THE BIOLOGICAL ACTIVITY OF SOME SIDEROCHROME DERIVATIVES

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SUMMARY

Semisynthetic derivatives of natural trihydroxamic acid iron chelates, or siderochromes, have been prepared and tested for biological activity. The activity of these compounds as growth factors for Arthrobacter JG-9 or as antagonists to the siderochrome antibiotic, albomycin, is relatively unaffected by structural alteration. However, their ability to function as ferric ionophores for the fungus, Ustilago sphaerogena, is very sensitive to minor structural variations. Branching at the β-position of the hydroxamate acyl function leads to loss of all biological activity.

Trihydroxamic acid iron chelates, or siderochromes, exhibit a number of interesting biological properties. Ferrichrome (Fig. 1) acts as a potent growth factor for some microorganisms, being active at concentrations as low as 10^{-9} M, and it also antagonizes the antibiotic action of the siderochrome, albomycin (1). Recently, we have shown that ferrichrome acts as a specific iron transport agent, or ferric ionophore, for the fungus, Ustilago sphaerogena (2). Other siderochromes vary in their biological activity, some being as active as ferrichrome and others, such as ferrichrome A, being devoid of activity. In an attempt to correlate chemical structure with biological activity, we have prepared structural analogs of ferrichrome and compared their activity in vivo.

MATERIALS AND METHODS

Crystalline ferrichrome and ferrichrome A were obtained from cultures of Ustilago sphaerogena (ATCC 12421) and the iron was removed from the chelates by previously described methods (3). Propionyl ferrichrome, in which the acetyl groups of ferrichrome have been replaced with propionyl groups, was prepared as follows. A solution of 120 mg of desferriferrichrome in 3 ml of N HCl was heated at 100° for 15 min, at which time a ferric chloride test was negative. The hydrolysate was taken to dryness under N_2 at 60°. The residue was dissolved in 0.5 ml pyridine and 300 µl of propionyl chloride added in 25 µl increments over a period of 1 hr, keeping the temperature at 0°. The product was taken to dryness, redissolved in 4.5 ml water, and the iron chelate formed by addition of excess ferrous sulfate. The chelate

was extracted into phenol-chloroform (1:1) and re-extracted into water after addition of ether. The compound was purified by passage through a small DEAE-cellulose column equilibrated with 0.05 M phosphate, pH 7, using water as eluting solvent. The concentration was determined spectrophotometrically, assuming ε_{425} to be 2895 $M^{-1}cm^{-1}$. Butyryl ferrichrome was prepared by a similar procedure using butyryl chloride as acylating agent.

The trimethyl ester of ferrichrome A was synthesized by overnight treatment of ferrichrome A with N methanolic HCl. Hexahydroferrichrome A was prepared by hydrogenation of desferriferrichrome A over Pd/charcoal until the absorbance at 260 nm reached a minimum value; its trimethyl ester was prepared as above. Ferrichrysin was synthesized by acidic deacylation of hexahydrodesferriferrichrome A followed by acetylation with acetic anhydride.

The growth factor activity of the compounds was determined for Arthrobacter JG-9 using the penicillin disc method (4). The ability of the compounds to antagonize the antibiotic action of albomycin was determined by the Bonifas test using B. subtilis (5). The activity of the compounds as ferric ionophores for Ustilago sphaerogena was determined by methods previously described (2).

RESULTS

The names, structures, and abbreviations of the siderochromes used in this study are summarized in Figure 1. The purity of the compounds was verified by paper chromatography and paper electrophoresis. All of the compounds with saturated acyl groups showed an absorption maximum at 425 nm, and this peak did not shift to higher wavelength at pH 2. The spectral stability of the chelates at low pH confirms that the compounds are trihydroxamic acid chelates (3). Because of the high biological potency of ferrichrome, which was used as starting material for the synthesis of PrFc and BuFc, it was necessary to establish that the latter compounds were completely free of acetyl groups. This was established by removal of the acyl groups by periodate reaction (3), extraction with ether, and examination of the extract by vapor phase chromatography. No acetic acid was detected by this procedure.

Growth factor activity. Table I shows the relative activity of the siderochromes as growth factors for Arthrobacter JG-9. The observation that PrFc and BuFc are almost as active as ferrichrome demonstrates that the nature of the acyl group is not critical for growth of this organism. The high activity of FChy shows that the presence of serine in place of glycine does not in itself significantly diminish activity. Therefore, the lack of activity of FcA, $FcAMe_3$, and $FcAMe_3H_6$, is probably not due to serine in the peptide ring, but rather due to steric factors, possibly the branched methyl groups at the β-position.

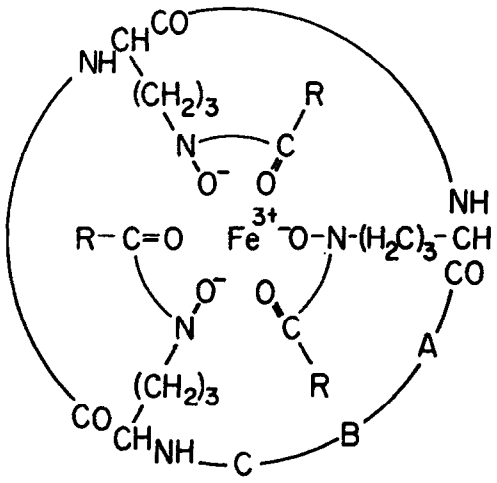

Figure 1. Structures of Siderochromes

Siderochrome	Abbrev.	A	B	C	R
Ferrichrome	Fc	glycine	glycine	glycine	CH_3-
Propionyl ferrichrome	PrFc	glycine	glycine	glycine	CH_3CH_2-
Butyryl ferrichrome	BuFc	glycine	glycine	glycine	$CH_3CH_2CH_2$-
Ferrichrome A	FcA	serine	serine	glycine	$^-OOCCH_2C(CH_3)=CH$-
Ferrichyrisin	FChy	serine	serine	glycine	CH_3-
Ferrichrome A Trimethyl ester	$FcAMe_3$	serine	serine	glycine	$CH_3OOCCH_2C(CH_3)=CH$-
Hexahydroferrichrome A Trimethylester	$FcAMe_3H_6$	serine	serine	glycine	$CH_3OOCCH_2CH(CH_3)CH_2$-

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Bonifas Assay. This assay is a measure of the ability of a compound to competitively antagonize the antibiotic activity of the siderochrome, albomycin, against B. subtilis. The relative activity of our compounds is shown in Table II. The results parallel the growth factor results and again demonstrate that the branched acyl groups almost completely abolish activity, whereas the smaller unbranched propionyl and butyryl groups, or the presence of serine in the peptide ring, have little effect.

Ionophoric activity. The relative rates of uptake of the trihydroxamic acid iron chelates by Ustilago sphaerogena are shown in Figure 2. Except for the butyryl derivative, all compounds exhibited significantly lower rates of uptake than ferrichrome. The uptake curve for PrFc (not shown)

TABLE I: Growth factor activity of siderochromes for Arthrobacter JG-9. All compounds were tested at a concentration of 0.1 mM using 1/4 inch pencillin discs on plates seeded with a 24 hr culture. The diameter of the growth zone was measured after 24 hr incubation at 25°.

Compound	Diameter of Growth Zone, mm
Fc	38
PrFc	34
BuFc	34
FChy	36
FcA	negative
$FcAMe_3$	negative
$FcAMe_3H_6$	negative

TABLE II: Antagonistic action of siderochromes against albomycin using the Bonifas test and B. subtilis as test organism (5). All compounds were tested at 0.1 mM concentration. Relative antagonistic action was compared to ferrichrome after incubation of the plates for 24 hr at 25°.

Compound	Bonifas test
Fc	+ + + +
PrFc	+ + + +
BuFc	+ + +
FChy	+ + +
FcA	negative
$FcAMe_3$	+
$FcAMe_3H_6$	+

was almost identical to that of FChy. In our previous work (2), we found that the uptake of FcA occurs at a low rate about the same as shown in Fig. 2 for $FcAMe_3$. The possibility that uptake of tracer iron may be due to

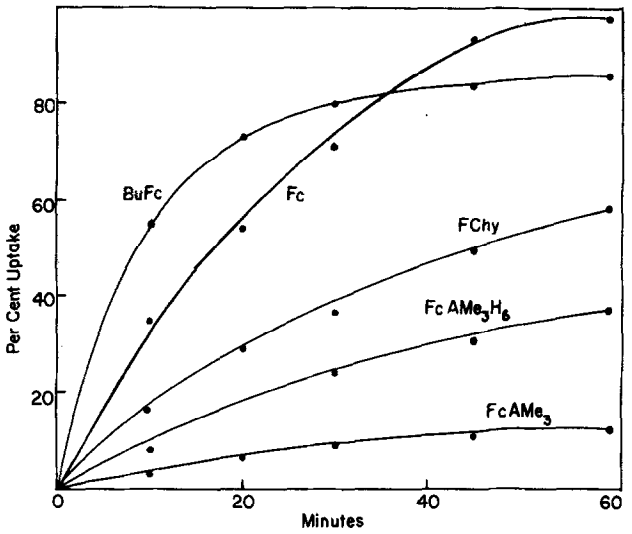

Figure 2. Rate of uptake of ^{59}Fe-labeled siderochromes by cell suspensions of Ustilago sphaerogena. The cell concentration was 17 mg/ml (dry weight) and the labeled substrates, 0.036 mM, were added at zero time. Uptake was followed at 30° and pH 7 by rapid centrifugation of the cells at 0° and determining loss of counts from the supernatant by scintillation counting (2).

exchange with endogenous desferriferrichrome cannot be excluded, except for the case of BuFc, which is taken up at a rate even faster than ferrichrome itself.

DISCUSSION

There is an increasing amount of evidence that many aerobic microorganisms possess a siderochrome transport system for sequestering iron from the environment. Such a system cannot be very specific for Arthrobacter JG-9. Mono- and dihydroxamic acids, and in some instances non-hydroxamate iron chelates, are known to satisfy the growth requirement of this organism, although many of these substances do not approach ferrichrome in their potency. Recently, it has been found that siderochrome requiring mutants of Salmonella typhimurium can also be satisfied by hydroxamic acid iron chelates of diverse structure (4).

Why, then, is ferrichrome A completely inactive as a growth factor for Arthrobacter JG-9? It is not due to the three charged carboxylate groups, because the trimethyl ester is also devoid of activity. The presence of α-β unsaturation in the hydroxamate acyl groups leads to en-

hanced stability of the iron chelate, which may be a factor in biological activity. However, our observation that the hydrogenated derivative, $FcAMe_3H_6$, is inactive rules against this possibility. Furthermore, the activity of FChy shows that Arthrobacter JG-9 is not sensitive to the presence of two serine residues in the peptide ring. The presence of alanine in the ring has recently been found not to affect the activity of siderochromes in a Salmonella typhimurium test system (4). We conclude that the bulkiness of the branched chain C-6 acyl groups of FcA, $FcAMe_3$ and $FcAMe_3H_6$ is responsible for the inactivity of these substances. The observation that PrFc and BuFc are only slightly less active than Fc suggests that the branched methyl group may be of primary importance in the lack of activity of $FcAMe_3$ and $FcAMe_3H_6$.

The antagonism of siderochromes against the antibiotic, albomycin, may reflect competition for an uptake system. The relative activities of our analogs as albomycin antagonists with B. subtilis (Table II) show that this organism, like Arthrobacter JG-9, is quite unspecific in its recognition of siderochromes. However, we again find that branched chain C-6 acyl groups lead to almost complete loss of activity.

In contrast to the lack of specificity of siderochromes in the tests described above, the ferrichrome transport system of U. sphaerogena is very sensitive to structural alteration. The replacement of two glycines by serines in the peptide ring (FChy) leads to a greatly diminished rate of uptake, showing that the structure of the peptide itself plays a role. Not only are branched chain C-6 siderochromes poor ionophores compared to ferrichrome, but even the change from acetyl to propionyl groups about the iron-chelating center adversely affects activity. We have no explanation why the BuFc exhibits an even higher initial rate of uptake than ferrichrome. The activity of BuFc is probably not due to non-specific surface binding because uptake is immediately and completely inhibited by mM azide, as was previously observed for the active uptake of ferrichrome by this organism.

The molecular mechanism of transport of low molecular weight ionophores has yet to be elucidated. Specific membrane proteins may be involved, although a siderochrome binding protein has not yet been demonstrated. If such a protein is involved in the siderochrome transport of U. sphaerogena, the results described in this paper suggest that steric factors play an important role in the specificity of binding.

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