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# STRUCTURE-ACTIVITY RELATIONSHIP OF CANTHARIDIN DERIVATIVES TO PROTEIN PHOSPHATASES 1, $2A_1$, AND 2B

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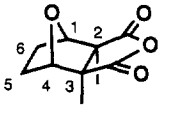
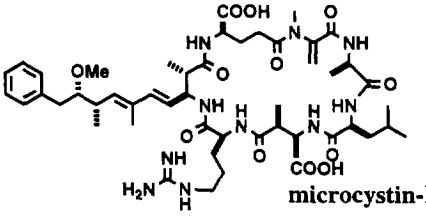
**Abstract:** The effects of structural modification of cantharidin on the inhibition of protein Ser/Thr phosphatases are described. Removal of the methyl substituents at C2 and C3 in cantharidin improved the inhibition of PP2B. In contrast, introduction of a substituent to C1/C4-position drastically decreased inhibition of PP1 and $PP2A_1$. PP2B was found to be quite tolerant to modifications at the C5 position.


Protein serine/threonine phosphatases (PP) play an important role in intracellular signal transduction.[1] The clinically useful immunosuppressants, FK-506 and cyclosporin A, are known to act through protein phosphatase 2B (PP2B, calcineurin).[2] FK-506 (and cyclosporin A) bind to an intracellular receptor protein known as an immunophilin, and this complex selectively inhibits PP2B leading to the suppression of T-cell proliferation. It is important to note, however, that neither FK-506 nor cyclosporin A alone can inhibit PP2B, as formation of the immunophilin complex is required. Various natural toxins and/or tumor promoters such as okadaic acid,[3] microcystin-LR,[4] tautomycin[5] and cantharidin[6] are also known to be strong protein phosphatase-inhibitors. While these compounds selectively inhibit protein phosphatase 1 (PP1) and/or 2A (PP2A), they are very weak inhibitors of PP2B. Thus it is of interest to find a direct inhibitor of PP2B. Such a compound could be a useful biological tool for studies of PP2B as well as a candidate therapeutic agent.

**Figure 1.**



**1**
**cantharidin**



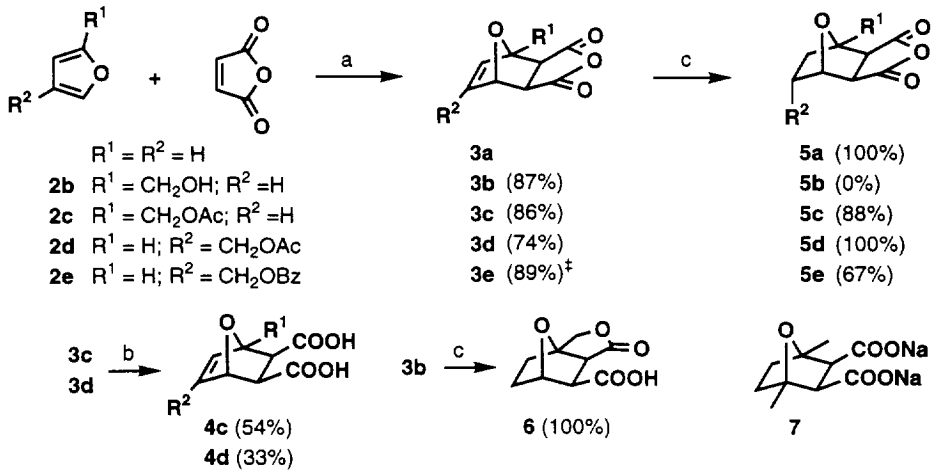
**microcystin-LR**

Based on the structure and selectivity of these natural products, as well as structural differences in these phosphatases, we undertook the challenge of designing a highly PP2B-selective inhibitor.[7] Because cantharidin (**1**) has a relatively simple structure, we chose it as our focus. The X-ray structure of the PP1-microcystin complex reveals an interaction between the two carboxylic acid groups of microcystin with the catalytic site of PP1 containing two metal ions. It is likely that the two carboxylic acid substituents in cantharidin also interact with this catalytic site, which is highly conserved through these three phosphatases. We postulated that it might be possible to change the specificity of cantharidin by introducing a substituent which favorably interacts with a neighboring non-conserved region of PP2B, but which also has an

unfavorable interaction with PP1 and PP2A. Although several cantharidin derivatives have been synthesized and tested for their ability to inhibit PP2A,[6a,8] to our knowledge, no systematic study of the structure-activity relationship including effects on PP1 and PP2B had been reported.[9] To build a model of the cantharidin-PP2B complex and to understand what part of cantharidin can be modified without loss of activity, we synthesized several cantharidin derivatives and measured their ability to inhibit PP1, $PP2A_1$ and PP2B.

The synthesis of each cantharidin derivatives was carried out as shown in Scheme 1.[10] Diels-Alder reaction of furan derivatives **2b-2e** with maleic anhydride gave the tricyclic compounds **3b-3e**.[11] These *exo-exo*-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride derivatives were stable in crystalline form, but significant amounts of retro-Diels-Alder products were detected when these derivatives were stored in diluted solution. Dicarboxylic acids **4c** and **4d**, obtained by the hydrolysis of **3c** and **3d**, were less susceptible to this retro-Diels-Alder reaction. Hydrogenation of **3a** and **3c-3e** afforded saturated products **5a** and **5c-5e**. In the case of alcohol **3b**, lactonization occurred to give **6** under the hydrogenation conditions.

**Scheme 1.**




**Reagents**: a: toluene, 23 °C (‡ 80 °C); b: aq.$NaHCO_3$-AcOEt, 23 °C; c: 10% Pd/C, $H_2$, THF, 23 °C

These cantharidin analogs were tested for their inhibition of PP1, $PP2A_1$, and PP2B at concentrations of 1 mM and 100 μM.[12] In addition to these synthetic samples, commercially available compounds **1**, **3a**, and **7** were also used for the following phosphatase assays. The results are summarized in Figure 2. As reported previously,[6a,b] cantharidin (**1**) strongly inhibited to PP1 and $PP2A_1$, but only very weak inhibition of PP2B was observed. Removal of the methyl substituents at C2 and C3 of cantharidin increased the activity of the resulting compounds (**3a** and **5a**) toward PP2B, suggesting that these substituents interact unfavorably only with PP2B. In contrast, introduction of a methyl group at C1 and C4 drastically decreased the activity of the resulting derivative (**7**) to all phosphatases, particularly in the case of PP1 and $PP2A_1$ where no inhibition was observed. It is noteworthy that compounds **7** still weakly inhibited PP2B. Compounds **3b**, **6**, **3c**, **4c**, and **5c** having a substituent at only one bridgehead position (C1/C4) also showed

decreased activity relative to the unsubstituted analogs **3a** and **5a**. Again, PP2B was more tolerant to this modification. These results suggest that there are more unfavorable interactions with one of the bridgehead positions on binding of these derivatives to PP1 and PP2A than on binding to PP2B. Substitution at the C1 or C4 position with a relatively small group may improve the selectivity of these derivatives for PP2B. Because the compounds tested here are racemic, however, we cannot distinguish which bridgehead position (C1 or C4) is most relevant at this time.

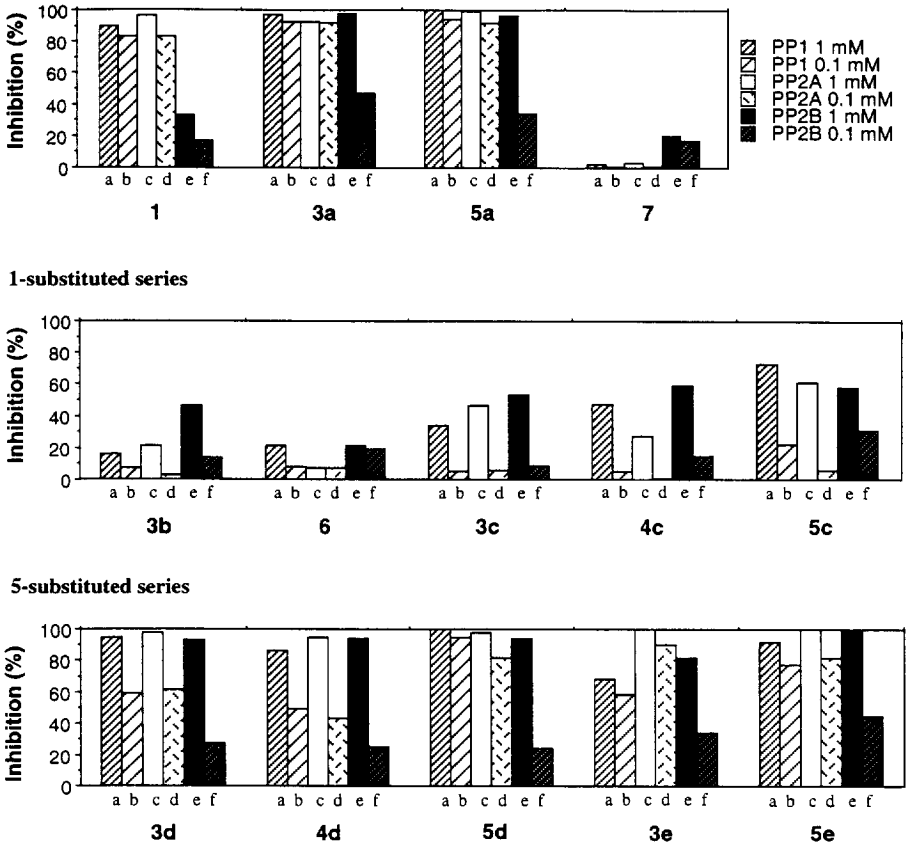



**Figure 2. Inhibition of phosphatase activity of PP1, PP2A$_1$, and PP2B with various cantharidin analogs.** Phosphatase activities were assayed in the presence or absence of the indicated test compound.[12] Inhibition is shown as % value based on the observed absorbance from the reaction without inhibitor (100%) and that without enzyme (0%). Concentration of the test compound was either 1mM (column a, c, e) or 0.1 mM (column b, d, f). Assays using PP1 (column a, b), PP2A$_1$ (column c, d), and PP2B (column e, f) as an enzyme were carried out for each compound.

Finally it was found that PP2B is quite tolerant to modifications at the C5 position. Compounds **3d**, **4d**, and **5d** inhibited all three phosphatases with an activity comparable to that of **3a** and **5a**. It is interesting that inhibition of PP1 and $PP2A_1$ by **3d** and **4d** was slightly weaker than that by **3a**. Furthermore, **3e** and **5e**, having the bulkier benzoyl substituent, also efficiently inhibited PP2B. These results suggest that further modification at the C5 position may afford a more specific and potent inhibitor of PP2B.

In summary we have assayed the inhibition of various cantharidin analogs to PP1, $PP2A_1$, and PP2B, and these results strongly indicate the direction of further structural modifications. Based on the structure-activity relationships described in this paper and the reported tertiary structures of PP2B and PP1, an effort to synthesize a highly selective inhibitor of PP2B using molecular modeling is currently in progress.

## References and Notes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9. After completion of this work, a report describing the ability of several 5-substituted cantharidin analogs including **5e** to inhibit PP2B appeared in a recent issue of this journal. This publication prompted us to report our preliminary results on the structure-activity relationship.
   Tatlock, J. H.; Linton, M. A.; Hou, X. J.; Kissinger, C. R.; Pelletier, L. A.; Showalter, R. E.; Tempczyk, A.; Villafranca, J. E. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1007.
10. Satisfactory IR, $^1$H-NMR, and mass spectral data were obtained for all synthetic compounds.
11. Synthesis of several *exo-exo*-7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic anhydride derivatives has already been reported, see: (a) Anet, F. A. L. *Tetrahedron Lett.*, **1962**, 1219. (b) Brown, G. M.; Dubreuil, P. *Can. J. Chem.* **1968**, *46*, 1577. (c) Imagawa, T.; Nakagawa, T.; Matsuura, K.; Akiyama, T.; Kawanishi, M. *Chemistry Lett.* **1981**, 903. (d) Pelter, A.; Singaram, B. *J. Chem. Soc. Perkin Trans. I* **1983**, 1383, and references cited therein.
12. Phosphatase assays were carried out according to the UBI (Upstate Biotechnology Inc.) protocol. Phosphatases, PP1 (rabbit skeletal muscle), $PP2A_1$ (rabbit skeletal muscle, composed of $\alpha$, $\beta$, and catalytic subunit), and PP2B (bovine brain), were purchased from UBI. For PP1 and $PP2A_1$ assays their Ser/Thr Phosphatase Assay Kit 1 was used. Free phosphate ion, released from a substrate phosphopeptide (KRpTIRR), was quantified by colorimetric analysis (630 nm) using the Malachite Green method. *p*-Nitrophenyl phosphate was used as a substrate for the PP2B assay. Reaction was measured by the absorption of a hydrolyzed product, *p*-nitrophenol (415 nm).


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