N-Bromosuccinimide Oxidation of Dipeptides and Their Amino Acids: Synthesis, Kinetics and Mechanistic Studies

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ABSTRACT: Dipeptides (DP), namely valyl–glycine (Val–Gly), alanyl–proline (Ala–Pro), and valyl–proline (Val–Pro) were synthesized by classical solution phase methods and characterized. The kinetics of oxidation of amino acids (AA) and DP by *N*-bromosuccinimide (NBS) was studied in the presence of perchlorate ions in acidic medium at 28°C. The reaction was followed spectrophotometrically at $\lambda_{max} = 240$ nm. The reactions follow identical kinetics, being first order each in [NBS], [AA], and [DP]. No effect on $[H^+]$, reduction product [succinimide], and ionic strength was observed. Effects of varying dielectric constant of the medium and addition of anions such as chloride and perchlorate were studied. Activation parameters have been computed. The oxidation products of the reaction were isolated and characterized. The proposed mechanism is consistent with the experimental results. An apparent correlation was noted between the rate of oxidation of AA and DP. © 2006 Wiley Periodicals, Inc. Int J Chem Kinet 38: 376–385, 2006

INTRODUCTION

Oxidative reactions play an important role in a variety of biochemical events ranging from normal metabolism to ageing and disease process [1,2]. Peptides and proteins represent major targets for modification in these reactions, and the identification of sites and structures of modification may lead to a mechanistic understanding and approaches for prevention. In this context, oxidation of α-amino acids is one of the well-documented biochemical processes. Several studies have been reported on the kinetics of oxidation of various substrates by NBS in different media [3–8]. Although, the kinetics of oxidation of amino acids and peptides with metal ions [9–11] reported, the studies on the oxidation of both amino acids and peptides by NBS have not been reported. The rate of oxidation of dipeptides and the corresponding amino acids is compared.

We have synthesized three dipeptides viz. valyl–glycine (Val–Gly), alanyl–proline (Ala–Pro), and valyl–proline (Val–Pro), which are fragments of elastic sequences [10] to elucidate the mechanism of these redox reactions.

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EXPERIMENTAL

All the amino acids used except glycine are of L-configuration unless otherwise specified. All *tert*-butyloxycarbonyl (Boc) amino acids, amino acid derivatives, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDCI), 1-hydrobenzotriazole (HOBt), trifluroacetic acid (TFA), and *N*-methylmorpholine (NMM) were purchased from Advanced Chem. Tech (Louisville, KY, USA). Thin-layer chromatography (TLC) was carried out on silica gel plates obtained from Whatmann Inc. with the following solvent systems: chloroform–methanol–acetic acid (95:5:3), R_f^1, chloroform–methanol–acetic acid (90:5:3), R_f^2, and chloroform–methanol–acetic acid (85:15:3), R_f^3.

The compounds on TLC plates were detected by UV light after spraying with ninhydrin or by chlorine/tolidine. The melting points were determined by using Thomas–Hoover melting point apparatus.

Boc-Val-Gly-OBzl. Boc-Val (3.5 g, 0.02 mol) and HOBt (3.37 g, 0.022 mol) in DMF (40 mL) was cooled to –15°C and EDCI (4.21 g, 0.22 mol) was added. After stirring for 20 min, a precooled solution of Gly-OBzl.Tos (6.78 g, 0.02 mol) and NMM (2.4 mL, 0.022 mol) in DMF (50 mL) was added and stirred overnight at room temperature. After evaporating DMF under reduced pressure, the residue was taken up by chloroform and extracted with 10% citric acid, water, and 5% sodium bicarbonate solution. The solvent was removed under reduced pressure and recrystallized from ether/ethyl acetate to obtain 6.34 g (87 %) of Boc-Val-Gly-OBzl. R_f^1 0.58, R_f^2 0.66, and R_f^3 0.72, mp 80°C (Lit [12] 80–82°C).

Val-Gly. Boc-Val-Gly-OBzl (0.015 mol) was saponified in methanol (50 mL) using 1 N NaOH (2.0 equivalent) for 2 h at room temperature. After evaporating the solvent under reduced pressure, the residue was taken up in water and washed with chloroform (3×25 mL). The aqueous layer was cooled and neutralized with cold 1 M HCl and extracted with chloroform (40 mL). The organic phase was washed with cold 0.1 M HCl, 50% saturated NaCl, and dried over Na_2SO_4. The solvent was removed in vacuo and triturated with ether, filtered, washed with ether, and dried to obtain 3.78 g (92%) of Boc-Val-Gly-OH. R_f^2 0.22 and R_f^3 0.34.

Boc-Val-Gly-OH (0.01 mol) was deblocked with TFA (10 mL/g of peptide) by stirring for 40 min. The solvent was removed under reduced pressure; the residue was triturated with ether and filtered, washed with ether to obtain TFA.Val-Gly-OH (100%).

Boc-Ala-Pro-OBzl. Boc-Ala-OH (4.1 g, 0.02 mol) was coupled to Pro-OBzl. HCl (4.8 g, 0.02 mol), using EDCI with HOBt and in the presence of NMM. The reaction was worked up the same as Boc-Val-Gly-OBzl to obtain 6.8 g (yield 88.2%) of Boc-Ala-Pro-OBzl. R_f^1 0.50 and R_f^2 0.63, mp 68°C (Lit [12] 67–69°C).

Ala-Pro. Boc-Ala-Pro-OBzl (5.8 g, 0.015 mol) was saponified in methanol (50 mL) using 1 N NaOH for 2 h at room temperature and worked up the same as Boc-Val-Gly-OH to obtain 4.2 g (yield 93.7%) of Boc-Ala-Pro-OH. R_f^2 0.24 and R_f^3 0.29. This was deblocked with TFA for 40 min to obtain TFA. Ala-Pro-OH (yield 100%).

Boc-Val-Pro-OBzl. Boc-Val-OH. (4.3 g 0.02 mol) was coupled to Pro-OBzl. HCl (4.8 g, 0.02 mol) using EDCI with HOBt and in the presence of NMM. The reaction was worked up the same as Boc-Val-Gly-OBzl to obtain 6.6 (yield 81.2%) of Boc-Val-Pro-OBzl. R_f^1 0.75 and R_f^2 0.84, mp 81°C (Lit [12] 80–82°C).

Val-Pro. Boc-Val-Pro-OBzl (6.1 g, 0.015 mol) was saponified in methanol (50 mL) using 1 N NaOH for 2 h at room temperature and worked up the same as Boc-Val-Gly-OH to obtain 4.4 g (yield 93.5%) of Boc-Val-Pro-OH. R_f^2 0.29 and R_f^3 0.33. This was deblocked with TFA for 40 min to obtain TFA. Val-Pro-OH (yield 100%).

Preparation of NBS Solution

An aqueous solution of NBS was prepared afresh each day from a GRS Merck sample of reagent, and its strength was checked by the iodometric method [13]. Solutions of AA and DP were prepared by dissolving the sample in H_2O of known strength. All other reagents were of analytical grade. Double-distilled water was used throughout the investigation.

Kinetic Procedure

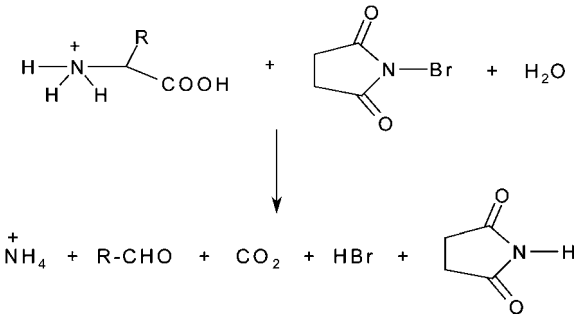
Solutions containing the requisite amount of substrate, perchloric acid (to maintain a known acid concentration), succinimide, mercuric acetate, and water (to keep the total volume constant) were placed in stoppered boiling tubes. The mixture was thermally equilibrated in a water bath at 28°C. To the solution in this tube was added an aliquot of pre-equilibrated NBS stock solution to give a known overall concentration. the progress of the reaction was monitored for two half-lives by measuring the absorbance of unreacted NBS at 240 nm using a spectrochem Elico SL 150 UV–Vis spectrophotometer. The reaction mixture containing $[NBS] = 1.0 \times 10^{-6}$, $[AA/DP] = 1.0 \times 10^{-4}$, $[HClO_4] = 0.01$ mol dm^{-3},

[succinimide] = 0.1 mol dm^{-3}, [$Hg(CH_3COO)_2$] = 0.0 01 mol dm^{-3} was quenched appropriately, plots of log(absorbance) vs. time were linear. The rate constants k_{obs} calculated from these plots were reproducible to within ±3% error.

Stoichiometry and Product Analysis

Mixtures containing AA/DP (0.0001 M), acid (0.01 M), and excess of *N*-bromosuccinimide (0.003 M) were kept for 24 h at 28°C. The unconsumed NBS was then determined; 1 mol of oxidant was sufficient to oxidize 1 mol of glycine/alanine/valine. Two moles of oxidant was sufficient to oxidize 1 mole of proline (AA) and 1 mole of Val–Gly (DP) and 3 mole of oxidant were sufficient to oxidize 1 mole of Ala–Pro and Val–Pro (DP) leading to aldehydes, carbon dioxide, ammonia, and succinimide. Based on these results, the following stoichiometric equations are suggested.

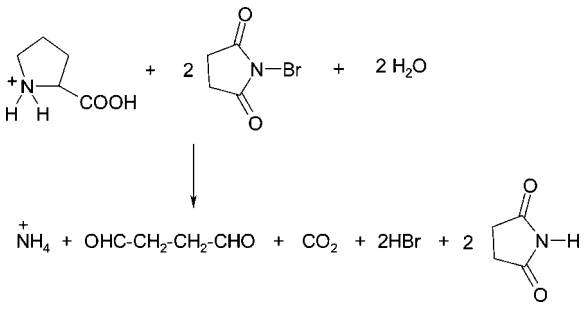
Stoichiometric Equations for Glycine (Gly), Alanine (Ala), and Valine (Val)



where
R = —H for glycine ; R = —CH_3 for alanine

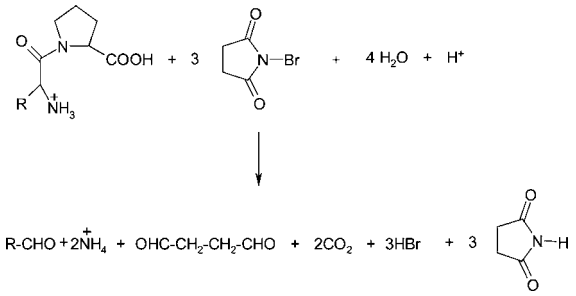
R = —$CH(CH_3)_2$ for valine

Stoichiometric Equations for Proline



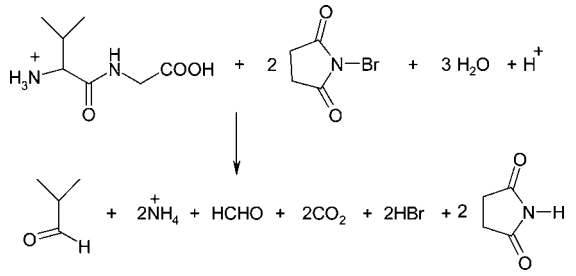
Stoichiometric Equations for Dipeptides (DP)

Alanyl–Proline (AP) and Valyl–Proline (VP)



where
R = —CH_3 for AP and R = —$CH(CH_3)_2$ for VP

Valyl–Glycine (VG)



After the reaction was completed, the reaction products were extracted with diethyl ether and subjected to column chromatography on silica gel (60–200 mesh) using a gradient elution (dichloromethane to chloroform). Aldehydes were analyzed qualitatively by gas chromatography. The (R_f) retention values of formaldehyde, acetaldehyde, isobutaraldehyde, and succinaldehyde are 6.0, 5.14, 27.4, and 31.9, respectively, which are identical with authentic samples. NH_3 and CO_2 were detected by the conventional method.

RESULTS

Effect of Varying Reactant Concentration on the Rate

All kinetic runs were performed under pseudo-first-order conditions with [AA] ≫ [NBS] and [DP] ≫ [NBS]. Plots of log [NBS] vs. time were linear even beyond 75% of the reaction, showing a first-order dependence of the rate on [NBS] (Table I) at constant $[succinimide]_0$, $[HClO_4]_0$,$[NaCl]_0$, and temperature, the rate increased with increase in $[AA]_o$ and in $[DP]_o$ (Table I). Plots of log k_{obs} vs. log [AA] (Fig. 1) were

Table I Effect of Varying Reactant Concentration on the Rate of Oxidation of Dipeptides and Their Amino Acids with $[HClO_4] = 0.01$ mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}, $[Hg(CH_3COO)_2] = 0.001$ mol dm^{-3} $T = 301$ K

$\times 10^6$[NBS] (mol dm^{-3})	$\times 10^4$[S] (mol dm^{-3})	$\times 10^5$ k_{obs} (s^{-1})						
		Gly	Ala	Val	Pro	Val–Pro	Ala–Pro	Val–Gly
0.6	1.0	10.81	11.64	12.40	15.88	08.57	08.50	06.20
0.8	1.0	10.66	11.62	12.37	15.70	08.76	08.45	06.33
1.0	1.0	10.61	11.61	12.79	15.88	08.68	08.56	06.47
1.2	1.0	10.24	11.58	12.89	15.20	08.53	08.66	06.55
1.4	1.0	10.10	11.48	12.85	15.45	08.71	08.70	06.73
0.6	0.6	07.73	08.05	08.24	09.59	05.02	05.18	03.89
0.6	0.8	08.18	09.23	10.33	12.47	06.87	06.89	05.98
0.6	1.0	10.61	11.46	12.79	15.88	08.68	08.56	06.47
0.6	1.2	12.07	13.72	15.86	18.82	09.77	10.82	08.85
0.6	1.4	14.20	16.39	17.25	20.86	10.55	12.62	10.20
0.6	1.6	16.24	17.85	19.15	22.36	11.48	14.29	12.72

linear with slopes of 1.00, 0.99, and 0.97 for Gly, Val, and Pro, respectively. Plots of log k_{obs} vs. log [DP] (Fig. 1) were linear with slopes of 1.05, 0.97, and 1.10 for Gly–Val, Ala–Pro, and Val–Pro, respectively.

Effect of $[HClO_4]$

Kinetic measurements were performed in $HClO_4$–$NaClO_4$ solution of different $[H^+]$. The effective $[H^+]$ used was evaluated with the aid of a calibration curve of $[HClO_4]$ vs. $[H^+]$. An increase in $[H^+]$ (0.1–2.0 M) had no effect on the rate.

Effect of Rate on Product and Added Salts

The effect on the rate of varying the concentration of succinimide (which is the reduction product of the oxidant) was investigated. An increase in [succinimide] (from 0.001 to 0.01 M) had no effect on the rate. Similarly, the effects of the anions $[Cl^-]$ (from 0.001 to 0.05 M) and ClO_4^- from (from 0.001 to 0.1 M) on the rate were insignificant. The reaction product succinimide had no effect on the reaction, indicating that the product is not involved in pre-equilibrium with the oxidant.

Effect of Varying Solvent Composition

The solvent composition of the medium was varied by adding methanol (0.0–40%), to the reaction mixture. The rate increased with increase in methanol content (Table II). The plots of log k_{obs} vs. $1/D$ (D = dielectric constant of the medium) were linear with positive slopes (Fig. 2). Measurements of rate constants were carried out in both the presence and absence of AA/DP with NBS. The rate constants were taken for the calculation of the effective k_{obs}, although the rate of oxidation of methanol in the absence of both AA and DP is negligible under the conditions employed.

Effect of Temperature

To determine the activation parameters, the reactions were carried out at different temperatures (22–34°C, Table III). The Arrhenius plots of log k_{obs} vs. $1/T$ (Fig. 3) were linear. The activation energies (E_a) were

Table II Effect of Varying Dielectric Constant on the Rate with $[NBS] = 1.0 \times 10^{-6}$ mol dm^{-3}, $[S] = 1.0 \times 10^{-4}$ mol dm^{-3}, $[Hg(CH_3COO)_2 = 1.0 \times 10^{-3}$ mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}, $[H^+] = 1.0 \times 10^{-2}$ mol dm^{-3}, $T = 301$ K

MeOH (%, v/v)	Dielectric Constant (D)	$\times 10^5 k_{obs}$(s^{-1})						
		Gly	Ala	Val	Pro	Val–Pro	Ala–Pro	Val–Gly
0	76.73	10.61	11.61	12.79	15.88	8.68	8.56	6.47
10	72.37	12.47	12.39	14.25	18.51	9.81	9.24	7.61
20	67.48	15.88	14.48	16.63	20.06	10.46	10.25	8.69
30	62.71	17.54	15.85	18.10	23.11	11.19	12.62	10.20
40	58.06	20.06	17.85	20.25	26.05	12.44	14.29	12.47

Table III Temperature Dependence of the Oxidation of Dipeptides and Their Amino Acids with $[NBS] = 1.0 \times 10^{-6}$mol dm^{-3}, $[S] = 1.0 \times 10^{-4}$mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}, $[H^+] = 1.0 \times 10^{-2}$mol dm^{-3}

Temperature (K)	$\times 10^3 1/T$ (K^{-1})	$\times 10^5 k_{obs}(s^{-1})$						
		Gly	Ala	Val	Pro	Val–Pro	Ala–Pro	Val–Gly
295	3.389	5.89	08.46	8.41	9.12	6.87	6.31	4.11
298	3.355	7.86	10.48	10.23	12.02	7.73	7.19	5.35
301	3.322	10.61	12.39	12.79	15.88	8.68	8.56	6.47
304	3.289	13.54	14.46	15.67	20.64	9.59	9.24	7.61
307	3.257	16.47	16.23	18.10	25.47	10.66	10.05	8.69

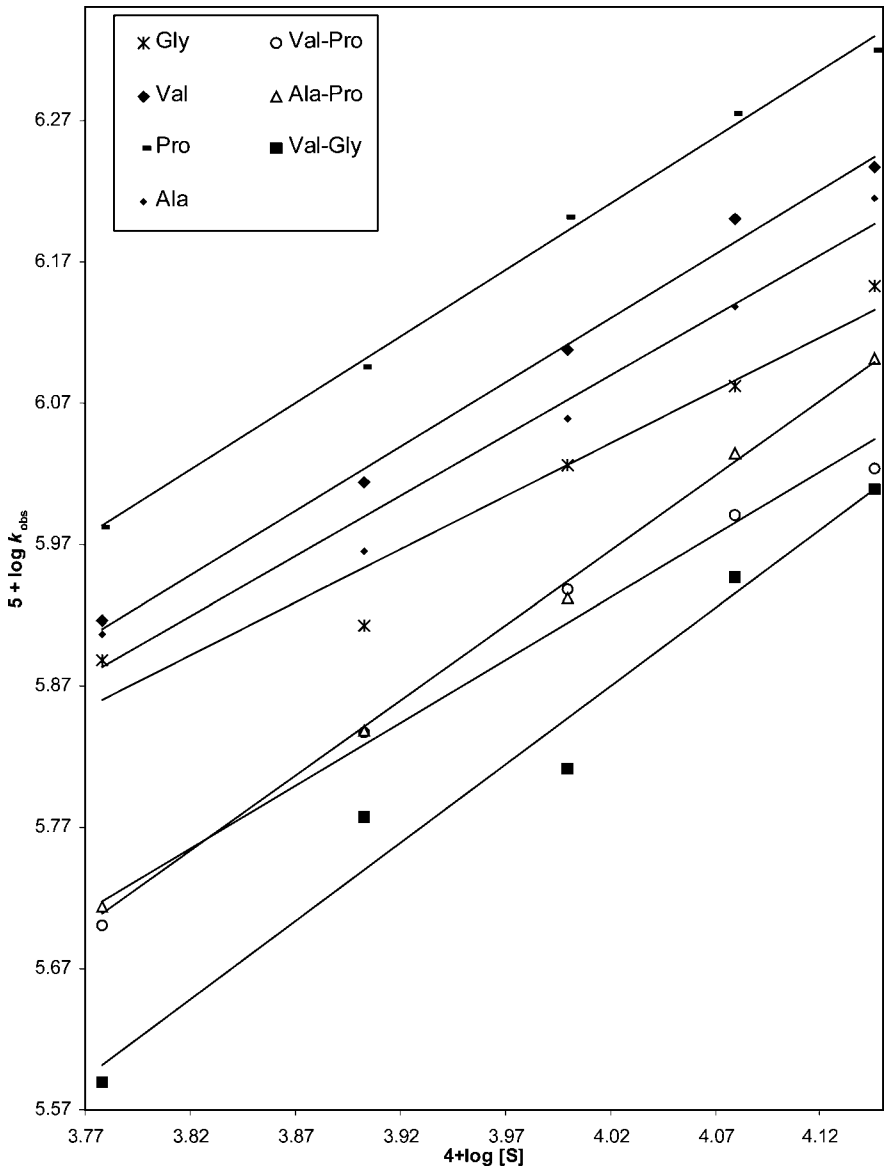


Figure 1 Effect of varying reactant concentration on the reaction rate with $[NBS] = 1.0 \times 10^{-6}$ mol dm^{-3} $[HClO_4] =$ 0.01 mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}, $[Hg(CH_3COO)_2] = 0.001$ mol dm^{-3}, $T = 28°C$.

Table IV Kinetic and Activation Data for the Oxidation of Dipeptides and Their Amino Acids with $[NBS] = 1.0 \times 10^{-6}$ mol dm^{-3}, $[S] = 1.0 \times 10^{-4}$ mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}, $[H^+] = 1.0 \times 10^{-2}$ mol dm^{-3}

Substrate	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	log A
Gly	70.25	67.75	−93.37	95.87	8.36
Val	50.38	49.24	−122.85	94.90	6.40
Pro	48.25	45.75	−167.91	96.29	4.57
Ala	64.78	62.27	−114.75	96.82	7.24
Val–Pro	25.87	23.36	−245.90	97.38	0.40
Ala–Pro	29.38	26.87	−233.78	97.24	1.03
Val–Gly	32.85	30.34	−224.94	98.05	1.5

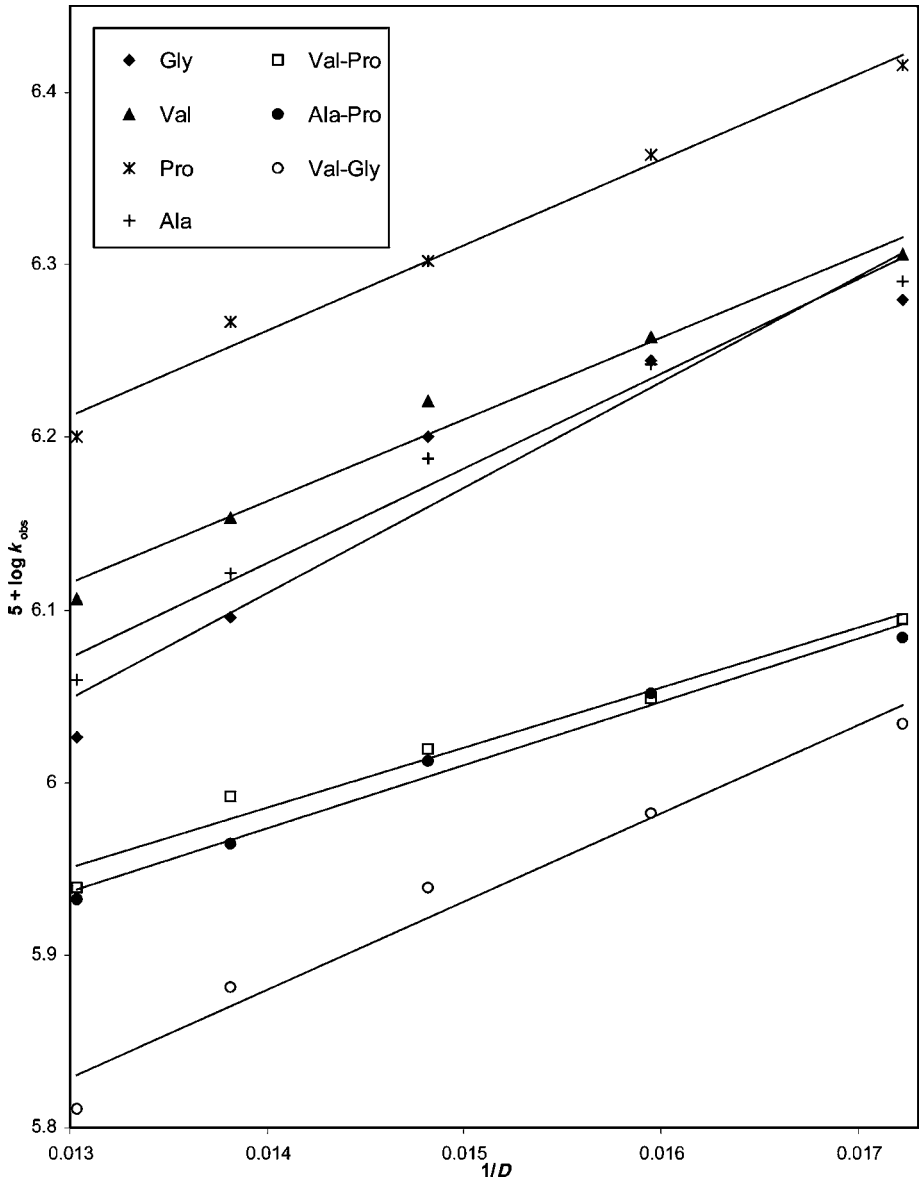


Figure 2 Effect of varying dielectric constant on the reaction rate, with $[NBS] = 1.0 \times 10^{-6}$ mol dm^{-3}, $[AA/DP] = 1.0 \times 10^{-4}$ mol dm^{-3}, $[Hg(CH_3COO)_2] = 1.0 \times 10^{-3}$ mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}, $[H^+] = 1.0 \times 10^{-2}$ mol dm^{-3}, $T = 28°C$.

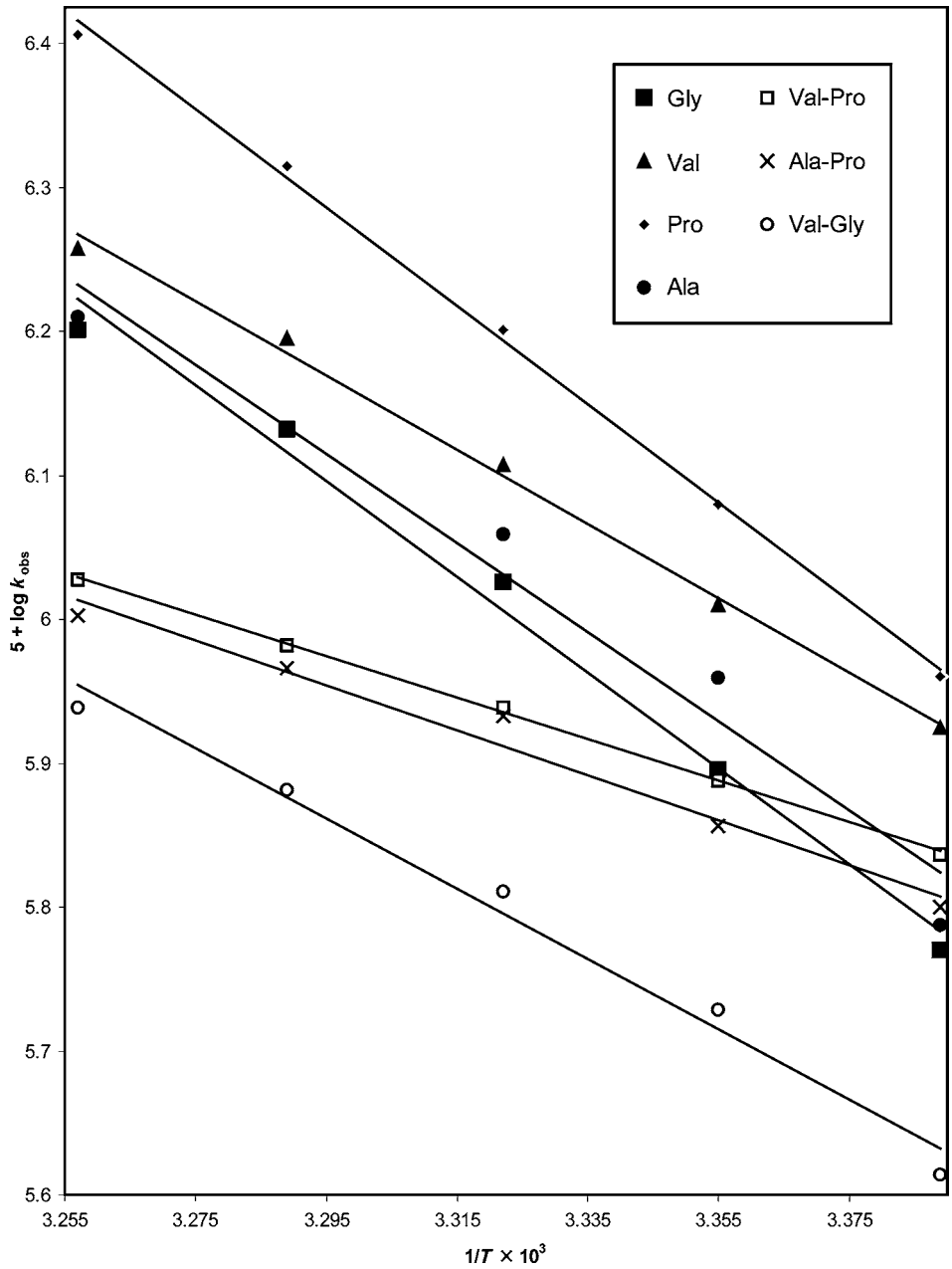


Figure 3 Temperature dependence of the oxidation of substrate by NBS, with $[NBS] = 1.0 \times 10^{-5}$ mol dm^{-3}, [AA/DP] = 1.0×10^{-4} mol dm^{-3}, $[HClO_4] = 1.0 \times 10^{-2}$ mol dm^{-3}, $[Hg(CH_3COO)_2] = 1.0 \times 10^{-3}$ mol dm^{-3}, [succinimide] = 0.1 mol dm^{-3}.

calculated from the slope of the plots, from these values, the thermodynamic parameters $\Delta H^{\#}$, $\Delta S^{\#}$, $\Delta G^{\#}$, and the frequency factor (log A) were evaluated (Table IV).

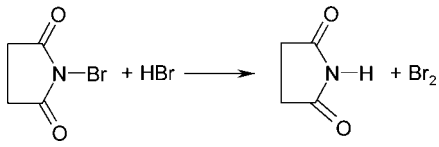
Test for Free Radicals

Addition of reaction mixture to aqueous acrylamide monomer solutions did not initiate polymerization, indicating the absence of in situ formation of free radical species in the reaction sequence.

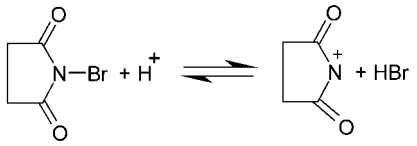
DISCUSSION

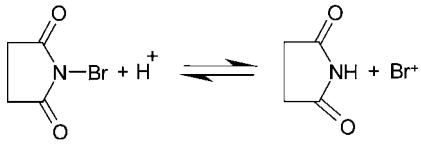
The results of the oxidation of amino acids and dipeptides, recorded here, have revealed that the reactions have identical kinetics and thus appear to have

common mechanism. Insignificant effect of mercuric acetate on reaction rate rules out its involvement in NBS oxidation and acts only as a scavenger [14,15] for any Br^- formed in the reaction. It suppresses completely the oxidation by Br_2, which would have been formed by the interaction of HBr and NBS as follows:



Mercuric acetate thus ensures the oxidation purely through NBS. NBS is known to exist in acidic media in the following equilibria:

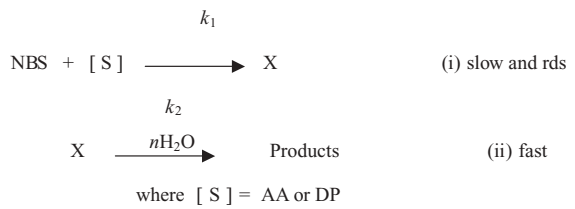




The NBS itself or protonated NBS, i.e., N^+BSH or Br^+ may be the possible oxidizing species in acidic media. In the presence of mercuric acetate protonated form of NBS, i.e., N^+BSH has been considered [16] as a reactive species of NBS in acidic medium.

In the present investigation, it was found that the added succinimide has a negligible effect on the rate of the reaction. This categorically excludes Br^+ as the oxidizing species. Hence, the active species may be NBS or N^+BSH. The order of the reaction with respect to $[H^+]$ is zero, and hence N^+BSH does not participate in the rate-determining step. All these factors indicate that NBS is the only possible oxidant species taking part in the reaction. In the light of the experimental results, a suitable mechanism has been proposed.

Scheme 1 accounts for the observed experimental results for AA and DP:



Scheme 1

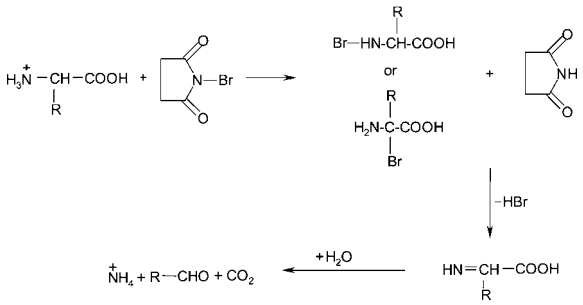
where [S] = AA or DP. Hence, rate = k_1[NBS] [AA or DP].

CONCLUSION

The rates of oxidation of amino acids and dipeptides by NBS were compared under identical experimental conditions, and it was found that the rates of oxidation of dipeptides were slower than those of free amino acids. The change in each case is due to the increased distance between the functional groups and consequently weaker electrostatic effects. Hence, the oxidation of dipeptides is expected to be slower than that of free amino acids. Further, an apparent correlation was noted between the rate of oxidation and hydrophobicity [17] of those sequences where increased hydrophobicity results in an increased rate of oxidation.

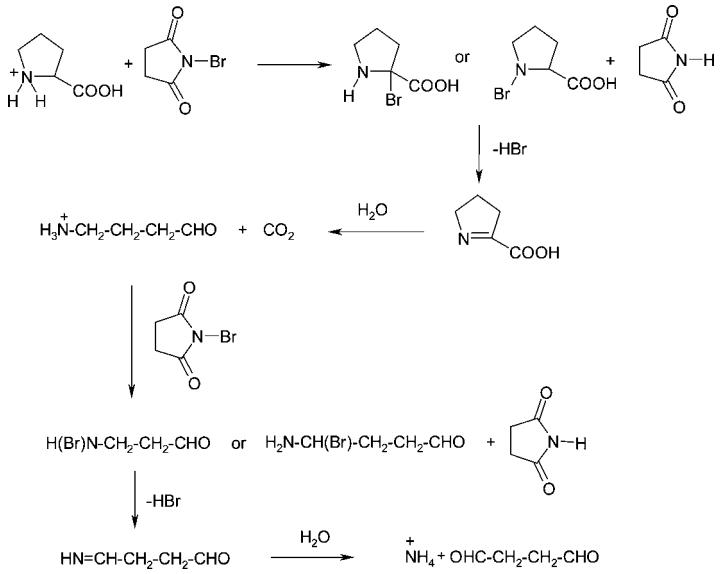
The most hydrophobic dipeptide, Val–Pro, oxidized at a faster rate than the less hydrophobic dipeptides Ala–Pro and Val–Gly. The probable reason for the increased oxidation rate for the more hydrophobic dipeptides is that the carboxylic groups are more destabilized, which enhances the rate of formation of a transition state complex with NBS, and the oxidation rate may be higher. Further, it was observed that the DP with Pro as C-terminus, Val–Pro, and Ala–Pro are more susceptible to oxidation than the DP with Gly as C-terminus (Val–Gly).

APPENDIX: MECHANISM FOR AMINO ACIDS

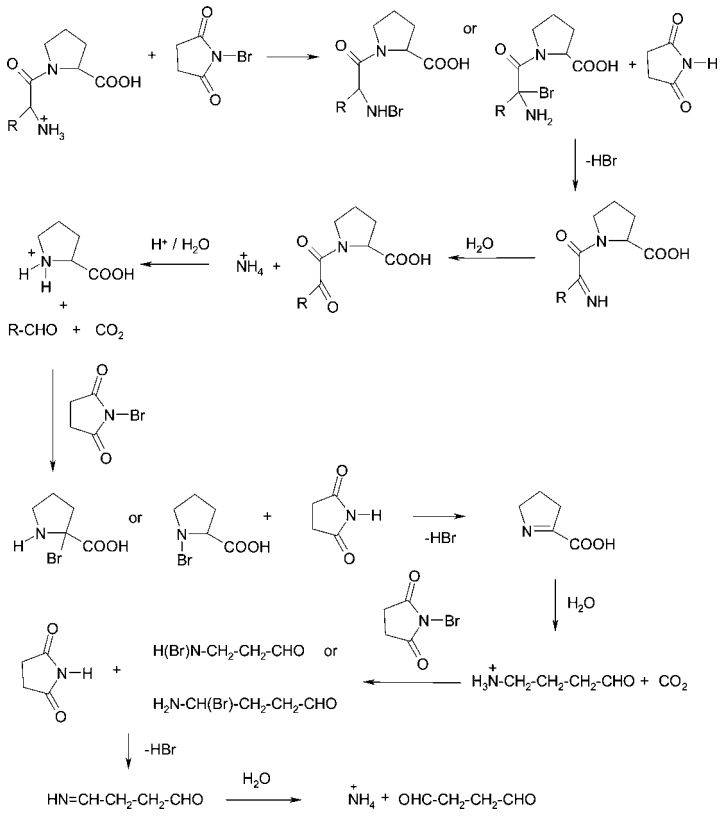


where R = —H for glycine; R = —CH_3 for alanine; R = —CH $(CH_3)_2$ for valine.

For Proline (Pro)

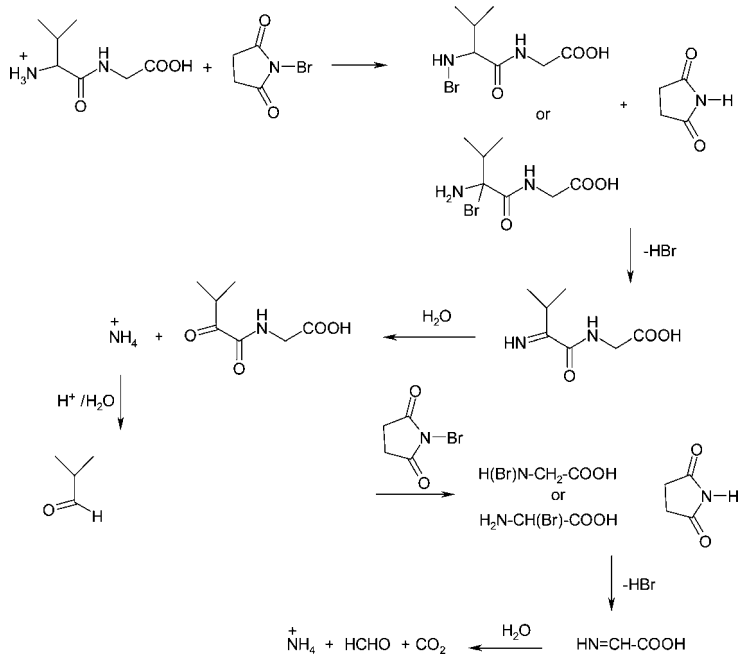


For Dipeptides (Val–Pro and Ala–Pro)



where R = —CH $(CH_3)_2$ for valyl-proline and R = —CH_3 for alanyl-proline.

For Val–Gly



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