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Cantharimides: A New Class of Modified Cantharidin Analogues Inhibiting Protein Phosphatases 1 and 2A

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Abstract—Cantharidin and its analogues have been of considerable interest as potent inhibitors of the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A). However, limited modifications to the parent compounds is tolerated. As part of an ongoing study we have developed a new series of cantharidin analogues, the cantharimides. Inhibition studies indicate that cantharimides possessing a D- or L-histidine, are more potent inhibitors of PP1 and PP2A (PP1 $IC_{50}=3.22\pm0.7$ μM; PP2A $IC_{50}=0.81\pm0.1$ μM and PP1 $IC_{50}=2.82\pm0.6$ μM; PP2A $IC_{50}=1.35\pm0.3$ μM, respectively) than norcantharidin (PP1 $IC_{50}=5.31\pm0.76$ μM; PP2A $IC_{50}=2.9\pm1.04$ μM) and essentially equipotent with cantharidin (PP1 $IC_{50}=3.6\pm0.42$ μM; PP2A $IC_{50}=0.36\pm0.08$ μM). Cantharimides with non-polar or acidic amino acid residues are only poor inhibitors of PP1 and PP2A.

The okadaic acid class of compounds is representative of the structurally diverse group of naturally occurring toxins that are potent inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A).[1] Regulation of cellular processes by modification of the levels of phosphorylated proteins is fundamental to a large number of, if not all, cellular functions.

The actual phosphorylation level is the result of a delicate balance between phosphatases and kinases. Kinases transfer a phosphate from ATP to a protein, typically phosphorylation at a serine, threonine or tyrosine residue. Phosphatases, on the other hand, remove the phosphate group, that is dephosphorylate the protein. In contrast to the multitude of protein kinases that have been discovered, relatively few protein phosphatases are known. In this regard, protein phosphatases were often considered as the poorer cousins of kinases. However, it is now acknowledged that the regulation of protein phosphorylation requires the coordinated control of both kinases and phosphatases and that the regulation of phosphatases is as complex and elegant as that of kinases. In many ways, the phosphorylation–dephosphorylation cycle can be regarded as a molecular 'on–off' switch, although this is probably an over simplification of the processes involved. The end result of these associated pathways is the regulation of many different cellular processes, as diverse as glycogen metabolism, calcium transport, muscle contraction, gene expression, protein synthesis, intracellular transport, phototransduction, cell cycle progression and apoptosis.[1]

In recent years, there has been intense interest in the development of potent and selective inhibitors of the serine/threonine class of protein phosphatases, PP1 and PP2A. These attempts have included the more complex toxins such as the Microcystin analogues developed by Chamberlin,[2] which resulted in moderate PP1 selectivity. Of all the known naturally occurring toxins, cantharidin represents by far the simplest synthetic target. Consequently, we and others have reported the synthesis of numerous analogues of cantharidin, and their inhibition of PP1 and PP2A.[3] However, these studies have only ever met with limited success. Cantharidin brooks little in the way of structural modification, with the majority of modifications at best maintaining the

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inherent potency and slight PP2A selectivity of cantharidin. Recently we reported the first successful anhydride modified cantharidin analogues that maintained potency, suggesting that we should re-examine our original hypothesis that a facile anhydride ring-opening was crucial for the inhibition of PP1 and PP2A.

Results and Discussion

Cantharidin (**1**) and tautomycin (**2**) (Fig. 1) are known to bind in the active sites of both PP1 and PP2A in the ring-opened dicarboxylic acid form.[4] However, we have recently shown that a series of ring-opened cantharidin analogues with only one free carboxylate (see Table 1) not only retained inhibitory activity against PP1 and PP2A but increased slightly the selectivity towards PP2A.[3a] As a result of this work, we have been forced to re-evaluate our understanding of the inhibition of PP1 and PP2A by cantharidin analogues. The first step in our re-evaluation was a closer examination of the crystal structure of PP1 and of a molecularly modelled structure of PP2A.

Our modelling analysis (data not shown) suggested that these ring-opened analogues bind in the active site and that the sole carboxylic acid residue still binds in the active site of PP1 and PP2A. The alkyl chain of ester is proximal to an acidic groove in the general vicinity of the protein's active site.[5] The major amino acids in this groove are acidic in nature and consequently we believed that compounds possessing the anhydride bridge of cantharidin (or the demethylated norcantharidin), a single carboxylic acid group and a basic residue would give rise to a new class of compounds with the ability to inhibit PP1 and PP2A.

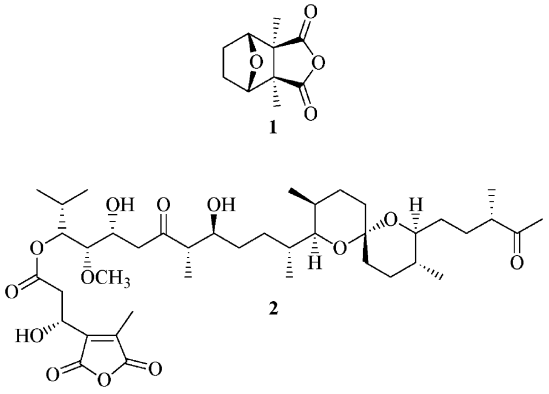


Figure 1.

Table 1. Inhibition of PP1 and PP2A by ring-opened cantharidin analogues

	PP1 (IC_{50} μM)[a]	PP2A (IC_{50} μM)[a]	PP2A/PP1
R = CH_3	4.71	0.41	11.5
R = CH_3CH_2	2.96	0.45	6.5
R = $CH_3CH_2CH_2$	4.82	0.47	10.2

[a]Average of three experiments in triplicate.

On the basis of our previous report and our modelling results, we set about the synthesis of a series of amino acid substituted norcantharidin analogues, the cantharimides. Herein, we report on our developments towards a new class of cantharidin analogues that inhibit both PP1 and PP2A (Scheme 1).

Experimental

The cantharimide analogues were synthesised in moderate to good yield by a modified Gabriel synthesis commencing from readily available norcantharidin (**3**). In a typical synthesis, 168 mg of **3** was placed in a thick-walled glass pressure vessel, followed by anhydrous toluene (10 mL), 1.0 equivalents anhydrous Et_3N and 1.0 equivalents of unprotected amino acid (either D or L).[6]

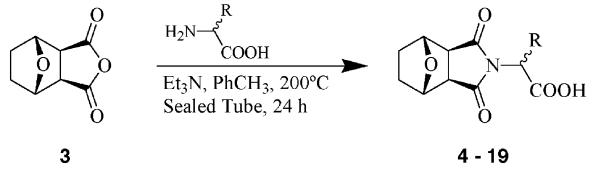
Workup comprised a $NaHCO_3$ wash, extraction with CH_2Cl_2, followed by acidification with concentrated HCl, and finally extraction with ethyl acetate. The crude products were then purified by column chromatography. Typical yields are shown in Table 2.

The simplicity of the chemical synthesis, and the ready availability of both D- and L-amino acids allowed examination of the effect of the different stereoisomers.

Biology

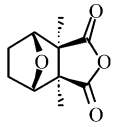
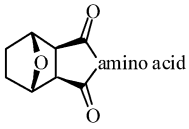
The cantharimides **4**–**19** were screened for their ability to inhibit PP1 and PP2A.[7] Cantharidin (**1**) and norcantharidin (**3**) were included as internal standard to ensure the relative validity of our protocol, and to allow the effects of differing assay conditions to be standardised.[8]

The results of the phosphatase inhibition study are shown in Table 2. We note that analogues **4**–**11** showed effectively no inhibitory effects at either PP1 or PP2A. Although **4** did show marginal activity at PP2A. However, the introduction of a bulky aromatic group, such as in **12** and **13** resulted in a significant increase, although still poor, in protein phosphatase inhibition (**12**: PP1 $IC_{50} = 770 \pm 146$ μM; PP2A $IC_{50} = 157 \pm 33$ μM and **13**: PP1 $IC_{50} = 312 \pm 153$ μM; PP2A $IC_{50} = 105 \pm 22$ μM). In terms of the active analogues, there appeared to be no overall discernable trend in the effect of a particular stereoisomer on phosphatase inhibition. In this context the L-isomer of tryptophan was more active at



Scheme 1. Synthesis of cantharimides.

Table 2. Inhibition of protein phosphatases 1 and 2A by compounds **1**, **3** and cantharimides **4**–**19**

Compd	Parent amino acid	Yield (%)	PP1 inhibition (IC_{50} μM)[a]	PP2A inhibition (IC_{50} μM)[a]	PP2A selectivity
1					
	Cantharidin	—	3.6±0.42	0.36±0.08	10
3	Norcantharidin	—	5.31±0.36	2.9±1.04	1.83
					
4	D-Ala	48	>1000	150±85	≥6
5	L-Ala	53	>1000	>1000	—
6	D-Phe	50	>1000	>1000	—
7	L-Phe	57	>1000	>1000	—
8	D-Leu	76	>1000	>1000	—
9	L-Leu	70	>1000	>1000	—
10	D-Ile	68	>1000	>1000	—
11	L-Ile	64	>1000	>1000	—
12	D-Try	31	770±146	157±33	4.9
13	L-Try	57	312±153	105±22	3.0
14	D-His	8	3.22±0.7	0.81±0.1	4.0
15	L-His	14	2.82±0.6	1.35±0.3	2.1
16	D-Tyr	21	101±34	112±10	0.9
17	L-Tyr	74	570±330	245±65	2.3
18	D-Glu	16	95±5	32±8	3.0
19	L-Met	32	>1000	>1000	—

[a]Average of three experiments in triplicate.

inhibiting both enzymes than the D-isomer, while the reverse trend was observed for the tyrosine analogue, and no significant stereoisomer effects were observed for the histamine analogue. It is interesting to note that these stereoisomer effects were similar for both enzymes, with the exception of alanine, where the D-isomer showed greater inhibition over the L-isomer, but only for PP2A.

Interestingly, there were also other trends emerging from within the more potent cantharimides. In addition to confirming our initial hypothesis that a basic amino acid residue would facilitate inhibition of PP1 and PP2A, we also noted that those cantharidimides possessing an easily ionisable group also showed moderate to poor activity.

Of all the cantharimides reported in Table 2, only **14** and **15** are potent inhibtors of PP1 and PP2A, essentially equipotent with cantharidin. Given the relative spatial orientation of the His side chain, carboxylic acid and tricyclic core in each of the enantiomers it is surprisingly there is almost no differentiation in binding of these enantiomers.

Molecular modelling

Cerius2–LigandFit[9] molecular modelling anaysis indicated the presence of two, high-scoring, binding modes that could explain our observed lack of enantiomer selectivity. Figure 2 highlights these two possible binding modes: head-to-head (Fig. 2a and b) and head-to-tail (Fig. 2c and d) for **14**/**15** and PP1.

Figure 2a illustrates the overlap of enantiomers **14** and **15** maximising interactions with the imide ring and histidine α-carbons (a head-to-head alignment). This results in poor carboxylate alignment and places the 7-Os on opposite sides and would be expected to result in a difference in inhibition of PP1 and PP2A, as it is known that the 7-O is crucial for inhibition (at least with PP2A), although, from Figure 2b, both **14** and **15** are able to occupy the active site with no obvious interferences from active-site residues.

Figure 2c shows the second high-scoring possibility generated by LigandFit. Surprisingly, in this instance **14** and **15** are presented to the enzyme in completely different orientations, essentially head-first or feet-first. Although this possibility at first appears contrary to expectations, it does allow for presentation of similar features in similar positions to the enzyme. Notable the carboxylate of **14** and the 7-O of **15** present themseleves in the same area of the protein (and vice versa). Additionally, docking in PP1's active site shows no obvious interferences (Fig. 2d).

Further examination of the proposed binding orientations of **14** and **15** are shown in Figure 3a–d below. According to Chamberlin's[5] model cantharidin binds (binding as cantharidic acid) with the carboxylates in close proximity to the divalent metals, and 7-O bridgehead proximal to Tyr-272 (and Tyr-261 in PP2A) within PP1′s

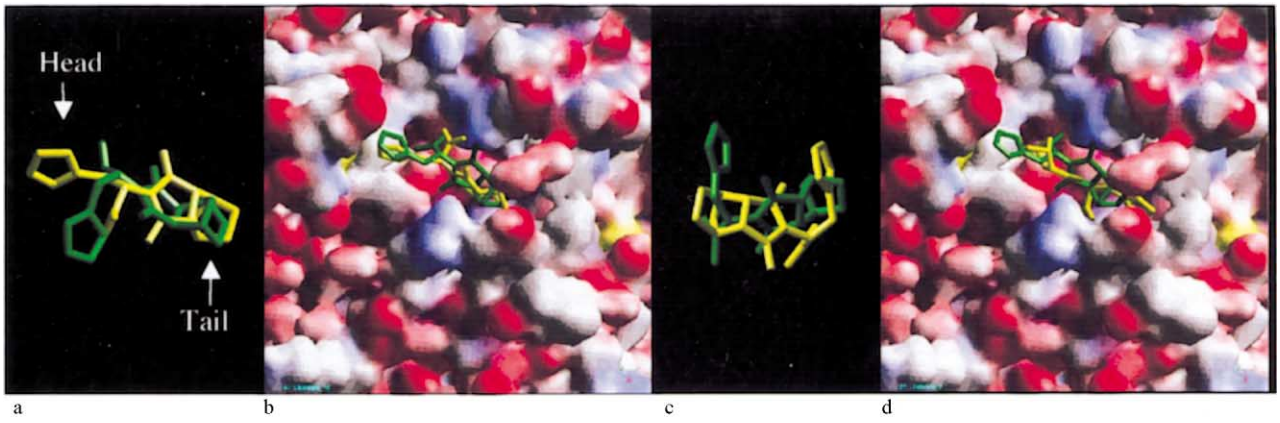


Figure 2. (a) Side-view of overlay of **14** (yellow) and **15** (green) overlaying to maximise interaction of imide and α-carbon with both **14** and **15** shown in orientation 1. (b) As (a), docked into the active site of PP1. (c) Top-view of overlay of alternate binding orientations for **14** (yellow) and **15** (green) with **14** in orientation 2 and **15** in orientation 1. (d) As (c), docked into the active site of PP1.

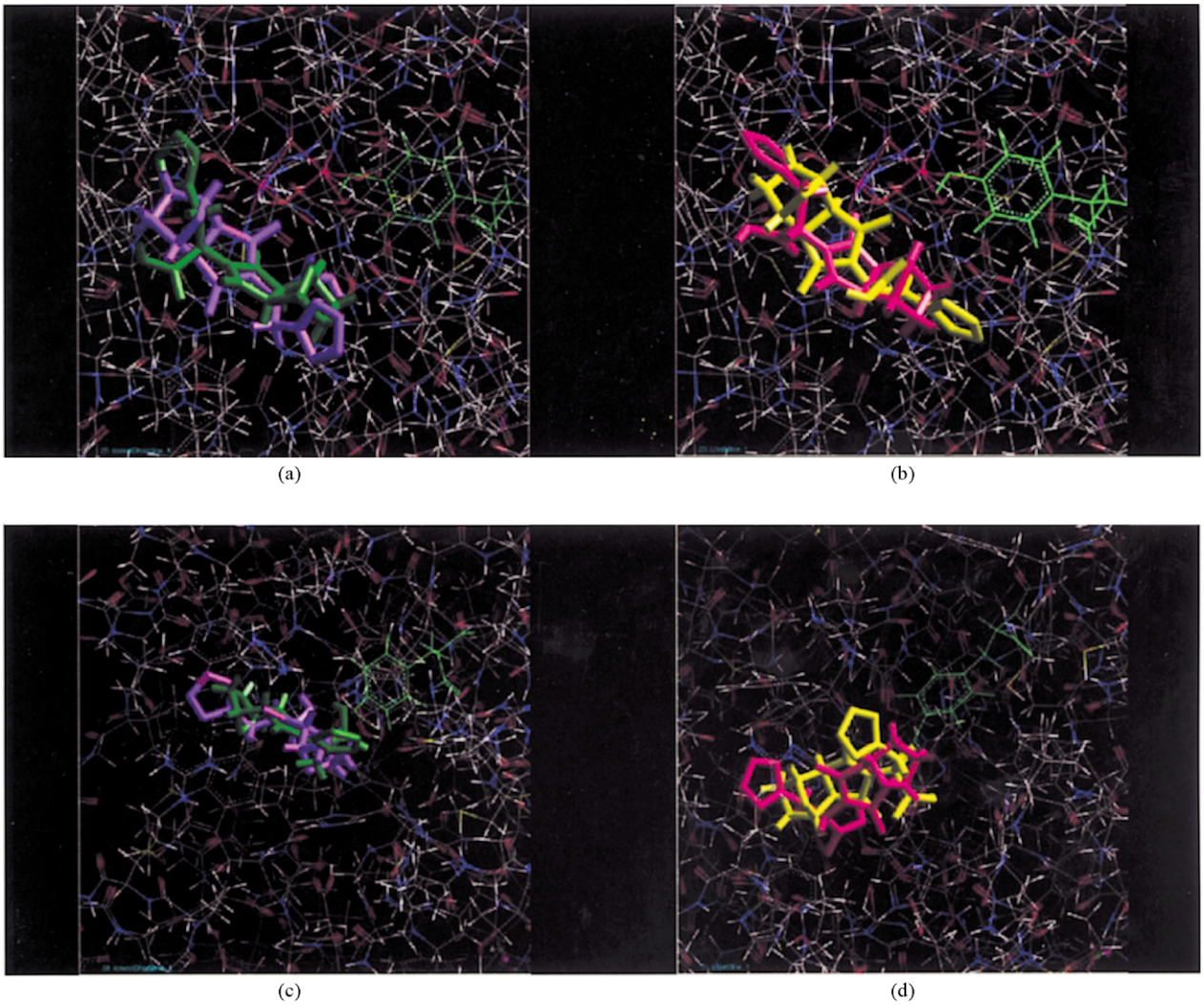


Figure 3. (a) The two highest scoring (LigandFit) binding possibilities of **14** in the active site of PP1. Tyr-272 is highlighted in light green. Orientation 1 (purple), Orientation 2 (green). (b) The two highest scoring (LigandFit) binding possibilities of **15** in the active site of PP1. Tyr-272 is highlighted in light green. Orientation 1 (pink), Orientation 2 (yellow). (c) The two highest scoring (LigandFit) binding possibilities of **14** in the active site of PP2A. Tyr-261 is highlighted in light green. Orientation 1 (purple), Orientation 2 (green). (d) The two highest scoring (LigandFit) binding possibilities of **15** in the active site of PP2A. Tyr-261 is highlighted in light green. Orientation 1 (pink), Orientation 2 (yellow).

Table 3. Key distances from **14** and **15** to key residues in PP1 and PP2A

Enzyme	Compd (orientation)	COOH→metals (Å)	O-Tyr272[a] (Å)
PP1	Cantharidic acid	7.383	4.715
	14 (1)	5.216 and 6.951	6.330
	14 (2)	**8.732 and 9.145**	**4.234**
	15 (1)	**5.510 and 8.388**	**4.339**
	15 (2)	6.033 and 7.314	5.499
PP2A	Compd (orientation)	COOH→metals (Å)	O-Tyr261 (Å)
	Cantharidic acid	4.274	4.292
	14 (1)	**3.019 and 3.401**	**3.763**
	14 (2)	3.109 and 5.653	6.637
	15 (1)	**2.918 and 3.448**	**3.829**
	15 (2)	5.159 and 3.280	6.024

[a]Highlighted table entries represent the most favourable binding orientations at PP1 and PP2A, based on LigandFit data.

active site (COOH→metals distance = 7.383 Å; O→Tyr-272 distance = 4.715 Å) and PP2A's (COOH→metals distance = 4.274 Å; O→Tyr-261 distance = 4.292 Å) active site, respectively (not shown).

With **14** and **15**, and PP1 calculations indicate that the two possible binding orientations result in little difference in the overall interactions with the divalent metals (**14**: orientation 1: 5.216 and 6.951 Å vs orientation 2: 8.732 and 9.145 Å; and **15**: orientation 1: 5.510 and 8.388 Å vs orientation 2: 6.033 and 7.314 Å). These values mirror those obtained for cantharidic acid. However, greater differences are observed when considering the differing orientations and their interactions with Tyr-272 (**14**: orientation 1: 6.330 Å vs orientation 2: 4.234 Å; and **15**: orientation 1: 4.339 Å vs orientation 2: 5.449 Å), again these values mirror those calculated for cantharidic acid. Thus, it appears on the basis of our modelling analysis that the most favourable interactions between **14** and PP1 occur in orientation 2 and with **15** and PP1 in orientation 1 (see Table 3).

Examination of the calculated interactions of **14** and **15**, and PP2A calculations suggest a different scenario. Again there is little difference in the overall interactions with the divalent metals (**14**: orientation 1: 3.019 and 3.401 Å vs orientation 2: 3.109 and 5.653 Å; and **15**: orientation 1: 2.918 and 3.448 Å vs orientation 2: 5.159 and 3.280 Å). There is also virtually no difference between the differing orientations and their interactions with Tyr-261 (**14**: orientation 1: 3.763 Å vs orientation 2: 6.637 Å; and **15**: orientation 1: 3.829 Å vs orientation 2: 6.024 Å), again these values mirror those calculated for cantharidic acid. Thus, it appears on the basis of our modelling analysis that the most favourable interactions between **14** and **15** and PP2A occur in orientation 1 (see Table 3).

Gratifyingly, our modelling analysis and biological data concur, with the determination of slight PP2A selectivity of both **14** and **15**.

Our data do not preclude other possible binding motifs, but are consistent with the data amassed in this area of study (Fig. 3).

Conclusions

A series of anhydride modified cantharidin analogues, the cantharimides, have been synthesised in moderate to good yields and examined for their ability to inhibit protein phosphatases 1 and 2A. As we had originally believed, only those analogues possessing basic amino acid substituents are potent inhibitors of PP1 and PP2A. Furthermore, the analogues reported herein represents the development of a new class of cantharidin analogues which are equipotent with cantharidin, and more potent than norcantharidin. Finally, we believe on the basis of our modeling analysis that this class of compounds will allow the development of selective PP1 and PP2A inhibitors. We will report, in due course, on our progress towards this goal.

Acknowledgements

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6. In a typical synthesis, D-Phe (165 mg, 1 mmol) was placed in a thick-walled glass pressure vessel along with anhydrous toluene (5 mL), anhydrous triethylamine (101 mg, 140 μL, 1 mmol) and norcantharidin (168 mg, 1 mmol) and a magnetic stirrer. The heterogeneous mixture was degassed, the tube sealed and the mixture heated to 200 °C behind a safety shield. After ca. 16 h, the homogeneous mixture was cooled to room temperature and opened cautiously. The contents were taken up in either ethylacetate or dichloromethane (25 mL) and washed with saturated $NaHCO_3$. The aqueous layer was then extracted with dichloromethane (2×10 mL) and acidified to pH2 by the dropwise addition of 6 M HCl, re-extracted with dichloromethane (2×10 mL), dried over Na_2SO_4. The solvent was removed in vacuuo to yield an oil. Purified by chromatotrom plate (solvent system: EtOAc/Hexanes 19:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.94 (CH_2), 2.92 (CH), 3.33 (CH_2), 4.10 (CH), 4.84 (CH), 6.9–7.30 (Ph), 9.81 (CO_2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.3, 29.15, 45.02, 51.82, 76.58, 126.17, 128.56, 129.38, 137.89, 170.08, 175.81.
7. Protein phosphatase assays were carried out essentially as described (Collins, E.; Sim, A. T. R. *Methods Mol. Biol.* **1998**, *93*, 79) using [^{32}P]-glycogen phosphorylase a as substrate and recombinant PP1 (Bernt, N. *Methods Mol. Biol.*, **1998**, *93*, 67) or partially purified (chicken skeletal muscle) PP2A catalytic subunits (Mackintosh, C. In *Protein Phosphorylation: A Practical Approach*; Hardie, D. G., Ed.; IRL, 1993). Briefly, enzyme activity was measured at 30 °C in a buffer (final volume of 30 μL) containing 50 mM Tris–HCl (pH 7.5), 1 mM EGTA, 0.1 mM EDTA, 5 mM caffeine, 0.1% mercaptoethanol, 0.3 mg/mL BSA. The concentration of PP1 or PP2A used was such that the reaction was limited to 15% dephosphorylation to ensure linearity. The reaction was started with the addition of 30 μg [^{32}P]-glycogen phosphorylase a and terminated after 20 min by the addition of 100 μL ice-cold 70% TCA. After 10 min on ice, the sample was centrifuged and a 100 μL aliquot of the supernatant was removed for scintillation counting of the [^{32}P] released during the reaction. Data is expressed as the IC_{50} concentration of the compound, which represents the concentration of compound required to produce 50% inhibition of protein phosphatase activity relative to a control (absence of inhibitor) incubation (100% activity).
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9. Conformational analysis and overlays were performed using CERIUS2.4.2 (MSI, San Diego, CA, 2000 release).