Initiation of lipid autoxidation by ABAP at pH 4–10 in SDS micelles†

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The rates of radical generation, R_i, by two water soluble initiators: 2,2′-azobis(2-methylpropionamidine) and 2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide], and the lipid soluble 2,2′-azobisisobutyronitrile were measured in an SDS micellar system over a pH range of 4–10. Enhanced values of R_i at low pH are attributed to Coulombic repulsion of protonated radicals.

In quantitative studies of the kinetics of lipid autoxidation a constant rate of formation of initiating free radicals is required.[1] This is usually provided by carbon-centered radicals thermally or photochemically generated from azo compounds. In the presence of oxygen, these radicals are rapidly converted to peroxyl radicals, $RO_2^{\bullet}$, and these attack a lipid molecule, LH:

$$RN{=}NR \rightarrow R^{\bullet} \rightarrow RO_2^{\bullet}\ (\text{rate} = R_g) \quad (1)$$

$$RO_2^{\bullet} + L\text{-}H \rightarrow ROOH + L^{\bullet}\ (\text{rate} = R_i) \quad (2)$$

Reactions (1) and (2) are called the initiation steps of the subsequent chain process. In the next step, the $L^{\bullet}$ radical reacts at a diffusion-controlled rate with O_2 to form a lipidperoxyl radical, $LO_2^{\bullet}$, which abstracts hydrogen atom from another lipid molecule to continue the chain.

$$L^{\bullet} + O_2 \rightarrow LO_2^{\bullet} \quad (3)$$

$$LO_2^{\bullet} + LH \rightarrow LO_2H + L^{\bullet}\ (\text{rate constant } k_p) \quad (4)$$

Reactions (3) and (4) constitute the chain propagation steps, and lead to the formation of hydroperoxides, often in high yields. Under 1 atmosphere air or oxygen and at 30 °C, reaction (4) with a rate constant 31 M^{-1} s^{-1} for methyl linoleate[2] is the rate determining step. The chain is terminated by the radical/radical reaction:

$$2LO_2^{\bullet} \rightarrow \text{non-radical products (rate constant } 2k_t) \quad (5)$$

The rate of autoxidation is given by eqn (I).

$$R_{ox} = -\frac{d[O_2]}{dt} = \frac{d[LO_2H]}{dt} = k_p\sqrt{\frac{R_i}{2k_t}}[LH] \quad (I)$$

Provided R_i and [LH] are known, a measurement of R_{ox} yields $k_p/(2k_t)^{1/2}$, the oxidizability of LH at the reaction temperature.[3,4]

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† Electronic supplementary information (ESI) available: Description of preparation of micelles, concentrations of initiators, inhibitors, buffers, details of measurements and equipment, rates of oxidation and rates of inhibited oxidation (Tables S1-S8), kinetic chain lengths (Table S9), plots of [PMHC] *vs.* time (Tables S10-S12, Figures S1-S3). See DOI: 10.1039/b715089j

For many years, studies of autoxidation mechanisms and kinetics were largely confined to homogeneous systems. Such processes are now well understood. However, more recently an increasing interest in biological systems has caused autoxidation research to focus on heterogeneous systems where the lipids were dispersed as micelles, liposomes, bilayers and other model membranes. The kinetics of autoxidation in many of these heterogeneous systems have been shown to follow the same rate law as for homogeneous solutions.[5]

In heterogeneous systems, water-soluble initiators that give charged radicals are generally employed. These radicals are commonly assumed to mimic the natural radicals present in biological systems. One of the most frequently used water-soluble azo initiator is 2,2′-azobis(amidinepropane) dihydrochloride, abbreviated as ABAP (Chart 1), initially invented for aqueous emulsion polymerization[6] and introduced for autoxidation studies in 1984.[7] At 37 °C and at pH 7, ABAP slowly decomposes and in the presence of air a constant flux of water-soluble positively charged peroxyl radicals is provided to initiate an autoxidation at a constant rate over several hours.‡

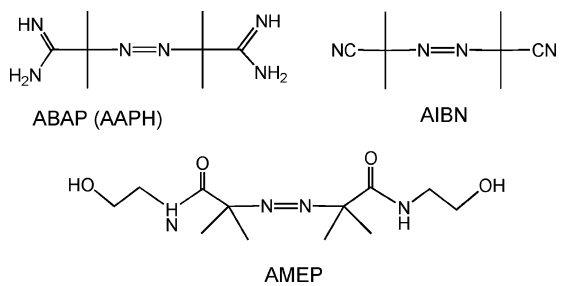

Chart 1 Structures of initiators: neutral form of 2,2′-azobis(2-amidinopropane) abbreviated ABAP or AAPH, 2,2′-azobisisobutyronitrile (AIBN) and 2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide] abbreviated as AMEP.

Knowing the rate of initiation is important in quantitative measurements of the antioxidant activity of chain breaking antioxidants. Recent studies on kinetic solvent effects have shown[8] that the reactions of electron deficient radicals $X^{\bullet}$ ($LO_2^{\bullet}$ and 2.2′-diphenyl-1-picrylhydrazyl, **dpph**$^{\bullet}$, which is isoelectronic with peroxyl) with phenols (most of which are chain-breaking antioxidants) in solvents that support phenol ionization can proceed *via* three nonexclusive mechanisms.[9] In non-polar solvents hydrogen atom transfer (HAT) and proton-coupled electron transfer (PCET) operate. However, in polar, ionizing solvents a small (usually very small) fraction of the phenol will be present as its anion and this anion can reduce electron-deficient radicals by the sequential proton-loss electron transfer, SPLET, mechanism.[8] In polar solvents all three mechanisms, HAT/PCET and SPLET can

operate. The HAT/SPLET competition depends on [ArO^-]/[ArOH] and usually [ArO^-] ≪ [ArOH] and the overall rate of the SPLET process will depend on the concentration of ionized form of phenol.[9] The ability of phenols to break autoxidation chains, expressed as the overall rate constant, k_{inh}, for the ArOH + $X^{\bullet}$ process is one of the most important parameters characterizing antioxidant activity. The SPLET mechanism can be much faster than the HAT/PCET processes and its relative importance is dependent on acid–base equilibria. Systematic studies on the role of pH and acid–base equilibria on the chemistry of chain-breaking antioxidants, and magnitude of k_{inh} would give valuable information about the overall antioxidant activity of phenols in non-homogeneous systems. However, for quantitative studies of the role of pH on the kinetics of inhibited and non-inhibited autoxidations, detailed information about initiation rates at various pH's is required. To our knowledge there are only three publications on this subject. Ito[10] studied the rates of radical decomposition, k_d, and non-radical hydrolysis of ABAP at 60 °C and observed a 30% decrease of k_d when the pH increased from 1 to 7, followed by a 10% decrease from pH 7 to 10. At pH > 7 hydrolysis was much faster than decomposition to radicals. Niki in his review on free radical initiators as sources of water- or lipid-soluble peroxyl radicals[11] claimed that the rate of ABAP decomposition was dependent to only a minor extent on solvent and pH. He supported that conclusion by experimental data on the rate of methyl linoleate autoxidation in an aqueous dispersion initiated by ABAP at 37 °C at pH's from 3 to 9.[12] Taking into account these two reports, the third, more recent work by Hanlon and Seybert[13] is surprising. These workers reported a strong *increase* of the rate of peroxide formation during the autoxidation of methyl linoleate in Triton X-100 micelles initiated with ABAP as the pH was increased from 5 to 9. In the same work, measurements of k_d as a function of pH gave almost the same results as had been reported by Ito: k_d at pH 7.5 was 40% lower than at pH 5.5. These contradictory results, *i.e.*, R_{ox} increases with pH whereas k_d decreases, were assumed to be due to a reversible addition of oxygen to the initiating carbon-centered amidinium radical and to a decrease of pK_a from 12 for parent ABAP molecule to 6.9 for its radical.

The cage effect makes the efficiency of radical formation always less than 1, commonly *ca.* 0.5. For this reason, the measurement of initiator decomposition does not provide the rate of initiation which can, however, be provided by the method of Boozer *et al*.[14] In this method, the duration of the induction period, τ_{ind}, is measured (see Fig. 1) for an autoxidation inhibited by an antioxidant, ArOH, having a known stoichiometric factor, n = the number of peroxyl radicals trapped by each ArOH molecule. Both, α-tocopherol and a synthetic analogue, 2,2,5,7,8-pentamethyl-6-chromanol (PMHC) have n values = 2, and are frequently used in such experiments. The value of R_i can be calculated from eqn (II), where $[ArOH]_0$ is the initial concentration of ArOH.

$$R_i = n\,[ArOH]_0/\tau_{ind} \qquad \text{(II)}$$

Listed in Table 1 are the results of our PMHC based measurements of R_i for the ABAP initiated autoxidation of methyl linoleate in SDS emulsions.§ We chose SDS to study the effect of pH because SDS micelles can be considered as a simple mimic of the amphiphilic environment of a phospholipid bilayer. For all three concentrations of PMHC consistent data were obtained: R_i decreases by a factor of about 2 from pH 4 to pH 6 and then is reasonably constant up to pH 9.

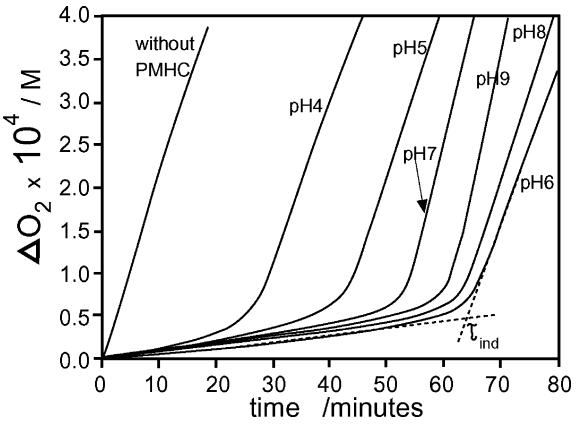

Fig. 1 Typical plots of oxygen uptake $\Delta[O_2] \times 10^4$/M during the inhibited (8.6 μM PMHC) and uninhibited autoxidation of SDS emulsions containing methyl linoleate at various pH 4–9, measured by an oxygen Clark type electrode. Induction time, τ_{ind}, was defined as it is shown on the plot. Concentration of ABAP was 10 mM in each experiment.

Table 1 Induction periods determined for autoxidation of methyl linoleate emulsion (SDS) at 37 °C and various pH for three concentrations of PMHC and calculated rates of initiation calculated [eqn (II)]. Values τ_{ind} are averages from the number of measurements given in parentheses

	[PMHC][a]						
	4.3		8.6		17.3		
pH	τ_{ind}[b]	R_i[c]	τ_{ind}[b]	R_i[c]	τ_{ind}[b]	R_i[c]	Mean R_i[c]
4	13.5 (4)	10.1	26.8 (6)	10.1	55.0 (6)	10.1	10.1 ± 0.1
5	19.0 (4)	7.5	45.5 (6)	6.3	89.7 (6)	6.4	6.8 ± 0.3
6	33.6 (8)	4.3	66.5 (6)	4.3	148.0 (6)	3.9	4.2 ± 0.2
7	28.0 (5)	5.1	52.5 (6)	5.5	104.8 (6)	5.5	5.4 ± 0.4[d]
8	29.1 (7)	4.9	62.5 (6)	4.6	122.9 (8)	4.7	4.7 ± 0.2
9	29.0 (10)	4.9	59.4 (7)	4.9	111.3 (8)	5.2	5.0 ± 0.3
10	29.3 (5)	4.9	55.3 (3)	5.2	99.5 (3)	5.8	5.3 ± 0.5

[a] μM. [b] minutes. [c] nM s^{-1}. [d] Values R_i calculated for 10 mM ABAP at 37 °C and pH 7.0 or 7.4 in various heterogeneous systems are: 13 nM s^{-1} in LDL,[15] 4.5 nM s^{-1} in LDL,[16] 5.8 nM s^{-1} for 11.3 mM ABAP in LDL,[17] 3.9 nM s^{-1} in soybean PC liposomes,[18] and 0.73 nM s^{-1}(at 30 °C) in SDS micelles of linoleic acid.[7a]

Most literature data on R_i for ABAP were made only at pH 7 or 7.4, our value R_i for 10 mM ABAP at pH 7 is in good to reasonable agreement with these data, see footnote *d* of Table 1. However, we did find data at pH 7.2 and 11 which allowed us to calculate R_i values for 10 mM ABAP of 2.2 and 1.29 nMs^{-1}, respectively.[18] These last two R_i, together with our own R_i values and Ito's results,[10] show that the rate of initiation by ABAP does not increase with increasing pH. The decrease of 60% in R_i from pH 4 to 6 may be followed by a small increase in R_i (10%) at higher pHs. Thus, our results are in agreement with Hanlon and Seybert data on ABAP decomposition rates (*i.e.* processes described by reaction (1) as well as non-radical decomposition) but raise question about the validity of their measurements of rates of peroxide formation [reaction (2)].

Table 2 Rates of non-inhibited autoxidation (R_{ox}) of methyl linoleate in SDS micelles initiated by 10 mM ABAP and rates of initiation (R_i) at various pH

pH	Number of determinations	$R_{ox} \times 10^7$/M s^{-1}	$R_i \times 10^9$/M s^{-1}
4	22	3.0 ± 0.2	1.5 ± 0.2
5	22	2.8 ± 0.3	1.2 ± 0.3
6	30	2.9 ± 0.2	1.3 ± 0.2
7	22	4.2 ± 0.8	2.9 ± 1.1
8	29	3.4 ± 0.3	1.8 ± 0.3
9	30	5.6 ± 0.8	5.1 ± 1.4
10	14	6.0 ± 0.6	5.7 ± 1.1

Since the induction period method for determining in R_i [14] has never been used over a wide pH range, it was necessary to validate this method [*i.e.* of eqn (II)] by comparison with other procedures in case pH affects phenol stoichiometric factors. A second method for determining in R_i is based on the measured rates of oxygen uptake for uninhibited autoxidations (see Fig. 1), eqn (I), which transforms to eqn (III).

$$R_i = \left(\frac{R_{ox}}{k_p/\sqrt{2k_t}\,[LH]}\right)^2 \qquad \text{(III)}$$

The oxidizability, $k_{p2}/(2k_t)^{1/2}$, for methyl linoleate at 37 °C was taken to be 0.033 (M s)$^{-1/2}$ in SDS micelles,[4a] and assumed to be pH independent. In eqn (III) the effective [LH] in SDS micelles was taken to be 0.24 M.¶ The results, R_{ox} and R_i rates at pH range 4–10 obtained by this method are presented in Table 2. The calculated changes of initiation rate with pH are in rather poor agreement with the phenol-based method of Boozer *et al*.[14] Specifically, the uninhibited oxidation method does not yield maximum R_i at pH 4 but rather at pH 9–10. Our results (Table 2) show 25% increase of R_{ox} at pH 8 and 50% increase at pH 9 compared to R_{ox} at pH 6. A similar increase of R_{ox} (*ca.* 25%) was observed by Mabrouk and Dugan[19] for methyl linoleate emulsion oxidation at 50 °C when the pH was increased from 6 to 8.[19] We suggest that the calculated R_i at the higher pH's arises because the oxidizability of the methyl linoleate micelles is not constant—partial hydrolysis of methyl linoleate occurs and the autoxidation of emulsion contaminated by free fatty acid is slightly faster.

In order to exclude the influence of pH on the Clark-type electrode employed in the inhibited and uninhibited oxidation (methods 1 and 2), we monitored the loss of PMHC during initiator decomposition in aerated SDS (method 3, see Supplementary Data). Assuming that one molecule of PMHC reacts with two peroxyls, the rate $R_g = R_i$ was determined:[15,20]

$$R = -2\mathrm{d}[\mathrm{PMHC}]/\mathrm{d}t \qquad \text{(IV)}$$

Because lipid was not present in the SDS micelles, there is no autoxidation, thus, pH effects on autoxidation rate are eliminated. The plots of [PMHC] *vs.* time gave straight lines for ABAP as well as for two other initiators, water-soluble AMEP and lipid soluble AIBN (see Chart 1 and Supplementary Data), and from the rates of PMHC loss [eqn (IV)] the values R_i listed in Table 3 were calculated.

The calculated changes in R_i with pH by this procedure appear to reflect the trends found by both the induction period method and the uninhibited autoxidation method, that is, to the R_i values is significantly greater at low pH and at high pH than at neutral pH (Fig. 2).

Table 3 Rates of effective generation of radicals (rates of initiation) by two water soluble azo initiators: neutral AMEP, and cationic ABAP and lipid soluble initiator AIBN at 37 °C in SDS emulsions at various pH.[a] The error of measurements is 15%

	$R_i \times 10^9$/M s^{-1}		
pH	ABAP[a]	AMEP[a]	AIBN[a]
4	13.2	5.15	6.37
5	9.59	3.85	4.52
6	4.84	2.15	2.95
7	3.64	1.58	3.40
8	9.64	4.01	2.96
9	9.02	4.13	5.64
10	8.18	6.70	12.5

[a] The initial concentrations of ABAP, AMEP, and AIBN (and $[PMHC]_0$ in parentheses), were 4.81 (**1.93**) mM, 68.1 (**2.00**) mM, and 9.88 (**1.70**) mM, respectively.

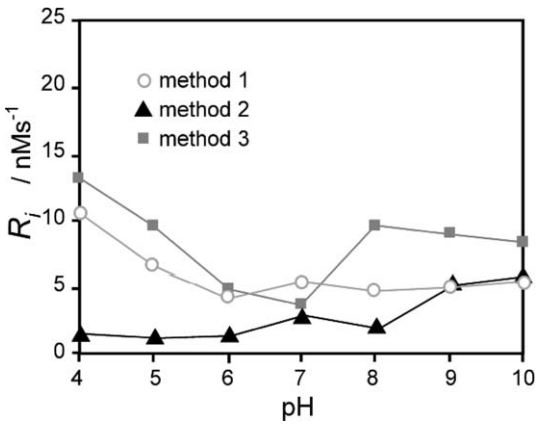

Fig. 2 Comparison of rates of initiation measured by induction time measurements (method 1, [ABAP] = 10 mM), uninhibited autoxidation (method 2, [ABAP] = 10 mM), and PMHC loss (method 3, [ABAP] = 9.9 mM).

Fig. 3 presents data for all three initiator decompositions, using method 3. The data obtained for AMEP can be compared for pH 7 only: $k_d = 0.18\ \mu s^{-1}$(pH 7), and efficiency of cage escape (e) is 0.1[15] thus, the rate of effective radical generation will be

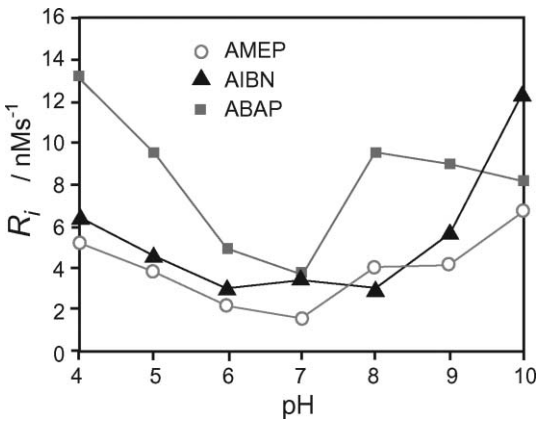

Fig. 3 Comparison of the rates of generation of radicals by 4.8 mM ABAP, 68 mM AMEP, and 9.9 mM AIBN at 37 °C in SDS emulsions at various pH measured by HPLC (method 3).

$R_g = 2ek_d[RN{=}NR] = 2 \times 0.1 \times 0.18 \times 10^{-6}\ s^{-1} \times 0.068\ M = 2.5\ nM\ s^{-1}$, in good agreement with 1.58 nM s^{-1} in this work (however, in the same work[15] R_i measured by tocopherol depletion for LDL autoxidation in aerated PBS at pH 7.4 was 1.5 nM s^{-1} for 43 mM AMEP). Unfortunately, we cannot compare our data for AIBN decomposition because we did not find any literature data for this initiator in a micellar system.

Hanlon and Seybert suggested,[13] that the large increase of the rate of LOOH formation observed in their experiments at pH > 7 could be caused by poor ability of the positively charged ABAP radicals to penetrate the micelles at pH < pK_a = 6.9.‖ If the explanation was valid, we should have observed a significant increase of R_i at the pH region 4–7 compared with R_i at pH 7–10 because in our system SDS micelles are negatively charged and Coulombic attraction between $^+RO_2^\bullet$ and SDS micelles should operate. However, over all the pH range the parent molecules of ABAP are also positively charged‖ and are attracted by SDS. Therefore no dramatic increase/decrease of R_i should be observed, in perfect agreement with our experiment. It seems that Hanlon and Seybert have not properly connected the rate of initiation with the rate of hydroperoxide formation. [LOOH] is not stoichiometrically connected with the amount of initiating radicals. On the basis of the R_i values (Table 1) and R_{ox} (Table 2) we were able to calculate the kinetic chain length of autoxidation $\nu = R_{ox}/R_i$, and ν increases from 29 at pH 4 to 113 at pH 10 (see Table S9). This means that one initiating radical leads to formation of 29 (at pH 4) and 113 (at pH 10) molecules of hydroperoxide. In ref. 13 Hanlon and Seybert also reported an inverse effect (*i.e.* 10-fold decrease of the rate of initiation) for negatively charged 4,4′-azobis(4-cyanopentanoic acid) for a pH increase from 4 to 7. We suppose that this inverse effect can be explained by a weak penetrating ability of this initiator and the radicals containing carboxylate anion, however, at a higher pH a decrease of initiation rate overlapped with an increase of the kinetic chain length, thus a plateau of the rate of LOOH formation was observed in their experiment.[13]

In conclusion, over the pH range of 5–8, which is of particular interest to biochemists and food technologists, R_i for ABAP can be regarded as constant. At lower pH's the bulk of our evidence (Tables 1 and 3) suggest that R_i is significantly greater than at neutral pH. This result we tentatively attribute to additional protonation of amidinium moieties of the ABAP, which could enhance Coulombic repulsion of the generated carbon-centered radicals thus leading to higher efficiency of cage escape (e) and an increase of R_i. At high pH, one phenol-based method indicates that R_i is almost the same as for neutral pH (Table 1) while the other indicates that it is larger (Table 3) as does the non-phenol method (Table 2). Fortunately, these very high pH's, as well as the very low pH's, are of little interests to free radical biochemistry.

Acknowledgements

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Notes and references

‡ Depending on database and choice of keywords, in the years 2000–2006 about 500 publications appeared in which ABAP is listed as a source of free radicals in lipid autoxidation processes. SCOPUS database for years 2000–2006 gives 550 papers with keywords "amidinepropane + oxidation", and 469 papers with keywords "AAPH + lipids". Most of them are in biochemistry, food chemistry, pharmacology and free radical journals. AAPH is another, frequently used abbreviation for the same initiator.

§ Experimental details are given in Electronic Supplementary Information. Briefly: methyl linoleate (99%), PMHC (97%) SDS (99%) and buffers were of highest purity, ultrapure water was used for preparations of solutions and traces of metals were removed from solutions by Chelex 100. Methyl linoleate in acetone was put in a glass ampoule and acetone was gently removed under nitrogen flow. Buffered 0.5 M SDS (5 ml) was added and the mixture was shaken on a Vortex for two minutes. For experiments with inhibitor (method 1), at that moment the appropriate amount (25–100 μL) of inhibitor (PMHC) dissolved in acetone was added with microliter syringe. In both methods, the induction period method (method 1) and in the uninhibited autoxidation method (method 2) the rates of autoxidation at 37 °C were monitored by oxygen uptake measurements conducted with a Clark type electrode and a 5300A Biological Oxygen Monitor (Yellow Springs Instruments). In method 3 the measurements of PMHC loss during 40 hours decomposition of ABAP, AMEP, and AIBN in SDS emulsions were done. Initial concentrations of compounds are given in a footnote to Table 3. Experimental details are placed in the Electronic Supplementary Information.

¶ The term [LH] in eqn (I) means the concentration of methyl linoleate in the non-polar phase (ref. 5). We calculated the effective [LH] in SDS emulsion by the method of Barclay *et al.*[3] In 5 mL of emulsion was 50 μL of lipid (0.151 mmol) and 2.5 mmol of SDS, the molar volume of SDS is 0.25 mL $mmol^{-1}$, thus the [LH] = 0.151 mmol/(2.5 mmol × 0.250 mL $mmol^{-1}$) = 0.24 M.

‖ pK_a for ABAP determined in water[21] is *ca.* 9.5. However, the pK_a 6.9 in ref. 13 denotes the acidity of radical that is considerably stronger acid than its parent compound.

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