Another interesting observation is that gel filtration of complexes of GAD with BAGA or BAP (oximes of the enzyme) and also of the complex of III, EI (24 hr, λ_{max} 380 nm), in all cases led to dephosphorylation of the coenzyme derivative. Reduction by $NaBH_4$ of ES and EI complexes followed by gel filtration also gave dephosphorylated products. On the other hand, the coenzyme cannot be separated from the native holoenzyme by gel filtration, nor is the phosphate split off when the coenzyme is converted to pyridoxamine phosphate by decarboxylative transamination with α-methylglutamate (Huntley and Metzler, 1968). Thus, a labilization of the phospho ester bond takes place for complexes of GAD in which the coenzyme is bound to a compound which partially or wholly occupies the substrate site. This fact suggests that the phosphate group of the coenzyme plays an active role in the transformations of substrate, perhaps by holding the coenzyme and substrate in a proper orientation.

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Structure of Schizokinen, an Iron-Transport Compound from *Bacillus megaterium**

Kary B. Mullis, J. R. Pollack,† and J. B. Neilands

ABSTRACT: Growth of *Bacillus megaterium* ATCC 19213 at limiting concentrations of iron induced the organism to excrete the iron-transport agent schizokinen. Hydrolysis of schizokinen with mineral acid yielded a novel organic hydroxylamine, characterized by synthesis as 1-amino-3-(*N*-hydroxyamino)propane. Oxidation with periodate afforded acetate as the acyl moiety of the hydroxamic acid bonds of schizokinen. Examination of the intact molecule by means of proton magnetic resonance spectroscopy enabled the conclusion that it is composed of a residue of citric acid symmetrically substituted in amide linkage to the amino groups of two residues of 1-amino-3-(*N*-hydroxy,*N*-acetyl)aminopropane. The ferric complex of schizokinen was prepared and shown to be an anion at neutral pH. A minor neutral component of unknown structure named schizokinen A appeared in the medium and could also be prepared from schizokinen by heating.

The technique of derepression of the biosynthesis of microbial iron-transport compounds by culture of various aerobic species at low levels of iron has enabled the isolation of a variety of substances, collectively termed siderochromes, which can be classed chemically as either hydroxamates or phenolates (Neilands, 1971). In the original observation of this phenomenon it was noted that *Bacillus megaterium* excreted a compound believed to be a member of the hydroxamate group (Garibaldi and Neilands, 1956). Later, Lankford

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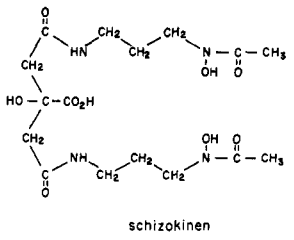
et al. (1966) described a factor, designated schizokinen (SK),[1] which was capable of reducing the division lag of bacteria and in a subsequent paper a substance with this property was isolated from *B. megaterium* and shown to be a hydroxamic acid (Byers *et al.*, 1967). An iron-transport role for SK seems assured since a strain of *B. megaterium* which cannot make the compound exhibits general siderochrome dependancy (Arceneaux and Lankford, 1966). In addition, SK serves as a potent growth factor for mutants of enteric bacteria carrying a lesion in their iron operon (Pollack, 1970).

In the present communication we propose as the structure of SK the formula shown in Figure 1, *viz.*, a symmetrical diamide conjugate of 2 moles of 1-amino-3-(*N*-hydroxy,*N*-acetyl)-aminopropane with citric acid.



schizokinen

FIGURE 1: Structure of schizokinen.

Experimental Section

Materials and Methods. Elemental analyses were performed by the microanalytical laboratory in the Chemistry Department, University of California, Berkeley. Iron was determined with a Model 303 Perkin-Elmer atomic absorption spectrophotometer. Ammonia was measured by distillation followed by nesslerization. Paper electrophoresis was carried out at pH 2.2 in 4% formic acid or at pH 7.0 in 0.1 M potassium phosphate buffer. Paper chromatography employed the following solvent systems: butanol–water–acetic acid (60:25:15, v/v), isopropyl alcohol–water (7:3, v/v), and methanol. Thin-layer chromatography was performed on Merck-Darmstadt silica gel plates. Developing sprays used were tetrazolium for hydroxylamines (Snow, 1954), the Altman reagent for citric acid (Smith, 1960), and 1% $FeCl_3$ in 0.05 N HCl for hydroxamic acids. Spectra were measured with the following instruments: infrared of KBr pellets, Perkin-Elmer 257; visible, Beckman DU-Gilford and Cary 14; proton magnetic resonance, Varian A-60; and optical rotatory dispersion, Cary 60. The solvents used for proton magnetic resonance measurements were deuterium oxide and d_6-dimethyl sulfoxide, the latter obtained from Merck Sharpe and Dohme of Canada. SK in culture supernatants were assayed by addition of 0.5 ml of the supernatant to 2.5 ml of 5 mM $Fe(ClO_3)_4$–0.1 M $HClO_4$ followed by measurement of the absorbancy at 500 nm (Atkin *et al.*, 1970).

Melting points were measured in capillaries and are uncorrected. The apparent pK_a values and neutral equivalents were determined at room temperature in water solution by use of the difunctional recording titrator (Neilands and Cannon, 1955).

Production and Isolation of SK. B. megaterium ATCC 19213 was obtained from the American Type Culture Collection and maintained on agar slants containing the following nutrients added per liter of distilled water: 1.0 g of K_2SO_4, 3.0 g of K_2HPO_4, 3.0 g of ammonium acetate, 20.0 g of sucrose, 800 mg of $MgSO_4 \cdot 7H_2O$, 8.6 mg of $ZnSO_4 \cdot 7H_2O$, 0.113 mg of $MnSO_4 \cdot H_2O$, and 15 g of agar. The pH was brought to 7.0 with concentrated ammonium hydroxide prior to sterilization of the medium at 15-lb steam pressure for 20 min. For production of SK the organism was grown on the above medium, minus agar, after supplementation with arginine hydrochloride at a level of 1.5 g/l.; this amino acid was found to increase significantly the yield. Heavy aeration was necessary for maximum yields, which varied from 1 to 3 g from an original 15 l. of nutrient solution. The cultures were incubated in a 37° room but due to the aeration the actual temperature in the carboys was somewhat lower than this; sterile distilled water was added to maintain constant volume. Growth was allowed to continue until production had ceased which, in the case of a 3% innoculum, was about 1 week. In starting a liquid culture from a single colony we supplemented the medium with about 1 μg/ml of SK as a means of avoiding long lag periods and concomitant risk of contamination.

After removing the cells from a 15-l. batch of medium the supernatant liquid was flash evaporated at 37° to 500 ml and the pH adjusted to 2 with concentrated HCl. Extraction with 1 l. of $CHCl_3$–phenol (1:1, w/w) removed the SK almost completely from the aqueous layer. Addition of 4 l. of ethyl ether to the organic phase and reextraction with 200 ml of distilled water transferred the SK to the aqueous phase. The latter was then concentrated to about 75 ml on a rotatory evaporator and applied to a 2.5 × 22 cm column of the acetate form of Dowex AG-2-X10. The column was washed with 100 ml of distilled water, which removed a very small amount of ferric ion positive material (schizokinen A, a neutral decomposition product of SK, the structure of which is unknown). The SK was eluted with 0.2 M NH_4Cl and was located, by means of the iron reagent, in about 250 ml of effluent. Ammonium salts were removed by repeating the above chloroform–phenol extraction procedure and the aqueous solution of SK so obtained was concentrated and further purified by passage through a 2.5 × 45 cm column of Bio-Gel P-2 polyacrylamide resin. The SK peak was centered at the 140-ml fraction. The proton magnetic resonance spectrum, neutral equivalent, and elemental composition of the iron complex, all of which are reported below, attest to the homogeneity of SK prepared in this way.

Results

Identification and Properties of SK. SK isolated as described above and dried under vacuum proved to be a clear, colorless glassy material which was extremely hygroscopic. The free acid was very soluble in water and in the lower alcohols. Cochromatography on paper with SK derived from an authentic sample of ferri-SK furnished by Dr. C. E. Lankford indicated identity of the two substances. In butanol–water–acetic acid and in isopropyl alcohol–water they gave R_F values of 0.60 and 0.64, respectively. Thin layer chromatography on silica gel in methanol indicated a mutual R_F of 0.60. Comparison of the infrared spectrum with that published by Byers *et al.* (1967) showed good agreement. Major peaks were located at 3420, 1630, 1435, 1075, and 800 cm^{-1}. SK subjected to electrophoresis at pH 2.2 remained stationary at the origin; at pH 7.0 and a field strength of 12 V/cm it moved 1.9 cm/hr to the anode. Electrometric titration showed a monovalent ionization with pK_a of 4.1 and a second buffer zone, which consumed 2 equiv of base, with a midpoint at pH 9.4.

[1] Abbreviations used are: SK, schizokinen; ferri-SK, ferric complex of schizokinen.

Ferri-SK. A solution of SK was standardized by titration with a known concentration of potassium hydroxide. To 300 μmoles of dissolved SK was added 300 μmoles of ferric chloride, which had been standardized with the same potassium hydroxide solution. The pH of the complex solution was raised to 7.0 with KOH, which required 4 equiv of base/mole of complex formed. Formation of the complex appeared to be complete at about pH 5–6. The ferri-SK was passed through Bio-Gel P-2 polyacrylamide resin where it separated into a major and a minor peak. The major peak was rechromatographed and moved as a single species. This material was crystallized by concentration and addition of ethanol, followed by cooling for several hours. The crystalline material was dried to constant weight *in vacuo* at 50°. It was allowed to rehydrate and found to absorb approximately 3 moles of water/mole of complex. *Anal.* Calcd for $C_{16}H_{24}FeN_4O_9(K^+)\cdot 3H_2O$ (565.4): C, 33.99; H, 5.35; N, 9.91. Found: C, 34.99; H, 5.61; N, 10.32.

An ammonium salt was prepared similarly and purified by elution from Dowex AG-2-X10 acetate with a 0.1–1.0 M gradient of ammonium chloride followed by gel filtration on Bio-Gel P-2. Addition of ether to a concentrated solution of the product in anhydrous methanol gave crystalline material which on exposure to the atmosphere for 2 hr was observed to take up exactly 2 moles of water. *Anal.* Calcd for $C_{16}H_{24}FeN_4O_9(NH_4^+)\cdot 2H_2O$ (526.3): C, 36.51; H, 6.13; Fe, 10.61; NH_4^+, 3.43. Found: C, 37.16; H, 6.02; Fe, 10.1; NH_4^+, 3.12.

At pH 7 the complex moved as an anion on paper electrophoresis, indicating the binding of an ionized citrate hydroxyl group to the iron. The potassium salt exhibited a visible absorption spectrum which was independent of pH in the range 9.5–5 but which diminished in intensity and shifted to the red at pH values <5. At neutral pH the absorption maximum was at 390 nm.

Hydroxyamino Constituent. The iron was extracted from 500 mg of ferri-SK with 8-hydroxyquinoline and the resulting SK was hydrolyzed with 10 ml of 6 N HCl for 18 hr at 105°. The hydrolysate was taken to dryness *in vacuo*, and the residue was dissolved in 5 ml of 1 N HCl and applied to a 2 × 31 cm column of Bio-Rex AG50W-X2H which had been equilibrated with 1 N HCl. The column was eluted with a linear gradient of 400 ml of 1 N HCl in the mixing chamber and 400 ml of 3 N HCl in the reservoir. The eluate was collected in 8-ml fractions, which were assayed for hydroxylamine reaction. Fractions 52–57, which contained the bulk of the activity, were pooled and taken to dryness *in vacuo*. The residue was crystallized from ethanol, yielding 25 mg of colorless needles, mp 142.5–143.5°.

A 9-mg sample of the hydrolytic fragment was dissolved in 5 ml of ethanol and treated with a stream of H_2 in the presence of 10 mg of platinum oxide catalyst. When the tetrazolium test became negative the catalyst was removed, the solution taken to dryness on the steam bath and the residue dissolved in 0.5 ml of 1 N NaOH and shaken with 0.1 ml of benzoyl chloride for 1 hr. After addition of 0.6 ml of 1 N NaOH the reaction was allowed to stand for 3 hr. The colorless precipitate was collected by filtration, washed with water, and recrystallized from methanol–water. The infrared spectrum and the melting point of 145–146° were essentially identical with those of *N,N*-dibenzoyl-1,3-diaminopropane (lit. (Shriner *et al.*, 1959) mp 147°).

Direct reductive hydrolysis of ferri-SK with 50% HI also gave 1,3-diaminopropane, identified by paper electrophoresis in 4% formic acid buffer.

A second 9.5-mg sample of the hydrolytic fragment was examined by electrometric titration and found to consume 2 equiv of NaOH, one with a pK_a of 4.6 and the other with a pK_a of 9.8. The molecular weight calculated from the second pK_a was 164, theoretical for 1-amino-3-(*N*-hydroxyamino)propane dihydrochloride, 163. This substance apparently has not been obtained previously by chemical synthesis or by isolation from natural products.

Acyl Substituent of the Hydroxamate Linkages. Treatment of SK with excess periodate by the method of Emery and Neilands (1962) eliminated the buffer zone at pH 9.4 and generated an equivalent amount of acid with pK_a of 4.8 ± 0.2. The latter was extracted and identified by paper chromatography and proton magnetic resonance spectroscopy as acetic acid. Concomitently with the periodate oxidation the solution of SK acquired an intense ultraviolet absorbance, with a peak at 267 nm, thus revealing the acetic acid as bound to an *N*-alkyl-substituted hydroxylamine (Emery and Neilands, 1962).

Citrate. Approximately 0.25 mmole of SK was hydrolyzed in 4 ml of 6 N HCl at 110° for 16 hr. The hydrolysate was evaporated to dryness and extracted several times with ether. The ether was concentrated and aliquots analyzed by thin-layer chromatography in 80% ethanol and paper chromatography in butanol–water–acetic acid with citric acid as standard. The spots were visualized by spraying with the Altman reagent which produced a pink spot from the citric acid standard and the sample, both of which had R_F values of 0.30 and 0.49 in the two solvent systems, respectively. No attempt was made to quantitate the yield of citric acid in view of the known propensity of this molecule to undergo extensive degradation in hot acid solution (Strassman *et al.*, 1968).

Synthesis of 1-Amino-3-(N-hydroxyamino)propane. 4-NITROBUTYRIC HYDRAZIDE HYDROCHLORIDE. 4-Nitromethylbutyrate was prepared from nitromethane and methyl acrylate by the method of Colonge and Pouchol (1962). The nitromethyl ester (0.75 mole, 110 g) was refluxed with 75 ml of ethanol and hydrazine hydrate (1.00 mole, 50 g) for 6 hr. The ethanol, water, and excess hydrazine were removed completely under vacuum and the residue was redissolved in very dry ethanol. The salt of the hydrazide was formed by passing dry HCl gas into the cooled ethanol solution until saturation was achieved. Excess HCl was removed under vacuum and crystallization then induced by a brief exposure to heat. The yield was 130 g, 90%.

ETHYL *N*-(3-NITROPROPYL)CARBAMATE. 4-Nitrobutyric hydrazide hydrochloride (0.1 mole, 19.3 g) was dissolved in 60 ml of cold 2.5 N HCl (0.15 mole) followed by addition of 60 ml of ethyl ether. Sodium nitrite (0.125 mole, 8.6 g) in 15 ml of water was added slowly with good stirring while keeping the temperature below 10°. The ether was separated and the aqueous phase extracted with another aliquot of ether; the combined ether extracts were washed with 5% $NaHCO_3$ and water. The ethereal solution of 4-nitrobutyric azide was dried over $CaCl_2$ and then filtered into 100 ml of dry ethanol. The ether and ethanol were distilled off slowly on a steam bath and the residual oil was dried overnight under vacuum. The yield was 11.8 g, or 66%, of a substance which on examination by proton magnetic resonance spectroscopy appeared to be pure ethyl *N*-(3-nitropropyl)carbamate.

ETHYL *N*-(3-HYDROXYAMINOPROPYL)CARBAMATE. Ethyl *N*-(3-nitropropyl)carbamate (0.067 mole, 11.8 g) and ammonium chloride (0.134 mole, 7.1 g) were dissolved in 200 ml of ethanol–water (1:1, v/v) and the solution was cooled to 5°. Zinc dust (0.134 mole, 8.7 g) was added in increments with efficient stirring and at a temperature below 10°. The reaction mixture was allowed to come to room temperature while

the sitrring was continued and the ZnO was filtered. The pH was found to be 7.4, indicating the hydroxyamino group to be in neutral form. The alcohol and water were removed at 40° on the rotary evaporator, the residue was extracted with ether, and the insoluble ammonium chloride was discarded. The ether was evaporated under vacuum to yield 10.8 g (*ca.* 100%) of an oily residue which was not further characterized.

1-AMINO-3-(*N*-HYDROXYAMINO)PROPANE. The oil obtained as above (10.8 g) was dissolved in 100 ml of 6 N HCl and refluxed for 18 hr. The aqueous acid was evaporated, and the residue was dissolved in methanol and crystallized to yield 2.8 g, or 26%, of the dihydrochloride of 1-amino-3-(*N*-hydroxyamino)propane. *Anal.* Calcd for $C_3H_{10}N_2O \cdot 2HCl$: C, 22.10; H, 7.42; Cl, 43.49; N, 17.18. Found: C, 22.70; H, 7.23; Cl, 42.74; N, 17.37. The product was shown to be identical with the natural substance by melting point and mixture melting point determinations, by infrared spectroscopy, and by paper chromatography in butanol–water–acetic acid (R_F 0.23).

Proton Magnetic Resonance Spectroscopy of SK. The 60-MHz proton magnetic resonance spectrum of SK in deuterium oxide is shown in Figure 2. Four protons appeared in a triplet (J = 6.5 Hz) at −221 Hz (relative to tetramethylsilane). This resonance was assigned to the methylene protons adjacent to the hydroxyamino groups. Another four-proton triplet (J = 6.5 Hz) appeared at −194 Hz, which became a quartet when the spectrum was repeated in dimethyl sulfoxide, a solvent in which amide hydrogens do not become deuterated. This resonance was assigned with confidence to the methylene protons adjacent to the amide groups. A broad singlet of four protons appeared at −160 Hz and was assigned to the methylene protons of citric acid. A sharp singlet of six protons occurred at 127 Hz, and was assigned to the acetate protons. A multiplet, which was not completely resolved, appeared at −110 Hz and was assigned to the inner methylene protons of the *n*-propyl chains.

In dimethyl sulfoxide the amide resonance appeared as a triplet representing two protons at −471 Hz (J = 6.5 Hz). At temperatures below 17° in the same solvent it was possible to observe the resonance due to the protons of the hydroxamic acid (Llinás *et al.*, 1970), although severe broadening due probably to proton exchange with the carboxyl group made accurate integration of the peak impracticable.

Molecular Weight of SK. An estimate of the molecular weight of SK was obtained by use of gel permeation chromatography. A small quantity of SK in 0.5 ml of water was applied to a 1 × 50 cm column of Sephadex G-10 equilibrated with 1% NaCl. The hydroxamic acid was eluted with 1% NaCl and appeared in the 19- to 24-ml fraction. The reference compounds ferrichrome A (mol wt 1054) and ferrichrome (mol wt 740) eluted at 15.4–18.9 and 19.5–25.0 ml, respectively. Since the ferrichromes have been shown to be very compact molecules (Llinás *et al.*, 1970), their radii of gyration are probably only a one-half or two-thirds of that of a linear species of similar molecular weight. Assuming SK to be a more open molecule, these data suggest that its molecular weight is probably in the range of 350–550.

A highly purified fraction of SK from Bio-Gel P-2 was lyophilized to a white powder. This material was dessicated further under vacuum in a weighing flask which could be quickly closed to avoid rapid absorption of water. The sample was weighed and dissolved in a known amount of water. An aliquot containing 16.7 mg, on titration to neutrality, consumed 37.7 μmoles of base thus yielding an equivalent weight of 443, which is in reasonable agreement with the theoretical value of 420.

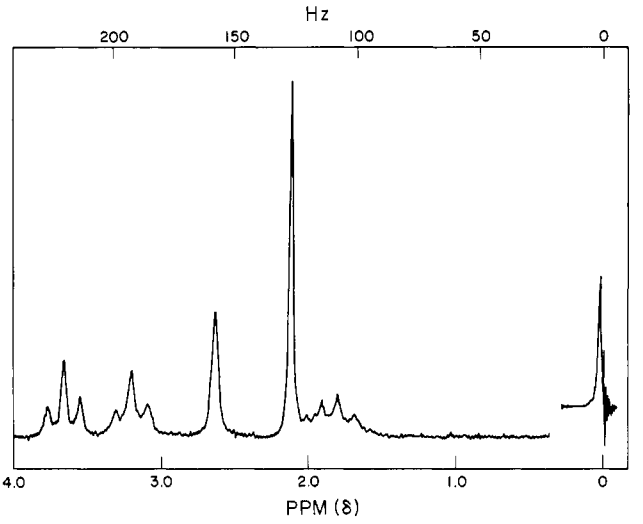


FIGURE 2: Proton magnetic resonance spectrum of schizokinen in deuterium oxide at 60 MHz.

Derivatives of SK. The ethyl ester of SK was obtained by treating the free acid overnight with anhydrous ethanol saturated with HCl gas. The solvent was removed on the rotary evaporator, and the residue was dissolved in distilled water and chromatographed on Bio-Gel P-2. The ethyl ester comprised 50% of the hydroxamate-positive material eluted from the column. The proton magnetic resonance spectrum resembled that of SK, except for the presence of an ethyl group which displayed a two proton quartet at −255 Hz, with J = 7 Hz, and a three proton triplet at −77 Hz, with J = 7 Hz. The broad singlet attributable to the citric acid residue in the spectrum of SK (Figure 2) appeared as a doublet centered at −166 Hz, with J = 4 Hz. In addition to the ethyl ester, another derivative of SK, which we have termed schizokinen A, was also formed in this reaction. It had an R_F of 0.74 in butanol–acetic acid–water, and was stationary when subjected to electrophoresis at pH 7.0. In these respects it appeared to be identical with the neutral compound eluted from Dowex AG-2 in the purification scheme for SK and to the compound which could be produced by boiling SK in water or in alcohol for several hours. Both formed characteristic ferric hydroxamate complexes.

Discussion

Gibson and Magrath (1969) have characterized aerobactin, a hydroxamic acid from *Aerobacter aerogenes*, as a conjugate of 6-(*N*-acetyl,*N*-hydroxyamino)-2-aminohexanoic acid and citric acid. The proton magnetic resonance spectrum of aerobactin between −100 and −300 Hz is similar to that of SK, differences appearing only as predicted by comparison of the two structures. Since citric acid bears three carboxyl groups, two possible diamide conjugates are possible. *viz.*, symmetrical and unsymmetrical. Since the methylene protons of the substituted citric acid in SK appear equivalent in the proton magnetic resonance spectrum, the substitution must be symmetrical. The same deduction could be made for aerobactin.

In addition to the information on symmetry, the proton magnetic resonance spectrum also demonstrates that the SK

molecule is comprised of citrate, 1-amino-3-(*N*-hydroxyamino)propane, and acetate in the ratio 1:2:2.

A mass spectrometric analysis of SK has not been attempted since previous experience indicated that this technique is of limited value for microbial iron-transport compounds of low volatility. A 0.5 mM solution of ferri-SK displayed no optical activity in the range 200–300 nm.

The neutral derivative of SK, schizokinen A, had properties compatible with its being a ketone in which the citrate carboxyl had condensed with one of the methylene carbons adjacent to the amide linkage forming a six-membered ring. Proton magnetic resonance showed the disappearance of half of these methylene protons relative to SK as would be expected. An infrared band in schizokinen A not present in SK appeared at 1710 cm^{-1} and sodium borohydride, sodium in ethanol, or dinitrophenylhydrazine all exhibited characteristic reactions with this derivative.

Titration of the neutral potassium salt of ferri-SK to pH 11 revealed no further ionizations, thus confirming that both hydroxamic acid groups are linked to the iron. However, since the visible absorption spectrum of ferri-SK is atypical of ferric hydroxamates, it is obvious that the iron is complexed with additional ligands. That the carboxyl group is coordinated to the metal ion is suggested by the observation that the neutral pH spectra of the ferri complexes of SK ethyl ester and schizokinen A are shifted to the red and have maxima at 425 nm. Warner and Weber (1953) showed that at pH values above 3 the hydroxyl group of citric acid participates in the complexation of ferric ion. This would provide the fourth proton titrated in the formation of the ferri-SK complex. A CPK model of ferri-SK also indicates that both the free carboxyl and hydroxyl groups of SK can bind to a central metal ion, although the resulting complex is extremely compact and is highly strained. The structures of the metal complexes of SK are to be examined by X-ray crystallography.

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