Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Medicinal Chemistry 41 (2006) 997–1002
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://france.elsevier.com/direct/ejmech

Short Communication

Synthesis and cytotoxicity of novel isomeric C-seco limonoids

Anitha Genupur [a], Josepha Lourdu Raj Jesu [a], Narasimhan Srinivasan [a,*], Anand Solomon Kamalakaran [b], Rajan Sundararajan Sundar [b]

[a] *Asthagiri Herbal Research Foundation, 7/1, Thirumazhisai Street, Sundaram Colony, East Tambaram, Chennai 600 059, Tamil Nadu, India*
[b] *Department of Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai 600025, Tamil Nadu, India*

Received in revised form 31 March 2006; accepted 3 April 2006
Available online 19 June 2006

Abstract

Attempts to cleave the C-ring in the bioactive limonoids characteristic of the species *Azadirachta indica* A. Juss using $BF_3{\cdot}OEt_2$ and $(C_4H_9)_4NBr$ resulted in novel isomeric C-seco limonoids. Structure related cytotoxic properties of the isomers and the native compounds have been studied using brine shrimp lethality bioassay (BSLB) method and molecular descriptors viz., theoretical and chromatographic hydrophobicity constants, oxidation state and molecular modelling studies. The O–O diad distances reveal the significance of the orientation of the furan ring in enhancing the cytotoxicity of the molecule.

Keywords: C-seco limonoids; Isomerisation; Cytotoxicity; Structure activity relationship

1. Introduction

The most comprehensively studied compounds with cyclic ether functionality are the C-seco limonoids which are characteristic of the species *Azadirachta indica* A. Juss [1] (Meliaceae). They possess high activity against herbivorous insects and are two to three orders of magnitude more active than the other classes [2]. Modification in the C-ring is archetypal of C-seco limonoids, the classic examples being the azadirachtin, nimbin and salannin group. Extensive studies on the semi-synthetic modifications of limonoids have been performed by various groups particularly on the photo oxidations, photolysis, oxidation of the furan ring, acetylation, reduction of enone, etc to interpret the structure activity relationship [3a–d]. However, attempts to study the possibility of cleavage of the C-ring were not made, in spite of the fact that the resulting product would mimic azadirachtin A in possessing free rotation about the C-8 and C-14 bond (Fig. 1).

Hence the cleavage of ether ring in nimbolide (C-seco limonoid) was performed by us wherein isonimbolide, a novel isomer of nimbolide was obtained [4] (Fig. 2).

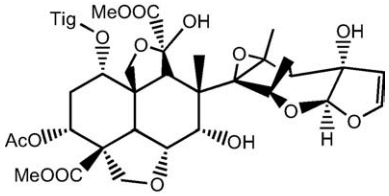
To ascertain the generality, the reaction was performed with other C-seco limonoids possessing different functional groups. The possible enhancement in the cytotoxic property of the novel isomers was studied using brine shrimp lethality bioassay (BSLB) method [5]. The technique has been used to screen, isolate and identify over 300 novel antitumour and pesticidal natural products and is found to have a positive correlation with human nasopharyngeal carcinoma cytotoxicity. Since then BSLB method has been used as a prescreen for a panel of six human solid tumour cell lines at the Cell Culture Laboratory of the Purdue Cancer Centre, Purdue University, USA [6]. Further, quantitative structure activity relationship (QSAR) using molecular descriptors, viz., hydrophobicity constants, oxidation state and molecular modelling studies have been studied in the present study. We report the results of our findings.

2. Chemistry

The common reagents cited in literature for the cleavage of ethers were the Bronsted acids like HBr in aqueous medium. For a highly sensitive natural product like nimbolide **1** the reaction conditions, however, proved to be vigorous ensuing multiple products that could not be isolated. Lewis acids with appropriate hardness had been a rewarding alternative.

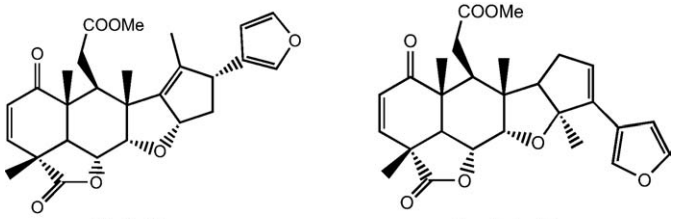
* Corresponding author.
E-mail address: narasimhan-s@yahoo.com (N. Srinivasan).

0223-5234/$ - see front matter © 2006 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2006.04.003



Azadirachtin A

Fig. 1. Structure of azadirachtin A.



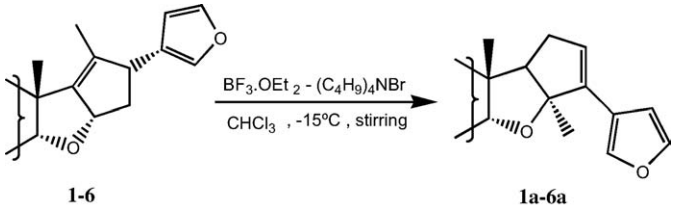
Nimbolide Isonimbolide

Fig. 2. Structures of nimbolide and isonimbolide.

$BF_3{\cdot}OEt_2$ in conjunction with a halide ion (tetraalkylammonium halide) is extremely useful for mild and regioselective cleavage of aliphatic and alicyclic ethers and for the removal of acetal protection in carbonyl compounds [7]. Nimbolide when subjected to ether cleavage using $BF_3{\cdot}OEt_2$ and $(C_4H_9)_4NBr$ in $CHCl_3$ at room temperature remained sensitive resulting in charring of the reaction mixture. Reduction of temperature to –15 °C yielded a non-polar product **1a**. Similarly, the C-ring in compounds **2**–**6** with interesting structural entities were subjected to the reaction (Fig. 3 and Scheme 1).

The structures of the products were authenticated by IR, mass, 1D and 2D NMR studies. In all the cases ring-opened products were not envisaged. However, structural isomers of the substrates were obtained. A perusal of the ^{13}C NMR of the products revealed that the carbon count remained unaltered. The substrate signals at 131, 144, 88, 41 and 49 ppm representing the D-ring were missing and new resonances at 96, 56, 31, 126 and 136 ppm were observed. Similarly in ^1H NMR disappearance of the signals at 5.5, 2.2, 2.1 and 3.7 ppm and appearance of new resonances at 2.41, 2.40, and 5.84 ppm was found. The connectivity of the protons at 2.41, 2.40, and 5.84 ppm to carbons resonating at 56, 31 and 126 ppm, respectively, were established using ^1H–^1H COSY and ^1H–^{13}C COSY. The peak at 136 ppm is assigned to the olefinic carbon with its counterpart resonating at 126 ppm. The quaternary carbon downfield at 96 ppm implied an oxygen-connected carbon. This suggests a possible modification in the D-ring which is supported by the downfield shift of the C-18 signal from 12 to 24 ppm in the product. In all the cases the reagent in stoichiometry was tolerated by the other functionalities like the enone, isolated double bond, C-12 ester, lactone, hydroxy, tigloyl/senicioxy groups. Also a second ether ring manifested as the F-ring in **5** and **6** did not undergo any reaction (Tables 1 and 2). The absence of peaks with 1:1 intensity at *m/z* 79 and 81 in the mass spectrum confirmed the absence of bromine atom. Furthermore, the structure of **1a** was unequivocally confirmed by X-ray crystallography.

In order to understand the mechanism, a blank experiment was conducted without $(C_4H_9)_4NBr$ which resulted in the recovery of the starting material as monitored from HPLC ascertaining the role of the quaternaryammonium bromide in the reaction. The mechanism probably involves co-ordination of the Lewis acid with ethereal oxygen of the C-ring of **1**–**6** followed by cleavage resulting in the formation of halo substituted BF_3 complex (**I**). The complex eliminates the halide ion to form a more stable allyl carbocation at the C-13 carbon (**II**). Deprotonation of H-17 results in intermediate **III**. The diene undergoes a 1,5 sigmatropic shift of hydrogen atom followed



Scheme 1. Synthetic procedure for the isomerisation of limonoids.

Table 1
Reaction profile

Run	Substrate	Product	Time (h)	Yield[a]
1	1	1a	15	52.35
2	2	2a	12	31.33
3	3	3a	6	25.35
4	4	4a	3	40.41
5	5	5a	8	46.66
6	6	6a	8	23.33

[a] Isolated yield.

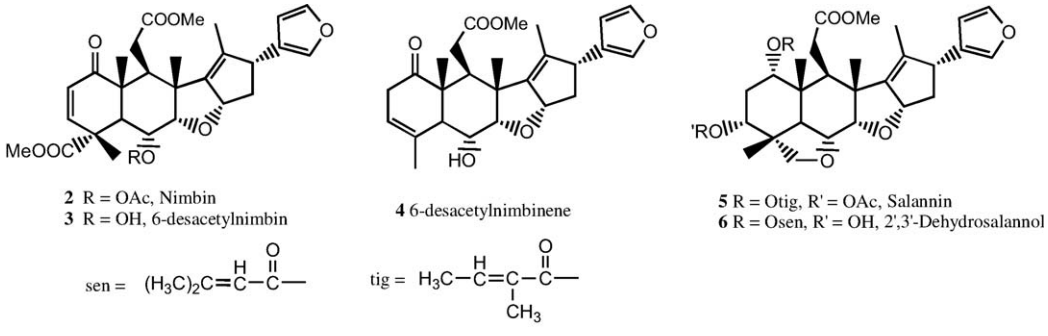


Fig. 3. Structures of limonoids.

Table 2
Carbon resonances of the product limonoids

Carbon	^{13}C (δ ppm)					
	1a	2a	3a	4a	5a	6a
1	200.62	201.78	202.70	213.45	72.58	71.68
2	130.83	125.98	126.87	25.07	30.71	29.62
3	149.49	147.58	148.38	128.35	71.27	71.27
4	43.76	42.32	47.95	188.50	44.89	42.98
5	47.14	46.94	43.59	41.08	42.51	42.10
6	73.42	68.24	66.41	66.61	73.06	72.76
7	77.92	79.84	83.58	84.17	85.62	84.94
8	52.86	49.88	49.34	49.41	47.34	48.13
9	44.10	41.29	43.11	40.00	42.28	41.78
10	46.57	48.90	49.58	50.62	52.08	51.33
11	31.20	33.79	33.74	33.19	32.17	31.64
12	173.71	174.84	175.11	174.97	173.47	173.02
13	96.07	94.81	95.60	95.50	96.07	95.73
14	56.22	55.98	57.39	57.50	57.72	57.33
15	31.47	31.27	31.70	32.31	31.69	31.34
16	126.25	126.20	126.83	126.83	128.33	127.70
17	136.97	137.05	137.47	130.50	136.42	136.90
18	24.59	24.22	24.98	24.73	26.27	25.76
19*	15.22	16.98	17.95	17.66	19.25	20.75
20	118.57	119.16	119.41	119.27	119.88	119.49
21	140.15	139.50	139.85	139.68	140.30	139.94
22	109.23	109.33	109.74	109.73	110.19	109.77
23	142.58	142.46	143.01	143.08	142.81	142.48
28	175.67	174.78	176.07	22.08	78.29	77.64
29*	18.53	17.09	17.52	14.76	20.84	14.25
30*	17.29	16.98	17.19	52.15	17.86	11.98
–OCH_3	51.89	52.69	52.15	–	51.67	51.73
–OCH_3	–	51.82	53.34	–	–	–
–	–	170.21	–	–	–	170.07
$OCOCH_3$						
–	–	20.62	–	–	–	27.44
$OCOCH_3$						
1′	–	–	–	–	165.14	166.46
2′	–	–	–	–	158.98	135.99
3′	–	–	–	–	115.89	129.09
4′	–	–	–	–	21.47	18.81
5′	–	–	–	–	21.47	17.27

by protonation at the C-14 position resulting in **V**. Successive intramolecular attack of the BF_3 complexed ethereal oxygen on the tertiary carbocation invokes a 180° rotation of the bond between C-8 and C-14 (**VI**) and subsequent ring closure to produce the rearranged product with intact cyclic ether accounting for the positional isomerisation (**1a**–**6a**). The plausible mechanism is depicted below (Scheme 2).

3. Results and discussion

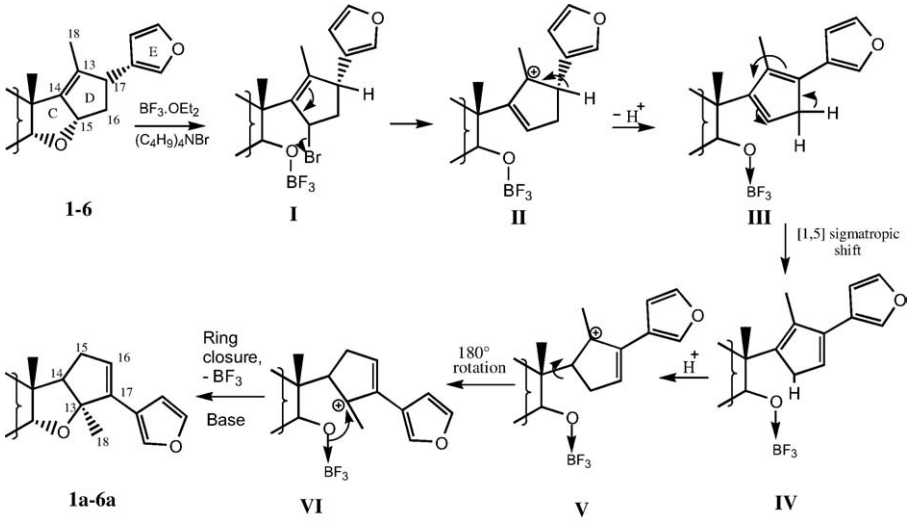
The cytotoxicity of the compounds **1**–**6** and **1a**–**6a** were determined using BSLB method [8]. ED_{50} values were calculated by processing the mortality values using Finney's probit analysis software [9]. Nimbolide is considered as the reference standard as it is established to be the most potent cytotoxic limonoid among several other limonoids of neem [10]. Among the substrates 6-desacetylnimbinene and salannin were more potent than nimbolide. The isomeric products exhibited better bioefficacy than their corresponding substrates except 6-desacetylnimbinene (**4**) as evinced from Table 3. The enhancement in the activity is highly significant in the case of nimbolide to isonimbolide.

A quantitative treatment of the bioactivity results was studied using following molecular descriptors.

Table 3
ED_{50} values of limonoids and their isomers

Run	Substrate		Run	Product		Ratio of activity
	Code	ED_{50}[a] (ppm)		Code	ED_{50}[a] (ppm)	
1	1	568.73	7	1a	20.55	0.96
2	2	15589.27	8	2a	4638.41	0.70
3	3	13376.25	9	3a	3027.49	0.77
4	4	522.14	10	4a	1181.76	–0.55
5	5	989.42	11	5a	309.18	0.68
6	6	277.04	12	6a	70.71	0.74

[a] Lower the ED_{50}, higher is the activity.



Scheme 2. Mechanism of the reaction.

3.1. Hydrophobicity constant

The hydrophobic character of a compound determines the ability of a compound to cross cell membranes and gains significance in receptor interactions. The chromatographic hydrophobicity constant (K'w) were calculated by modifying the method of Luco et al. [11] using the formula $\log K'\varphi = \log K'\mathrm{w} - S\varphi_{AN}$ where $K'\varphi$ is the capacity factor and φ_{AN} is the volume of acetonitrile used for the HPLC analysis. S is the slope obtained from a plot of $\log K'\varphi$ vs. φ_{AN}. The theoretical hydrophobicity constant (Clog P) was calculated by modifying the method of Leo et al. [12] using the SMILES notation software.

The Clog P values and $\log K'$w values did not fall in an identical range [13]. The change in the $\log K'$w values between substrates and products were random while a positive correlation was seen in Clog P with the products possessing higher values than the substrates (Fig. 4).

3.2. Oxidation state

Oxidation state (O) of limonoids [14] were determined by counting –1 for each C–H bond and +1 for each carbon bonded to a heteroatom. The sum of these counts is divided by the total number of carbon atoms. This is illustrated as $O = \sum(o + 3c - h)/n$. No logical correlation was observed which allows presumption that the position of the oxygen may be critical rather than the oxidation state (Table 4).

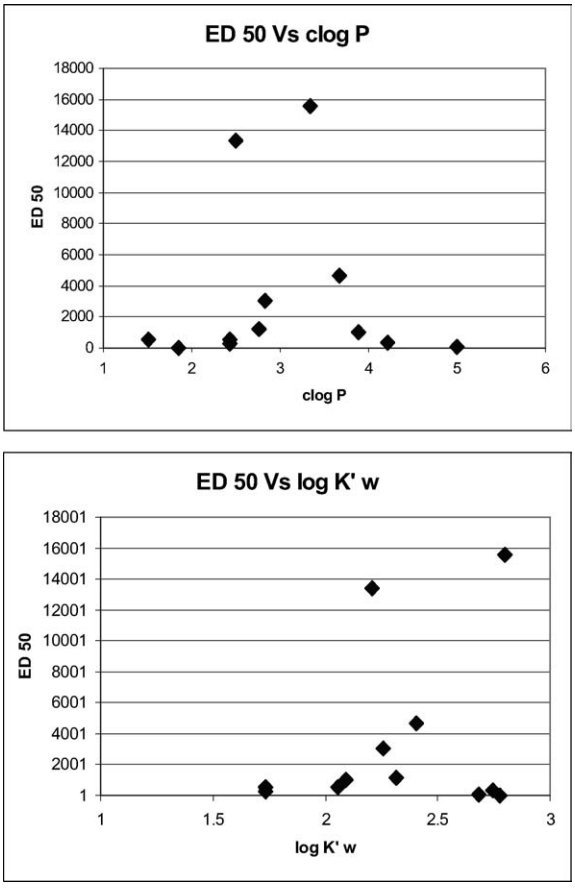


Fig. 4.

Table 4
Molecular descriptors of limonoids and their isomers

Run	Substrate				
	Code	O[a]	Rt[b]	Hydrophobicity constant	
				log K'w[c]	Clog P[d]
1	1	–0.703	21.66	2.0556	1.517
2	2	–0.733	24.37	2.7985	3.340
3	3	–0.750	20.31	2.2073	2.504
4	4	–0.807	22.80	1.7329	2.429
5	5	–0.875	22.02	2.0930	3.885
6	6	–0.807	30.50	1.7329	2.429
7	1a	–0.703	23.84	2.7773	1.847
8	2a	–0.733	31.11	2.4032	3.670
9	3a	–0.750	25.71	2.2571	2.834
10	4a	–0.807	24.93	2.3136	2.759
11	5a	–0.875	25.17	2.7438	4.215
12	6a	–0.807	38.78	2.6814	5.004

[a] Oxidation state of limonoids.
[b] Retention time were obtained from HPLC analysis.
[c] Experimental hydrophobicity constant.
[d] Theoretical hydrophobicity constant.

3.3. Molecular modelling

Any bioactivity is the result of ligand receptor interactions. Binding of the ligand to the receptor site operates through the lock and key mechanism which may be stabilised through hydrogen bonds involving electronegative oxygen atoms in the present case. The models of the limonoids were built using the module BUILDER incorporated in 'INSIGHT II' software package loaded on an 'OCTANE' silicon graphics work station using X-ray structure of closely related compounds. The built model was brought to a minimised energy using consistent force field 91 (CFF91) incorporated in the software 'DISCOVER'.

The interaction site involving oxygen atoms can involve a diad, triad or a tetrad. As a first approximation the O–O distances between two oxygen atoms (diad) were considered for the study [15a–b]. These distances between all the oxygen atoms were calculated and grouped into different ranges and the total number of diad distances in each range was obtained. The values showed a trend in frequency range between 4.5 and 5.5 Å between the isomers and the naturally available compounds. A plot of the ED_{50} vs. number of diads gives a linear relationship in this frequency range alone with the number of occurrences increasing from the substrate to its product. Analysis of the functional groups in this frequency range depicts interaction between the $-OCH_3$ group about the C-12 carbon and the C-ring oxygen in the substrates with an additional interaction between the C-ring oxygen and furan ring oxygen in the product. This ascertains the role of the orientation of the furan in enhancing the cytotoxicity of the compounds (Table 5 and Fig. 5).

The substrates are functionalised in the A-ring and the F-ring. The variations in the A-ring are an enone (nimbolide, nimbin, 6-desacetylnimbin), isolated double bond at C-3,4 positions and a ketone at C-1 (6-desacetylnimbinene) and oxygen

Table 5
Frequency of O–O diads

Run	Code	Distance (Å)										
		1.5–2.5	2.5–3.5	3.5–4.5	4.5–5.5	5.5–6.5	6.5–7.5	7.5–8.5	8.5–9.5	9.5–10.5	10.5–11.5	11.5–12.5
1	1	2	3	0	2	4	4	2	3	0	1	0
2	1a	2	4	0	4	4	3	3	2	1	1	0
3	2	3	4	4	3	6	4	4	5	1	2	0
4	2a	4	5	3	6	5	3	5	3	1	1	0
5	3	2	5	1	1	4	2	3	4	0	1	0
6	3a	3	5	1	3	6	3	4	2	1	1	0
7	4	2	3	2	3	5	5	4	2	1	1	0
8	4a	2	1	0	7	7	2	2	4	1	1	0
9	5	3	3	1	4	5	7	5	2	1	2	0
10	5a	4	1	0	10	5	7	1	4	2	3	0
11	6	1	3	0	1	3	4	1	2	0	0	0
12	6a	1	2	0	1	0	1	2	1	0	1	1

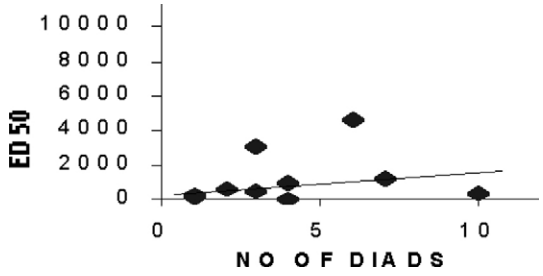


Fig. 5. Plot of cytotoxic activity vs. number of O–O diads.

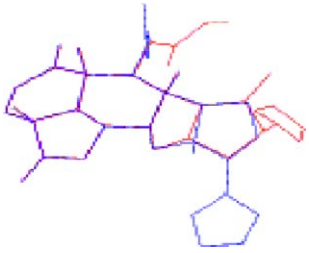


Fig. 6. Superimposed diagrams of cristal structures of nimbolide **1** and isonimbolide **1a** drawn using X-ray crystallographic structures on an OCTANE SILICON Graphics Work station.

substitutions at C-1 and C-3 positions (salannin and 2′,3′-dehydrosalannol) while those of the F-ring are a lactone in nimbolide, an ether in salannin and 2′,3′-dehydrosalannol. Since observed change in activity does not correlate with a single substitution, a combination of pharmacophores is responsible in driving the activity, wherein a suitable orientation of the furan ring aids in enhancing the bioefficacy further (Fig. 6).

4. Conclusion

An examination of the cytotoxic activity of the novel C-seco isomers synthesised from natural molecules determined through BSLB method provides a structure activity correlate, which may be summarised as follows. The novel isomers prove to be more potent than the naturally available limonoids. Theoretical hydrophobicity constant showed a positive correlation while the change in the chromatographic hydrophobicity constant for the substrates and the products did not follow any pattern. While the position of the oxygen is important rather than the oxidation state, hydrogen bonding may play a vital role in the bioactivity as is evinced from the trend exhibited by the active compounds in the O–O diad ranges between 4.5 and 5.5 Å which emphasises the significance of the orientation of the furan ring in enhancing the activity. Thus, in future such molecules with rearranged skeleton in the C- and the D-rings can be semi-synthetically made to generate better drugs to combat cancer and related diseases.

5. Experimental section

Reactions were performed in oven-dried glassware closed with a calcium chloride guard tube. Analytical TLC was performed on silica gel plates precoated with fluorescent indicator and visualised with ceric ammonium molybdate stain. NMR was recorded at ambient temperature with $CDCl_3$ as solvent and TMS as the internal standard. Chemical shifts are represented in ppm and coupling constants in Hz. *Artemia salina* were obtained from National Institute of Ocean Technology, Chennai, Tamil Nadu, India and the experiments were performed using sterilised sea water. HPLC analysis was performed on a Shimadzu LC-10ATVP liquid chromatograph fitted with a Luna 5 μm column and UV–VIS detector using acetonitrile/water (60:40) system as the eluent at a flow rate of 0.5 ml/min.

5.1. Isolation of tetranortriterpenoids (*1*–*6*)

Nimbolide [16], 2′,3′-dehydrosalannol [17] and 6-desacetylnimbinene [18] were isolated from neem leaves. Nimbin, salannin and 6-desacetylnimbin were isolated from the neem seed oil as reported in the literature and were confirmed by superimposable ^{1}H NMR and ^{13}C NMR with literature.

5.2. Synthesis of C-seco isomers

To a 250 ml round bottomed flask fitted with a guard tube, 0.21 mmol of substrate was added and dissolved in 100 ml of chloroform (AR) at −15° C. To this solution, 0.22 mmol (70 mg) of tetrabutylammonium bromide and 0.22 mmol (0.02 ml) of borontrifluoride etherate were added. The reaction mixture was stirred for the stipulated time. As the temperature

was allowed to attain room temperature the reaction mixture turned to pale green colour from yellow. The completion of the reaction was monitored using TLC and neutralised with solid sodium bicarbonate. The reaction mixture was filtered and concentrated under reduced pressure. Flash column chromatography of the crude product using silica gel (70–325 mesh) column packed in hexane/ethyl acetate furnished the pure product. Isonimbolide **1a** – MF $C_{27}H_{30}O_7$; MW 466; M.Pt. 228–235 °C; UV (MeOH) λ_{max} 208 nm; mass (*m*/*z*) 466 [M^+]; isonimbin **2a** – MF $C_{30}H_{36}O_9$; MW 540; M.Pt. 212–215 °C; UV (MeOH) λ_{max} 217 nm; mass (*m*/*z*) 541 [M^+ + 1]. 6-Isodesacetylnimbin **3a** – MF $C_{28}H_{34}O_8$; MW 498; M. Pt. 160–162 °C; UV (MeOH) λ_{max} 216, 237 nm; mass (*m*/*z*) 499 [M^+ + 1]. 6-Isodesacetylnimbinene **4a** – MF $C_{26}H_{32}O_6$; MW 440; M.Pt. 74–76 °C (sublimes); UV (MeOH) λ_{max} 209, 225 nm; mass (*m*/*z*) 441 [M^+]. 2′,3′-Isodehydrosalannol **5a** – MF $C_{32}H_{42}O_8$; MW 555; M.Pt. 70–75 °C; UV (MeOH) λ_{max} 216, 220, 224 nm; mass (*m*/*z*) 555 [M^+]. Isosalannin **6a** – MF $C_{34}H_{44}O_9$; MW 597; M.Pt. 75–80 °C (sublimes); UV (MeOH) λ_{max} 209 nm; mass (*m*/*z*) 597 [M^+].

5.3. Evaluation of cytotoxicity using BSLB method

5.3.1. Hatching

Dried cysts are exposed to sea water (1 g cyst per l) at 28 °C, under conditions of continuous illumination and strong aeration. The nauplii emerge approximately after 12 hours.

5.3.2. Assay

After 12 hours of hatching, the prototropic nauplii were collected using a micropipette from the lighted side and concentrated in a small beaker. To the vials used for the test were added 5 ml of sterile seawater, 10 nauplii aged 12 hours and the test compound. The mortality was determined by counting the number of dead organisms in the II/III instar when they exhibit greatest sensitivity to the test compounds. To determine whether the organisms died out of starvation, a control was maintained. However a hatched brine shrimp nauplii can survive for up to 48 hours without food.

Acknowledgements

One of the authors A.S.K. wishes to thank Council of Scientific and Industrial Research, New Delhi for the award of Senior Research Fellowship.

References

[1] T.R. Govindachari, Curr. Sci. 63 (1992) 117.
[2] T.R. Govindachari, N.S. Narasimhan, G. Suresh, P.D. Partho, G. Gopalakrishnan, J. Chem. Ecol. 22 (1996) 8.
[3] a, J.P. Andrew, J. Shaun, E.D. Morgan, M.S.J. Simmonds, B.M. Walley, J. Chem. Ecol. 23 (1997) 2841. b. S.R. Rojatkar, D.D. Sawaikar, B.R. Patewad, S. Gurunath, P.P. Pujar, B.A. Nagasampagi, Nat. Prod. Lett. 11 (1998) 181. c. E.D. Morgan, A.P. Jarvis, G.R. Jones, Arkivoc. 1 (2000) 312. d. G. Geetha, N.D. Pradeep, V. Kasinath, M.S.R. Sivaramakrishanan, R. Malathi, S.S.R. Rajan, Tet. Lett. 42 (2001) 6577.
[4] K. Anand Solomon, R. Malathy, S.S. Rajan, G. Anitha, J. Josepha Lourdu Raj, S. Narasimhan, G. Suresh, G. Gopalakrishnan, Acta Crystallogr. C61 (2005) 70.
[5] A.S. Michael, C.G. Thompson, H. Abramovitz, Science 123 (1956) 464.
[6] J.L. McLaughlin, C.J. Chang, D.L. Smith, Studies in Nat, Prod. Chem. 9 (1991) 388.
[7] A.K. Mandal, P.Y. Shrotri, A.D. Ghogare, Synthesis (Mass.) (1986) 221.
[8] J.L. Carbello, Z.L. Hernandez, P. Perez, M.D. Garcia, BMC Biotechnolgy 2 (2002) 17.
[9] D.J. Finney, Probit Analysis, III Ed, University Press Cambridge, UK, 1971.
[10] E. Cohen, C.E. Quistad, G.B. Casida, Life Sci. 58 (1996) 1075.
[11] J.M. Luco, E.M. Sosa, J.C. Cesco, E.C. Tonn, O.S. Giordano, Pestic. Sci. 41 (1994) 1.
[12] A.J. Leo, C. Hansch, P.G. Sammes, J.B. Taylor, The rational design mechanistic study and therapeutic application of chemical compounds, in: Comprehensive Medicinal Chemistry: 4, Pergamon Press, Oxford Press, England, 1990, p. 295.
[13] R. Lipinski, Adv. Drug Del. Rev. 23 (1997) 3.
[14] M.S. Das, G.F.D. Dasliva, O.R. Gottlief, D.L. Dreyer, Biochem. Syst. Ecol. 12 (1984) 299.
[15] a, R. Malathi, Ph.D. thesis, University of Madras, 2002. b. G. Suresh, Geetha Gopalakrishnan, S. Daniel Wesley, N.D. Pradeep Singh, R. Malathi, S.S. Rajan, J. Food and Agricultural Chemistry 50 (2002) 4484.
[16] D.E.U. Ekong, Chem. Commun. (1967) 808.
[17] H.S. Garg, D.S. Bhakuni, Phytochemistry 24 (1985) 866.
[18] W. Kraus, R. Cramer, Chem. Ber. 114 (1981) 2375.