Mechanism of Inhibition of H^+, K^+-ATPase by Sodium 2-[[4-(3-Methoxypropoxy)-3-methylpyridin-2-yl]methylsulfinyl]-1*H*-benzimidazole (E3810)

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Sodium 2-[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]methylsulfinyl]-1*H*-benzimidazole (E3810) and omeprazole inhibit gastric acid secretion through inhibition of the activity of H^+, K^+-ATPase present in parietal cell membrane vesicles, by chemical modification of SH groups in the enzyme molecule. In order to clarify the mechanism of the chemical modification, reaction products of E3810 and omeprazole with 2-mercaptoethanol under acidic conditions (pH 3, 4, 5, 6) were isolated by HPLC, and subjected to structural analysis by UV, ^{1}H-NMR and mass spectrometry. E3810 and omeprazole appeared to undergo two kinds of reactions, affording disulfide-type products (type I reaction) and sulfide-type products (type II reaction). The rates of these reactions were determined by HPLC, and the stability of the products in the presence and absence of glutathione was investigated. In the case of E3810, type I reaction was found to proceed faster than type II reaction at every pH value studied. The type I reaction of E3810 was faster than that of omeprazole. The rate of type I reaction decreased at pH 5 and 6, especially for omeprazole, and the contribution of type II reaction increased as the pH of the reaction mixture was increased. The sulfide-type modification products were stable, whereas the formation of the disulfide-type modification products was reversed by the action of endogenous SH compounds such as glutathione. These results suggest that higher inhibitory activity of E3810 against gastric acid secretion and faster recovery of the enzyme activity after inhibition by E3810 can be expected, as compared with those of omeprazole.

Key words H^+, K^+-ATPase; SH group-modification; E3810; omeprazole

H^+, K^+-ATPase transports H^+ ions to the outside of the parietal cells of the gastric mucosa and transports K^+ to the inside of these cells in conjugation with hydrolysis of ATP. The pH difference between the intracellular and extracellular space attained by the transport of H^+ by this enzyme is as great as 6. Both sodium 2-[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]methylsulfinyl]-1*H*-benzimidazole (E3810) and omeprazole (Fig. 1) are considered to modify the SH groups of this enzyme under acidic conditions, leading to the inhibition of gastric acid secretion.[1–5] It has been reported that the rate of acid-activation of the H^+, K^+-ATPase inhibitor is the main factor determining the potency of the inhibitory activity.[6] It was also found that E3810 and omeprazole bind to Cys residues of H^+, K^+-ATPase.[7–9] Therefore, it is very important to investigate the nature of the chemical reactions between the inhibitors and SH groups.

In this study, we examined the reactions of E3810 and omeprazole with 2-mercaptoethanol at pH 3—6 by HPLC, since 2-mercaptoethanol can be regarded as a model of the Cys residues of H^+, K^+-ATPase, and performed structural analysis of the reaction products.

It has been reported in experiments using gastric gland of rabbits that the inhibition by E3810 and omeprazole is reversed after the addition of glutathione and that the activity of H^+, K^+-ATPase after inhibition by E3810 recovered faster than that after inhibition by omeprazole.[5] If the product formed by the modification of SH groups is stable, the recovery of the enzyme activity from the inhibition should be slow. Therefore, we compared the stability of the reaction products of 2-mercaptoethanol with E3810 and omeprazole.

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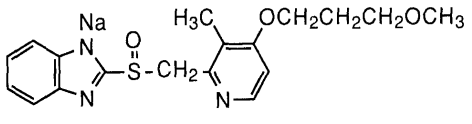
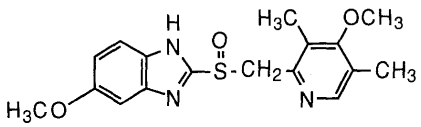
Materials and Methods

Reaction of E3810 and Omeprazole with 2-Mercaptoethanol E3810 (lot. No. 88041501 and lot. No. 90060703, synthesized at Eisai Chemical Co., Ibaraki, Japan) or omeprazole (lot. No. 87061411, synthesized at Eisai Chemical Co.) was added to 0.1 M citrate buffer containing 0.2% 2-mercaptoethanol and 2% acetonitrile (pH 3—6), and the solution was incubated at 25 °C for about 15 min in the case of E3810 or about 40 min in the case of omeprazole, then subjected to HPLC analysis under the following conditions.

Operating Conditions for HPLC Apparatus: pump, CCPM (Tosoh, Tokyo, Japan); pump controller, PX-8010 (Tosoh, Tokyo, Japan); sample processor, WISP 710B (Millipore Waters, Massachusetts, U.S.A.); detector and integrator, System Gold 168 Detector (Beckman, California, U.S.A.). Column: Nucleosil $5C_{18}$ 4.6 mm i.d., 150 mm length. Column temperature: about 25 °C. Mobile phase solvent A: acetonitrile, water and 70% perchloric acid (100:900:1). Mobile phase solvent B: acetonitrile, water and 70% perchloric acid (600:400:1). Gradient program: linear gradient, 0 min (solvent A: 100%, solvent B: 0%)→40 min (solvent A: 0%, solvent B: 100%). Flow rate: 1 ml/min. Detection:



E3810



Omeprazole

Fig. 1. Chemical Structures of E3810 and Omeprazole

UV_{290nm}. Volume of injection: 20 μl.

Structural Analysis of Reaction Products The reaction products of E3810 or omeprazole with 2-mercaptoethanol were isolated by HPLC and TLC, and their UV (U-3500 and 330 spectrophotometer, Hitachi, Tokyo, Japan), ^{1}H-NMR (JNM-GX400, JEOL, Tokyo, Japan) and mass (JMS-HX100, JEOL) spectra were obtained. Based on these spectra, the structures of the reaction products were determined.

Measurement of Stability of Modified SH Group Products The modified SH group products formed by the reaction of 2-mercaptoethanol with E3810 or omeprazole were added to 0.1 M citrate buffer containing 1% acetonitrile (pH 3—6) or 0.1 M citrate buffer containing 1% acetonitrile and 0.1% glutathione (pH 3—6), incubated at 25 °C for about 15 min, and subjected to analysis by HPLC under the operating conditions described above in order to investigate their stability.

Results

Reaction of E3810 with 2-Mercaptoethanol HPLC chromatograms of the reaction mixture of E3810 and 2-mercaptoethanol are shown in Fig. 2. Four reaction products were formed. The time courses of the changes in peak area of these reaction products are shown in Fig. 3. At pH 3 and 4, reaction product 4 was mainly produced. As the pH of the reaction mixture was increased, the

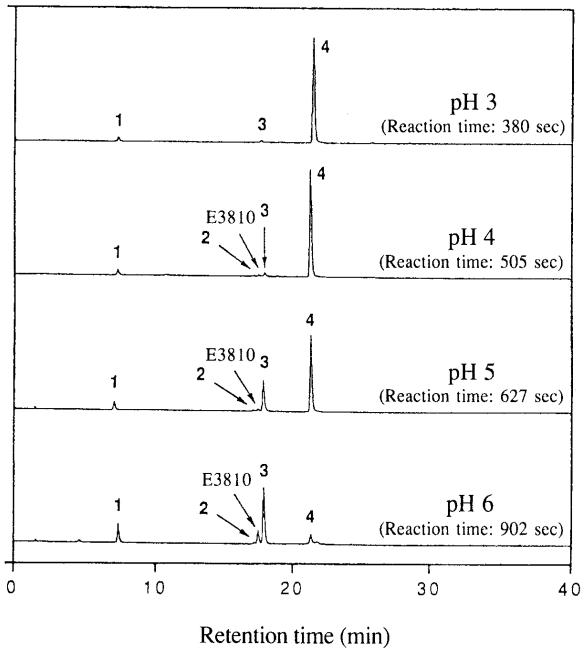


Fig. 2. HPLC Chromatograms of Reaction Mixture of E3810 and 2-Mercaptoethanol

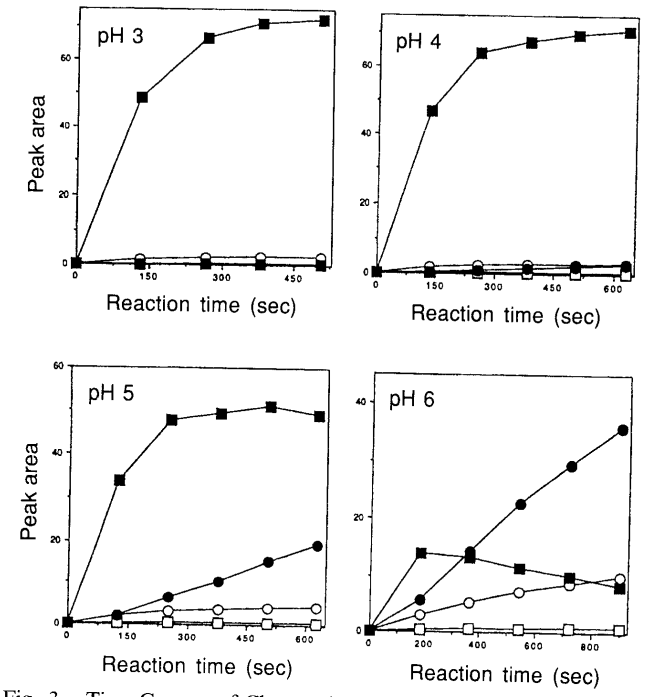


Fig. 3. Time Courses of Changes in Peak Area of Reaction Products of E3810 with 2-Mercaptoethanol

○, reaction product 1; □, reaction product 2; ●, reaction product 3; ■, reaction product 4.

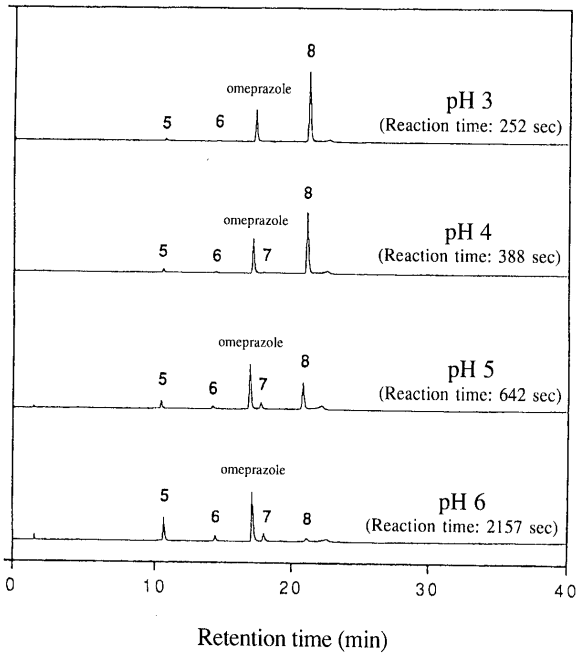


Fig. 4. HPLC Chromatograms of Reaction Mixture of Omeprazole and 2-Mercaptoethanol

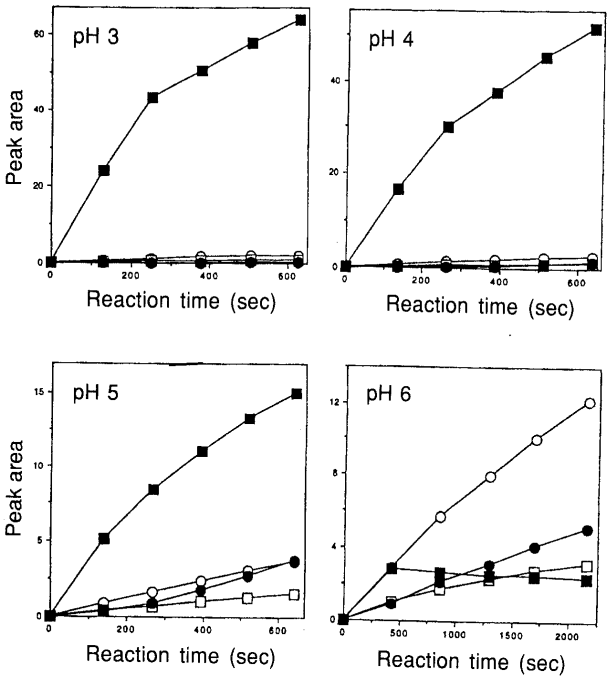
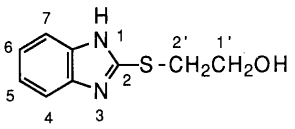
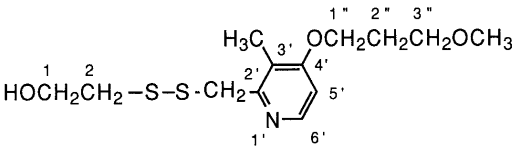


Fig. 5. Time Courses of Changes in Peak Area of Reaction Products of Omeprazole with 2-Mercaptoethanol

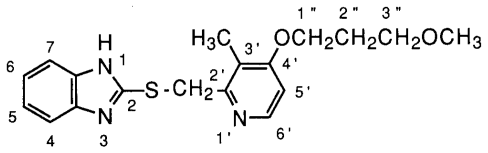
○, reaction product 5; □, reaction product 6; ●, reaction product 7; ■, reaction product 8.



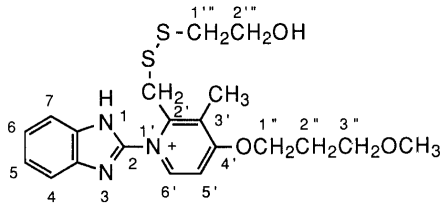
Reaction Product 1



Reaction Product 2

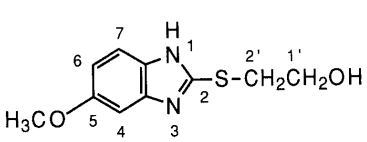


Reaction Product 3

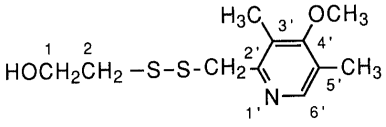


Reaction Product 4

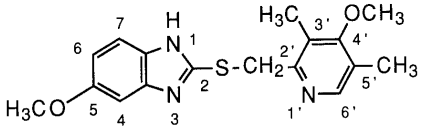
Fig. 6. Chemical Structures of Reaction Products of E3810 with 2-Mercaptoethanol



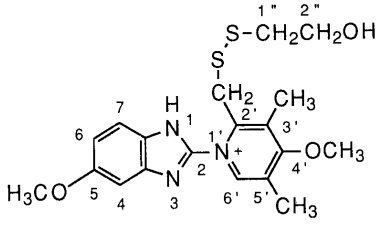
Reaction Product 5



Reaction Product 6



Reaction Product 7



Reaction Product 8

Fig. 7. Chemical Structures of Reaction Products of Omeprazole with 2-Mercaptoethanol

amounts of reaction products 1 and 3 increased.

Reaction of Omeprazole with 2-Mercaptoethanol HPLC chromatograms of the reaction mixture of omeprazole and 2-mercaptoethanol are shown in Fig. 4. Four reaction products were formed. The time courses of the changes in peak area of these reaction products are shown in Fig. 5. At pH 3 and 4, reaction product 8 was mainly produced. As the pH of the reaction mixture was increased, the amounts of other reaction products increased. At pH 6, reaction product 5 was the main product.

Structural Analysis of Reaction Products The chemical structures of the reaction products of E3810 or omeprazole with 2-mercaptoethanol were determined on the basis of the UV, ^{1}H-NMR and mass spectra, and are shown in Figs. 6 and 7.

The spectra data of the reaction products were as follows.

Reaction Product 1. 2-(2-Benzimidazolylthio)ethanol UV ($CH_3CN:H_2O=1:4$) λ_{max} nm: 284, 291. ^{1}H-NMR (CD_3OD) δ: 3.42 (2H, t, $J=6$ Hz, H-2′), 3.89 (2H, t, $J=6$ Hz, H-1′), 7.21 (2H, m (AA′BB′), H-5 and H-6), 7.49 (2H, m (AA′BB′), H-4 and H-7). MS m/z: 195 (MH^+).

Reaction Product 2. 2-[4-(3-Methoxypropoxy)-3-methylpyridin-2-yl]methyldisulfanylethanol UV (CH_3CN: H_2O: hydrochloric acid = 150 : 850 : 2) λ_{max} nm: 239, 268. ^{1}H-NMR (CD_3OD) δ: 2.22 (2H, quint., $J=6$ Hz, H-2″), 2.42 (3H, s, CH_3-3′), 2.83 (2H, t, $J=6$ Hz, H-2), 3.39 (3H, s, CH_3O-3″), 3.65 (2H, t, $J=6$ Hz, H-3″), 3.81 (2H, t, $J=6$ Hz, H-1), 4.31 (2H, s, CH_2-2′), 4.51 (2H, t, $J=6$ Hz, H-1″), 7.58 (1H, d, $J=6$ Hz, H-5′), 8.58 (1H, d, $J=6$ Hz, H-6′). MS m/z: 304 (MH^+).

Reaction Product 3. 2-[4-(3-Methoxypropoxy)-3-methylpyridin-2-yl]methylthiobenzimidazole UV (CH_3OH) λ_{max} nm: 290. ^{1}H-NMR (CD_3OD) δ: 2.12 (2H, quint., $J=6$ Hz, H-2″), 2.28 (3H, s, CH_3-3′), 3.38 (3H, s, CH_3O-3″), 3.62

(2H, t, J=6 Hz, H-3″), 4.22 (2H, t, J=6 Hz, H-1″), 4.67 (2H, s, CH_2-2′), 6.99 (1H, d, J=6 Hz, H-5′), 7.25 (2H, m (AA′BB′), H-5 and H-6), 7.53 (2H, m (AA′BB′), H-4 and H-7), 8.27 (1H, d, J=6 Hz, H-6′). MS m/z: 344 (MH^+).

Reaction Product 4. 1-(Benzimidazol-2-yl)-2-(2-hydroxyethyldisulfanylmethyl)-4-(3-methoxypropoxy)pyridinium UV (CH_3CN : H_2O : hydrochloric acid = 250 : 750 : 2) λ_{max} nm: 252, 280. ^{1}H-NMR (CD_3OD) δ: 2.29 (2H, quint., J=6 Hz, H-2″), 2.60 (3H, s, CH_3-3′), 2.63 (2H, t, J=6 Hz, H-1‴), 3.42 (3H, s, CH_3O-3″), 3.60 (2H, t, J=6 Hz, H-2‴), 3.69 (2H, t, J=6 Hz, H-3″), 4.61 (2H, s, CH_2-2′), 4.69 (2H, t, J=6 Hz, H-1″), 7.51 (2H, m (AA′BB′), H-5 and H-6), 7.78 (2H, m (AA′BB′), H-4 and H-7), 7.82 (1H, d, J=7 Hz, H-5′), 9.07 (1H, d, J=7 Hz, H-6′). MS m/z: 420 (M^+).

Reaction Product 5. 2-(5-Methoxybenzimidazol-2-ylthio)ethanol UV (CH_3CN : H_2O = 1 : 4) λ_{max} nm: 297. ^{1}H-NMR (CD_3OD) δ: 3.38 (2H, t, J=6 Hz, H-2′), 3.86 (2H, t, J=6 Hz, H-1′), 3.87 (3H, s, CH_3O-5), 6.86 (1H, dd, J=9, 3 Hz, H-6), 7.01 (1H, br, H-7), 7.37 (1H, br, H-4). MS m/z: 225 (MH^+).

Reaction Product 6. 2-(4-Methoxy-3,5-dimethylpyridin-2-yl)methyldisulfanylethanol UV (CH_3CN : H_2O : hydrochloric acid = 150 : 850 : 1) λ_{max} nm: 279. ^{1}H-NMR (CD_3OD) δ: 2.48 (3H, s, CH_3-3′ or 5′), 2.51 (3H, s, CH_3-3′ or 5′), 2.83 (2H, t, J=6 Hz, H-2), 3.80 (2H, t, J=6 Hz, H-1), 4.16 (3H, s, CH_3O-4′), 4.31 (2H, s, CH_2-2′), 8.54 (1H, s, H-6′). MS m/z: 260 (MH^+).

Reaction Product 7. 5-Methoxy-2-(4-methoxy-3,5-dimethylpyridin-2-yl)methylthiobenzimidazole UV (CH_3OH) λ_{max} nm: 307. ^{1}H-NMR (CD_3OD) δ: 2.30 (3H, s, CH_3-3′ or 5′), 2.31 (3H, s, CH_3-3′ or 5′), 3.81 (3H, s, CH_3O-5), 3.86 (3H, s, CH_3O-4′), 4.60 (2H, s, CH_2-2′), 6.89 (1H, dd, J=9, 2 Hz, H-6), 7.05 (1H, br, H-7), 7.42 (1H, d, J=9 Hz, H-4), 8.19 (1H, s, H-6′). MS m/z: 330 (MH^+).

Reaction Product 8. 2-(2-Hydroxyethyldisulfanylmethyl)-4-methoxy-1-(5-methoxybenzimidazol-2-yl)-3,5-dimethylpyridinium UV (CH_3CN : H_2O : hydrochloric acid = 100 : 400 : 1) λ_{max} nm: 285. ^{1}H-NMR (CD_3OD) δ: 2.58 (1H, dt, J=16, 6 Hz, H-1″), 2.62 (1H, dt, J=16, 6 Hz, H-1″), 2.62 (6H, s, CH_3-3′, CH_3-5′), 3.59 (2H, t, J=6 Hz, H-2″), 3.93 (3H, s, CH_3O-5), 4.44 (3H, s, CH_3O-4′), 4.59 (2H, s, CH_2-2′), 7.13 (1H, dd, J=9, 3 Hz, H-6), 7.25 (1H, d, J=3 Hz, H-7), 7.68 (1H, d, J=9 Hz, H-4), 9.01 (1H, s, H-6′). MS m/z: 406 (M^+).

Stability of Modified SH Group Products The stability of the reaction products 1 and 4, derived from E3810, and products 5 and 8, from omeprazole, were investigated in the presence and absence of glutathione. The results are shown in Figs. 8 and 9.

Reaction products 1 and 5 were stable in the presence and absence of glutathione at all pH values studied. In contrast, reaction products 4 and 8 decomposed in the presence of glutathione at pH 5 and 6.

Discussion

The sulfenic acid has been proposed as a reactive intermediate in the chemical modification of SH groups of H^+, K^+-ATPase by omeprazole.[3] The reaction products 3 and 4 formed from E3810 and the reaction products 7 and 8 formed from omeprazole were considered to be produced by reaction with such a sulfenic acid intermediate. These reactions are referred to as type I reactions in this report (Fig. 10). The reaction products 1, 2, 5 and 6, however, were considered to be formed by another mechanism. We propose here a new mechanism, presented in Fig. 11, which we designate as type II reaction.

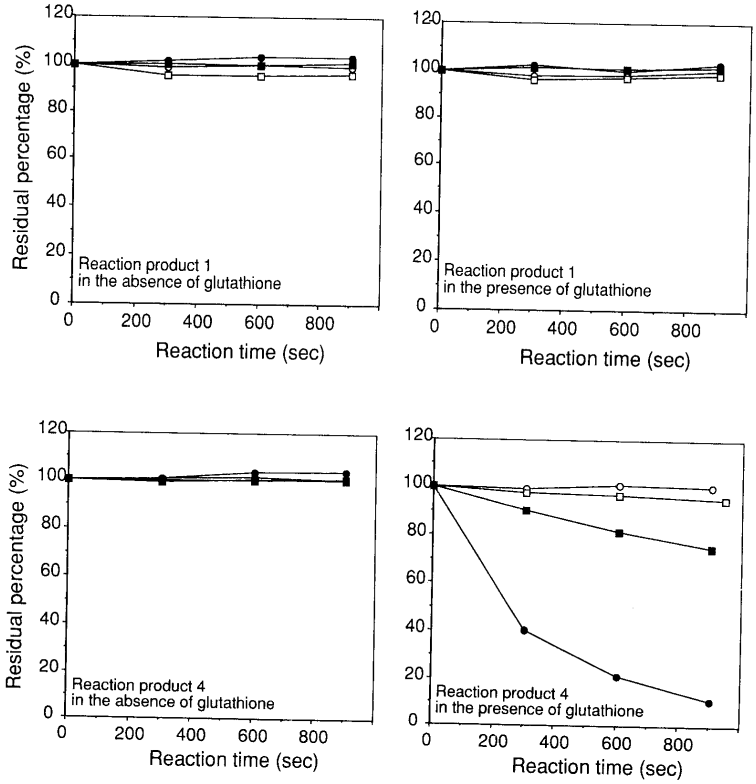


Fig. 8. Stability of Reaction Products 1 and 4

○, pH 3; □, pH 4; ●, pH 5; ■, pH 6.

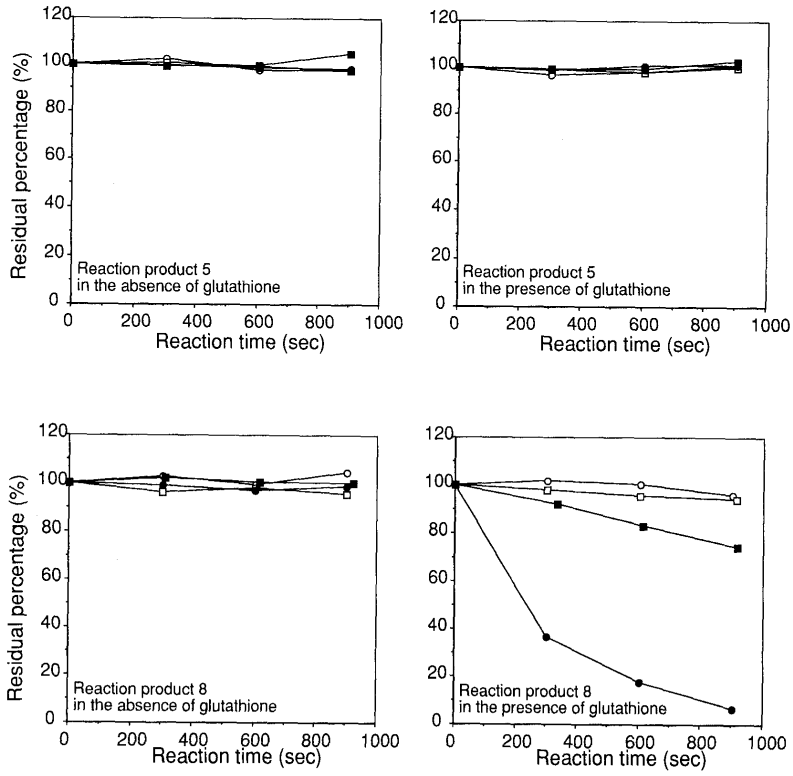


Fig. 9. Stability of Reaction Products 5 and 8

○, pH 3; □, pH 4; ●, pH 5; ■, pH 6.

Thus, E3810 and omeprazole undergo two different SH group-modification reactions. If we accept 2-mercaptoethanol as a model of Cys residues of H^+, K^+-ATPase, the structures of reaction products 1, 4, 5 and 8 suggest that type I and type II reactions cause –S–S– linking and –S– linking between the inhibitors and the enzyme, respectively. Reaction products 2, 3, 6 and 7 are produced subsequently due to the excess of 2-mercaptoethanol.

The velocity of type I and type II reactions depended on the pH of the reaction mixture. Type I reaction is initiated by an intramolecular nucleophilic attack of the N atom in the pyridine ring, while type II reaction is initiated by a nucleophilic attack of the extramolecular SH group, affording disulfide-type and sulfide-type modification products, respectively. In the case of E3810, type I reaction was found to proceed faster than type II reaction at all pH values studied.

The type I reaction of E3810 proceeded faster than that of omeprazole (Figs. 3 and 5). This result indicates that the inhibition of H^+, K^+-ATPase by E3810 should occur more promptly than that by omeprazole. The rate of type I reaction decreased at higher pH (pH 5 and 6), and the contribution of type II reaction increased as the pH of the reaction mixture was increased. The decrease in the velocity of type I reaction at pH 5 and 6 was more marked in the case of omeprazole. In contrast to E3810, the contribution of type II reaction was greater than that of type I reaction at pH 6 in the case of omeprazole.

In the experiments on the stability of the sulfide-type products and disulfide-type products, reaction products 1 and 5, which are sulfide-type modification products, were found to be stable irrespective of the presence or absence of glutathione at all pH values tested. In contrast, reaction products 4 and 8, which are disulfide-type modification products, were found to be unstable at pH 5 and 6, and underwent reductive cleavage of the disulfide bond by glutathione. This type of product was stable at pH 3 and 4. It was demonstrated that reaction products 3 and 7 were produced from reaction products 4 and 8, respectively (Fig. 12). These results indicate that the sulfide-type modification products are stable, suggesting no reversibility of the enzyme inhibition, whereas the enzyme inhibition by the disulfide-type modification products is expected to be reversed by the action of endogenous SH compounds such as glutathione. Reaction products 3 and 7 are considered to be produced in the reversal of the modifications by endogenous glutathione. In fact, it has been reported that reaction product 3 is a main metabolite of E3810.[10] Thus, the enzyme activity is expected to be restored more quickly after inhibition by type I modification reaction than after inhibition by type II modification reaction. Because H^+, K^+-ATPase operates in a highly acidic condition which is generated by excess gastric acid secretion, H^+, K^+-ATPase inhibition is expected to occur mainly by type I reaction. However, it was found that about half of the H^+, K^+-ATPase activity, inhibited by omeprazole at pH 6.1, was restored by addition of 2-mercaptoethanol.[1] Accordingly, it was considered that type II reaction contributes partially to H^+, K^+-ATPase inhibition as a result of gastric pH increase caused by inhibition of the proton pump.

Clinically desirable characteristics of H^+, K^+-ATPase inhibitors are considered to be primarily an immediate inhibition of the enzyme activity of H^+, K^+-ATPase and secondly a duration of action that is not unnecessarily long, with reversibility of the enzyme inhibition. Peptic

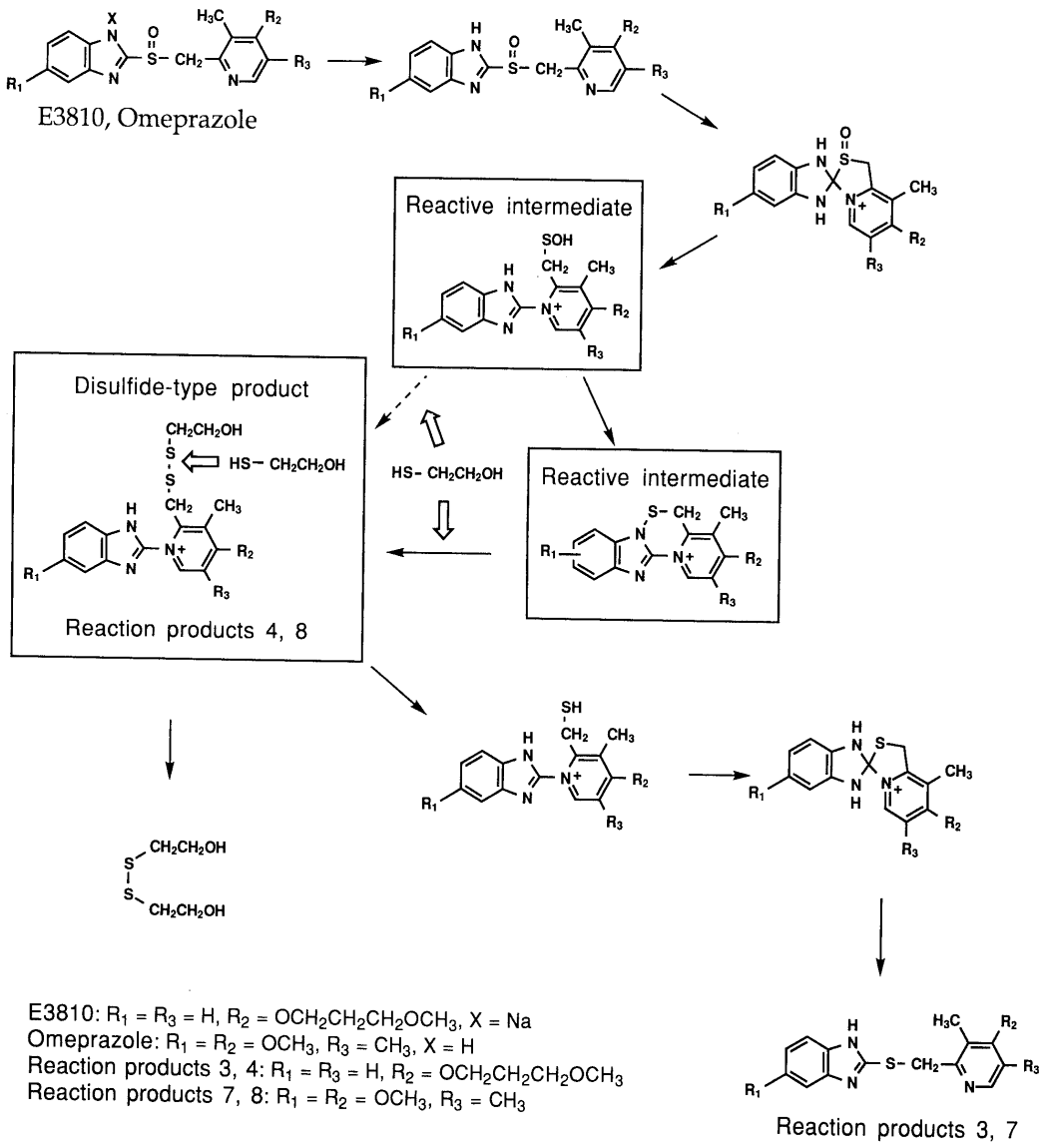


Fig. 10. Type I SH Group-Modification Reaction of E3810 and Omeprazole

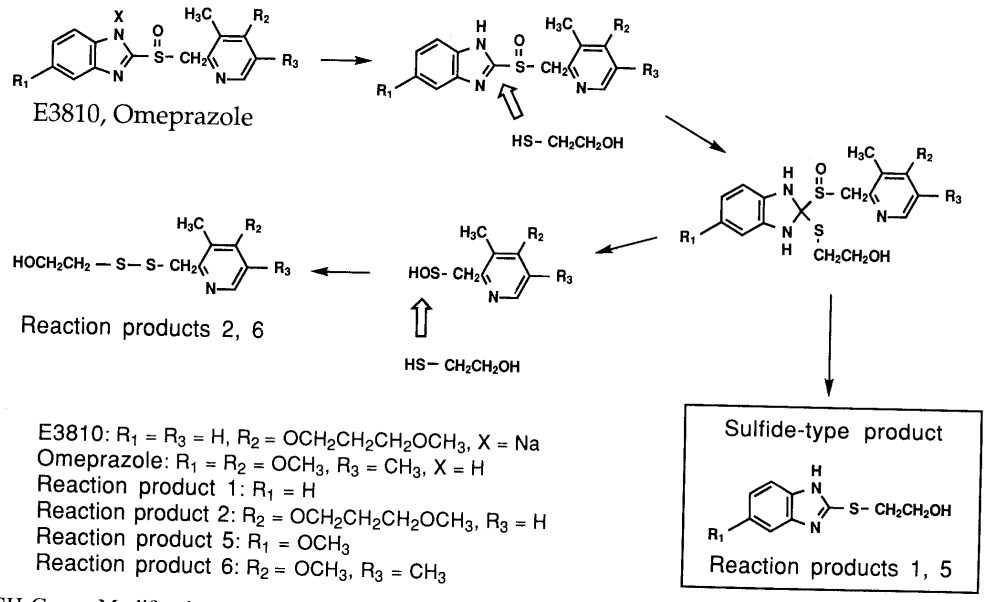


Fig. 11. Type II SH Group-Modification Reaction of E3810 and Omeprazole

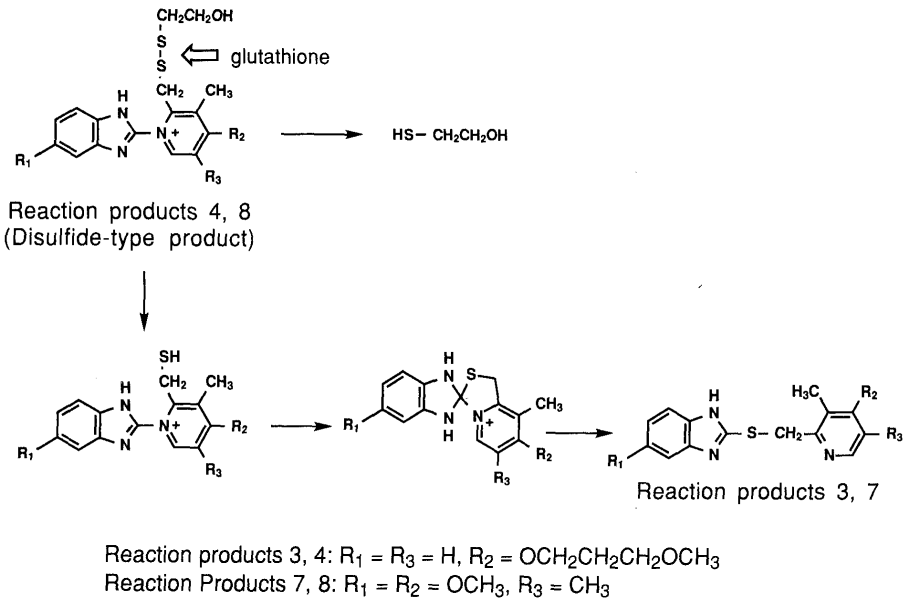


Fig. 12. Mechanism of Reversibility of Disulfide-Type Modification by Glutathione

ulcer is induced by an excess gastric acid secretion. Type I reaction, which is enhanced in an acidic condition, would inhibit H^+, K^+-ATPase, and the restoration of the enzyme activity would be accelerated with increasing pH. Therefore, H^+, K^+-ATPase inhibition by type I reaction is considered to be more desirable than that by type II reaction. It is suggested from the results of the present study that E3810 causes faster inhibition of H^+, K^+-ATPase than omeprazole owing to type I reaction. The disulfide-type modification, which is generated by type I reaction, would be rapidly reversed by endogenous glutathione, so that the duration of enzyme inhibition by E3810 is expected to be shorter than that by omeprazole.

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