Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: David E. Williams, Frank Loganzo, Lauren Whitney, Jenny Togias, Richard Harrison, Maya P. Singh, Leonard A. McDonald, Selvaluxmy Kathirgamanathar, Veranja Karunaratne & Raymond J. Andersen (2011) Depsides isolated from the Sri Lankan lichen *Parmotrema* sp. exhibit selective Plk1 inhibitory activity, Pharmaceutical Biology, 49:3, 296-301, DOI: 10.3109/13880209.2010.517540

To link to this article: https://doi.org/10.3109/13880209.2010.517540

Published online: 01 Feb 2011.

Pharmaceutical Biology, 2011; 49(3): 296–301

ISSN 1388-0209 print/ISSN 1744-5116 online
DOI: 10.3109/13880209.2010.517540

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RESEARCH ARTICLE

Depsides isolated from the Sri Lankan lichen *Parmotrema* sp. exhibit selective Plk1 inhibitory activity

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Abstract

Context: Mitotic kinase enzymes regulate critical stages of mitosis and are amenable to pharmacological inhibition. Since natural products have been a rich source of antimitotic inhibitors, we postulated that natural products would also provide effective inhibitors of mitotic kinases.

Objective: To explore unique marine and terrestrial natural product sources for new anticancer drug leads, we screened our natural product extract library for polo-like kinase-1 (Plk1) kinase inhibitors.

Materials and methods: Extracts of the lichen *Parmotrema* sp. (Parmeliaceae) exhibited *in vitro* inhibitory activity. Bioassay-guided fractionation of the *Parmotrema* sp. extract led to the isolation of depside inhibitors.

Results: A new depside **1** has been isolated from the Sri Lankan lichen *Parmotrema* sp. along with the known metabolites **2** (β-collatolic acid) and **3** (β-alectoronic acid). The structure of depside **1** was elucidated by spectroscopic analysis. The three depsides **1**–**3** exhibited moderate inhibition of purified recombinant Plk1 kinase with IC_{50} of 2.8, 0.7, and 1.7 μM, respectively, at 1 μM ATP. Inhibitory activity was also observed at high concentrations of ATP, suggesting the potential for activity in a cellular environment. The depsides were also tested against a panel of 23 other recombinant kinases and were found to possess up to 30-fold selectivity toward Plk1.

Discussion and conclusion: These data suggest that the depsides **1**–**3** may serve as core structures that can be further explored as potential inhibitors of Plk1 and other kinases.

Keywords: Mitotic kinases, purified recombinant Plk1 kinase, differential activity

Introduction

Antimitotic drugs are commonly used in cancer therapy (Jiang et al., 2006). These agents primarily affect tubulin dynamics and interfere with cellular structures such as the mitotic spindle, which regulates the faithful segregation of chromosomes during cell division. An alternative approach to attenuate cell growth is to target other mediators of the cell division process, including mitotic kinases (Blagden & de Bono, 2005). These enzymes regulate critical stages of mitosis and are amenable to pharmacological inhibition. Natural products, including taxanes, vincas, and other agents, have been a rich source of antimitotic inhibitors. Hence, we postulated that natural products might also be effective inhibitors of mitotic kinases.

Polo-like kinase-1 (Plk1) is a serine/threonine kinase that has several critical functions in cell division, including initiation of mitosis, centrosome maturation, spindle

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(Received 25 October 2009; revised 08 August 2010; accepted 18 August 2010)

assembly, anaphase regulation, and cytokinesis (Barr et al., 2004). These normal functions are compromised when the Plk1 pathway is deregulated. Plk1 overexpression can induce tumor growth in mouse models (Smith et al., 1997). In patients, Plk1 protein or mRNA is reportedly overexpressed in many solid tumors, including those derived from colon (Takahashi et al., 2003), pancreas (Gray et al., 2004), prostate (Weichert et al., 2004), ovary (Takai et al., 2001), head and neck (Knecht et al., 1999), non-Hodgkin's lymphoma (Mito et al., 2005), and others. For several of these tumor types, a positive correlation is observed between higher Plk1 expression, increased invasive potential, and reduced patient survival. Experimental reduction of Plk1 protein markedly impairs tumor cell growth *in vitro* and *in vivo*. Plk1 depletion using small interfering RNA (siRNA), antisense oligonucleotides, or neutralizing antibodies results in mitotic arrest and apoptosis of cancer cell lines (Liu & Erikson, 2003; Spänkuch et al., 2004). Hence, these depletion studies supported the hypothesis that inhibition of Plk1 may be useful for the treatment of cancers.

Multiple reports of Plk1 inhibitors have been emerging (McInnes et al., 2006; Strebhardt & Ullrich, 2006). Wortmannin, a well-documented inhibitor of phosphoinositol-3-kinases (PI3K), inhibits Plk1 kinase activity (Liu et al., 2007). Significantly, several highly potent and selective small molecule inhibitors of Plk1 were recently reported (Lansing et al., 2007; Steegmaier et al., 2007). As molecular tools, these agents provide insight into Plk1 function in mitosis and other processes. Moreover, these inhibitors provide proof-of-concept that attenuation of Plk1 kinase activity effectively drives cells into mitotic arrest resulting in cell death. These data suggest that Plk1 kinase inhibitors may be useful in the treatment of cancers that depend upon Plk1 for cell division.

As part of a collaborative program designed to explore unique marine and terrestrial natural product sources for new anticancer drug leads, we screened our natural product extract library for Plk1 kinase inhibitors and found that extracts of the lichen *Parmotrema* sp. (Parmeliaceae), collected in Sri Lanka exhibited *in vitro* inhibitory activity. Bioassay-guided fractionation of the *Parmotrema* sp. extract led to the isolation of the inhibitors **1**, β-collatolic acid (**2**) (Rangaswami & Rao, 1955), and β-alectoronic acid (**3**) (Krivoshchekova et al., 1983) (Figure 1). The structure of the new metabolite **1**, along with the biological activities of despides **1**–**3**, is described below.

Materials and methods

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-600 spectrometer (East Milton, ON, Canada) with a 5 mm CPTCI cryoprobe. ^{1}H chemical shifts are referenced to the residual acetone-d_6 signal (δ 2.05 ppm) and ^{13}C chemical shifts are referenced to the acetone-d_6 solvent peak (δ 29.92 ppm). Low- and high-resolution ESI-QIT-MS were recorded on a Bruker-Hewlett Packard 1100 Esquire-LC system mass spectrometer.

Merck Type 5554 silica gel plates and Whatman MKC18F plates were used for analytical thin-layer chromatography. Reversed-phase HPLC purifications were performed on a Waters 600E System Controller liquid chromatograph attached to a Waters 996 photodiode array detector. All solvents used for HPLC were of Fisher HPLC grade.

For the Plk1 enzyme assay, recombinant human Plk1 was expressed in *Escherichia coli* cells and purified by sequential chromatography on NiNTA, HQ/CM, and size-exclusion columns to >95% purity. Plk1 enzyme activity was assessed in a DELFIA format assay as follows. Plk1 enzyme was diluted in PKB buffer (20 mM HEPES, 10 mM $MgCl_2$, 2 mM L-cysteine) and added to a 384-well streptavidin plate. Compounds to be evaluated were added to the reaction as 50× DMSO stocks in 0.5 µL. After 5-min preincubation of compound with enzyme, 5 µL of 5× peptide/ATP mixture was added to each well. Peptide substrate was derived from the C-terminal domain of Myt1 protein, including the T495 phosphorylation site (Btn-SFPSFEPRNLLSLFEDTLDPT). Reactions were incubated for 90 min at room temperature. Final reaction conditions were: 5 ng (2.9 nM or 200 ng/mL) Plk1, 0.5 µM MYT1 peptide, 10 µM ATP, 20 mM HEPES, 10 mM $MgCl_2$, 2 mM L-cysteine, 2% DMSO. In ATP-dependent experiments, final ATP concentrations were 1, 10, 100, or 1000 µM. Plates were washed twice with 75 µL of Tris-buffered saline + 0.05% Tween-20 (TBST) in an automated plate washer. A mixture of primary and secondary antibodies was added (anti-phosphothreonine, Cell Signaling Technologies (Danvers, MA), diluted to 1:4000, plus anti-

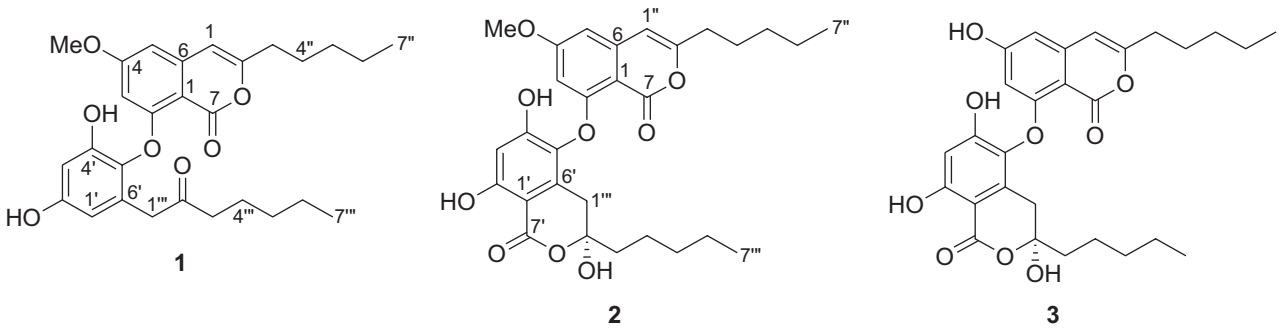

Figure 1. The structures of depsides **1**–**3**.

rabbit-IgG-Eu, Perkin Elmer (Waltham, MA), diluted to 1:5000 in DELFIA assay buffer). Antibodies were incubated on the plate for 60 min at room temperature. Plates were washed twice with TBST. DELFIA enhancement solution was added, incubated for 30 min, and then plates were read in a PerkinElmer Victor5 microplate reader. Signal-to-noise ratio was calculated from the ratio of +ATP/–ATP wells. The inhibitory effect of compounds on Plk1 kinase activity was determined by calculating the decrease in signal between DMSO and control versus compound-treated wells. IC_{50} values were calculated using the Excel add-in module XLfit, model 203 (IDBS, Guildford, UK). Statistical analyses were conducted with a two-tailed *t*-test to determine differences between IC_{50} values. All compounds were resuspended as 10 mM stocks in 100% DMSO and serially diluted into DMSO for assays. BI3536 was provided by Wyeth Chemical Sciences (Pearl River, NY). Wortmannin (catalog no. 681675) and staurosporine (catalog no. 569397) were purchased from Calbiochem/EMD Chemicals (Gibbstown, NJ).

For the kinase selectivity profiling assay, all buffer salts and reagents were purchased from Sigma (St. Louis, MO), and were of the highest purity available. Kinases were purchased from Invitrogen (Carlsbad, CA) and were used without further purification. Fluorescently labeled peptide substrates were purchased from Anaspec (Freemont, CA). The peptide sequence was optimized for each kinase. The structural identity of the three depsidones was confirmed by mass spectrometry before sample testing. Compound plates were prepared by pipetting an 11-point dose–response titration in DMSO with final assay concentrations ranging from 10 μM to 5 nM. Assay plates were prepared with 150 nL addition of compound, followed by 7.5 μL of assay buffer (50 mM HEPES, 10 mM $MgCl_2$, 0.005% Brij, 5 mM 2-mercaptoethanol) containing substrate and ATP, followed by 7.5 μL of buffer-containing enzyme. All reactions were performed at 1.5 μM peptide substrate ([S] << K_m) and at the experimentally determined K_m of ATP for each kinase. All final enzyme concentrations were below 50 nM, and in most cases were 10 nM or less. The reactions were incubated at room temperature for 1 to 2 h depending on kinase activity, but in all cases exhibited between 20% to 30% substrate phosphorylation. The reactions were quenched by the addition of 15 μL of stop buffer (100 mM HEPES, 20 mM EDTA, 0.005% Brij, and 0.2% Caliper coating reagent). Substrates and products were separated on the Caliper LC3000 using standard separation protocols. The percentage inhibition was calculated for each compound concentration and the IC_{50} was determined using Equation (1).

$$\%\,\text{Inhibition} = \left(\text{Max} + \frac{(\text{Max} - \text{Min})}{1 + \left(\frac{\text{Conc}}{\text{IC}_{50}} \right)^{\text{Hill}}} \right) \quad (1)$$

Specimens of *Parmotrema* sp. were found growing on a rock at 3500 ft, near Labukelle, Central Province, Sri Lanka. A herbarium specimen has been deposited in the Department of Chemistry, University of Peradeniya (LB-11).

Specimens of *Parmotrema* sp. (2.3 g) were repeatedly extracted with MeOH to give a MeOH extract (100 mg) that was fractionated via silica gel MPLC (eluent: 100% CH_2Cl_2 to 100% MeOH) to give a fraction (39.8 mg) that exhibited inhibitory activity along with the known and widely distributed depside atranorin (2.8 mg) (Jayaprakasha & Rao, 2000). The active fraction was further fractionated on a Sephadex LH-20 using MeOH as eluent to generate two active fractions, which after reversed-phase HPLC, using a CSC-Inertsil 150A/ODS2, 5 μm 25 × 0.94 cm column with 13:7 MeCN/(0.05% TFA/H_2O) as eluent, gave pure samples of three depsides, the new compound **1** (0.8 mg) and the known metabolites **2** (2.9 mg) and **3** (1.1 mg).

Results

From the MeOH extracts of the lichen *Parmotrema* sp., one new depside, compound **1**, along with the known compounds β-collatolic acid (**2**) (Rangaswami & Rao, 1955) and β-alectoronic acid (**3**) (Krivoshchekova et al., 1983) were isolated (Figure 1).

The physical and spectroscopic data obtained for compound **1** are as follows:

Depsidone 1: Isolated as a purple powder; UV (MeOH) λmax (log ε) 206 (4.5), 244 (4.6), 279 (4.5), 326 (4.0) nm; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; positive ion HRESIMS $[M+Na]^+$ *m*/*z* 505.2170 (calculated for $C_{28}H_{34}O_7Na$, 505.2202).

The depsides **1**–**3** were assayed in a Plk1 enzyme assay at various ATP concentrations to evaluate the potency and ATP dependence of these inhibitors against Plk1. The K_m(ATP) for Plk1 was determined to be ~6 μM under the conditions of this assay (data not shown). Compounds were tested at 1, 10, 100, and 1000 μM ATP to span a range of ATP concentrations from near-K_m to elevated levels that may mimic the high ATP concentrations present in cells where inhibitors must compete with ATP for binding to kinases. The IC_{50} values for the depsides at varied ATP concentrations are indicated in Table 2. At lower ATP concentrations (1 μM), the IC_{50} for depsides **1**–**3** were 2.8, 0.7, and 1.7 μM, respectively. Notably, minimal shift in IC_{50} was observed at high ATP concentrations for the three depsides. Table 3 indicates the ratio of IC_{50} at each ATP concentration compared with the IC_{50} at 1 μM ATP. At 1000 μM ATP, the IC_{50} of depsides **1** and **2** increased by only 36-fold, while the IC_{50} of depside **3** changed minimally (1.4×). The ATP dependence of the depsides were compared with other compounds known to inhibit Plk1, including wortmannin, BI 2536, and staurosporine. Wortmannin is predicted to be an irreversible inhibitor of Plk1, as observed for PI3K kinases (Liu et al., 2007), and hence would be expected to display minimal

Table 1. Comparison of the ^{1}H and ^{13}C NMR data for depsides **1** and **2** recorded at 600 MHz in acetone-d_6.

Atom No.	^{1}H		^{13}C	
	1	**2**	**1**	**2**
1			103.7	103.9
2			162.5	162.1[a]
3	6.13 (d J=2.3 Hz)	6.27 (bs)	101.7	101.6
4			166.2	166.4
5	6.61 (d J=2.3 Hz)	6.68 (s)	102.3	103.1
6			143.2	143.4
7			159.1	159.4[a]
1′	6.33 (d J=2.8 Hz)		110.2	101.8
2′			156.3	162.2
3′	6.47 (d J=2.8 Hz)	6.49 (s)	103.8	103.2
4′			151.1	157.8
5′			133.7*	133.3[b]
6′			131.0	133.2[b]
7′				169.9
1″	6.36 (s)	6.40 (s)	103.6	103.6
2″			159.9	160.1
3″	2.50 (t J=7.5 Hz)	2.52 (t J=7.5 Hz)	33.9	33.9
4″	1.69 (m)	1.70 (m)	27.4	27.3
5″	1.39	1.39	32.0	32.0
6″	1.39	1.39	23.2	23.1
7″	0.91 (t J=7.0 Hz)	0.92 (t J=7.0 Hz)	14.3	14.3
1‴	3.56 (bs)	3.133.08	44.5	32.1 br
2‴			207.2	105.8
3‴	2.37 (t J=7.5 Hz)	1.92 (m)	42.7	41.2
4‴	1.35 (tt J=7.5, 7.5 Hz)	1.46	24.1	24.0
5‴	1.09 (m)	1.28	32.0	32.5
6‴	1.18 (qt J=7.3 Hz)	1.28	23.2	23.2
7‴	0.81 (t J=7.3 Hz)	0.85 (br)	14.3	14.3
OMe (C4)	3.79 (s)	3.80 (s)	56.1	56.3
OH (C2′)	8.32 (s)	11.36 (s)		
OH (C4′)	8.48 (s)	9.78 (bs)[a]		
OH (C2‴)		no[a]		

no—Not observed when run in acetone-d_6.
[a,b]Assignments within a column are interchangeable.
*Not observed in the 1D ^{13}C NMR spectrum.

ATP dependence as a result of covalent binding. BI 2536 is reported to be an ATP competitive inhibitor; however, the compound is known to effectively inhibit Plk1 in cells and therefore is capable of action under high cellular ATP concentrations (Lansing et al., 2007; Steegmaier et al., 2007). These two Plk1 inhibitors remained potent even at 1000 μM ATP (Tables 2 and 3). In contrast, staurosporine, a generic ATP competitive kinase inhibitor with submicromolar activity against Plk1 at low ATP

Table 2. Inhibition of recombinant Plk1 kinase by depsides **1**–**3** and reference compounds.

Compound	IC_{50} (nM)			
	1 μM ATP	10 μM ATP	100 μM ATP	1000 μM ATP
1	2787 ± 556 (4)	7959 ± 1809 (5)	11503 ± 2990 (3)*	8739 ± 246 (5)
2	740 ± 179 (4)	2275 ± 402 (6)	5888 ± 1022 (6)	4727 ± 1384 (5)*
3	1704 ± 782 (3)	1444 ± 164 (5)	3127 ± 1096 (5)	2385 ± 615 (6)
Wortmannin	11 ± 1 (4)	17 ± 3 (6)	28 ± 5 (6)	54 ± 8 (6)*
BI 2536	4 ± 1 (4)	7 ± 1 (6)	12 ± 2 (5)	22 ± 5 (5)
Staurosporine	634 ± 46 (4)	1826 ± 245 (4)*	6152 ± 1438 (4)*	>30000 (4)*

Data are mean IC_{50} (nM) ± SEM (number of determinations). IC_{50} (μM) at 10 μM ATP corresponds to the following IC_{50} (μg/mL): **1**, 3.8 μg/mL; **2**, 1.2 μg/mL; **3**, 0.7 μg/mL.
*Statistically significant difference in IC_{50} from 1 μM ATP (P<0.01).

concentrations, was clearly displaced under conditions of high ATP (IC_{50} ratio = 47× at 1000 μM ATP). These data suggest that the depsides either associate with Plk1 at a site other than the ATP-binding site (non-ATP competitive) or associate at the ATP-binding site (ATP competitive) but have a slow dissociation rate. Although these depsides are only moderately potent against purified Plk1 enzyme, they retain micromolar activity even at high ATP concentrations, suggesting that they could overcome elevated cellular levels of ATP.

The depsides **1**–**3** were also tested against a panel of 23 kinases to compare their selectivity profile among kinases (Table 4). These kinases are a small representative subset of different kinase classes spanning the kinome. The enzyme data suggest that the depsides possess differential activity between Plk1 and several other kinases, in many cases by more than an order of magnitude. While depsides **2** and **3** inhibited Plk1 with IC_{50} < 3 μM, they showed minimal activity against diverse kinases (IC_{50} > 10 μM against ~14 of the 23 kinases tested). Moreover, for depside **3** the selectivity ratio between Plk1 and many other kinases was >35-fold. Depside **1** was the least potent against Plk1 (2–11 μM, at varied ATP concentrations) and showed the correspondingly lowest activity against the kinase panel.

Discussion

Depside **1** was obtained as a purple powder that gave a [M+Na]+ ion in the HRESIMS at m/z 505.217 that was appropriate for a molecular formula of $C_{28}H_{34}O_7$ differing from that of the known compound **2** by loss of CO_2. Comparison of the ^{13}C NMR spectrum of depside **1** with that of depside **2** (Table 1) indicated that decarboxylation had taken place at C-7′ in depside **1** since the C-7′ lactone carbonyl and the C-2‴ hemiketal resonances in depside **2** were no longer present but instead a ketone carbon was

observed resonating at δ 207.2 ppm in depside **1**. Careful analysis of the gCOSY, gHSQC, and gHMBC data confirmed the structure of depside **1** (Figure 2) as the decarboxylated adduct of β-collatolic acid (**2**) (Rangaswami & Rao, 1955).

A new finding was the biological activity of these three depsides against Plk1 and a panel of diverse kinases. Depsides **1**–**3** were found to inhibit recombinant purified Plk1 kinase activity at micromolar levels. These compounds retain activity even at high ATP concentrations, consistent with ATP competitive inhibitors possessing a slow off-rate. In addition, the depsides possessed differential activity against a small panel of diverse kinases. Moreover, these data suggest some preferential activity of the depsides for Plk1 versus other kinases. For example, all three depsides inhibited the mitotic kinase aurora B at equivalent potency as Plk1. Of the kinases tested, aurora is the most closely related to Plk1. In addition, moderate activity was also observed against other mitotic kinases, including Cdk2 and Cdk1. Interestingly, depsides **2** and **3** also inhibited the non-receptor tyrosine kinases (NRTK) Hck, Fyn, and Abl with IC_{50} of 3–10 μM. In contrast, minimal activity was observed against other NRTKs such as Src and Lyn (IC_{50} > 50 μM). These kinases are structurally distant from Plk1 in the kinome, but these data suggest related binding sites for the depsides on the serine–threonine kinase Plk1 and some tyrosine kinases, and may allow the use of the depsides as useful probes to interrogate these unrelated kinases.

Table 3. ATP dependence of depsides **1**–**3** and reference compounds.

	Ratio of IC_{50} (compared with IC_{50} at 1 μM ATP)			
Compound	1 μM ATP	10 μM ATP	100 μM ATP	1000 μM ATP
1	1	2.9	4.1	3.1
2	1	3.1	8.0	6.4
3	1	0.8	1.8	1.4
Wortmannin	1	1.5	2.5	4.7
BI 2536	1	1.9	3.2	5.9
Staurosporine	1	2.9	9.7	47.3

The inhibitory activity of the depsides **1**–**3** against Plk1 and other kinases suggests that these are likely to bind to the ATP pocket of kinases. The relative ATP independence of these entities also suggests tight binding that may be beneficial in a high ATP cellular environment. The depsides were tested for their ability to inhibit phosphorylation of the Plk1 substrate Myt1 in a cellular assay; however, no inhibition was observed up to 30 μM (data not shown). Hence, these depsides may either have low

Table 4. Kinase selectivity profile of depsides **1**–**3**.

	1		**2**		**3**	
		IC_{50} ratio		IC_{50} ratio		IC_{50} ratio
Kinase	IC_{50} (μM)	Kinase/Plk1	IC_{50} (μM)	Kinase/Plk1	IC_{50} (μM)	Kinase/Plk1
Plk1 (10 μM ATP)	8.0		2.2		1.4	
Aurora B	11.3	1.4	2.7	1.2	2.0	1.4
HCK	13.6	1.7	3.6	1.6	3.5	2.5
CDK2/cyclin A	11.3	1.4	2.7	1.2	3.5	2.5
CDK1/cyclin B	>50	>6.3	6.8	3.1	6.2	4.4
CK1 gamma 1	>50	>6.3	nd		6.4	4.5
ABL1	>50	>6.3	4.7	2.1	6.6	4.7
FYN	>50	>6.3	10.4	4.7	6.7	4.8
PKB-alpha	>50	>3.1	13.4	6.1	6.9	5.0
ERK2	>50	>6.3	8.7	4.0	7.6	5.4
GCK	>50	>6.3	12.9	5.9	10.2	7.3
PKA	>50	>6.3	8.5	3.9	10.5	7.5
RSK1	>50	>6.3	11.4	5.2	14.0	10.0
VEGFR2	>50	>6.3	11.7	5.3	19.0	13.6
PDGFR-alpha	>50	>6.3	14.9	6.8	23.3	16.7
Src	>50	>6.3	>50	>22.7	>50	>35.7
ROCK1	>50	>6.3	26.6	12.1	>50	>35.7
PKC-beta	>50	>6.3	26.3	12.0	>50	>35.7
PKC-alpha	>50	>6.3	30.8	14.0	>50	>35.7
P38 alpha	>50	>6.3	13.3	6.0	>50	>35.7
MET	>50	>6.3	>50	>22.7	>50	>35.7
LYN A	>50	>6.3	nd		>50	>35.7
IKK-alpha	>50	>6.3	nd		>50	>35.7
CHK1	>50	>6.3	>50	22.7	>50	>35.7

nd—Not determined.
Values are IC_{50} (μM), or the ratio of IC_{50} kinase/IC_{50} Plk1.
Data are sorted by IC_{50} for depside **3**.
Results are representative of two independent kinase profiling screens.

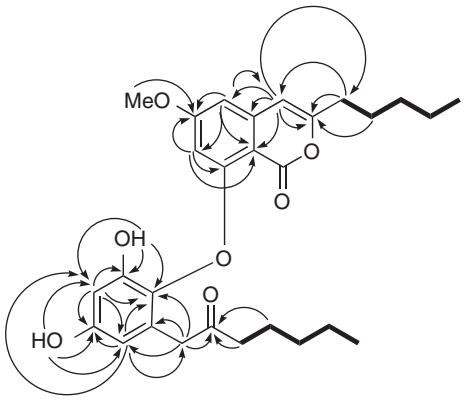

Figure 2. gCOSY connectivities (in bold) and key gHMBC (arrow) correlations used to assign the structure of depside **1**.

cell membrane permeability or lack sufficient enzyme potency to possess cell activity.

Conclusion

Our data suggest that depsides **2** and **3** have similar kinase inhibition profiles. The subtle replacement of a hydroxyl in depside **3** by a methoxy residue in depside **2** suggests that this site imparts minimal effect on kinase binding. In contrast, the structural differences between depside **1** compared with depsides **2** and **3** are sufficient to reduce potency by more than 5–10-fold against a panel of kinases. The depsides are less potent than other reported Plk1 inhibitors such as wortmannin and BI 2536, which inhibit Plk1 at low nanomolar concentrations; however, they represent a new structural class of Plk1 inhibitors. These data suggest that the depsides **1**–**3** may serve as core structures that can be further explored as potential inhibitors of Plk1 and other kinases.

Declaration of interest

The research was supported by National Cancer Institute-National Cooperative Drug Discovery Group grant CA 67786. The authors alone are responsible for the content and writing of the paper.

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