



A thiabendazole sulfonamide shows potent inhibitory activity against mammalian and nematode α -carbonic anhydrases \star

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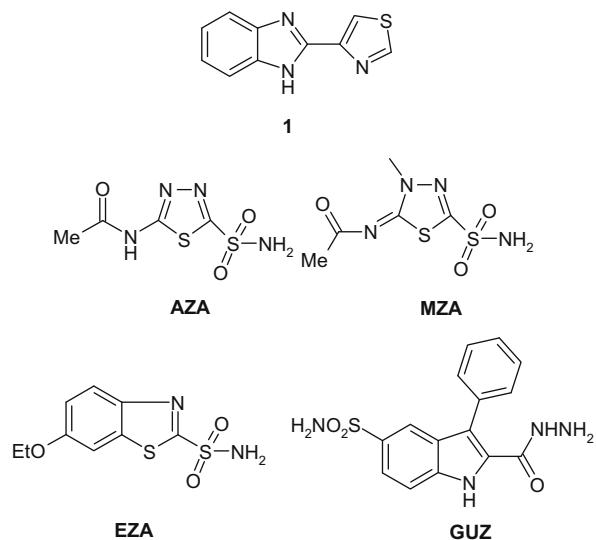
ABSTRACT

A sulfonamide derivative of the antihelmintic drug thiabendazole was prepared and investigated for inhibition of the zinc enzyme carbonic anhydrase CA (EC 4.2.1.1). Mammalian isoforms CA I–XIV and the nematode enzyme of *Caenorhabditis elegans* CAH-4b were included in this study. Thiabendazole-5-sulfonamide was a very effective inhibitor of CAH-4b and CA IX (K_i s of 6.4–9.5 nm) and also inhibited effectively isozymes CA I, II, IV–VII, and XII, with K_i s in the range of 17.8–73.2 nM. The high resolution X-ray crystal structure of its adduct with isozyme II evidenced the structural elements responsible for this potent inhibitory activity.

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Thiabendazole **1** is a broad-spectrum antihelmintic used for the treatment of parasitic infections in animals and humans and as an agricultural fungicide for postharvest treatment of fruits and vegetables, as it inhibits the growth of many pathogenic and saprobic fungi, such as among others *Aspergillus* spp., *Cladosporium cladosporioides*, *Cunninghamella echinulata*, *Fusarium roseum*, *Gliocladium* sp., *Mortierella isabellina*, *Mucor plumbeus*, *Rhizopus arrhizus*, and *Trichothecium roseum*.^{1,2} The mechanism of action of this drug is poorly understood but at least its antihelmintic actions seems to be due to its selective binding to the parasite β -tubulin with subsequent prevention of microtubule formation.³

Helminths and other nematodes infect 25% of the world's population.⁴ In addition to the widespread parasitic nematode species *Onchocerca volvulus*, *Wuchereria bancrofti*, and *Brugia malayi*, for which few effective therapies are presently known,⁵ *Mansonella perstans* filariasis is widely present in Africa and equatorial America.^{5a} *Strongyloides stercoralis*, an intestinal nematode acquired in the tropics or subtropics, is emerging as a widespread infection in the Western countries.^{5b} Although there are effective drugs for some of these infections (thiabendazole is effective against larva migrans, mebendazole for ascariasis, trichiuriasis and hookworms,



albendazole for inoperable cases of cystic hydatid disease, diethylcarbamazine for *Toxocara* induced visceral larva migrans and loiasis, ivermectin for onchocerciasis, praziquantel for schistosomiasis and niridazole for *Dracunculus medinensis*)^{4,5} the widespread resistance to many of them,⁶ both in humans and animals, may lead to

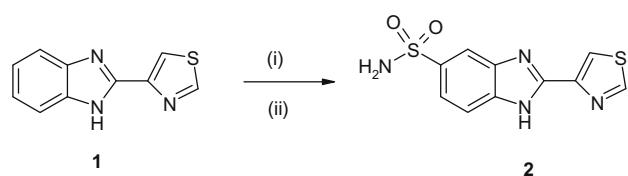
\star The X-ray coordinates of the hCA II-thiabendazole-5-sulfonamide adduct are available in PDB with the ID 3FFP.

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serious medical problems. It is thus critically important to design agents targeting other metabolic pathways in these organisms, which may circumvent the resistance/toxicity problems of the currently used antihelmintics. One of the enzymes present in many worm species is the carbonic anhydrase, CA (EC 4.2.1.1).^{5c} Indeed, CAs are widespread all over the phylogenetic tree, with several different evolutionarily unrelated gene families encoding them.^{7–10} CAs are catalysts for the interconversion of carbon dioxide and bicarbonate and are involved in pH regulation and function in several metabolic pathways.^{7–10} In mammals there are 16 CAs known to date. These include several cytosolic isoforms (CA I–III, CA VII, and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial forms (CA VA and VB), as well as one secreted CA isozyme, CA VI. Three acatalytic isozymes are also known, that is, CA VIII, CA X, and CA XI.^{7–10} In mammals, these enzymes are involved in crucial physiological processes connected with respiration and transport of CO₂/bicarbonate between metabolizing tissues and lungs, pH and CO₂ homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureogenesis), bone resorption, calcification, tumorigenicity, and several other physiologic/pathologic processes.^{7–13}

Sulfonamide CA inhibitors (CAIs) such as acetazolamide **AZA**, methazolamide **MZA** or ethoxzolamide **EZA** among others, are clinically used agents for the management of a variety of disorders connected to CA disbalances, such as glaucoma^{7,8}, in the treatment of edema due to congestive heart failure,¹¹ or for drug-induced edema; as mountain sickness drugs,¹¹ whereas other agents of this pharmacological class show applications as anticonvulsants,¹² antiobesity,⁷ or antitumor drugs/tumor diagnostic agents.^{7,10} As there are few isoforms-selective inhibitors to date, many new sulfonamides (such as **GUZ** exemplified here)¹⁴ are constantly being reported in the search of derivatives with better inhibition profiles as compared to the promiscuous, first generation inhibitors such as **AZA**, **MZA**, or **EZA**.⁷

There are only a few reports regarding the CAs present in nematodes.^{5c} An α -CA, subject to environmental pH regulation denominated CAH-4b (or ceCA) was recently cloned and characterized in the model organism *Caenorhabditis elegans* (which contains at least 6 CA isoforms) by Hall et al.¹³ In addition, DeRosa et al. investigated another α -CA isoform homologous to *C. elegans* CAH-6 (78% homology) and hCA III (55% homology) for its involvement in exsheathment of *Ostertagia ostertagi* nematodes, that infect intestines of cattle.¹⁵ Although DeRosa et al. did not directly confirm that this CA isoform was involved in the process of exsheathment, the transcriptional regulation of the enzyme suggests that it may function in developmental processes related to this.¹⁵ Furthermore, the strong α -CA inhibitor ethoxzolamide (**EZA**) is useful as an anti-infective in cattle infected with *Ostertagia ostertagi*.¹⁵ CAH-4b was shown by our group to be susceptible to inhibition by sulfonamides such as **AZA**, **MZA**, and **EZA**.¹³ Thus, considering the fact that nematodes contain CAs in their genome (although scarcely investigated to date) and the fact that these enzymes seem to be susceptible to inhibition by sulfonamides, we decided to incorporate the sulfamoyl moiety responsible for the binding to the zinc ion from the CA active site, in the molecule of the antihelminthic compound **1**. The rationale for this drug design study is the following one: (i) we hypothesize that the thiabendazole scaffold (responsible for the antihelminthic biological activity of **1**) of the sulfonamide derivative may bind to β -tubulin similarly with the parent derivative **1**; (ii) the additional sulfonamide group should lead to interactions with CAs present in the parasite. Obviously, we are also interested in this new sulfonamide scaffold for its interaction with mammalian CA isoforms, which as mentioned above, constitute drug targets for many types of applications.



Scheme 1. Preparation of sulfonamide **2** from thiabendazole **1**. Reagents and conditions: (i) ClSO₃H at 0 °C; (ii) aqueous NH₃, room temperature.

2-(4'-Thiazolyl)-1*H*-benzimidazole (thiabendazole) **1** was treated with chlorosulfonic acid and the obtained sulfonyl chloride with ammonia, leading to the 5-sulfamoyl derivative **2** (Scheme 1).¹⁶ Only one sulfonamide isomer (**2**) has been obtained in good yield, by the sulfamoylation illustrated in Scheme 1, without the need to protect/deprotect the endocyclic NH from the benzimidazole ring. Sulfonamide **2** has been investigated for the inhibition of mammalian CA isozymes hCA I–XIV as well as the nematode CA from *C. elegans*, ceCA (Table 1).¹⁷ Inhibition data of the standard compounds **AZA**, **MZA**, and **EZA**,⁷ as well as that of the bicyclic sulfonamide **GUZ**¹⁴ are also provided in Table 1, for comparison reasons.^{18–22}

The following should be noted regarding the inhibition data of Table 1:

- (i) Thiabendazole-5-sulfonamide **2** behaved as a strong inhibitor of isoforms hCA I, IV, VB, VI, IX, XII, and ceCA, with inhibition constants in the range of 6.4–50.9 nM. Especially potent inhibition ($K_i < 10$ nM) was observed for hCA IX and the nematode enzyme ceCA. Indeed, **2** is the most potent nematode CA inhibitor evidenced so far,¹³ being 3.7-fold more efficient than **AZA**, the compound showing the second most potent inhibition against ceCA, discovered in our first study on this enzyme.¹³
- (ii) Isoforms hCA II, hCA VA, and hCA VII were also efficiently inhibited by **2**, with K_i values in the range of 72.5–73.2 nM. These data are indeed very interesting, since usually CA II and VII are the isoforms with the highest affinity for sulfonamide inhibitors such as **AZA**, **MZA**, and **EZA** (Table 1). In the case of **2**, other isoforms than hCA II and VII are much better inhibited, as shown above.

Table 1

Inhibition data with some of the clinically used sulfonamides **AZA**–**GUZ** and **2** against mammalian isozymes I–XIV (the isoforms CA VIII, X, and XI are devoid of catalytic activity and probably do not bind sulfonamides as they do not contain Zn(II) ions) and nematode enzyme ceCA⁷

Isozyme*	K _i ** (nM)				
	AZA	MZA	EZA	GUZ	2
hCA I ^a	250	50	25	7.5	50.9
hCA II ^a	12	14	8	7.2	72.5
hCA III ^a	2.0 × 10 ⁵	7.0 × 10 ⁵	1.1 × 10 ⁶	1.4 × 10 ⁶	34,500
hCA IV ^a	74	6200	93	9000	48.9
hCA VA ^a	63	65	25	1100	72.7
hCA VB ^a	54	62	19	1100	40.9
hCA VI ^a	11	10	43	2650	25.0
hCA VII ^a	2.5	2.1	0.8	89	73.2
hCA IX ^b	25	27	34	102	6.4
hCA XII ^b	5.7	3.4	22	110	17.8
mCA XIII ^a	17	19	50	2633	4850
hCA XIV ^a	41	43	25	48	424
ceCA	35	42	40	339	9.5

^a Full length enzyme.

^b Catalytic domain.

* h, human; m, murine isozyme.

** Mean value from at least three different measurements.¹⁴ Errors were in the range of $\pm 5\%$ of the obtained value (data not shown).

(iii) mCA XIII and hCA XIV were weakly inhibited by **2** (K_i s in the range of 424–4850 nM), whereas hCA III was the least inhibited isoform among those investigated here, with a K_i of 34.5 μ M.

It may be observed that **2**, similarly to **GUZ**, shows an inhibition profile against various CA isozymes quite different from those of the first generation CA inhibitors such as **AZA**, **MZA**, and **EZA**. Its strong ceCA versus CA II inhibition is obviously due to interactions with different amino acid residues within the two active sites, which have a rather low homology (of around 60%).¹³ These interactions are more favorable with those of the nematode enzyme as compared to the human one (see discussion later in the text). However, as the X-ray crystal structure of ceCA is not yet available, only hypothesis of this differential binding can be made. It should be also noted that whereas **GUZ** is a weak ceCA inhibitor, **2** is a very potent one (Table 1).

In order to have more insights regarding the interaction of the new sulfonamide **2** with the CA active site, the X-ray crystal structure of its adduct with isoform hCA II has been resolved. Crystals of the hCA II–**2** adduct were isomorphous with those of the native protein,^{23,24} allowing for the determination of the crystallographic structure by difference Fourier techniques. The refined structure presented a good geometry with r.m.s.d. from ideal bond lengths and angles of 0.016 \AA and 1.6°, respectively (Table 2). The overall quality of the model was good with all residues in the allowed regions of the Ramachandran plot. Refinement statistics are summarized in Table 2. Inspection of the electron density maps at various stages of the refinement, showed features compatible with the presence of one molecule of inhibitor **2** bound within the active site (Fig. 1). Interactions between the protein and Zn^{2+} ion were entirely preserved in the adduct (data not shown), as in all other hCA II–sulfonamide/sulfamate/sulfamide complexes investigated so far.²³ A careful analysis of the three-dimensional structure of the complex revealed a compact binding between the inhibitor and the enzyme active site, similarly to what was observed earlier for other such complexes, with the tetrahedral geometry of the Zn^{2+} binding site and the key hydrogen bonds between the SO_2NH_2 moiety of the inhibitor and enzyme active site all retained (Figs. 1 and 2).²³ In particular, the ionized nitrogen atom of the sulfonamide group of **2** is coordinated to the zinc ion at a distance of 1.90 \AA . This nitrogen is also hydrogen bonded to the hydroxyl group of Thr199 (N–Thr199OG = 2.79 \AA), which in turn interacts with the Glu106OE1 (N–Glu106OG = 2.79 \AA),

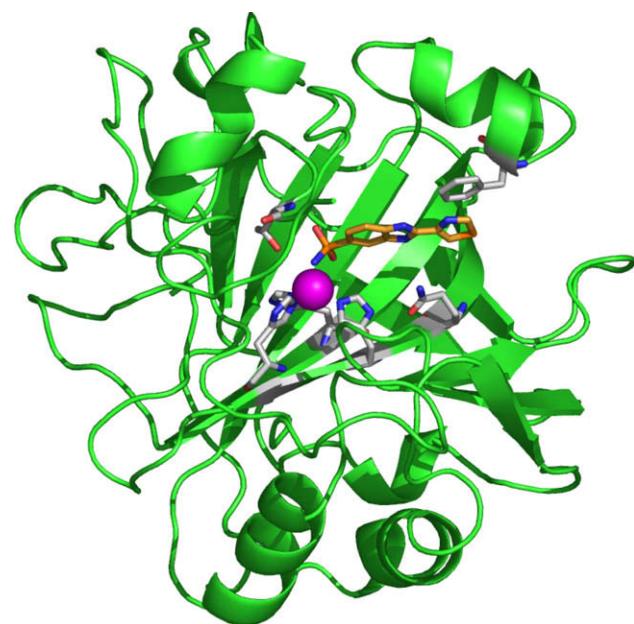


Figure 1. The hCA II–**2** adduct. The enzyme backbone is shown in green, catalytic Zn^{2+} ion as the violet sphere with its three histidine ligands (His94, 96, and 119) evidenced. Inhibitor **2** is shown in gold, whereas amino acid residues involved in its binding (Thr199, Gln92, and Phe131) shown in stick model and CPK colors.

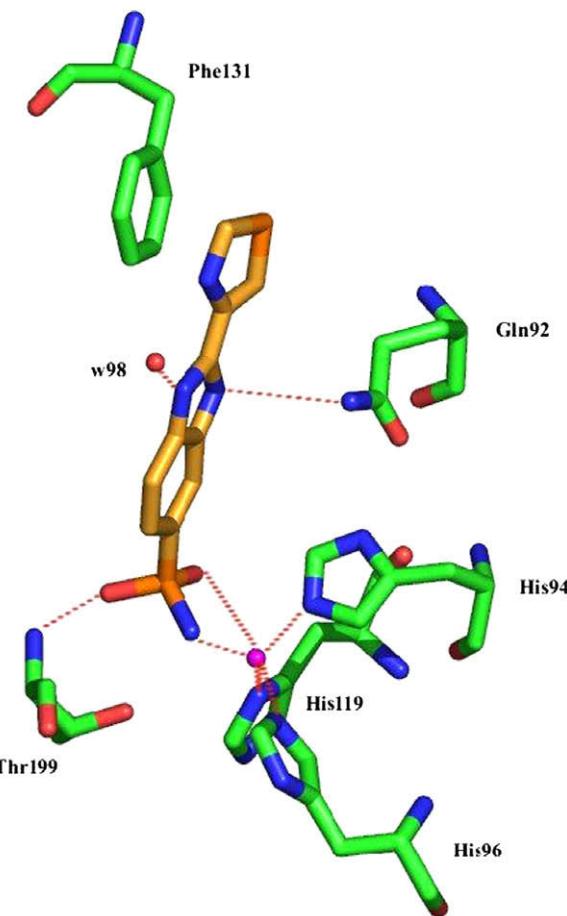


Figure 2. Detailed interactions between **2** (in gold) bound within the hCA II active site and amino acid residues/water molecule involved in its binding, Thr199, Gln92, Phe131, and W98. The Zn^{2+} ion (violet) and its three His ligands (green) are also shown.

Table 2
Crystallographic parameters and refinement statistics of the hCA II–**2** adduct

Parameter	Value
<i>Crystal parameter</i>	
Space group	$P2_1$
Cell parameters	$a = 42.2 \text{\AA}$ $b = 41.4 \text{\AA}$ $c = 72.2 \text{\AA}$ $\beta = 104.5^\circ$
<i>Data collection statistics (20.0–1.81 \AA)</i>	
No. of total reflections	43,090
No. of unique reflections	22,187
Completeness (%) ^a	99.6 (100.0)
$F2/\text{sig}$ ($F2$)	20.9 (3.0)
R -sym (%)	5.5 (31.5)
<i>Refinement statistics (20.0–1.81 \AA)</i>	
R -factor (%)	18.7
R -free (%) ^b	24.0
Rmsd of bonds from ideality (\AA)	0.016
Rmsd of angles from ideality (°)	1.6

^a Values in parenthesis relate to the highest resolution shell (1.87–1.81 \AA).

^b Calculated using 5% of data.



Figure 3. Superposition of the hCA II-**2** adduct (gold, PDB file 3FFP) with the hCA II-**GUZ** adduct (magenta, PDB file 3B4F).^{14a} The protein backbones of the two adducts (green ribbon) are completely superimposable, as are the Zn(II) ions (violet sphere) and their His ligands. Residues Thr199 and Phe131 are also evidenced (CPK colors).

atom (2.50 Å, data not shown). One oxygen atom of the sulfonamide moiety is 3.08 Å away from the catalytic Zn²⁺ ion, whereas the second one participates in a hydrogen bond (of 2.92 Å) with the backbone amide group of Thr199.^{8,13,17} The benzimidazole scaffold of **2** fills the active site channel of the enzyme, participating in several hydrophobic contacts (data not shown) and two strong hydrogen bonds, which stabilize the complex. The endocyclic NH group of the inhibitor makes a hydrogen bond (of 2.70 Å) with a water molecule (W98) whereas the second benzimidazole nitrogen makes a second hydrogen bond (of 3.50 Å) with the CONH₂ moiety of Gln92, an amino acid involved in the binding of many types of inhibitors to the CA II active site.²⁹ The thiazole ring substituting the scaffold of **2** is orientated towards the hydrophobic half of the CA II active⁷ site and makes a π–π stacking with the phenyl moiety of Phe131. Such an interaction has been observed earlier in other hCA II-sulfonamide adducts.^{23,29} These multiple interactions between the hCA II active site and **2** explain its rather effective hCA II inhibitory activity, with a *K*_i of 72.5 nM (Table 1).

Figure 3 shows a superposition between the hCA II adducts of sulfonamide **2** reported here and that of the bicyclic indolesulfonamide **GUZ** reported earlier by this group.¹⁴ These two sulfonamides possess a rather similar bicyclic scaffold (benzimidazole in **2** and indole in **GUZ**) and the sulfamoyl zinc-binding group is also attached in the 5-position in both of them. However, the two compounds differ markedly with regard to the substituents present in the terminal part of the molecule (a phenyl and CONHNH₂ moiety in **GUZ**, and a thiazole ring in **2**). Indeed, the two compounds bind in a very different manner to the enzyme, with only their SO₂NH moieties being superimposable (Fig. 3). Thus, the bicyclic scaffolds of the two inhibitors are tilted by almost 90° with respect to one another, and they also orient towards different parts of the active site: **GUZ** is orientated towards the hydrophilic, whereas the scaffold of **2** towards the hydrophobic half of the CA II active site. Furthermore, the thiazolyl moiety of **2** and the Ph/CONHNH₂ ones of **GUZ** lie in very different parts of the active site when these sulfonamides are bound to the enzyme. Thus, even if these compounds are structurally not very different, their binding to the enzyme varies a lot, and this is reflected

by the 10-times better inhibitory activity of **GUZ** compared to **2** towards CA II (Table 1). Indeed, **GUZ** makes many more favorable interactions with hCA II as compared to **2**.

As the entrance of the active site cavity in the various CA isoforms is the region with least conserved amino acid residues among the investigated isozymes,^{7,23} this may explain the diverse inhibition profiles observed with sulfonamides as discussed here (Table 1). Indeed, compounds without a bulky tail orientated toward the exit of the active site cavity, such as **AZA**, **MZA**, and **EZA**, indistinctly inhibit all CA isoforms with high efficacy (usually with inhibition constants <100 nM), leading to promiscuous, unselective CAIs. On the contrary, compounds possessing bulkier moieties that bind towards the exit/edge of the active site cavity (such as **GUZ** and **2**) may interact differently with the various amino acid residues present in those regions show levels of selectivity for inhibition of certain CA isozymes, as exemplified here by these compounds. However, as the X-ray crystal structure is not available yet for all CA isoforms (i.e., CA VB, VI, VII, IX, XIII, and XV)⁷ the nature and number of these amino acid residues responsible for the selective inhibition of CA isozymes is still unknown.

In conclusion, we have prepared a sulfonamide derivative of the antihelmintic drug thiabendazole and investigated its ability to inhibit mammalian CA isoforms (CA I–XIV) and the nematode enzyme CAH-4b from *C. elegans*. Thiabendazole-5-sulfonamide was a very effective inhibitor of CAH-4b and CA IX (*K*_is of 6.4–9.5 nM) and also inhibited effectively isozymes CA I, II, IV–VII, and XII, with *K*_is in the range of 17.8–73.2 nM. The high resolution X-ray crystal structure of its adduct with isozyme II evidenced the structural elements responsible for this potent inhibitory activity.

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16. Thiabendazole (1.97 mmol) was cooled to 0 °C in a crushed ice bath and treated dropwise with chlorosulfonic acid (0.65 mL, 9.8 mmol), under stirring. The resulted homogeneous mixture was heated under stirring at 70–80 °C for 5 h, cooled and poured on ice. The crude sulfonyl chloride was treated with ammonia solution (33%) when the sticky precipitate dissolved. The mixture was stirred overnight at 70 °C in the laminar hood in order to remove excess of ammonia until the precipitation of the desired sulfonamide, which was filtrated and air-dried. The crude compound was purified by titration in ethanol, leading to a gray solid with 56% yield. ¹H NMR (DMSO-*d*₆) δ ppm: 7.32 (s, 2H, SO₂NH₂); 7.73 (s, 2H, Ar); 8.08 (s, 1H, Ar); 8.58 (s, 1H, CH(5')); 9.39 (s, 1H, CH(2')). MS (electrospray): MH⁺ = 281.2; MNa⁺ = 303.17.

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24. The hCA II-2 adduct was crystallized as previously described.²³ Diffraction data were collected under cryogenic conditions (100 K) on a CCD Detector KM4 CCD/Sapphire using Cu K α radiation (1.5418 Å). The unit cell dimensions were determined to be: a = 42.2 Å, b = 41.4 Å, c = 72.2 Å, and α = γ = 90°, β = 104.5° in the space group P2₁. Data were processed with CrysAlis RED (Oxford Diffraction 2006).²⁵ The structure was analyzed by difference Fourier technique, using the PDB file 1CA2 as starting model. The refinement was carried out with the program REFMAC5²⁶; model building and map inspections were performed using the COOT program.²⁷ The correctness of stereochemistry was finally checked using PROCHECK.²⁸ Coordinates and structure factors have been deposited with the Protein Data Bank (Accession Code 3FFP).

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