Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 4111–4115

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Biological Investigation and Structure–Activity Relationship Studies on Azadirone from *Azadirachta indica* A. Juss☆

Srinivas Nanduri,[a,*] Siva Sanjeeva Rao Thunuguntla,[a] Vijay Kumar Nyavanandi,[a] Sridevi Kasu,[a] P. Mahesh Kumar,[a] P. Sai Ram,[a] Sriram Rajagopal,[b] R. Ajaya Kumar,[b] Dhanvanthri S. Deevi,[b] R. Rajagopalan[b] and A. Venkateswarlu[a]

[a]*Discovery Chemistry, Dr. Reddy's Laboratories Ltd., Discovery Research, Bollaram Road, Miyapur, Hyderabad 500 050, India*
[b]*Discovery Biology, Dr. Reddy's Laboratories Ltd., Discovery Research, Bollaram Road, Miyapur, Hyderabad 500 050, India*

Received 4 June 2003; accepted 11 August 2003

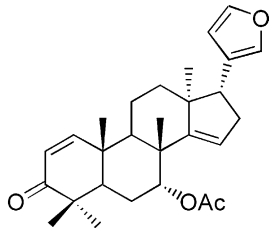
Abstract—Azadirone **1**, a limonoidal constituent of *Azadirachta indica* is found to possess potent cytotoxic activity against a panel of human cancer cell lines in our in vitro studies. In vitro screening of a number of semi-synthetic analogues of **1** revealed that the α,β-unsaturated enone moiety or its equivalent conjugated system in A-ring, C-7 acetyloxy/chloroacetyloxy or keto group in B-ring and the furan moiety are responsible for the activity of **1** and its analogues. Compound **1** and two of the semi-synthetic analogues **10** and **13** were found to possess good in vivo antitumor activity in modified hollow fiber animal models.

Introduction

Limonoids, the triterpene derivatives present in Meliaceae and Rutaceae families have attracted much attention owing to their marked insect antifeedant,[1] growth regulatory[2] properties and a variety of medicinal effects including antibacterial, antifungal,[3–5] antimalarial,[6–9] antipyretic[10,11] and antiinflammatory[10,12] activities. Anti-HIV properties of limonin and nomilin on infected human mononuclear cells were recently reported.[13] The cytotoxic activity and structure–activity relationships of various limonoids belonging to Meliaceae and Rutaceae families against the murine P388 lymphocytic leukemia cell lines were reviewed.[14] Promising cytotoxic activity of citrus limonoids against colon, breast and other cancers were reported.[15–17] Antitumoral activity of Haperforine A from *Harrisonia perforate* was described.[18] Potent cytotoxic activity of a number of limonoids present in *Melia* azedarach,[19–22] *Melia volkensii*[23–25] and *Melia* toosendan[26] were reported. Nimbolide and 28-deoxonimbolide were identified as cytotoxic constituents of *Azadirachta indica* leaves.[27]

As a part of our study to identify novel cytotoxic agents of plant origin, the activity of azadirone **1** was studied. Azadirone, a limonoidal triterpene was first isolated from nim oil in 1967[28] and subsequently its structure was elucidated.[29] In the present study, **1** was isolated from neem flowers for the first time. While there are no reports in literature about the biological activity of **1**, the present study revealed **1** to be a potent cytotoxic agent with good in vitro and in vivo activities. The investigation was also extended to explore the structural requirements for the activity of **1** and to synthesize novel semi-synthetic analogues with improved activity. The details of the study are presented in this communication (Fig. 1).

Compound **1** was isolated from the methanolic extract of dry neem flowers as a major constituent (0.05–0.1% yield) and identified as azadirone based on extensive spectral studies and comparison with published



Azadirone **1**

Figure 1.

☆DRL Publication No.: 297.

*Corresponding author. Tel.: +91-40-2304-5439; fax: +91-40-2304-5438; e-mail: nandurisrinivas@drreddys.com

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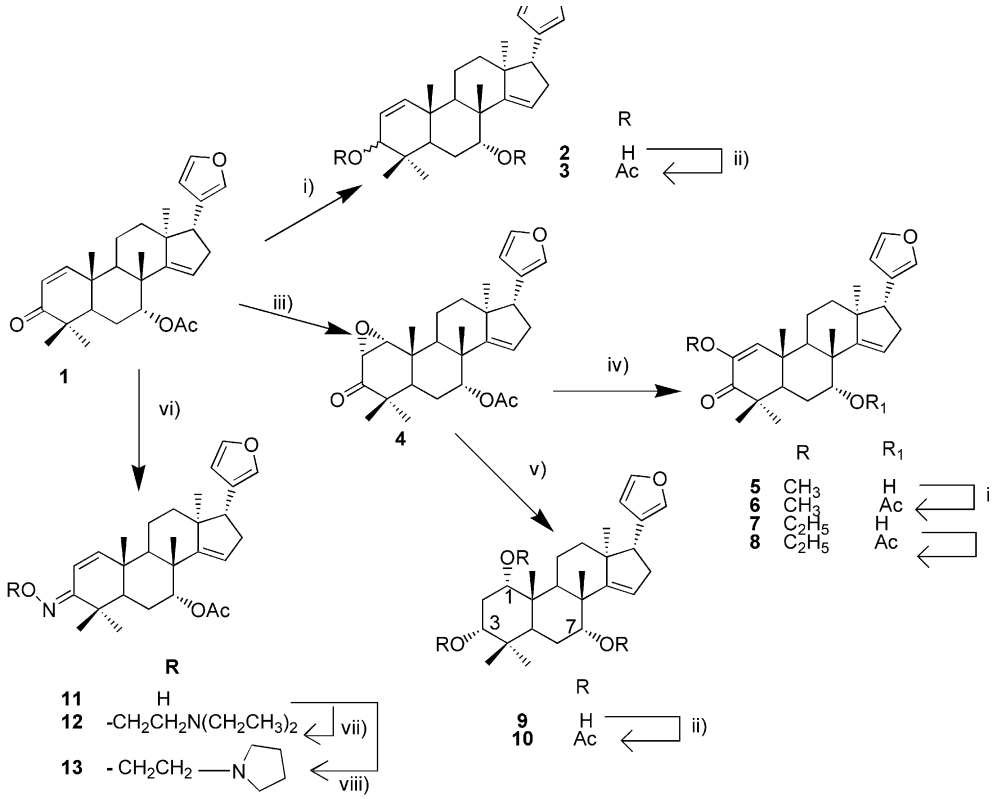
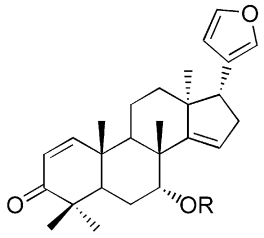
doi:10.1016/j.bmcl.2003.08.030



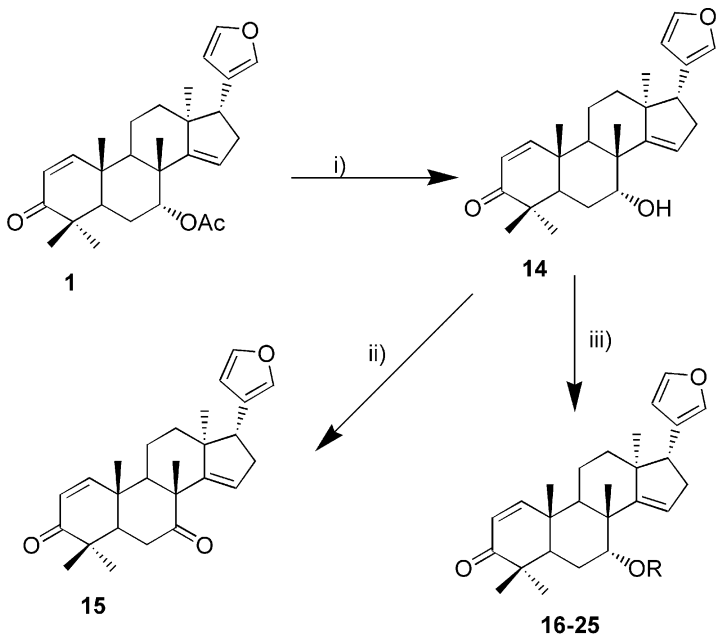
Scheme 1. (i) DIBAL-H/benzene/rt/2 h; (ii) Ac_2O/pyridine/DMAP/2 h; (iii) H_2O_2/2 N NaOH/MeOH/2 h; (iv) 10% alcoholic NaOH/24 h; (v) $LiAlH_4$/THF/reflux/24 h; (vi) $H_2NOH{\cdot}HCl$/EtOH/pyridine/reflux/3 h; (vii) $ClCH_2CH_2N(CH_2CH_3)_2{\cdot}HCl/K_2CO_3$/acetone/reflux/8 h; (viii) 1-(2-chloroethyl)pyrrolidine$\cdot HCl/K_2CO_3$/acetone/reflux/8 h.



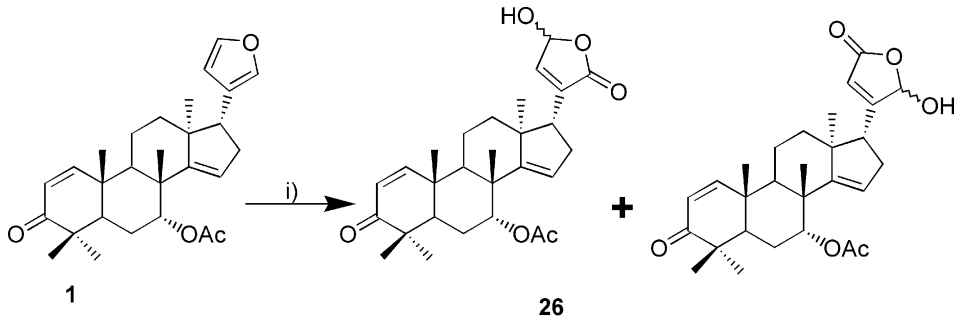
Cpd. No	R	Conditions
16	CH_2CH_3	NaH/C_2H_5I / THF / reflux / 24 h
17	$COCF_3$	TFAA / DCC / DMAP / toluene / reflux / 24 h
18	$COCH_2CH_3$	$(C_3H_7CO)_2O$ / pyridine / DMAP / DCM / r.t. / 1 h
19	$COCH_2CH_2CH_3$	$(C_3H_7CO)_2O$ / pyridine & DMAP / DCM / r.t. / 1 h
20	$COCH(CH_3)_2$	$[(CH_3)_2CHCO]_2O$ / pyridine / DMAP / DCM / r.t. / 1 h
21	$COCH{=}CH\text{-}C_6H_5$	Cinnamic acid / DCC / DMAP / toluene / reflux / 24 h
22	$COCH{=}CH\text{-}(3,4\text{-di } OCH_3)C_6H_3$	Caffeic acid / DCC / DMAP / toluene / reflux / 24 h
23	$COCH_2NHBoc$	N-Boc glycine / DCC / DMAP / toluene / reflux / 8 h
24	$CONHC_6H_5$	PhNCO / DMAP / toluene / reflux / 4 h
25	$COCH_2Cl$	$ClCH_2COCl$ / TEA & DMAP / DCM / r.t. / 12 h

Figure 2. C-7 derivatives of azadirone.

results.[29] Semi-synthetic analogues of **1** were prepared by modifying the α,β-unsaturated enone moiety in A-ring (Scheme 1), C-7 acetyloxy group in B-ring (Scheme 2), and the furan moiety (Scheme 3). Compound **1** and its analogues were evaluated for their in vitro cytotoxic activity against eight cancer cells representing different human cancers using SRB assay according to NCI protocol.[30] In the preliminary screening, the percent growth of the cancer cell lines at 10 μM concentration of the compounds was measured (Table 1). Compounds showing 50% or less growth in the preliminary screening were selected, subjected to dose response studies with concentrations ranging from 100 to 0.1 μM and the GI_{50}'s were calculated (Table 2).



Scheme 2. (i) EtOH/Aq. NaOH/reflux/2 h; (ii) PDC/DCM/8 h; (iii) Conditions given in Figure 2.



Scheme 3. (i) *m*-CPBA/NaOAc/DCW/rt/5 h.

In the above in vitro studies, **1** exhibited potent activity against breast, melanoma and prostate cell lines having GI_{50} values in sub micro molar concentrations. To study the influence of α,β-unsaturated enone moiety in A ring of **1**, **3** (3-acetyloxy derivative of **1**,), and **4** (1α,2α-epoxy derivative of **1**) were synthesized (Scheme 1). Compound **3** was prepared by reducing **1** with DIBAL-H to give **2**, which was further acetylated to obtain **3**. Selective epoxidation of C-1,2 olefin bond gave **4**. Further, 2-alkoxy derivatives **6** and **8** were prepared by refluxing **4** in methyl and ethyl alcohols in the presence of alkali and acetylation of the resulting 2-alkoxy 7-deacetyl derivatives **5** and **7**. C-3 oxime derivative **11** and its corresponding ethers **12** and **13** were synthesized by usual methods.

In the preliminary screening, **3** and **4** exhibited loss of activity indicating that the α,β-unsaturated enone moiety plays an important role for the activity of **1**. Compounds 2-methoxy and 2-ethoxy derivatives **6** and **8**, respectively, showed moderate activity in the preliminary assay. In further evaluation, while **6** exhibited loss of activity against all the cell lines, **8** showed good activity against breast, CNS and colon cell lines. When the enone moiety was converted to its C-3 oxime as in **11** moderate activity was observed in the preliminary screening. However, activity was found to improve considerably with the oxime ether derivatives **12** and **13**. These observations clearly show that α,β-unsaturated enone moiety or its equivalent conjugated moieties in A ring can confer substantial cytotoxic activity.

Compound **4** was further utilized as a key intermediate in the synthesis of **9**, a compound with 1,3-oxygenation pattern in A ring. Compound **9** was obtained by reductive opening of the epoxide in **4** with $LiAlH_4$, which on further acetylation gave **10**, the corresponding triacetyl derivative. Compound **10**, exhibited good in vitro cytotoxic activity against most of the cell lines.

Modifications carried out in B ring (Scheme 2) include hydrolysis of C-7 acetyloxy moiety leading to the corresponding hydroxy derivative **14**, which on further oxidation gave 7-keto derivative (**15**). Several C-7 ester and ether derivatives (**16**–**25**) of **14** were synthesized and are listed in Figure 2.

The screening results indicated that **14** exhibited good activity against breast, CNS, melanoma and prostate

Table 1. In vitro cytotoxic activities of azadirone derivatives (preliminary screening)

Compd	Cytotoxicity (% growth at 10^{-5} M concentration)							
	Breast	CNS	Colon	Lung	Melanoma	Ovarian	Prostate	Renal
	MCF-7/ADR	U251	SW620	H522	M14	SKOV3	DU145	A498
1	−18	−4	29	29	−6	30	−6	23
2	76	100	79.6	97	100	100	100	88.2
3	78	46	59	86	76	19.3	74	69
4	59.3	100	100	69.6	100	100	63.1	42.7
6	58	63	35	82	67	38	47	50
7	17	54	36	57	67	67	84	52
8	51	70	65	52	40	61	35	33
10	38	−24	−60	8	−22	42	−19	−5
11	55	5	47	44	38	87	56	20
12	28	5	23	26	35	−7	46	7
13	22	−24	−51	−13.9	26	−35	32	−16
14	36	46	100	100	15.8	64	46	65
15	30	27	3	43	7	43	7	9
16	66	63	81	69	61	100	76	45
17	54	52	60	75	86	49	77	59
18	29	40	51	48	39	61	67	67
19	57	15	49	40	59	90	68	43
20	57	40	61	54	95	84	69	74
21	55	41	86	69	41	86	81	48
22	66	77	62	63	39	85	25	5
23	62	53	67	53	58	48	63	33
24	55	71	64	51	74	100	76	100
25	−2	22	12	5	−3	14	13	−8
26	−13	44.3	100	73.3	71.3	41.6	24.8	70.3

Table 2. In vitro cytotoxic activities of azadirone derivatives

Compd	Cytotoxicity (GI_{50} μM)[a]							
	Breast	CNS	Colon	Lung	Melanoma	Ovarian	Prostate	Renal
	MCF-7/ADR	U251	SW620	H522	M14	SKOV3	DU145	A498
1	0.06	2.5	7	6	0.08	5	0.05	2
6	95	20	20	40	40	35	35	40
8	8	5	5	30	20	40	50	40
10	4	30	3	40	5	9	4	15
12	6	4	4	3	4	3	7	8
13	3	4	3	4	5	4	6	4
14	5	3.5	>100	>100	9	>100	9.5	>100
15	3	6	3	25	0.6	7.5	6	4
23	60	50	20	15	20	65	20	12
25	3	6	5	4	5	5	5	4

[a]Cytotoxicity GI_{50} values are the concentrations corresponding to 50% growth inhibition.

cell lines with GI_{50}'s in micro molar concentrations and showed loss of activity against other cell lines. However, the corresponding 7-keto derivative **15** showed good activity against all the cell lines. The biological evaluation of C-7 ester and ether derivatives of **14** showed the following interesting pattern. Replacement of the acetyl moiety with ethyl substituent (**16**) led to loss of activity indicating that an ester linkage at C-7 is essential. Changing of the acetyl to trifluoroacetyl (**17**) resulted in loss of activity. Moderate activity was observed with propionyl moiety (**18**) which decreased with butyryl (**19**) and isobutyryl (**20**) derivatives. More bulky groups like cinnamoyl, glycinyl, and carbamoyl derivatives (**21**–**24**) also showed loss of activity. Interestingly, activity was found to be retained with C-7 chloroacetyloxy derivative (**25**). The above results clearly indicate that a keto group or an ester linkage at C-7 can result in good cytotoxic activity and bulkier esters reduce the activity.

As **26**, a mixture of hydroxy butenolide derivatives of **1**, showed loss of activity (Table 1), it is inferred that the furan moiety in **1** is also very important for the cytotoxic activity of **1** (Scheme 1).

In summary, the above studies resulted in **10**, **12**, **13**, **15**, and **25** with good in vitro anticancer activity. Of these, **10** and **13** along with azadirone **1**, which exhibited good cytotoxic activity and possess unique structural features were evaluated for their in vivo potential in Swiss Albino Mice using modified hollow fiber assay.[31,32] Poly vinylidine fluoride hollow fibers containing different types of human cancer cell lines were implanted intraperitoneally (ip) and subcutaneously (sc) into the mice. The compounds were administered by IP route at two different doses for 4 days. The doses were determined based on the MTD values of the compounds. The percentage growth inhibition of cancer cells determined in

Table 3. In vivo hollow fiber assay results of azadirone derivatives

Compd	Dose (mg/kg)	sc Score	ip Score	Total score
1	75	12	14	36
	150	24	24	48
10	75	6	12	18
	150	24	24	48
13	50	8	14	22
	100	22	24	46

both the compartments was an index of the compound's anticancer potency. Compounds inhibiting 50% or more growth of cancer cells compared to the vehicle treated control cells were considered active and a score of 2 was assigned at the dose tested. Each compound was tested against 12 human cancer cell lines. The results obtained are summarized in Table 3. The results indicated that the compounds are moderately active at low doses and fairly active at higher doses.

In conclusion, we have identified azadirone **1**, present in neem flowers, as a potent cytotoxic agent with good in vitro and in vivo activity. Semi-synthetic studies performed and cytotoxic activity screening of the derived analogues resulted in **10** and **13** with good activity. The studies also revealed that the α,β-unsaturated enone moiety or its equivalent conjugated system of A-ring, C-7 acetyloxy/chloroacetyloxy or keto group of B-ring and the furan moiety are the structural requirements for the potent activity of **1** and its analogues.

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