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Cantharidin analogues: synthesis and evaluation of growth inhibition in a panel of selected tumour cell lines

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Abstract

Diels–Alder addition of furans (furan, furfuryl alcohol, and 3-bromofuran) to maelic anhydride yields three distinct 5,6-dehydronorcantharidins. Hydrogenation of (4,10-dioxatricyclo[5.2.1.0]decane-3,5-dione) (**4a**), in dry ethanol affords the monoester (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic aid monoethyl ester) (**6**). Subsequent transesterification affords a series of monoesters (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monomethyl ester (**7**)), 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monopropyl ester (**8**), (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monohexyl ester (**9**)) and differentially substituted diesters (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid 2-ethyl ester 3-isopropyl ester) (**10**), and (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid 2-ethyl ester 3-phenyl ester) (**11**). Analogues were firstly screened for their ability to inhibit protein phosphatases 1 (PP1) and 2A (PP2A) as the lead compounds cantharidin (**1**) and norcantharidin (**2**) are known PP1 and PP2A inhibitors. Only analogues **4a**, **6**–**8** displayed good PP1 and PP2A inhibition (PP1 IC_{50}'s = 2.0, 2.96, 4.71, and 4.82 μM, respectively; PP2A IC_{50}'s = 0.2, 0.45, 0.41, and

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0.47 μM, respectively). All analogues were also screened for their anti-cancer potential against a panel of tumour cell lines, HL60, L1210, SW480, WiDr, HT29, HCT116, A2780, ADDP, and 143B, producing GI_{50} values ranging from 6 μM to >1000 μM. Analogues possessing good PP1 and/or PP2A inhibition also returned moderate to good anti-cancer activity. Analogues with substituents directly attached to the intact bicyclo[2.2.1]heptane skeleton were poor to moderate anti-cancer agents. This correlates well with their lack of PP1 or PP2A activity. Analogues capable of undergoing a facile ring opening of the anhydride or with a single carboxylate were good PP1 and PP2A inhibitors, largely correlating to the observed anti-cancer activity in all cases, except **11**. Analogue **11**, whist neither a PP1 nor a PP2A inhibitor shows anti-cancer activity comparable to **1** and **2**. We believe that intracellular esterases generate the corresponding dicarboxylate, which is a potent PP1 and PP2A inhibitor, and that it is this species which is responsible for the observed anti-cancer activity.

Keywords: Cantharidin; Tumour cells; Growth inhibition

1. Introduction

The Meloidae family of Coleoptera (beetles) has been known since antiquity to produce a potent defensive agent, cantharidin (exo, exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) (**1**) (Fig. 1) [1]. During mating the male beetle deposits a spermatophore containing μg of cantharidin in the females spermatophoral receptacle, a copulatory gift that is then used to protect the fertilised eggs from predation [2]. Cantharidin is found in over 1500 species of beetles including *Lytta vesicatoria* (L.) which occurs around the Mediterranean area, *Lytta tenuicollis* (Pallas) in India, *Mylabris* spp. in India and China, and *Epicauta* spp. in Asia and North America [1].

Cantharidin has been used as a medicinal agent for over 2000 years and is listed as a drug under the name of Mylabris in the medical monograph *Materia Medica* published in 77 AD [3]. Some of the most ancient Chinese prescriptions (306–168 BC) refer to the use of Mylabris for the treatment of furuncles and piles [3]. In more recent times cantharidin has been used topically (0.7%) in the treatment of warts [4]. Of the medicinal uses of cantharidin the most important is its anti-cancer activity.

The first recorded use of cantharidin as an anti-cancer agent was in 1264 [3]. In more recent studies including our own, cantharidin has been shown to be active in cervical, tongue, ginival, mucoepidermoid carcinoma, adenocystic carcinoma,

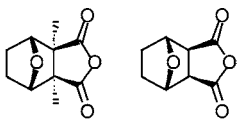


Fig. 1.

neuroblastoma, bone, leukaemia, ovarian, and colon cancer cell lines cells producing GI_{50} values in the range of 1.3–15 μM [3,5–8]. Interestingly, clinical trials involving cantharidin have shown this agent to induce haemopoiesis, this is in contrast with most other chemotherapy drugs that induce the dose-limiting effect of myelosuppression. Although cantharidin is cytotoxic to cancer cells and stimulatory on the bone marrow, the renal toxicity of this drug has prevented its use in mainstream oncology.

Norcantharidin (**2**), the demethylated analogue of cantharidin also possesses anticancer activity and stimulates the bone marrow, however, the nephrotoxicity associated with cantharidin treatment is absent [3,9]. Norcantharidin is active in vitro against several tumour cell lines including cervical, hepatoma, ovarian, laryngocarcinoma, colon, osteocarcinoma, and leukaemia cell lines [3–5,7,8].

Both cantharidin and norcantharidin are known protein phosphatase 1 (PP1) and protein phosphates 2A (PP2A) inhibitors [10,11]. Of all the known naturally occurring protein phosphatase inhibitors, cantharidin is structurally the simplest. PP1 and PP2A via reversible phosphorylation modulate numerous cellular signal transduction events, moderating such diverse functions as neurotransmission, muscle contraction, glycogen synthesis, T-cell activation, and cell proliferation [12–16]. It is therefore, not surprising that there have been considerable efforts directed towards the synthesis of more potent and selective cantharidin analogues [10,17]. We have recently described, in detail, all the known cantharidin SAR data [10,11]. Briefly, no modification of the bicyclo[2.2.1] skeleton is permissible, although the presence of double bond (5,6-ene) has little effect on activity (but does reduce analogue stability); the 7-oxa bridge [18], and the ability to generate a minimum of one free carboxylate at the enzymes active site are required to maintain activity [19,20]. Importantly, we and others have shown that hydrolysis of the anhydride is very facile in aqueous media [10]. As part of our ongoing investigations we have been exploring synthetic methodologies towards the development of a series of cantharidin analogues with our primary interest in obtaining SAR data pertaining to the known anti-cancer activity of cantharidin and norcantharidin.

2. Experimental

2.1. Materials

All reagents were of commercial quality and were used as received (Aldrich). Solvents were dried and purified using standard techniques. Reactions were monitored by TLC, on aluminum plates coated with silica gel with fluorescent indicator (Merck 60 F_{254}). Unless otherwise noted, NMR spectra were recorded in $CDCl_3$ at 300 MHz for ^1H and at 75 MHz for ^{13}C (Bruker Advance 300MX). Elemental analyses were determined by the University of Queensland Microanalysis Service. Mass spectra (*m*/*e*) were obtained in the EI (70 eV) mode at the Organic Mass Spectrometry Facility at the University of Tasmania using a Kratos Analytical Concept ISQ high-resolution mass spectrometer.

2.2. Chemistry

2.2.1. 4,10-Dioxa-tricyclo[5.2.1.0]dec-8-ene-3,5-dione (**4a**)

Furan (20 mL, 275 mmol) and maleic anhydride (5 g, 51 mmol) were stirred together in ether (10 mL) at room temperature (ca. 30 °C) for 48 h after which the white precipitate was collected. Yielded 8.259 g, 97%.

^{1}H NMR ($CDCl_3$): δ 3.15 (2H, s), 5.43 (2H, s), 6.55 (2H, s).

^{13}C NMR ($CDCl_3$): δ 49.4 (2C), 82.90 (2C), 137.67 (2C), 170.55 (2C).

2.2.2. 8-Bromo-4,10-dioxa-tricyclo[5.2.1.0]dec-8-ene-3,5-dione (**4b**)

3-Bromofuran (1.00 g, 0.61 mL, 6.8 mmol) and maleic anhydride (0.67 g, 6.8 mmol) were stirred together in ether (10 mL) at room temperature (ca 30 °C) for 48 h after which the white precipitate was collected. Yielded 0.807 g, 48.1%. mp 130–132 °C.

^{1}H NMR ($CDCl_3$): δ 3.35 (1H, s), 3.46 (1H, d), 5.22 (1H, s), 5.37 (1H, s), 6.70 (1H, d).

^{13}C NMR ($CDCl_3$): δ 49.45, 50.86, 85.15, 86.93, 127.37, 137.39, 170.60, 170.92.

2.2.3. 1-Hydroxymethyl-4,10-dioxa-tricyclo[5.2.1.0]dec-8-ene-3,5-dione (**4c**) and 4-Oxo-3,10-dioxa-tricyclo[5.2.1.0]decane-6-carboxylic acid (**5**)

Maleic anhydride (5 g, 51 mmol) was dissolved in ether (50 mL). To this was added furfuryl alcohol (4.4 mL, 51 mmol). The solution was stirred for 24 h at room temperature (ca. 30 °C) after which the off-white precipitate was filtered and washed with ice-cold ether (2 × 20 mL). Note that prolonged standing at room temperature leads to decomposition. Consequently the adduct (5.00 g, 25.5 mmol) was dissolved in ice-cold methanol (150 mL) and 10%-Pd/C added and the resultant suspension hydrogenated at 60 psi for 24 h at room temperature. After this time the suspension was filtered through a celite pad, the pad washed with ice-cold ether (2 × 20 mL), and the solvent removed *in vacuo* to yield a white solid (3.23 g, 32% two steps).

Compound ***4c***, *mp*: *82–83 °C*. ^{1}H NMR: δ 2.17 (1H, br s), 3.25 (1H, d), 3.34 (1H, d), 4.19 (2H, d), 5.42 (1H, d), 6.58 (1H, dd), 6.62 (1H, d).

^{13}C NMR: δ 48.79, 50.83, 59.64, 81.62, 92.07, 136.99, 137.87, 176.18, 175.23.

Compound ***5***, *mp*: *187–188 °C*. ^{1}H NMR: d 1.73 (2H, m), 1.91 (2H, m), 3.14 (1H, d), 3.35 (1H, d), 4.55 (2H, s), 4.73 (1H, d).

^{13}C NMR: d 28.17, 29.87, 52.74, 53.31, 69.95, 81.51, 91.26, 174.33, 176.59.

2.2.4. 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monoethyl ester (**6**)

Anhydride **4a** (1.000 g, 6 mmol) was dissolved in dry ethanol (25 mL) and 10%-Pd/C (0.100 g) added and the mixture hydrogenated at 4 atmospheres for 17 h. After this time the spent catalyst was filtered off and the solvent removed. Recrystallisation from ethyl acetate afforded an off-white solid. Yielded 0.813 g, 63%. Mp 113–114 °C.

^{1}H NMR ($CDCl_3$): δ 1.20 (3H, t), 1.50 (2H, m), 1.70 (2H, m), 2.97 (2H, dd), 4.00 (2H, q), 4.8 (1H, d), 4.90 (1H, d).

^{13}C NMR ($CDCl_3$): δ 14.75, 29.64, 29.68, 52.97 (2C), 61.83, 79.06, 79.30, 171.53, 176.86.

*2.2.5. 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monomethyl ester (**7**)*

The synthesis of **7** was carried out as described for **6** with hydrogenation in dry methanol. Recrystallisation from ethyl acetate afforded an off-white solid. Yielded 0.547 g, 48%. Mp 140–141 °C.

^{1}H NMR($CDCl_3$): δ 1.51 (2H, m), 1.79 (2H, m), 3.00 (2H, m), 3.64 (3H, s), 4.88 (1H, s), 4.93 (1H, s).

^{13}C NMR ($CDCl_3$): δ 29.58, 29.65, 52.84 (2C), 52.95, 78.99, 79.33, 172.16, 176.14.

*2.2.6. 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monopropyl ester (**8**)*

The synthesis of **8** was carried out described for **6** with hydrogenation in dry propan-1-ol. Recrystallisation from ethyl acetate afforded an off-white solid. Yielded 1.111 g, 81%. Mp 78–80 °C.

^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t), 1.50 (2H, m), 1.60 (2H, m), 1.80 (2H, m), 2.99 (2H, m), 4.00 (2H, m), 4.87 (1H, d), 4.94 (1H, d).

^{13}C NMR (DMSO-d_6): δ 11.03, 22.44, 29.58, 29.65, 52.97 (2C), 67.56, 79.07, 79.42, 172.16, 176.14.

*2.2.7. 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid monohexyl ester (**9**)*

The synthesis of **9** was carried out described for **6** with hydrogenation in dry hexanol. Recrystallisation from ethyl acetate afforded an off-white solid. Yielded 0.547 g, 48%. Mp 78–80 °C.

^{1}H NMR (DMSO-d_6): δ 0.88–1.51 (13H, m), 2.99 (2H, s), 3.39 (2H, m), 3.91 (2H, m), 4.68 (2H, s).

^{13}C NMR (DMSO-d_6): δ 13.86, 22.01, 22.18, 25.07, 25.22, 27.93, 28.52, 28.55, 30.94, 32.54, 51.30, 51.92, 60.76, 63.86, 77.54, 77.74, 171.07, 172.20 (2 isomers).

*2.2.8. 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid 2-ethyl ester 3-isopropyl ester (**10**)*

Monoester **6** (0.510 g, 3 mmol) was dissolved in dry isopropyl alcohol (10 mL) and a catalytic amount of *p*-toluenesulfonyl chloride was added, and the mixture refluxed for 3 h. After this time the solvent was removed. Column chromatography (hexane/ethyl acetate) and recrystallisation from ethyl acetate afforded an off-white solid. Yielded 0.49 g, 64%.

^{1}H NMR ($CDCl_3$): δ 1.50 (9H, m), 1.79 (4H, m), 3.57 (1H, s), 3.60 (1H, s), 4.00 (1H, sept), 4.11 (2H, q), 4.90 (1H, m).

^{13}C NMR ($CDCl_3$): δ 10.14, 21.75, 21.86, 28.98, 29.13, 52.34, 52.46, 60.94, 66.75, 78.34, 78.76, 171.15, 171.23,

*2.2.9. 7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid 2-ethyl ester 3-phenyl ester (**11**)*

Monoester **6** (1.000 g, 4.6 mmol) was dissolved in benzyl bromide (8 mL) added together with 2.5 g finely powered KOH and the mixture refluxed for 3 h. After cooling the solution was diluted with 100 mL of water and extracted with chloroform (2×40 ml). The extract was washed with water (1×50 ml) and then dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was placed under

high vacuum and purification was initially carried out using flash chromatography, using a solvent system of hexane ethyl acetate. The diastereomers were further purified by chromatography with a solvent system of 99:1 hexane: ethyl acetate. Yielded 1.09 g, 78%.

11a. ^{1}H NMR ($CDCl_3$): δ 1.24 (3H, t), 1.84 (2H, m), 1.87 (2H, m), 3.21 (1H, s), 3.23 (1H, s), 4.08 (2H, q), 4.89 (2H, m), 5.22 (2H, s), 7.37–7.42 (5H, m).

^{13}C NMR ($CDCl_3$): δ 13.95, 28.95, 28.98, 50.92, 51.11, 66.82, 77.40, 77.81, 80.56, 127.53, 128.14 (2C), 128.30, 128.69, 138.15, 170.74, 172.05.

11b. ^{1}H NMR ($CDCl_3$): δ 1.25 (3H, t), 1.55 (2H, m), 1.57 (2H, m), 3.12 (1H, s), 3.13 (1H, s), 4.14 (2H, q), 4.83 (2H, m), 5.13 (2H, s), 7.29–7.34 (5H, m).

^{13}C NMR ($CDCl_3$): δ 14.17, 29.18 (2C), 51.03, 51.22, 66.91, 77.92 (2C), 77.81, 80.66, 128.18, 128.24, 128.32, 128.41, 128.65, 147.24,171.03, 172.14.

11c. ^{1}H NMR ($CDCl_3$): δ 1.15 (3H, t), 1.49 (4H, m), 2.98 (2H, s), 4.01 (2H, q), 4.90 (1H, s), 4.91 (1H, s), 5.01 (2H, s), 7.28–7.30 (5H, m).

^{13}C NMR ($CDCl_3$): δ 13.97, 29.00 (2C), 51.92, 52.15, 66.62, 78.35, 78.41 (2C), 128.16, 128.27, 128.33, 128.44 (2C), 151.19, 170.75, 170.82.

11d. ^{1}H NMR ($CDCl_3$): δ 1.28 (3H, t), 1.78 (2H, m), 1.80 (2H, m), 3.21 (1H, s), 3.23 (1H, s), 4.20 (2H, q), 4.56 (2H, m), 5.19 (2H, s), 7.35–7.39 (5H, m).

^{13}C NMR ($CDCl_3$): δ 13.67, 28.62 (2C), 50.63, 50.84, 64.65, 72.25, 77.49 (2C), 127.23 (2C), 127.37 (2C), 127.97, 140.64, 170.44, 171.62.

2.2.10. Cell culture and stock solutions

Stock solutions were prepared as follows and stored at −20 °C: Cantharidin (Biomol, USA) as a 12 mM solution in dimethylsulphoxide (DMSO); norcantharidin and cantharidin analogues as 10 mM solutions in phosphate buffered saline. 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 Dulbecco's modified Eagle's medium (Trace Biosciences, Australia) supplemented with 5% foetal bovine serum and 10 mM sodium bicarbonate. HT29 (human colon carcinoma), WiDr (human colon carcinoma), and SW480 (human colon carcinoma) cells were similarly maintained, however, they were supplemented with 10% foetal bovine serum. L1210 (murine lymphocytic leukemia), HL60 (human promyelocytic leukemia) and HCT116 (human colon carcinoma) cells were maintained in RPMI 1640 (Trace Biosciences) supplemented with 10% foetal bovine serum. ADDP (cisplatin resistant A2780) cells were similarly maintained however, they were 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).

2.2.11. In vitro growth inhibition 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 1,000 cells/well for L1210, 5000 for HL60, and 2500–2800 cells/well for the remaining 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 72 h drug exposure growth inhibitory effects were evaluated using the MTT

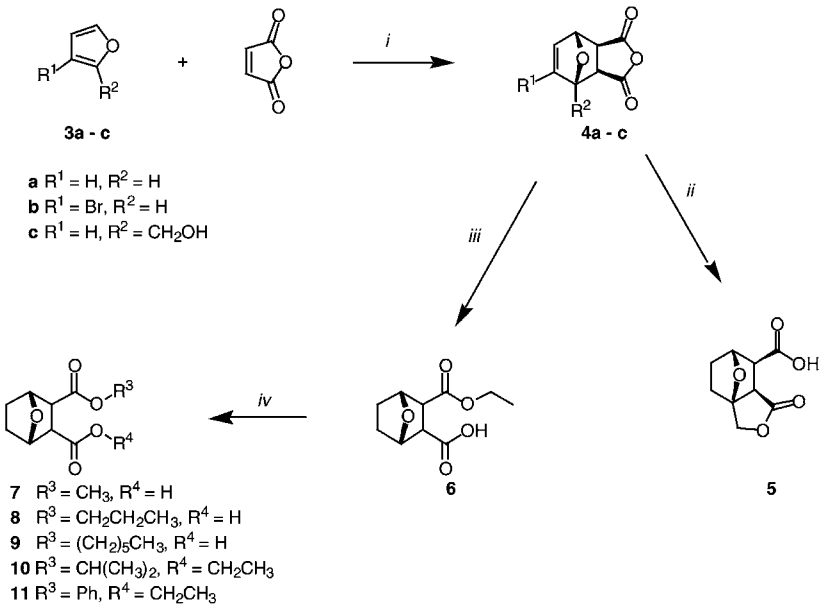
(3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay and absorbance read at 540 nm. The GI_{50} was the drug concentration at which cell growth is 50% inhibited based on the difference between the optical density values on day 0 and those at the end of drug exposure [23].

2.2.12. Protein phosphatase inhibition

A nonradioactive in vitro assay detailed by Gupta (1997) [24] was adopted to measure PP1 and PP2A enzyme activity in the presence of inhibitors. Serine/threonine protein phosphatase assay kit, purified PP1 (rabbit skeletal muscle), PP2A (human red blood cells), and a hexapeptide (Lys–Arg–pThr–Ile–Arg) substrate were purchased from Upstate Biotechnology (Lake Placid, NY). The concentration of PP2A, PP1, and substrate used in the assay was 0.3 mU/well, 30 mU/well, and 200 μM, respectively. The reactions were initiated by addition of substrate (5 μL) to a mixture containing enzyme (5 μL), reaction buffer (10 μL; 50 mM Tris–HCl, pH 7.0, 100 μM $CaCl_2$), and inhibitor (10 μL), producing a total reaction volume of 30 μL/well and incubated at RT for 60 min. Immediately prior to addition of substrate, the enzyme and inhibitor were preincubated for 10 min. Reactions were halted via addition of a malachite green solution (50 μL), and absorbance readings were taken at 650 nm after 10 min development time. Samples were blanked against wells containing enzyme (5 μL), and buffer (25 μL). Initial inhibitor dilutions were made in DMSO and subsequent dilutions were made in distilled deionised H_2O. Enzyme dilutions were made with buffer containing 20 mM MOPS, pH 7.5, 0.15 M NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 2 mM EGTA, 0.1 mM $MnCl_2$, 1 mM DTT, 10% glycerol, and 0.1 mg/ml serum albumin. A dose response curve of percentage enzyme activity versus drug concentration was produced from which an IC_{50} value was calculated indicating the concentration of drug required to inhibit enzyme activity by 50%. Data represent the means (±SEM) IC_{50} of three independent replicates.

3. Results and discussion

Herein we report the synthesis and biological evaluation of two series of norcantharidin analogues (Scheme 1). In the first series we investigated the effects of subtle skeletal modifications upon the compounds anti-cancer activity. Room temperature Diels–Alder addition of furans **3a**–**c** and maleic anhydride yielded unsubstituted **4a** (90%), similar conditions allowed the generation of the 5-bromo analogue **4b** (48%), and the bridgehead methanol analogue **4c** (61%). Stirring **4c** in methanolic HCl yielded the carboxy-lactone **5** arising from the opening of the anhydride and the acid catalysed lactonisation with the bridgehead CH_2OH (64%). Interestingly hydrogenation (H_2, 4 atm, 10%-Pd/C) of **4a** in super-dry ethanol yielded the ring opened monoester **6** as the major reaction product (as a mixture of stereoisomers), which after purification was transesterified (*p*-TosOH, ROH) to yield either the series of monoesters **7**–**9**, or diesters **10** and **11**. Compounds were typically purified by column chromatography followed, where appropriate, by recrystallisation.



Scheme 1. Reagents and conditions. (i) Diethylether, RT, 24 h; (ii) MeOH/HCl, RT 3 h; (iii) 10% Pd–C, H_2 (4 atm), EtOH; (iv) *p*-TosOH, ROH, 80 °C.

Eggelete [21] has shown the hydrogenation of **4a** to be solvent dependent, using acetone hydrogenation occurs solely at the 5,6-double bond, whereas with ethanol or ethyl acetate both the 5,6-double bond and one of the C=O's are reduced to afford the saturated hydroxy lactone as a mixture of two stereoisomers. We have recently explored this chemistry, finding that the major product isolated in the case of 'super-dry' ethanol was the saturated monoethyl ester **6**. We also found this reaction worked well with methanol and propanol, only after scrupulous drying of each alcohol (Mg/I_2). It is conceivable with **5** that the first formed product is the corresponding monoethyl ester, as in **6**, followed by an intramolecular transesterification, however, it is also possible to generate **5** directly via intramolecular alcohol trapping. Neither our work nor that of Yadav et al. [22] in the synthesis of advance intermediates in the synthesis of Taxol have explored the exact nature of the reaction mechanism.

The PP1 and PP2A inhibitory data, and the growth inhibitory profile of **1**–**11** are shown in Table 1. For comparative purposes cantharidin (**1**) and norcantharidin (**2**) have also been included.

It is apparent, in view of the protein phosphatase inhibition data presented in Table 1 that direct supply of analogues with a free carboxylate is crucial to ensure good inhibition. This is highlighted by the marked differences in inhibition between **8** (PP1 $IC_{50} = 4.82\,\mu M$; PP2A $IC_{50} = 0.47\,\mu M$) and **11** (PP1 $IC_{50} > 200\,\mu M$; PP2A $IC_{50} > 200\,\mu M$) after esterification of the sole remaining carboxylate. These data are in accord with previous studies in this area. From Table 1 it is also apparent that cantharidin (**1**) is a potent broad-spectrum anti-cancer agent returning low μM

Table 1
Growth inhibition of Cantharidin (**1**), norcantharidin (**2**) 5,6-dehydronorcantharidin (**4a**), skeletally modified norcantharidin analogues (**4b**, **4c**, **5**) monoester analogues (**6**–**9**), diester analogues (**10**, **11**), and in a panel of tumour cell lines after 72 h continuous exposure

Compound	IC_{50} μM		GI_{50} μM (Cell line)[a]								
	PP1	PP2A	HL60	L1210	HT29	WiDr	SW480	HCT118	A2780	ADDP	143B
1	1.78	0.26	10 ± 2	15 ± 2	7 ± 0.5	6 ± 0.5	13 ± 5	9 ± 1.1	10 ± 2	11 ± 1	10 ± 1
2	1.98	0.37	25 ± 2	13 ± 0.3	33 ± 7	36 ± 5	33 ± 7	24 ± 4	40 ± 11	47 ± 3	43 ± 9
4a	2.0	0.2	177 ± 3	185 ± 51	183 ± 20	198 ± 53	155 ± 9	160 ± 10	157 ± 9	183 ± 17	248 ± 29
4b	>200	>200	—	>1000	>1000	>1000	>1000	—	—	—	—
4c	>200	>200	—	490 ± 90	>1000	>1000	750 ± 41	—	—	—	—
5[b]	—	—	—	—	—	—	—	—	—	—	—
6	2.96	0.45	333 ± 88	443 ± 92	243 ± 39	450 ± 60	310 ± 20	266 ± 9	185 ± 5	330 ± 39	437 ± 37
7	4.71	0.41	117 ± 9	263 ± 19	105 ± 5	257 ± 34	150 ± 17	76 ± 14	100 ± 10	180 ± 8	118 ± 8
8	4.82	0.47	270 ± 15	313 ± 3	15 ± 4	33 ± 6	118 ± 13	75 ± 5	110 ± 10	315 ± 65	450 ± 50
9	>200	>200	—	500 ± 123	450 ± 76	575 ± 61	260 ± 45	—	—	—	—
10	>200	>200	—	>1000	>1000	>1000	>1000				
11	>200	>200	—	23 ± 4	24 ± 4	38 ± 9	38 ± 6	18 ± 5	16 ± 3		

[a] GI_{50} calculated after 72 h of continuous drug exposure relative to untreated controls. Values are the means (±SEM) of three to four experiments.
[b] Insoluble in the testing medium. —, Not determined.

potencies (average of ca. 10 μM) across all cell lines examined. A similar trend was noted for norcantharidin (**2**), albeit at a slightly higher GI_{50} value (average of ca. 33 μM). Analogue **4a** shows moderate anti-cancer activity with GI_{50}'s ranging from 155–248 μM. Neither **4b** nor **4c** displayed any such activity. Of **4a**–**4c**, only **4a** displays any notable PP1 and/or PP2A inhibition (PP1 $IC_{50} = 2.0\,\mu M$; PP2A $IC_{50} = 0.2\,\mu M$); the 5-bromo (**4b**) and the 4-CH_2OH (**4c**) substituents are known to be detrimental to PP1 and PP2A inhibition [10]. Analogues **6**–**8** displayed both moderate PP1 (IC_{50}'s = 2.96, 4.71 and 4.82 μM respectively) and PP2A (IC_{50}'s = 0.45, 0.41, and 0.47 μM, respectively) inhibition and displayed poor to moderate anti-cancer activity against the cell lines illustrated. The only notable exception being **8**, which showed both good potency and selectivity for the colon cell lines: HT29, WiDr and HCT116 at 15, 33, and 75 μM, respectively. Of all the other analogues screened only poor GI_{50} values were observed, excepting compounds **5** and **11**. With these latter analogues, no data was obtainable for **5** being insoluble in the testing media, whilst **11** displays GI_{50} values comparable to **2** against L1210 ($23 \pm 4\,\mu M$), HT29 ($24 \pm 4\,\mu M$), WiDr ($38 \pm 9\,\mu M$), and SW480 ($38 \pm 6\,\mu M$) cell lines and comparable to **1** against HCT116 ($18 \pm 5\,\mu M$) and A2780 ($16 \pm 3\,\mu M$) cell lines. In light of the poor PP1 and PP2A inhibition it is somewhat surprising that **11** shows comparable anti-cancer activity. While this lack of enzyme (protein phosphatase) inhibition potentially indicates an alternative mode of anti-cancer activity, it does not preclude operation via protein phosphatase inhibition. In this regard the diester linkages of **11** are likely to have undergone hydrolytic degradation by intracellular esterases resulting in the intracellular formation of the dicarboxylate and subsequent inhibition of PP1 and PP2A. Cell line to cell line variations in the concentrations of intracellular esterases and subtle variations in cell permeability would also account for the differences in growth inhibition of **11** compared with **2** (note that **2** will yield the identical dicarboxylate on facile ring opening of the anhydride). This phenomenon also explains the lack of diastereometric discrimination in the growth inhibitory profiles of **11a**–**11b**. Further to this, all of the mono and diester compounds would also be amenable to similar enzymatic degradation, however, their lack of membrane permeability may have precluded the initiation of this reaction.

From the data obtained it appears that a free carboxylate is detrimental to cytotoxicity in the panel of cell lines examined, and in the one other case where the carboxylate was masked by a second ester group, compound **10**, this analogue also showed GI_{50} values >1000 μM suggesting that the phenyl group of **11** is crucial for activity.

It is noteworthy that the synthesis of **11** is not stereocontrolled and as such was tested as a mixture of stereoisomers. In an attempt to examine potential stereochemical influences on the observed cytotoxicity we further purified **11** into four diastereoisomers by flash chromatography and re-examined the cytotoxicity of each of these diastereoisomers in turn. These data are displayed in Table 2. From the data in Table 2 it is apparent that the cytotoxicity of **11** is independent of stereochemistry. This in turn suggests that the binding site of these isomers is highly tolerant of the spatial orientation of the side-chains and how they are presented within the active site.

Table 2
Growth inhibition of diastereoisomers of **11** in a panel of tumour cell lines after 72 h continuous exposure

GI_{50} (μM)[a]					
Cell line	**11**	**11a**	**11b**	**11c**	**11d**
L1210	23 ± 4	26 ± 4	19 ± 4	32 ± 4	23 ± 3
HT29	24 ± 4	20 ± 2	18 ± 1	21 ± 2	16 ± 2
WiDr	38 ± 9	27 ± 4	24 ± 3	27 ± 7	24 ± 5
SW480	38 ± 6	28 ± 3	30 ± 2	30 ± 1	26 ± 3
HCT116	18 ± 5	20 ± 2	17 ± 3	21 ± 3	18 ± 4
A2780	16 ± 3	14 ± 2	16 ± 2	15 ± 3	14 ± 1

[a] GI_{50} calculated after 72 h of continuous drug exposure relative to untreated controls. Values are the mean (±SEM) of three to four experiments.

4. Conclusions

As can be seen from the data presented subtle modifications of cantharidin's skeleton permitted the development of a new series of analogues with significant alterations in their cytotoxicities against a panel of cell lines. Of the analogues examined only the ethyl phenyl diester (**11**) showed promise as a lead compound in this area. This compound is not a potent protein phosphatase inhibitor suggesting an alternative mode of action to that of cantharidin and norcantharidin. However, intracellular degradation of the ester linkages of **11** may well have produced such a compound. Additionally our studies do not preclude the possibility of bio-modification by the culture media, although we have previously re-isolated cantharidin from assay media unchanged [18]. We are currently working towards the development of more potent and selective analogues of this nature and will report the outcome of these studies in due course.

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