Kinetics of Ozonation. 2. Amino Acids and Model Compounds in Water and Comparisons to Rates in Nonpolar Solvents

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Abstract: Absolute rates of reaction of amino acids and model compounds with ozone have been measured in aqueous buffer solutions. For the *less reactive* amino acids and for amines, the rates of reaction are proportional to the amount of free (i.e., unprotonated) amine present and therefore are relatively slow below pH 7. The rates of reaction of the corresponding amides are also slow. Rates of reaction of the *more reactive* amino acids at pH 7.0 are in the order of cysteine > tryptophan > methionine > tyrosine > histidine. 3-Hexenoic acid, a model of a polyunsaturated fatty acid (PUFA), is similar in reactivity to methionine or tyrosine. These data imply that the more reactive amino acids, whether free or in polypeptides, must be considered as possible targets for the reaction of ozone in vivo, along with the more usually considered PUFA. Which of these types of target molecules might receive the most damage in vivo probably depends on accessibility and cellular architecture, and these factors, plus the possibility of co-oxidation and other complexities, require further study.

Ozone is the most ubiquitous and toxic of the world's pollutants; yet despite a vast investment in the study of its organic chemistry[1] and its biological effects,[2] the molecular-level basis of the destruction caused by ozone in biological systems is largely unknown.[3] This paper continues our efforts[4] to provide a detailed set of rate constants for the reaction of ozone with biological molecules. Our aim is to provide a set of data that can be used to model the rates of attack of potential target molecules in vivo.

Two types of molecules have been of primary concern in biochemical studies of ozone: polyunsaturated fatty acids (PUFA) and amino acids, either free or in polypeptides or enzymes.[3] Several authors have reported qualitative data on the reactivities of proteins and amino acids in aqueous solution,[5,6] but studies of PUFA and model compounds have been conducted almost exclusively in aprotic, non-interacting solvents,[4,7,8] However, recently Hoigné and Bader[9,10] have reported rate constants for ozonation of several amino acids and alkenes in aqueous media at a range of pH values.

The present work is in part an extension of the work of Hoigné and Bader, expanding the list of amino acids for which rate constants are available to include all of the common amino acids, some derivatized amino acids, and some related compounds for comparison. Of particular importance, we have been able to measure rates faster than those determined by Hoigné and Bader, providing data for more reactive amino acids and for a model of a PUFA, *trans*-3-hexenoic acid, in the same aqueous media. We also compare the rate constants for reaction of the substances in water with those in nonpolar, non-interacting solvents.[7]

Experimental Section

Purification of Materials. Buffer solutions in distilled, deionized water were prepared by adding 0.2 M H_3PO_4 to 0.2 M Na_2HPO_4 until the desired pH was achieved. Although the buffering capacity of the solutions varied, the concentration of phosphate was held constant. Solutions with pH values ≥8 were prepared from 0.2 M borate.

All amino acids and derivatives were puchased from either Sigma or ICN and were used without further purification. Most of the other compounds were purchased from Aldrich. The liquids were distilled before use, and the solids were used without further purification. Materials from other suppliers included acrylonitrile (Kodak), maleic acid (Fisher), tetrahydrofuran (Baker), 2-propanol (MCB), ethanol (AAPER), and sucrose (Baker).

Kinetic Measurements. Stopped-flow spectrometry was carried out as described in Part 1 of this series,[4] using a Nortech stopped-flow spectrophotometer (Model SF-3L, 0.2-mm path length, mixing/dead time 6 ms) interfaced to an On-Line Instrument Systems Model 3820 data system.[11] Loss of ozone was monitored at 285 nm for five or more half-lives when the reactions were rapid enough to permit this. Except for some of the very slow reactions, all measurements were repeated at least seven times on the same solution. With the exception of the runs conducted to measure temperature dependence, all data were collected between 22 and 24 °C; most were carried out at 23.0 ± 0.1 °C.

Ozone solutions were prepared by bubbling a stream of ozone in oxygen into acidified (H_2SO_4) water. Equal volumes of substrate solutions were prepared in 0.2 M buffer so the resultant solution after mixing in the stopped-flow spectrometer was 0.1 M buffer at a pH close to that of the initial substrate solution. The pH values after mixing were taken on a Horizon Model 5998-10 pH meter calibrated with pH 8.0, 7.0, and 4.0 standard buffer solutions. Measurements were made either directly on the effluent from the stopped-flow instrument or on an identical mixture of the solutions. Addition of ozone does not affect the pH. Stopped-flow measurements carried out in the absence of substrate indicated that the background rate of ozone degradation at pH ≤8 occurs at a rate of less than 0.01 s^{-1} and can be neglected. At pH values below 4, no loss of ozone was observed in the first 1000 s.

We have used the pK values for the substrates we studied that are compiled in the *CRC Handbook of Chemistry and Physics*;[12] although some variation in pK values is reflected in the literature, the differences affect our calculated rate constants only slightly. Values not in that listing are referenced individually.

Relative Rates. The rates of reaction of some compounds with ozone were measured by competitive experiments. Two different protocols were used. In the first a solution of ozone was added slowly to a stirred solution of two or more amino acids and the resulting solution was lyophilized, taken up in citrate buffer, and analyzed by a standard amino acid analysis.[13]

(1) Bailey, P. S. "Ozonation in Organic Chemistry"; Academic Press: New York, 1978; Vol. I;f 1982, Vol. II, and references therein.
(2) Menzel, D. B. "Free Radicals in Biology"; Pryor, W. A., Ed.; Academic Press: New York, New York, 1976; Vol. II, Chapter 6.
(3) (a) Pryor, W. A.; Dooley, M. M.; Church, D. F. "Advances in Modern Environmental Toxicology"; Lee, S. E., Mustafa, M. G., Mehlman, M. A., Eds.; Princeton Scientific Publishers: Princeton, New Jersey, 1982; Vol. V, Chapter 2. (b) Mudd, J. B.; Banerjee, S. K.; Dooley, M. M.; Knight, K. L. "Gaseous Air Pollutants and Plant Metabolism"; Koziol, M. J., Whatley, F. R., Eds.; Butterworths: London, 1984; Chapter 8.
(4) Pryor, W. A.; Giamalva, D.; Church, D. F. *J. Am. Chem. Soc.* **1983**, *105*, 6858-6861.
(5) Mudd, J. B.; Leavitt, R.; Ongun, A.; McManus, T. T. *Atmos. Environ.* **1969**, *3*, 669-682.
(6) (a) Previero, A.; Scoffone, E.; Pajetta, P.; Benassi, C. A. *Gazz. Chim. Ital.* **1963**, *93*, 841-848, 849-858. (b) Previero, A.; Scoffone, E. *Gazz. Chim. Ital.* **1963**, *93*, 859-866. (c) Previero, A.; Bordignon, E. *Gazz. Chim. Ital.* **1964**, *94*, 630-638.
(7) The term "non-interacting" indicates a nonprotonic solvent that cannot convert the carbonyl oxide from a Criegee ozonation into a stabilized derivative. See ref 1, Vol. I, p 83.
(8) (a) Razumovskii, S. D.; Zaikov, G. E. *J. Org. Chem. USSR (Engl. Transl.)* **1972**, *8*, 468-472. (b) Williamson, D. G.; Cvetanovic, R. J. *J. Am. Chem. Soc.* **1968**, *90*, 3668-3672. (c) Williamson, D. G.; Cvetanovic, R. J. *J. Am. Chem. Soc.* **1968**, *90*, 4248-4252. (d) Carles, J.; Fliszár, S. *Adv. Chem. Ser.* **1972**, *112*, 35-49.
(9) Hoigné, J.; Bader, H. *Water Res.* **1983**, *17*, 185-194.
(10) Hoigné, J.; Bader, H. *Water Res.* **1983**, *17*, 173-183.
(11) On-Line Instrument Systems, Jefferson, GA.
(12) "The CRC Handbook of Chemistry and Physics", 62nd edition; Weast, R. C., Ed.; CRC Press: Boca Raton, Florida, 1981; p C-724.

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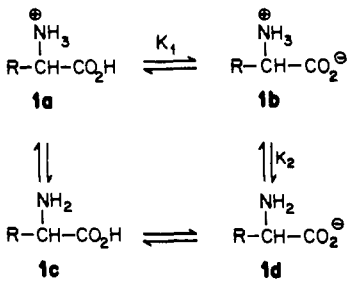

Figure 1. The ionization of a simple amino acid.

Alternatively, an ozone solution was added to a solution of tryptophan and one other substrate. The solution was diluted to a standard volume, and the ultraviolet spectrum was recorded on either a Varian Cary 219 or a Beckman 24 spectrophotometer. From the spectra, the amount of kynurenine present was determined and taken as the amount of tryptophan consumed in the reaction.[14,15] The relative reactivity can be deduced from a series of such runs in which initial tryptophan and ozone concentrations are kept constant while the amount of the other substrate, X, is varied. Equation 1 can then be used to calculate relative reactivity, where the initial and final values for tryptophan are taken as the amount of kynurenine formed in the presence of excess ozone and the difference between this value and the amount of kynurenine formed in a given run, respectively. The initial concentration of X is known, and the final

$$\frac{k_{TRP}}{k_X} = \frac{\ln (TRP_0/TRP_t)}{\ln (X_0/X_t)} \quad (1)$$

concentration of X may be measured indirectly. For a reaction with 1:1 stoichiometry (which we assume), the loss of X is equal to the difference between the total amount of ozone and the amount of tryptophan consumed. Thus eq 1 may be expressed as eq 2, where A_n is the absorbance of a paritcular run, A_f is the absorbance of the reaction with excess ozone, and A_0 is the absorbance resulting from a run with no added substrate X (which is proportional to the concentration of ozone). In this ex-

$$\frac{k_{TRP}}{k_X} = \frac{\ln [A_f/(A_f - A_n)]}{\ln \left\{ X_0 \Big/ \left[X_0 - TRP_0 \left(\frac{A_0 - A_n}{A_f} \right) \right] \right\}} \quad (2)$$

pression X_0 is a concentration term while A_n, A_0, and A_f are in absorbance units. The term $[TRP]_0$, the initial concentration of tryptophan, converts the absorbance terms in the denominator to concentration units.

Results

Typical rates of reaction of ozone with some of the substrates studied at a variety of pH values are listed in Table I. Data similar to these were collected for most of the compounds studied and are summarized in Tables II–IV.

In aqueous solution, a simple amino acid (that is, an amino acid with a non-ionizing side chain) exists in four forms due to the ionization of the carboxylate and amino groups. These forms **1a–d** are illustrated in Figure 1. Since carboxylic acids and protonated amines react only slowly with ozone,[9,10,16–18] we collected data at pH values greater than K_1, the dissociation constant of the carboxylic acid group. In this pH range only forms **1b** and **1d** are present and K_2 represents the ratio of free amine **1d** to protonated amine **1b**.[19,20]

Table I. Rates of Reaction of Some Amino Acids and Amines with Ozone in Water

substrate	pH	k,[a] M^{-1} s^{-1}
glycine	2.4	0.09 ± 0.05
	4.1	0.9 ± 0.1
	4.9	2.2 ± 0.1
	5.8	47 ± 4.3
	6.0	99 ± 5.8
	6.4	135 ± 15.3
	6.9	329 ± 13
	7.0	370 ± 27
	7.0	375 ± 25
	7.0	427 ± 12
	7.2	675 ± 43
	7.6	1326 ± 65
proline	1.9	0.5 ± 0.2
	2.2	0.7 ± 0.1
	3.5	1.85 ± 0.1
	4.1	2.37 ± 0.14
	5.1	82.2 ± 3.5
	5.4	115 ± 7.0
	5.6	150 ± 7.0
	6.0	295 ± 10
	7.0	2125 ± 150
	7.6	5985 ± 640
	8.2	17100 ± 1000
butylamine	5.75	0.9 ± 0.1
	6.1	2.2 ± 0.1
	6.7	8.8 ± 0.4
	8.0	203 ± 6.1
tert-butylamine	2.4	0.15 ± 0.05
	6.4	2.1 ± 0.1
	7.5	21.3 ± 1.0
histidine	2.0	38 ± 1.5
	3.5	473 ± 33
	4.6	20400 ± 1400
	5.6	84400 ± 4000
	7.0	203000 ± 19000
cysteine	1.75	25000 ± 2300
	2.2	65900 ± 5800
	2.8	135000 ± 26000
	3.6	511000 ± 130000

[a] This is the total rate constant as defined in eq 7.

The mathematical treatment of the data was done in a manner similar to that of Hoigné and Bader.[9] The rate of reaction of an ionizable compound with ozone may be expressed as the sum of the rates of the two forms, as illustrated in eq 3.

$$\frac{-d[O_3]}{dt} = [O_3](k_{HB}[HB] + k_B[B]) \quad (3)$$

$$k' = \frac{-d[O_3]}{[O_3]dt} = k_{HB}[HB] + \frac{k_B K_2 [HB]}{[H^+]} \quad (4)$$

$$\frac{k'}{[HB]} = k_{HB} + \frac{k_B K_2}{[H^+]} \quad (5)$$

(In the equations below, B refers to a free base and HB refers to the protonated form; positive and negative charges have been omitted.) Inserting the ionization constant and rearranging yields eq 4 and 5, where k' is the total observed pseudo-unimolecular rate constant. A plot of $k'/[HB]$ vs. $K_2/[H^+]$ will have a slope of k_B and an intercept of k_{HB}. The same treatment may be used to evaluate an equilibrated mixture of a neutral compound and its deprotonated form. Values of k_{HB} and k_B calculated in this way are listed in Tables II and III.

For the first 16 entries in Table II and some entries in Table III, k_{HB} is near zero, confirming that neither the carboxylic acid functionality not the protonated amine reacts with ozone at a significant rate in aqueous buffers at 25 °C. A comparison of the amino acids to other primary alkyl amines (Table III, entries 1-3) indicates that while the total rate of reaction of amino acids

(13) Analyses were performed on a Beckman 119 amino acid analyzer equipped with a 0.9 × 48 cm column of AA-15 resin, as described in Beckman Technical Bulletin A-TB-116, July 1974.

(14) Under acidic conditions the reaction of tryptophan and ozone affords near-quantitative yields of *N*-formylkynurenine which is rapidly hydrolyzed under the reaction conditions. See ref 15.

(15) Sakiyama, F.; Masuda, N.; Nakazawa, T.; Katsuragi, Y. *Chem. Lett.* **1978**, 893–896.

(16) Yamamoto, Y.; Niki, E.; Kamiya, Y. *J. Chem. Soc. Jpn.* **1982**, *55*, 2677–2678.

(17) Niki, E.; Yamamoto, Y.; Saito, T.; Nagano, K.; Yokoi, S.; Kamiya, Y. *J. Chem. Soc. Jpn.* **1983**, *56*, 223–228.

(18) Reference 1, Vol. II, Chapter VII, and references therein.

(19) While this is not strictly true, the correction is negligible above pH 2. See ref 20.

(20) Edsall, J. T.; Wyman, J. "Biological Chemistry"; Academic Press: New York, New York, 1958; Vol. I, Chapters 8 and 9.

Table II. Rates of Reaction of Amino Acids with Ozone as a Function of pH

substrate	pK_2	no. of points	pH range	k_{HB}[a]	$k_B \times 10^{-4}$[a]
leucine	9.74	3	4.9–7.0	0.0 ± 0.5	5.3 ± 0.03
isoleucine	9.76	3	3.3–7.0	2.3 ± 1.9	5.6 ± 0.1
alanine	9.87	6	4.1–7.0	0.7 ± 5.2	7.6 ± 0.1
valine	9.72	6	3.9–8.1	−2.0 ± 5.0[b]	6.8 ± 0.02
glycine	9.78	12	2.4–7.6	32.6 ± 12.6	20.8 ± 0.1
phenylalanine	9.24	4	2.3–7.0	535 ± 774	38.2 ± 13
proline	10.6	11	1.9–8.2	300 ± 560	430 ± 14
glutamic acid	9.47[c]	2	4.3–6.8	0.2	2.6
glutamine	9.28	3	3.4–7.0	0.9 ± 0.6	2.6 ± 0.8
aspartic acid	9.82	2	4.2–6.8	1.0	4.1
asparagine	8.85	3	3.9–7.2	−5.8 ± 6.1[b]	4.2 ± 0.05
arginine	8.99	4	3.5–7.0	60 ± 61	5.7 ± 0.7
threonine	9.10	4	2.3–7.0	5.8 ± 6.3	4.5 ± 0.2
serine	9.21	4	2.6–7.0	7.1 ± 4.3	12.9 ± 0.2
lysine	9.18[c]	3	4.0–7.0	6.6 ± 6.9	3.1 ± 0.2
histidine	6.00[d]	5	2.2–7.0	2700 ± 6000	21.2 ± 0.1
cysteine	8.14[d]	4	1.7–3.6	(4.2 ± 1.4) × 10^4	(2.4 ± 0.1) × 10^6
methionine[e]	9.21		2.4–7.0	4 × 10^6	
tryptophan[e]	9.38		2.4–7.0	7 × 10^6	

[a] Rate constants are in M^{-1} s^{-1}. [b] This value is derived from eq 5. The negative value is a mathematical artifact and is in all cases within one standard deviation of zero. [c] The pK values were from ref 20. [d] The pK value for the side chain was used. [e] These reactions were independent of pH. The rates were determined by relative reactivities.

Table III. Rates of Reaction of Amines and Other Amino Acid Models with Ozone as a Function of pH

	pK	no. of points	pH range	k_{HB}[a]	$k_B \times 10^{-4}$[a]
butylamine	10.77	4	5.7–8.0	−0.7 ± 0.4[b]	11.7 ± 0.02
tert-butylamine	10.83	3	2.4–7.5	0.1 ± 0.1	4.5 ± 0.01
sec-butylamine	10.63[c]	6	5.4–7.9	0.1 ± 0.1	5.2 ± 0.04
benzylamine	9.33	3	2.5–7.4	387 ± 415	6.3 ± 3.5
diethylamine	10.49	4	4.1–7.5	11.5 ± 6.4	62.2 ± 0.7
triethylamine	11.01	4	5.3–7.1	5.2 ± 4.5	213 ± 3.4
imidazole	6.95	7	2.3–7.2	214 ± 700	23.7 ± 0.4
4-methylimidazole	7.52	5	2.2–6.9	1720 ± 1600	313 ± 0.7
N-α-acetyllysine	10.53[d]	3	6.0–7.1	1.1 ± 1.1	10.3 ± 0.24
N-ε-acetyllysine	9.46[d]	4	3.2–6.9	1.2 ± 1.4	2.4 ± 0.1
N-α-acetylhistidine	7.2[e]	4	4.5–5.9	−480 ± 800[b]	84.5 ± 1.5
glutathione	8.75[f]	5	1.9–3.1	(2 ± 0.6) × 10^4	(4 ± 0.6) × 10^6
methionine sulfoxide	9.21[g]	6	2.3–7.4	94 ± 215	6.6 ± 1.4
methione sulfone	9.21[g]	5	3.2–7.4	−37 ± 73[b]	15.2 ± 0.5
maleic acid	6.07[h]	5	2.1–7.9	−6300 ± 14000[b]	2.4 ± 0.02
2-hexenoic acid	6.69[i]	3	2.4–7.2	−10000 ± 60000[b]	34.1 ± 0.02
pivalic acid		2	5.1–5.8	<1 × 10^{-3}	~2 × 10^{-3}

[a] Rate constants are in M^{-1} s^{-1}. [b] See footnote *b*, Table II. [c] This pK is that of 2-propanol. [d] This pK value is from ref 24. [e] This pK value is from ref 25a. [f] This pK value is from ref 20. [g] This pK is that of methionine. [h] This is pK_2. [i] This is the pK of crotonic acid.

with ozone in this pH range is higher than that of simple amines, k_{HB} and k_B are similar; differences in total rates are due to the higher ionization constants of simple amines. This is illustrated in Figure 2 in which the rates of reaction of primary alkyl amines and amino acids with hydrocarbon side chains are plotted vs. pH − pK. It should be noted that pH − pK may be expressed in other equivalent forms (eq 6) and, where pH ≪ pK, eq 7 holds true.

$$\mathrm{pH} - pK = \log \frac{[\mathrm{B}]}{[\mathrm{HB}]} = \log \frac{K_2}{[\mathrm{H^+}]} \quad (6)$$

$$k = \frac{k'}{[\mathrm{HB}] + [\mathrm{B}]} \approx \frac{k'}{[\mathrm{HB}]} \quad (7)$$

Figure 2 does not include data for proline, glycine, or phenylalanine. Phenylalanine reacts somewhat faster than the other hydrocarbon-like amino acids, presumably due to the reactivity of the aromatic ring and the benzylic hydrogens.[21-24] Proline, a secondary amine, reacts at a rate faster than primary amines,

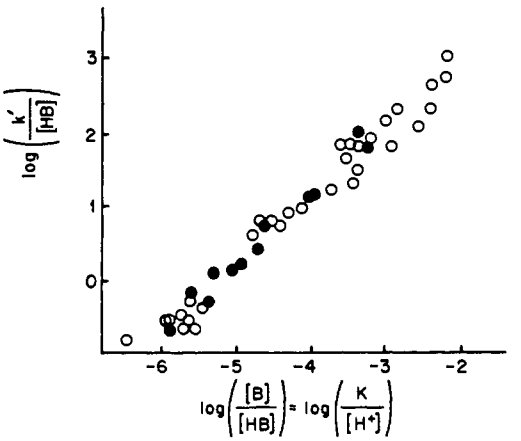

Figure 2. Rates of reaction of ozone with amines vs. concentration of nonprotonated species in solution: (○) hydrocarbon-like amino acids, (●) primary alkyl amines.

as expected.[9,10,16-18] Glycine reacts about four times as fast as the average hydrocarbon-like amino acid. Although glycine is atypical in that it has no alkyl group, the reason for its greater reactivity is not clear. Hoigné and Bader also found glycine to be more reactive toward ozone than was alanine.[9]

(21) See, for instance, ref 22–24.
(22) Nakagawa, T. W.; Andrews, L. T.; Keefer, R. M. *J. Am. Chem. Soc.* **1960**, *82*, 269–276.
(23) Hellman, T. M.; Hamilton, G. A. *J. Am. Chem. Soc.* **1974**, *96*, 1530–1535.
(24) Pryor, W. A.; Ohto, N.; Church, D. F. *J. Am. Chem. Soc.* **1983**, *105*, 3614–3622.

Table IV. Rates of Reaction of Some Miscellaneous Compounds with Ozone in Water

substrate	pH	k, M^{-1} s^{-1}
ethanol	2.1	0.45 ± 0.1
	3.4	0.59 ± 0.1
	7.0	1.4 ± 0.1
	7.9	3.6 ± 0.2
2-propanol	2.2	2.5 ± 0.4
	3.1	2.8 ± 0.3
	5.9	2.9 ± 0.2
	7.0	3.5 ± 0.3
1-butanol	2.1	0.6 ± 0.1
	3.2	0.6 ± 0.1
	5.8	0.7 ± 0.1
	7.2	1.1 ± 0.1
sucrose	2.1	0.5 ± 0.1
	3.5	1.1 ± 0.1
	6.9	2.8 ± 0.1
	7.8	15.6 ± 1.0
tetrahydrofuran	2.2	6.1 ± 0.4
	2.7	6.8 ± 1.0
	6.0	7.4 ± 0.7
	7.0	7.8 ± 0.7
	8.1	14.3 ± 1.8
acrylonitrile	6.2	830 ± 32
	7.0	870 ± 115
	7.9	830 ± 33
N,*N*-dimethylacetamide	2.5	0.05 ± 0.05
	3.3	0.5 ± 0.1
	7.0	0.7 ± 0.1
N-methylacetamide	7.2	0.6 ± 0.1
dimethyl sulfoxide	2.3	8.2 ± 0.5
	4.5	7.5 ± 0.4
	6.0	7.2 ± 0.4
	7.0	8.1 ± 0.7
N-acetylglycine	3.7	0.3 ± 0.1
	6.5	1.7 ± 0.2
N-acetylserine	5.7	1.5 ± 0.1
	6.3	5.8 ± 0.5
	6.8	13.5 ± 0.5

Of the amino acids that have functionalized side chains, some have k_{HB} and k_B values that are not significantly different from the hydrocarbon-like amino acids (entries 8–16 in Table II). Of these, glutamic and aspartic acids and the amides glutamine, asparagine, and arginine have side chains containing groups known to react very slowly with ozone.[9,16-18]

Serine and threonine, the two hydroxy amino acids, also behave like the hydrocarbon amino acids. These data, as well as those of Hoigné and Bader,[9] demonstrate that the rates of reaction of alcohols with ozone are slow relative to the rates of free amines (see Table IV, entries 1 and 2). As expected, therefore, the rate of reaction of *N*-acetylserine with ozone, while exhibiting some pH dependence, is over an order of magnitude slower than that of serine at any pH.[25]

Lysine contains a second amino group and, although the ionization constant is one pK unit lower for the α-amino group, interpretation of the data could still be ambiguous. To clarify the site of reaction, the rates of reaction of both *N*-α-acetyllysine and *N*-ϵ-acetyllysine with ozone were measured (Table III, entries 9 and 10).[26] The calculated second-order rate constant k_B for *N*-α-acetyllysine is virtually identical with that of *n*-butylamine, while that of the ϵ-acetylated derivative is virtually identical to that of lysine and the hydrocarbon amino acids (see Figure 3).

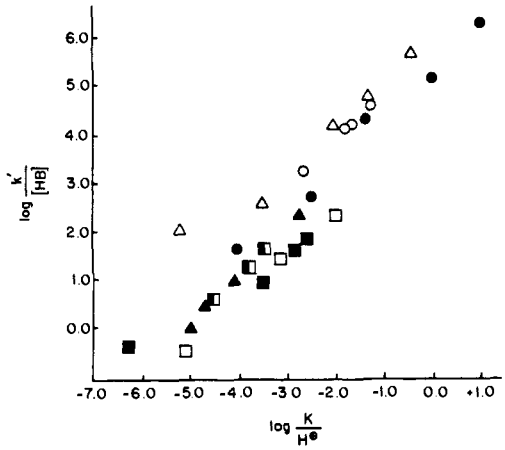

Figure 3. The effect of pH on some rates of reaction with ozone: (●) histidine, (○) *N*-acetylhistidine, (△) 4-methylimidazole, (■) *N*-α-acetyllysine, (◧) *N*-ϵ-acetyllysine, (▲) *n*-butylamine.

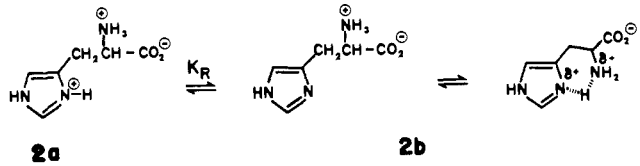

Figure 4. The ionization of the imidazole ring of histidine.

Two amino acids, histidine, and cysteine, have moieties in their side chains with ionization constants intermediate between those of carboxylic acids and those of their α-amino groups. The pK values of the imidazole ring of histidine (pK = 6.0) and the sulfhydryl group of cysteine (pK = 8.14) were used to calculate k_{HB} and K_B for these compounds. Although histidine is far more reactive at any pH studied than the hydrocarbon-like amino acids, its value of k_B is only slightly greater, implying that the rate of reaction of an imidazole group is similar to that of a primary amine. Indeed, k_B for the reaction of imidazole with ozone is 2.4 × 10^5 M^{-1} s^{-1}, virtually identical with that of histidine. 4-Methylimidazole, however, yields a rate constant an order of magnitude higher, due to the electron-donating ability of the methyl group. The value of k_B for *N*-α-acetylhistidine is intermediate between those of histidine and 4-methylimidazole. The reactivity of these compounds is illustrated graphically in Figure 3.

Of the four compounds containing an imidazole ring, histidine is the least reactive toward ozone and also has the lowest pK. Both of these facts can be explained by the interactions of the protonated α-amino group with the imidazole ring, an effect noted in other reactions of histidine.[27] As illustrated in Figure 4, hydrogen bonding to a ring nitrogen may serve to stabilize the monoprotonated form **2b**, thus lowering K_R. Similarly, this electron withdrawal from the ring serves to deactivate it toward the electrophilic attack of ozone.

The pH dependence of the reaction of cysteine with ozone indicates that the site of reaction is at the sulfhydryl rather than the amino group. Over the pH range used, the change in cysteine involves going from the neutral form to the thiolate ion. The extrapolated k_{HB} value for CysSH is 4 × 10^4 M^{-1} s^{-1}, but the actual measured rates at low pH indicate that the rate constant is nearer 2 × 10^4 M^{-1} s^{-1}. Like the other compounds, the total rate increases by about one order of magnitude per pH unit, so we were unable to measure a rate at a pH higher than 3.6. These data yield a calculated k_B value for $CysS^-$, the thiolate anion, to be about 2.5 × 10^{10} M^{-1} s^{-1}. Similar values of k_{HB} and k_B are obtained for

(25) Although alcohols are also ionizable, their pK values are too far above the pH range dealt with here to meaningfully separate the rate constants of the two forms.

(26) Hermann, V. P.; Lemke, K. *Hoppe-Seyler's Z. Physiol. Chem.* **1968**, *349*, 390–394.

(27) (a) Ohe, M.; Kajita, A. *J. Biochem.* **1977**, *81*, 431–434. (b) Elvidge, J. A.; Jones, J. R.; Salih, R.; Shandala, M.; Taylor, S. E. *J. Chem. Soc., Perkin Trans. 2* **1980**, 477–451.

Table V. Rates of Amino Acids with Ozone Measured by Techniques Other Than Stopped Flow

substrate	method: stopped flow, M^{-1} s^{-1}	competition with tryptophan, k_{rel}[a] pH 2.6	pH 6.0	pH 7.0
tryptophan	$>3 \times 10^6$	1	1	1
methionine		0.64	0.4	0.6; 0.8[b]
tyrosine			0.07	0.4[c]
histidine	*d*			0.04[b]
3-hexenoic acid	1.9×10^6	0.i5	0.26	0.24

[a] These values, unless otherwise stated, were measured by the UV method described in the Experimental Section at 23 ± 1 °C. [b] These values were determined by amino acid analysis as described in the Experimental Section. [c] The pH was 7.2 for this experiment. [d] The rate varies as a function of pH. See Tables I and II.

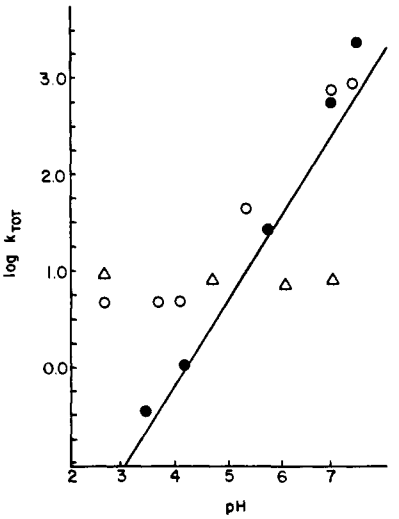

Figure 5. The effect of pH on some rates of reaction with ozone: (○) methionine sulfoxide, (●) methionine sulfone, (△) dimethyl sulfoxide. The solid line represents glycine.

glutathione, a tripeptide containing a cysteinyl residue.

We were unable to determine rates of reaction of three amino acids by the stopped-flow technique. The reactions of methionine and tryptophan with ozone were too fast at all pH values, and the loss of ozone with time could not be followed spectrophotometrically in the presence of tyrosine or tryptophan due to interfering absorbances. We have used two approaches to measure these rate constants, both of which depend on the measurement of relative reactivities. These methods, described in the experimental section, yield similar results and can be put on an absolute scale by competitive experiments in which one or both of these substrates are allowed to react with ozone in the presence of histidine or 3-hexenoic acid. The rates obtained by these competitive techniques are collected in Table V.

The rate constants appearing in Table V are for the total reaction, with no attempt to differentiate k_{HB} and k_B. However, there are no significant differences in rates of reaction of tryptophan, methionine, or 3-hexenoic acid with ozone from pH 2.4 to 7.0. This is not unreasonable since the reactions take place at the indole ring, at sulfur, and at the double bond, respectively, none of which is near the site of ionization. This was verified in the case of methionine by an examination of the reactions of methionine sulfoxide, methionine sulfone, and dimethyl sulfoxide (Me_2SO) with ozone. These data are illustrated graphically in Figure 5. Me_2SO reacts slowly with ozone with no significant pH dependence. Methionine sulfone, with reaction at sulfur completely blocked, reacts like a typical hydrocarbon-like amino acid. The behavior of methionine sulfoxide, however, may be approximated by the sum of the rates of methionine sulfone and Me_2SO. At low pH, where the rate of reaction of a hydrocarbon-like amino acid is negligible, the rate of reaction is near

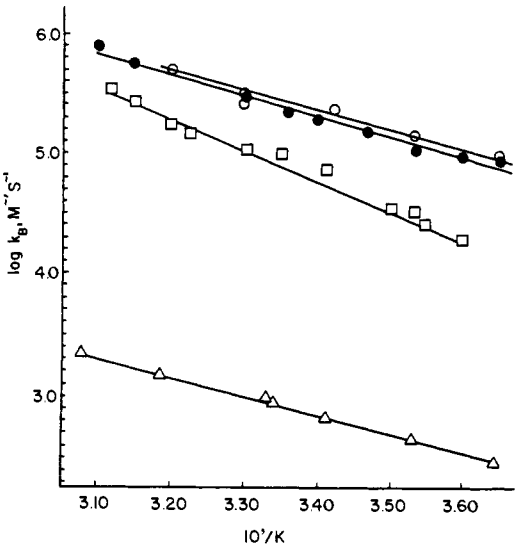

Figure 6. The effect of temperature on some rates of reaction with ozone: (○) imidazole, (●) glycine, (□) alanine, (△) acrylonitrile.

Table VI. Activation Parameters for the Reaction of Some Compounds with Ozone

substrate	solvent	E_a, kcal mol^{-1}	log A/s	ref
glycine[a]	water	7.9	11.2	this work
alanine[a]	water	10.7	12.7	this work
imidazole[a]	water	6.4	10.1	this work
acrylonitrile[a]	water	8.3	7.4	this work
acrylonitrile	CCl_4	5.8	6.4	4
trichloroethylene	water	8.4	7.2	32
trichloroethylene	CCl_4	9.0 (8.1)	7.1 (6.3)	32 (4)

[a] Standard deviations in E_a and log A/s are less than 0.2 kcal mol^{-1} and 0.3 log A/s unit, but due to the approximations made in correcting the pK of these substrates for changes in temperature, these data are probably less accurate than their standard deviations indicate.

that of Me_2SO. At higher pH the rate of reaction of a sulfoxide is small compared to that of an amine, and the rate of reaction of methionine sulfoxide is near that of methionine sulfone.

The rate of reaction of tyrosine varies with pH due to the ionization of the phenolic group, as Hoigné and Bader have demonstrated with other phenols.[9] Our data are not sufficient to quantitate the reactivities of the ionized and un-ionized forms but do provide a total rate of reaction near pH 7.

For purposes of comparison, the rates of reaction of some other compounds with ozone were measured, and these data are included in Tables III and IV. Of these, pivalic acid and the amides *N*-acetylglycine, *N*,*N*-dimethylacetamide, and *N*-methylacetamide exhibit very low reactivity toward ozone, as expected. Of the four alkenes studied, those in which the double bond is conjugated to a carboxylate group, maleic acid and 2-hexenoic acid, are found to have rate constants that are pH dependent, with the anionic form reacting faster. The rates of reaction of 3-hexenoic acid, in which the double bond is not in conjugation with the carboxylate group, and acrylonitrile, which is not ionizable, do not vary with pH. This indicates, not surprisingly, that the reaction takes place at the double bond.

The rates of reaction of ozone with imidazole, acrylonitrile, glycine, and alanine were measured at several temperatures to determine activation parameters. Evaluation of the data is complicated by the effect of temperature on the pK of both the substrates and the phosphate buffer systems. Literature values were used for the pK of alanine[28] and glycine[29] where possible; values at other temperatures and pK values for imidazole were calculated from the Perrin equation.[30] Phosphate buffer solutions undergo

(28) Smith, P. K.; Taylor, A. C.; Smith, E. R. B. *J. Biol. Chem.* **1937**, *122*, 109–122.
(29) (a) Owen, B. B. *J. Am. Chem. Soc.* **1934** *56*, 24–27. (b) King, E. J. *J. Am. Chem. Soc.* **1951**, *73*, 155–159.

only small pH changes with changes in temperature so that extrapolations of pH values from literature sources were used and are probably accurate to three significant figures.[20] The rate of reaction of acrylonitrile with ozone is independent of pH (see Table IV).

In the analysis of activation parameters, k_{HB} was assumed to be zero and the activation parameters therefore reflect changes in k_B. Plots of log k vs. $1/T$ appear in Figure 6; the derived values of E_a and log A/s are given in Table VI with some other values from the literature.

Discussion

The data reported herein are wholly consistent with Bailey's mechanism for the ozonation of amines, in which the initial attack of ozone takes place at nitrogen (eq 8).[18] Of particular significance is the rate of reaction of *tert*-butylamine relative to primary alkyl amines with α-hydrogens. For steric reasons the attack at nitrogen

$$R_3N: + O_3 \rightarrow R_3N^+—O—O—O^- \rightarrow \text{products} \qquad (8)$$

would be expected to be faster for *n*-butylamine and slowest for *tert*-butylamine, but the differences should be relatively small. If ozone attack takes place at the α-hydrogen atoms, the order of attack should be benzylamine > *sec*-butylamine > *n*-butylamine ≫ *tert*-butylamine. Since the differences in rates are small and the relative rates are in the order predicted by steric considerations alone, these reactions must proceed entirely or in large part via attack of ozone at nitrogen.

We also provide activation parameters for the reaction of ozone with some compounds in water. For acrylonitrile, both log A/s and E_a are somewhat higher in water than in CCl_4,[4] but still not inconsistent with a concerted [2 + 3] cycloaddition mechanism in both solvents.[4,31] These data are comparable to an earlier study in which the activation parameters for the reaction of ozone with trichloroethylene were found to change even less in going from CCl_4 to aqueous solvent.[32,33] Arrhenius parameters determined for alanine and glycine are, as expected,[18] consistent with a less ordered transition state than is true for olefins. The activation parameters for imidazole also suggest that the rate-determining step is less ordered than would be expected for a [2 + 3] cycloaddition. Possible pathways consistent with these data include initial π or σ complex formation with the aromatic system (eq 9),[34,35] electrophilic attack by ozone on nitrogen in a manner similar to the reaction of simple amines (eq 8),[18] or an electron transfer from the nitrogen lone pair to ozone (eq 10); our data cannot distinguish between these alternatives.

$$Ar—H + O_3 \rightarrow [\text{CT complex}] \rightarrow \rightarrow \text{products} \qquad (9)$$

$$R_3N: + O_3 \rightarrow R_3N^+\cdot + O_3^-\cdot \rightarrow \rightarrow \text{products} \qquad (10)$$

Hoigné and Bader have provided data indicating that the rate of reaction of an alkene is about 5 times as fast in water as in CCl_4. We find this to be the case for acrylonitrile and maleic acid, but the difference becomes less when more reactive alkenes are considered. Styrene[4] and allylbenzene[36] react at half the rate in CCl_4 as in water;[10] 1-hexene in CCl_4[4] reacts at half the rate of 1-hexene-4-ol in water and at about the same rate as 1-hexene-3-ol in water.[10]

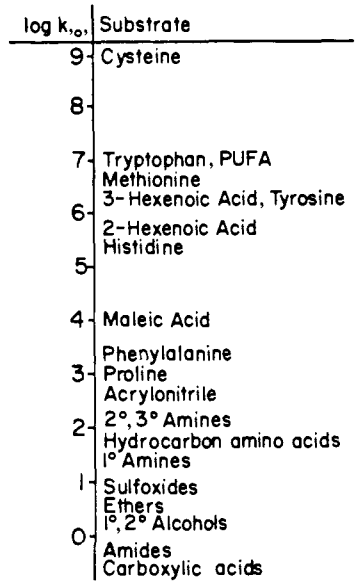

Figure 7. Rate constants for the reaction of ozone with representative compounds in water at pH 7.0.

Some earlier qualitative studies of the reactivities of amino acids toward ozone have been conducted. Mudd et al., in a qualitative rate study of amino acids in aqueous solutions, reported the order of reaction to be cysteine > tryptophan > methionine > tyrosine > histidine > cystine > phenylalanine ≫ all others.[5] In formic acid, Previero et al. have reported amino acid reactivities toward ozone to be tryptophan > methionine ≫ cysteine > tyrosine ≫ all others (including histidine).[6] Our data are consistent with these reports if the pH dependence of the rates is considered. At low pH we observe the order reported by Previero et al., while at high pH we obtain the order of reactivity reported by Mudd et al. Hoigné and Bader have provided some quantitative rates of reaction which also are consistent with our work and that of other authors.[5,6,10] These data allow for a comparison of the reactivities of ozone with a wide variety of organic compounds in water. Approximate rates of reaction at pH 7 are illustrated in Figure 7.

Biological Effects. From the rates illustrated in Figure 6, some aspects of the effects of ozone on biological systems may be deduced. The reactivity of most of the amino acids toward ozone is attributable to the α-amino group. In polypeptides and proteins, where the α-amino groups are tied up in amide linkages, these residues may be expected to have very low reactivities toward ozone. On the other hand, some amino acids possess side-chain groups that are highly reactive toward ozone; these amino acids are cysteine, tryptophan, and methionine (and possibly tyrosine, histidine, and cystine), all of which at pH 7 have rate constants greater than 10^6.

In view of these data, it is not surprising that in cases where the effects of ozone on proteins and polypeptides have been investigated it is the more reactive residues that are affected.[3] This work and that of Mudd et al.[5] demonstrate that glutathione, a peptide containing a cysteine residue, reacts rapidly with ozone. Some sulfhydryl-containing enzymes have also been shown to be rapidly deactivated by ozone.[37] Also, in a number of proteins ultraviolet spectra show that tryptophan and tyrosine residues are ozonated, often with loss of enzymatic activity.[38,39] The reaction of ozone with tyrosine may prove particularly significant bio-

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(31) Huisgen, R. *Angew. Chem., Int. Ed. Engl.* **1963**, *2*, 633–645.

(32) Tarunin, B. I.; Aleksandrov, Y. A.; Perepletchikova, V. N.; Baklanov, N. V. *Kinet. Catal. (Engl. Transl.)* **1977**, *18*, 1091–1093.

(33) Also, for the ozonation of benzene and some alkylbenzenes E_a has been shown to decrease and log A/s increase in going from CCl_4 to acetic acid solvent (ref 22). Activation parameters in water are at least qualitatively similar to those in acetic acid (see ref 10).

(34) Reference 1, Vol. II, Chapter 5, and references therein.

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(38) Giese, A. C.; Leighton, H. L.; Bailey, R. *Arch. Biochem. Biophys.* **1952**, *40*, 71–84.

(39) (a) Kuroda, M.; Sakiyama, F.; Narita, K. *J. Biochem. (Tokyo)* **1975**, *78*, 641–651. (b) Tamaoki, H.; Sakiyama, F.; Narita, K. *J. Biochem. (Tokyo)* **1978**, *83*, 771–781.

logically since ozonations of phenolic solutions have been shown to produce hydroxyl radicals.[40] Methionine also has been shown to be susceptible to ozonation.[41,42]

Our data indicate that the more reactive amino acid residues and PUFA are comparable in reactivity toward ozone. However, the proportion of attack on amino acids and PUFA in vivo cannot be deduced from rate data alone, since these rate studies do not take the effects of the microenvironment into account. The cellular geometry undoubtedly determines the accessibility of ozone to a potential substrate, and perhaps also the nature of the damage done by ozonation of the substrate. Additionally, the possibility of a single molecule of ozone causing damage to more than one substrate must be considered. It is known that ozone initiates the autoxidation of PUFA and this can cause the cooxidation of other materials, such as proteins, that are present. Furthermore, PUFA autoxidation produces harmful byproducts such as malonaldehyde that can crosslink proteins.[43] It is also possible that harmful byproducts such as singlet oxygen or oxidized derivatives of amines (such as nitro or nitroso compounds, hydroxylamines, or imino acids) could be produced from the ozonation of proteins or peptides,[18] which could cause further damage. Thus, although this compilation of rate constants does not allow one to rank the biological risk of all the potential target molecules, it does serve as a guide for further studies.

Acknowledgment. This work was supported in part by grants from the NIH (HL-16029) and the National Science Foundation. We would like to thank Dr. E. W. Blakeney for performing the amino acid analyses.

Registry No. Leucine, 61-90-5; isoleucine, 73-32-5; alanine, 56-41-7; valine, 72-18-4; glycine, 56-40-6; phenylalanine, 63-91-2; proline, 147-85-3; glutamic acid, 56-86-0; glutamine, 56-85-9; aspartic acid, 56-84-8; asparasine, 70-47-3; arainine, 74-79-3; threonine, 72-19-5; serine, 56-45-1; lysine, 56-87-1; histidine, 71-00-1; cysteine, 52-90-4; methionine, 63-68-3; trystophan, 73-22-3; butylamine, 109-73-9; *tert*-butylamine, 75-64-9; *sec*-butylamine, 13852-84-6; benzylamine, 100-46-9; diethylamine, 109-89-7; triethylamine, 121-44-8; imidazole, 288-32-4; 4-methylimidazole, 822-36-6; *N*-α-acetyllysine, 1946-82-3; *N*-ε-acetyllysine, 692-04-6; *N*-α-acetylhistidine, 2497-02-1; glutathione, 70-18-8; methionine sulfoxide, 454-41-1; methionine sulfone, 1118-85-0; maleic acid, 110-16-7; 2-hexenoic acid, 1191-04-4; pivalic acid, 75-98-9; ethanol, 64-17-5; 2-propanol, 67-63-0; 1-butanol, 71-36-3; sucrose, 57-50-1; tetrahydrofuran, 109-99-9; acrylonitrile, 107-13-1; *N*,*N*-dimethylacetamide, 127-19-5; *N*-methylacetamide, 79-16-3; dimethyl sulfoxide, 67-68-5; *N*-acetylglycine, 543-24-8; *N*-acetylserine, 16354-58-8; tyrosine, 60-18-4; 3-hexenoic acid, 4219-24-3.

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(43) Chio, K. S.; Tappel, A. L. *Biochemistry* **1969**, *8*, 2827-2832, and references therein.

Kinetics and Mechanism of Aromatic Thallation. Identification and Proof of Competing Electrophilic and Electron-Transfer Pathways

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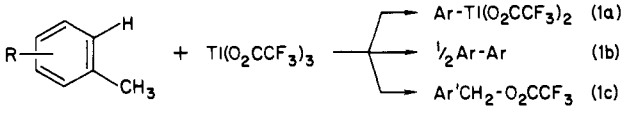
Abstract: The unusual occurrence of simultaneous electrophilic (two-electron) and electron-transfer (one-electron) pathways during the thallation of the homologous methylbenzenes $ArCH_3$ is demonstrated by (1) the careful analysis and identification of three major types of products, (2) the complete dissection of the complex kinetics, and (3) the identification of the reactive intermediates by time-resolved UV-vis and ESR spectroscopy. Side-chain substitution S, dimerization D, and oxidative nuclear substitution O derive from the radical cation $ArCH_3^{+\cdot}$ produced as a common intermediate by electron transfer from the methylbenzene to thallium(III) trifluoroacetate in trifluoroacetic acid. The importance of $ArCH_3^{+\cdot}$, which is detected by both its electronic and ESR spectra, decreases in the following order, hexamethylbenzene > pentamethylbenzene > durene ≫ mesitylene, with a concomitant rise in electrophilic nuclear thallation R to account for the complete material balance. The striking color changes that accompany thallation are identified as charge-transfer transitions in the series of transient 1:1 π-complexes of the methylbenzene donors and the thallium(III) acceptor. Quantitative spectrophotometry employing the Benesi-Hildebrand analysis establishes the cationic $Tl(O_2CCF_3)_2^+$ formed by the dissociation of a single trifluoroacetate ligand from the parent thallium tris(trifluoroacetate) as the active electron acceptor. The complete analysis of the complex kinetics including kinetic isotope effects which accompany the nuclear thallation R of mesitylene as well as the side-chain substitution S of hexamethylbenzene shows that the cationic $Tl(O_2CCF_3)_2^+$ also serves the dual function as the active electrophile and the active oxidant, respectively. The close competition between these apparently disparate pathways is quantitatively evaluated by the second-order rate constants which differ by less than an order of magnitude. Therefore, the thallation of aromatic hydrocarbons represents one of the few systems in which such dual pathways, electrophilic and free radical, apparently occur side by side under the same experimental conditions of solvent, temperature, etc. Accordingly, it represents an unusual opportunity to delineate two-electron (concerted, electrophilic) from one-electron (stepwise, free radical) mechanisms—especially as to whether they represent parallel or sequential events.

The pioneering studies of Taylor, McKillop, and co-workers[1,2] have established the utility of thallium tris(trifluoroacetate) (TTFA) as a reagent in organic chemistry, particularly to effect a wide variety of aromatic transformations. In this regard, at least three major reactions have been identified in aromatic thallation: namely, (a) nuclear substitution, (b) biaryl coupling, and (c) side-chain substitution, e.g.



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