Decomposition of *N*-Nitrosopeptides in Strong Acids

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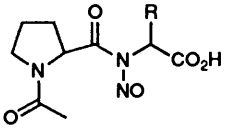
Decomposition of the *N*-nitrosodipeptides obtained from *N*-(*N'*-acetyl-L-prolyl)glycine and *N*-(*N'*-acetyl-L-propyl)-L-alanine in aqueous acid at 25 °C involves both deamination and denitrosation. Both reactions occur concurrently *via* different conjugate acid intermediates, with denitrosation being predominant at high acidity. Acidity dependences and inverse solvent deuterium isotope effects [$k(H_2SO_4)/k(D_2SO_4)$ *ca.* 0.7] suggest that deamination involves rate limiting attack by H_2O on an *O*-conjugate acid, formed in a rapid pre-equilibrium. For denitrosation, H^+ transfer to the amide *N*-atom is considered rate limiting because of the substantial normal solvent deuterium isotope effects [$k(H_2SO_4)/k(D_2SO_4)$ *ca.* 2.5] and Bunnett ω values in the range −0.1 to −0.5: the *N*-conjugate acid formed breaks down rapidly to products. Both the kinetics and mechanisms for decomposition of these *N*-nitrosodipeptides are very similar to those of alicyclic *N*-nitrosamides.

N-Nitrosopeptides were first reported in 1984[1,2] and a full account of the synthesis and spectroscopic properties of several examples from *N*-acyldipeptides has been published recently.[3] The chemical properties of *N*-nitrosopeptides are less well described, but they should be similar to *N*-nitrosamides, their closest structural analogues. *N*-Nitrosamides are fairly labile compounds which rearrange to unstable diazo esters on heating,[4] are readily hydrolysed with deamination in neutral and alkaline media[5] and undergo concurrent denitrosation and deamination (hydrolysis) in strong acids.[6] Both the thermal rearrangement and heterolytic deamination reactions generate diazoalkanes, and *N*-nitrosamides therefore have some applications as alkylating agents.[7]

Our interest in the chemistry of *N*-nitrosopeptides stems from their potential involvement in dietary related cancers. Nitrosation reactions proceed in the stomach[8] and are likely to involve proteins and peptides because of their common dietary occurrence. The incidence of colon cancer correlates with dietary protein intake[9] and *N*-(*N'*-acetyl-L-propyl)-*N*-nitrosoglycine (**1a**) exhibits a broad spectrum of genotoxic properties characteristic of an alkylating agent.[10–13]

In this paper, kinetic studies are reported for the decomposition of the *N*-nitroso derivatives of *N*-(*N'*-acetyl-L-propyl)glycine (**1a**) and *N*-(*N'*-acetyl-L-propyl)-L-alanine (**1b**)



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a R = H **b** R = Me

in aqueous $HClO_4$ and H_2SO_4, along with product studies, acidity dependences and solvent deuterium isotope effects. Probable mechanisms for the acid catalysed decomposition of these compounds are deduced and compared with similar reactions of *N*-nitrosamides.

Experimental

Substrates and Reagents.—The *N*-nitrosodipeptides (**1a** and **1b**) were synthesised by aprotic nitrosation of the benzyl esters of the corresponding dipeptides, followed by removal of the benzyl ester groups by catalytic hydrogenolysis; both were obtained as crystalline solids with microanalyses and spectroscopic properties consistent with the assigned structures. Details of these syntheses, and the spectroscopic characterisation of **1a** and **1b** have been published recently.[3]

Reaction solutions were prepared from analytical grade $HClO_4$ (72% w/w) and H_2SO_4 (98% w/w), D_2SO_4 (96% w/w, MSD) and D_2O (n_D = 0.999, Aldrich). Their acidity was determined by titration against carbonate-free NaOH solutions (standardised with analytical grade benzoic acid) using either phenolphthalein or 4-nitrophenol as indicator. Analytical reagent grade $NaNO_2$ was vacuum dried but reagent grade sulphanilamide and urea were used as supplied.

Kinetics.—These experiments were usually carried out in the thermostatted cuvettes of either a Pye-Unicam SP8-500 or a Cecil CE599 UV/VIS spectrophotometer. Decomposition was followed by the time-dependent decrease in absorption of the reaction solutions at λ *ca.* 237 or 403 nm using initial substrate concentrations of *ca.* 5×10^{-5} or 5×10^{-3} mol dm^{-3}, respectively. These wavelengths correspond to absorption maxima for both *N*-nitrosodipeptides.[3] The higher wavelength was used for reactions in the absence of urea or sulphanilamide at acidities where the extent of nitrous acid formation was sufficiently extensive to interfere with substrate quantification at λ *ca.* 237 nm. Observed pseudo-first-order rate coefficients [eqn. (1)], were

$$\text{Rate} = k_0[N\text{-Nitrosodipeptide}] \quad (1)$$

calculated from the experimental results by least-squares linear regression analysis (unweighted) of $\ln(A_t - A_\infty)$ *versus* time where A_t and A_∞ are absorbances at times t and infinity, respectively. For relatively rapid reactions ($k_0 > 10^{-3}$ s^{-1}, $t_{\frac{1}{2}} < 10$ min), A_∞ was determined experimentally after 10 half-lives. For slower reactions, A_∞ was calculated to ±1% limits from experimental data over 3–4 half-lives using eqn. (2), where

$$A_\infty = \{A_{t_2}^2 - A_{t_1} \cdot A_{t_3}\}/\{2A_{t_2} - (A_{t_1} + A_{t_2})\} \quad (2)$$

$t_1 - t_2 = t_2 - t_3$ and A_{t_1} *etc.* are the corresponding absorbances. All of the kinetic experiments gave excellent pseudo-first-order behaviour and k_0 values were reproducible to ±10%. Experiments with deuteriated solvents were carried out on the same scale as those with regular media.

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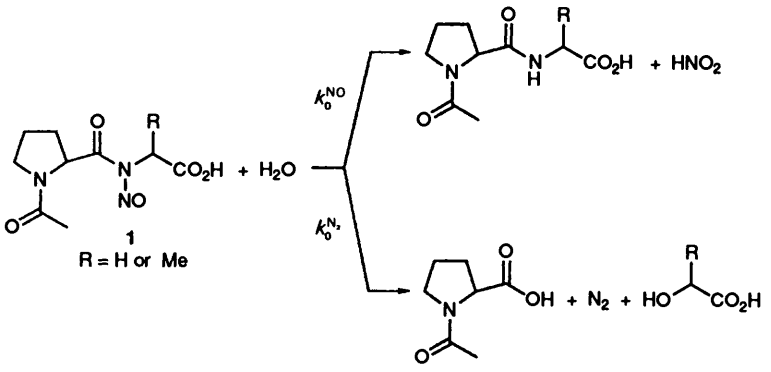


Scheme 1 Concurrent denitrosation and deamination of *N*-nitrosodipeptides in acidic media

Table 1 Effect of HNO_2 traps on reaction rates and percentage denitrosation for the decomposition of **1a** and **1b** in aqueous $HClO_4$ at 25 °C: initial [**1a**] or [**1b**] *ca.* 5×10^{-5}–5×10^{-3} mol dm^{-3}

$[HClO_4]$/mol dm^{-3}	$[HNO_2$ trap]/mol dm^{-3}	$k_0/10^{-5}$ s^{-1}	$[HNO_2]_\infty$ (%)	$k_0^{NO}/10^{-5}$ s^{-1}	$k_0^{N_2}/10^{-5}$ s^{-1}
1a					
4.70	None	37.0			
4.70	0.01 Sulphanilamide	36.1	81.7	29.3	6.8
4.70	0.10 Sulphanilamide	39.3	81.1	32.1	7.2
5.36	None	60.3			
5.33	0.10 Sulphanilamide	60.0	83.0	49.8	10.2
5.64	None	80.0			
5.66	0.05 Urea	81.7			
1b					
1.20	None	29.2			
1.20	0.05 Urea	29.4			
2.06	None	34.6			
1.94	0.05 Sulphanilamide	33.8	7.10	2.40	31.4
2.06	0.05 Urea	34.8			
2.88	None	40.7			
2.88	0.05 Urea	41.1			
6.13	None	146			
6.13	0.005 Sulphanilamide	150	75.3	113	37
6.14	0.05 Sulphanilamide	146	73.3	107	39

Product Analyses.—To evaluate the individual contribution from each pathway to the overall decomposition rate, accurate product analysis is required for each kinetic experiment. This was conveniently made by measuring the yield of HNO_2 released from a known initial *N*-nitrosodipeptide concentration by *in situ* diazotisation of sulphanilamide and subsequent reaction with *N*-1-naphthylethylene diamine to form an azo dye, which was then estimated colorimetrically at λ_{max} 541 nm (Shinn's method).[14] The yield of HNO_2 was deduced from an azo dye calibration curve generated with standard $NaNO_2$ solutions. Thus, the *N*-nitrosodipeptide substrate (to give an initial concentration of *ca.* 10^{-3} mol dm^{-3}) was added gravimetrically to a duplicate reaction solution containing an excess of the sulphanilamide reagent. From this solution several portions were assayed for HNO_2 after 10 half-lives. No corrections were made for spontaneous decomposition of either HNO_2 or the diazonium ion from sulphanilamide produced *in situ*, because these have been shown to be unnecessary.[6] The concentration of azo dye formed was found to be independent of the sulphanilamide concentration (provided it was in excess of the *N*-nitrosodipeptide substrate), and duplicate assays agreed to ±2%.

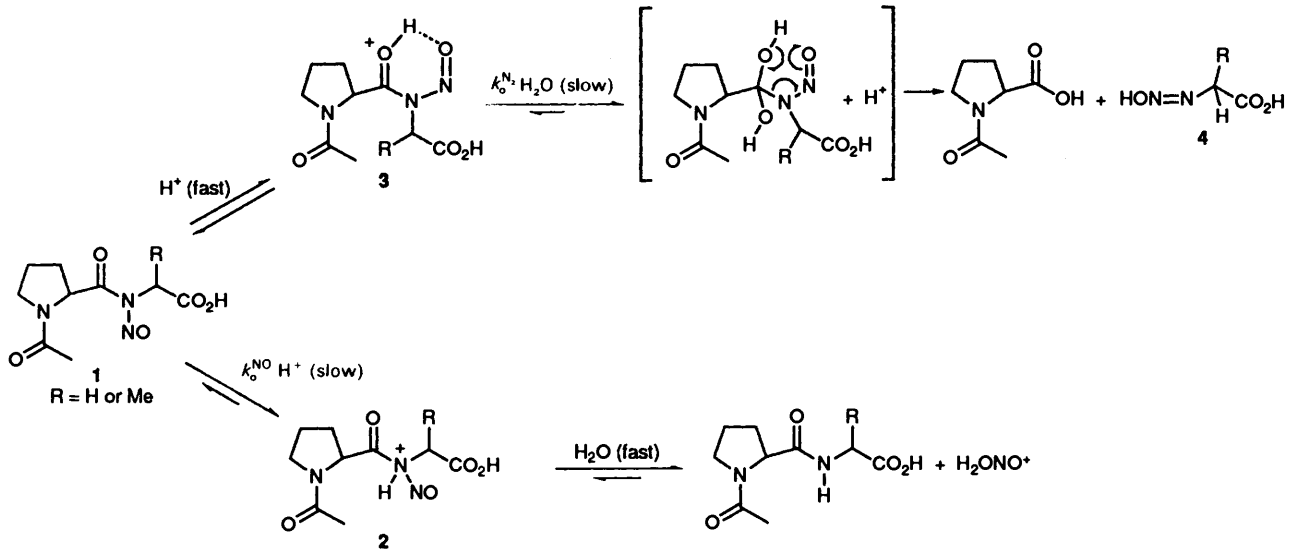
The products formed by the deamination pathway in strong acids were not specifically identified. For decomposition in aqueous buffers, however, where denitrosation is negligible, **1a** gave quantitative yields of *N*-acetyl-L-proline and glycolic acid and **1b** gave *N*-acetyl-L-proline and lactic acid similarly.[15] It was assumed that the same products result from deamination in strong acids.

Results

Both *N*-nitrosodipeptides **1a** and **1b** decomposed readily in strongly acidic media at 25 °C. Their overall rates of decomposition followed pseudo-first-order kinetics, eqn. (1), and the reaction products showed that two independent pathways were operative (Scheme 1). One pathway (k_0^{NO}) involved loss of the *N*-nitroso group to regenerate the parent dipeptide and the other ($k_0^{N_2}$) an hydrolysis resulting in cleavage of the peptide C–N bond and therefore deamination. This behaviour is reminiscent of that found earlier for the decomposition of *N*-nitrosamides in strong acids.[6] Values of k_0 were independent of the presence of an HNO_2 trap (either urea or sulphanilamide) as were the amounts of HNO_2 released in the presence of excess sulphanilamide (Table 1). This demonstrates that reversibility of the denitrosation reaction is not important and, as for *N*-nitrosamides, that denitrosation and deamination result from concurrent first order processes.

The overall decomposition rates of **1a** and **1b** were assumed to be the sum of denitrosation and deamination pathways [*i.e.* eqn. (3) applies] with the negligible contribution from purely

$$k_0 = k_0^{NO} + k_0^{N_2} \quad (3)$$



Scheme 2 Mechanisms for concurrent denitrosation and deamination of *N*-nitrosodipeptides in aqueous acid

Table 2 Decomposition rates (k_0, k_0^{NO} and $k_0^{N_2}$) and percentage denitrosation for **1a** in aqueous $HClO_4$ and H_2SO_4 at 25 °C: initial [**1a**] *ca.* 5×10^{-3} mol dm^{-3}; [sulphanilamide] = 5×10^{-2} mol dm^{-3}

Acid/mol dm^{-3}	$k_0/10^{-5}$ s^{-1}	$[HNO_2]_\infty$ (%)	$k_0^{NO}/10^{-5}$ s^{-1}	$k_0^{N_2}/10^{-5}$ s^{-1}
$HClO_4$				
1.20	4.63	22.2	1.03	3.6
2.34	10.2	45.1	4.60	5.6
3.29	18.7	63.8	11.9	6.8
4.05	21.9	76.5	16.8	5.1
4.70	39.3	81.1	32.1	7.2
5.33	60.0	83.0	49.8	10.2
5.93	93.2	89.1	83.0	10.2
H_2SO_4				
2.12	10.5	26.2	2.75	7.7
3.36	25.1	50.8	12.8	12.3
4.03	38.1	59.8	22.8	15.3
4.77	58.9	75.4	44.4	14.5
5.81	107	80.1	85.7	21.3
7.22	226	88.8	200	26.0
7.84	437	94.1	411	25.8
8.73	832	97.3	810	22.0

thermal decomposition. This can be justified by the much slower decomposition of **1a** and **1b** in organic solvents.[15] The extent of denitrosation was determined colorimetrically using Shinn's procedure[14] after trapping the HNO_2 released *in situ* with sulphanilamide (see Experimental). Because the decomposition of **1a** and **1b** is relatively rapid and the diazonium ion from sulphanilamide is stable in strong acids,[6] the reactions were allowed to reach completion (> 10 half lives) before coupling to produce the azo dye. The rate coefficient for denitrosation k_0^{NO} was readily calculated from k_0 and the final yield of HNO_2 relative to the initial *N*-nitrosodipeptide concentration, eqn. (4).

$$k_0^{NO} = k_0[HNO_2]_\infty/[N\text{-nitrosodipeptide}]_0 \quad (4)$$

The rate coefficient for deamination ($k_0^{N_2}$) was obtained by difference [eqn. (5)]. Experimental values of k_0 and percentage

$$k_0^{N_2} = k_0 - k_0^{NO} \quad (5)$$

$[HNO_2]_\infty$ for the decomposition in aqueous $HClO_4$ and H_2SO_4 at 25 °C of **1a** and **1b** are listed in Tables 2 and 3, respectively, together with the calculated values of k_0^{NO} and $k_0^{N_2}$.

Acidity Dependences.—From Table 2, it is apparent that deamination of **1a** predominates at low $[HClO_4]$ and $[H_2SO_4]$, but this reaction is overtaken by denitrosation at $[HClO_4]$ *ca.* 2.5 mol dm^{-3} and $[H_2SO_4]$ *ca.* 3.3 mol dm^{-3}. It follows that denitrosation is more strongly acid catalysed than deamination. This difference is more easily seen from plots of log k_0^{NO} and log $k_0^{N_2}$ *versus* the H_A acidity function[16] shown for the decomposition of **1a** in aqueous $HClO_4$ in Fig. 1. The plot is reasonably linear for denitrosation with slope 0.98 whereas the best straight line through points showing substantial scatter for deamination has slope *ca.* 0.26. Comparable results, summarised in Table 4, are obtained for catalysis by aqueous H_2SO_4 where the slope of log k_0^{NO} and log $k_0^{N_2}$ *versus* $-H_A$ are 1.17 and *ca.* 0.26 (with poor linearity), respectively. Further comparison of the data in Table 2 shows that at similar H_A values, decomposition of **1a** is slightly faster (*ca.* 1.6-fold) in aqueous H_2SO_4 than $HClO_4$ mainly because of an increased rate of deamination.

Very similar acidity dependences (Table 4) also apply to the denitrosation and deamination data for compound **1b** from Table 3. Thus, plots of log k_0^{NO} *versus* $-H_A$ are linear with slopes of 1.06 and 1.00 for aqueous $HClO_4$ and H_2SO_4, respectively,

Table 3 Decomposition rates (k_0, k_0^{NO} and $k_0^{N_2}$) and percentage denitrosation for **1b** in aqueous $HClO_4$ and H_2SO_4 at 25 °C: initial [**1b**] *ca.* 5×10^{-3} mol dm^{-3}; [sulphanilamide] = 5×10^{-2} mol dm^{-3}

Acid/mol dm^{-3}	$k_0/10^{-5}$ s^{-1}	$[HNO_2]_\infty$ (%)	$k_0^{NO}/10^{-5}$ s^{-1}	$k_0^{N_2}/10^{-5}$ s^{-1}
$HClO_4$				
1.11	27.7	2.61	0.72	27.0
1.94	33.8	7.10	2.40	31.4
2.52	38.5	10.3	3.98	34.5
3.15	44.1	17.2	7.59	36.5
3.81	50.8	28.5	14.5	36.3
4.25	59.7	34.4	20.5	39.2
5.11	75.5	52.2	39.4	36.1
5.68	108	69.2	74.7	33.3
6.03	156	71.6	112	44.3
6.55	200	78.0	156	44.0
H_2SO_4				
1.27	39.0	11.2	4.37	34.6
1.83	51.3	18.0	9.23	42.1
2.49	57.5	27.4	15.8	41.7
3.36	79.4	25.0	19.9	59.5
4.03	87.1	32.0	27.9	59.2
4.77	135	39.8	53.7	81.3
5.85	251	46.0	115	136
6.65	501	57.3	287	214
7.22	661	64.4	426	235
7.84	1000	74.1	741	259

Table 4 Acidity function (H_A) dependences for the denitrosation and deamination of **1a** and **1b** in aqueous $HClO_4$ and H_2SO_4 at 25 °C

N-Nitrosodipeptide	Acid	d log k_0^{NO}/ d $(-H_A)$	d log $k_0^{N_2}$/ d $(-H_A)$
1a	$HClO_4$	0.98	0.26
1a	H_2SO_4	1.17	0.26
1b	$HClO_4$	1.06	0.10
1b	H_2SO_4	1.00	0.24

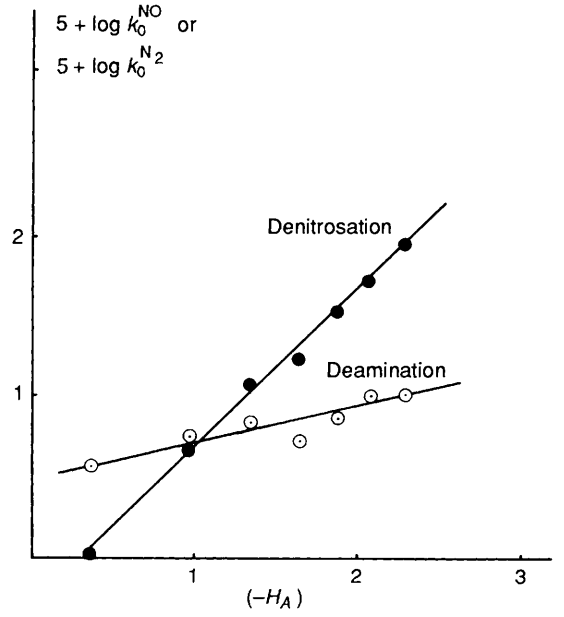


Fig. 1 Plot of log k_0^{NO} and log $k_0^{N_2}$ *versus* $-H_A$ for the decomposition of **1a** in aqueous $HClO_4$ at 25 °C

whereas the plots of log $k_0^{N_2}$ *versus* $-H_A$ have much lower slopes (*ca.* 0.1–0.24) and show substantial scatter. Like **1a**, compound **1b** decomposes more rapidly (*ca.* 3 fold) in aqueous H_2SO_4 than $HClO_4$, but this arises from faster denitrosation as well as faster deamination.

Otherwise, the main difference between the two *N*-nitrosodipeptide substrates is the higher lability of compound **1b** regardless of the solvent acid. For a given H_A value, **1b** decomposes *ca.* eight times faster than **1a** and most of this difference stems from faster deamination. Interestingly, **1b** undergoes faster deamination than **1a** even at the lowest solvent acidities (compare Tables 2 and 3) which suggests that **1b** is an intrinsically less stable compound.

Solvent Deuterium Isotope Effects.—Rates of decomposition and product ratios were briefly examined for **1a** in D_2SO_4 at 25 °C to evaluate the solvent deuterium isotope effects by comparison with H_2SO_4. These results, including the calculated values of k_0^{NO}, $k_0^{N_2}$ and the isotopic rate ratios, are reported in Table 5. Clearly, the denitrosation of **1a** is accompanied by a normal isotope effect [$k_0^{NO}(H_2SO_4) > k_0^{NO}(D_2SO_4)$] whereas deamination is accompanied by an inverse isotope effect [$k_0^{N_2}(H_2SO_4) < k_0^{N_2}(D_2SO_4)$].

Discussion

Both the products and the kinetic dependences show that decomposition of the *N*-nitrosodipeptides **1a** and **1b** in aqueous acids proceeds by at least two concurrent pathways (Scheme 1), each of which has first order dependence on substrate concentration. One pathway (k_0^{NO}) involves denitrosation to regenerate the parent dipeptide. The other ($k_0^{N_2}$) results in cleavage of the peptide C–N bond and subsequent deamination.

Denitrosation is more strongly acid catalysed (Table 4) and is the dominant pathway in concentrated $HClO_4$ and H_2SO_4 (Tables 2 and 3). Values of k_0^{NO} are independent of added sulphanilamide or urea (Table 1) which suggests that release of nitrous acid (NO^+) is rapid. An earlier step on the reaction path must therefore be rate limiting and the solvent deuterium isotope effect (Table 5) confirms that H^+ transfer from the solvent to the *N*-nitrosodipeptide **1a** is slow. At a given H_A value, the denitrosations of **1a** and **1b** proceed at virtually the same rates in aqueous $HClO_4$ and at similar but faster rates for both compounds in aqueous H_2SO_4. The additional methyl substituent of **1b** should have little effect on the H^+ transfer, and

Table 5 Decomposition of **1a** in D_2SO_4 at 25 °C and solvent deuterium isotope effects: initial [**1a**] *ca.* 5×10^{-3} mol dm^{-3}; [sulphanilamide] = 5×10^{-2} mol dm^{-3}

$[D_2SO_4]$/mol dm^{-3}	$k_0/10^{-5}$ s^{-1}	$[HNO_2]_\infty$ (%)	$k_0^{NO}/10^{-5}$ s^{-1}	$k_0^{N_2}/10^{-5}$ s^{-1}	$k_0^{NO}(H_2SO_4)/k_0^{NO}(D_2SO_4)$	$k_0^{N_2}(H_2SO_4)/k_0^{N_2}(D_2SO_4)$
5.27	40.5	52.1	21.1	19.4	2.5	0.87
7.16	136	65.7	89.4	46.6	2.6	0.54

the generally faster denitrosations in H_2SO_4 can be attributed to general acid catalysis. Bunnett and Olsen[17] have shown that variation of reaction rates with a_{H_2O} gives useful information about the rate limiting step and the role of water for acid catalysed reactions. All of the relevant Bunnett and Olsen plots of $\log k_0^{NO} + H_A$ *versus* $\log a_{H_2O}$ (the preferred correlation for slow H^+ transfers)[17] for the denitrosation of **1a** and **1b** in both aqueous $HClO_4$ and H_2SO_4 are reasonably linear with gradients (ω values) between -0.1 and -0.5. These ω values are entirely consistent with slow H^+ transfer[18] and they imply a common mechanism for the denitrosation of both **1a** and **1b**.

Deamination predominates in dilute $HClO_4$ and H_2SO_4 (Tables 2 and 3) and is the only significant decomposition pathway at pH > 0. It is also acid catalysed, but to a much lesser extent than denitrosation (Table 4). In fact, values of $k_0^{N_2}$ for both **1a** and **1b** correlate better with stoichiometric $[HClO_4]$ and $[H_2SO_4]$ than the H_A acidity function. The deamination pathway involves hydrolytic cleavage of the peptide C–N bond, which for regular peptides in acidic conditions usually involves rate limiting nucleophilic attack by water on the conjugate acid with rapid expulsion of the amino fragment. The same mechanism should prevail for the *N*-nitrosodipeptides **1a** and **1b**, particularly as the diazotic acid (**4**, Scheme 2) is a good leaving group. The experimental data support this conclusion. The inverse solvent isotope effect for **1a** (Table 5) shows that H^+ transfer to form a conjugate acid intermediate is rapid, and the reduced acidity dependences [$d \log k_0^{N_2}/d(-H_A)$ *ca.* 0.1–0.26] are indicative of a slow bimolecular reaction with H_2O. Unfortunately, the Bunnett and Olsen plots[17] for the deamination of **1a** and **1b** show pronounced curvature and the information they provide about the role of H_2O in the rate limiting step is difficult to interpret. However, faster deaminations of both **1a** and **1b** in aqueous H_2SO_4 than $HClO_4$ are indicative of HSO_4^- catalysis (and therefore rapid expulsion of the diazotic acid **4**) because other nucleophiles produce rate enhancements at pH > 0.[15]

The different solvent isotope effects and acidity dependences for denitrosation and deamination exclude a common rate limiting step for both reactions. The different solvent isotope effects for **1a** also eliminate mechanisms whereby denitrosation and deamination proceed *via* a common conjugate acid intermediate. If formation of this conjugate acid is slow for denitrosation [$k_0^{NO}(H_2SO_4) > k_0^{NO}(D_2SO_4)$] but a subsquent higher energy step is rate limiting for deamination [$k_0^{N_2}(H_2SO_4) < k_0^{N_2}(D_2SO_4)$], then the common conjugate acid intermediate would preferentially decompose *via* the lower energy, product-forming pathway to give the parent dipeptide and nitrous acid (NO^+) exclusively. Thus, denitrosation and deamination must proceed *via* two independent pathways involving unique conjugate acid intermediates. The structure of each of these conjugate acids is conjectural, but protonation of either the peptide *O*-atom or *N*-atom seems most probable. An analogous deduction was drawn earlier for the decomposition of *N*-butyl-*N*-nitrosoacetamide[6a] which also occurs *via* concurrent, acid-catalysed denitrosation and deamination pathways with very similar acidity dependences and solvent isotope effects to those found for the *N*-nitrosodipeptides **1a** and **1b**. Extending the argument for *N*-butyl-*N*-nitroso acetamide[6a] to *N*-nitrosodipeptides **1a** and **1b** leads to the conclusions that (i) denitrosation involves slow formation of the peptide *N*-conjugate acid **2** which then rapidly expels nitrous acid (NO^+), and (ii) deamination involves rapid formation of the peptide *O*-conjugate acid **3** which then undergoes a rate-limiting reaction with water followed by a rapid expulsion of the diazotic acid **4** (Scheme 2). There seem to be few mechanistic differences between the acid catalysed decompositions of *N*-nitrosodipeptides and alicyclic *N*-nitrosamides.

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