to two factors: firstly, the existence of both *E* and *Z* isomers about the exocyclic double bond, and secondly, slow rotation on the NMR chemical shift timescale about the adjacent C–N bond in

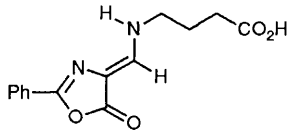


1A

1B



1C



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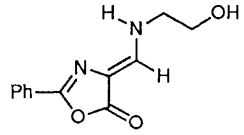

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Table 1 Chemical shift assignments (δ) for the three forms of phenyloxazolone **1** in [^{2}H$_6$]dimethyl sulphoxide solution at 27 °C

Signal assignment	1A	1B	1C
CO_2H	11.99	11.99	11.99
NH	8.79	8.59	8.44
Alkenic proton	7.54	7.23	7.89
Phenyl group—			
Ortho protons	7.8–7.9	7.8–7.9	7.8–7.9
Meta and *para* protons	7.45–7.55	7.45–7.55	7.45–7.55
C-1′ Methylene protons	3.36	3.82	3.36
C-2′ Methylene protons	1.58	1.64	1.58
C-3′ Methylene protons	1.30	1.40	1.30
C-4′ Methylene protons	1.53	1.60	1.53
C-5′ Methylene protons	2.22	2.23	2.22

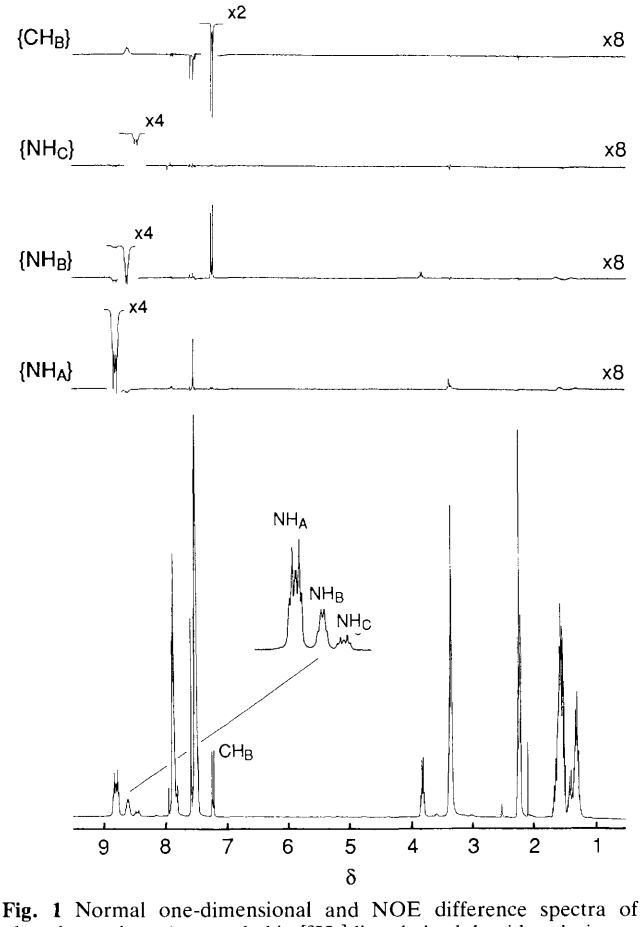

Fig. 1 Normal one-dimensional and NOE difference spectra of phenyloxazolone **1**, recorded in [^{2}H$_6$]dimethyl sulphoxide solution at 27 °C and 300 MHz. Preirradiation targets are indicated at the left of each trace, and vertical expansions are also indicated. Relevent NOE enhancements and coupling constants measured from these spectra are: enhancement $CH_B(NH_B)$ = 13%, enhancement $NH_B(CH_B)$ = *ca.* 5%, $J(NH_B, CH_B)$ = 7.3 Hz, $J(NH_A, CH_A)$ = $J(NH_C, CH_C)$ = 14.6 Hz. The proportions of the three species, estimated by integration of the three NH signals, are **1A**, 68%, **1B**, 24% and **1C**, 8%. See text for further discussion.

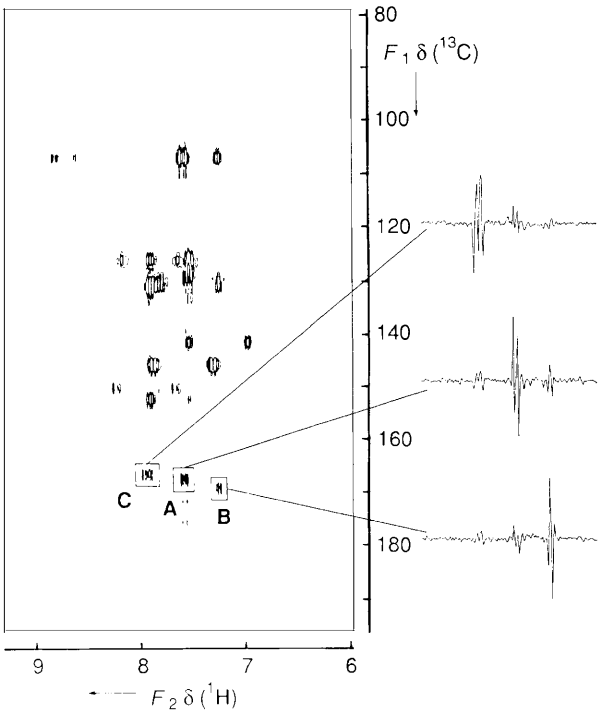

Fig. 2 Part of the two-dimensional, long-range HMQC spectrum of phenyloxazolone **1**, recorded in [$^2H_6$]dimethyl sulphoxide solution at 27 °C and 500 MHz. Positive and negative contours are plotted without distinction. The three cross peaks between the alkenic ^1H signal and the C-5 ^{13}C signal for each of species **1A**, **1B** and **1C** are highlighted and labelled on the spectrum, and the F_2 cross-sections corresponding to each of these cross peaks are also shown. See text for further discussion.

the side chain. The latter is not unexpected, since this C–N bond must have substantial double bond character due to delocalisation of the nitrogen lone pair into the conjugated ring system. The *E* and *Z* isomers may undergo slow equilibration within the solution (although we found no evidence to support this, even at higher temperatures), or they may arise due to equilibration during the preparation of the compound. Compound **1** was synthesised by reaction of 4-ethoxymethylene-4,5-dihydro-5-oxo-2-phenyloxazole with 6-aminocaproic acid using the method of Mäkelä.[4]

The NH signal at δ 8.59 (NH_B) is clearly the only one to arise from a rotamer in which the NH and alkenic CH protons are *cis* to one another. This may be deduced from the strong NOE enhancements and smaller coupling constant between this NH and its alkenic CH coupling partner (see Fig. 1). However, the distinction between *E* and *Z* isomers about the double bond cannot be made using ^1H NMR alone, because there are no protons in the heterocyclic ring. To resolve this issue, we measured the value of ^3J (^{13}C,^1H) for the coupling between the alkenic proton and the carbonyl ring carbon C-5 for each species; this is expected to be significantly larger for the *E* isomers than for the *Z*,[5] much as *trans* $^3J(^1H,^1H)$ values for mono- or di-substituted alkenes are larger than the corrresponding *cis* coupling constants.[6] Note that, of the three alkenic proton signals, only that due to species **1B** is clearly visible in the control spectrum shown in Fig. 1 (at δ 7.23, indicated CH_B). The others are overlapped with the phenyl signals, but were easily located in the DQF-COSY spectrum.

Measurement of long-range heteronuclear coupling constants is itself non-trivial, and in the present work we used a new data-fitting approach recently developed by Titman *et al.*[7,8] The starting point for this method is a two-dimensional (^{13}C, ^1H) shift correlation spectrum, recorded using the HMQC (heteronuclear multiple quantum coherence) pulse sequence under conditions optimised for detection of long-range (^{13}C, ^1H) couplings.[9,10] Cross-sections taken parallel to F_2, *i.e.* in the ^1H dimension, through cross peaks in such a spectrum show multiplet structures in which each line has a different phase (see examples in Fig. 2), making it impossible to analyse the data 'by eye'. These phase properties arise owing to unavoidable modulations of the proton signals by the evolution of ^1H chemical shifts and (^1H, ^1H) couplings during a necessary fixed delay set to 50 ms that occurs in the pulse sequence. However, it can be shown that the contribution of the heteronuclear coupling to such a cross peak multiplet section is simply to duplicate the 'proton-only' contribution by an *anti*-phase splitting; in other words, it produces one copy of the 'proton-only' contribution that is positive and one that is negative, with an offset between the two copies equal to the heteronuclear coupling constant.

Suitable templates for these 'proton-only' contributions can be constructed from a conventional absorption mode ^1H spectrum, by excising the relevant multiplet and imposing the necessary phase modulations using appropriate manipulations of the corresponding FID calculated by inverse Fourier transformation. The resulting template is then used to reconstruct the experimental cross peak multiplet section from the HMQC spectrum. Various displacements between the *anti*-phase copies of the template are tried in a least-squares fitting procedure, until the displacement that gives the best fit between reconstructed and experimental cross peak sections is found. This displacement corresponds to the value of the heteronuclear coupling constant. We have shown previously that this method recovers heteronuclear coupling constants with high reliability, even from quite noisy data, and is free from the distortions that arise in many other methods when lines are broad. Further details may be found in reference 8.

Fig. 2 shows a region of the two-dimensional long-range HMQC spectrum of compound **1**. The cross peaks due to coupling between the alkenic proton and C-5 have been highlighted for each of the three species. The values of $^3J(^{13}C,^1H)$ found by applying the fitting method to the appropriate F_2 cross sections (also shown in Fig. 2) were, for **1A**, 1.6 Hz, for **1B**, 2.1 Hz and for **1C**, 9.1 Hz. It is therefore clear that species **1A** and **1B** are *Z* isomers about the exocyclic double bond, while species **1C** is an *E* isomer; this information was the vital piece required to determine that the structural identity of the three species is as shown in the scheme. In this instance, two of the three alkenic CH signals required for use as templates were overlapped with signals from the phenyl group in the normal one-dimensional ^1H spectrum. The template multiplets were therefore obtained from suitable F_2 cross-sections through the relevant (NH, CH) cross peaks in a phase-sensitive TOCSY spectrum[11,12] of **1**, run using the DIPSI-2 mixing sequence[13,14] and *z*-filtration[15,16] to ensure pure absorption phase. Further details may be found in reference 8.

With this information at hand, some other features in the NOE difference spectra of Fig. 1 can also be explained. Since there is saturation transfer between NH_A and NH_B and also between CH_A and CH_B, species **1A** and **1B** are clearly interconverting at a rate comparable with the NMR T_1 timescale (*i.e.* the rate constants for exchange are in the order of 0.1–1 s^{-1}). The small enhancement of CH_A on preirradiation of NH_A (1.6%) arises because, even though the protons have a *trans* relationship in species **A**, proton CH_A has no close proton neighbours so that its relaxation by NH_A still represents an appreciable fraction of its total relaxation. The appearance of this enhancement entirely on the upfield component of the CH_B doublet must be due to a superimposed partly suppressed selective population transfer (SPT) effect.[17]

These experiments were carried out using dimethyl sulphoxide solutions in order to obtain sufficiently concentrated samples, but one-dimensional ^1H spectra show that an

essentially identical situation exists in aqueous solution (buffered at pH 7.0 with NaH_2PO_4). Assignments from the dimethyl sulphoxide spectra were translated to aqueous solution by running a series of spectra in water–dimethyl sulphoxide mixtures. Furthermore, other closely related compounds (**2** and **3**) show essentially identical patterns of signals, and may therefore be taken to exist as a similar mixture in solution.

In summary, this work shows the usefulness of the present method for measuring long-range heteronuclear coupling constants, and also highlights the previously unreported structural heterogeneity of these important haptens. The species we have identified are certain also to coexist under conditions used for physical studies of the interaction of phOx with antibodies. The derivatisation of surface lysine residues in the preparation of immunogens is performed by essentially the same synthetic method used for the amino-caproate conjugate, and a similar isomeric composition may be expected *in vivo*. Efforts to resolve the isomers by crystallisation and HPLC have not been successful in our hands, and given the demonstration above that species **A** and **B** readily interconvert, the use of heterogeneous structures in immunochemistry may be inescapable.

Do previous studies require reinterpretation? Preferential binding of an antibody to one isomeric species would compromise affinity measurements. However, in equilibrium dialysis and fluorescence quenching measurements made on a series of monoclonal anti-phOx antibodies, deviations of the data from a simple equilibrium model were not noted. Confronting the immune system with multiple structural forms might be expected to enhance the diversity of its response. However, certain inbred mouse strains exhibit an immune response of extraordinary homogeneity;[4] the majority of phOx-specific monoclonal antibodies sampled one week after administration of a conjugated immunogen are of a single, identical covalent structure.[18] Both observations support the view that the aromatic rings of phOx constitute the most significant antigenic moiety, and that the exact structure of the linkage to the carrier, wherein lie the isomeric differences discussed here, has only a minor influence on immune recognition.

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