Iron(III)-Chelating Resins. 3.[1] Synthesis, Iron(III)-Chelating Properties, and in Vitro Antibacterial Activity of Compounds Containing 3-Hydroxy-2-methyl-4(1*H*)-pyridinone Ligands

Min-Hua Feng, Leen van der Does,* and Adriaan Bantjes

Department of Chemical Technology, Biomaterials Section, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

Received February 1, 1993•

The synthesis, iron(III)-chelating properties, and antibacterial activity of several compounds containing the 3-hydroxy-2-methyl-4(1*H*)-pyridinone (HMP) moiety are described. Using the HMP derivatives iron(III) could be mobilized from iron(III)-binding proteins at physiological pH with a rate order of transferrin > lactoferrin > ferritin. Addition of HMP-containing compounds to a growth medium at a concentration of 20 mM/L resulted in a complete inhibition of the growth of *Escherichia coli* and about 90% inhibition for *Listeria inocua* after 7 h of incubation at 37 °C. After inhibition of bacteria growth by the HMP derivatives growth started again when ferric ions were added to the medium, which implies that the antibacterial activity is due to a limitation of iron available to the organisms.

Introduction

3-Hydroxy-2-methyl-4(1*H*)-pyridinone (HMP) derivatives are of considerable interest because they may be used as orally active iron(III) chelators in the treatment of iron overload and acute iron poisoning.[2-6] Other potential uses of the chelators include iron(III) mobilization from mammalian iron(III)-binding proteins such as transferrin, lactoferrin, and ferritin,[7-9] removal of other metal ions such as Ga(III), In(III), and Al(III),[10,11] and bacterial growth inhibition.[12,13]

Recently, we reported the preparation and properties of iron(III)-chelating systems in which iron(III) chelators had been immobilized onto Sepharose gels.[1,14] In order to get iron(III)-chelating systems with a higher chelator density and stability, we decided to study the polymerization of monomers with 3-hydroxy-2-methyl-4(1*H*)-pyridinone groups as iron(III)-chelating ligands.[15] For this purpose, two HMP derivatives with a polymerizable group were synthesized, and several related HMP compounds were also prepared for comparison.

The HMP monomers are new compounds, so that the complexing behavior for iron(III) of the two HMP monomers and the related HMP compounds was investigated. We were also interested in the ability of the HMP derivatives to mobilize iron(III) at physiological pH from transferrin, lactoferrin, and ferritin. These iron(III)-binding proteins are the main proteins for iron sequestration, transport, delivery, and storage in the body, and the mobilization of iron from the iron-binding proteins at physiological pH is of great importance for many applications such as iron removal from food, treatment of iron overload *in vivo*, preparation of pure iron-free proteins, *etc.*[1,4,16] Because the iron(III)-binding proteins have shown an inhibitory effect on the growth of a wide variety of microbiological species when not fully saturated with iron,[17,18] the synthetic compounds containing the HMP moiety were also investigated for their effect on bacterial growth.

This paper describes the synthesis of the two HMP monomers and of the related HMP compounds. Their iron(III)-chelating properties and the mobilization of iron(III) from transferrin, lactoferrin, and ferritin in *vitro* are also reported. In addition, results will be given of growth studies with the bacteria *Escherichia coli* and *Listeria inocua*.

Results and Discussion

Chemical Synthesis. Scheme I shows the procedures for the preparation of HMP derivatives, starting from 3-hydroxy-2-methyl-4-pyrone (1). Two HMP derivatives (3 and 5) were prepared to investigate the effect of blocking of the OH group on the iron(III)-chelating properties and on the antibacterial activity.

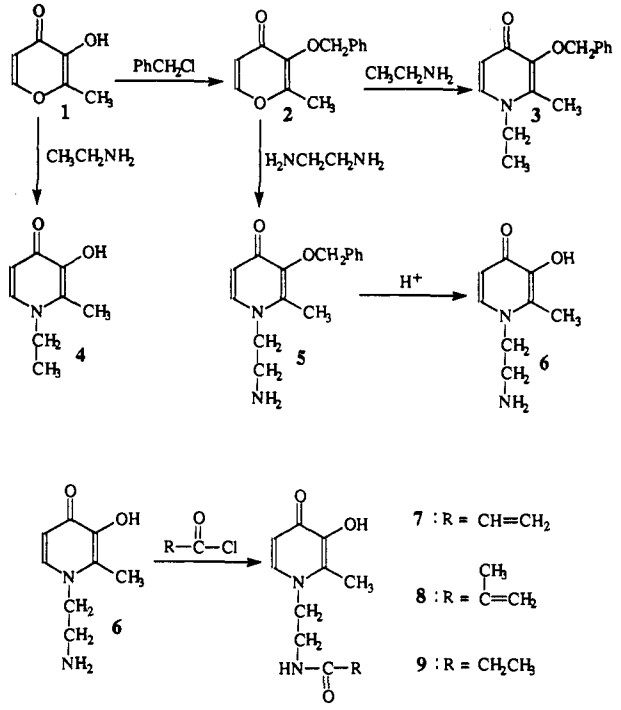
Iron(III) Chelation Examination. In relation to the iron(III)-chelating properties of the HMP derivatives it was observed that all the HMP derivatives formed complexes with iron(III), except compounds 3 and 5. The complexes were orange red at physiological pH, while the color changed to deep red over the pH range from 11.0 to 2.0. The Job plots[19] of the two HMP monomers (7 and 8) and a related HMP compound (9) with iron(III) were measured at pH 7.2 (Figure 1). The complexes with a maximal absorbance at 456 nm possess a chelator/iron(III) stoichiometry of approximately 3:1. The HMP chelator iron(III) complexes (3:1) were titrated with HCl or NaOH, and their pH titration curves revealed that the complexes are stable over the pH range 5–11 (Figure 2). Because pK_{a1} and pK_{a2} of HMP derivatives are about 3.6 and 10.0, respectively,[19] the 3:1 chelator–iron(III) complexes are easily identified as neutral complexes at physiological pH.

Iron(III) mobilization from lactoferrin by the HMP derivatives was examined at physiological pH. Table I gives the percentage of iron(III) mobilized from lactoferrin after 24 h of dialysis at room temperature, which was determined spectrophotometrically by measuring the visible absorbance of the dialysate and by using the extinction coefficient of the chelator–iron(III) complex. It can be seen from Table I that a large amount of iron was mobilized from lactoferrin by the unprotected HMP derivatives. The protected compounds 3 and 5 were almost ineffective, which indicates that the hydroxy group was

* To whom correspondence should be addressed.
• Abstract published in *Advance ACS Abstracts*, August 15, 1993.

0022-2623/93/1836-2822$04.00/0

Scheme I. Synthesis of Compounds Containing HMP Ligands



essential for the iron-chelating properties of the HMP-containing compounds.

The compounds in Table I with an unprotected OH group have different substituents on the ring N atom. It might be possible that substituents at the ring-nitrogen would influence the chelating properties of the chelators. However, it has been reported that the bulk of the N-substituent at the ring could be varied without appreciably influencing the affinity for iron(III).[20] In our study, a marked difference of iron mobilization ability among the unprotected HMP chelators was not observed.

The HMP derivatives are also capable of mobilizing iron from other iron(III) binding proteins. Results of iron(III) mobilization from transferrin, lactoferrin, and ferritin by compound **7** at physiological pH is shown in Figure 3 (the behavior of compounds **8** and **9** was similar). Substantial iron mobilization from transferrin, lactoferrin, and ferritin took place, and the amount of iron mobilized from the proteins by the HMP chelator increased progressively with time. Iron(III) in transferrin was more rapidly mobilized than from lactoferrin and ferritin, which is consistent with previously reported results with other chelators.[21,22] It can also be seen from Figure 3 that iron(III) mobilization was much slower from ferritin, and this observation is in agreement with results reported by Hider et al. using ferritin and transferrin.[9,23]

Microbiological Evaluation. The effects of the HMP derivatives on the growth of *E. coli* and *L. inocua* are summarized in Table II. The growth of *E. coli* was completely inhibited by all unprotected HMP derivatives after 7 h of incubation, whereas all of the HMP derivatives tested had a considerable antibacterial activity against *L. inocua*. As has already been mentioned, compounds **3** and **5** had almost no iron-chelating properties (Table I). However, these compounds did show antibacterial activity, probably because of different reaction conditions. For the bacterial growth study compounds **3** and **5** were sterilized during 15 min at 121 °C, which may have resulted in partial removal of the protecting benzyl groups.

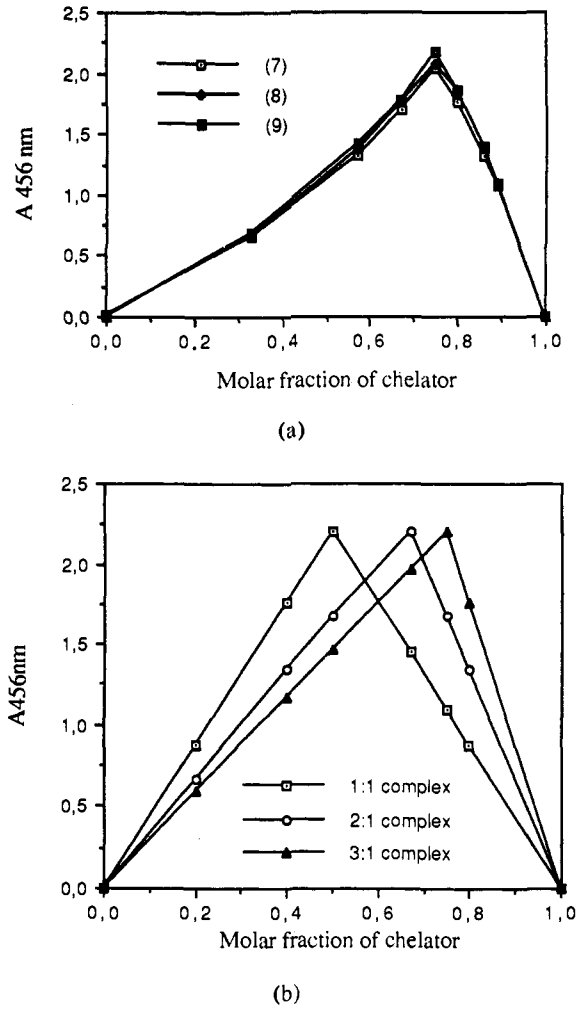


Figure 1. (a) Job plots of the HMP chelators with iron(III) at pH 7.2. (b) Theoretical Job plots for 1:1, 2:1, and 3:1 chelator–iron(III) complexes.

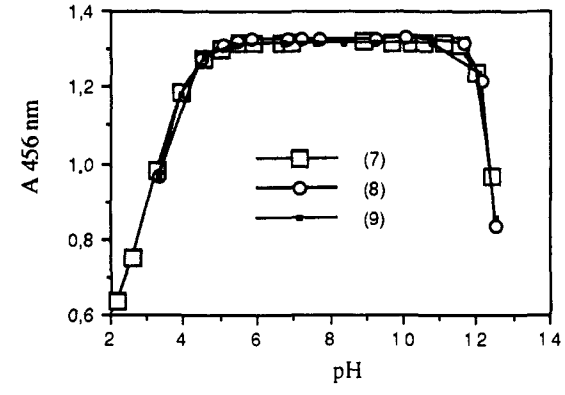


Figure 2. pH titration curves of the HMP chelator–iron(III) complexes.

In order to investigate in more detail the effect of iron(III) on growth inhibition induced by the HMP derivatives, bacteria were incubated using three different growth conditions: (1) BHI medium alone, (2) BHI medium supplemented with a HMP derivative, and (3) BHI medium supplemented with the HMP derivative and extra iron(III) calculated to saturate the HMP derivative.

As can be seen from Figure 4 and 5, addition of the HMP derivative to the medium produced a pronounced growth inhibition although the effect on *L. inocua* was less than on *E. coli*. Addition of ferric ions in the amount required to saturate the HMP derivative abolished the

Table I. Iron Mobilization from Lactoferrin at Physiological pH after 24 h

2-methyl-4(1*H*)-pyridinone	code	iron mobilized (%)
3-(benzyloxy)-1-ethyl-	**3**	2
1-ethyl-3-hydroxy-	**4**	71
1-(β-aminoethyl)-3-(benzyloxy)-	**5**	4
1-(β-aminoethyl)-3-hydroxy-	**6**	60
1-(β-(acrylamido)ethyl)-3-hydroxy-	**7**	62
3-hydroxy-1-(β-(methacrylamido)ethyl)-	**8**	63
3-hydroxy-1-(β-propylamido)ethyl)-	**9**	61
blank control	/	0.2

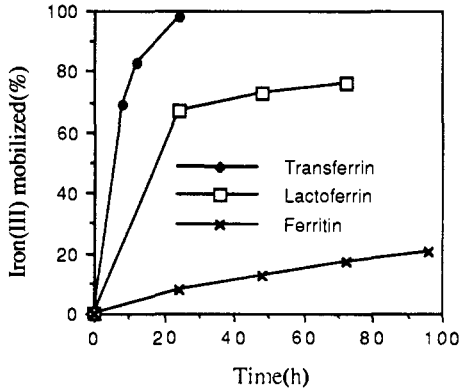


Figure 3. Iron(III) mobilization from transferrin, lactoferrin, and ferritin.

Table II. Effect of HMP Derivatives on Growth of *E. coli* and *L. inocua*[a]

HMP deriv added	growth inhibition (%) *E. coli*	*L. inocua*
3	53	72
4	100	89
5	0	34
6	100	93
7	100	87
8	100	90
9	100	86

[a] The experiments were carried out with HMP derivatives at a concentration of 20 mM. Assay tubes were incubated at 37 °C for 7 h, and the visible numbers (CFU) per milliliter at 0 h were 2×10^5 for *E. coli* and 5×10^5 for *L. inocua*.

growth inhibition by the HMP derivative on both *E. coli* and *L. inocua*. Although the growth was inhibited by the presence of the HMP derivative, the addition of iron(III) after 48 h (Figure 4) resulted in an increase of the growth. This implies that the HMP derivative did not kill the bacteria but only had an inhibitory effect on the growth.

The minimum inhibitory concentrations (MIC) of the HMP derivatives for *E. coli* and *L. inocua* are shown in Table III, and it is obvious that the HMP derivatives were more active against *E. coli* than against *L. inocua*. The compounds bearing an unprotected hydroxy group were found to be superior to the protected analogues in the growth inhibition. The antibacterial activities of the unprotected HMP ligands were generally equal to each other in molar concentration as expected.

Effects of iron deprivation on bacterial growth have been reviewed.[24-26] Compounds containing HMP ligands with a high affinity for iron(III) may be useful in inhibiting growth of a variety of bacteria by limiting the amount of available iron(III). As noted in Table II, when the unprotected HMP derivatives were added to the BHI medium at a final concentration of 20 mM/L, growth of *E. coli* was completely inhibited and the growth of *L. inocua*

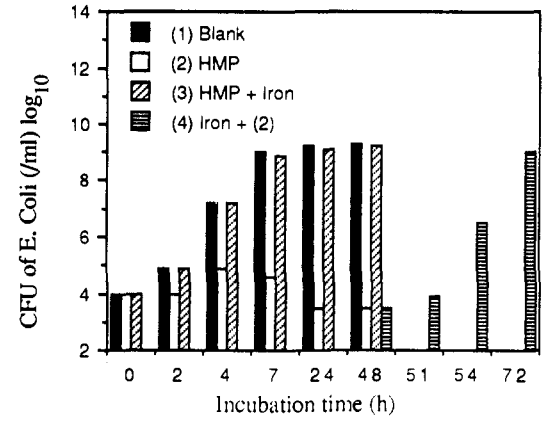


Figure 4. Growth of *E. coli* using various conditions. The experiments were conducted at 37 °C with (1) medium alone, (2) medium + compound 7 at 10 mM, and (3) medium + compound 7 at 10 mM + Fe^{3+} at 3.3 mM. (4) Fe^{3+} was added in the medium (2) containing HMP after 48 h of incubation.

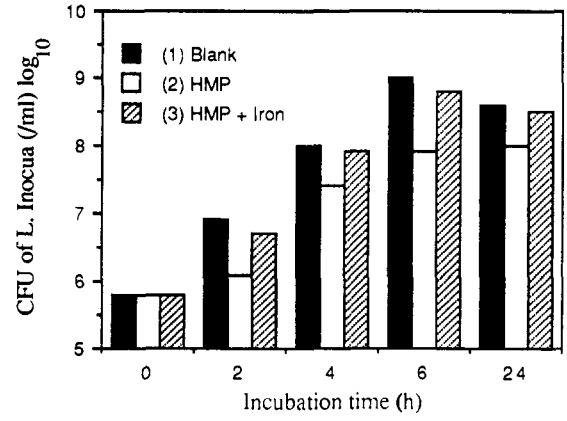


Figure 5. Growth of *L. inocua* using various conditions. The experiments were conducted at 37 °C with (1) medium alone, (2) medium + compound 7 at 40 mM, and (3) medium + compound 7 at 40 mM + Fe^{3+} at 13.3 mM.

Table III. MIC of HMP Derivatives for *E. coli* and *L. inocua*

HMP deriv	MIC (mmol/L) *E. coli*	*L. inocua*
3	50	50
4	5	25
5	50	50
6	5	25
7	5	25
8	5	25
9	5	25

was substantially reduced after 7 h incubation at 37 °C. The activities of the HMP derivatives were comparable to those of desferrioxamine and apolactoferrrin.[27,28]

Our results show that the growth of the Gram-positive bacteria of *L. inocua* was less inhibited than that of the Gram-negative *E. coli* by all unprotected HMP derivatives. This difference between *E. coli* bacteria and other bacteria has also been reported for the growth inhibition by apolactoferrin.[28-30] Besides, it seems impossible to completely inhibit the growth of *L. inocua* by the HMP derivatives even when increasing the concentration of the chelators to 40 mM (Figure 5). Theoretically, the addition of 40 mmol of the HMP derivative per liter would lead to chelation of 13.4 mmol of iron(III), while the iron concentration in the medium was about 0.037 mmol per liter (determined by AAS). This means that the capacity of the chelator present in the medium was about 360 times

the iron(III) concentration. Compounds with HMP ligands form very stable water-soluble iron(III) complexes at a physiological pH with stability constants of the 3:1 ligand/iron(III) complexes in the region of 10^{36}–10^{37} as reported.[31]

It is possible that the growth of *L. inocua* was not completely inhibited if part of the iron chelated by the HMP derivatives was still available for the bacteria or that only a small amount of iron was sufficient for the bacterial growth. There might be another explanation for the difference between the behavior of the two bacteria types when using the same HMP derivative. It is believed that certain Gram-positive bacteria can develop iron-transporting systems with a high affinity for iron(III),[26,32] which might compete with the HMP derivative for iron. However, at present there is little biochemical information regarding the nature of the iron-transporting system utilized by Gram-positive bacteria.

The basis for the antibacterial activity of the compounds containing HMP ligands appears to be sequestration of iron required for bacteria growth and replication, as was also found for apolactoferrin and desferrioxamine. The ability of these HMP derivatives to mobilize iron(III) from iron(III)-binding proteins and to inhibit the growth of bacteria makes them potentially useful in applications such as treatment of iron overload in blood, inhibition of bacterial growth in food, and preparation of polymeric iron(III) chelating agents.

Conclusions

Compounds containing the 3-hydroxy-2-methyl-4(1*H*)-pyridinone (HMP) moiety were synthesized and evaluated chemically and biologically. The HMP derivatives showed a high affinity for iron(III) and were able to mobilize iron(III) from transferrin, lactoferrin, and ferritin at physiological pH. The HMP-containing compounds had an inhibitory effect on the growth of the bacteria of *E. coli* and *L. inocua*, and the inhibitory effects were related to their iron(III)-chelating properties.

Experimental Section

Materials. 3-Hydroxy-2-methyl-4-pyrone (1) was purchased from Aldrich Chemical Co. Human serum transferrin, bovine milk lactoferrin, and horse spleen ferritin were obtained from Sigma Chemical Co. and used without further purification. All other chemical reagents were used as received from Aldrich or Merck. *E. coli* CA5 and *L. inocua* 6B bacteria cultures were provided by NIZO (Netherlands Institute for Dairy Research, Ede).

Melting points were taken in open capillary tubes by using a Buchi apparatus and are uncorrected. Elemental analyses were carried out by TNO, Zeist, The Netherlands. ^{1}H-NMR spectra were obtained with a Nicolet 200-MHz spectrophotometer. UV–vis spectra were recorded using a Uvikon 930 spectrophotometer. Infrared spectra were obtained with a Bio-Rad FTS-60 FT-IR spectrophotometer. Iron contents in proteins and in growth media were determined by atomic absorption spectrophotometry (AAS) with a Perkin-Elmer Zeeman 5000 atomic absorption spectrophotometer.

Synthesis of HMP Derivatives. 3-(Benzyloxy)-1-ethyl-2-methyl-4(1*H*)-pyridinone (3), 1-ethyl-3-hydroxy-2-methyl-4(1*H*)-pyridinone (4), 1-(β-aminoethyl)-3-(benzyloxy)-2-methyl-4(1*H*)-pyridinone (5), and 1-(β-aminoethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone (6) (Scheme I) were prepared from 3-hydroxy-2-methyl-4-pyrone (1) in our laboratory as described previously by us and other workers.[1,19,33]

1-(β-(Acrylamido)ethyl)- (7), 1-(β-(methacrylamido)ethyl)- (8) and 1-(β-(propylamido)ethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinones (9) were synthesized by reacting 1-(β-aminoethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone·2HCl with acryloyl, methacryloyl, and propionyl chloride, respectively (Scheme I).

1-(β-(Acrylamido)ethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone (7). To a flask with a magnetic stirrer and a dropping funnel were added 1-(β-aminoethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone·2HCl (0.1 mol), triethylamine (0.36 mol), and water (100 mL). A solution of acryloyl chloride (0.12 mol) in acetonitrile (10 mL) was added dropwise to the mixture in 2 h (ice bath), and then the mixture was stirred for 4 h at room temperature. The reaction mixture was evaporated in vacuum, and the resulting solid was washed with chloroform and recrystallized from methanol (10.0 g, 45% yield), mp 176–8 °C. Anal. Calcd for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.00; H, 6.35; N, 12.45. ^{1}H NMR (DMSO-d_6) δ: 8.30 (1 H, t), 7.40 (1 H, d), 6.10 (3 H, m), 5.6 (1 H, q), 3.0–5.5 (1 H, s), 4.00 (2 H, t), 3.4 (2 H, q), 2.30 (3 H, s).

3-Hydroxy-1-(β-(methacrylamido)ethyl)-2-methyl-4(1*H*)-pyridinone (8) was synthesized by the same procedure as used for the preparation of compound 7 (44% yield), mp 127–9 °C. Anal. Calcd for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83; N, 11.86. Found: C, 61.33; H, 6.79; N, 11.95. ^{1}H NMR (DMSO-d_6) δ: 8.10 (1 H, t), 7.40 (1 H, d), 6.10 (1 H, d), 5.6 (1 H, s), 5.3 (1 H, s), 3.0–5.5 (1 H, s), 4.00 (2 H, t), 3.4 (2 H, q), 2.30 (3 H, s), 1.8 (3 H, s).

1-(β-(Propylamido)ethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone (9) was prepared using propionyl chloride in a way similar to that used in the preparation of compound 7 (35% yield), mp 149–51 °C. Anal. Calcd for $C_{11}H_{16}N_2O_3$: C, 58.91; H, 7.19; N, 12.49. Found: C, 58.01; H, 7.30; N, 12.32. ^{1}H NMR (DMSO-d_6) δ: 8.00 (1 H, t), 7.40 (1 H, d), 6.10 (1 H, d), 3.0–5.5 (1 H, s), 4.00 (2 H, t), 3.30 (2 H, q), 2.30 (3 H, s), 2.0 (2 H, q), 1.00 (3 H, t).

Iron(III) Chelation Studies of the HMP Derivatives. All solutions were freshly prepared, and pH adjustments were carried out using 10 M HCl and 5 M NaOH. pH titration curves of chelator–iron(III) complexes were carried out with mixtures of a chelator solution (25 mL, 2 mM) and ferric chloride solution (25 mL, 0.5 mM). The mixtures were titrated with HCl or NaOH solution, and the absorbances at 456 nm in the mixture at different pH values were measured. Job plots of the chelators with iron(III) were obtained at pH 7.2 using 4 × PBS (phosphate buffered saline). A 0–5-mL portion of a chelator solution (4 mM) and 5–0 mL of iron(III) chloride solution (2 mM) were mixed at different molar ratios to a final volume of 5 mL. Then, 5 mL of 4 × PBS (pH 7.2) was added, and the absorbances at 456 nm in the solutions were measured. Theoretical Job plots were calculated based on 1:1, 2:1, and 3:1 chelator–iron(III) complexes.

Iron Mobilization from Lactoferrin. A 100% iron(III)-saturated lactoferrin solution was prepared by adding an appropriate amount of a freshly prepared aqueous solution of $FeCl_3 \cdot 6H_2O$ into a lactoferrin solution in 0.1 M sodium bicarbonate, and the lactoferrin solution was incubated overnight at room temperature. The iron(III) concentration of the lactoferrin solution was 0.78 mM. For iron(III) mobilization from the lactoferrin solution at physiological pH, 1.0 mL of the lactoferrin solution was dialyzed against 4 mL of one of the HMP derivatives solutions (2 mM in 4 × PBS) at room temperature for 24 h. The absorbance of the resulting iron(III) chelator complexes at 456 nm in the dialyzate was measured, and the amount of iron(III) mobilized was calculated from the corresponding calibration curves of the chelator–iron(III) complexes. In the cases of compounds **3** and **5**, the same amount of compound **6** was added to the dialyzate after the dialysis for measuring the visible absorbances of the dialyzates. For comparison, the procedure was also carried out using a blank control.

Iron Mobilization from Transferrin, Lactoferrin, and Ferritin. For comparing the iron mobilization from different iron-binding proteins, some of the HMP derivatives were examined for their ability to mobilize iron from transferrin, lactoferrin, and ferritin. A 100% iron(III)-saturated human transferrin or bovine lactoferrin solution was prepared by incubation of transferrin or lactoferrin solution (0.13 mM) in PBS (pH 7.4) containing $NaHCO_3$ (10 mM) with an appropriate amount of a freshly prepared solution of iron(III) citrate in PBS (pH 7.4) overnight at room temperature. A 0.10-mL sample of horse spleen ferritin solution (Sigma) was diluted with PBS (pH

7.4) to 50 mL. The iron concentration of the prepared ferritin solution was 1.02 mM as measured by AAS.

A 10-mL portion of the transferrin or lactoferrin solution enclosed in a Visking dialysis bag was dialyzed against 20 mL of an HMP derivative solution (2 mM in PBS) at room temperature. A 1.0-mL portion of the ferritin solution was dialyzed against 4 mL of the HMP derivative solution (2 mM in PBS) at room temperature. The absorbance of the resulting iron(III)–chelator complexes at 456 nm in the dialyzate was measured. The amount of iron mobilized was calculated from corresponding calibration curves of the chelator–iron(III) complexes. For comparison, the procedure was carried out using a blank control.

Determination of Bacterial Growth. The HMP derivatives were tested in vitro for their antibacterial activity against Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*L. inocua*) by the method of Bishop et al.[29] with a slight modification. The data were expressed as the percentage of growth inhibition (GI) calculated using the formula given by Bishop et al.[29]

$$\text{growth inhibition (\%)} = \frac{\Delta\text{CFU 0–7 h control} - \Delta\text{CFU 0–7 h experimental}}{\Delta\text{CFU 0–7 h control}}$$

where

ΔCFU 0–7 h control = CFU at 7 h − CFU at 0 h for the control assay

ΔCFU 0–7 h experimental = CFU at 7 h − CFU at 0 h for the experimental assay

CFU = colony-forming units per milliliter

Maintenance of Bacteria Cultures. The *E. coli* and *L. inocua* cultures were maintained using a reported method[29] except that brain heart infusion (BHI, Difco) agar was used.

Growth Inhibition Assay. For assay, stock cultures (BHI slants) were streaked for growth on BHI plates and incubated at 37 °C for 24 h. A single loopful of bacteria was transferred to 10 mL of BHI medium and incubated at 37 °C for 1.0 h. A 1.0-mL sample of the culture was transferred to 200 mL of BHI medium and incubated for 2 h at 37 °C in a water bath with shaking. A 1.0-mL portion of the culture was transferred to the experimental tubes containing BHI medium. The final volume in all assay tubes was 10.0 mL. Plate counts (CFU) were determined on the cultures using the standard plate counting technique as previously described.[34]

Minimum Inhibitory Concentrations (MIC). Minimum inhibitory concentrations (MIC) for *E. coli* were determined on BHI agar after 18 h of incubation at 37 °C, according to the standard agar dilution technique, using 2-fold dilution and with visual inspection of bacterial growth.[35] Plates were seeded with a multipoint inoculator at a density of 10^4–10^5 colony-forming units (CFU) per spot. MIC of 90% inhibition for *L. inocua* was measured after 6 h of incubation at 37 °C with a standard tube dilution method.[36] The tubes were seeded at a density of 10^4–10^5 colony-forming units (CFU) per milliliter.

Acknowledgment. The authors wish to thank Dr. A. H. Weerkamp of the Microbiology Department of NIZO for his interest and helpful suggestions and for supplying bacteria cultures. We appreciate the technical assistance and advice received from Ir. H. v. d. Beld of our department.

References

(1) Feng, M.; van der Does, L.; Bantjes, A. Iron(III) chelating resins. II. 3-Hydroxy-4(1H)-pyridinones-Sepharose gels. *J. Biomater. Sci., Polym. Ed.* **1992**, *4*(2), 145–154.
(2) Porter, J. B.; Hoyes, K. P.; Abeysinghe, R. D.; Brooks, P. N.; Huehns, E. R.; Hider, R. C. Comparison of the subacute toxicity and efficacy of 3-hydroxypyridin-4-one iron chelators in overloaded and non-overloaded mice. *Blood* **1991**, *78*(10), 2727–2734.
(3) Porter, J. B.; Morgan, J.; Hoyes, K. P.; Burke, L. C.; Huehns, E. R.; Hider, R. C. Relative oral efficacy and acute toxicity of hydroxypyridin-4-one iron chelators in mice. *Blood* **1990**, *76*(11), 2389–2396.
(4) Kontoghiorghes, G. J.; Sheppard, L.; Hoffbrand, A. V.; Charalambous, J.; Tikerpae, J.; Pippard, M. J. Iron chelation studies using desferrioxamine and the potential oral chelator, 1,2-dimethyl-3-hydroxypyrid-4-one, in normal and iron loaded rats. *J. Clin. Pathol.* **1987**, *40*, 404–408.
(5) Kontoghiorghes, G. J.; Hoffbrand, A. V. Orally active α-ketohydroxypyridine iron chelators intended for clinical use: in vivo studies in rabbits. *Br. J. Haematol.* **1986**, 62, 607–613.
(6) Kontoghiorghes, G. J. New orally active iron chelators. *Lancet* **1985**, *1*(8432), 817.
(7) O'Connell, M. J.; Ward, R. J.; Baum, H.; Peters, T. J. Iron release from haemosiderin and ferritin by therapeutic and physiological chelators. *Biochem. J.* **1989**, *260*, 903–907.
(8) Kontoghiorghes, G. J. Iron mobilization from lactoferrin by chelators at physiological pH. *Biochim. Biophys. Acta* **1986**, *882*, 267–270.
(9) Hider, R. C.; Kontoghiorghes, G. J.; Silver, J. Pharmaceutical compositions. U.K. Patent 2118176, 1983.
(10) Clevette, D. J.; Lyster, D. M.; Nelson, W. O.; Rihela, T.; Webb, G. A.; Orvig, C. Solution chemistry of gallium and indium 3-hydroxy-4-pyridinone complexes in vitro and in vivo. *Inorg. Chem.* **1990**, *29*, 667–672.
(11) Nelson, W. O.; Rettig, S. J.; Orvig, C. The exoclathrate $Al(C_7H_8NO_2)_3 \cdot 12H_2O$. A facial geometry imposed by extensive hydrogen bonding with the ice structure. *J. Am. Chem. Soc.* **1987**, *109*, 4121–4123.
(12) Sakagami, K.; Iwamatsu, K.; Atsumi, K.; Hatanaka, M. Synthetic Cephalosporines. VII. Synthesis and antibacterial activity of 7-[(Z)-2-(2-aminothiazol-4-yl)-2-(3-(3-hydroxy-4-pyridon-1-yl)-3-carboxypropoxyimino)acetamido]-3-(1,2,3-thiadiazol-5-yl)-thiomethyl-3-cephem-4-carboxylic acid and its related compounds. *Chem. Pharm. Bull.* **1990**, *38*(12), 3476–3479.
(13) Sakagami, K.; Iwamatsu, K.; AtsumiK.; Hatanaka, M. Synthetic Cephalosporines. VI. Synthesis and antibacterial activity of 7-[(Z)-2-(2-aminothiazol-4-yl)-2-(1-carboxy-1-methyl)ethoxy-iminoacetamido]-3-(3-hydroxy-4-pyridon-1-yl)methyl-3-cephem-4-carboxylic acid and related compounds. *Chem. Pharm. Bull.* **1990**, *38*(8), 2271–2273.
(14) Feng, M.; van der Does, L.; Bantjes, A. Iron(III) chelating resins. I. Preparation and properties of Sepharose-desferrioxamine gels. *J. Biomater. Sci., Polym. Ed.* **1992**, *4*(2), 99–105.
(15) (a) Feng, M.; van der Does, L.; Bantjes, A. Iron(III) chelating resins. IV. Crosslinked copolymers beads of 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1H)-pyridinone (AHMP) with 2-hydroxyethyl methacrylate (HEMA). To be published. (b) Feng, M.; van der Does, L.; Bantjes, A. Iron(III) chelating resins. V. Crosslinked copolymers of 1-(β-acrylamidoethyl)-3-hydroxy-2-methyl-4(1H)-pyridinone (AHMP) with N,N-dimethylacrylamide (DMAA) for iron(III) chelation studies. To be published.
(16) Morgen, E. H. Studies on the mechanism of iron release from transferrin. *Biochim. Biophys. Acta* **1979**, *580*, 312–326.
(17) Reiter, B.; Oram, J. D. Bacterial inhibition in milk and other biological fluids. *Nature (London)* **1967**, *216*, 328–330.
(18) Rogers, H. J. Bacterial iron metabolism and host resistance. In *Microbiology*; Schlessiger, D., Ed.; American Society for Microbiology: Washington, D.C., 1974; pp 289–298.
(19) Kontoghiorghes, G. J.; Sheppard, L.; Chambers, S. New synthetic approach and iron chelating studies of 1-alkyl-2-methyl-3-hydroxypyrid-4-ones. *Arzneim.-Forsch./Drug Res.* **1987**, *37*(II), Nr. 10, 1099–1102.
(20) Brady, M. C.; Lilley, K. S.; Treffry, A.; Harrison, P. M.; Hider, R. C.; Taylor, P. M. Release of iron from ferritin molecules and their iron-cores by 3-hydroxypyridinone chelators in vitro. *J. Inorg. Biochem.* **1989**, *35*, 9–22.
(21) Aisen, P.; Leibman, A. Lactoferrin and transferrin: A comparative study. *Biochim. Biophys. Acta* **1972**, *257*, 314–323.
(22) Kontoghiorghes, G. J. 2-Hydroxypyridine-N-oxides: effective new chelators in iron mobilisation. *Biochim. Biophys. Acta* **1987**, *924*, 13–18.
(23) Hider, R. C.; Kontoghiorghes, G. J.; Silver, J. Pharmaceutical compositions. U.K. Patent 2136807, 1984.
(24) Bullen, J. J.; Rogers, H. J.; Griffith, E. Bacterial iron metabolism in infection and immunity. In *Microbial iron metabolism*; Neilands, J. B., Ed.; Academic Press: New York, 1974.
(25) Bullen, J. J.; Rogers, H. J.; Griffith, E. Role of iron in bacterial infection. *Curr. Top. Microbiol. Immunol.* **1978**, *80*, 1.
(26) Rosenberg, H.; Young, I. Iron transport in the enteric bacteria. In *Microbial iron metabolism*; Neilands, J. B., Ed.; Academic Press: New York, 1974.
(27) van Asbeck, B. S.; Marcelis, J. H.; van Kats, H.; Marx, J. J.; Verhoef, J. In vitro antibacterial activity of the iron chelator deferoxamine. In *Curr. Chemother. Immunother., Proc. Int. Congr. Chemother., 12th*, Periti, P., Gialdroni grassi, G., Eds.; Am. Soc. Microbiol.: Washington, D.C., 1981; Pub. 1982, Vol. 1, pp 94–6.
(28) Nonnecke, B.; Smith, K. L. Inhibition of mastitic bacteria by bovine milk apolactoferrin evaluated by in vitro microassay of bacterial growth. *J. Dairy Sci.* **1984**, *67*, 606–613.
(29) Bishop, J. G.; Schanbacher, F. L.; Ferguson, L. C.; Smith, K. L. In vitro growth inhibition of mastitis-causing coliform bacteria by

bovine apolactoferrin and reversal of inhibition by citrate and high concentrations of apolactoferrin. *Infect. Immun.* **1976**, *14*(4), 911–918.
(30) Batish, V. K.; Chander, H.; Zumdegni, K. C.; Bhatia, K. L.; Singh, R. S. Antibacterial activity of lactoferrin against some common food-borne pathogenic organisms. *Aust. J. Dairy Technol.* **1988**, *43*(1), 16–18.
(31) Motekaitis, R. J.; Martell, A. E. Stabilities of the iron(III) chelates of 1,2-dimethyl-3-hydroxy-4-pyridinone and related ligands. *Inorg. Chim. Acta* **1991**, *183*, 71–80.
(32) Maskell, J. P. The functional interchangeability of enterobacterial and staphylococcal iron chelators. *Antonie van Leeuwenhoek* **1980**, *46*, 343.
(33) Kontoghiorghes, G. J.; Sheppard, L. Simple synthesis of the potent iron chelators 1-Alkyl-3-hydroxy-2-methylpyrid-4-ones. *Inorg. Chim. Acta* **1987**, *136*, L11–L12.
(34) *Standard methods for the examination of dairy products*, 13th ed.; American Public Health Association: New York, 1972.
(35) Barry, A. L. In *The antimicrobic susceptibility test: Principles and Practices*; Kimpton, H., Ed.; Lea & Febiger: Philadelphia, 1976; pp 76–91.
(36) National Committee for Clinical Laboratory Standards, Tentative standard: Standard methods for dilution antimicrobial susceptibility tests for bacteria which grow aerobically, Publication M7-T, Vol. 3, No. 2, Villanova, PA, 1983.