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Letter to the editor

Anhydride modified cantharidin analogues. Is ring opening important in the inhibition of protein phosphatase 2A?

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Abstract – A series of anhydride modified cantharidin analogues have been synthesised and screened for their ability to inhibit protein phosphatase 2A. Surprisingly only analogues capable of undergoing a facile ring opening of the anhydride moiety displayed any significant inhibition. Subsequent NMR experiments indicated that 7-oxobicyclo[2.2.1]heptane-2,3-dicarboxylic acid was the major (sole) species under assay conditions. The ability of these modified anhydro-cantharidin analogues to inhibit protein phosphatase 2A varies from 4 (**16**) to 100% (**8**) at 100 μM test concentration. © 2000 Éditions scientifiques et médicales Elsevier SAS

protein phosphatase 2A / cantharidin / anhydride ring opening

1. Introduction

Reversible phosphorylation plays a pivotal role in cellular signal transduction, moderating such diverse functions as neurotransmission, muscle contraction, glycogen synthesis, T-cell activation and cell proliferation [1–5].

Serine/threonine phosphatases, which are responsible for protein dephosphorylation, comprise a unique class of enzymes consisting of four primary subclasses based on their differences in substrate specificity and environmental requirements. Of the serine/threonine phosphatases, protein phosphatases 1 and 2A (PP1 and PP2A, respectively) share sequence identity between both enzyme sub-units (50% for residues 23–292; 43% overall) and, are present in all eukaryotic cells and are together responsible for 90% of all cellular dephosphorylation. Knowledge of structure and subsequent correlation of binding function for both PP1 and PP2A would therefore provide a vital link toward understanding signal transduction mechanisms.

Okadaic acid (**1**), [6] calyculin A (**2**), [7] microcystin-LR (**3**) [8] and tautomycin (**4**) [9] are representative of the structurally diverse group of compounds comprising the okadaic acid class of compounds. All are potent protein phosphatase 1 and 2A inhibitors (*figure 1*). Recently Quinn et al. [10] have used molecular modelling to reconcile the apparent anomalies between the structural diversity of the okadaic acid class of compounds and the similarities in the binding at both PP1 and PP2A, e.g. **1** PP1 $IC_{50} = 60$ nM, PP2A $IC_{50} = 1$ nM; **2** PP1 $IC_{50} = 0.5–1.0$ nM, PP2A $IC_{50} = 2.0$ nM. The okadaic acid class of compounds are potent and competitive inhibitors of both PP1 and PP2A.

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However with the exception of okadaic acid (**1**), cantharidin (**5**) [11] and thyrisferyl 23-acetate (**6**) [12] (being slightly PP2A selective) they exhibit poor selectivity. To date there has only been one compound reported to possess selectivity towards either PP1 or PP2A, i.e. fostrecin (**7**) (*figure 2*). More recently, Chamberlin has reported the synthesis of a series of PP1 selective inhibitors [13].

Fostrecin (**7**) exhibits $>40\,000$-fold selectivity for PP2A ($IC_{50} = 3.4$ nM) over PP1 [14]. Fostrecin is active against leukemia (L1210, $IC_{50} = 0.46$ μM), lung, breast, and ovarian cancer and displays efficacious in vivo anti-tumour activity against L1210 leukemia in mice [15]. In addition to its phosphatase inhibition fostrecin also inhibits topoisomerase II ($IC_{50} = 40$ μM) [16] in vitro through a novel, non-DNA-strand cleaving mechanism, but does not induce G_2 arrest like other topoisomerase II inhibitors [17]. Fostrecin, however, is a more potent inhibitor of protein phosphatase 2A than topoisomerase II. As a

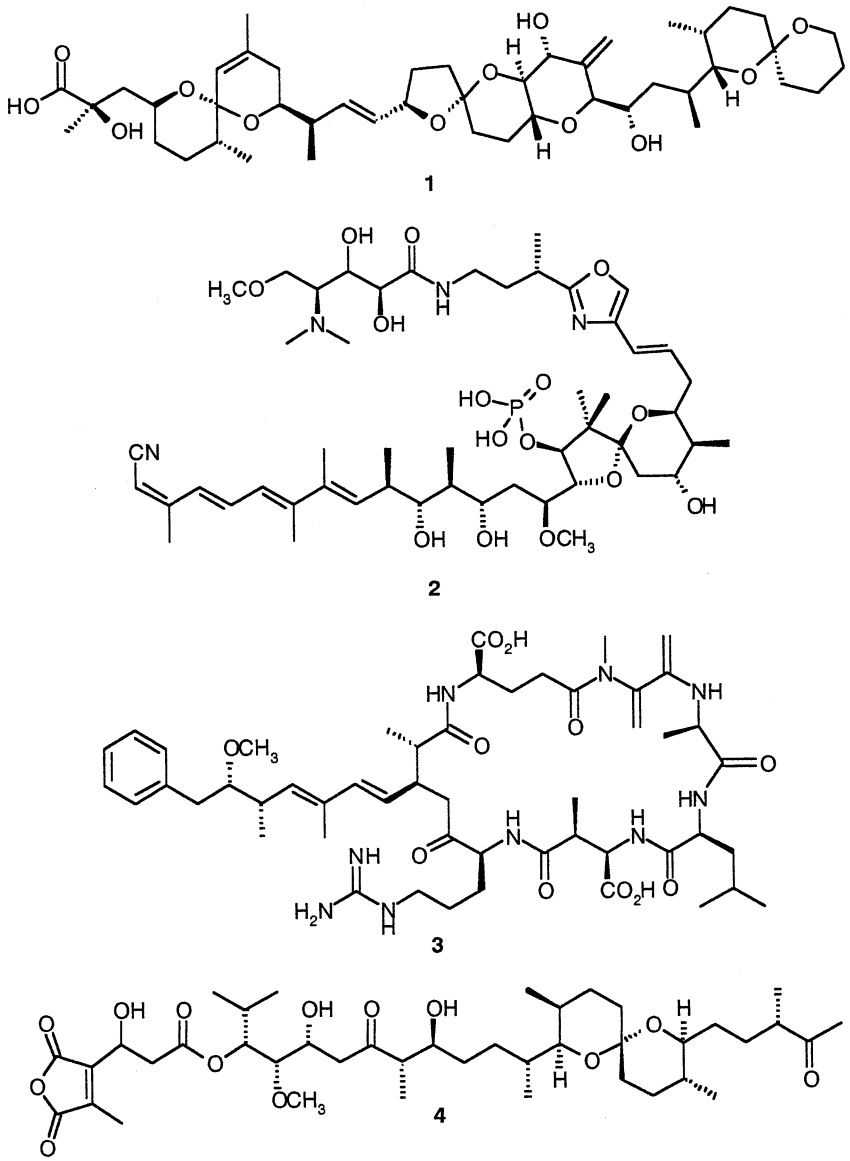


Figure 1. Representative examples of the okadaic acid class of compounds.

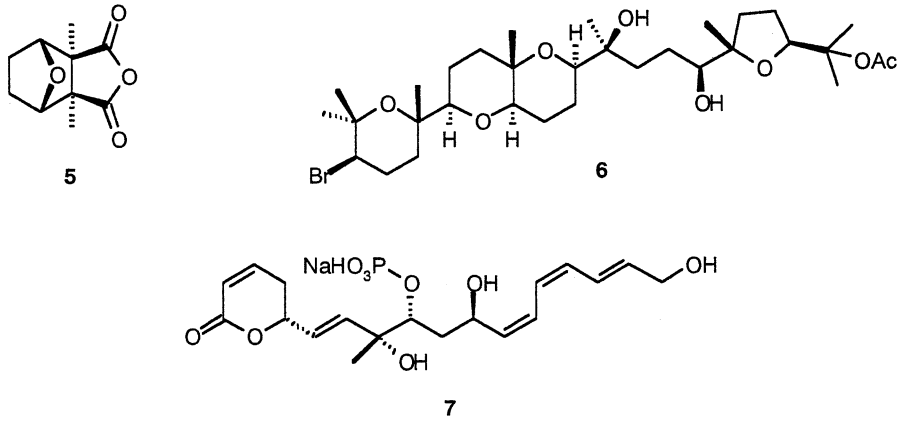


Figure 2. Small molecule inhibitors of protein phosphatases 1 and 2A.

consequence of the reported PP2A selectivity and anti-cancer properties of fostrecin, we have renewed our interest in developing small, potent and cell-permeable inhibitors of protein phosphatase 1 and 2A. One of our current approaches involves development of our current understanding into how cantharidin acts at each respective enzyme. The work reported herein focuses on PP2A.

Cantharidin (*exo*, *exo*-2,3-dimethyl-7-oxobicyclo-[2.2.1]heptane-2,3-dicarboxylic acid anhydride) (**5**), in the form of the dried body of the Chinese blister beetles: *Mylabris phalerata* or *M. cichorii* [18, 19] has been used by the Chinese as a natural remedy for the past 2000 years. Western medicine decreed cantharidin to be too toxic for medicinal purposes in the early 1900s [20]. However, its purported aphrodisiac qualities (the active ingredient of 'Spanish Fly'), and its widespread occurrence in cattle feed still results in numerous human and livestock poisonings [21].

Li and Casida [22], and previous work in this laboratory [23] has assisted in the delineation of certain features that are crucial for the inhibition of PP2A by cantharidin analogues (*figure 3*).

To date structure activity studies have concentrated on the addition (and subsequent modification) of alkyl side-chains and modifications of the 7-position (the bicycloheptane bridgehead), all of these studies have resulted in a decrease in PP2A inhibition. We have previously reported the effect of bridgehead modification and that data indicated that this is a H-bonding site, crucial for inhibition of PP2A [23]. In this instance we were interested in determining other key hydrogen bonding sites; with this in mind we set

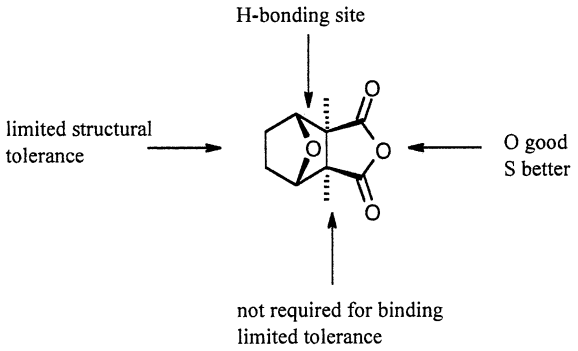


Figure 3. Structural modifications and features permissible whilst still maintaining inhibition of protein phosphatase 2A.

about synthesising a series of anhydride modified cantharidin analogues.

2. Results and discussion

A series of anhydride modified cantharidin analogues were synthesised [24] (*figures 4* and *5*), and then subjected to a PP2A inhibition screen to determine their ability to inhibit this enzyme. In this instance compounds were screened in triplicate at a single dose (100 μM). These results are shown in *table I*, compounds **8** and **9** are included for reference purposes being key intermediates in the synthesis as well as known potent inhibitors of PP2A at the test concentration.

Synthesis of compounds **8**–**16** was accomplished by standard Diels–Alder chemistry, and all the products

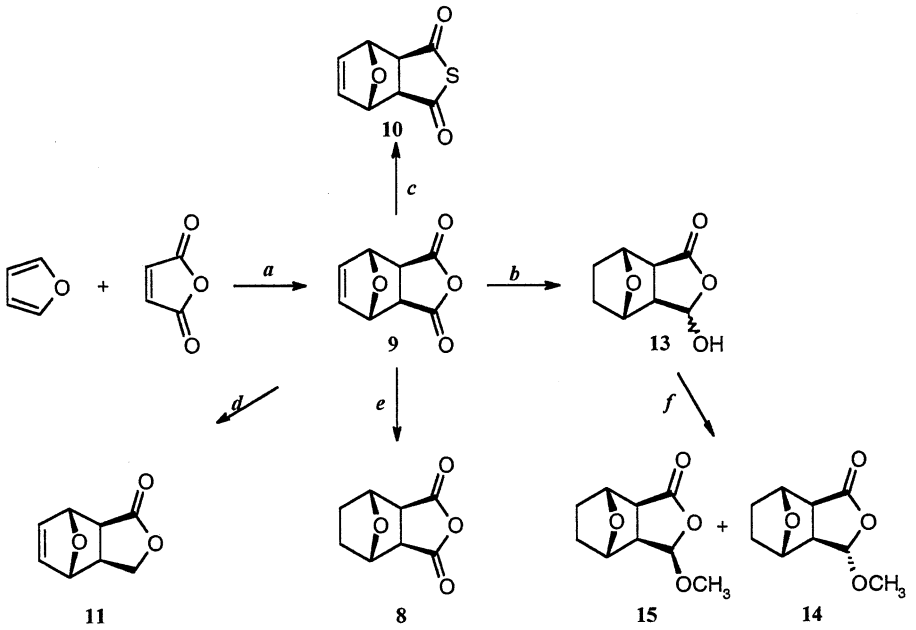


Figure 4. Reagents and conditions: (*a*) Furan:maleic anhydride (5:1), Et_2O, 2 days, RT; (*b*) H_2/10% Pd-C, EtOH, 12 h; (*c*) $Na_2S{\cdot}9H_2O$, 2h, RT; (*d*) $NaBH_4$, then HCl; (*e*) H_2/10% Pd-C, acetone, 12 h; (*f*) *p*-TosOH, MeOH reflux 2 h, silica gel chromatography ($CHCl_3$).

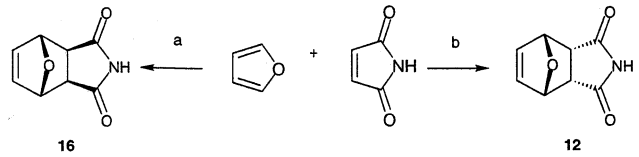


Figure 5. Reagents and conditions: (*a*) Furan:maleimide (5:1), Et_2O, 7 days, in dark, 75%, *exo* product; (*b*) Furan: maleimide (5:1), Et_2O (or H_2O), sealed tube 12 h, 90 °C, 66%, *endo* product.

were purified by a mixture of column chromatography and recrystallisation. All compounds returned data as reported in the literature [24].

Of the nine compounds listed in *table I*, only **8**, **9** and **10** show any significant inhibition of PPP2A at 100, 97 and 97%, respectively. Interestingly the replacement of the anhydride oxygen atom of **9** with nitrogen (compounds **12** and **16**) results in a complete loss of inhibition (97–15% and 4%, compounds **9**, **12** and **16,** respectively), whilst no such decrease is observed upon introduction of a sulfur atom (**10**). Indeed no modifications of the cyclic anhydride, except as noted for **10**, are tolerated, and consequently result in no inhibition of PP2A. We had originally believed that replacement of the O-atom with N (as N–H and N–R, where R = alkyl or aryl) would furnish us with a better understanding of the H-bonding requirements of these cantharidin analogues at PP2A, with these replacements allowing us to probe potential H-bonding acceptor/donor effects.

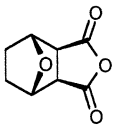
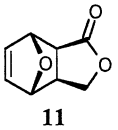
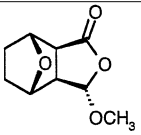
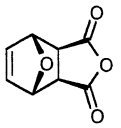
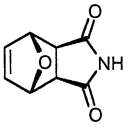
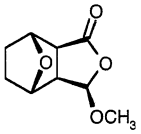
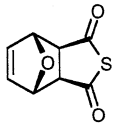
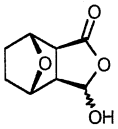
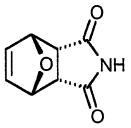
Previously we have shown that **9** undergoes a rapid conversion to the dicarboxylic acid under assay conditions, these results were also confirmed by ^{1}H-NMR spectroscopy which showed a very facile opening to the dicarboxylic acid **23** (*figure 6*) [23].

We thus examined the stability of our non-active analogues, additionally we also examined the reported inhibition values in the literature for a series of anhydride and dicarboxylic acid compounds. The result of the literature evaluation of the effect of ring opening of the anhydride group on inhibition of PP2A is shown in *table II*.

In the case of the anhydride modified analogues presented in *table I* (**11**–**16**), we examined their hydrolytic stability and noted that they were stable under assay conditions, showing no decomposition even after prolonged exposure (typically no decomposition after 12 h). Indeed **12** can be synthesised via the Diels–Alder reaction in water (at reflux) [24]. This behaviour is contrary to that found for example with the potent inhibitor **9**, as can be seen from *figure 6.*

As can be seen from *table II* the PP2A inhibition values differ when the anhydrides (all entries with the

Table I. The inhibition of protein phosphatase 2A by anhydride modified cantharidin analogues.

Compound	Inhibition of PP2A (%)[a]	Compound	Inhibition of PP2A (%)	Compound	Inhibition of PP2A (%)
 **8**	100	 **11**	21	 **14**	11
 **9**	97	 **12**	15	 **15**	9
 **10**	97	 **13**	11	 **16**	4

[a]Compounds were screened using 11.8 μg of partially purified protein phosphatase 2A from mouse brain and compounds were screened at 100 μM (compounds **8-16**).

exception of 5) are compared with those of their ring opened dicarboxylic acid partners. The general trend is that the dicarboxylic acid partner exhibits poorer PP2A inhibition values. This effect is most noticeable with the more flexible analogues: entries 2 (97–80%); 3 (48.1–17.2%); 4 (24–12.2%); and 7 (61–43%). In the case of entry 3, it is most probable that this greater reduction in binding (48.1–17.2%) is a combination of no hydrogen bonding site at what was formally the 7-O atom (of cantharidin) and the generation of the dicarboxylic acid. Interestingly Pombo-Villiar [25] reported no such decrease in the case of the corresponding bis-sodium salt [26]. The reason for this apparent anomaly is unclear as, under assay conditions generation of the dicarboxylic acid would be expected.

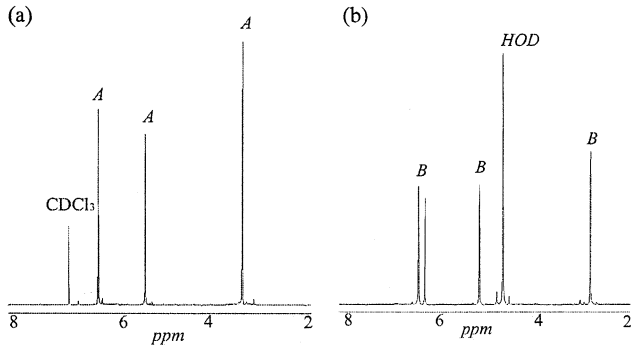
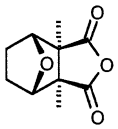
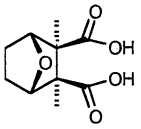
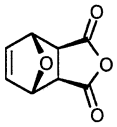
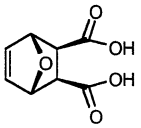
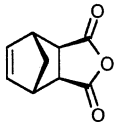
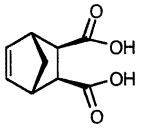
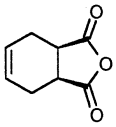
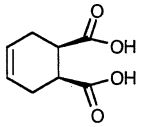
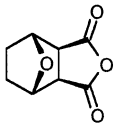
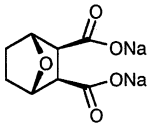
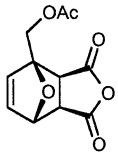
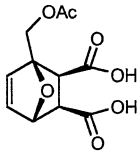
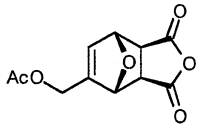
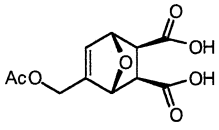


Figure 6. ^{1}H-NMR spectra obtained (a) with **9** in $CDCl_3$ and, (b) with **9** in D_2O after 10 min (similar to the conditions used during the measurement of PP2A inhibition). Peaks annotated *A* are assigned to the cyclic anhydride **9**, peaks annotated *B* are assigned to the corresponding dicarboxylic acid **23**.

The data in *table II* suggest that it is therefore important that we consider not only the ability of these compounds to ring open as the sole determining ability to inhibit PP2A, but other additional factors must also be in play. The most significant difference between the anhydride and dicarboxylic acid is the increased conformational flexibility, with the dicarboxylic acid presenting essentially four low energy conformations to the receptor prior to binding, as opposed to essentially one presented by the anhydride (*figure 7*).

Although the crystal structure of PP2A has yet to be solved data are available from the closely related PP1 [28] (and for PP2A in the form of a molecularly modelled structure) [29]. In the case of PP1, the

Table II. Effects of anhydride to dicarboxylic acid modification on the inhibition of PP2A.

Entry	Anhydride	Inhibition of PP2A	Carboxylic acid	Inhibition of PP2A	Ref.
1	 **5**	IC_{50} = 40 nM 92-95%	 **22**	IC_{50} = 53 nM 92-95%	22 24
2	 **9**	97%	 **23**	80%	23
3	 **17**	48.1%	 **24**	17.2%	23
4	 **18**	24%	 **25**	12.2%	23
5	 **19**	IC_{50} = 0.2μM	 **26**	IC_{50} = 0.17 μM	25
6	 **20**	5%	 **27**	0%	26
7	 **21**	61%	 **28**	43%	26

presence of Fe^{2+} and Mn^{2+} in the active site goes some way to explaining the increased toxicity of endothal thioanhydride with respect to **9** (with the former being three times more potent (LD_{50} = 0.31 mg/kg compared with 1.0 mg/kg, respectively) [30]. The softer S-atom displays a greater affinity for the metals in the active site compared with the harder O-atom. It is thus possible to visualise preliminary binding of the anhydride units to the two divalent metals (in the case of PP1) [28]. This initial binding then allows for either a metal hydride mediated nucleophilic attack from one of the water molecules present in the active site or direct nucleophilic attack from Tyr-272.

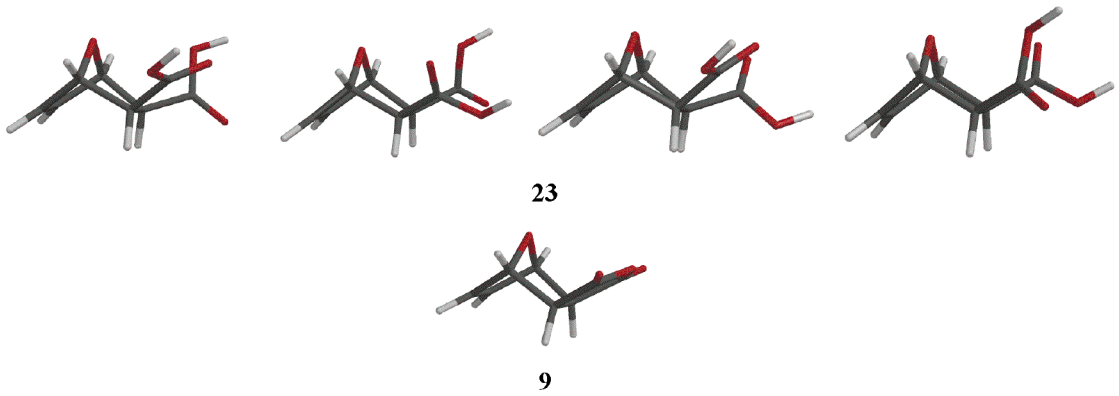


Figure 7. Molecular modelling [27] representations of the four minimum energy conformations of dicarboxylic acid **23**, and the minimum energy conformation of anhydride **9**.

3. Conclusions

The data presented herein indicate that a facile ring opening to the corresponding dicarboxylic acid species allows greater binding to PP2A. The apparent difference in the inhibition of PP2A between the anhydride and the dicarboxylic acid probably arises from a prior organisation, allowing rapid nucleophilic attack, followed by a rapid ring opening and subsequent binding to the protein. In addition Sugiyama et al. [31], have recently reported that tautomycin (**4**) binds to PP1 and PP2A in its hydrolysed dicarboxylic acid form, thus strengthening our belief that the ability of anhydrides to ring open to the dicarboxylic acid is a key step in the inhibition of PP2A by cantharidin analogues.

4. Experimental

Protein phosphatase 2A was partially purified and assayed in a modification of the method previously reported by Nishiwaky et al. [32].

Protein phosphatase 2A was isolated from mouse brain by DEAE-cellulose column chromatography using 50 mM Tris–HCl buffer (pH 7.4). Partially purified PP2A was eluted with buffer containing 0.2 M NaCl. Enzyme activity was measured in 50 mM Tris–HCl buffer (pH 7.0) containing 100 μM EDTA, 5 mM caffeine, 0.1% 2-mercaptoethanol, 0.6 mg/mL BSA and [^{32}P]-phosphorylase *a*. Inhibition of protein phosphatase activity was determined by incubation of [^{32}P]-phosphorylase *a* (5 μg) protein phosphatase 2A (11.8 μg) and 100 μM of cantharidin or cantharidin derivative (**8**–**16**) for 10 min at 30 °C in 100 μL volume in triplicate. The reaction was terminated by addition of 100 μL of ice-cold 50% trichloroacetic acid. After centrifugation an aliquot (150 μL) of supernatant was counted in Amersham Aquasol scintillant. Data are expressed as percent inhibition with respect to a control (absence of competing compound) incubation.

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