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Conformational analysis of a secondary hydroxamic acid in aqueous solution by NOE spectroscopy

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Hydroxamic acids are metal-binding compounds used by micro-organisms and possess applications in medicine and industry. Hydroxamic acids favor two conformations, *E* and *Z*; metal binding is limited to the *Z* conformation. The *Z* conformation may be identifiable by NOE spectroscopy, but analysis is complicated by the potential for long-range coupling as well as for relayed NOEs due to conformational switching. In this report, we re-examine the reported conformational preference of *N*-methyl acetohydroxamic acid (NMHA) in D_2O using NOE spectroscopy. We find that the favored conformation of NMHA in aqueous solution is the *E* conformation, contrary to an earlier report. NOE build-up curves are proposed as a valuable tool to probe conformational behavior in similar systems. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: NMR; ^{1}H; conformations; NOE spectroscopy; hydroxamic acids; NOE build-up curve; long-range coupling; relayed NOE

Introduction

The hydroxamic acid is a naturally occurring metal ligand with high affinity for ferric iron. The relevance of hydroxamic acids to medicine, microbial iron harvesting, and industrial metal-binding applications has prompted the development of various model systems.[1–13] Hydroxamic acids favor two conformations, the so-called *E* and *Z* (antiperiplanar and synperiplanar; Fig. 1).[13] The hydroxamate unit must exist in the *Z* conformation in order to function as a bidentate metal ligand. The conformations differ in stability, with a rotational barrier that is high enough to permit resolution of both species by NMR.

Assignment of *E* and *Z* forms has been made most frequently using NMR chemical shift trends.[4,13–15] The propensity for hydroxamic acids to aggregate in nonpolar media selectively stabilizes the *Z* form at higher concentrations, which has been used as a basis for assignment.[4b,7,9] Computational studies of differential stabilities of *E* and *Z* have also been used to support assignment of major and minor conformations.[5,15] One report has used NOE studies to assign conformations.[10] Some inconsistencies exist in *E* and *Z* assignments, however. For example, *E* and *Z* have each been assigned by different workers as predominant for *N*-alkyl hydroxamic acids in water.[5,14,15]

We report here the conformational preferences of *N*-methyl acetohydroxamic acid (NMHA; Fig. 1) in D_2O, based on NOE data at 281 K. NMHA exists in the expected two states, in a 76 : 24 ratio, consistent with the prior report.[5] The earlier report assigned the major conformation as *Z* based on computational analysis of primary hydroxamic acids. NOE data indicate that the favored conformation is *E*. This result has implications for conformational characterization and metal-binding behavior of hydroxamic acids in aqueous solution.

Experimental

All reagents were purchased from Thermo Fisher Scientific, Waltham, MA, USA or from Sigma-Aldrich Corporation, St. Louis, MO, USA. All reagents were used without further purification. A synthesis of hydroxamic acids reported by Lee and Miller was modified to afford NMHA (Scheme 1).[16] Paraformaldehyde (0.616 g, 0.0205 mol) was stirred in 20 ml Millipore water to dissolve. *O*-benzylhydroxylamine (3.683 g, 0.0231 mol) was added, and the pH was adjusted to ~7 with 3 M NaOH. The resulting two-phase mixture was stirred at room temperature for 75 min. The mixture was extracted three times with CH_2Cl_2. The CH_2Cl_2 extracts were pooled and extracted twice with 0.5 M citric acid, once with water, and once with brine. The organic layer was dried with sodium sulfate and evaporated to yield 2.54 g (91.6%) of oxime **I** as a clear oil. ^{1}H NMR ($CDCl_3$): 5.1 (s, 2H), 6.41 (d, 1H), 7.03 (d, 1H), 7.27–7.34 (m, 5H); ^{13}C NMR: 76.1, 78.0, 128.0, 128.3, 128.4, 137.5; IR (neat): 3065, 3032, 2926, 1612, 1455, 1362, 1024 cm^{-1}; MS: *m/z* 136.0759 (M + H).

Oven dried glassware was used for the second reaction. Oxime **I** (2.37 g, 0.0175 mol) was dissolved in glacial acetic acid (50 ml). Acetic anhydride (2.0 ml, 0.021 mol) was added, followed by sodium cyanoborohydride (1.367 g, 0.02175 mol). The reaction was stirred at ambient temperature for 2 h, placed in a separatory funnel with 50 ml methylene chloride, and then extracted twice with 1 M potassium carbonate, twice with water, and once with brine. The organic layer was dried with sodium sulfate and evaporated. The product (**II**) was purified by chromatography on silica using 9 : 1 hexanes/acetone to yield 0.699 g (19.0%) of *O*-benzyl-*N*-methyl acetohydroxamic acid as a clear oil. ^{1}H NMR ($CDCl_3$): 2.05 (s, 3H), 3.18 (s, 3H), 4.81 (s, 2H), 7.37 (s, 5H); ^{13}C NMR: 20.2, 33.4, 76.2, 128.7, 129.0, 129.3, 134.5, 172.7; IR (neat): 3033, 2935, 1667, 1456, 1417, 1384 cm^{-1}; MS: *m/z* 180.1027 (M + H).

Acid washed glassware was used for the third reaction and for all handling of NMHA solutions. *O*-benzyl-*N*-methyl acetohydroxamic acid **II** (0.699 g, 0.00390 mol) was placed in a Parr

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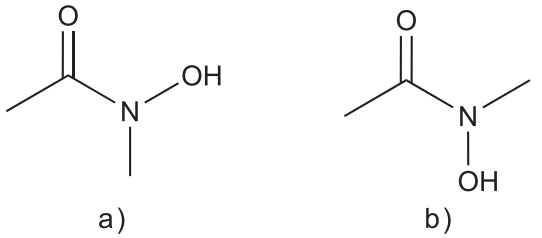
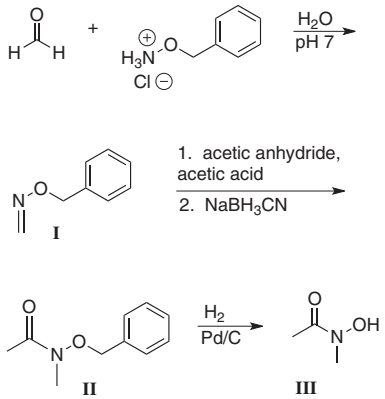


Figure 1. *N*-methyl acetohydroxamic acid in (a) *Z* conformation and (b) *E* conformation.



Scheme 1. Synthesis of *N*-methyl acetohydroxamic acid.

hydrogenation flask with 5% Pd on C (0.099 g) and methanol (10 ml). The mixture was shaken under 30 psi of H_2 for 45 min. The methanol mixture containing the catalyst and NMHA (**III**) was centrifuged in acid washed capped centrifuge tubes to pellet the catalyst. The supernatant was removed, and the Parr vessel and catalyst were rinsed with methanol, after which the suspension was centrifuged and separated as before. The rinse and centrifugation were repeated four times, and the combined methanol supernatant fractions were evaporated under dry nitrogen to yield 0.230g (66.3%) of NMHA as a faintly yellow oil. ^{1}H NMR (D_2O): see Table 1; ^{13}C NMR: see Table 1; IR (neat): 3400 (br), 3181 (br), 2918, 1621, 1427, 1392, 1202 cm^{-1}; MS: *m/z* 179.1035 (2M + H).

NMR studies used a Bruker Avance III 400 MHz spectrometer running Topspin 3.1 software (Bruker BioSpin Corporation, Billerica, MA, USA). All liquid handling was carried out with auto-pipettes. NMR tubes were acid washed and oven dried. NMR samples were prepared under N_2 and were then parafilmed. NMHA was dissolved in low-paramagnetic D_2O (Cambridge Isotope Laboratories, Cambridge, MA, USA; used without further purification) to a concentration of 0.035 M, and a second sample was prepared at 0.0035 M by dilution of the first sample. The specimen used for NOE studies (0.035 M) was septum sealed and degassed using three freeze/pump/thaw cycles. D_2O spectra were externally referenced to 25 mM sodium 3-(trimethylsilyl)-1-propanesulfonate in D_2O. NMR temperature was controlled for all data collection. NMR temperature was calibrated using a commercial methanol standard.

One-dimensional proton spectra were collected using 16 scans and the Bruker ZG30 pulse program. NOE spectra were collected using 1056 scans and the Bruker SELNOGP pulse program. A sweep width of 20.0255 ppm and 65 536 data points were used for both 1D and NOE data collections. Irradiation for NOE spectra was centered on either the major or the minor *N*-methyl proton frequency. Mixing times for NOE spectra ranged from 0.02 to 1.0 s. NOE spectra took approximately 2 h to acquire. Apodization that provided 0.3 Hz line broadening was applied to NOE data. NOE spectra were phased to render the irradiation signal a negative peak. NOE spectra were referenced by applying the spectrum reference value from the 1D spectrum to each SELNOGP data set.

Integral intensities for the NOE build-up curve were determined using Topspin 3.1 software. The acetyl region was expanded, and an integral region was defined starting at 2.120 ppm, continuing across the acetyl region, and ending at 2.065 ppm. The overall acetyl integral was then cut at 2.092 ppm, the chemical shift at which baseline resolution between signals for the two conformations was apparent at the longest mixing times. Integral area at higher chemical shift was assigned to the major conformation, and integral area at lower chemical shift was assigned to the minor conformation. Integral areas were located under the 'Integrals' tab of the main spectral window; the 'Integral[abs]' value was recorded.

Every individual integral value was normalized to (divided by) the integral area of the minor conformation at 0.6 ms mixing time; the latter integral had the largest value of the entire series (Fig. 4).

Table 1. Chemical shifts of protons and carbons of NMHA in *E* and *Z* conformations

	N–$\underline{C}H_3$ (ppm)	N–$\underline{CH}_3$ (ppm)	$CH_3\underline{C}(O)$ (ppm)	$\underline{CH}_3C(O)$ (ppm)	$\underline{C}(O)$ (ppm)
E	3.22	32.41	2.11	15.60	170.42
Z	3.36	35.78	2.09	16.37	166.24

Results and Discussion

^{1}H NMR spectra of 0.035 and 0.0035 M NMHA showed no discernable difference in *E*:*Z* ratio. These data were interpreted to mean that aggregation was not occurring at either concentration. The higher concentration sample was therefore used for NOE studies. All signals appeared to be singlets. ^{1}H NMR peaks for hydroxamic acids are often broad around 300 K because of conformational exchange.[5,7,10] Data were therefore collected at 281 K to obtain maximum signal resolution and minimize exchange.

Signals from the *N*-methyl group were better resolved than the acetyl methyl signals. Accordingly, for NOE experiments, irradiation was centered on the major or minor *N*-methyl frequency. The proximity of acetyl and *N*-methyl protons in the *Z* conformation suggested that an observable NOE in the acetyl signal would be unique to the *Z* form.

Analysis of hydroxamic acids by NOE spectroscopy is complicated by conformational interconversion. Polarization created in one form can be carried into the other form, with the result that chemical exchange NOEs may be observed.[10] Because chemical exchange NOEs would grow in at later times than direct NOEs, build-up curves were prepared to differentiate direct from chemical exchange NOEs. Each *N*-methyl resonance was irradiated across a range of mixing times, and the response in the acetyl region was recorded.

Irradiation of the minor *N*-methyl signal (3.36 ppm) generated a response in the minor acetyl signal (2.09 ppm) in a time-dependent

fashion (Fig. 2). At longer mixing times (≥0.2 s), a second response was observed in the major acetyl signal (2.11 ppm). The later appearance of the signal at 2.11 suggested that this response was a relayed NOE, a result of conformational switching from *Z* to *E*.

The major *N*-methyl resonance at 3.22 ppm was also irradiated in a series of SELNOGP experiments with incremented mixing times (Fig. 3). Interpretation was complicated by the presence of weak long-range coupling between acetyl and *N*-methyl protons, because COSY artifacts can appear in NOESY spectra. This phenomenon appears in Fig. 3 as an antiphase component at 2.11 ppm at shorter mixing times (up to 0.4 s). Presence of coupling was confirmed by 2D COSY spectroscopy (not shown).

An all-positive response in the minor acetyl frequency (2.09 ppm) does appear around 0.2 s (Fig. 3), at the same time when we believe a relayed NOE is seen at 2.11 ppm in irradiation of the minor *N*-methyl resonance (Fig. 2). As stated previously, we believe this result is consistent with relay of polarization via conformational exchange. We believe the same relay phenomenon is responsible for the growth of positive intensity in the major acetyl frequency at 2.11 ppm in Fig. 3. This phenomenon is not predominant until mixing times exceed 0.4 s, which we infer is correlated with polarization relayed *back* from the minor acetyl (the initial relay in this series of experiments).

Growth curves of the responses to irradiation of the minor *N*-methyl resonance (3.36 ppm; data shown in Fig. 2) are shown in Fig. 4. The intensity of the response at 2.09 ppm (the minor

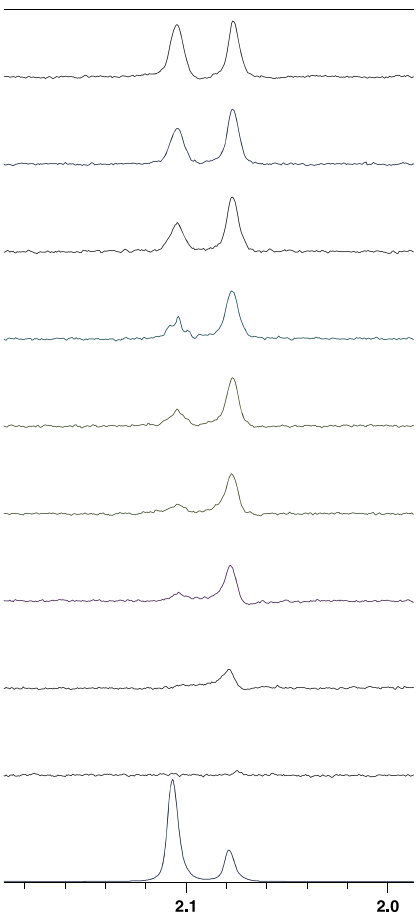


Figure 2. Result of irradiation of minor *N*-methyl resonance at 3.36 ppm (acetyl region shown); from bottom: 1D proton spectrum; SELNOGP spectra, mixing times: 0.02, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 1.0 s.

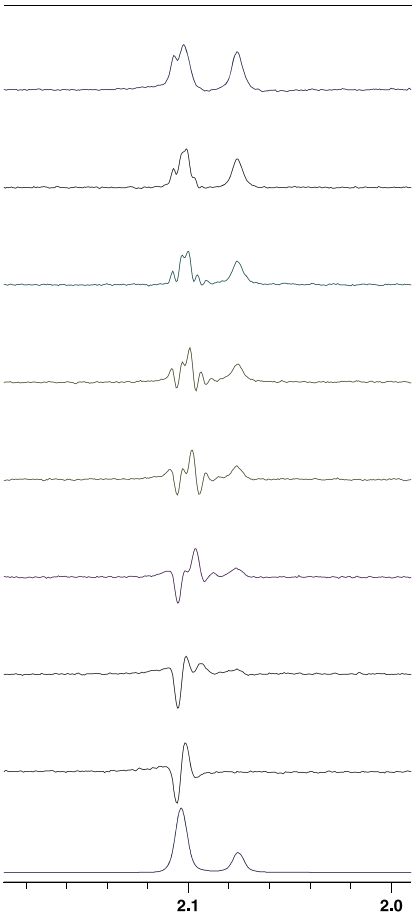


Figure 3. Result of irradiation of major *N*-methyl resonance at 3.22 ppm (acetyl region shown); from bottom: 1D proton spectrum; SELNOGP spectra, mixing times: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 1.0 s.

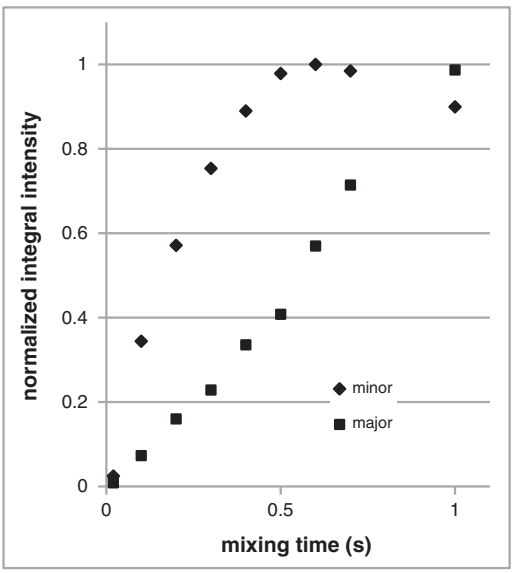


Figure 4. NOE growth curves for responses at 2.09 and 2.11 ppm following irradiation at 3.36 ppm. Minor conformer, 2.09 ppm; major conformer, 2.11 ppm.

conformation) grows rapidly in the shortest mixing times, indicating a direct NOE between the minor *N*-methyl and minor acetyl signals. This result is expected to be unique to the *Z* conformation, because the methyl groups are too far separated in the *E* isomer to show Overhauser enhancement. In contrast, the signal at 2.11 ppm (the major conformation) shows only slowly increasing NOE enhancement at early timepoints, which we believe is due to relayed polarization. The rate of increase of peak intensity at 2.11 ppm increases with mixing time, consistent with increased likelihood of conformational switching over time. Conversely, the growth rate of NOE intensity at 2.09 ppm decreases with mixing time, which is also consistent with relayed polarization due to conformational switching. At the longest mixing time (1 s), relative intensities of peaks at 2.09 and 2.11 ppm have reversed, with the major species at that timepoint being the major conformation in solution.

Taken in total, these data are all supportive of a favored conformation of *E* for NMHA in D_2O. The minor species is thus assigned as the *Z* conformation.

Conclusions

Results of NOE studies indicate that the *E* conformation is favored for NMHA in D_2O. The presence of long-range coupling in at least the *Z* conformation is observed. Relayed NOEs due to conformational switching are also evident at longer mixing times. Data from a range of mixing times clarify the structural predilections of NMHA, which has now been shown to favor the *E* conformation in all solvents except dimethyl sulfoxide.[4b] NOE build-up curves have proven to be an effective way to differentiate the effects of direct and relayed NOEs. Additional conformational analysis of NMHA and homologs will aid in the design of effective hydroxamic acids for medical and commercial applications.

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