N–O Bond Fission as the Rate-Determining Step in the Aqueous Conversion of *N*-Peptidyl-*O*-(*p*-nitrobenzoyl)hydroxylamines to *p*-Nitrobenzoic Acid and Peptidylhydroxamic Acids

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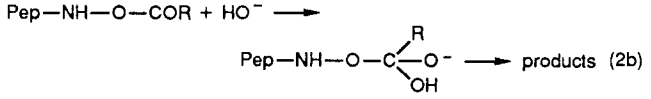
N-Acetyl-, *N*-alanyl-alanyl-, *N*-alanyl-prolyl-, and *N*-Boc-alanyl-prolyl-*O*-(*p*-nitrobenzoyl) hydroxylamines, compounds that are mechanism-based irreversible inactivators of some proteolytic enzymes, are degraded in aqueous buffers at neutral pH to *p*-nitrobenzoic acid and either the corresponding *N*-acylhydroxamic acid or products of its further degradation such as the diketopiperazine. At neutral pH, the reactants exist as the monoanion, as a result of the acidity of the –CO–NH–O– linkage. The *p*-nitrobenzoic acid formed in a mixture of 50% $H_2{}^{18}O$ and 50% $H_2{}^{16}O$ contains less than 2% ^{18}O, which shows that nucleophilic attack of water at the ester carbonyl is not occurring in the degradation. The decomposition of the *N*-alanyl-prolyl derivative, labeled with ^{15}N at the N–O nitrogen, exhibits a kinetic isotope effect $k_{14}/k_{15} = 1.092 \pm 0.056$, suggesting that N–O fission is occurring in the rate-determining step of the degradation. Kinetic solvent isotope effects of 1.02–1.15 are inconsistent with an expectation of factors around 2 or greater for spontaneous hydrolysis of the ester linkage. All derivatives have $\Delta H^* = 24$–27 kcal/mol and $\Delta S^* = +4$–7 eu, consistent with unimolecular fission of the substrate N–O to generate *p*-nitrobenzoate ion and the acyl nitrene. The nitrene must suffer nucleophilic attack at nitrogen very rapidly, producing the hydroxamic acid as the initial product. In the peptide derivatives, further reaction to the cyclized products results.

N-Peptidyl-*O*-acylhydroxylamines are irreversible inhibitors of some proteases, functioning by a mechanism that is currently under investigation.[1-4] In the present report, evidence is presented on the mechanism of the related hydrolytic reaction (eq 1). The degradation was studied at neutral pH, where the reactants (pK_a ca. 5–6)[5] exist as monoanions.

$$\text{Pep–N}^-\text{–O–COR} \rightarrow \text{RCOO}^- + \text{Pep–NHOH} \quad (1)$$

The two most attractive mechanisms for the reaction are a simple ester hydrolysis reaction (eq 2) and a nitrene-generating α-elimination reaction[6-12] (eq 3). The two

$$\text{Pep–N}^-\text{–O–COR} + \text{HOH} \rightarrow \text{Pep–NH–O–COR} + \text{HO}^- \quad (2a)$$



(2b)

$$\text{Pep–N}^-\text{–O–COR} \rightarrow \text{RCOO}^- + \text{Pep–N} \quad (3a)$$

$$\text{Pep–N} + \text{HOH} \rightarrow \text{Pep–NH–OH} \quad (3b)$$

mechanisms predict different outcomes for four experiments: (a) conduct of the reaction in ^{18}O-labeled water; (b) measurement of the rate with substrate labeled at N–O with ^{15}N; (c) determination of the enthalpy and entropy of activation; (d) solvent isotope effect.

The mechanism of eq 2 predicts (a) incorporation of ^{18}O into the product carboxylate, (b) no effect of ^{15}N substitution, (c) an enthalpy of activation typical of ester-cleavage reactions[13] (10–15 kcal/mol) and an entropy of activation somewhat negative (from incorporation of a water molecule and concentration of charge, leading to restricted solvation), and (d) a solvent isotope effect of 2–10 for water attack at ester carbonyl.[17]

The mechanism of eq 3 predicts (a) no incorporation of ^{18}O into product carboxylate, (b) a primary nitrogen isotope effect of several percent,[14] (c) an enthalpy of activation possibly as large as the heterolytic bond energy of the N–O bond and a positive entropy of activation (reflecting the unimolecular fragmentation), and (d) a solvent isotope effect near unity, since the unimolecular transition state would experience no stabilization through catalytic hydrogen bridging.

This paper reports the indicated experiments. The results allow a choice between the mechanistic possibilities.

Table I. Structural Assignments of Selected Chemical Shifts of Carbons from ^{13}C NMR Spectra of Ac-NHO-Nbz and Related Compounds in Solutions Containing 1/1 d_5-DMSO/Water (as 0.1 M HCl or 0.1 NaOH)

	structural elements (chemical shifts, $\delta_{HCl}/\delta_{NaOH}$)		
compd	CH_3	CO	CO (NbzOH)
Ac-NHO-Nbz[a]	19.2/20.0	167.3/168.7	163.0/163.8
Ac-NHOH	20.1/20.3	170.8/166.2	
AcOH	21.6/25.0	173.3/180.9	
NbzOH			166.8/172.4
$MeNH_2$	28.3[c]		
Ac-NHO-Nbz[b]	20.0[c]	167.9[c]	168.7[c]

[a] See top spectrum of Figure 1. [b] After degradation, see bottom spectrum of Figure 1. [c] In a solution of d_5-DMSO/sodium phosphate buffer (pH 7.0; 1/1)

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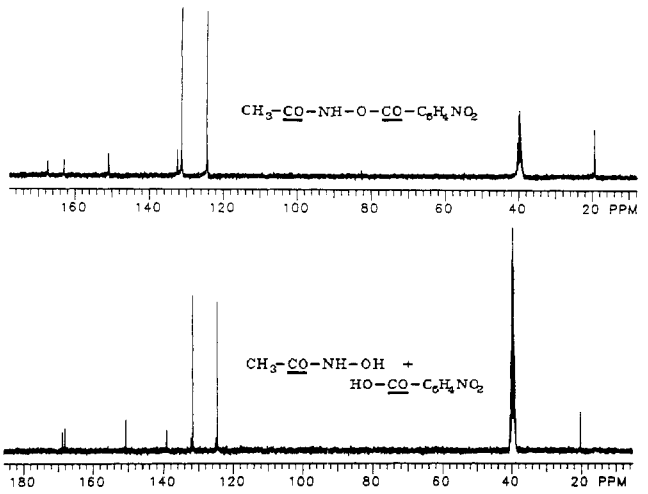

Figure 1. Natural abundance ^{13}C NMR spectra of *N*-acetyl-*O*-(*p*-nitrobenzoyl)hydroxylamine. Top: substrate in a mixture of d_6-dimethyl sulfoxide/sodium phosphate buffer solution (pH 7.0; 1/1). Bottom: substrate solution after 5 days of hydrolytic degradation and addition of d_6-DMSO to dissolve precipitated NbzOH.

Table II. Results of Mass Spectrometric Analysis after Degradation of *N*-Acyl-*O*-(*p*-nitrobenzoyl)hydroxylamines

N-acyl reside	$^{18}O/^{16}O$ ratio in water[a]	ion peak, % abundance 167	168	169	enrichment, %
alanyl-alanyl	1/1	100	9.5	1.5	<2.0
alanyl-prolyl	1/1	100	9.3	7.5	5.6
Boc-alanyl-prolyl	1/1	100	10.8	2.0	<2.0
acetyl	1/1	100	12.6	2.1	<2.0
controls					
p-nitrobenzoic acid	0/1	100	9.6	2.1	
p-nitrobenzoyl chloride	1/3	100	10.3	26.1	25
p-nitrobenzoyl chloride	3/1	31.3	4.6	100	76

[a] Ratio of $H_2{}^{18}O$ (97–99% at. % ^{18}O) to ordinary water.

Results

Product Studies. The ^{13}C NMR spectra of the product solutions for the aqueous degradation of *N*-acetyl-*O*-(*p*-nitrobenzoyl)hydroxylamine (Ac-NHO-Nbz) and *N*-alanyl-prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine (Ala-Pro-NHO-Nbz) indicate the nature of the products. Table I lists structural assignments of ^{13}C NMR resonances of Ac-NHO-Nbz and related compounds. Comparison (Figure 1) with the spectra of products from Ac-NHO-Nbz clearly demonstrates that neither AcOH nor $MeNH_2$ are formed, as would have been expected from Ac–N hydrolysis or Lossen rearrangement to methyl isocyanate followed by hydrolysis and decarboxylation. The products from Ac-NHO-Nbz are simply acetohydroxamic acid and *p*-nitrobenzoic acid (NbzOH), as shown by Figure 1, while for the peptidyl derivative a mixture of products is obtained, including the diketopiperazine. Previous studies[1-3] by TLC showed that here also the hydroxamic acid is initially formed and that the other products observed in the NMR spectrum are secondary conversion products of the hydroxamic acid.

^{18}O Tracer Study by Mass Spectrometry. Table II shows the result of mass spectrometric determination of the ^{18}O content of the NbzOH formed in water solutions containing oxygen label. In every case except the Ala-Pro derivative, no incorporation was detectable, while with this derivative, the level of label was about 5–6%, or 11% of the possible incorporation.

Table III. First-Order Rate Constants and Isotope Effects for Degradation of Boc-Ala-Pro-*NH-ONbz in 0.091 M Sodium Phosphate Buffer (pH 7.0) and 9.1% MeOH at 54.2 ± 0.2 °C

substrate label[a]	$10^3k_o \pm$ SD, s^{-1}	no. of determinations	k_{14}/k_{15}
^{14}N	1.786 ± 0.034	5	1.040 ± 0.028
^{15}N	1.717 ± 0.033	5	
^{14}N	1.667 ± 0.097	5	1.042 ± 0.063
^{15}N	1.578 ± 0.040	5	
^{14}N	1.741 ± 0.017	4	1.043 ± 0.017
^{15}N	1.670 ± 0.021	4	

[a] ^{14}N = ordinary material; ^{15}N = 48 ± 2 at. % ^{15}N.

Table IV. Solvent Isotope Effects for the Degradation of RNHONbz in Aqueous Buffers[a]

compd	solvent isotope effect k_H/k_D	conditions
Ala-Pro-NHO-Nbz	1.017 ± 0.02 (N = 5)	40 mM sodium phosphate buffer, pH = 7.6, μ = 0.125, 30 °C
Ala-Ala-NHO-Nbz	1.108 ± 0.025 (N = 5)	40 mM sodium phosphate buffer, pH = 7.6, μ = 0.125, 30 °C

[a] Individual rate constants are given in the supplementary material.

Table V. First-Order Rate Constants and Activation Parameters for Degradation of RNHONbz in Aqueous Buffers

R	10^3k_o + SD, s^{-1}	$\Delta H^{\ddagger} \pm$ SD, kcal/mol	$\Delta S^{\ddagger} \pm$ SD, cal/(K mol)
acetyl	0.15 (55 °C)[a]	27.3 ± 0.6	+6.3 ± 0.2
Ala-Ala	3.16 (50 °C)[b]	24.3 ± 0.8	+4.7 ± 0.1
Ala-Pro	0.32 (50 °C)[b]	26.1 ± 0.6	+6.1 ± 0.2

[a] Sodium borate buffer, 0.04 M, pH 9.1, 7.4% acetonitrile [b] Sodium phosphate buffer, 0.04 M, pH 7.6, 7.4% acetonitrile.

^{18}O Tracer Study by NMR. Figure 2 shows the carbonyl ^{13}C NMR spectra for the NbzOH formed in the degradation of Ac-NHO-Nbz and Ala-Pro-NHO-Nbz, as well as the product of hydrolysis of Nbz–Cl in the same isotopic water mixture. This last spectrum shows the expected[15] ^{18}O-induced shift in the ^{13}C chemical shift of 0.025 ppm, with both isotopic peaks of about equal intensity since both isotopes were present in equal amounts in the water. The spectrum of the Ac-NHO-Nbz product shows no ^{18}O incorporation, while the spectrum for the Ala-Pro product shows a small ^{18}O peak, consistent with the mass spectrometric observation of 5–6% labeling.

^{15}N Kinetic Isotope Effect. Parallel determinations of the first-order rate constants for generation of NbzOH from Ala-Pro-NHO-Nbz with 48 ± 2% ^{15}N (by mass spectrometry) at N–O and material with natural abundance of nitrogen at this position gave, in phosphate buffer (0.091 M, pH 7.0) at 54 °C, on three different days

k_{14}/k_{15} = 1.040 ± 0.028 (N = 5); 1.042 ± 0.063 (N = 5); 1.043 ± 0.017 (N = 4)

The data are shown in Table III. The second value was rejected because of the large standard deviation. Taking

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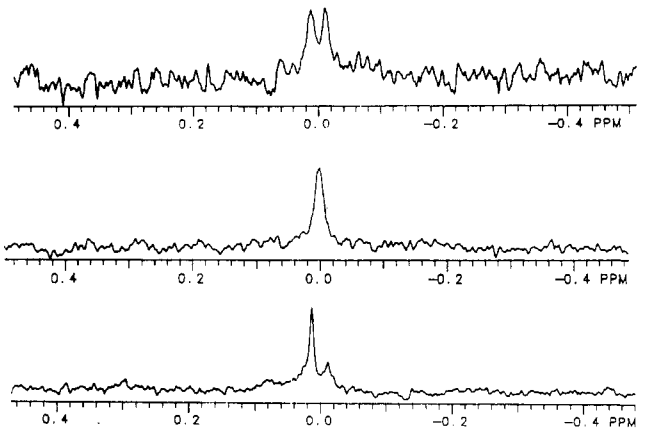

Figure 2. ^{13}C NMR signal for the carboxyl carbon of *p*-nitrobenzoate ion after degradation of various acyl derivative in a 1:1 mixture of ordinary water and 97–99% $H_2{}^{18}O$. Top: product from *p*-nitrobenzoyl chloride, showing the expected[15] ^{18}O-induced chemical shift of 0.025 ± 0.001 ppm; the two labeled products are in nearly equal concentration. Middle: product from *N*-acetyl-*O*-(*p*-nitrobenzyl)hydroxylamine. No oxygen has been incorporated from the water. Bottom: Product from *N*-alanyl-prolyl-*O*-(*p*-nitrobenzyl)hydroxylamine. The small ^{18}O signal indicates 5–6% label, about 11% of the possible incorporation.

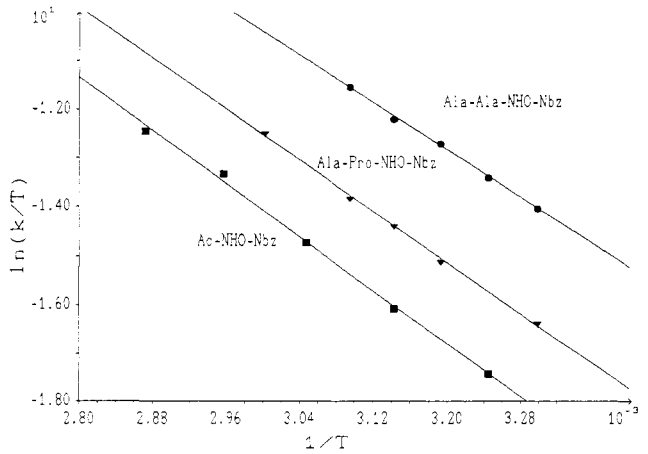

Figure 3. Eyring plots of the temperature dependence of the first-order rate constants of degradation of *N*-acyl-*O*-(*p*-nitrobenzoyl)hydroxylamines in aqueous buffers (see legend of Table IV; individual rate constants are given in supplementary material).

the error in the mass spectrometric determination of the label enrichment at ±2%, the corrected value of the nitrogen isotope effect was estimated at 1.092 ± 0.056.

Solvent Isotope Effects. Table IV shows the ratio of decomposition rate constants in HOH and DOD for all three derivatives.

Activation Parameters. Table V gives the values of the activation parameters for all three derivatives; a typical Eyring plot appears in Figure 3. All enthalpies lie in the range 24–27 kcal/mol and all entropies in the range +4–7 eu.

pH Dependence. Figure 4 shows the pH dependence of the first-order rate constant for decomposition of *N*-acetyl-*O*-(*p*-nitrobenzoyl)hydroxylamine between pH 2.8 and 8.6 at 65 °C. The pK_a of 4.59 ± 0.12 derived from these data is in good agreement with the equilibrium value of 4.87 ± 0.05 determined at 30 °C.

Discussion

All experimental results are in agreement with the mechanism of eq 3, with N–O fission (eq 3a) as the rate-determining step. The NMR and MS ^{18}O tracer studies

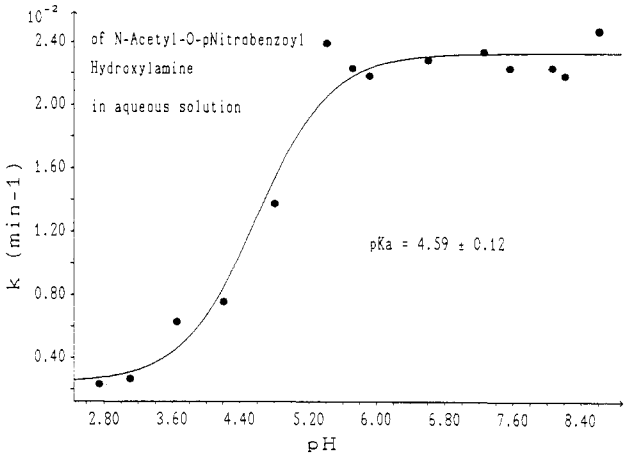

Figure 4. pH dependence of the first-order rate constants of the decomposition of *N*-acetyl-*O*-Nbz at 65 °C (individual rate constants are given in supplementary material).

indicate that no more than 11% of the reaction for any substrate proceeds with incorporation of solvent oxygen into the product *p*-nitrobenzoate ion. The values of the activation parameters (a relatively large $\Delta H^{\ddagger}$, a positive $\Delta S^{\ddagger}$) are consistent with unimolecular bond fission of eq 3a as rate-limiting. The large primary nitrogen isotope effect (1.09 ± 0.06) is also a clear indicator of this step as rate-limiting. It is of course true that other mechanisms involving rate-limiting O–N fission, not leading to ^{18}O incorporation, would also be consistent with the data.

It is not necessarily true that the same mechanism as seen in free solutions prevails in the active sites of enzymes for which these compounds are irreversible inactivators.[1-4] However, in view of the findings reported here, nitrene formation, followed by reaction of the nitrene with an enzymic functional group is a rational hypothesis for the mechanism of irreversible inhibition.

Experimental Section

Materials. Hydroxylamine[^{15}N] hydrochloride (99 at. % ^{15}N, ICN Biomedicals), [^{18}O]water (97–99 at. %, ICN Biomedicals), acetonitrile-d_2 (99.5 at. % ^2H, Stohler Isotope Chemicals), dimethyl sulfoxide-d_6 (99.5 at. % ^2H, Stohler Isotope Chemicals), and *p*-Nitrobenzoyl chloride (Merck Darmstadt) were used. All other reagents were analytical grade. Deuterium oxide was from Berlin-Chemie (99.86% D, lot 010287).

Substrates. *N*-Peptidyl-*O*-(*p*-nitrobenzoyl)hydroxylamines were synthesized as previously described.[1] The N-protected peptide methyl esters were treated in dry methanol in the presence of 1 equiv of sodium methoxide with a 10-fold excess of hydroxylamine, which was prepared from hydroxylamine hydrochloride and sodium methoxide in methanol. The resulting hydroxamic acids were purified analogously to Boc amino acids. O-Acylation with *p*-nitrobenzoyl chloride was achieved under Schotten–Baumann conditions or in tetrahydrofuran with triethylamine as base and pyridine as catalyst. After extraction of the crude products, the diacylhydroxylamines were crystallized from methanol/petroleum ether or ethyl acetate/petroleum ether. The Boc protecting group was removed by using HCl/glacial acetic acid to give *N*-alanyl-alanyl-*O*-(*p*-nitrobenzoyl)- and *N*-alanyl-Prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine hydrochloride. Yields were always between 60 and 85% of the theoretical value. *N*-Acetyl-*O*-(*p*-nitrobenzoyl)hydroxylamine: mp 159–160 °C (dec). Anal. Calcd for $C_9H_8N_2O_5$ (M_r 224.16): C, 48.22; H, 3.60: N, 12.50. Found: C, 48.62; H, 3.76; N, 12.26. *N*-Alanyl-prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine hydrochloride: mp 148–149 °C (dec). Anal. Calcd. for $C_{15}H_{19}N_4O_6Cl$ (M_r 386.79): C, 46.58; H, 4.95; N, 14.49. Found: C, 46.70; H, 4.91; N, 13.80. *N*-Alanyl-Alanyl-*O*-(*p*-nitrobenzoyl)hydroxylamine hydrochloride: mp 140–141 °C (dec). Anal. Calcd for $C_{13}H_{17}N_4O_6Cl$ (M_r 360.75): C, 43.28; H, 4.75; N, 15.53. Found: C, 43.32; H, 4.67; N, 16.4. *N*-(*tert*-Butyloxycarbonyl)Alanyl-prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine:

mp 121–122 °C (dec). Anal. Calcd for $C_{20}H_{26}N_4O_8$ (M_r 450.45): C, 53.33; H, 5.82; N, 12.44. Found: C, 53.13; H, 5.93; N, 12.25.

For the synthesis of [^{15}N]-Boc-alanyl-prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine, Boc-alanyl-prolyl-methyl ester was treated in MeOH with ^{15}N enriched hydroxylamine (containing methanol solution). Mass spectrometric analysis gave 48 ± 2% ^{15}N enrichment in the final diacylated product.

Kinetics. The previously employed spectrophotometric procedure[1,2] was followed. The temperature dependence, the pH dependence, and solvent isotope effects for the decomposition of *N*-acyl-*O*-(*p*-nitrobenzoyl)hydroxylamines were measured with a Carl-Zeiss-Jena micropressor-controlled Specord M 40 spectrophotometer equipped with a jacketed cell compartment, containing electrical heater and temperature control. Temperatures were precise within ±0.1 °C. Data collected and stored in an internal buffer were analyzed with software packages provided on an application ROM for the instrument. The nitrogen isotope effect was determined with a Cary 118 spectrophotometer equipped with a jacketed cell holder and interfaced to a Zenith 158 personal computer for data acquisition. The temperature was maintained with a Lauda K4R circulating water bath and was monitored with a thermistor for direct electronic reading near the sample holder. Reactions were carried out in 1.0-cm Teflon-stoppered silica cells. For kinetic runs at temperatures distant from room temperature, sufficient thermal equilibration time was allowed. After the reaction was initiated by addition of the substrate-containing sample to the thermally equilibrated cell, the first 5 min of data aquisition was ignored for calculations. Stock solutions were made in H_2O (*N*-alanyl-alanyl-*O*-(*p*-nitrobenzoyl)- and *N*-alanyl-prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine), in methanol (*N*-Boc-alanyl-prolyl-*O*-(*p*-nitrobenzoyl)hydroxylamine), and acetonitrile (*N*-acetyl-*O*-(*p*-nitrobenzoyl)hydroxylamine. Final concentrations were achieved by dilution of the stock solution in the buffer-containing UV cell. All substrate solutions were (1.0–1.3) × 10^{-4} M. Reactions were monitored by following the absorbance of *p*-nitrobenzoic acid.[1,2] CHES and sodium phosphate buffer solutions were prepared in HOH and DOD as previously described;[17] KCl was used to maintain an ionic strength of 0.125. Data were collected over at least 4–6 half-times, and rate constants were calculated by nonlinear regression programs (Gauss–Newton–approximation method) on a Zenith 158 personal computer or a Hewlett-Packard 2598 A desktop computer.

pK_a Determinations. The UV maximum of 0.1 mM buffered solutions of *N*-acyl-*O*-(*p*-nitrobenzoyl)hydroxylamines shifted from 263 to 268 nm between pH 2.2 and pH 8.6. This allowed calculations of the pK_a's as previously described.[2]

NMR Spectra. ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer operating at 75.43 MHz, equipped with a 5-mm probe, thermally equilibrated at 20 ± 1 °C. In a typical ^{18}O trapping experiment, a 500-Hz sweep width, 90° pulse angle, 8.02-s acquisition time, and 8.5K data block were used. Protons were broad-band decoupled, and 1500–3000 transients accumulated. As standard parameters for natural-abundance ^{13}C product analysis, 0.6-s acquisition time, 16 500-Hz sweep width, 90° pulse angle, a 20K data block, and 2000–5000 transients were used. TMS was used as external standard.

Product Analysis. Solutions (30–60 mM) of diacyl hydroxamic acids were prepared in 0.5 mL of acetonitrile or 0.5 mL of dimethyl sulfoxide, 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.0) was added, and the spectra were recorded. For the degradation studies, similar solutions were prepared and stored in 2.0-mL glass vials for 2–5 days at 37 °C in a shaking water bath. Before NMR analysis, organic solvent was added to undissolved precipitated *p*-nitrobenzoic acid. In ^{18}O trapping experiments, 10 mM solutions in acetonitrile were made, and 0.25 mL of 0.2 M sodium phosphate buffer (pH 7.0) and 0.25 mL of [^{18}O]water were added. Reference samples contained 0, 25, or 75% [^{18}O]-water. The solutions were kept in a shaking water bath at 50 °C. After 18–24 h, 0.1–0.2 mL of 10% HCl was added. A few crystals of precipitating *p*-nitrobenzoic acid were collected by centrifugation of the solution at 5000 rpm through a filter-containing plastic tube (Centrex, Keene, NH), dried, and supplied for mass spectrometric analysis. Residual solutions were transferred to 5-mm NMR tubes, and the carbonyl region was inspected.

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Registry No. 4-(O_2N)C_6H_4COONHAc, 123206-53-1; H-Ala-Ala-NHOCOC_6H_4-4-NO_2, 87620-98-2; H-Ala-Pro-NHOCOC_6H_4-4-NO_2, 87621-00-9; BOC-Ala-Ala-NHOCOC_6H_4-4-NO_2, 87620-99-3; H-Ala-Ala-NHOCOC_6H_4-4-NO_2·HCl, 123206-54-2; H-Ala-Pro-NHOCOC_6H_4-4-NO_2·HCl, 86030-65-1; HONH$_2$·HCl, 5470-11-1; AcNHOH, 546-88-3; 4-(O_2N)C_6H_4COCl, 122-04-3; BOC-Ala-Ala-OMe, 19794-10-6; BOC-Ala-Pro-OMe, 33300-71-9; BOC-Ala-Pro-^{15}NHOCOC_6H_4-4-NO_2, 123206-55-3; AcOH, 64-19-7; 4-(O_2N)C_6H_4COOH, 62-23-7; $MeNH_2$, 74-89-5.

Supplementary Material Available: First-order rate constants for decomposition and solvent isotope effects for selected compounds (3 pages). Ordering information is given on any current masthead page.

1,3-Dipolar Cycloadditions between Nitrile Oxides and Substituted 7-Oxabicyclo[2.2.1]heptenes

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1,3-Dipolar cycloadditions between aromatic nitrile oxides and a series of 7-oxabicyclo[2.2.1]heptenes have been studied. The reactivity of these systems is compared to that of related bicyclo[2.2.1]heptenes.

Bicyclic derivatives such as 1[1] add soft electrophiles in a regio- and stereoselective manner influenced by the substituents at C-2; however, this is not so clearly established for Diels–Alder cycloadditions.[2] Thus, this remote control appears to be highly reaction dependent. On the other hand, the effect of an oxygen bridge in position 7 should also be taken into account when comparing the higher reactivity of these systems toward electrophiles with that of the corresponding methylene analogues.[3] The

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(1) These substrates are versatile synthetic intermediates; see: Auberson, Y.; Vogel, P. *Helv. Chim. Acta* **1989**, *72*, 278 and references therein.
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