

Metal-coordinating substrate analogs as inhibitors of metalloenzymes

[zinc metalloenzymes/S → Co(II) charge transfer/metal-directed inhibition/mercaptan and phosphoramidate inhibitors/magnetic circular dichroism]

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ABSTRACT A group of active-site metal coordinating inhibitors of zinc proteases (carboxypeptidase A, thermolysin, *Bacillus cereus* neutral protease, and angiotensin-converting enzyme) have been synthesized and their properties investigated. Their general structures are R—SH and R—NH—PO₂(Oφ)H, where —S⁻ or —O⁻ serve as metal ligands and R refers to an amino acid or peptide group designed to interact with substrate recognition sites. These inhibitors can be extremely potent; thus, *N*-(2-mercaptoacetyl)-D-phenylalanine, e.g., inhibits carboxypeptidase A with a $K_{i,app}$ of 2.2×10^{-7} M. The spectral response of cobalt(II)-substituted thermolysin or carboxypeptidase A to the sulfur-containing inhibitors signals the direct interaction of the mercaptan with the metal. An S → Co(II) charge transfer band is generated near 340 nm and is detected by absorption, circular dichroism, and magnetic circular dichroism. The cobalt(II) spectra indicate both inner sphere coordination with sulfur and 4-coordination in the enzyme-inhibitor complex. Thus, the metal undergoes a simple substitution reaction, the inhibitor most likely displacing water at the fourth coordination site.

Potent active-site-directed inhibitors with substrate-like properties, referred to variously as "transition-state analogs," "affinity labels," and "suicide inhibitors," have proven valuable in the exploration of structure-function relationships of many (nonmetallo) enzymes (1-11). Compounds incorporating two groups of high affinity—one of them designed to bind preferentially to the enzyme like a substrate, the other to interact selectively with the active-site metal—would be expected similarly to markedly inhibit metalloenzymes selectively. Inhibitors with such characteristics have been synthesized and have been postulated, but not proven, to interact with active-site metal atoms (12-16).

We have designed and investigated the mode of action of a group of such metal coordinating substrate analogs of the general structure R—S⁻ and R—NH—PO₂(Oφ)⁻, all of which inhibit zinc proteases. They incorporate the anions —S⁻ or —P—O⁻, which bind to active-site metals, and substrate-like moieties consisting of either amino acids or peptides R, which interact with the recognition site of the enzyme (Fig. 1). The data to be presented illustrate the potency, specificity, and general usefulness of these agents when interacting with a number of zinc enzymes and their cobalt-substituted derivatives, the latter providing spectral evidence that their —S⁻ groups, in particular, are coordinated directly to the metal.

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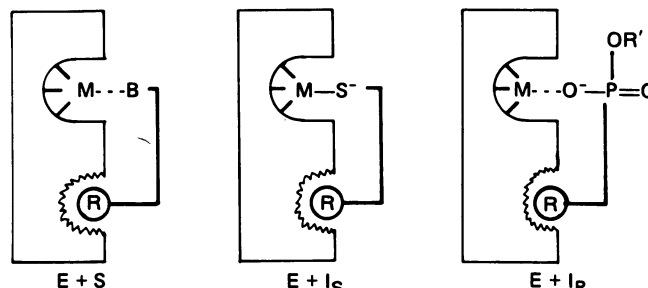


FIG. 1. Diagram illustrating the interaction of substrates or metal-binding inhibitors with the active site of metalloenzymes. One or more coordinating positions of the metal M is available to interact with the scissile group B of the substrate or an anionic group of an inhibitor.

METHODS AND MATERIALS

Carboxypeptidase A_α (Sigma) was purified further by affinity chromatography (17), thermolysin (Sigma) was recrystallized from sodium bromide (18), and *Bacillus cereus* neutral protease (Worthington) was purified to homogeneity by phosphoramidate affinity chromatography (13). Angiotensin-converting enzyme was prepared from rabbit lung (19). 2-Mercapto-3-phenylpropionic acid was synthesized from 2-bromo-3-phenylpropionic acid (Pfaltz and Bauer, Stamford, CT) and sodium thiobenzoate (16).

The 2-mercaptoacetyl (HSAc) derivatives of amino acids and peptides were synthesized by using the *N*-hydroxysuccinimide ester of *S*-acetylmercaptoacetic acid. Acylation of peptide or amino acid methyl esters by this activated ester, followed by simultaneous hydrolysis of both the thiol and alkyl ester function of the products, gave the desired crystalline derivatives. The phosphoramidates were synthesized from the requisite peptide or amino acid methyl or ethyl esters with diphenylphosphorochloridate (13) to give the crystalline diphenylphosphoryl alkyl esters. Prior to use, the monophenylphosphoryl derivatives bearing a free COOH-terminal carboxyl group were prepared by base hydrolysis of the parent diphenylphosphorylated peptide esters (13, 20). Details of these syntheses will be reported elsewhere.

Thermolysin and *B. cereus* neutral protease activities were measured spectrophotometrically with 0.2 mM *N*-(2-furanacryloyl)-glycyl-L-leucyl-L-alanine, pH 7.5/50 mM Hepes (13, 18). Carboxypeptidase activity was monitored spectrophotometrically at 334 nm with 0.2 mM *N*-(2-furanacryloyl)-glycyl-L-phenylalanine, pH 7.5/50 mM Tris-HCl/0.1 M NaCl (21).

Abbreviations: MCD, magnetic circular dichroism; CD, circular dichroism; HSAc, 2-mercaptoacetyl.

Angiotensin-converting enzyme activity was measured with 0.1 mM *N*-(2-furanacryloyl)-*L*-phenylalanyl-glycylglycine, pH 7.5/50 mM Hepes/0.3 M NaCl (22). Inhibition constants were established by varying inhibitor concentration at a fixed substrate concentration under conditions where $[S] \ll K_m$, optimal for the detection of inhibition but not its mode. Plots of k_{obs-1} against inhibitor concentration were linear. $K_{i,app}$ was obtained from the extrapolated intercept on the abscissa. Activity was measured at 25°C at enzyme concentrations below 20 nM. Zinc-free thermolysin (18) and zinc-free carboxypeptidase A (23) were prepared as described. Protein concentrations were measured by absorbance at 280 nm by using $6.64 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for thermolysin, $6.42 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for carboxypeptidase A, and $5.60 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for *B. cereus* neutral protease. Angiotensin-converting enzyme concentrations were measured by the method of Lowry *et al.* (24). Precautions were taken to avoid contamination with adventitious metal ions (25), and all buffer and substrate solutions were extracted with 0.1% dithizon in CCl_4 . Deionized distilled water was used throughout.

Absorption spectra were measured with a Varian 219 spectrophotometer. Circular dichroic (CD) and magnetic circular dichroic (MCD) spectra were measured with a Cary 61 recording spectropolarimeter equipped with a superconducting magnet. MCD spectra were obtained at 4.0 tesla (40 kG).

RESULTS AND DISCUSSION

Compounds containing a mercaptan, R-SH, or a phenylphosphoryl group variously inhibit the zinc metalloenzymes bovine carboxypeptidase A, thermolysin, *B. cereus* neutral protease, and angiotensin-converting enzyme (Table 1). Addition of Zn^{2+} ions, $\approx 1 \mu\text{M}$, does not affect the competitive inhibition by any of these agents.

Perturbation of the $d \rightarrow d$ transitions of cobalt-substituted carboxypeptidase A and thermolysin (18, 26) gives direct spectral evidence that these agents inhibit by binding to the metal atom at the active site. Thus, HSAC-D-Phe alters the symmetry or coordination geometry (or both) of the cobalt atom of carboxypeptidase A, as is apparent from the spectral shifts and the approximately 2-fold increase of intensity in the visible region of the spectrum (Fig. 2). These features are also reflected in the CD and MCD spectra, the latter indicating, in addition, that the metal remains coordinated tetrahedrally (27). The interaction generates a distinct new transition, λ_{max} 338 nm, $\epsilon \approx 900 \text{ M}^{-1} \text{ cm}^{-1}$, which is apparent also in the CD spectrum as a negative shoulder at 338 nm and in the MCD spectrum (Fig. 2) as an intense positive band at 350 nm. We assign it to the formation of a sulfur \rightarrow cobalt(II) charge transfer band. The spectrum of the corresponding HSAC-D-Leu complex is similar.

The interaction with these inhibitors is stoichiometric, 1 mole of inhibitor per mole of enzyme resulting in maximal spectral effects. The addition of a large excess of known competitive inhibitors, either D,L-benzylsuccinic acid, $K_{i,app} \approx 1.1 \times 10^{-6} \text{ M}$ (14), or 2-amino-3-phenylethylphosphonate, $K_{i,app} \approx 10^{-6} \text{ M}$, to the [E-Co(II)-HSAC-D-Phe] complex abolishes the charge transfer band (Fig. 2); the resulting spectra are identical to those for the D,L-benzylsuccinic acid or 2-amino-3-phenylethylphosphonate enzyme complexes alone. Thus, these agents obviously displace and substitute for the thiol inhibitor, thereby eliminating the S \rightarrow Co(II) charge transfer.

HSAC-L-Phe-L-Ala binds stoichiometrically to cobalt(II)-substituted thermolysin, as shown by spectral perturbations in the ligand field region (Fig. 3), but the spectral charge-transfer effects differ somewhat. A major absorption band in the near-ultraviolet region overlaps that of tryptophan and tyrosine.

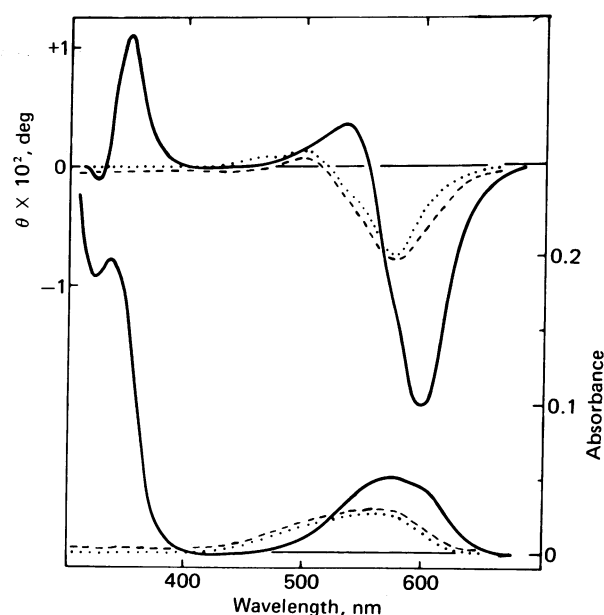


FIG. 2. Electronic absorption spectra (Upper) and MCD spectra (Lower) of cobalt(II)-substituted carboxypeptidase A ($2.8 \times 10^{-4} \text{ M}$) at pH 7.5 in 1.0 M NaCl/50 mM Hepes in the absence (.....) and presence (—) of the inhibitor HSAC-D-Phe ($2.8 \times 10^{-4} \text{ M}$). The cobalt enzyme, containing 0.84 equivalent of Co(II), was prepared by adding aqueous cobalt sulfate to the apoenzyme. Addition of D,L-benzylsuccinic acid to a final concentration of $3.3 \times 10^{-3} \text{ M}$ to the HSAC-D-Phe-inhibited enzyme (---) illustrates displacement of the mercaptan inhibitor. MCD is at 4 tesla.

However, MCD resolves a large positive maximum centered at 328 nm. This is assigned to S \rightarrow Co(II) charge transfer, which is eliminated by addition of a large excess of the phosphoramidate inhibitor monophenylphosphoryl-L-Phe-L-Ala to the thermo-

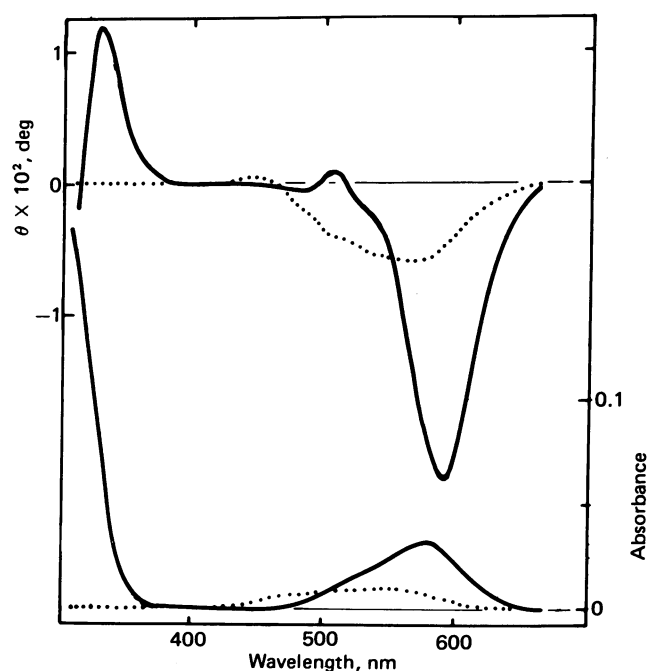


FIG. 3. Electronic absorption spectra (Upper) and MCD spectra (Lower) of cobalt(II)-substituted thermolysin ($4.2 \times 10^{-4} \text{ M}$) at pH 7.5 in 50 mM Hepes/10 mM Ca^{2+} /2.5 M NaBr in the absence (.....) and presence (—) of HSAC-L-Phe-L-Ala ($4.2 \times 10^{-4} \text{ M}$). The cobalt enzyme was prepared by adding 0.75 equivalent of aqueous cobalt sulfate to the apoenzyme. MCD is at 4 tesla.

lysine-mercaptan complex. The resultant spectrum is identical to that obtained when monophenylphosphoryl-L-Phe-L-Ala alone is added to the enzyme.

Affinity labels and similar agents for (nonmetallo) enzymes characteristically encompass both substrate-like and highly reactive moieties. These account for their remarkably effective inhibitory capacity. Preferential binding to substrate-recognition sites of the active center directs the inhibitor towards the active site, which it modifies chemically, thereby precluding substrate binding or catalysis or both. Classically, serine-specific alkylating agents exemplify active-site-directed affinity labels of (nonmetallo) enzymes. When a metal ion is at the active site of an enzyme, a somewhat analogous strategy for the study of such a component can be devised.

Simple anions or chelating agents inhibit virtually all metalloenzymes, a circumstance characteristic of the class (28). The incorporation of metal ligands into substrate analogs, however, is restricted to the design of metal coordinating inhibitors that form *mixed complexes*, E·M·I, so that the interaction of the metal atom with the ligand of the inhibitor can be detected *in situ*. Potent bifunctional inhibitors of zinc proteases are now available, having in common potentially coordinating anionic groups that are combined with substrate-like moieties. Thus, L-benzylsuccinic acid, one of the best inhibitors of carboxypeptidase A (12), is thought to coordinate with the metal via one of its carboxylate groups whereas the aromatic ring and the second carboxyl group determine specificity. Other examples include mercaptan inhibitors of carboxypeptidases A and B (16), as well as alkyl mercaptans—e.g., D-(3-mercapto-2-methylpropanoyl)-L-proline (14)—which are potent inhibitors of the zinc dipeptidyl carboxypeptidase, angiotensin-converting enzyme. Similarly, hydroxamates (15)—e.g., Ac-N(OH)-Leu—or phosphoramidates (13, 29)—e.g., monophenylphosphoryl-L-phenylalanyl-L-phenylalanine—are potent inhibitors of thermolysin and other zinc neutral proteases and have already served as ligands for use in affinity chromatography (13).

Although there seems to be little doubt about the existence of metal coordinating inhibitors directed both at a metal and at substrate recognition sites, experimental evidence for this dual mode of action is very sparse. In fact, to our knowledge, the only direct evidence for the coordination of such an inhibitor with an active-site metal is confined to the interaction of phosphoramidon, *N*-(α -L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, with the zinc atom of thermolysin (30). X-ray crystallographic analysis of the phosphoramidon complex with thermolysin shows that one of the phosphite oxygen atoms of the inhibitor is 2 Å from the metal, displacing water at the fourth coordination site (31). The peptide moiety of the inhibitor interacts with the hydrophobic substrate domain, thereby directing the anion to bind to the metal.

The present investigation was designed to extend these studies to other similar agents by different approaches and, if possible, to provide criteria and procedures to quickly verify or reject such a mechanism of inhibition when postulated.

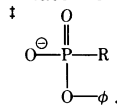
Table 1 demonstrates that the thiol peptides studied potently inhibit the zinc proteases examined as a direct function of the active-site-directed features that they incorporate. For example, just as a hydrophobic side chain adjacent to a free carboxyl group determines specificity of substrate binding to carboxypeptidase A, addition of a methyl (2-mercapto-propionic acid) or benzyl (2-mercapto-3-phenylpropionic acid) group in the α position of mercaptoacetic acid progressively increases affinity. In contrast, a hydrophilic carboxyl group in the side-chain position, as in thiomalic acid, markedly curtails it. HSAc-D-Phe, which incorporates all essential specificity determinants of a substrate in addition to the critical anionic ligand, R-S⁻, is the most potent carboxypeptidase inhibitor in this group, $K_{iapp} \approx 2.2 \times 10^{-7}$ M. As would be expected, blocking of the α -carboxyl group, a determinant of carboxypeptidase A specificity, as in HSAc-L-Phe-NH₂, virtually abolishes inhibition.

Table 1. Inhibition constants for zinc proteases*

Inhibitor†	K_{iapp} , μ M			
	Carboxypeptidase A	Thermolysin	Neutral protease	Angiotensin-converting enzyme
Mercaptans				
HS-CH ₂ -COOH	9.0	>10,000	—	—
HS-CH(CH ₃)-COOH	5.1	>10,000	>10,000	8.6
HS-CH(CH ₂ φ)-COOH	1.2	>10,000	—	—
HS-CH-(CH ₂ -COOH)-COOH	>300	>10,000	—	—
Phosphoramidates‡				
R =				
L-Phe	2.1	>80	20	370
D-Phe	10	>300	—	—
L-Phe-DL-Trp	160	0.32	0.23	—
L-Phe-L-Phe	>1000	3.8	0.80	1.0
L-Phe-L-Ala	>1000	0.52	0.12	2.2
L-Phe-NH ₂	530	4.0	0.27	460

* Measured at pH 7.5. —, No measurement made.

† Racemic mixtures unless indicated otherwise.



Simple mercaptans (Table 1, first four entries) do not inhibit the endopeptidases thermolysin and *B. cereus* neutral protease, enzymes with similar substrate specificities. However, the 2-mercaptoacetylated dipeptides (i.e., compounds designed to satisfy specificity requirements to yield metal coordinating inhibitors) exhibit K_{iapp} values as low as 3.4×10^{-7} M. Mercaptoacetylated amino acids and peptides are all excellent inhibitors of angiotensin-converting enzyme, HSAc-L-Phe being most effective among those examined. S-Acetylation, of course, severely curtails inhibition of all these enzymes, consistent with the proposed metal mercaptide formation.

Joint incorporation of elements of both site and metal specificity also renders the phosphoramidates excellent inhibitors (Table 1). Thus, the phosphorylated dipeptides are particularly good inhibitors of the endopeptidases, including angiotensin-converting enzyme; they are, however, much less effective towards carboxypeptidase A. The K_{iapp} values of the most effective phosphoramidate inhibitors of thermolysin are on par with phosphoramidon, $K_{iapp} = 88$ nM, and they thus mimic it. The failure of Zn^{2+} to reverse the inhibition by any of the agents in Table 1 mitigates against metal removal as the basis of inhibition.

We have used absorption, CD, and MCD spectra of Co(II)-substituted enzymes extensively to relate structure to the function of metalloenzymes (18, 26–28). In the present instance, the spectra signal the direct interaction of these inhibitors with the active-site cobalt atom, as is evident particularly with the mercaptans. The transition at ≈ 340 nm detected by absorption, CD, and MCD spectroscopy is due to $S \rightarrow Co(II)$ charge transfer, as established both in model cobalt-sulfur complex ions and in cobalt(II)-substituted metalloproteins in which cysteine residues serve as metal ligands. Thus, $[Co(S_2-o\text{-xyl})_2]^{2-}$ (31), $[Co(SC_6H_5)_4]^{2-}$ (32), and cobalt(II) complexes of various mercaptoacylamino acids (33), as well as Co(II)-substituted stellacyanin, azurin, plastocyanin (34), and β -lactamase II (35), all exhibit such $S \rightarrow Co(II)$ charge transfer bands with ϵ values ≈ 1000 $M^{-1} cm^{-1}$. In plastocyanin, the mercaptan of a cysteine residue is a metal ligand (36). Similarly, Co(II) rubredoxin absorbs intensely at 350 nm, $\epsilon \approx 9400$ $M^{-1} cm^{-1}$, consistent with eight $S \rightarrow Co(II)$ charge transfer transitions of $\epsilon \approx 1200$ per bond (37). Similar transitions are also observed in both the cobalt-substituted alcohol dehydrogenase from horse liver (38) and that from yeast (39). In all cases the absorptivity of this charge transfer band is ≈ 1000 $M^{-1} cm^{-1}$ per $S \rightarrow Co(II)$ bond, consistent with the present mercaptan inhibitors of carboxypeptidase A and thermolysin, $\epsilon \approx 900$ $M^{-1} cm^{-1}$.

The addition of an excess of other inhibitors (e.g., D,L-benzylsuccinate) that compete with the mercaptan inhibitor for the enzyme eliminates all near-ultraviolet absorption and optical activity, consistent with their displacing the mercaptan. The competitive inhibitors used here (i.e., D,L-benzylsuccinate and a phosphonate for carboxypeptidase A and a phosphoramidate for thermolysin) are also thought to interact with the metal.

Collectively, these data demonstrate active-site-directed inhibition of these metalloenzymes by metal ligands, as represented schematically in Fig. 4. Direct interaction of their anionic ligand with the metal and specificity engendered by the substrate analog moiety of these inhibitors generate the multiple interactions that result in the selectivity and high affinity observed. The cobalt spectra indicate both inner sphere coordination with sulfur and 4-coordination in the enzyme-inhibitor complex. The MCD spectra, one of the best indices of overall coordination geometry and number (27), are most consistent with tetrahedral coordination in the enzyme-inhibitor complexes here examined. Thus, when binding the mercaptans,

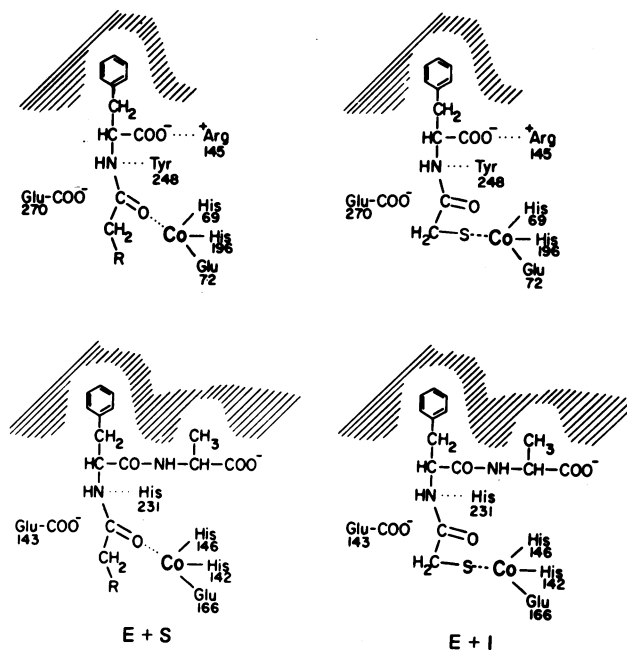


FIG. 4. Representation of the binding of mercaptan inhibitors to the active sites of carboxypeptidase A (Upper) and thermolysin (Lower) and comparison to proposed modes of substrate binding inferred from x-ray crystallographic studies.

the metal appears to undergo a simple displacement reaction involving a water molecule. However, the data characterizing the interaction of these inhibitors cannot be extrapolated to that of substrates in all details.

Substrate-binding modes proposed for thermolysin and carboxypeptidase A (40, 41) generally indicate that the zinc atom activates the substrate by polarizing the susceptible carbonyl bond via Lewis acid catalysis; the remainder of the substrate is thought to align with enzyme subsites, thereby affecting interaction of the carbonyl oxygen with the zinc atom. The agents under discussion appear to inhibit by directing an effective anionic ligand, $-O^-$ or $-S^-$, to the metal to substitute for the carbonyl oxygen of the substrate. However, the data do not reveal the coordination geometry of possible transition state intermediates, which might become apparent from analogous studies of veritable substrates.

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