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Anhydride Modified Cantharidin Analogues: Synthesis, Inhibition of Protein Phosphatases 1 and 2A and Anticancer Activity

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Abstract—Two series of anhydride modified cantharidin analogues were synthesised and screened for their phosphatase inhibition (PP1 and PP2A) and cytotoxicity in various cancer cell lines (*Ovarian* A2780, ADDP; *Osteosarcoma* 143B; and *Colon* HCT116 and HT29). One series was synthesised by a novel, high yielding one-pot hydrogenation-ring-opening-esterification procedure, the other by acid catalysed acetal formation. Analogues **5**–**7** and **9** displayed moderate PP2A selectivity (ca. 5- to 20-fold) and inhibition typically in the low μM range (comparable, in some cases to cantharidin). The anticancer activity of these analogues varied with the cell line under study; however, many of them showed selective cytotoxicity for the colon tumour cell lines.

Cantharidin (*exo*,*exo*-2,3-dimethyl-7-oxobicyclo[2.2.1]-heptane-2,3-dicarboxylic acid anhydride) (**1**) (Fig. 1), in the form of the dried body of the Chinese blister beetles: *Mylabris phalerata* or *M. cichorii*[1] has been used by the Chinese as a natural remedy for the past 2000 years. Western medicine decreed cantharidin to be too toxic in the early 1900s.[2] Cantharidin has important antitumour properties and has been used as an anticancer agent by the Chinese for the treatment of hepatoma and oesophageal carcinoma.

The first recorded use as an antitumour agent was in 1264.[1] A unique feature of cantharidin observed during clinical trials was the stimulation of the bone marrow production of white cells, which is in contrast to most other anticancer drugs that readily induce myelosuppression. Despite such qualities, the nephrotoxicity of cantharidin has prevented it from entering mainstream oncology. Norcantharidin (**11**), the demethylated analogue of cantharidin, also possesses anticancer activity and stimulates the bone marrow, but without the urinary toxicity.[1] Both agents are known protein phosphatase 1 (PP1) and 2A (PP2A) inhibitors.[3]

There has been intense interest in developing potent and selective inhibitors of PP1 and PP2A in recent years. These attempts have included numerous analogues of cantharidin (so far with little success)[4] and the more complex toxins such as the Microcystin analogues developed by Chamberlin,[5] which resulted in moderate PP1 selectivity. We, too, have had an ongoing interest in cantharidin analogues, an interest that was further heightened with the discovery of fostriecin (**2**) (Fig. 1) as a selective and potent PP2A inhibitor with the additional benefit of being a potent anticancer agent.

The anticancer activity of fostriecin (**2**) has been linked to its selective inhibition of PP2A. Fostriecin exhibits >40 000-fold selectivity for PP2A (IC_{50} 3.4 nM) over PP1.[6] As with other protein phosphatase inhibitors (okadaic acid, calyculin A), fostriecin abrogates the G_2 checkpoint of the cell cycle, which forces the cell prematurely into mitosis with inadequate spindle

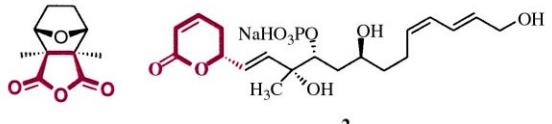

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Figure 1.

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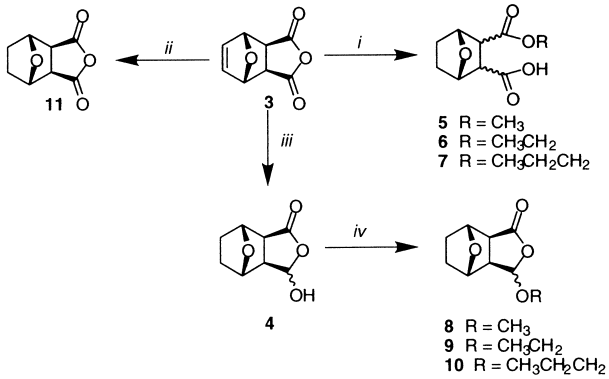
formation and poorly replicated DNA.[6] Fostriecin is active against leukemia (L1210, IC_{50} 0.46 μM), lung, breast, and ovarian cancer cells and displays efficacious in vivo antitumour activity against L1210 leukemia in mice and is currently undergoing phase I clinical trials at NCI as an anticancer agent. In addition to its phosphatase inhibition fostriecin also inhibits topoisomerase II (IC_{50} 40 μM) in vitro through a novel, non-DNA-strand cleaving mechanism, but does not induce G_2 arrest like other topoisomerase II inhibitors.[7] Fostriecin, however, is a more potent inhibitor of protein phosphatase than topoisomerase II.

As can be seen from Figure 1 there is a superficial structural commonality between **1** and **2**. Intrigued by this, we set out to synthesise a series of fostriecin/cantharidin 'cross' analogues. We believed that the synthesis of such analogues would allow the development of oxidatively stable fostriecin analogues possessing the stability and bone marrow stimulatory properties of **1** (and **11**), whilst possessing the potent phosphatases inhibition and anticancer properties of fostriecin. Additionally, we felt that these modifications may allow the introduction of a hydrophobic tail, more in line with the more potent okadaic acid class of compounds.[5] In the first instance, we were primarily interested in the addition of an aliphatic side chain within the five-membered ring of cantharidin, followed by both phosphatase (PP1 and PP2A) and anticancer screening in an attempt to define the biological action of these agents. We have recently observed that the lactone (**4**) (and other cantharidin analogues, not shown) showed selective cytotoxicity towards colon tumour cell lines compared with leukemia, ovarian, and osteosarcoma cell lines.[8] We were thus encouraged to develop a series of cantharidin/fostriecin analogues as potential anticancer agents. Herein we wish to report some of our preliminary results in the areas of protein phosphatase inhibition and anticancer activity.

Chemistry

We based our original synthesis of **4** on the work of Egglette, starting from the room temperature Diels–Alder addition of furan and maleic anhydride, affording anhydride **3** in excellent yield.[9] However, we found that using the literature protocol failed to generate even the moderate quantities of **4** required for subsequent synthetic manipulations. Closer examination of the products obtained indicated that the major product isolated was not the expected lactol **4**,[10] but the hydrogenated, ring-opened, esterified analogue **6** (see Scheme 1).[11] Subsequently, we examined the generality of this reaction and found that: The reaction proceeds smoothly in the cases of methanol, ethanol and propanol. Results with extended-chain alcohols will be reported in due course. Additionally, the reaction only proceeds in this manner if 'super-dry' alcohols are used (doubly distilled from Mg/I_2).[11,12]

In cases where rigorous drying conditions were not enforced, **4** was the sole product isolated. Treatment of



Scheme 1. (i) 10% Pd-C, H_2 4 atm, 'super-dry ROH';[11] (ii) 10% Pd-C, H_2 4 atm, acetone, 24 h; (iii) 10% Pd-C, H_2 4 atm, wet EtOH; (iv) ROH, cat *p*-TosOH, reflux 2 h.

4 with catalytic *p*-TosOH and methanol (ethanol or propanol) at reflux for 2 h followed by standard work up, resulted in the clean generation of a series of acetal-lactones **8**–**10**.

Biology

The cantharidin analogues, **5**–**10**, were screened for their ability to inhibit protein phosphatase 1 and protein phosphatase 2A.[13] Cantharidin (**1**) and norcantharidin (**11**) were included as internal standards to ensure the relative validity of our protocol. The results of the phosphatase inhibition study are shown in Table 1. We note that analogues **5**–**7** show excellent potency compared with **1** and **11**, as well as improving (albeit slightly) on PP2A selectivity.

Note that with analogues **5**–**7**, we have been able to maintain the low micromolar inhibition of PP1 and PP2A previously observed with cantharidin, and an increase, albeit a modest one, over PP2A selectivity. This is in keeping with other results, which indicated that "a ring-opened anhydride derivative" was necessary to promote efficient binding with PP2A.[14] Analogues **8**–**10** showed marginal or no protein phosphatase activity. However, **9** displayed greater PP2A selectivity than any of the analogues generated herein. We are currently investigating the origin of the PP2A selectivity of **9**.

Table 1. Inhibition of protein phosphatases 1 and 2A by compounds **1** and **5**–**11**[a]

Compound	PP1 inhibition IC_{50} (μM)	PP2A inhibition IC_{50} (μM)	PP2A selectivity
1	1.78	0.26	6.8
5	4.71	0.41	11.5
6	2.96	0.45	6.6
7	4.82	0.47	10.3
8	>1000	>1000	ND
9	746	55	13.6
10	>1000	>1000	ND
11	1.98	0.37	5.5

[a]Average of three experiments in triplicate.

Table 2. $IC_{50}(\mu M)$[a] values of tumour cell lines after 72 h continuous exposure to test compounds **1**, **5**–**11**

Tumour type/ cell line	1	5	6	7	8	9	10	11
A2780[b]	10±2	100±10	185±5	110±10	538±83	100±20	375±176	50±0
ADDP[b]	11±1.2	180±8	330±39	315±65	323±40	107±9	535±120	47±3
143B[c]	10±1.2	118±8	437±37	450±50	>1000	103±17	>1000	43±9
HCT116[d]	9±1	76±14	266±9	75±5	143±23	80±17	195±5	24±4
HT29[d]	6.4±0.7	105±5	243±39	15±4.5	28±1	26±8	41±11	33±7

[a]IC_{50} is the concentration that induces 50% growth inhibition compared with untreated control cells.
[b]Ovarian.
[c]Osteosarcoma.
[d]Colon.

In view of the anticancer activity and clinical use of cantharidin, norcantharidin, and fostriecin we also conducted cytotoxicity studies in a number of tumour cell lines, and the results from these studies are shown in Table 2.

Cytotoxicity was evaluated using the MTT assay.[15] From Table 2 it was apparent that cantharidin (**1**) and norcantharidin (**11**) were potent, but nonselective anticancer agents. In the first series of modifications that we have carried out, compounds **5**–**7** had the effect of an overall decrease in the cytotoxic potency against the cell lines illustrated. The only notable exception being **7**, which showed both moderate potency and selectivity for the colon cell lines: HCT116 and HT29 at 75 and 15 μM, respectively.

In the second series of analogues **8**–**10**, the cytotoxic potency of **8** and **10** in A2780, ADDP, and 143B cell lines was lower than that observed for the first series of analogues. However, the potency of **9** was typically comparable or better than that of the first series of analogues (A2780 100 ± 20 μM; ADDP 107 ± 9 μM; 143B 103 ± 17 μM). Interestingly, as observed for analogue **7**, analogues **8**–**10** showed a marked increase in cytotoxicity in the colon cell lines particularly the HT29 cells, where cytotoxicity resembled that observed for norcantharidin (**11**). It is noteworthy that in HT29 cells, the presence of the alkyl chain (methyl, ethyl or propyl) appeared to have little effect on cytotoxicity. Again with compounds **8**–**10**, irrespective of the magnitude of their cytotoxicity the trend observed (low-high-low) parallelled the observed ability of these compounds to inhibit protein phosphatases. Most interestingly, compound **9** showed reasonable potency across the cell lines screened, increasing in the case of colon cells, and displayed moderate phosphatase inhibition with moderate PP2A selectivity.

Finally, it was apparent that in both series of compounds, the highest cytotoxicity was observed for those analogues showing the greatest selectivity for PP2A over PP1. This observation notwithstanding, it is inherently difficult to correlate in vitro protein phosphatase inhibition with cellular cytotoxicity, as the latter will potentially be influenced by not only the degree of protein phosphatase inhibition but also by intracellular drug uptake, intracellular drug metabolism, and variations in intracellular protein phosphatase activity. Furthermore, we cannot preclude the involvement of other intracellular targets.

Conclusions

We have developed a novel one-pot reaction for the synthesis of mono-esterified analogues of cantharidin which displayed both potency and levels of selectivity at PP2A comparable to cantharidin. The corresponding acetal lactones exhibited almost no protein phosphatase inhibition, with the exception of **9** reinforcing the belief that the ring-opened form of cantharidin is the active species. Additionally, the analogues tested showed promise as selective anticolon-cancer agents. Those agents exhibiting greater inhibition of PP2A also exhibit greater cytotoxicity. This study does not rule out any other possible mode of action, but it is intriguing to think that selective inhibition of PP2A may give rise to more potent and organ/tissue specific anticancer agents.

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10. It is known that this hydrogenation is solvent dependent, with the olefin only being reduced if the hydrogenation is carried out in either acetone or ethyl acetate as the solvent.
11. A typical synthesis of **6** is as follows: anhydride **11** (5.028 g, 0.03 mol) was dissolved in dry ethanol (doubly distilled after reflux over Mg/I_2), 10%-Pd/C (500 mg) added and the mixture was shaken under 4 atmos of hydrogen at room temperature overnight (typically 17 h). The catalyst was filtered off and the solvent removed in vacuo. The crude solid was recrystallised from ethyl acetate yielding a white solid (4.089 g, 63%). Mp 108–110 °C. ^{1}H NMR ($CDCl_3$) δ 1.2 (t, 3H), 1.5 (m, 2H), 1.7 (m, 2H), 2.97 (q, 4H), 4.0 (q, 2H), 4.8 (d, 1H), 4.9 (d, 1H); ^{13}C NMR ($CDCl_3$) δ 14.75, 29.64, 29.68, 52.97, 61.83, 79.06, 79.3, 171.86, 176.86. A typical synthesis of **8** is as follows: hydroxylactone (**4**) (0.85 g, 0.005 mol) was dissolved in ethanol (10 mL), and a catalytic quantity of *p*-TosOH (ca. 20 mg) was added and the mixture was then refluxed for 2 h. After cooling, the solvent was removed in vacuo, and the resulting oil taken up in chloroform (20 mL) and washed successively with saturated $NaHCO_3$ (2×10 mL); water (10 mL), dried over Na_2SO_4. Removal of the solvent (in vacuo) yielded upon standing an off-white solid (0.723 g, 73% as a mixture of diasteromers). Mp 50–52 °C. ^{1}H NMR ($CDCl_3$) δ 1.2 (m, 3H), 1.5 (m, 2H), 1.7 (m, 2H), 2.4 (d, 1H), 2.8 (d, 1H), 3.5–3.8 (m, 2H), 4.6 (d, 1H), 4.8 (d, 1H), 5.2 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 15.0, 28.5, 29.3, 51.0, 51.7, 66.0, 80.2, 80.9, 107.0, 176.0.
12. The use of distilled or reagent grade alcohols result in the formation of **4**. Additionally, **4** appears to be the major product isolated when the reaction is conducted in higher alcohols; e.g., hexanol.
13. Protein phosphatase assays were carried out essentially as described (Collins, E.; Sim, ATR. *Methods Mol. Biol.* **1998**, *93*, 79–102) using [^{32}P]-glycogen phosphorylase *a* as substrate and recombinant PP1 (Bernt, N. *Methods Mol. Biol.* **1998**, *93*, 67–78) 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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15. **Cell culture and stock solutions**. Stock solutions were prepared as follows and stored at −20 °C: cantharidin (Biomol, USA) and cantharidin analogues as 10 mM solutions in phosphate buffered saline (PBS). All cell lines were cultured at 37 °C, under 5% CO_2 in air. The cell lines A2780 (human ovarian carcinoma) and 143B (human osteocarcinoma) were maintained in DMEM (Trace Biosciences, Australia) supplemented with 5% foetal bovine serum and 10 mM sodium bicarbonate. HT29 (human colon carcinoma) cells were maintained in DMEM supplemented with 10% foetal bovine serum and 10 mM sodium bicarbonate. HCT116 (human colon carcinoma) cells were maintained in RPMI 1640 (Trace Biosciences, Australia) supplemented with 10% foetal bovine serum. ADDP (cisplatin resistant A2780) cells were maintained in RPMI supplemented with 5% foetal bovine serum. All culture media was further supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and glutamine (4 mM). The doubling time for each cell line was 14 h for HCT116, 20–24 h for HT29 cells, 13–15 h 143B cells, 20–24 h for A2780, and 14–16 h for ADDP cells. Cells were passaged every 3–7 days and all cell lines were routinely tested and found to be mycoplasma free. **Cytotoxicity assay**. Cells in logarithmic growth were transferred to 96-well plates. Cytotoxicity was determined by plating cells in triplicate in 100 μL medium at a density of 2500–3500 cells/well for all cell lines. On day 0 (24 h after plating) when the cells were in logarithmic growth, 100 μL medium with or without the test agent was added to each well. After drug exposure growth inhibitory effects were evaluated using the MTT (3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay and absorbance read at 540 nm (Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1998**, *48*, 589). The IC_{50} was the drug concentration at which cell growth is 50% inhibited based on the difference between the optical density values on day 0 and those at the end of drug exposure (Bergman, A. M.; Ruiz van Haperen, V. W.; Veerman, G.; Kuiper, C. M.; Peters, G. J. *Clin. Cancer Res.* **1996**, *2*, 521).