Enzymatic Halogenation of Pyrazoles and Pyridine Derivatives

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Pyrazole, 1-methylpyrazole and 3-methylpyrazole are chlorinated by the enzyme chloroperoxidase from *Caldariomyces fumago*, in the presence of potassium chloride and hydrogen peroxide at *p*H 2.7, yielding the corresponding 4-chloro derivatives in good yields. A 4*H*-pyrazole is proposed as an intermediate in this reaction. 2-Aminopyridine was converted regiospecifically by the same enzyme into 2-amino-3-chloropyridine, and 8-hydroxyquinoline gave its 5,7-dibromo-derivative in very good yield when bromide ion was used as the halide substrate.

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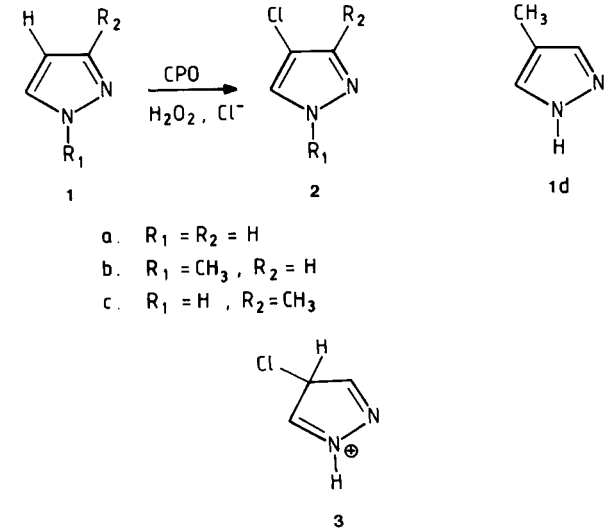
Chloroperoxidase from *Caldariomyces fumago* (CPO; EC 1.11.1.10; chloride: hydrogen peroxide oxidoreductase) is a well-known enzyme, capable of halogenating a great variety of organic compounds by means of hydrogen peroxide and chloride, bromide or iodide ions [1]. However, until recently only a few heterocyclic substrates have been studied: thiazole [2], antipyrine [3], NADH [4] and barbituric acid and some of its derivatives [5]. Since we have an ongoing interest in the use of enzymes in heterocyclic organic synthesis [5,6] we studied the enzymatic chlorination of pyrazole (**1a**), 1-methylpyrazole (**1b**), 3-methylpyrazole (**1c**) and 2-aminopyridine (**4**) and the bromination of 8-hydroxyquinoline (**6**) [7]. The publication of our results was induced by the appearance of a study on the CPO-mediated halogenation of nucleic bases, in which also pyrazole was mentioned as one of the substrates used [8].



Scheme I

Conversion of pyrazole (**1a**) and two derivatives thereof by CPO/H_2O_2/Cl^-. 4-Methylpyrazole (**1d**) is not a substrate for the enzyme. The 4*H*-pyrazole **3** is postulated as an intermediate in the reaction (see text).

When pyrazole (**1a**, see Scheme I) was incubated with CPO under conditions which are optimal for the enzyme (0.1 *M* orthophosphoric acid/potassium hydroxide, *p*H 2.7, 20 mmolar potassium chloride, 0.24 mmolar hydrogen peroxide) and the reaction was monitored by uv-spectroscopy we observed that the maximum in the uv-spectrum shifted rapidly from 214 to 218 nm (see Figure). This uv-absorption slowly decreased but readdition of fresh enzyme caused a rapid shift of the λ max to 224 nm; this

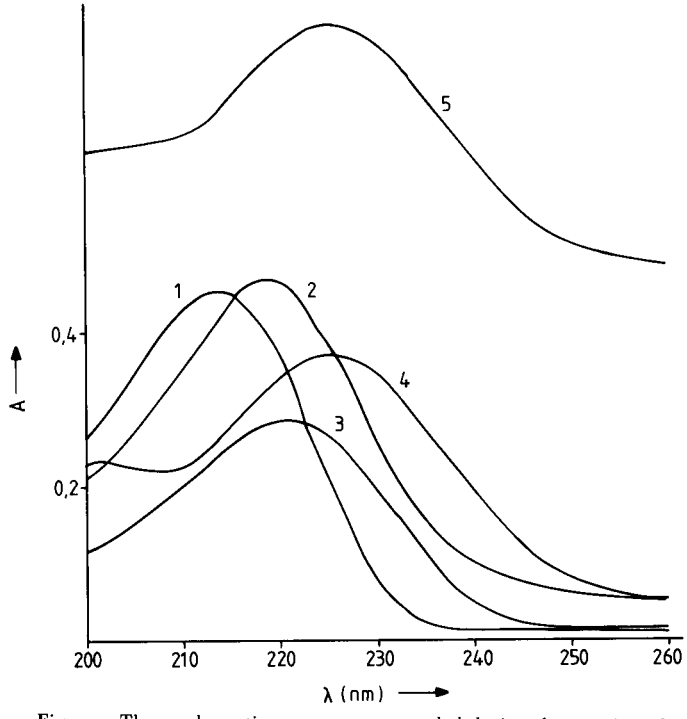


Figure. The uv-absorption spectra, as recorded during the reaction of pyrazole (**1a**) with CPO. The quartz cuvette was filled with 2.5 ml of a solution containing 0.1 mmolar **1a**, 0.24 mmolar hydrogen peroxide, 20 mmolar potassium chloride and 0.1 *M* orthophosphoric acid/potassium hydroxide, *p*H 2.7. CPO (280 ng) was added to this and the uv-spectra were recorded at appropriate time intervals. Trace 1: t = 0 (pure **1a**); trace 2: t = 0.5 minute; trace 3: t = 2.5 hours. Additional CPO (840 ng) was subsequently added. Trace 4: t = 3.0 hours (reaction is complete); Trace 5: authentic **2a** (offset + 0.4 Abs).

peak did not change on further addition of enzyme. After isolation the product appeared to be 4-chloropyrazole (**2a**). The structure of the intermediate with λ max = 218 nm is possibly the positively charged 4-chloro-4*H*-pyrazole **3**, since 4*H*-pyrazolium salts are known to have maximal uv-absorption around 220 nm [9]. 4*H*-pyrazoles have been observed as intermediates during reactions of pyrazoles with chlorine or *t*-butyl hypochlorite, but isomerize to 3*H*-pyrazoles or form trimers when they contain hydrogen atoms attached to the ring carbon atoms [10]. Attempts to detect **3** by gc/ms inspection of the enzymatic reaction mixture failed; only **2a** was found [11]. Monitoring the enzymatic reaction by ^{1}H-nmr spectroscopy in deuterium oxide was also not successful, since only signals of starting material **1a** and product **2a** were observed. Probably the concentration of intermediate **3** is too low for detection; the fact that **1a** and **2a** only became visible after a 30 minute pulse period indicates that this method is unfavourable for this particular study. Although it is reasonable to assume that 4*H*-pyrazoles are short living intermediates in halogenation reactions of 1*H*-pyrazoles, more concrete evidence is needed.

CPO reacts with the substituted pyrazoles 1-methylpyrazole (**1b**) and 3-methylpyrazole (**1c**) in a comparable manner as with **1a**, giving the 4-chloro-derivatives **2b** respectively and **2c** in very good yields (see Table). It is interesting that the regiochemistry of this reaction is the same as in the CPO-mediated halogenation of 1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazol-3-one (antipyrine) [3]. As expected, 4-methylpyrazole (**1d**) is not a suitable substrate for chloroperoxidase, because position 4 is blocked.

Table

Compound	Yield (%) CPO/H_2O_2/X^-	HOCl
4-Chloropyrazole (**2a**)	68	20
4-Chloro-1-methylpyrazole (**2b**)	83	68
4-Chloro-3-methylpyrazole (**2c**)	91	67
2-Amino-3-chloropyridine (**5**)	18	11
5,7-Dibromo-8-hydroxyquinoline (**7**)	79 [a]	–

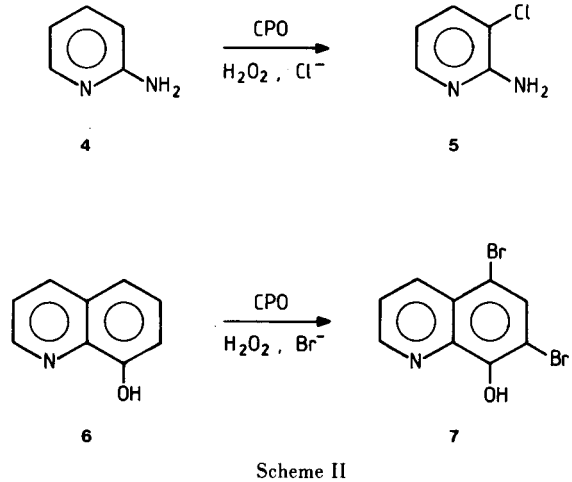
Yields of the CPO-mediated chlorination of several heterocyclic substrates, compared to those obtained with hypochlorous acid. See Experimental for details; yields were determined by glc unless indicated otherwise.

[a] Yield of isolated product.

Because there is some evidence that CPO generates free hypochlorous acid, which could act as the active chlorinating species in solution [12], we also studied the chlorination of pyrazoles with this reagent. We found that hypochlorous acid, when slowly added to solutions of **1** at *p*H 2.7, indeed reacts with **1a-c** giving **2a-c**, but only in moderate yields (see Table).

The non-enzymatic chlorination of pyrazoles as published in the literature usually takes place by bubbling an appropriate amount of dry chlorine gas into a solution of the compound in tetrachloromethane or by treatment with sulfuryl chloride [13]. In the first case **2a** was synthesized with a yield of 55%. The yield of **2c** was not given and **2b** was prepared by a different route. Itoh *et al.* [8] converted **1a** by means of CPO giving **2a** and its bromo analogue in respective yields of 7 and 14%. Their low yields are most probably due to their unconventional workup procedure and the high concentration of hydrogen peroxide they use, which is known to inactivate CPO [14].

We believe that our enzymatic procedure is competitive with the non-enzymatic synthesis, since the yields are higher and the products obtained are more pure.



Scheme II

Reactions of CPO/H_2O_2/X^- (X = Cl or Br) with two pyridine derivatives, *e.g.* 2-aminopyridine (**4**) and 8-hydroxyquinoline (**6**).

2-Aminopyridine (**4**, see Scheme II) was converted by CPO into 2-amino-3-chloropyridine (**5**). No 5-chloro- or 3,5-dichloro-2-aminopyridine could be detected in the reaction mixture. This regiospecific chlorination was slow and inactivation of the enzyme occurred during incubation. It was not possible to obtain complete conversion by repeatedly adding fresh enzyme to the reaction mixture. This is in part due to the very low stability of **5** under the conditions used; the reaction mixture immediately turns brown upon neutralization. The structure of the product was proved by mass spectroscopy and ^{1}H-nmr. When the hypochlorous acid method was applied **5** was obtained in very low yield together with starting material (see Table). Compound **5** is described in the literature [15] and was synthesized by aminolysis of 2,3-dichloropyridine or from 2-amino-4-bromo-3-chloropyridine by reduction with hydrogen; yields are not given. The regiospecific enzymatic chlorination at C-3 described here contrasts interestingly with the different regiospecificity observed in

the chlorination of **4** in 20% sulfuric acid using chlorine, 2-amino-5-chloropyridine being obtained in 54% yield [16]. The same product was also obtained when using hydrogen peroxide or potassium chlorate in concentrated hydrochloric acid [17].

8-Hydroxyquinoline (**6**, see Scheme II) was found to be a more suitable substrate for the enzyme than **4**. When reacting with CPO, hydrogen peroxide and potassium bromide, 5,7-dibromo-8-hydroxyquinoline (**7**) was formed in a yield of 79%. Compound **7** was already synthesized previously in high yields by reacting **6** with bromine [18]. However, our enzymatic synthesis can be performed much more smoothly, without the need of molecular halogens and/or hypohalous acid. For this reason, the scope of enzymatic halogenations will be intensively studied at our laboratory.

EXPERIMENTAL

Chloroperoxidase from *Caldariomyces fumago* (the crude type) was obtained from Sigma Chemical Company or from the Laboratory of Biochemistry, University of Amsterdam, The Netherlands (Dr. Wever). Monochlorodimedon was also purchased from Sigma. 1-Methylpyrazole and the chlorinated pyrazoles were synthesized according to Hüttel [19,13]. Sodium hypochlorite was purchased from Janssen Chimica and was assayed by injecting an appropriate amount of the solution in 0.1 mmolar monochlorodimedon at *p*H 2.7. The difference in A_{278} is proportional to the hypochlorite concentration, using $\Delta\epsilon = 12{,}200\ M^{-1}.cm^{-1}$. All chemicals were of the highest commercial grade, but 2-aminopyridine and 8-hydroxyquinoline were purified, by sublimation and recrystallization from ethanol/water, respectively.

The uv-spectra and kinetic measurements were performed on an Aminco-Chance DW-2 split-beam spectrophotometer. Mass spectra were recorded on an AEI MS 902 instrument or a VG-Micromass 7070 F apparatus (direct probe mode). The gc/ms spectra were obtained using the VG-Micromass 7070 F gas chromatograph-mass spectrometer. The reaction of pyrazole with CPO was monitored with a Bruker CXP-300 ^{1}H-nmr spectrometer operating at 300.066 MHz, with suppression of the HOD signal. Gas chromatography was performed on a Varian 3000 apparatus, equipped with a fused silica capillary column (L = 30 m); the stationary phase was DB17, film thickness 0.25 μm.

Enzymatic Halogenation.

For uv-monitoring of the enzymatic halogenation the reaction was performed as follows. A solution (2.44 ml) containing 0.1 mmolar (0.05 mmolar for **6**) of organic substrate and 20 mmolar potassium halide in 0.1 *M* orthophosphoric acid/potassium hydroxide, *p*H 2.7 was brought into a 3.0 ml quartz cuvette. Sixty μl of 10 mmolar hydrogen peroxide in doubly distilled water (final concentration 0.24 mmolar) and 280 μg CPO [20] were added and the uv-spectra were recorded at 25°. It was necessary in the reaction of **1a** to add extra 840 ng of CPO after 2.5 hours. For glc analysis the procedure was as follows: 2.5 ml of a solution containing 0.5 mmolar organic substrate and 20 mmolar potassium halide in 0.1 *M* orthophosphoric acid/potassium hydroxide, *p*H 2.7 was brought into a 10 ml test tube. Three hundred μl of 10 mmolar hydrogen peroxide in doubly distilled water (final concentration 1.07 mmolar) and 1.5 μg of CPO were added. After 20 minutes incubation at 25° a 0.5 μl sample was taken and directly injected into the gas chromatograph. It was necessary in the reaction of **1a** to add extra 1.5 μg of CPO after 60 minutes and again after 120 minutes; after 135 minutes a sample was taken for analysis. The glc oven temperature was 120° for **1a-c** and 160° for **4**. The adjusted retention times were: **1a**, 1.0 minute; **1b**, 0.6 minute; **1c**, 2.6 minutes; **2a**, 4.4 minutes; **2b**, 2.1 minutes; **2c**, 6.5 minutes; **4**, 1.2 minutes; **5**, 1.9 minutes. Analysis of **7** with glc or hplc was not possible due to its strong interactions with all tested column materials.

Isolation of Enzymatic Products.

The reaction with the relatively apolar compounds like the pyrazoles and 8-hydroxyquinoline were carried out as follows. To a solution of the substrate (0.5 mmolar) in 0.1 *M* potassium phosphate buffer (*p*H 2.7) which contained 20 mmolar potassium halide and 1.07 mmolar hydrogen peroxide was added 0.6 μg of CPO per ml of reaction medium. The conversion of the substrate was monitored by means of uv-spectroscopy or glc. In the case of **1a-c** the reaction mixture was worked up by adjusting the *p*H of the solution to 6.0 by adding a dilute solution of potassium hydroxide. The reaction medium was extracted continuously overnight with distilled diethyl ether and after drying and evaporation of the ether, the pure product was isolated and characterized. In the case of **6** the product separates as a greyish precipitate during the reaction which can be easily filtered off, dried and analyzed.

Since **5** is too-water-soluble for direct extraction with organic solvents at low *p*H and unstable at neutral *p*H the halogenation reaction was performed in the following way. The standard reaction medium described above was replaced by dilute hydrochloric acid (*p*H 2.7) containing 5 mmolar potassium chloride and 1.07 mmolar hydrogen peroxide. CPO, 0.6 μg per ml of reaction medium was injected into the solution. During the reaction some extra hydrogen peroxide and CPO were added. After 3 hours incubation the reaction mixture was lyophilized and the product was extracted from the solid material with distilled methanol. A more or less salt-free product was obtained which could be analyzed.

Analysis of the data for **5** was; ms: m/e 128-130 (100%), 101-103 (27), 93 (43), 66 (19); ^{1}H-nmr (deuteriochloroform): δ 4.8 (br s, NH_2), δ 6.62 (dd, H_5, $J_{4,5}$ = 8.1 Hz, $J_{5,6}$ = 5.2 Hz), δ 7.48 (dd, H_4, $J_{4,6}$ = 1.5 Hz), δ 7.97 (dd, H_6).

GC/MS Study on the Enzymatic Chlorination of Pyrazole.

The enzymatic reaction was performed as described in isolation of the enzymatic products. The ethereal solution of the extracted product was injected into the gas chromatograph. The column dimensions were 2 mm ID x 1.5 m, filling 3% OV-17 on Chromosorb WHP 100/120. The initial temperature (100°) was maintained for 1 minute, and then raised to 250° with a rate of 8°/minute. The adjusted retention times were: pyrazole, 1.5 minute; 4-chloropyrazole, 2.1 minutes; 4-bromopyrazole, 4.0 minutes.

The ^{1}H-NMR Study on the Enzymatic Chlorination of Pyrazole.

To 5 ml of deuterium oxide was added 5 μl of 1 *M* sulfuric acid (in deuterium oxide), 100 μl of 10 mmolar pyrazole (in deuterium oxide) and 7.4 mg of potassium chloride (Solution A). The apparent *p*H of this solution was 2.53, corresponding to a *p*D of 2.93 [21], well within the optimal activity region of the enzyme [1]. Hydrogen peroxide (30%) was diluted with deuterium oxide to a final concentration of 10 mmolar (Solution B).

Solution A (2.44 ml) was mixed with 60 μl of Solution B and 330 ng of CPO was added. Part of this mixture was placed in a nmr-tube and spectra were recorded using 30 minute-pulse periods. When the reaction was complete the apparent *p*H of the solution was checked and proved to be 2.67.

Reactions with Hypochlorous Acid.

To 2.5 ml of a 0.5 mmolar solution of substrate in buffer at *p*H 2.7 was added 1 equivalent of hypochlorous acid in 4 portions with stirring. After 15 minutes incubation, the reaction mixture was analyzed as described above.

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