(ATGCAT), 53263-13-1; d(GCGCGC), 76186-50-0; d(CGCGCGCG), 89991-79-7; poly(dG-dC), 36786-90-0; actinomycin D, 50-76-0.

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Haloperoxidase-Catalyzed Halogenation of Nitrogen-Containing Aromatic Heterocycles Represented by Nucleic Bases

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ABSTRACT: The enzymatic halogenation of nitrogen-containing aromatic heterocycles catalyzed by two different types of haloperoxidases, the chloroperoxidase of *Caldariomyces fumago* (heme type) and the bromoperoxidase of *Corallina pilulifera* (non-heme type), has been studied. Chloroperoxidase catalyzed the chlorination of uracil and pyrazole, the bromination of cytosine, uracil, thymine, cytidine, 2'-deoxyuridine, guanosine, and pyrazole, and the iodination of uracil and pyrazole to yield the respective halogenated products. The bromoperoxidase also catalyzed the bromination of cytosine, uracil, cytidine, and pyrazole and the iodination of uracil and pyrazole to form the same products as in the chloroperoxidase reactions. A slight difference in the reactivity toward these substrates was observed between the two haloperoxidases. The results of product and halogenation intermediate analyses suggested that the bromination reaction of the bromoperoxidase occurs at the active site of the enzyme. On the contrary, the halogenation by the chloroperoxidase was found to involve the formation of a molecular halogen and its release into the solution. On the basis of the results, we discussed the abilities of the haloperoxidases as halogenating reagents.

Enzyme-catalyzed halogenation is a common biological phenomenon. Previously, various halometabolites, including chloramphenicol, pyrrolnitrin, etc., were isolated from microbial sources and identified (Neidleman, 1975). In the marine environment, many halogenated compounds such as bromophenols have been found in marine plants (Faulkner, 1970; Fenical, 1974, 1975). In mammals, enzymatic halogenations are important in the biosynthesis of the thyroxine hormone and in biological defense mechanisms (Morrison & Schonbaum, 1976).

Detailed studies of the enzymes participating in the biological formation of halometabolites have been few, being limited to those on the chloroperoxidase of the fungus *Caldariomyces fumago* (Shaw & Hager, 1959, 1961; Shaw et al., 1959) and the thyroid peroxidase involved in the synthesis of thyroxine (Morrison & Schonbaum, 1976). Hager and coworkers presented information on the enzymic properties (Morris & Hager, 1966), kinetic mechanism (Hager et al., 1966), and reaction mechanism (Libby et al., 1982) of the chloroperoxidase. In the haloperoxidase reaction, a halide anion (X^-, X; Cl, Br, I) is activated to the halonium cation (X^+) through hydrogen peroxide dependent oxidation, and then the halonium cation is transferred to a halogen acceptor molecule. There have been many reports concerning chloroperoxidase substrates, as follows: β-keto acids (Shaw & Hager, 1961); cyclic β-diketones (Hager et al., 1966); steroids (Neidleman et al., 1966; Neidleman & Oberc, 1968; Levine et al., 1968; Neidleman & Levine, 1968); substituted phenols such as tyrosine (Taurog & Howells, 1966) and anisole (Brown & Hager, 1967); thiols (Silverstein & Hager, 1974), thiazoles (Neidleman et al., 1969); alkenes (Geigert et al., 1983a); alkynes; cycloalkanes (Geigert et al., 1983b,c); and several

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α,β-unsaturated carboxylic acids (Yamada et al., 1985a). The wide substrate specificity and nonstereospecificity of the chloroperoxidase reaction can be explained in terms of molecular halogen (X_2) addition chemistry in water, which is released from the enzyme in the presence of the halide anion and hydrogen peroxide (Yamada et al., 1985a).

Furthermore, some bromoperoxidases, which are specific for I^- and Br^-, have been found in and isolated from marine algae such as *Rhodomela* (Ahern et al., 1980), *Rhipocephalus* (Baden & Corbett, 1980), *Penicillus* (Baden & Corbett, 1980; Manthey & Hager, 1981), and *Corallina* and *Amphiroa* (Yamada et al., 1985b; Itoh et al., 1985, 1986). The enzymes in algae probably participate in the synthesis of halogenated compounds. In further studies on marine bromoperoxidase, we found that the enzyme of *Corallina pilulifera* is a different type of haloperoxidase (non-heme type: NH type) from other haloperoxidases (heme type: H type) including chloroperoxidase, the bromoperoxidases of *Rhodomela*, *Rhipocephalus*, and *Penicillus*, lactoperoxidase, myeloperoxidase, etc. Therefore, we proposed that haloperoxidases should be classified into two groups: one of H-type enzymes and the other of NH-type enzymes (Itoh et al., 1985, 1986). Recently, another NH-type bromoperoxidase was reported in a brown alga, *Ascophyllum nodosum* (Wever et al., 1985; de Boer et al., 1986).

In order to apply haloperoxidases to the chemical halogenation process, we have studied the enzymatic halogenation of various aromatic heterocycles with two different types of haloperoxidases. It was found that several nucleic bases were utilized as substrates by the chloroperoxidase and bromoperoxidase. Then, we identified the reaction products derived from these substrates and compared the enzymatic reactions to the chemical reactions. This paper presents the substrate specificities, reaction mechanisms, and enzyme stabilities of the two haloperoxidases, and also their abilities as biochemical halogenating reagents are discussed.

Experimental Procedures

Chemicals. Cytosine, uracil, thymine, cytidine, adenosine, and guanosine were purchased from Kohjin Co., Japan. 5-Chlorouracil, 5-iodouracil, 5-bromocytidine, 2′-deoxyuridine, purine, pyrimidine, and pyrazole were obtained from Nakarai Chemicals Ltd., Japan. 5-Bromocytosine, 5-bromouracil, 5-bromo-2′-deoxyuridine, 8-bromoguanosine, and 4-iodopyrazole were obtained from Sigma Chemical Co. and Aldrich Chemical Co. Cellulofine GC-700, a gel filtration medium, was purchased from Seikagaku Kogyo Co., Japan, DEAE-Sepharose CL-4B and Sephadex LH-20 were from Pharmacia Fine Chemicals, Sweden, and Amberlite XAD-2 was from Rohm & Hass Corp. Thin-layer chromatoplates (TLC plates; silica gel 60 F_{254}) were obtained from Merck Japan Ltd. Chlorine gas was obtained by the reaction of concentrated HCl with potassium permanganate. The gas was passed through water and concentrated sulfuric acid to remove HCl and water, respectively. Other reagents used were all of analytical grade.

Preparation of Chloroperoxidase. Chloroperoxidase was obtained from *Caldariomyces fumago* CMI 89362 grown on fructose–salts medium (Pikard & Hashimoto, 1980; Yamada et al., 1985a). Following removal of the mycelium by filtration, the culture medium (ca. 2.5 L, 79 500 units) was treated with cold ethanol (−20 °C) to remove the gelatinous black pigment by the method of Morris and Hager (1966). This enzyme preparation was stocked and used for the halogenation reactions with substrates. Further purification was carried out to obtain a homogeneous enzyme preparation. The enzyme solution from the above step, which had been dialyzed against 20 mM potassium phosphate buffer (pH 6.0), was subjected to DEAE-Sepharose column chromatography (3.5 × 28 cm). After the loaded column was washed with the buffer, the enzyme was eluted with a linear gradient of KCl, from 0 to 0.6 M, in the buffer. The active fractions were collected and concentrated to about 20 mL by evaporation at 35 °C under reduced pressure. The enzyme solution was applied on a Cellulofine GC-700 column (2.1 × 110 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 6.0). Following elution with the buffer, the active fractions were collected. This rapid purification method (94% yield, 75 000 units) gave an enzyme with an A_{410}/A_{280} purity ratio of 1.28. The enzyme solution was used for the halogenation intermediate analysis of the chloroperoxidase reaction.

Preparation of Bromoperoxidase. Bromoperoxidase was obtained from stocked samples of *Corallina pilulifera*, which were collected on the coast of Takahama (Fukui Prefecture, Japan) in April 1985, according to the previously reported purification procedures (Yamada et al., 1985b; Itoh et al., 1985). The purity of the enzyme was checked by disc gel electrophoresis. Unless otherwise indicated, the partially purified enzyme was used for the halogenation reactions with substrates and the homogeneous enzyme for the analysis of the halogenation intermediate of the bromoperoxidase reaction.

Enzyme Assay. Haloperoxidase activities were measured spectrophotometrically according to the methods in the previous reports (Yamada et al., 1985a,b). In all cases, 1 unit of enzyme activity was defined as the amount of enzyme which converts 1 μmol of a substrate to the product in 1 min at 25 °C.

Enzymatic Reaction. The reaction mixture for the chloroperoxidase reaction comprised 1 mmol of potassium phosphate buffer (pH 3.0), 0.2 mmol of KCl, KBr, or KI, 0.2 mmol of hydrogen peroxide (final concentration), 0.1 mmol of each substrate (20 μmol for guanosine), and 40 units of chloroperoxidase in a total volume of 10 mL, in a 30-mL flask. The reaction was kept going by adding 0.1 mmol of hydrogen peroxide at 30-min intervals and allowed to proceed for 3 h at 30 °C.

The bromoperoxidase reactions were carried out in the same manner as in the case of the chloroperoxidase reactions, except that the reaction mixture contained 1 mmol of potassium phosphate buffer (pH 6.0), 0.4 mmol of KBr or KI, 0.2 mmol of hydrogen peroxide (final concentration), and 12.5 units of bromoperoxidase. In all cases, a control run was done without the enzyme.

Reaction Mixture Analysis. Aliquots of a reaction mixture (5–10 μL) were subjected to analytical high-performance liquid chromatography (HPLC). HPLC was performed with a Shimadzu LC-5A system equipped with an M & S pack C18 column (reversed-phase column, 4.6 × 150 mm; M & S Instruments Inc., Japan) at a flow rate of 1.0 mL/min, using the following solvent systems: (a) 0.2 M potassium phosphate buffer (pH 6.0)/methanol, 9:1 (by volume); (b) 0.2 M potassium phosphate buffer (pH 6.0)/methanol, 17:3; and (c) 0.2 M potassium phosphate buffer (pH 6.0)/methanol, 4:1. Unless otherwise indicated, the decrease of the substrate and the formation of the product were measured at 254 nm on HPLC, except in the case of pyrazole at 230 nm. The substrates showed the following retention times (r_t): uracil, 2.3 min; cytosine, 2.0 min; thymine, 3.7 min; cytidine, 2.4 min; 2′-deoxyuridine, 3.6 min; adenine, 4.6 min; purine, 4.5 min (solvent a); guanosine 3.1 min; adenosine, 6.4 min (solvent b); and pyrazole, 3.9 min (solvent c).

Simultaneously, aliquots of the reaction mixture (5–10 μL) were applied to a silica gel plate, which was developed with one of the following solvent systems (by volume): (a) 1-butanol/ethanol/H_2O, 2:1:1 (for cytosine, uracil, thymine, and guanosine); (b) ethanol (for adenosine); (c) methanol (for cytidine and adenine); and (d) 1-butanol/methanol, 4:1 (for 2′-deoxyuridine). The products and substrates on the chromatograms were detected with ultraviolet light or iodine vapor.

For volatile substrates such as pyridine and pyrimidine, aliquots of the reaction mixture (2 μL) were subjected to gas chromatography on a coiled column (2 m × 3 mm) packed with KOH/Carbowax 20M (1 + 0.4%, 60–80 mesh). As a carrier, nitrogen gas was used at a flow rate of 50 mL/min. For each substrate, the following injection and column temperatures were used, respectively: 170 and 90 °C for pyridine (r_t = 1.0 min); 200 and 100 °C for pyrimidine (r_t = 1.2 min).

Analysis of Products. NMR spectra were measured with a JEOL-FX 100 (100-MHz) spectrometer with tetramethylsilane as a reference. Gas chromatography–mass spectrometry (GC–MS) was performed on a glass column (1 m × 4 mm) packed with silicone OV-1 (3%, 80–100 mesh) at 20 eV in a Hitachi M-80 mass spectrometer. In-beam electron-impact mass spectrometry (EI-MS) was carried out at 20 eV with the same instrument. Confirmation of the identification of the products was performed by cochromatography of authentic standards on HPLC and TLC, whenever they were available.

Isolation of Products. Cytosine (222 mg, 2 mmol) was dissolved in a reaction mixture comprising 20 mmol of potassium phosphate buffer (pH 3.0), 4 mmol of KBr, 4 mmol of hydrogen peroxide (final concentration), and 800 units of chloroperoxidase in a total volume of 200 mL. The reaction was kept going by adding 2 mmol of hydrogen peroxide at 30-min intervals and continued for 3 h at 30 °C. In further experiments, all reactions were initiated and kept going by the addition of 0.5 volume of hydrogen peroxide at 30-min intervals and continued for 3 h at 30 °C. After the reaction, the pH of the reaction mixture was adjusted to 6.5, and then the mixture was evaporated to dryness. The product was extracted with a 90% ethanol solution, evaporated, and then dissolved in a 0.05 N HCl solution. This solution was applied on a Dowex 50×8 (H-form) column (1.7 × 23 cm), which was eluted with 1 N HCl. The product was rechromatographed on the Dowex 50×8 column. This procedure afforded 210 mg of a white powder: ^{1}H NMR (Me_2SO-d_6) δ 8.25 (1 H, s); EI-MS m/z (M^+ 191, 189) 161, 149, 147, 121, 119, 110, 93, 83, 67.

Isolation of the product from uracil was carried out in a slightly different manner from in the case of cytosine. The reaction mixture comprised 2 mmol (224 mg) of uracil, 20 mmol of potassium phosphate buffer (pH 3.0), 5 mmol of KBr, 4 mmol of hydrogen peroxide, and 1000 units of chloroperoxidase in a total volume of 200 mL. After the reaction, the mixture (adjusted to pH 7.0) was evaporated, and the product was extracted with a 80% ethanol solution. Following evaporation of the extract to dryness, the product was dissolved in 20% methanol, applied on a Sephadex LH-20 column (2 × 70 cm), and then eluted with 20% methanol. This chromatography was repeated once more under the same conditions. This procedure afforded 40 mg of a white product: ^{1}H NMR (Me_2SO-d_6) δ 7.83 (1 H, s); EI-MS m/z (M^+ 192, 190) 149, 147, 122, 120, 106, 104, 93, 91, 81, 79.

The reaction mixture for thymine comprised 1 mmol (126 mg) of thymine, 10 mmol of potassium phosphate buffer (pH 3.0), 2.5 mmol of KBr, 2 mmol of hydrogen peroxide, and 600 units of chloroperoxidase in a total volume of 100 mL. After incubation, the solution was subjected to Amberlite XAD-2 column chromatography (1.5 × 70 cm). Following washing of the column with water, the product was eluted in a stepwise manner with 20% and 40% methanol solutions. The product obtained in the above step was further purified by preparative HPLC on a YMC D-ODS-5 column (reversed-phase column, 20 × 250 mm; Yamamura Chemical Lab. Co., Japan) using 15% methanol as the solvent. The product was unstable under dry conditions, so it was stored in a solution until analysis: EI-MS m/z (M^+ 224, 222) 207, 205, 179, 177, 163, 161, 153, 151, 143, 136, 134, 126, 115, 108, 106, 89.

Cytidine (243 mg, 1 mmol) was dissolved in a reaction mixture comprising 10 mmol of potassium phosphate buffer (pH 3.0), 2 mmol of KBr, 2 mmol of hydrogen peroxide, and 800 units of chloroperoxidase in a total volume of 100 mL. After the reaction, the solution (adjusted to pH 7.0) was applied on an Amberlite XAD-2 column (3.2 × 30 cm). The column was washed with water, and then the product was eluted in a stepwise manner with 25% and 50% methanol solutions. Evaporation of the fractions gave 220 mg of a white product: ^{1}H NMR (Me_2SO-d_6) δ 3.55–3.75 (2 H, br m, 5′-H) 3.75–4.05 (3 H, br m, 2′-, 3′-, and 4′-H), 5.71 (1 H, d, 1′-H), 8.40 (1 H, s, 6-H); EI-MS m/z (M^+ + 1, 324, 322) 242, 240, 231, 229, 220, 218, 192, 190, 175, 173, 147, 133, 120, 110, 95.

The product from 2′-deoxyuridine was isolated from a reaction mixture comprising 10 mmol of potassium phosphate buffer (pH 3.0), 1 mmol of substrate (114 mg), 2 mmol of KBr, 2 mmol of hydrogen peroxide, and 800 units of chloroperoxidase in a total volume of 100 mL. The HPLC analysis data showed that three products (r_t = 5.1, 7.8, and 10.2 min) were formed. After the reaction, the mixture was subjected to Amberlite XAD-2 column (1.5 × 70 cm) chromatography. Following washing of the column with water, the products were separately eluted with a linear gradient, 0–50% of methanol. Then, the three products were separately purified by preparative HPLC on the same column as above with 10% methanol. However, two products (r_t = 5.1 and 7.8 min, on analytical HPLC) were unstable on drying, so they were dissolved in water until analysis. The fractions containing the last product (r_t = 10.2 min, on analytical HPLC) gave 10 mg of a white powder: ^{1}H NMR (Me_2SO-d_6) δ 2.03–2.23 (2 H, m, 2′-H), 3.50–3.70 (2 H, m, 5′-H), 3.70–3.90 (1 H, m, 4′-H), 4.10–4.30 (1 H, m, 3′-H), 6.11 (1 H, t, 1′-H), 8.40 (1 H, s, 6-H); EI-MS m/z (M^+ 308, 306) 192, 190, 149, 147, 122, 120, 117, 97.

Pyrazole (136 mg, 2 mmol) was dissolved in reaction mixture comprising 20 mmol of potassium phosphate buffer (pH 3.0), 6 mmol of KCl, 4 mmol of hydrogen peroxide, and 800 units of chloroperoxidase in a total volume of 200 mL. After incubation, the mixture was applied on an Amberlite XAD-2 (3.2 × 30 cm) column, and the column was washed with water. The product was eluted in a stepwise manner with 50%, 75%, and 100% methanol. The fractions containing the product were collected and concentrated by evaporation. The product was further purified by Sephadex LH-20 column chromatography (1.6 × 35 cm) with 20% methanol as the eluent. Evaporation of the fractions gave 15 mg of a faint brownish product: ^{1}H NMR ($CDCl_3$) δ 7.57 (2 H, s, 3- and 5-H); GC–MS m/z (M^+ 104, 102) 77, 75.

The brominated product of pyrazole was obtained by means of the bromoperoxidase reaction. The reaction mixture comprised 5 mmol of potassium phosphate buffer (pH 6.0), 2.5 mmol of KBr, 1 mmol of hydrogen peroxide, 0.5 mmol (34 mg) of pyrazole, and 40 units of bromoperoxidase in a total

volume of 50 mL. The product was purified in the same manner as in the case of the chloroperoxidase reaction with pyrazole. After purification by means of sequential column chromatographies on Amberlite XAD-2 and Sephadex LH-20, 10 mg of a white product was obtained: ^{1}H NMR ($CDCl_3$) δ 7.60 (2 H, s, 3- and 5-H); GC–MS m/z (M^+ 148, 146) 121, 119, 94, 92.

Results

Substrate Specificity of Haloperoxidases toward Nitrogen-Containing Aromatic Heterocycles. We tested 12 substances, i.e., 6 pyrimidine bases, 4 purine bases, pyridine, and pyrazole, as substrates for the chloroperoxidase and bromoperoxidase reactions. The chloroperoxidase acted on cytosine, uracil, adenine, cytidine, adenosine, guanosine, and pyrazole when KCl was used as the halide. The enzyme also catalyzed the bromination of cytosine, uracil, thymine, cytidine, 2′-deoxyuridine, guanosine, and pyrazole and the iodination of uracil and pyrazole. However, nonsubstituted pyrimidine, purine and pyridine were not utilized as substrates. It was apparent that the substitution of hydrogens in pyrimidine and purine bases by amino or carbonyl groups was necessary for the halogen acceptor of the chloroperoxidase reactions.

In the case of the bromoperoxidase, cytosine, uracil, cytidine, and pyrazole were brominated, and uracil and pyrazole were iodinated. The bromoperoxidase showed a similar tendency of substrate specificity to that of the chloroperoxidase. Of course, the enzyme had no ability to catalyze the chlorination reaction. When the bromination reactions of the two haloperoxidases were compared, a difference between the chloroperoxidase and bromoperoxidase became obvious. The bromoperoxidase did not utilize 2′-deoxyuridine or guanosine as a substrate. This indicated that the enzyme does not have wide substrate specificity as observed in the case of the chloroperoxidase-catalyzed bromination.

Identification of Products from Cytosine. The EI-MS spectrum of the brominated product obtained through the chloroperoxidase reaction showed molecular ion peaks (m/z 191 and 189) split into a 1:1 distribution by the bromine isotopes of m/z 81 and 79, which corresponded to bromocytosine. The NMR spectrum of the product showed a single peak of a methine proton (8.25 ppm), and this indicated that the 5-position hydrogen was replaced by bromine. HPLC analysis data with a standard sample proved that the product was 5-bromocytosine.

The product obtained through the bromoperoxidase reaction was completely identical with the product of the chloroperoxidase reaction.

The chlorinated products from cytosine could not be identified because they were unstable.

Identification of Products from Uracil. The chlorinated compound produced through the chloroperoxidase reaction showed the same retention time (r_t = 3.9 min) on HPLC and the same R_f value (0.74) on TLC (solvent a) as standard 5-chlorouracil. Therefore, it was identified as 5-chlorouracil.

On EI-MS analysis, the brominated product, which was obtained through the chloroperoxidase reaction, indicated the molecular ions of bromouracil (m/z 192 and 190). The NMR spectrum of the product and HPLC analysis confirmed that the product was 5-bromouracil.

The product of the bromoperoxidase reaction was the same as that of the chloroperoxidase reaction.

The iodinated compound formed through the chloroperoxidase or bromoperoxidase reaction was found to be completely identical with authentic 5-iodouracil on HPLC (r_t = 6.4 min) and TLC (R_f = 0.80, solvent a) analyses.

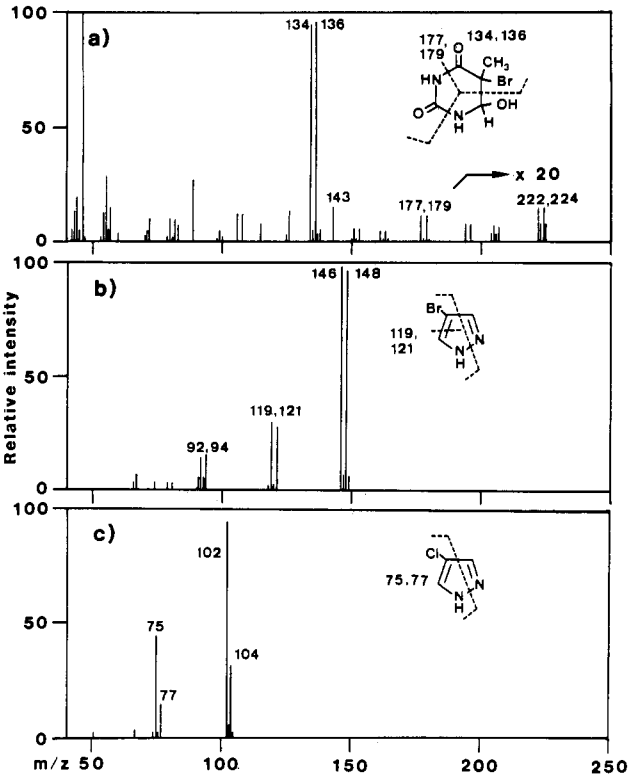


FIGURE 1: Mass spectra of (a) 5-bromo-6-hydroxy-5,6-dihydrothymine (EI-MS), (b) 4-bromopyrazole (GC–MS), and (c) 4-bromopyrazole (GC–MS) through the haloperoxidase reactions.

Identification of Product from Thymine. Figure 1a shows the EI-MS spectrum of the brominated product derived from thymine by chloroperoxidase reaction. In the spectrum, molecular ion peaks at m/z 224 and 222, and other fragment ion peaks (m/z 207 and 205, M^+ – OH; 179 and 177, M^+ – CH·OH·NH; 136 and 134, M^+ – CH·OH·NH·CO·NH; 126, M^+ – OH·Br), were observed. These ion peaks corresponded to a bromohydrin compound derived from thymine. The analysis data of fragment ion peaks gave proof of additions of bromine at the 5-position and a hydroxyl group at the 6-position of thymine. From these results, the product was supposed to be 5-bromo-6-hydroxy-5,6-dihydrothymine.

In the bromoperoxidase reaction, the complete consumption of the substrate and no formation of 5-bromo-6-hydroxy-5,6-dihydrothymine were observed.

Identification of Products from Cytidine. The EI-MS spectrum of the product obtained on chloroperoxidase bromination showed molecular ion peaks (m/z 324 and 322; M^+ + 1) and strong peaks at m/z 192 and 190 due to the fragment ion of bromocytosine. The NMR spectrum and HPLC analysis data confirmed that the product was 5-bromocytidine.

The product obtained through the bromoperoxidase reaction was the same as that in the case of the chloroperoxidase reaction and an authentic sample.

We could not isolate the chlorinated compounds from the chloroperoxidase reaction mixture because of their instability.

Identification of Products from 2′-Deoxyuridine. 2′-Deoxyuridine was brominated by the chloroperoxidase reaction to yield three products, as shown in Figure 2c. However, we could not clarify the structures of the first two products because they were unstable on drying. On EI-MS analysis, the last product showed molecular ion peaks at m/z 308 and 306 that corresponded to bromo-2′-deoxyuridine. The NMR spectrum of the product was the same as that of standard 5-bromo-2′-deoxyuridine. HPLC analysis also supported this identification.

Table I: Reaction Products from Various Nitrogen-Containing Heterocycles on Haloperoxidase-Catalyzed Halogenation[a]

substrate	chloroperoxidase, halide			bromoperoxidase, halide	
	Cl^-	Br^-	I^-	Br^-	I^-
cytosine	not identified (6.6, 9.1, 11.5)	5-bromocytosine (3.8)	nr	5-bromocytosine (3.8)	slight conversion (4.9)
uracil	5-chlorouracil (3.9)	5-bromouracil (4.5)	5-iodouracil (6.4)	5-bromouracil (4.5)	5-iodouracil (6.4)
thymine	slight conversion	5-bromo-6-hydroxy-5,6-dihydrothymine (9.3)	nr	decomposition	nr
adenine	not isolated (7.6)	nr	nr	nr	nr
cytidine	not isolated (6.1, 9.2)	5-bromocytidine (6.1)	nr	5-bromocytidine (6.1)	nr
2′-deoxyuridine	nr[b]	two unstable products (5.1, 7.8) 5-bromo-2′-deoxyuridine (10.2)	nr	nr	nr
adenosine	not isolated (7.8)	nr	nr	nr	nr
guanosine	decomposition	8-bromoguanosine (11.0)	nr	nr	nr
pyrazole	4-chloropyrazole (13.4)	4-bromopyrazole (17.8) minor product (24.2)	4-iodopyrazole (27.5)	4-bromopyrazole (17.8)	4-iodopyrazole (27.5)

[a] Pyridine, pyrimidine, and purine were not used as substrates for chloroperoxidase and bromoperoxidase. The retention time (minutes) on HPLC is shown in parentheses, and the solvent system is described under Experimental Procedures. [b] nr, no reaction.

No conversion of 2′-deoxyuridine through the bromoperoxidase reaction was observed.

Identification of Products from Adenine, Adenosine, and Guanosine. Chloroperoxidase catalyzed the chlorination of adenine and adenosine to give unknown compounds, which were very unstable and gradually reverted to the substrate in a few hours. Therefore, we did not attempt to isolate these products.

In the chlorination reaction of guanosine, the substrate was completely consumed and decomposed into unknown substances.

The brominated compound derived from guanosine through the chloroperoxidase reaction was found to be completely identical with authentic 8-bromoguanosine on HPLC and TLC analyses.

The bromoperoxidase showed no reaction with guanosine.

Identification of Products from Pyrazole. Figure 1c shows the GC–MS spectrum of chlorinated product derived from chloroperoxidase reaction with pyrazole. The molecular ion peaks at m/z 104 and 102 with a 3:1 distribution were due to the isotopes of the chlorine atom of m/z 35 and 37. This suggested that the product had one chlorine atom in the pyrazole ring. In the NMR spectrum, no existence of spin–spin coupling of protons was observed. This indicated that the 4-position hydrogen of pyrazole was substituted by chlorine. Hence, the product was identified as 4-chloropyrazole.

The bromination reactions of chloroperoxidase and bromoperoxidase yielded the same product from pyrazole. GC–MS analysis of the product showed molecular ion peaks at m/z 148 and 146 split into a 1:1 distribution that corresponded to bromopyrazole, as shown in Figure 1b. The single peak at 7.60 ppm and the lack of spin–spin coupling in the NMR spectrum were similar to in the case of 4-chloropyrazole. Therefore, the product was identified as 4-bromopyrazole.

The iodinated products obtained through the chloroperoxidase and bromoperoxidase reactions were found to be completely identical with standard 4-iodopyrazole on HPLC analysis.

The results as to the identification of the products are summarized in Table I.

Comparison of the Chloroperoxidase and Bromoperoxidase Reactions with Chemical Halogenating Reagents. The chloroperoxidase-catalyzed chlorinations of uracil and cytosine were compared with the molecular chlorine reactions. Figure 2 shows the results of HPLC analysis of the products obtained from uracil and cytosine after bubbling of molecular chlorine for several seconds into solutions containing each substrate.

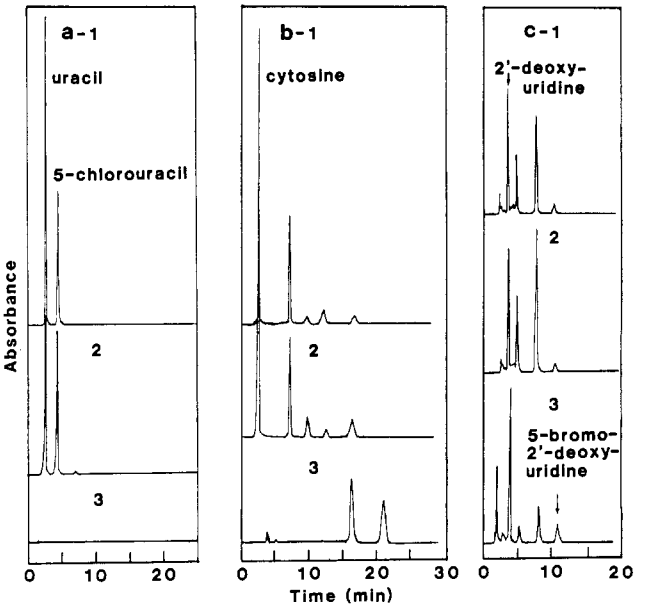


FIGURE 2: HPLC elution profiles of the products obtained on chloroperoxidase-catalyzed chlorination and chlorine gas reaction with (a) uracil and (b) cytosine and chloroperoxidase-catalyzed bromination and molecular bromine reaction with (c) 2′-deoxyuridine. The enzymatic reaction conditions were given under Experimental Procedures. The chlorine gas reaction was performed by bubbling chlorine gas into solutions containing 5 mmol of potassium phosphate buffer (pH 3.0) and 0.5 mmol of each substrate in a total volume of 50 mL. The molecular bromine reaction was performed by the addition of bromine (24 mg, 150 μmol as Br_2 in 0.5 mL of ethanol) over 10 min, with gentle stirring, to a solution comprising 2 mmol potassium phosphate buffer (pH 3.0 or 6.0) and 0.2 mmol of 2′-deoxyuridine in a total volume of 20 mL. Detection of the product on HPLC was carried out at various wavelengths, in the range of 205–254 nm. The elution profiles shown in this figure were obtained at 254 nm for cytosine and uracil and at 230 nm for 2′-deoxyuridine. (a-1 and b-1) Chloroperoxidase reaction (chlorination); (a-2 and b-2) chlorine gas reaction for 5 s; (a-3) chlorine gas reaction for 2 min; (b-3) chlorine gas reaction for 30 s; (c-1) chloroperoxidase reaction (bromination); and (c-2 and c-3) molecular bromine reaction at pH 3.0 and 6.0, respectively.

The analysis data were identical with those for the chloroperoxidase reaction. However, cytosine was converted into unknown products on further incubation with chlorine (Figure 2b-3). Uracil decomposed into unknown substances, which did not absorb light in the ultraviolet region, on long time reaction with molecular chlorine (Figure 2a-3).

Figure 2c shows the results of HPLC analysis of the products derived from 2′-deoxyurudine through the molecular bromine reaction, at pH 3.0 and 6.0, and the chloroperoxidase

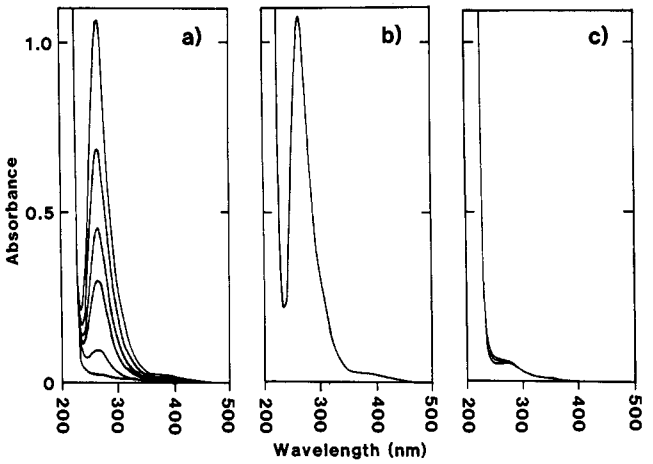


FIGURE 3: Comparison of the absorption spectra of (a) the chloroperoxidase- and (c) bromoperoxidase-generated products with that in the case of (b) molecular bromine in an aqueous solution. (a) The reaction mixture comprised 0.1 mmol of potassium phosphate buffer (pH 3.0), 0.1 mmol of KBr, 0.5 μmol of H_2O_2, and 0.2 unit of chloroperoxidase in a total volume of 1.0 mL. The reaction was initiated by adding H_2O_2, and the spectrum was measured at 2-min intervals at 25 °C. (b) The solution comprised 0.1 mmol of potassium phosphate buffer (pH 3.0 and 6.0), 0.1 mmol of KBr, and 60 (pH 3.0) or 90 nmol of bromine (pH 6.0) in a total volume of 1.0 mL. (c) The reaction mixture comprised 0.1 mmol of potassium phosphate buffer (pH 6.0), 0.1 mmol of KBr, varying amounts of H_2O_2, from 50 nmol to 1 μmol, and 0.2, 0.4, or 0.8 unit of bromoperoxidase in a total volume of 1.0 mL. The measurements were performed in the same manner as described in (a).

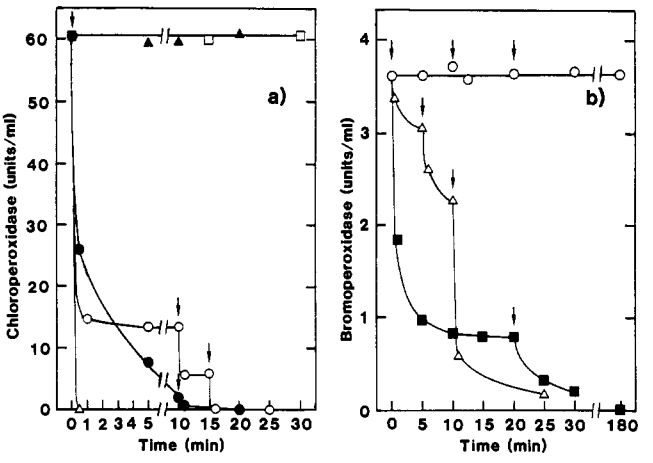


FIGURE 4: Enzyme stabilities of (a) chloroperoxidase and (b) bromoperoxidase in reaction mixtures without a halogen acceptor and on exposure to molecular bromine. The chloroperoxidase (80 μg) in 1.0 mL of 0.1 M potassium phosphate buffer (pH 3.0) and the bromoperoxidase (720 μg) in the same buffer (pH 6.0) were incubated with the following additives, and then the remaining activities were measured: (□) 20 μmol of KBr or KCl; (▲) 5 μmol of H_2O_2; (△) 0.6 μmol of Br_2 in (a) and 3.0 μmol of Br_2 in (b) (the arrows indicate the additions of 0.6, 1.2, and 1.2 μmol of Br_2, in that order); (○) 20 μmol of KBr and 0.15 μmol of H_2O_2 in (a) (the arrows indicate the additions of 0.05 μmol of H_2O_2) and 20 μmol of KBr and 20 μmol of H_2O_2 in (b) (the arrows indicate the additions of 5, 5, and 10 μmol of H_2O_2, in that order); (●) 20 μmol of KCl and 2 μmol of H_2O_2 (the arrows indicate the additions of 1 μmol of H_2O_2); (■) 3.0 μmol of Br_2 (the arrows indicate the additions of 2.4 and 0.6 μmol of Br_2, in that order).

reaction. Molecular bromine acted on 2′-deoxyuridine to give the same products as in the case of chloroperoxidase bromination. The similarity of molecular chlorine and bromine reactions to the chloroperoxidase halogenations was coincident with the results of a previous study (Yamada et al., 1985a). Considering that bromoperoxidase was inactive toward 2′-deoxyuridine, it was supposed that the brominating reactions of bromoperoxidase did not involve the formation of molecular bromine and its release into the solution.

Analysis of the Brominating Intermediate of the Bromoperoxidase Reaction. Bromine is known to form a tribromide ion complex, which absorbs light in the ultraviolet region (Katzin, 1952), and hypobromous acid in an aqueous solution.

$$Br_2 \xrightleftharpoons{Br^-,\ H_2O} Br_3^- + HOBr$$

Libby et al. (1982) reported the formation of molecular bromine from bromide ions through the chloroperoxidase reaction. Figure 3 shows the ultraviolet spectra of the enzymatic products of chloroperoxidase and bromoperoxidase compared with those in the case of a bromine solution under the same conditions. In the case of chloroperoxidase, the formation of tribromide ions was obvious. However, the content of tribromide ions in the solution was observed to gradually decrease with time (Figure 3a). This was considered to be due to the H_2O_2 oxidation by HOBr (Griffin, 1983). For bromoperoxidase, the formation of tribromide ions was carefully investigated through measurements at different hydrogen peroxide concentrations with various enzyme concentrations from 0.2 to 0.8 unit/mL of the mixture. However, no appreciable generation of tribromide ions in the reaction mixture was observed (Figure 3c).

Stability of Chloroperoxidase and Bromoperoxidase under Reaction Conditions. Molecular halogens are known to be very active chemical species. Therefore, it was thought that molecular halogen released in the chloroperoxidase reaction mixture may cause damage to the chloroperoxidase itself.

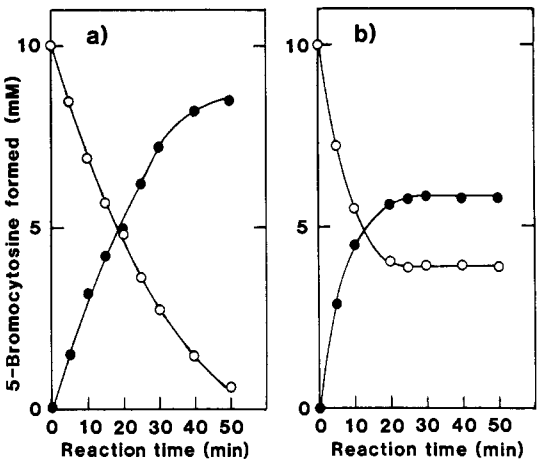


FIGURE 5: 5-Bromocytosine formation through (a) bromoperoxidase- and (b) chloroperoxidase-catalyzed bromination. The reaction mixtures comprised 1 mmol of potassium phosphate buffer (pH 6.0) for bromoperoxidase and (pH 3.0) for chloroperoxidase, 0.4 mmol of KBr, 0.26 mmol of H_2O_2, and 5 units of bromoperoxidase or 10 units of chloroperoxidase in a total volume of 10 mL. The reaction was started and continued by adding 20 μmol of H_2O_2 at 4-min intervals. (○) Cytosine concentration; (●) 5-bromocytosine formed.

Figure 4 shows the remaining chloroperoxidase and bromoperoxidase activities in the presence of halide ions and hydrogen peroxide without a halogen acceptor or on exposure to molecular bromine. Surprisingly, the chloroperoxidase rapidly lost its activity during the reaction. On the contrary, bromoperoxidase was stable for at least 3-h reaction. It became apparent that molecular bromine irreversibly denatured both the enzyme. That no bromoperoxidase activity was lost during the incubation with bromide ions and hydrogen peroxide suggested that the enzyme did not release active species such as molecular bromine into the solution.

Production of 5-Bromocytosine by the Haloperoxidase Reaction. Figure 5 shows the formation of 5-bromocytosine

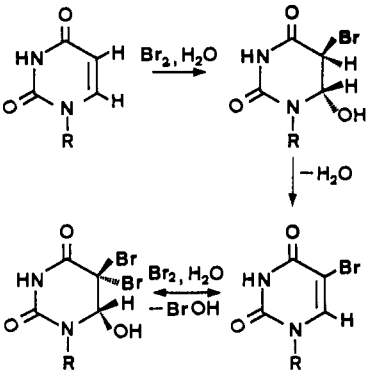
from cytosine through bromoperoxidase- and chloroperoxidase-catalyzed brominations. The reactions were continued for 50 min by feeding hydrogen peroxide at 4-min intervals. In the case of the bromoperoxidase reaction, almost linear conversion of cytosine to 5-bromocytosine was observed. However, as the reaction proceeded, a small amount of a byproduct (r_t = 8.2 min, on HPLC) was detected. This indicated that complete equimolar conversion of cytosine could not be attained. On the other hand, the bromination of cytosine by the chloroperoxidase did not completely proceed. This phenomenon was considered to be due to the irreversible denaturation of the chloroperoxidase.

Discussion

This is the first report concerning the enzymatic halogenations of nucleic bases and related compounds. Halogenated nucleic bases, nucleosides, and nucleotides are known to be anticancer or antivirus agents which inhibit the synthesis of DNA or RNA in a living cell system (Santi et al., 1979; Renis, 1980). In the field of microbiology, some halogenated compounds have been isolated as biological active compounds (Neidleman, 1975). Our results suggested the possibility of the participation of haloperoxidases in the syntheses of these compounds and the application of haloperoxidases to the enzymatic halogenation process. Table I summarizes the substrate specificites of two haloperoxidases, the chloroperoxidase of *Caldariomyces fumago* and the bromoperoxidase of *Corallina pilulifera*, for nucleic acid related substances. The enzymes were examined in this study as biological halogenating reagents because both enzymes have been well studied and are very stable and readily available (Morris & Hager, 1966; Hager et al., 1966; Libby et al., 1982; Itoh et al., 1985, 1986). However, the most important point of the experiments was the clarification of the catalytic difference between the chloroperoxidase and bromoperoxidase. The chloroperoxidase is an H-type enzyme, and most haloperoxidases of other origin are this type of enzyme, while the bromoperoxidase of *C. pilulifera* is an NH-type enzyme (Itoh et al., 1986). Therefore, we supposed that the bromoperoxidase had a different specificity and reaction mechanism from those of the chloroperoxidase.

In the previous study, we revealed that the chloroperoxidase reaction is the same as that of molecular bromine using *trans*-cinnamic acid and its derivatives, and systematized the substrates of the chloroperoxidase (Yamada et al., 1985a). The results indicated that the substrate specificity of chloroperoxidase depends on the nucleophilicity of the substrate. As shown in Figures 2 and 3, there is no doubt that the chloroperoxidase reactions are identical with those of molecular chlorine and bromine in the case of nitrogen-containing heterocycles. Bromoperoxidase-catalyzed bromination also occurs via the intervention of a bromonium cation intermediate (Br^+), not a radical (Yamada et al., 1985b; Itoh et al., 1986). Therefore, the substrate specificities of those two haloperoxidases toward these nitrogen-containing aromatic heterocycles are likely. The abilities of pyridine, pyrimidine, and purine as nucleophiles are low because π-electron density is reduced by the electronegative nitrogen atom. In addition, the coordination of electrophilic molecular halogen to the nitrogen atom increases the electronegativity of nitrogen atom, which results in less reactivity toward electrophiles. However, the presence of an electron-attracting group in the heterocycles reduces the electron density of the nitrogen atom. Therefore, the coordination of molecular halogen to nitrogen atom hardly occurs. The substrate specificities of the chloroperoxidase and bromoperoxidase can be explained in this way.

Scheme I



Substitutions of hydrogen at the 5-position of uracil and cytosine at the 4-position of pyrazole by halogen are likely, because of the general resonance. Bromination of cytosine and uracil by bromine in an aqueous solution was examined in detail by Taguchi and Wang (1979) and Tee and Berks (1980), respectively. The first step in the bromination reaction is the formation of unstable bromohydrin derivatives, which are subsequently converted to 5-bromocytosine or 5-bromouracil derivatives. They reported that in the presence of excess bromine, 5,5-dibromo-6-hydroxy-5,6-dihydrocytosine or a similar uracil derivative is produced. However, these products also readily revert to 5-bromocytosine or 5-bromouracil derivatives through the nucleophilic or electrophilic elimination of HOBr (Scheme I). On enzymatic bromination of thymine, we isolated unstable 5-bromo-6-hydroxy-5,6-dihydrothymine. The formation of this product could be explained in the same manner. In the case of the bromination of 2′-deoxyuridine, unstable products (Figure 2c) were produced, which probably corresponded to 5-bromo-6-hydroxy-5,6-dihydro or 5,5-dibromo-6-hydroxy-5,6-dihydro derivatives. The EI-MS spectra of the compounds (data not shown) indicated the presence of fragment ion peaks of 5-bromo-2′-deoxyuridine and the absence of molecular ion peaks. The retention times of these products on HPLC supported the existence of a hydroxyl group in the molecular structures of the products.

As to the chlorination reaction of chloroperoxidase, we isolated only 5-chlorouracil and 4-chloropyrazole. Adenine and adenosine were observed to be converted into unknown compounds, but they were not isolated because they were unstable. The chemical chlorinations of cytosine and uracil were complicated. It was found that long-time reaction led to the formation of two products from cytosine which showed long retention times on HPLC with a reversed-phase column (Figure 2b-3). Hence, these products cannot be explained in terms of the formation of chlorohydrin derivatives. In the case of uracil, disappearance of the products was observed. This indicated the decomposition of the ring structure of uracil. The saturation at the 5- and 6-positions of the pyrimidine residue probably led to straining of the plane structure of the molecule and the cleavage of the ring would occur instead of elimination of HOCl.

The difference in reactivity between the bromoperoxidase and chloroperoxidase should be due to the dissimilarity of reaction mechanisms of the enzymes. The bromoperoxidase did not utilize 2′-deoxyuridine or guanosine as a substrate. Thus, the reactivity of the bromoperoxidase was lower than that in the case of chloroperoxidase bromination, in other words, lower than that of molecular bromine in an aqueous solution. It appeared that bromoperoxidase-catalyzed bromination did not involve the formation of molecular bromine. Hence, the bromination occurs at the active site of the bro-

moperoxidase. We speculated the presence of a bromonium cation (Br^+) intermediate in the enzyme which exists at the active site of the bromoperoxidase and acts on the halogen acceptor.

From the viewpoint of application of haloperoxidases to the enzymatic process, the chloroperoxidase has some disadvantages. Figure 5 shows that the chloroperoxidase-catalyzed bromination of cytosine stopped within a short time. This phenomenon was considered to be due to denaturation of the chloroperoxidase. Molecular bromine probably acts on several amino acid residues such as cysteine, histidine, tryptophan, and tyrosine (Morrison & Schonbaum, 1976) and also on coenzymes, e.g., NAD^+, riboflavin, CoA, pyridoxal, etc. Therefore, there is little possibility of using the chloroperoxidase with other enzyme systems, for example, the hydrogen peroxide generating system of oxidases.

Another disadvantage of the chloroperoxidase was its high peroxidase activity. We previously reported the formation of tribromophenols from phenol using bromoperoxidase (Yamada et al., 1985b). However, the chloroperoxidase catalyzed not only the bromination but also the oxidation of phenol, to yield large amounts of a brown pigment.[1] In several cases, we often detected minor byproduct formation, which probably depended on the peroxidative reaction of the chloroperoxidase.

Although bromoperoxidase has no ability to catalyze the chlorination reaction and its enzyme reactivity is lower than that of the chloroperoxidase, it is superior to the chloroperoxidase in the following points: (1) its high stability under the reaction conditions; (2) the low formation of minor byproducts; and (3) its possible combination with other enzyme systems. Further studies on the stereospecificity and other substrates for the bromoperoxidase are in progress.

Acknowledgments

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Registry No. Chloroperoxidase, 9055-20-3; bromoperoxidase, 69279-19-2; cytosine, 71-30-7; uracil, 66-22-8; thymine, 65-71-4; adenine, 73-24-5; cytidine, 65-46-3; 2′-deoxyuridine, 951-78-0; adenosine, 58-61-7; guanosine, 118-00-3; pyrazole, 288-13-1; 5-chlorouracil, 1820-81-1; 4-chloropyrazole, 15878-00-9; 5-bromocytosine, 2240-25-7; 5-bromouracil, 51-20-7; 5-bromo-6-hydroxy-5,6-dihydrothymine, 1195-73-9; 5-bromocytidine, 3066-86-2; 5-bromo-2′-deoxyuridine, 59-14-3; 8-bromoguanosine, 4016-63-1; 4-bromopyrazole, 2075-45-8; 5-iodouracil, 696-07-1; 4-iodopyrazole, 3469-69-0.

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[1] N. Itoh, unpublished results.