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Synthesis and biological evaluation of norcantharidin analogues: Towards PP1 selectivity

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Abstract—Simple modifications to the anhydride moiety of norcantharidin have lead to the development of a series of analogues displaying modest PP1 inhibition (low μM IC_{50}s) comparable to that of norcantharidin (PP1 $IC_{50} = 10.3 \pm 1.37$ μM). However, unlike norcantharidin, which is a potent inhibitor of PP2A ($IC_{50} = 2.69 \pm 1.37$ μM), these analogues show reduced PP2A inhibitory action resulting in the development of selective PP1 inhibitory compounds. Data indicates that the introduction of two *ortho*-disposed substituents on an aromatic ring, or *para*-substituent favours PP1 inhibition over PP2A inhibition. Introduction of a *p*-morphilinoaniline substituent, **35**, affords an inhibitor displaying PP1 $IC_{50} = 6.5 \pm 2.3$ μM; and PP2A $IC_{50} = 7.9 \pm 0.82$ μM (PP1/PP2A = 0.82); and a 2,4,6-trimethylaniline, **23**, displaying PP1 $IC_{50} = 48 \pm 9$; and PP2A IC_5 85 ± 3 μM (PP1/PP2A = 0.56). The latter shows a 7-fold improvement in PP1 versus PP2A selectivity when compared with norcantharidin. Subsequent analysis of **23** and **35** as potential PP2B inhibitors revealed modest inhibition with IC_{50}s of 89 ± 6 and 42 ± 3 μM, respectively, and returned with PP1/PP2B selectivities of 0.54 and 0.15. Thus, these analogues are the simplest and most selective PP1 inhibitors retaining potency reported to date.

1. Introduction

The intricately linked protein phosphorylation and dephosphorylation cycle is regulated by a fine balance between kinases and the phosphatases, respectively.[1–6] The prior notion that phosphatases existed merely as on–off switches moderating the action of kinases is a vast oversimplification of their role, especially in the cell cycle.[7–9] Further to this, it is now known that phosphatases have a vital and delicate role to play in a myriad of biological processes.[7] Within the phosphatase family, the serine/threonine phosphatases comprising of PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7 are responsible for the vast majority of dephosphorylation events in living organisms. Of these phosphatases, PP1 and PP2A account for >90% of phosphatase activity and as such have received the greatest attention.[7–9]

Both PP1 and PP2A are strongly inhibited by a number of naturally occurring toxins: okadaic acid (**1**) (PP1 $IC_{50} = 60$ nM; PP2A $IC_{50} = 1$ nM);[10] calyculin A (**2**) (PP1 $IC_{50} = 0.5$–1.0 nM; PP2A $IC_{50} = 2.0$ nM);[11] microcystin-LR (**3**) (PP1 $IC_{50} = 1.7$ nM; PP2A $IC_{50} = 0.04$ nM);[12] tautomycin (**4**) (PP1 $IC_{50} = 0.3$ nM; PP2A $IC_{50} = 1$ nM);[13] tautomycetin (**5**) (PP1 $IC_{50} = 1.6$ nM; PP2A $IC_{50} = 62$ nM)[14] and fostriecin (**6**) (PP1 $IC_{50} = 131{,}000$ nM; PP2A $IC_{50} = 3.4$ nM)[15] are exemplars of these toxins. Of these only fostriecin exhibits noteworthy PP2A selectivity (~40,000-fold). PP1 selectivity has proved more problematic to achieve with poor PP1 selectivity reported for tautomycin (~4-fold), tautomycetin (~40-fold), a modified microcystin-LA analogue developed by Chamberlin (~7-fold)[16] and phospatidic acid (>100-fold).[17]

The discovery of a selective PP1 inhibitor has the potential to initiate a number of biochemical studies as to this protein's exact role. However, the advent of tautomycetin and phosphatidic acid has resulted in only modest progress thus far. As with a number of members of the okadaic acid class of compounds, tautomycetin displays antibiotic properties,[18] has been used as an

Keywords: Cantharidin; Norcantharidin; Small molecule protein phosphatase inhibitors; PP1; PP2A; PP2B.
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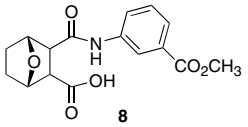
immunosuppressive agent[19,20] and induces morphological changes (bleb formation) in human leukaemia cells (K562).[21] Studies with tautomycetin have highlighted a role for PP1 as a positive regulator of Raf-1.[22] The observed growth inhibition by tautomycetin in a range of colorectal cancer cell lines (HT-15, HT-29 and DLD-1) is believed to be acquired via Raf-1 modulation.[23] It is known that tautomycetin has potential application as an anti-rejection/immunomodulatory agent potentiating the effects of cyclosporine following organ transplantation, suggesting a possible role for PP1, and obviously a potential new drug target for small molecule PP1 specific inhibitors (Fig. 1).

Of all the known inhibitors of PP1 and PP2A, cantharidin (**7**) is structurally the simplest and displays modest PP2A selectivity (PP1 $IC_{50} = 1.78\ \mu M$; PP2A $IC_{50} = 0.26\ \mu M$),[24] it has however tolerated little in the way of structural modifications that impart either improved potency or selectivity. Sodeoka has had excellent success utilising the norcantharidin (**11**) framework as a protein phosphatase inhibitor core in the design and synthesis of selective PP2B analogues.[25,26] Our group has had very modest success in this area with modified norcantharidin analogues;[27–35] however, in this instance the improvements in PP2A versus PP1 selectivity were the result of a loss of both PP1 and PP2A inhibitory activity, with PP1 potency less affected. Given our past and continuing interest in elegantly simple modification of the synthetically amenable PP1 and PP2A inhibitor, norcantharidin, we have continued our efforts to develop more potent and selective inhibitors of both PP1 and PP2A.[27–35] In this paper, we report our latest developments in this area and take small steps towards the first true small molecule inhibitors displaying PP1 selectivity.

2. Results and discussion

Our initial synthetic efforts focused on the simple manipulation of **8**, a norcantharidin (**11**) analogue derived from anhydride ring opening.[29]



8

PP1 $IC_{50} = 16 \pm 2\ \mu M$

PP2A $IC_{50} = 7.7 \pm 1.3\ \mu M$

Thus, a series of analogues based on **8** were prepared in excellent yields via the *exo*-selective Diels–Alder[36,37] addition of furan and maleic anhydride followed by simple hydrogenation (Scheme 1).[34] Treatment of norcantharidin in THF at room temperature with a series of substituted aromatic amines afforded the corresponding amide-acid analogues **12** in good to excellent yields (46–90%). Subsequent biological evaluation against PP1 and PP2A gave rise to the data shown in Table 1.

Commencing with the parent aniline (**12**), we note a modest level of enzyme inhibitory activity when compared with that of norcantharidin **11** and cantharidin **6** (PP1 IC_{50}s of 10.3 ± 1.37; $7.6 \pm 0.78\ \mu M$ and PP2A

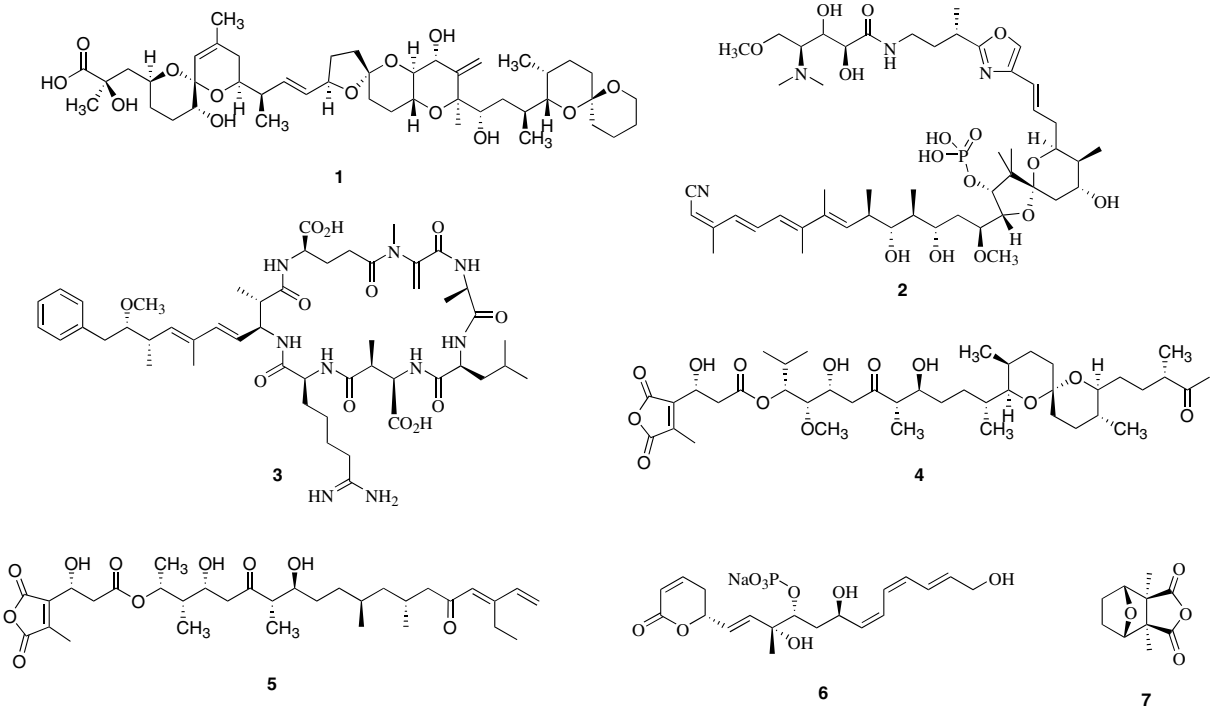
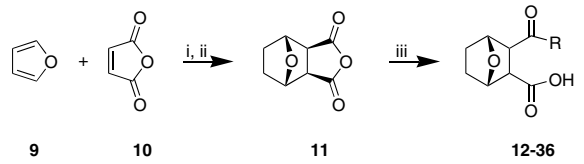


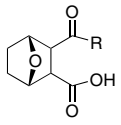
Figure 1. Chemical structures of archetypal protein phosphatase 1 and 2 A inhibitors: (1) okadaic acid; (2) calyculin A; (3) microcystin-LR; (4) tautomycin; (5) tautomycetin; (6) fostrecin and (7) cantharidin.



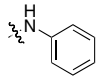
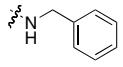
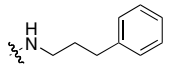
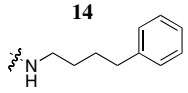
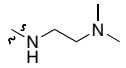
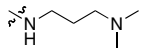
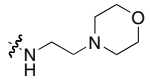
9 10 11 12-36

Scheme 1. Synthesis of amide-acid analogues of norcantharidin. Reagents and conditions: (i) rt, 24 h, Et_2O; (ii) 4 atm H_2, 10% Pd–C, 3 days; (iii) RNH_2, THF, rt 16, or RNH_2, 60 °C, 16 h.

Table 1. Protein phosphatase 1 and 2A inhibition by amide-acid analogues **12–18**



12-18

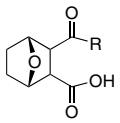
Entry	R	PP1 IC_{50}[a] (μM)	PP2A IC_{50}[a] (μM)	PP1/PP2A
1	 **12**	24.3 ± 2.8	7.7 ± 0.8	3.2
2	 **13**	35 ± 0	12 ± 0	2.9
3	 **14**	25 ± 0	25 ± 4	1
4	 **15**	75 ± 12	95 ± 5	0.79
5	 **16**	67 ± 7	>100	<0.67
6	 **17**	43 ± 7	57 ± 2	0.75
7	 **18**	30 ± 3	43 ± 1	0.70

[a] Mean ± SEM of three experiments conducted in duplicate.

IC_{50}s of 2.69 ± 0.15; 0.99 ± 0.12 μM, respectively). However, moving the pendant aromatic ring distal to the amide function caused a decrease in potency for both enzymes, with the benzyl-(**13**) and propylphenyl-(**14**) substituents essentially equipotent (Table 1, entries 2 and 3), further extension with the butylphenyl-(**15**) analogue produced a 4-fold decrease in PP1 potency and a 7-fold decrease in PP2A potency, relative to **12**. More interestingly, these analogues display a change in enzyme selectivity, viz the parent is ~3-fold PP2A selective, as is the norm for norcantharidin analogues.[27–35,38–40] However, extension of the aromatic ring progressively switched the enzyme selectivity in favour of PP1 (Table 1, entries 2–4). Although this increase in relative PP1 selectivity is at the cost of PP1 potency, as previously observed,[27] we were intrigued with these observations and were keen to further examine the requirements for PP1 selectivity. Accordingly we synthesized analogues lacking the aromatic ring, but possessing a moderately sized alkyl chain at this point. These alkyl chains typically possessed a terminal functional group bearing lone pairs of electrons to mimic the electron rich nature of the aromatic ring. As can be seen from Table 1 (entry 5), a terminal *N*,*N*-dimethylaminoethyl (**16**) substituent effectively lost PP2A inhibition whilst displaying a modest improvement in PP1 inhibition relative to **15**, consequently PP1 selectivity was again improved. Further elongation of this chain, *N*,*N*-dimethylaminopropyl (**17**), improved the inhibition of both PP1 and PP2A but at the cost of PP1 selectivity. Viewing continued extension of this side chain as being counterproductive, and most likely increasing both PP1 and PP2A potency we sought to encapsulate the terminal dimethyl groups and amine functionality within an ethylmorpholino moiety (**18**). In this instance we were again rewarded with an increase in both PP1 and PP2A potency while maintaining PP1 selectivity (Table 1, entry 7). While the observed PP1 selectivities are at best modest, they also represent some of the highest PP1 selectivities reported to date.

Suitably encouraged about the possibility of developing a family of PP1 selective inhibitors, we again turned our attention to aromatic amines. In this series of compound we commenced by placing the alkyl chain on the aromatic ring, that is, we utilised a series of alkyl substituted anilines as the nucleophiles to open norcantharidin's, (**11**), anhydride ring affording **19–35**. Introduction of an *ortho*-ethyl (**19**) returned modest PP1 and PP2A inhibition, but also PP2A selectivity; however, the corresponding *meta*-ethyne side chain (**20**) induced a modest increase in potency and marginal PP1 selectivity (Table 2, entry 2). A series of methyl substituted analogues (**21–23**) displayed PP1 selectivity only when both *ortho*-positions were occupied (Table 2, entries 3 and 5). Compound **22** with a 2,3-dimethyl disposition lost both potency and returned PP2A selectivity suggesting that for PP1 selectivity the aromatic ring must be moved out of the amide plane or possess a considerable degree of hindered rotation. Indeed, molecular modeling analysis (MacSpartan*Pro*, data not shown) indicates that most PP1 selective analogues all have the phenyl ring twisting such that it is orthogonally disposed to the amide moiety. Analogue **23** is one of the most selective small molecule PP1 inhibitors yet reported. In attempts to investigate this possibility further, we sought to bulk up the alkyl chains examined and turned our attention to *tert*-butyl analogues **24** and **25**, interestingly neither of these analogues displayed notable PP1 selectivity, nor PP2A selectivity (Table 2, entries 6 and 7). It therefore appears that the presence of a bulky side chain in the

Table 2. Protein phosphatase 1 and 2A inhibition by amide-acid analogues **19**–**35**



19-35

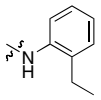
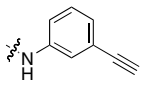
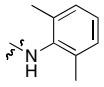
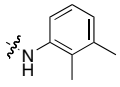
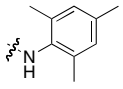
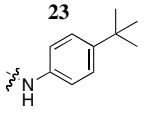
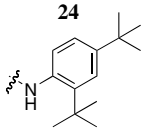
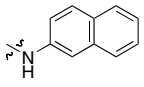
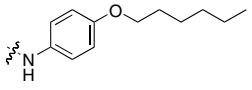
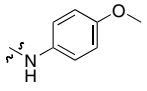
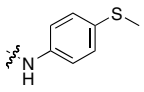
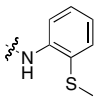
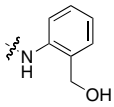
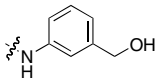
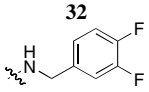
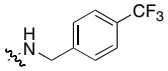
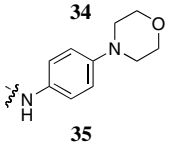
Entry	R	PP1 IC_{50}[a] (μM)	PP2A IC_{50}[a] (μM)	PP1/PP2A
1	 **19**	51 ± 5	24 ± 3	2.12
2	 **20**	17 ± 0.7	18 ± 0.9	0.94
3	 **21**	56 ± 10	72 ± 2	0.78
4	 **22**	17 ± 0.7	18 ± 0.9	0.94
5	 **23**	48 ± 9	85 ± 3	0.56
6	 **24**	10 ± 0	11 ± 1	0.91
7	 **25**	8.2 ± 2	8.2 ± 0.7	1
8	 **26**	14 ± 2	15 ± 1	0.93
9	 **27**	11 ± 5	15 ± 2	0.73
10	 **28**	15 ± 1	16 ± 1	0.94
11	 **29**	10 ± 4	14 ± 1	0.71

Table 2 (*continued*)

Entry	R	PP1 IC_{50}[a] (μM)	PP2A IC_{50}[a] (μM)	PP1/PP2A
12		23 ± 1	33 ± 2	0.70
	30			
13		36 ± 2	39 ± 2	0.92
	31			
14		15 ± 0.3	7 ± 1.5	2.14
	32			
15		26 ± 2	26 ± 2	1
	33			
16		16 ± 3	20 ± 1	0.8
	34			
17		6.5 ± 2.3	7.9 ± 0.4	0.82
	35			

[a] Mean ± SEM of three experiments conducted in duplicate.

para position also leads towards parity and marginal PP1 selectivity; this is further evidenced by the introduction of a 2-naphthyl (**26**), a *para*-O*n*C_6H_{14} (**27**), a *para*-OCH_3 (**28**) and a *para*-SCH_3 (**29**) substituent, in which all analogues returned modest PP1 selectivity (Table 2, entries 8–11). The SCH_3 moiety within which was also introduced as an *ortho* substituent (**30**) resulted in a slight loss in potency at both PP1 and PP2A, but essentially no change in PP1 selectivity (cf. Table 2, entries 11 and 12).

Attempts to introduce other functionality to the aromatic ring was met with mixed success, an *ortho*-CH_2OH (**31**) afforded marginal PP1 selectivity, but the same substituent *meta*-disposed (**32**) returned inherent PP2A selectivity albeit with an improvement in potency. The introduction of fluorine substituents, **33** and **34** gave two compounds with good potency and a drift towards PP1 selectivity, most notably with the *para*-CF_3 substituent; in this latter case the observed selectivity is better than the *para*-*tert*-butyl **24**, suggesting that a combination of *para*-substituents' bulk and an electron withdrawing group may aid the introduction of PP1 selectivity. Our explorations in this area gave rise to the *para*-morpholino (**35**) substituted analogue that displayed excellent PP1 inhibition (IC_{50} < 10 μM, Table 2 entry 17) and selectivity similar to **34**.

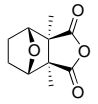
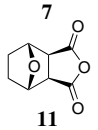
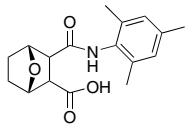
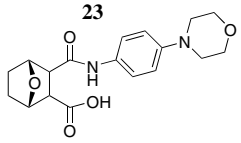
Having determined the relative PP1 versus PP2A selectivity, we felt it prudent to examine selectivity against the related PP2B. In this instance we restricted our examination to the two parent compounds, cantharidin (**7**) and norcantharidin (**11**), and the two most PP1 selective analogues generated herein, compounds **23** and **35**. The data are presented in Table 3.

Of the four analogues examined for PP2B inhibition, only the parent cantharidin displayed essentially no activity returning an IC_{50} > 100 μM. Removal of the methyl substituents in the analogous norcantharidin results in a modest level of PP2B inhibition (IC_{50} = 31 ± 1 μM), and we also note that analogues **23** and **35** are at best modest inhibitors (IC_{50}s = 89 ± 6 and 42 ± 3 μM, respectively). By virtue of higher levels of PP1 inhibition, **35** also displays the best PP1/PP2B selectivity (0.15).

3. Conclusions

As part of our on-going investigations towards the development of PP1 and PP2A selective norcantharidin analogues, we have synthesized a series of norcantharidin analogues displaying PP1 selectivity. In the course of this work, we have modified the inherent selectivity of our lead compound (**12**, ~3-fold PP2A selective) and developed an analogue with slight decrease in PP1 potency, but a 5-fold decrease in PP2A selectivity (**22**, ~0.5-fold PP2A selective), hence the increased PP1 selectivity is at the expense only of PP2A potency. As a result of this study, we have for the first time developed a series of PP1 selective analogues, representing a

Table 3. Protein phosphatase 1, 2A and 2B inhibition by cantharidin (**7**), norcantharidin (**11**) and amide-acid analogues **23** and **35**

Compound	PP1 IC_{50} (μM)	PP2A IC_{50} (μM)	PP2B IC_{50} (μM)	PP1/PP2A	PP1/PP2B
 **7**	7.6 ± 0.78	0.99 ± 0.12	>100	7.7	>0.07
 **11**	10.3 ± 1.37	2.69 ± 0.12	31 ± 1	3.8	0.33
 **23**	48 ± 9	85 ± 3	89 ± 6	0.56	0.54
 **35**	6.5 ± 2.3	7.9 ± 0.4	42 ± 3	0.82	0.15

[a]Mean ± SEM of three experiments conducted in duplicate.

potentially important advance in the development of new, novel and selective PP1 inhibitors. Future work further exploring this PP1 binding domain will be reported in due course. Importantly these analogues also display for PP1 over PP2B.

4. Experimental

4.1. General

All starting materials were purchased from Aldrich Chemical Company and Lancaster Synthesis. Solvents were bulk, and distilled from glass prior to use. ^{1}H and ^{13}C spectra were recorded on a Bruker Advance AMX 300 MHz spectrometer at 300.13 and 75.48 MHz, respectively. Chemical shifts are relative to TMS as internal standard. All compounds returned satisfactory analyses. High resolution mass spectrometry was conducted at the University of Wollongong, Australia. All compounds returned satisfactory micro analysis and/or high resolution mass spectral data.

4.2. Protein phosphatase inhibition

Assays were performed according to previously reported PP1, PP2A and PP2B assays.[6] Protein phosphatases PP1 (rabbit skeletal muscle), PP2A (human red blood cells) and PP2B (bovine brain) were purchased from Upstate Biotechnology (Lake Placid, NY). Protein phosphatase assays were carried out according to the manufacturer's protocol in the presence or absence of an appropriate concentration of test compound. For PP1 and PP2A, a serine/threonine assay was used in which free phosphate ion released from a substrate phosphopeptide (Lys-Arg-pThr-Ile-Arg) was quantified by colorimetric analysis using the Malachite Green method. PP1, PP2A and substrate used in the assay were 30 mU/well, 0.3 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 room temperature for 60 min. Reactions were halted via addition of a malachite green solution (50 μL), and the absorbance readings were taken at 650 nm after 10 min development time. 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. For the PP2B assay, *para*-nitrophenyl phosphate (pNPP) was used as a substrate. The reaction was initiated by addition of substrate (12 μL, 2.4 mM) to a mixture (8 μL) containing 57 nM PP2B, 50 mM Tris–HCl, pH 7.0, 100 μM $CaCl_2$, $NiCl_2$, 0.3 mg/mL BSA and 0.25 μM calmodulin, and inhibitor (10 μL), producing a total reaction volume of 30 μL/well and incubated at 37 °C for 60 min. For each of the enzymatic assays, the enzyme and inhibitor were preincubated prior to addition of substrate. Samples were blanked against wells containing enzyme and buffer only. Initial inhibitor dilutions were made in DMSO and subsequent dilutions were made in distilled deionised H_2O. 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 mean (±SEM) IC_{50} of three independent experiments performed in duplicate.

4.3. 3-Phenylcarbamoyl-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (12)

^{1}H NMR (DMSO-d_6): 9.64 (1H, s, NH), 7.50 (1H, d, J = 7.6 Hz), 7.25 (1H, t, J = 7.6 Hz, 1H), 7.01 (1H, t, J = 6.7 Hz), 4.76 (s, 1H), 4.62 (1H, d, J = 3.9 Hz), 3.03 (1H, d, J = 9.4 Hz), 2.92 (1H, d, J = 9.4 Hz), 1.48–1.59 (4H, m).

^{13}C NMR (DMSO-d_6): 173.1, 170.1, 140.1, 129.4 (2C), 123.8, 120.1 (2C), 79.6, 77.8, 54.4, 52.6, 29.8, 29.3.

MP: 170 °C.

HRMS (M+H) calcd 262.1079, found 262.1085.

4.4. 3-Benzylcarbamoyl-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (13)

^{1}H NMR: (DMSO-d_6): 7.93 (1H, t, J = 5.7Hz), 7.29–7.21 (5H, m), 4.74 (1H, d J = 2.4 Hz), 4.50 (1H, d J = 2.4 Hz), 4.21 (2H, sept, J = 6.1Hz), 2.92 (1H, d, J = 9.7Hz), 2.84 (1H, d, J = 9.6Hz), 1.56–1.43 (4H, m).

^{13}C NMR: (DMSO-d_6): 172.3, 170.5, 139.4, 128.1, 127.1, 126.6, 78.78, 76.70, 52.9, 51.2, 42.1, 28.9, 28.3.

MP: 162–163 °C.

HRMS (M+H) calcd 276.1236, found 276.1238.

4.5. 3-(3-Phenylpropylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (14)

^{1}H NMR: (DMSO-d_6): 7.34 (1H, t, J = 5.3Hz), 7.15–7.28 (5H, m), 4.71 (1H, d, J = 2.3Hz), 4.47 (1H, d, J = 4.3Hz), 2.98 (2H, q, J = 6.2 Hz), 2.52 (2H, t, J = 7.5Hz), 1.64 (2H, quin, J = 7.3Hz), 1.43–1.56 (4H, m).

^{13}C NMR: (DMSO-d_6): 172.2, 170.3, 141.7, 128.2, 128.1, 125.6, 78.6, 76.7, 53.0, 51.5, 38.9, 32.5, 30.7, 28.8, 28.6.

MP: 135–137 °C.

HRMS (M+H) calcd 304.1549, found 304.1541.

4.6. 3-(4-Phenylbutylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (15)

^{1}H NMR: (DMSO-d_6): 7.30 (1H, t), 7.15–7.29 (5H, m), 4.69 (1H, d), 4.43 (1H, d, J = 2.3Hz), 2.98 (2H, q, J = 6.2Hz), 2.51(2H, quin, J = 7.3 Hz), 1.35–1.60 (8H, m).

^{13}C NMR: (DMSO-d_6): 172.7, 170.7, 142.1, 128.2, 125.6, 78.5, 77.0, 53.3, 52.3, 49.3, 38.2, 34.7, 28.5, 28.3.

MP: 105–107 °C.

HRMS (M+H) calcd 318.1705, found 318.1712.

4.7. 3-(2-Dimethylaminoethylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (16)

^{1}H NMR: ($CDCl_3$): 7.34 (1H, t, J = 5.5 Hz), 5.00 (1H, d, J = 4.65 Hz), 4.76 (1H, d, J = 2.1 Hz) 4.67 (1H, d, J = 4.8 Hz), 3.57 (2H, q, J = 6.3 Hz) 3.07 (1H, d, J = 9.6 Hz), 2.96 (1H, d, J = 9.6 Hz), 2.43 (2H, t, J = 6.8 Hz), 2.22 (6H, s) 1.58–1.50 (4H, m).

^{13}C NMR: ($CDCl_3$): 172.3, 170.3, 78.7, 76.7, 53.0, 51.4, 49.4. 44.8, 36.3, 28.8, 28.3.

MP: 45–47 °C.

HRMS (M+H) calcd 257.1501, found 257.1509.

4.8. 3-(3-Dimethylaminopropylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (17)

^{1}H NMR: ($CDCl_3$): 7.34 (1H, t, J = 5.5 Hz), 5.02 (1H, d, J = 4.5 Hz), 4.80 (1H, d, J = 2.0 Hz) 4.70 (1H, d, J = 4.2 Hz), 3.57 (2H, q, J = 7.4 Hz), 3.10 (1H, d, J = 9.6 Hz), 2.96 (1H, d, J = 9.6 Hz), 2.60 (2H, t, J = 6.7 Hz), 2.22 (6H, s), 1.66 (2H, quin, J = 7.4 Hz), 1.58–1.50 (4H, m).

^{13}C NMR: ($CDCl_3$): 175.5, 174.3, 78.5, 76.3, 53.0, 51.2, 49.2. 44.7, 36.7, 28.5, 28.1, 24.9.

MP: 42–44 °C.

HRMS (M+H) calcd 271.1658, found 271.1666.

4.9. 3-(2-Morpholin-4-ylethylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (18)

^{1}H NMR: (DMSO-d_6): 4.65 (1H, m), 3.77 (2H, m), 3.51 (2H, m), 3.16 (1H, d, J = 9.6 Hz), 3.00 (1H, d, J = 9.5 Hz), 2.51 (2H, m), 1.52 (2H, m).

^{13}C NMR: (DMSO-d_6): 176.1, 173.5, 82.0, 81.5, 56.7, 52.3, 51.8, 47.8, 32.8, 32.2, 30.5, 30.3.

MP: 116–119 °C.

HRMS: $C_{14}H_{22}N_2O_5$ requires 299.1607; Found 299.1610.

4.10. 3-(2-Ethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (19)

^{1}H NMR: (DMSO-d_6): 11.85 (1H, COOH), 8.70 (1H, NH), 7.65 (1H, d, J = 7.6Hz), 7.02–7.19 (3H, m), 4.85 (1H, d, J = 4.12Hz), 4.71 (1H, d, J = 4.32Hz), 3.07 (2H, q, J = 5.8Hz), 2.54 (2H, q, J = 7.5Hz), 1.53–1.64 (4H, m), 1.23 (3H, t, J = 7.6 Hz).

^{13}C NMR: (DMSO-d_6): 172.0, 169.3, 135.7, 135.1, 128.2, 125.8, 124.4, 123.2, 79.0, 77.0, 54.1, 51.7, 28.4, 23.5, 14.0.

MP: 171–172 °C.

HRMS (M+H) calcd 290.1392, found 290.1388.

4.11. 3-(3-Ethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (20)

^{1}H NMR (DMSO-d_6): 11.1 (brs, 1H), 8.78 (1H, s, NH), 7.34 (1H, d, J = 7.7 Hz), 7.00 (1H, t, J = 7.7 Hz), 6.92 (1H, d, J = 7.2 Hz), 4.81 (1H, s), 4.69 (1H, d, J = 4.2 Hz), 3.08 (1H, d, J = 9.7 Hz), 3.01 (1H, d, J = 9.7 Hz), 2.22 (3H, s), 2.05 (3H, s), 1.52–1.59 (4H, m).

^{13}C NMR: (DMSO-d_6): 173.2, 170.1, 137.4, 137.2, 129.9, 126.0, 125.9, 122.7, 79.9, 77.9, 54.7, 52.7, 29.6, 29.3, 21.1, 14.3.

MP: 177 °C.

HRMS (M+H) calcd 286.1079, found 286.1083.

4.12. 3-(2,6-Dimethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (21)

^{1}H NMR: (DMSO-d_6): 11.86 (1H, COOH), 8.96 (1H, NH), 7.02 (3H, s), 4.78 (1H, d J = 3.6 Hz), 4.60 (1H, d J = 4.7 Hz), 3.15 (1H, d J = 9.6 Hz), 2.90 (1H, d J = 9.6 Hz), 2.13 (6H, s), 1.52–1.59 (4H, m).

^{13}C NMR: (DMSO-d_6): 172.2, 168.8, 135.2, 135.1, 127.4, 126.1, 79.3, 76.6, 52.9, 51.0, 29.0, 28.5, 18.1.

MP: 193–195 °C.

HRMS (M+H) calcd 290.1392, found 290.1396.

4.13. 3-(2,3-Dimethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (22)

^{1}H NMR: (DMSO-d_6): 10.9 (1H, brs), 9.75 (1H, s, NH), 7.73 (1H, s), 7.43 (1H, d, J = 8.1 Hz), 7.27 (1H, t, J = 7.8 Hz), 7.11 (1H, d, J = 7.3 Hz), 4.76 (1H, s), 4.63 (1H, d, J = 3.8), 4.10 (1H, s), 3.03 (2H, d, J = 9.6 Hz), 2.93 (2H, d, J = 9.6 Hz), 1.52–1.54 (4H, m).

^{13}C NMR: (DMSO-d_6): 171.9, 169.4, 139.3, 128.8, 126.1, 122.3, 121.8, 119.9, 83.3, 79.4, 78.4, 77.0, 53.5, 51.8, 28.8, 28.3.

MP: 166 °C.

HRMS (M+H) calcd 290.1392, found 290.1400.

4.14. 3-(2,4,6-Trimethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (23)

^{1}H NMR (DMSO-d_6): 11.85 (1H, brs), 8.87 (1H, s, 1H, NH), 6.82 (2H, s), 4.77 (1H, s), 4.58 (1H, d, J = 4.5 Hz), 3.12 (1H, d, J = 9.4 Hz), 2.88 (1H, d, J = 9.4 Hz), 2.19 (3H, s), 2.08 (6H, s), 1.51–1.66 (4H, m).

^{13}C NMR (DMSO-d_6): 172.2, 168.8, 135.0, 134.9, 132.4, 128.0, 79.3, 76.6, 52.9, 50.9, 29.0, 28.5, 18.0, 17.9 (2C).

MP: 187 °C.

HRMS (M+H) calcd 304.1549, found 304.1553.

4.15. 3-(4-*tert*-Butylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (24)

^{1}H NMR (DMSO-d_6): 11.89 (1H, brs), 9.48 (1H, s, 1H, NH), 7.42 (2H, d, J = 8.4 Hz), 7.26 (2H, d, J = 8.4 Hz), 4.77 (1H, s, 1H, CH), 4.61 (1H, d, J = 3.3 Hz), 3.03 (1H, d, J = 9.6 Hz), 2.91 (1H, d, J = 9.6 Hz), 1.45–1.60 (4H, m), 1.24 (9H, s).

^{13}C NMR (DMSO-d_6): 173.1, 169.9, 146.1, 137.5, 126.0 (2C), 119.8, 119.7, 54.4, 52.5, 34.8, 32.0 (3C), 29.8, 29.3.

MP: 179 °C.

HRMS (M+H) calcd 318.1705, found 318.1699.

4.16. 3-(2,4-Di-*tert*-Butylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (25)

^{1}H NMR (DMSO-d_6): 1.25 (18H, s), 1.49–1.62 (4H, m), 2.89 (2H, d, J = 9.5 Hz), 3.04 (2H, d, J = 9.5 Hz), 4.61 (1H, d, J = 4.0 Hz, 1H, CH), 4.84 (1H, s, 1H, CH), 7.05 (1H, s, 1H, CH–Ar), 7.39 (2H, s, 2H, 2× CH–Ar), 9.50 (1H, s, 1H, NH), 10.9 (1H, brs, 1H, COOH).

^{13}C NMR (DMSO-d_6): 173.2, 169.9, 151.4, 139.6, 117.5, 114.3, 79.8, 77.8, 54.6, 52.3, 35.3, 32.1 (6C), 29.8, 29.4.

MP: 182 °C.

HRMS (M+H) calcd 374.2331, found 374.2335.

4.17. 3-(Naphthalen-2-ylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (26)

^{1}H NMR (DMSO-d_6): 10.95 (1H, brs), 9.82 (1H, s), 7.74–7.83 (3H, s), 7.36–7.53 (3H, m), 4.79 (1H, s), 4.67 (1H, s), 3.17 (2H, d, J = 9.9 Hz), 2.96 (2H, d, J = 9.9 Hz), 1.51–1.62 (4H, m).

^{13}C NMR (DMSO-d_6): 173.1, 170.5, 137.7, 134.3, 130.5, 129.0, 128.3, 128.0, 127.2, 125.3, 120.9, 116.0, 79.6, 77.8, 54.5, 52.7, 29.8, 29.3.

MP: 237–238 °C.

HRMS (M+H) calcd 312.1236, found 312.1242.

4.18. 3-(4-Hexyloxyphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (27)

^{1}H NMR (DMSO-d_6): 10.85 (1H, brs), 9.41 (1H, s), 7.39 (2H, d, J = 8.9 Hz), 6.82 (2H, d, J = 8.9 Hz), 4.84 (1H, d, J = 3.8 Hz), 4.61 (1H, d, J = 3.8 Hz), 3.89 (2H, t, J = 6.5 Hz), 3.01 (1H, d, J = 9.6 Hz), 2.91 (1H, d, J = 9.6 Hz), 1.51–1.67 (6H, m), 1.34–1.36 (4H, m), 0.88 (3H, t, J = 6.9 Hz).

^{13}C NMR (DMSO-d_6): 173.1, 169.7, 155.4, 133.2, 121.7 (2C), 115.2 (2C), 79.6, 77.7, 68.5, 54.3, 52.5, 29.8, 29.3 (2C), 28.6, 22.8, 14.8.

MP: 156 °C.

HRMS (M+H) calcd 362.1968, found 362.1965.

4.19. 3-(4-Methoxyphenylcarbamoyl)-7-oxabicyclo[2.2.1] heptane-2-carboxylic acid (28)

^{1}H NMR (DMSO-d_6): 9.39 (1H, s, NH), 7.40 (2H, d, J = 8.9 Hz), 6.83 (2H, d, J = 8.9 Hz), 4.76 (1H, d, J = 3.7 Hz), 4.60 (1H, d, J = 3.7 Hz), 3.69 (3H, s), 3.09 (1H, d, J = 9.6 Hz), 2.90 (1H, d, J = 9.6 Hz), 1.51–1.56 (4H, m).

^{13}C NMR (DMSO-d_6): 173.1, 169.7, 156.0, 133.3, 121.7 (2C), 114.6 (2C), 79.6, 77.8, 56.1, 54.4, 52.5, 29.8, 29.3.

MP: 159 °C.

HRMS (M+H) calcd 292.1185, found 292.1188.

4.20. 3-(4-Methylsulfanylphenylcarbamoyl)-7-oxabicyclo [2.2.1]heptane-2-carboxylic acid (29)

^{1}H NMR (DMSO-d_6): 11.9 (1H, s), 9.61 (1H, s, NH), 7.47 (2H, d, J = 8.4 Hz), 7.19 (2H, d, J = 8.4 Hz), 4.84 (1H, d, J = 3.9 Hz), 4.62 (1H, d, J = 3.9 Hz), 3.03 (1H, d, J = 9.6 Hz), 2.92 (1H, d, J = 9.6 Hz), 2.42 (3H, s), 1.48–1.60 (4H, m).

^{13}C NMR (DMSO-d_6): 173.0, 170.1, 137.8, 132.2, 128.1 (2C), 120.8 (2C), 79.5, 77.7, 54.3, 52.6, 29.8, 29.3, 16.7.

MP: 172–173 °C.

HRMS (M+H) calcd 308.0957, found 308.0962.

4.21. 3-(2-Methylsulfanylphenylcarbamoyl)-7-oxabicyclo [2.2.1]heptane-2-carboxylic acid (30)

^{1}H NMR (DMSO-d_6): 1.57 (m, 4H, 2× CH_2), 2.37 (s, 3H, CH_3), 3.09 (s, 2H, 2× CH), 4.75 (d, J = 4.2 Hz, 1H, CH), 4.88 (s, 1H, CH), 7.08 (t, J = 7.5 Hz, 2H, 2× CH–Ar), 7.19 (t, J = 7.5 Hz, 1H, CH–Ar), 7.38 (d, J = 7.8 Hz, 1H, CH–Ar), 7.84 (d, J = 7.8 Hz, 1H, CH–Ar), 8.98 (s, 1H, NH), 12.2 (s, 1H, COOH).

^{13}C NMR (DMSO-d_6): 16.5 (CH_3), 28.3 (CH_2), 28.4 (CH_2), 51.8 (CH), 54.6 (CH), 77.0 (CH), 78.9 (CH), 122.2 (C–Ar), 124.5 (CH–Ar), 126.6 (CH–Ar), 128.2 (C–Ar), 129.7 (CH–Ar), 137.1 (CH–Ar), 169.6 (CON), 172.0 (COOH).

MP: 135 °C.

HRMS (M+H) calcd 308.0957, found 308.0952.

4.22. 3-(2-Hydroxymethylphenylcarbamoyl)-7-oxabicyclo [2.2.1]heptane-2-carboxylic acid (31)

^{1}H NMR (DMSO-d_6): 8.99 (1H, s, NH), 7.63 (1H, d, J = 7.9 Hz), 7.32 (1H, d, J = 7.4 Hz), 7.19 (1H, t, J = 7.1 Hz), 7.06 (1H, t, J = 7.1 Hz), 5.22 (1H, brs), 4.69 (1H, d, J = 4.2 Hz), 4.45 (2H, s), 4.08 (1H, s), 3.03 (2H, t, J = 10.3 Hz), 1.51–1.62 (4H, m, 4H).

^{13}C NMR (DMSO-d_6): 172.7, 169.9, 136.4, 133.9, 127.9, 127.4, 124.4, 123.5, 79.3, 77.6, 60.7, 54.7, 52.4, 29.1, 29.0.

MP: 145–146 °C.

HRMS (M+H) calcd 292.1185, found 292.1190.

4.23. 3-(3-Hydroxymethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (32)

^{1}H NMR: (DMSO-d_6): 10.9 (1H, brs), 9.54 (1H, s, NH), 7.49 (1H, s), 7.37 (1H, d, J = 8.0 Hz), 7.19 (1H, t, J = 7.7 Hz), 7.76 (1H, d, J = 7.4 Hz), 5.11 (1H, brs), 4.75 (1H, d, J = 3.8 Hz), 4.62 (1H, d, J = 3.8 Hz), 4.44 (2H, s), 3.05 (1H, d, J = 9.6 Hz), 2.92 (1H, d, J = 9.6 Hz), 1.49–1.60 (4H, m).

^{13}C NMR: (DMSO-d_6): 173.1, 170.1, 143.9, 140.0, 129.1, 121.9, 118.4, 118.2, 79.6, 77.8, 63.7, 54.5, 52.5, 29.8, 29.3.

MP: 132 °C.

HRMS (M+H) calcd 292.1185, found 292.1183.

4.24. 3-(3,4-Difluorophenylcarbamoyl)-7-oxabicyclo[2.2.1] heptane-2-carboxylic acid (33)

^{1}H NMR: (DMSO-d_6): 9.88 (1H, s, NH), 7.69 (1H, m), 7.30 (1H, m), 7.19 (1H, m), 4.76 (1H, br), 4.63 (1H, br), 2.97 (2H, m), 1.67–1.47 (4H, m).

^{13}C NMR: (DMSO-d_6): 172.0, 169.6, 150.5, 147.3, 143.5, 136.1, 117.2, 108.0, 78.4, 76.9, 53.2, 51.8, 28.8, 28.3.

MP: 174–176 °C.

HRMS (M+H) calcd 298.0891, found 298.0893.

4.25. 3-(4-Trifluoromethylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (34)

^{1}H NMR: (DMSO-d_6): 11.97 (1H, brs), 10.01 (1H, s, NH), 7.73 (2H, d, J = 8.4 Hz), 7.61 (2H, d, J = 8.4 Hz), 4.77 (1H, br), 4.66 (1H, br), 3.07 (1H, d, J = 9.6 Hz), 2.96 (1H, d, J = 9.6 Hz), 1.58–1.50 (4H, m).

^{13}C NMR: (DMSO-d_6): 172.0, 169.8, 142.7, 126.1–123.5 (d, J = 894.0 Hz), 125.8 (q, J = 15.0 Hz), 123.5, 123.1, 122.7, 122.5, 122.3 (sextet, J = 60 Hz), 78.4, 76.9, 53.4, 51.8, 28.8, 28.3.

MP: 189–191 °C.

HRMS (M+H) calcd 330.0953, found 330.0955.

4.26. 3-(4-Morpholin-4-ylphenylcarbamoyl)-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid (35)

^{1}H NMR (DMSO-d_6): 10.9 (1H, brs), 9.35 (s, 1H, NH), 7.36 (1H, d, J = 8.9 Hz), 6.85 (1H, d, J = 8.9 Hz), 4.76 (1H, d, J = 2.1 Hz), 4.60 (1H, d, J = 3.7 Hz), 2.88–3.02 (6H, m, 6H), 1.47–1.56 (4H, m).

^{13}C NMR (DMSO-d_6): 173.1, 169.6, 148.0, 132.5, 121.2 (2C), 116.3 (2C), 79.6, 77.7, 67.0, 54.4, 52.5, 50.0, 29.8, 29.3.

HRMS (M+H) calcd 347.1607, found 347.1611.

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