Synthesis, characterization and properties of chromium(III) complex [Cr(SA)(en)$_2$]Cl $\cdot$ 2H$_2$O

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Abstract

The reaction of chromium(III) chloride, salicylic acid (SA) and ethylenediamine (en) led to the formation of chromium complex [Cr(SA)(en)$_2$]Cl $\cdot$ 2H$_2$O(1). The crystal structure belongs to monoclinic system with the space group P2(1), $R_1 = 0.0358$. In this compound, Cr(III) atom is six-coordinated in octahedral coordination geometry by one phenolic hydroxyl oxygen, one carboxylate oxygen from the salicylic acid and four nitrogen atoms from two ethylenediamine molecules, respectively. The transfer manners of Cr(III) from the title compound to the low-molecular-mass chelator, ethylenediamine-$N,N,N^0,N^0$-tetraacetic acid (EDTA) and the iron-binding protein apovotransferrin (apoOTf) were followed by a combination of UV–visible (UV–Vis) and fluorescence spectra in 0.01 M Hepes at pH 7.4. The results show that Cr(III) can be transferred from the complex to apovotransferrin with the retention of the salicylate acted as a synergistic anion.

Keywords: Chromium(III); Salicylic acid; Apovotransferrin; Transfer

1. Introduction

In the last 15 years, nutritional studies have suggested that chromium(III) may have an essential role in mammals. Chromium is known to activate enzymes, maintain protein stability and enhance carbohydrate metabolism [1–5]. Chromium(III) picolinate, Cr(pic)$_3$, has been the most thoroughly studied of these synthetic products and has become a very popular nutritional supplement [6]. However, the effect of Cr(pic)$_3$ has been extremely contentious. Of particular concern are recent reports that the complex can efficiently cleave DNA in the presence of biological reducing agents and can induce strand breaks in chromosomes of intact cells [7–10]. Hence, a search has been underway to identify the biologically active form of chromium, that is, the biomolecule that binds chromium(III) and possesses an intrinsic function associated with insulin action in mammals [11,12].

Up to date, the mechanisms of absorption of chromic ions are uncertain. Little is known of the fate of Cr(III) intaken orally. Essentially no data exist on the forms of chromium(III) in food as a result of its very low concentration [13]. The fate of chromium(III) once it enters the bloodstream is better elucidated. The iron-transport protein transferrin has been proposed to serve as the major chromium transport agent [6]. Vincent has studied the interaction of Cr(pic)$_3$ with transferrin by UV–Vis spectrum [14,15]. In order to explore the mechanism of chromium(III) absorption, one novel chromium(III) complex [Cr(SA)(en)$_2$]Cl $\cdot$ 2H$_2$O was synthesized, and then the manner, in which chromium is transferred from the complex to apovotransferrin (apoOTf), was followed by a combination of UV–visible (UV–Vis) and fluorescence spectra. As a control experiment, EDTA was employed first as a simple competition agent at different temperature. The ligand...
salicylic acid was chosen originally because of its low toxicity and the similar structure with that of aspirin.

2. Experiments

2.1. Materials and physical measurements

All manipulations were performed under aerobic conditions, and all chemicals were all analytical grade reagents and were used without further purification. Apovotransferrin was obtained from Sigma (Lot 115H7080). Deionized water was used throughout. All glassware, including absorption and fluorescence cuvettes (1 cm), were routinely soaked in 1 M HNO₃ and then rinsed with deionized water.

UV–visible (UV–Vis) spectra were measured with a HP8453 UV–Vis spectrophotometer. Fluorescence spectra were measured with a Hitachi 850 fluorescence spectrophotometer. The temperature of the solutions was maintained at 15 or 37 °C by a jacketed cell holder connected to an external circulating water bath (Shimadzu TB-85 or Huber). Chemicals for carbon, hydrogen, and nitrogen were determined by microanalysis using a Perkin–Elmer 240B elemental analyzer. The X-ray data were collected on a Smart Apex CCDX diffractometer.

2.2. Preparations of protein samples

Apovotransferrin was purified according to the literature [16]. The final concentration was determined from the absorbance at 278 nm using an extinction coefficient of 91 200 M⁻¹ cm⁻¹.

2.3. Synthesis of the complex

CrCl₃·6H₂O (0.67 g, 2.5 mM), salicylic acid (SA) (0.34 g, 2.5 mM) and granular (Mesh size 20) zinc (0.10 g, 1.5 mM) was added into methanol (30 mL) and then was refluxed for 1 h. To the mixture ethylenediamine (en) was refluxed for 15 min as a pink precipitate formed in >70% yield. The precipitate was filtered, washed with methanol and dried. The dried powder was dissolved in water and an X-ray quality crystal was obtained after two days at room temperature.

2.3.1. Analysis

Calc. for C₁₁H₂₄O₅N₄ClCr (%): C, 34.78; H, 6.32; N, 14.76. Found (%): C, 34.57; H, 6.23; N, 14.83.

2.4. X-ray structure determination of CrC₁₁H₂₄O₅N₄Cl₂H₂O

A single crystal of dimensions 0.40 × 0.20 × 0.20 mm was mounted on a glass fiber and used for data collection. Cell constants and an orientation matrix for date collection were obtained by least-squares refinement of diffraction date from reflections with 2.44–26.81° using a Bruker SMART APEX CCD automatic diffractometer. Date were collected at 293 K using Mo Ka radiation (λ = 0.71073 Å) and the ω-scan technique and corrected for Lorentz and polarization effect (SADABS).

The structure was solved by direct methods (SHELX97) [17] and subsequent differences Fourier map and then refined on F² by a full-matrix least-squares procedure using anisotropic displacement parameters [18]. After several cycles of refinement, the hydrogen atoms of ethylenediamine and salicylic acid legends were located at their calculated positions and were refined using a riding model, with Csp³–H = 0.97 Å, Csp²–H = 0.93 Å and Nsp²–H = 0.90 Å, and with Ueq(H) = 1.5 Ueq(parent atom). The hydrogen atoms of the water solvate molecules were located in a difference Fourier map and constrained to ride on their parent atoms, with O–H = 0.85 Å and Ueq(H) = 1.2 Ueq(O). Atomic scattering factors are from International Tables for X-ray Crystallography [19] and molecular graphics from SHELXL [20]. A summary of the crystal date, experiment details and refinement results is given in Table 1.

3. Results and discussion

3.1. Description of the structure of [Cr(SA)(en)₂]Cl·2H₂O

The structure of [Cr(SA)(en)₂]Cl·2H₂O was determined by X-ray crystallography. The perspective structure and the atomic numbering schemes for the chromium complex are shown in Fig. 1. Selected bond lengths and angles are given in Table 2.
The main structure of the title compound is made up of one [Cr(SA)(en)\(_2\)]\(^{+}\) complex cation, one Cl\(^-\) and two water solvate molecules. The chromium(III) center is an octahedron with four nitrogen atoms (Cr–N, 2.064–2.095 Å) from two en molecules, one phenolic hydroxyl oxygen (Cr–O, 1.903 Å) and carboxylate oxygen (Cr–O, 1.941 Å) from SA ligand. These two oxygen atoms and two nitrogen atoms occupy the equatorial position and the other nitrogen atoms occupy the axial position. The complex unit consists of one six-member ring (C–C–O–Cr–O–C) and two five-member rings (C–N–Cr–N–C) in the molecule.

3.2. Fluorescence and UV–Vis spectra of the complex

In Fig. 2, the characteristic absorption peak of the salicylic acid occurs at 298 nm (curve a), while in the complex this peak exhibits red shift to 326 nm (curve b). The electronic spectrum in the visible region of the complex (curve c, attributed to d–d transitions) is altered in intensity and shifts in position of the adsorption bands relative to the corresponding Cr(III) aquo ions.

Salicylic acid contains carboxylic group and phenolic hydroxyl (HOOC\(_6\)H\(_4\)OH, the pK values of SA are 2.97 and 13.40 for the carboxylic and phenol groups) [21]. Fig. 3 displays the fluorescence spectrum of salicylic acid in 0.01 M Hepes buffer at pH 7.4, with a maximum emission peak near 410 nm (curve b). With the coordination

<table>
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<th>Table 2</th>
<th>Selected bond lengths (Å), angles (°)</th>
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</tr>
<tr>
<td>N3–Cr1–N2</td>
<td>91.87(11)</td>
</tr>
</tbody>
</table>

The main structure of the title compound is made up of one [Cr(SA)(en)\(_2\)]\(^{+}\) complex cation, one Cl\(^-\) and two water solvate molecules. The chromium(III) center is an octahedron with four nitrogen atoms (Cr–N, 2.064–2.095 Å) from two en molecules, one phenolic hydroxyl oxygen (Cr–O, 1.903 Å) and carboxylate oxygen (Cr–O, 1.941 Å) from SA ligand. These two oxygen atoms and two nitrogen atoms occupy the equatorial position and the other nitrogen atoms occupy the axial position. The complex unit consists of one six-member ring (C–C–O–Cr–O–C) and two five-member rings (C–N–Cr–N–C) in the molecule. [Cr(SA)(en)\(_2\)]Cl\(_2\) \cdot 2H\(_2\)O belongs to monoclinic system with the space group \(P2(1)\), \(a = 8.354(2)\) Å, \(b = 9.856(3)\) Å, \(c = 10.120(3)\) Å, \(\beta = 94.045(3)\)°, \(V = 831.2(4)\) Å\(^3\) and \(Z = 2\). There are four kinds of intermolecular hydrogen bonds formed which stabilize the conformation with the distance in the ranges of 3.068–3.114 Å for O–H· · ·O, 3.165 Å for O–H· · ·Cl, 2.907–3.012 Å for N–H· · ·O and 3.268–3.600 Å for N–H· · ·Cl, respectively.
of salicylic acid to Cr(III) the fluorescence intensity at 410 nm is quenched and the title complex hardly has any fluorescence in the same condition, as shown in Fig. 3 (curve a).

3.3. Transfer of chromium

3.3.1. The competition with EDTA

Chromium levels in tissues and biological fluids are extremely low, generally within an order of magnitude of the detection limits of current analytical techniques. The common bioinorganic probes for Cr(III) are of limited utility [6]. As described above, the spectra are sensitive to the changes of the coordination sphere Cr(III) center for [Cr(SA)(en)2]⁺, especially such as the dissociation of SA, give rise to significant changes in the complex’s absorption and fluorescence spectra. Therefore it enables us to follow the reactivity of [Cr(SA)(en)2]⁺ towards biomolecule in a low concentration by those spectra. In order to research the interaction of the complex with transferrin, EDTA was employed as a simple competitive ligand first.

To examine the transfer of Cr(III) from [Cr(SA)(en)2]⁺ to EDTA, 10 equiv. of EDTA was added to 10 µM [Cr(SA)(en)2]⁺ in 0.01 M Hepes, pH 7.4. The reaction mixture was stored at 37 °C. Both the UV–Vis spectra and fluorescence spectra were monitored as a function of time until the spectra became constant with time. The UV–Vis spectra are shown in Fig. 4. It can be seen that the absorption peaks at 326 nm for the coordinated SA decrease and the peaks at 298 nm for free SA increase gradually. There are two isosbestic points at 310 nm and 280 nm, respectively. Curves of absorbance at 326 nm and 298 nm, A326 vs. time and A298 vs. time for the reaction are shown in Fig. 4 inset. It is obvious that the complex [Cr(SA)(en)2]Cl is decomposed by EDTA, meanwhile, salicylic acid ligand is free from Cr(III) gradually. The rate constant is calculated to be \( (2.20 \pm 0.05) \times 10^2 \text{ M}^{-1} \text{ h}^{-1} \).

The fluorescence spectra are shown in Fig. 5. Sample was excited at 280 nm and the emission was monitored from 350 nm to 480 nm. The characteristic fluorescence intensity of SA at 410 nm increase gradually. Curve of \( F_{410} \) vs. time for the reactions at different temperature are displayed in Fig. 5 inset, the rate constant at 37 °C is \( (1.90 \pm 0.03) \times 10^2 \text{ M}^{-1} \text{ h}^{-1} \). It shows that SA ligand is released from the complex gradually. In contrast, the absorbance spectra of the complex [Cr(SA)(en)2]Cl is stable in Hepes buffer at 37 °C. Furthermore, the rate constants obtained from the two methods are in good agreement, so it well indicates that Cr(III) are combined to EDTA, and SA or en ligands are competitively replaced. This procedure can be briefly illustrated by the following sketch:

\[
[\text{Cr(SA)(en)2}]^+ \xrightarrow{\text{EDTA}} \text{Cr(EDTA)} + \text{SA} + 2\text{en}
\]

3.3.2. The competition with apoovotransferrin (apoOTf)

The manner in which chromium is transported in organism is unknown. The iron-transport protein transferrin, the second most abundant protein in blood serum, has been proposed to serve as the major chromium transport agent. A molecule of transferrin (~80 kDa) is made up of two similar halves, the N- and C-lobes, joined by a bridging peptide. Each lobe is comprised of two domains, the N I and N II for the N-lobe and C I and C II for C-lobe, which form a cleft containing a high-affinity binding site for ferric ion and other ions. The ferric ion is bound in a distorted coordination to four protein ligands (in human transferrin by Asp63, Tyr95, Tyr188 and His249 for the N-lobe, or by

![Fig. 4. Absorption spectra at different time for the mixture of 10 µM [Cr(SA)(en)2]⁺ with 10 equiv. of EDTA, 37 °C, 0.01 M Hepes, pH 7.4, (h) 1, 0; 2, 50; 3, 100; 4, 150 h. Inset: curves of absorbance at 296 nm and 326 nm with time.](image)

![Fig. 5. Fluorescence changes with time (a → d) for the mixture of 10 µM [Cr(SA)(en)2]⁺ with 10 equiv. of EDTA at 15 °C in 0.01 M Hepes, pH 7.4, (h) 1, 0; b, 4; c, 30; d, 50 h. Inset: curves of fluorescence at 410 nm with time at different temperature, 1, 37 °C; 2, 15 °C.](image)
Asp392, Tyr426, Tyr517 and His585 for the C-lobe) and two ligands from a synergistically bound, bidentate anion \([22–24]\). Binding constants of chromium to transferrin were reported to be \(K_1 = 1.42 \times 10^{10} \text{M}^{-1}\) and \(K_2 = 2.06 \times 10^{7} \text{M}^{-1}\). In this paper, apoovotransferrin (apoOTf) was utilized in place of serum transferrin because of its ready availability in quantity and its cost; the binding properties of apoOTf are nearly identical to serum transferrin \([14]\). ApoOTf can bind Fe(III) and other metal ions tightly \([25,26]\) in the presence of synergistically bound anion that is usually carbonate. The UV–Vis spectra of Cr(III)-OTf reported previously are similar to that of Cr(III)-saturated transferrin \([15]\). The addition of Cr(III) to apoOTf results in enhancement of the intensity of the UV absorption bands at ca. 240 and 291 nm (arising in part from tyrosine residues which serve as metal ligands) as a result of Cr(III) binding \([15]\).

Furthermore, fluorescence quenching measurements can also be used to monitor metal binding \([23]\). The fluorescence is dominated by emission from Trp residues, but the fluorescence may be quenched when a cation, such as a transition metal or a lanthanide ion, is bound nearby. In the paper, titration of CrCl\(_3\) to apoOTf was monitored by fluorescence spectra at 20 °C in 0.01 M Hepes buffer at pH 7.4. When the metal–protein binding had been equilibrated after 30 min, a fluorescence spectrum was recorded and a new aliquot of titration was added. To correct for dilution during each titration and normalize the results from different titration, the fluorescence intensity was converted to molar fluorescence intensity (FM) by dividing the fluorescence intensity by the analytical concentration of apoOTf, as shown in Fig. 6. Highly effective quenching occurred at 336 nm in this titration, fluorescence intensity of apoOTf decreased to about 50%. From Fig. 6, it can be seen that one apoOTf molecule binds approximately 2 equiv. of Cr(III). Such behavior arises from the interaction of the Cr(III) with the hydrophobic pockets in the protein structure (harboring the tryptophan residues), thus leading to the energy transfer quenching of tryptophan fluorescence. Whereas quenching was scarcely observed in the titration of salicylic acid to apoOTf in the same condition, meanwhile, the fluorescence intensity at 410 nm increased regularly with the increasing of concentration of salicylic acid. Thus, for apoOTf, both changes in fluorescence spectrum (336 nm) and the UV–Vis spectrum at ca. 240 and 291 nm can be used to investigate the transfer of chromium from the complex \([\text{Cr(SA)(en)2}]\text{Cl}\) to protein. For convenience, some characteristic fluorescence peaks or UV–Vis absorption peaks appeared in this paper are listed in Table 3.

First, solution of 18 \(\mu\text{M} [\text{Cr(SA)(en)2}]^+\) with 1 equiv. of apoOTf in 0.01 M Hepes, pH 7.4 was excited at 280 nm at 37 °C. The fluorescence of apoOTf at 336 nm (characteristic of tryptophan residue) is quenched with time slowly (a → c), as shown in Fig. 7. Curve of \(F_{336}\) vs. time for the reaction is given in Fig. 7 inset. The quenching phenomenon at 336 nm results from the decomposition of the complex and combination of Cr(III) to the protein. The rate constant at 37 °C is calculated to be \((5.02 \pm 0.05) \times 10^2 \text{M}^{-1} \text{h}^{-1}\). The peak at 410 nm does not appear as

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<td><strong>Characteristic fluorescence peaks or UV–Vis absorption peaks</strong></td>
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<td>Fluorescence peak (\lambda_{\text{max}}) (nm)</td>
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<tr>
<td>Free-salicylate</td>
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<tr>
<td>Coordinated-salicylate</td>
</tr>
<tr>
<td>apoOTf</td>
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Fig. 7. Fluorescence spectra at different time (a → c) for the reaction of 18 \(\mu\text{M} [\text{Cr(SA)(en)2}]^+\) with 1 equiv. of apoOTf at 37 °C in 0.01 M Hepes at pH 7.4. Inset: the changes of fluorescence at 336 nm with time.

Fig. 6. Titration curve for the addition of CrCl\(_3\) to apoOTf at 20 °C in 0.01 M Hepes, pH 7.4.
expectation, although the same concentration of SA with apoOTf exhibits a strong fluorescence peak at 410 nm (shown in Fig. 8).

Fig. 9 shows the difference UV spectra of the mixture of 18 µM [Cr(SA)(en) 2] + with 1 equiv. of apoOTf at 37 °C blanked as protein solution. With time going it reveals a significant enhancement in the absorbance at 291 and 240 nm. To be surprising, the peak at 326 nm nearly does not decrease. Curve of $A_{291}$ vs. time for the reaction is shown in Fig. 9 inset. The increase may be attributed to the binding of Cr(III) to residues of the protein. The rate constant at 37 °C is calculated to be $(5.02 \pm 0.05) \times 10^2 \text{M}^{-1}\text{h}^{-1}$. It is well consistent with the result from fluorescence spectra approximately, meaning that Cr(III) has transferred from the title complex to apoOTf and the Cr–apoOTf bonds are formed.

However, neither concomitant appearance of free salicylate fluorescence at 410 nm nor any decrease in the intensity of the Cr(III)-salicylate charge transfer band at 326 nm are observed throughout this competition reaction. These interesting results allow us to infer that SA ligand is not replaced by apoOTf in the transfer of Cr(III) to the protein. This may bring about some questions, such as the fate of en and SA ligands. To identify it, some control experiments were carried out.

It is impossible to observe directly the free en ligands in the reaction mixture only by UV–Vis spectra or fluorescence spectra. In this paper [Cr(en) 3]Cl 3 was employed to react with apoOTf in an attempt to uncover the fate of en ligands. Two equivalents of [Cr(en) 3]Cl 3 was added to 14 µM apoOTf in 0.01 M Hepes, pH 7.4. Sample was allowed to incubate at 37 °C. Both the UV–Vis spectra and fluorescence spectra were monitored as a function of time. The UV–Vis spectra changes are depicted in Fig. 10. Curve of $A_{291}$ vs. time for the reaction is shown in Fig. 10 inset. The spectra also show enhancement of the intensity of the UV–Vis absorption bands at ca. 240 and 291 nm. The rate constant at 37 °C is calculated to be $(2.15 \pm 0.03) \times 10^3 \text{M}^{-1}\text{h}^{-1}$. When the chromium complexes (CrCl 3 [15], [Cr(en) 3]Cl 3 and [Cr(SA)(en) 2]Cl) react with apoOTf, they all exhibit very similar UV–Vis spectra in the range of 240–300 nm, and either of the fluorescence intensity of apoOTf at 336 nm in those three reactions is quenched gradually. Based on the coordination demands or binding abilities of the apoOTf site, it can be proposed...
that two en ligands are dissociated during the transfer of Cr(III) from [Cr(SA)(en)2]Cl to apoOTf.

Then, 1 equiv. of [Cr(SA)(en)2]Cl was pre-incubated with 18 μM apoOTf in 0.01 M Hepes, pH 7.4 at 37 °C for 8 days (period I). Then excessive EDTA was added to the mixture and was stored at 37 °C again (period II). Both the UV–Vis spectra and fluorescence spectra were monitored as a function of time through the course, as shown in Figs. 11 and 12, respectively. Sample is referred for simplicity as [Cr(SA)-apoOTf] + EDTA. In period II with the addition of 250–2500 equiv. of EDTA, the Cr(III)-salicylate charge transfer band (λmax = 326 nm) and the UV–Vis absorption bands at ca. 240 and 291 nm all decrease; the peaks at 298 nm for the free SA appear (Fig. 11). Meanwhile, the quenched fluorescence intensity of apoOTf at 336 nm is restored, with the appearance of fluorescence of free SA at 410 nm (Fig. 12). All these phenomena clearly show the coordinated SA to Cr(III) bound by apoOTf is replaced by excessive EDTA in period II and apoOTf or SA molecules are free from Cr(III). In other words, a very stable complex [Cr(SA)-apoOTf] is formed in period I and then destroyed by excessive EDTA in period II.

A number of literatures have indicated that dozens of organic anions (including salicylate) could substitute for carbonate as the synergistically bound anions in iron–transferrin complex [27,28]. A carboxylate group and a proximal polar group (hydroxy, keto, amino, or carboxylate) within 6.3–7.0 Å of the carboxylate are required for synergistic binding. Dubach and his coworkers used EPR spectra to examine the mode of synergistic anion binding. It is proposed that these anions behave as bidentate ligands, with coordination to the iron through both the carboxylate and proximal polar group [29]. Salicylate–Fe(III)–transferrin complex shows characteristic charge transfer band in the visible spectrum with λmax = 450 nm, implying direct metal–anion bonding. The salicylate anion readily forms a chelate ring and is proposed to have the same function as the synergistically bound anion. This theory is favored by the results obtained in this paper. However, en molecular does not fit for the demands of synergistically bound, and it is easily replaced by apoOTf. Based on the discussion above, it can be inferred in reason that salicylate serves as the role of synergistically bound anion when Cr(III) is transferred from the title complex to the protein and the salicylate–Cr(III)–transferrin ternary complex is formed. Perhaps the summing-up can be illustrated in Scheme 1.

Vincent and co-workers [14] has studied the interaction of Cr(pic)3 with apoOTf in 5% DMSO and 95% Tris (0.05 M, pH 7.5) by UV–Vis spectrum. It showed that the Cr(III) could not be transferred from Cr(pic)3 to apoOTf unless the metal was reduced to the Cr(II) level. In contrast, [Cr(SA)(en)2]3+ is relatively easy to release [Cr(SA)]3+ to apoOTf and forms a very stable complex salicylate–Cr(III)–transferrin.
4. Conclusions

This paper describes the synthesis and characterization of the complex \([\text{Cr(SA)(en)}_2]\text{Cl} \cdot 2\text{H}_2\text{O}\) and the reaction with apoovotransferrin. The mechanisms of transfer of chromium from the complex to EDTA and apoOTf are different. Competition studies show that Cr(III) can be transferred from the complex to apoovotransferrin, with the retention of the salicylate, it is inferred first by the author that a very stable ternary complex salicylate–Cr(III)–transferrin was formed. The use of salicylic acid is desirable for further studies to test the role of chromium metabolism.

5. Abbreviations

- Hepes: \(N\)-2-hydroxyethyl-piperazine-\(N'\)-2-ethansulfonic acid
- EDTA: ethylenediamine-\(N,N,N',N'\)-tetraacetic acid
- apoOTf: apoovotransferrin
- SA: salicylic acid
- en: ethylenediamine

6. Supplementary materials

Crystallographic data for the compound has been deposited with the Cambridge Crystallographic Data Centre, CCDC Nos. 289525. Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK, fax: +44 1223 336 033, e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk.

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