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Potent memapsin 2 (β-secretase) inhibitors: Design, synthesis, protein-ligand X-ray structure, and in vivo evaluation

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Abstract—Structure-based design, synthesis, and biological evaluation of a series of peptidomimetic β-secretase inhibitors incorporating hydroxyethylamine isosteres are described. We have identified inhibitor **24** which has shown exceedingly potent activity in memapsin 2 enzyme inhibitory (K_i 1.8 nM) and cellular (IC_{50} = 1 nM in Chinese hamster ovary cells) assays. Inhibitor **24** has also shown very impressive in vivo properties (up to 65% reduction of plasma Aβ) in transgenic mice. The X-ray structure of protein-ligand complex of memapsin 2 revealed critical interactions in the memapsin 2 active site.

Memapsin 2 (β-secretase, BACE 1) has become a major target for the development of inhibitor drugs for the treatment of Alzheimer Disease (AD).[1] Memapsin 2 is the first protease that cleaves β-amyloid precursor protein (APP) in the pathway leading to the generation of amyloid-β peptide (Aβ) in the brain.[2,3] The excess level of Aβ results in the formation of amyloid plaques and neurofibrillary tangles. The neurotoxicity of Aβ ultimately leads to neuronal death, brain inflammation, dementia, and AD.[4] Consequently, inhibition of memapsin 2 has emerged as an excellent therapeutic target for the intervention of AD. On the basis of initial kinetics and substrate specificity information, we designed a number of potent inhibitors incorporating a nonhydrolyzable Leu-Ala hydroxyethylene dipeptide isostere.[5,6] One of the early key inhibitors was OM99-2 (**1**, Fig. 1). It has shown potent inhibitory activity (K_i = 1.6 nM) for human memapsin 2.[6a] An X-ray crys-

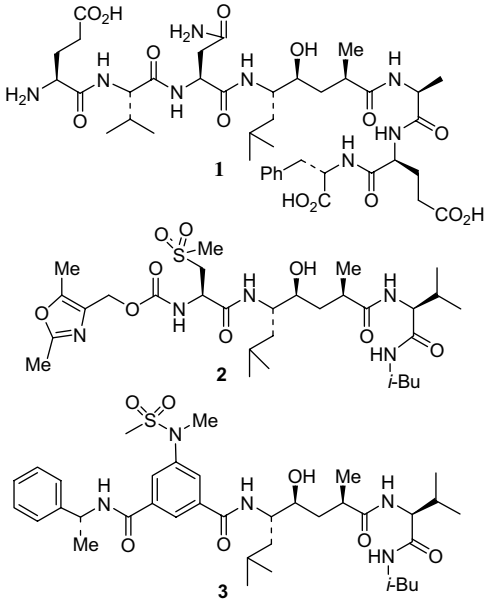

Figure 1. Structure of memapsin 2 inhibitors **1**–**3**.

Keywords: Alzheimer; Design; Synthesis; Inhibitor; Memapsin 2; β-Secretase.
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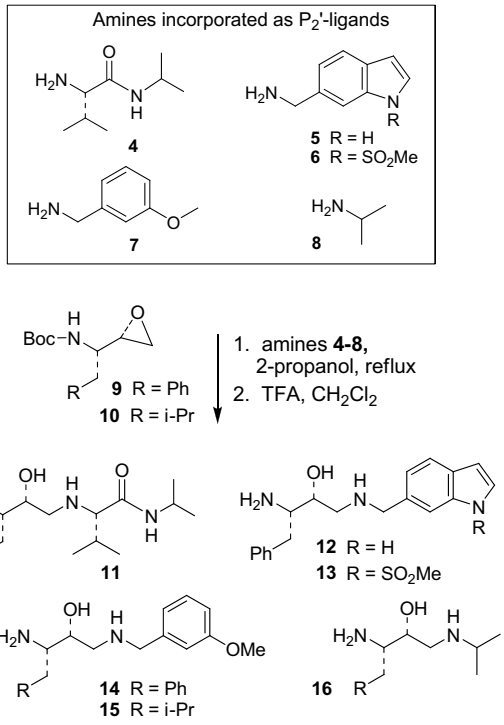
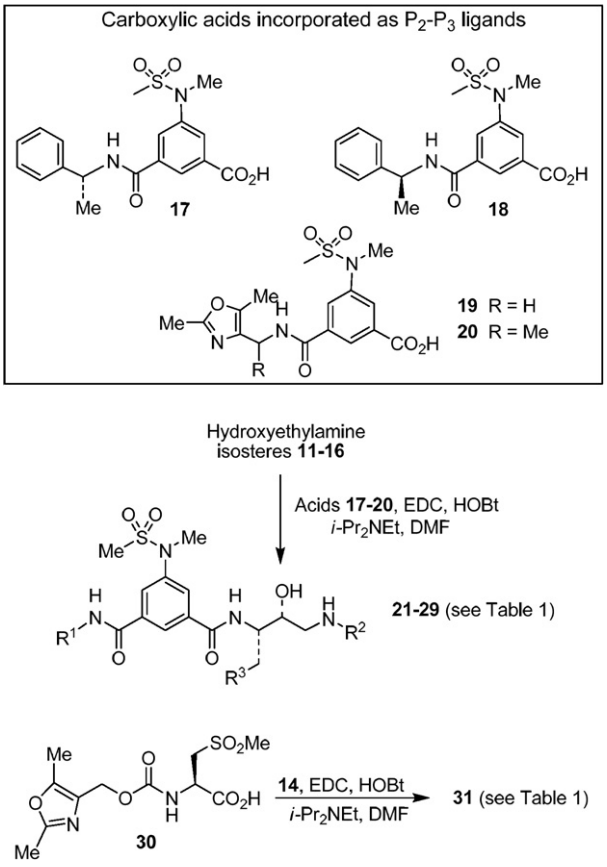
tal structure of **1**-bound memapsin 2 provided critical molecular insight and drug-design templates for the memapsin 2 active site.[7] Based upon the crystallographic information and preliminary structure–activity relationship studies, we then designed a number of highly potent and selective inhibitors.[8] Inhibitor **2** incorporating methylsulfonyl alanine as the P_1-ligand and oxazolylmethyl urethane as the P_3-ligand exhibited excellent potency (K_i = 0.12 nM against memapsin 2) and selectivity over BACE-2 (3800-fold) and cathepsin D (2500-fold).[8] However, its cellular inhibitory potency was in the low micromolar range (IC_{50} of 1.4 μM) in Chinese hamster ovary cells.

To further improve inhibitor properties, we subsequently developed inhibitors incorporating substituted isophthalamides as P_2–P_3 ligands in combination with the Leu-Ala hydroxyethylene dipeptide isostere.[9] As shown in Figure 1, inhibitor **3** has exhibited excellent memapsin 2 inhibitory potency (K_i = 1.1 nM), good cellular inhibitory activity (IC_{50} = 39 nM), and has shown a 30% reduction of $A\beta_{40}$ production in transgenic mice after a single intraperitoneal administration (8 mg/kg).[9] In our continuing effort to further improve inhibitor properties, we have also investigated inhibitors incorporating a variety of other isosteres. Recently, a number of reports incorporating isophthalamide-based P_2-ligands in memapsin 2 inhibitor design have appeared in the literature.[10] Herein, we now report the design and synthesis of a series of potent memapsin 2 inhibitors incorporating a substituted isophthalamide as P_2–P_3 ligands in combination with hydroxyethylamine dipeptide isosteres. A number of inhibitors have displayed excellent memapsin 2 inhibitory potency. We have identified



Scheme 1. Synthesis of hydroxyethylamine substituted isosteres.



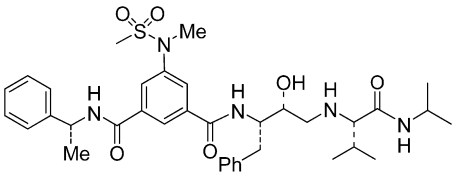
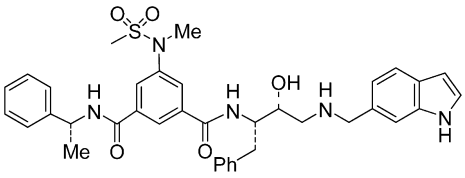
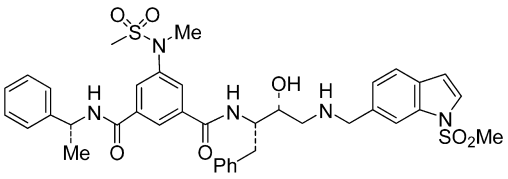
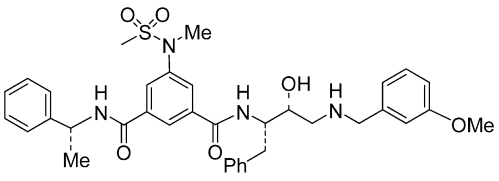
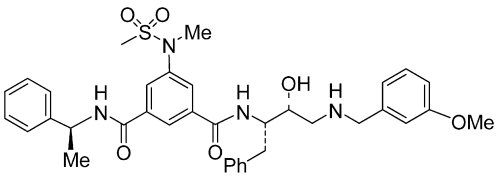
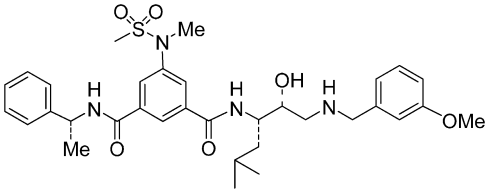
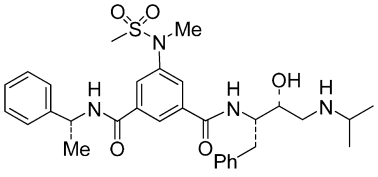
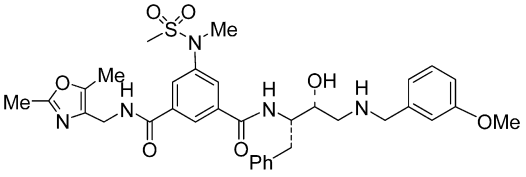
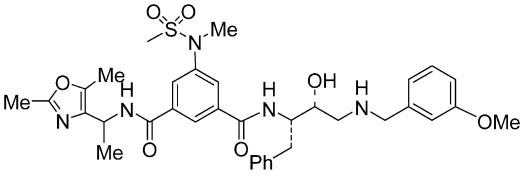
Scheme 2. Synthesis of memapsin 2 inhibitors.

an inhibitor (**24**) which has shown excellent enzyme and cellular inhibitory activity, good selectivity over BACE-2 and cathepsin D, and excellent in vivo properties in transgenic mice. A high resolution protein-ligand X-ray structure of this inhibitor also provided important molecular insight into ligand-binding site interactions for further molecular design.

The synthesis of substituted hydroxyethylamine isosteres is shown in Scheme 1. Reactions of amines **4**–**8** with known[11] epoxides **9** and **10** in isopropanol at reflux provided the respective aminoalcohol. Exposure of the resulting aminoalcohol to trifluoroacetic acid (20% in CH_2Cl_2) at 23 °C for 2 h removed the BOC-group and afforded the corresponding diamines **11**–**16** in excellent yields (80–85%) in a two-step sequence. The synthesis of various inhibitors containing the hydroxyethylamine isosteres is shown in Scheme 2. Substituted aminoisophthalamide derivatives **17**–**20** were prepared as described previously.[9,10] Coupling of these acids with various amines **11**–**16** using EDC/HOBt in the presence of *i*-Pr_2NEt furnished respective inhibitors (**21**–**29**) in 45–55% yields. For synthesis of inhibitor **31**, the requisite acid **30** was prepared by *m*-CPBA oxidation of the corresponding known sulfide.[8] Coupling of acid **30** with amine **14** as described above afforded inhibitor **31** (47% yield).

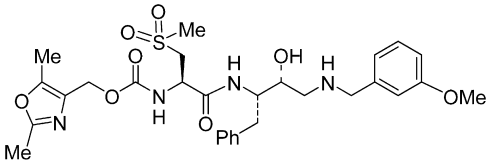
Structure and inhibitory potency of various inhibitors are shown in Table 1. The memapsin 2 inhibitory activ-

Table 1. Structures and potencies of memapsin 2 inhibitors

Entry	Inhibitor structure	Compound	K_i (nM, memapsin 2)	Cellular IC_{50} (μM)
1		**21**	564	nd
2		**22**	230	0.62
3		**23**	27	0.2
4		**24** (GRL-8234)	1.8	0.001
5		**25**	617	nd
6		**26**	916	nd
7		**27**	425	1.1
8		**28**	552	nd
9		**29**	894	nd

(*continued on next page*)

Table 1 (*continued*)

Entry	Inhibitor structure	Compound	K_i (nM, memapsin 2)	Cellular IC_{50} (μM)
10		**31**	15,000	nd

ity of various inhibitors was determined using established assay protocols described previously.[12] As it can be seen, size and substituents on the hydroxyethylamine isosteres are important for potency. Inhibitor **21** with a (*R*)-α-methylbenzyl isophthalamide derivative as the P_2–P_3 ligand and a valine-derived P_2'-ligand exhibited modest enzyme inhibitory activity (entry 1). An unsubstituted indolylmethyl group as the P_2'-ligand has shown a K_i value of 230 nM and a cellular IC_{50} value of 620 nM in Chinese hamster ovary cells.[13] In an effort to promote interaction with the residues in the S_2'-subsite, we incorporated a *N*-methylsulfonamide functionality on the indole nitrogen. The corresponding inhibitor **23**, has shown a significant improvement in memapsin 2 inhibitory activity (K_i = 27 nM) and cellular activity (IC_{50} = 200 nM) compared to inhibitor **22**. Incorporation of a 3-methoxybenzyl derivative as the P_2'-ligand provided a very potent inhibitor **24** (GRL-8234). It exhibited a K_i value of 1.8 nM, thus a 15-fold potency enhancement over the indole derivative **23**. Furthermore, inhibitor **24** has shown a very impressive cellular memapsin 2 inhibitory activity with an average IC_{50} value of 1 nM (5 determinations). Inhibitor **25** with a P_3-(*S*)-α-methylbenzylamide is notably less potent than inhibitor **24** with a P_3-(*R*)-isomer. Inhibitor **26** with a P_1-leucine side chain has shown significant reduction in potency, thus indicating the preference for a more sterically demanding P_1-phenylmethyl substituent. The lipophilic 3-methoxybenzyl P_2'-ligand is very critical for the enhanced cellular inhibitory properties of inhibitor **24**. As can be seen, replacement of this P_2'-ligand with an isopropyl group resulted in inhibitor **27** (K_i = 425 nM; IC_{50} = 1.1 μM) with a significant reduction of potency. In particular, there is a 1000-fold reduction in cellular inhibitory activity compared to **24**. P_3-oxazolylmethyl amide derivatives (compounds **28** and **29**) have shown only moderate activity compared to α-methylbenzyl derivative **24**. Compound **31** with a methylsulfonyl alanine as the P_2-ligand along with a oxazolylmethyl urethane as the P_3-ligand (entry 10) exhibited no appreciable activity. This is in marked contrast to inhibitor **2** with a hydroxyethylene isostere.[8]

Inhibitor selectivity over other aspartyl proteases may be important, particularly over memapsin 1 (BACE 2) and cathepsin D. Memapsin 1 has specificity similar to memapsin 2 and it is presumed to have important physiological functions.[14] Cathepsin D is also important as it is involved in cellular protein catabolism.[15] We have evaluated the selectivity properties of inhibitor **24** against recombinant memapsin 1 and human cathepsin D. It is more than 39-fold selective over memapsin 1 and more than 23-fold selective over cathepsin D. It exhibited an encouraging IC_{50} value against CYP3A4 (696 nM).

We examined the in vivo efficacy to inhibit Aβ production in transgenic mice by inhibitor GRL-8234. The compound was injected intraperitoneally to Tg2576 mice[16] and the plasma was sampled immediately prior to and 3 h post-administration. Treatment with compound **24** resulted in a 65% reduction of $Aβ_{40}$ in plasma at 3 h after a single administration of 8 mg/kg (Fig. 2). Some of the decrease is likely originated from the reduction of Aβ in the brain since Aβ in young Tg2576 mice is almost entirely produced in the brain[17] and then transferred to the plasma. Also, the plasma Aβ has been shown to correlate well with brain Aβ in memapsin 2 inhibition using Tg2576 mice.[13,18] The inhibition of memapsin 2 activity in vivo was significant compared to the relative $Aβ_{40}$ in vehicle-treated control animals collected at 3 h post-administration (p = 0.000012) and relative to the baseline $Aβ_{40}$ levels for the treated group (p = 0.0072). As observed for compound **3**[9] and the prototype inhibitor OM003-DR9[13] the plasma reduction by GRL-8234 was of a magnitude that indicates inhibition in brain $Aβ_{40}$ production in Tg2576 mice of this age. Inhibition of brain $Aβ_{40}$ by 15–20% at 2 h post-administration of a single 4 mg/kg dose was confirmed, and further studies in rats indicate robust brain penetration of GRL-8234. The details of this investigation will be published elsewhere.

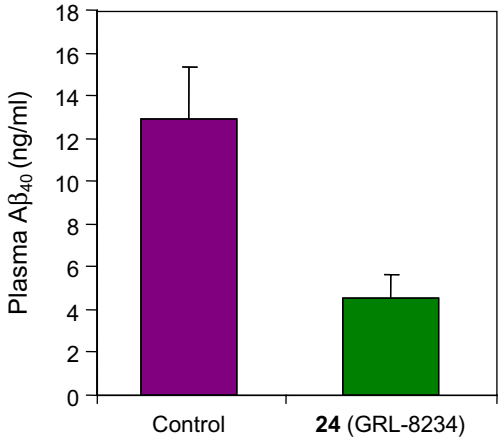


Figure 2. Inhibitor **24** (GRL-8234) reduced $Aβ_{40}$ production following a single intraperitoneal administration at 8 mg/kg. Plasma $Aβ_{40}$ assessed at 3 h post-injection was significantly lower than control group (p = 0.0072). Error bars are s.e.m. from n = 5 animals per group.

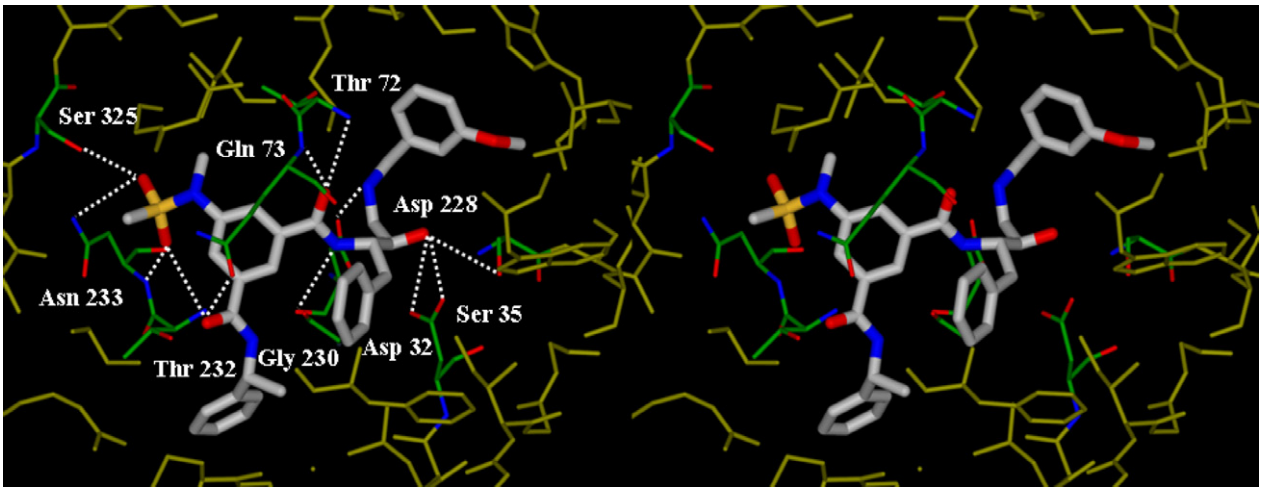


Figure 3. Stereoview of the X-ray crystal structure of **24** (thick stick with gray carbon) with memapsin 2. Hydrogen bonds between the inhibitor and memapsin 2 are shown in white dotted lines.

To obtain molecular insight into the binding properties of inhibitor **24**, we determined the protein-ligand X-ray structure of **24**-bound memapsin 2.[19] As shown in Figure 3, the transition-state hydroxyl group forms two hydrogen bonds (2.4 and 3.3 Å bond distances) with the active site aspartic acid residue, Asp32. The hydroxyethylamine nitrogen forms another tight hydrogen bond with catalytic residue Asp228 (2.6 Å bond distance). The P_3-phenyl ring occupies a unique position which spans S_3- and S_4-subsites and causes a significant positional shift of the protein loop containing residues from 8 to 13 (the 10's loop) located in the S_3/S_4 pocket. This conformational change to accommodate the bound inhibitor identifies a flexible part of the active site cleft which can be further exploited for ligand design. This binding pocket consists of residues 12–14, 229–232, and 335. These residues make direct contact with the phenyl ring at P_3. The P_2-sulfonamide fits well into the S_2 pocket and makes extensive interactions with memapsin 2. One of the sulfonamide oxygen atoms is within hydrogen bonding distance with the Asn233 side chain nitrogen and the Ser325 side chain oxygen (3.3 and 3.2 Å bond distance, respectively). The same sulfonamide oxygen also makes ionic interactions with the guanidine side chain of Arg235, which is believed to confer selectivity of GRL-8234 over cathepsin D, an important human aspartic protease which has a valine at this position. The Arg235 side chain appeared to be quite flexible and adopted a different conformation than our previously reported structures.[7,9] The other sulfonamide oxygen makes hydrogen bonds to Thr232 and Asn233 main chain nitrogen atoms at 3.3 and 2.9 Å bond distances, respectively. The terminal methoxybenzene makes non-polar interactions with residues in the hydrophobic P_2' binding-site which includes Gly34, Ile126, Tyr198, and the flap residues Pro70, Tyr71, and Thr72. This may explain its enhanced binding affinity over indole **22** or indolesulfonamide derivative **23**.

In summary, we have investigated various structural elements of memapsin 2 inhibitors containing hydroxyethylamine dipeptide isosteres and substituted isophthalamides as P_2–P_3 ligands. These research efforts have resulted in inhibitors with improved potency and cellular inhibitory properties compared to inhibitors with hydroxyethylene isosteres. Inhibitor **24** has shown excellent enzyme inhibitory activity as well as cellular inhibitory potency in Chinese hamster ovary cells. It has also exhibited very impressive in vivo properties. An intraperitoneal administration of inhibitor **24** to Tg2576 mice resulted in a 65% reduction of $A\beta_{40}$ production at 3 h after a single dose of 8 mg/kg. A protein-ligand X-ray crystal structure of **24**-bound memapsin 2 has provided important molecular insight into the ligand-binding site interactions. This information has provided the basis for further design and improvement of inhibitor properties that are currently in progress.

Acknowledgment

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12. Memapsin 2 inhibition was measured using recombinant enzyme produced from *E. coli* expression as described in Ref. 2a. A fluorogenic substrate Arg-Glu(EDANS)-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys (Dabcyl)-Arg was used with 0.47 μM of the enzyme in 0.1 M Na-acetate + 5% dimethylsulfoxide, pH 4.5, at 37 °C. The excitation wavelength was 350 nm and the emission wavelength was 490 nm.
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19. The protein-ligand X-ray structure of **24**—bound memapsin 2 has been deposited in PDB (PDB ID: 2vkm). Recombinant human BACE was expressed and purified as previously described. Crystals of free BACE (APO form) were grown using a hanging drop vapor diffusion method. Monoclinic crystals suitable for structure determination were obtained at 13% PEG 8000, pH 6.5, and 17 °C with a protein concentration of 18 mg/ml. The crystals grow to full size (about 0.25 mm × 0.35 mm × 0.05 mm) in 4–5 weeks. Inhibitor **24**-memapsin 2 complex crystal were prepared by soaking the free memapsin 2 crystal in concentrated inhibitor solution for 2 days.
Memapsin 2/inhibitor crystal was soaked in the mother liquor plus 20% (v/v) glycerol and quickly frozen under a cryogenic nitrogen gas stream. Cryogenic diffraction data from a single crystal was recorded on a Mar 345 image plate mounted on a MSC-Rigaku RU-300 X-ray generator. The structure was determined by molecular replacement implemented with the program AmoRe using the previously determined memapsin 2 structure (PDB ID: 1M4H) as a search model.[20] Rotation and translation functions followed by the rigid-body refinement with data from 15 Å to 3.5 Å resolution in space group P21 gave unambiguous solutions for the four memapsin 2 molecules in the asymmetric unit. A random selection of 8% of reflections was set aside as the test set for cross-validation during the refinement. The refined model has well-defined electron density for the inhibitor and its corresponding structure was built into the active site. The four molecules in the crystallographic asymmetric unit have essentially identical structures. The crystal form was determined to be monoclinic with a resolution of 2.05 Å. The unit cell parameters are $a = 86.7$ Å, $b = 130.3$ Å, $c = 87.7$ Å, $\beta = 97.4°$.
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