Appendix

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Original article

Synthesis of friedelan triterpenoid analogs with DNA topoisomerase IIα inhibitory activity and their molecular docking studies

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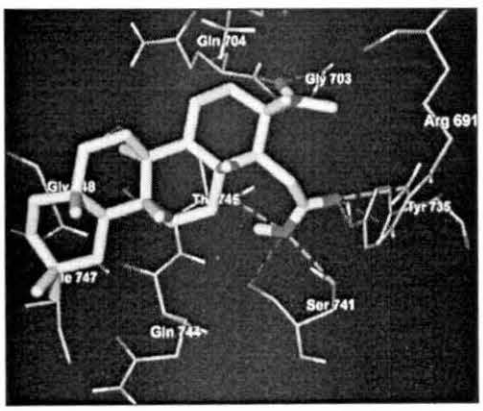
HIGHLIGHTS

- Very first report of toisomerase inhibitory activity of the friedelan derivatives.
- 3D molecular docking on topoisomerase IIα (1 bgw, PDB).
- Prediction of nature of interactions of the drug molecules with enzyme.
- Helpful for drug designing and mechanism of drug resistance phenomena.
- Prediction of dose dependent inhibition activity against topoisomerase IIα.

GRAPHICAL ABSTRACT



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ABSTRACT

Five highly oxygenated friedelan derivatives (**3a**, **3b**, **4**, **5a** and **5b**) were synthesized. The structures of these compounds were established on the basis of spectral (IR, 1D and 2D NMR, MS *etc.*) and chemical data. The molecules, including the parent compounds were screened for three-dimensional (3D) molecular docking on the crystal structure of topoisomerase IIα (1 bgw for topoisomerase IIα, PDB). Compounds **3a** and **5a** showed a dose dependent inhibition of catalytic activity of human topoisomerase IIα.

1. Introduction

The discovery of lead for pharmaceutical investigations requires identification of new molecules that are able to interact with and modify a biological target [1,2]. Natural products represent one of the most relevant approaches to this goal. Natural products are

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produced in living organisms by the activity of biosynthetic enzymes. They are thus recognized by the enzymes at specific binding sites complementary in shape and physicochemical properties. Natural products may possess the imprint for binding to the therapeutic target proteins containing the ligand binding motif similar to the biosynthetic enzyme [1]. Therefore, it is important to identify novel compounds those are complementary to biological structure space.

Triterpenoids are a large, ubiquitous and structurally diverse group of natural products that exhibit nearly 200 diverse skeletons [8]. Most significant triterpenoids are 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles, or 6-6-6-6-6 pentacycles [3] with physiological functions allied with chemical protection of plants [4]. Triterpenes, highly oxidized at ring A have been reported to possess a wide spectrum of biological activities [5].

Topoisomerases are ubiquitous enzymes that direct and modify the topological state of DNA [6,7]. They play crucial role in several aspects of DNA metabolism like replication, transcription, recombination and chromosomal segregation at mitosis [6,7]. These enzymes act by sequential breakage and reunion of either one DNA strand (Topoisomerase I) or both DNA strands (Topoisomerase II) [6,7]. All vertebrates have two highly similar, though functionally discrete, Topo II isoforms, α and β [8]. Multiple studies have shown the Topo IIα levels increase during cell proliferation and this enzyme appears to be the isoform involved in mitosis [9,10]. The pharmacological inhibition of this enzyme has therefore been identified as target for anticancer drug development [9,10]. To date several topoisomerase inhibitors have been identified. Among them plant-derived camptothecin and podophyllotoxins/etoposides have notable therapeutic efficacy as anti-tumor drugs. Despite their numerous applications, one cannot ignore the toxicity associated to these compounds [11,12]. Therefore, the development of newer drugs with lesser side effects, more chemical stability and better efficacy is indispensable.

Pentacyclic triterpenes, such as betulinic, boswellic, ursolic, and oleanolic acids are highly abundant in many edible fruits and vegetables. They are reported to inhibit cultured human melanoma, neuroblastoma, malignant brain tumor and leukemic cells [13,14]. They inhibit topoisomerases I and IIα by contending with DNA for topoisomerase binding sites, thus preventing topoisomerase-DNA cleavable complex formation [3,13,14]. The general pentacyclic ring structure of triterpenoids has been reported to be essential for topoisomerase inhibitory activity [15]. However, the structure itself is inadequate for inhibition and the nature and arrangement of the side groups/functionality are the key factors [15].

Although, friedelan group of triterpenoids are 6-6-6-6-6 pentacycles and are wide spread in nature, surprisingly modern "lead research" on friedelan skeleton is not much prevalent. In recent times only few works on the transformative reactions on friedelin (**1**) have been reported [4,16,17]. Some recent studies have indicated the *in vitro* anti-tumor activity of some of hemisynthetic friedelin derivatives [4,16,17], but the mechanisms through which these compounds achieve this effect has not yet been elucidated. Moreover, the systematic studies on transformative reactions and biological activity of cerin (**2**) are limited.

In the present study structurally modified friedelan derivatives, highly oxidized on ring A were synthesized from friedelin (**1**) and rare cerin (**2**) and characterized by IR, 1D, 2D NMR and MS. The 3D molecular docking of these molecules on crystal structure of topoisomerase IIα (1 bgw for topoisomerases IIα, PDB) [18] was performed to evaluate the binding energies as well as their mode of interaction. Finally, the molecules were tested for their ability to inhibit the catalytic activity of topoisomerases IIα.

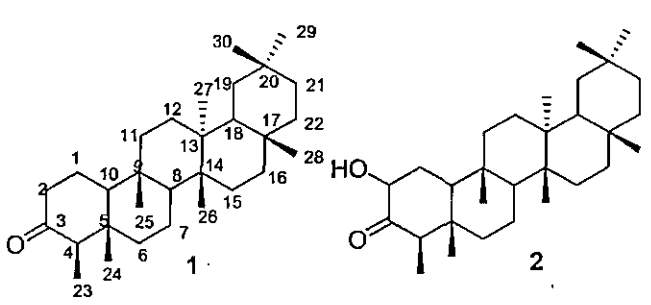

Fig. 1. Chemical structures of triterpenoids from *Quercus suber* (1) friedelin and (2) cerin.

2. Results and discussion

2.1. Chemistry

We have synthesized some hemisynthetic friedelan compounds (**3a**, **3b**, **4**, **5a** and **5b**) by simple chemical modifications of triterpenes **1** and **2** (Fig. 1) isolated from *Quercus suber* (Cork).

Two different schemes (Schemes 1 and 2) were used for their synthesis. Both these schemes are quite suitable for possible large-scale applications. The conversions mainly focused on the oxidative cleavage of the ring A of the natural triterpenoids. The oxidative cleavage of **1** and **2** produced 2,3-secofriedelan-2,3-dioic acid (**5a**) and 4-oxa-3,4-secofriedelan-3-oic acid (**3a**), respectively. A controlled lead tetra acetate (LTA) oxidation on both **3a** and **5a** at room temperature selectively produced an A-nor-lactone, **4** (Schemes 1 and 2) with 68% yield.

Friedelin, **1** and cerin, **2** were isolated from cork by using soxhlet apparatus. Cerin was obtained as slightly yellowish crystals of melting point (mp) 260–261 °C. Oxidation of **2** in glacial acetic acid in presence of anhydrous CrO_3, followed by evaporation of the solvent at reduced pressure gave a yellow gummy residue (Scheme 1). Purification of the residue over a column of silica gel gave white powdered compound, **3a** of melting point (mp) 214–215 °C, which upon methylation with diazomethane afforded the corresponding methyl ester **3b** of mp 167–168 °C. In the IR spectrum compound **3a** showed peaks at 3079, 1734 (H-bonded >C=O), 1696 (carbonyl of COOH group), 1465 (C–O), 1419, 1302, 1074 and 899 cm^{-1}. ^1H NMR spectra of compound **3a** gave signals for the presence of eight tertiary methyls at δ_H 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23). Two methylene hydrogens at C_1 appeared at δ_H 1.90 (dd, 1H, $J_{1eq10ax}$ = 4.0 Hz, J_{gem} = 15.6 Hz, H-1) and 2.35 (1H, dd, $J_{1ax10ax}$ = 6.6 Hz, J_{gem} = 15.6 Hz, H-1). C_{10} axial hydrogen atom appeared at δ_H 2.30 (dd, 1H, $J_{10ax1eq}$ = 4.0 Hz, $J_{10ax1ax}$ = 6.6 Hz, H-10) and the carboxyl hydrogen appeared as a singlet at δ_H 9.96 (s, 1H, COOH). ^{13}C spectral data are tabulated in Table 1. On the basis of the above data, structure of compound **3a** was established as 4-oxa-3,4-secofriedelan-3-oic acid. Compound **3a** on esterification with diazomethane gave the corresponding ester, **3b** with 94% yield. In its ^1H NMR spectrum it gave a sharp singlet at δ_H 3.67 (s, 3H, $-OCOCH_3$) due to the ester methyl and all other signals were in good correlation to the proposed structure of **3b**. The IR, NMR data of **3a** and **3b** were comparable to that reported in literature [19–21].

In an another attempt to synthesize friedelan derivatives highly oxidized at ring A, the oxidation of **1** was undertaken with ammonium vanadate in concentrated HNO_3-glacial acetic acid at 0 °C. Purification of the reaction mixture through column chromatography yielded a white powdered compound, **5a** (Scheme 2). Compound **5a** on esterification by diazomethane yielded the

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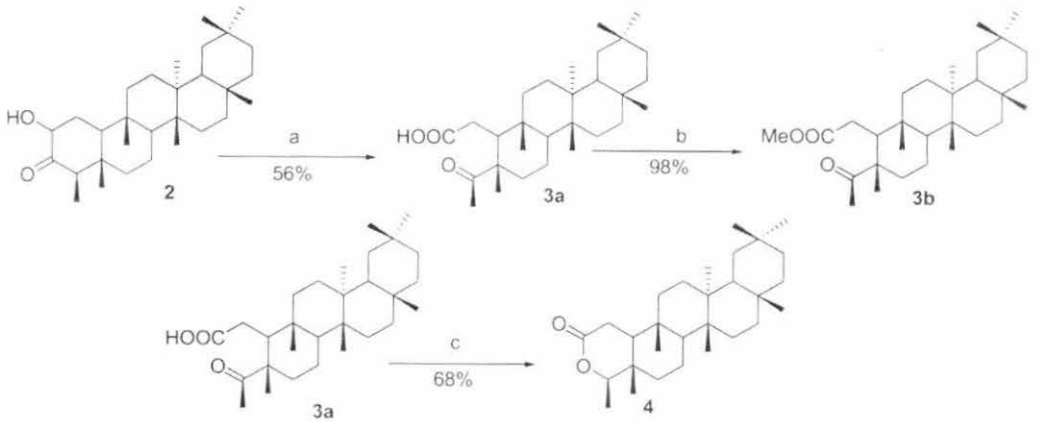


Scheme 1. Oxidative ring cleavage of compound **2**. Reagents and conditions a, Dry CrO_3, glacial AcOH, ice cold water, $CHCl_3$, anhyd. $Al_2(SO_4)_3$; b, Diethyl ether, CH_2N_2, glacial AcOH, ice cold water, $CHCl_3$, anhyd. $Al_2(SO_4)_3$; c, Lead tetra acetate, glacial AcOH, ice cold water, $CHCl_3$, anhyd. $Al_2(SO_4)_3$.

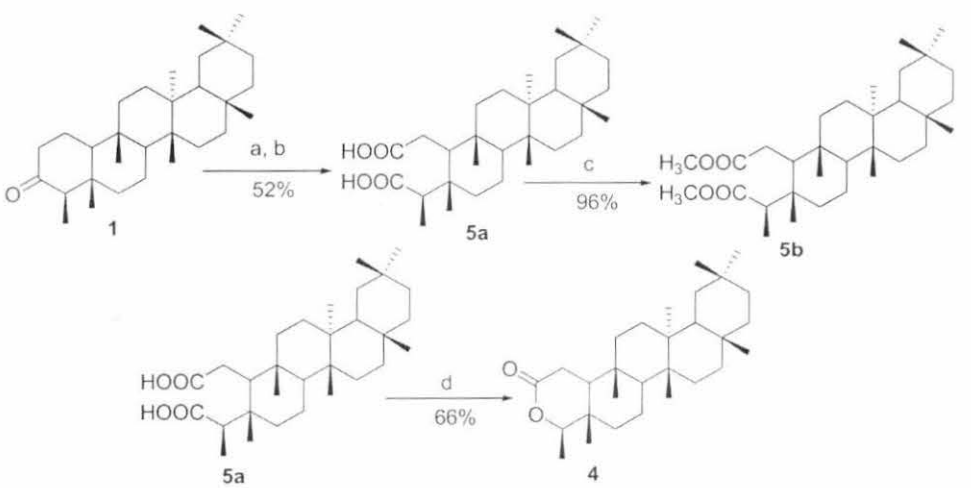
corresponding ester **5b** exclusively of mp 167–169 °C. The IR, MS and NMR (both ^1H and ^{13}C) data of **5a** and **5b** were comparable with the data reported in the literature [19–21].

Further oxidation of **3a** and **5a** separately with LTA in glacial acetic acid furnished the same compound **4**, as white powder of mp 266–267 °C. The molecular formula of the compound, as determined by ESI (*m/z* 429 M+1) and TOF (*m/z* 428 M^+) MS was $C_{29}H_{48}O_2$. This molecular formula was also corroborated by ^1H and ^{13}C NMR spectroscopic data (Table 1). The ^{13}C and DEPT spectroscopic data of **4** revealed the presence of 29 carbon signals including an ester carbonyl (δ_c 172.1), eight primary, ten secondary, four tertiary and seven quaternary carbon atoms. The six degrees of unsaturation inherent in the molecular formula of **4**, coupled with the NMR data showed the presence of one carbonyl group and five rings in the molecule **4**. These results indicated that a probable cyclization of the nor-seco acid **3a** and seco-diacid **5a** had occurred during the LTA oxidation and the nor-lactone, **4** had been generated.

In the IR spectrum, compound **4** gave peaks at 2939, 2866, 1730 (six membered lactone), 1459 (C-O), 1388 (CH-CH_3), 1241 and 1082 cm^{-1}. In its ^1H NMR spectrum it showed seven tertiary methyl signals at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) and 1.17 (s, 3H, H_3-28) (Fig. 1). Another secondary methyl group at C_4 appeared at δ_H 1.20 (d, 3H, J = 9 Hz, H_3-23). C_4-H appeared as a quartet at δ_H 4.05 (1H, q, J = 6.3 Hz). C_1-Hs are deshielded due to the magnetic anisotropy induced by the neighboring carbonyl group at C_2 and each appeared as a doublet of a doublet (dd) centered at δ_H 2.55 (1H, dd, J = 6.6 and 12.3 Hz, αH) and δ_H 2.40 (1H, dd, J = 6.6 and 12.9 Hz, βH). The slight difference in the observed 'J' value may be due to the unequal coupling of the axial and equatorial hydrogens on that carbon. H_{10} (α) appeared as a singlet at δ_H 1.56 (1H, s). All this data is in good agreement with that for friedelan skeleton.

The COSY, NOESY and HMBC spectra of **4** allowed assignment of all the proton and carbon signals. All the ^{13}C data is presented in Table 1. All the above evidence supports the final compound as a nor-lactone. The probability of formation of the other possible six membered lactone (2-oxafriedelan-3-one) was ruled out because of the greater deshielding nature of H_4 (δ_H 4.05, 1H, q, J = 6.3 Hz). Nevertheless as compound **4** had been formed from a 2,3-seco compound (either **3a** or **5a**), there remains every possibility that the stereochemistry at C_4 had been changed, which may give rise to structure II (Fig. 2). Thus, the probable structures of the compound are either I or II (Fig. 2).

The exact stereochemistry at C_4 was confirmed by 2D NMR techniques. The NOESY spectrum of compound **4** gave significant



Scheme 2. Oxidative transformation of compound **1**. Reagents and conditions: a, Glacial CH_3COOH, Vanadium pentaoxide, HNO_3 cold H_2O, Chloroform, Na_2SO_4 (Anhy.); b, H_2O_2; c, CH_2N_2, dry ether, glacial acetic acid, Na_2SO_4(Anhy.); d, LTA, glacial CH_3COOH, $CHCl_3$, Na_2SO_4 (Anhy.).

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Table 1
^{13}C NMR data of parent and different hemisynthetic friedelan derivatives.

Position	δ_C				
	1	3a	4	1[a]	3a[a]
1	22.3	32.9	34.9	22.3	32.9
2	41.5	176.2	172.0	41.5	178.2
3	213.2			213.3	
4	58.3	193.3	87.6	58.2	233.9
5	42.2	54.7	53.9	42.1	53.4
6	41.3	37.2	39.2	41.3	37.5
7	18.2	18.2	18.6	18.2	17.5
8	53.1	51.5	52.8	53.1	52.5
9	37.5	38.2	37.2	37.4	38.2
10	59.5	50.0	76.6	59.4	49.8
11	35.7	34.3	35.3	35.6	34.4
12	30.5	29.5	30.0	30.5	29.9
13	39.7	40.5	39.7	39.7	39.6
14	38.3	38.6	36.1	38.3	38.3
15	32.5	32.6	32.2	32.4	32.3
16	36.0	35.4	35.8	36.0	35.9
17	30.0	29.7	29.9	30.0	29.9
18	42.8	50.1	42.7	42.7	48.8
19	35.4	36.8	35.0	35.3	35.8
20	28.2	28.0	28.1	28.1	28.1
21	32.8	31.9	32.7	32.7	32.8
22	39.3	38.9	38.1	39.2	39.2
23	6.8	25.3	16.6	6.8	25.3
24	14.7	26.8	12.4	14.6	17.6
25	18.0	18.7	17.5	17.9	17.8
26	20.2	16.1	20.7	20.2	20.2
27	18.7	20.9	16.6	18.6	18.7
28	32.1	33.9	32.1	32.1	32.2
29	35.0	34.7	34.1	35.0	34.9
30	31.8	31.7	31.8	31.7	31.8

[a] Reported data in literature.

information to this end. All NOE cross peaks have opposite phase to the diagonal, indicating that these arose from positive NOE enhancement as anticipated for a molecule of the size (having M^+ 428) under ambient conditions. In the NOESY spectrum, H_4 at δ_H 4.05 (1H, q, $J = 6.3$ Hz) showed two important correlations between H_{10} at δ_H 1.56 (1H, s) and H_6 (α) δ_H 1.09 (1H, m). In addition, cross peaks were observed by the NOE effects due to H_{10} at δ_H 1.56 (1H, s) with H_6 (α) δ_H 1.09 (1H, m). The above data established the stereochemistry of C_4-H as alpha and hence structure I (Fig. 2) is the exact structure of **4**. Thus compound **4** is 3-oxafriedelan-2-one.

2.2. 3D molecular docking studies of friedelan analogs

To determine whether the friedelan analogs have potential as topoisomerase inhibitor, the parent compounds (**1** and **2**) and their hemisynthetic derivatives (**3a**, **4** and **5a**) were docked into the central catalytic domain of the enzyme (1 bgw PDB for topoisomerase IIα) by using AutoDock 4.

A previously modeled lupane bound structure was used as a starting point for calculating the lowest energy conformation of the bound ligands. The amino acids ARG 1016, HIS 1012, TYR 805, LYS 812 and LYS 713, have been shown to be present in the triterpenoids (lupane skeleton) binding domain of Topo IIα [22]. An initial docking was therefore performed considering these amino acid residues as flexible for binding of the present triterpenoid derivatives. Although, the calculated binding energies are negative for all friedelan derivatives (ligands) against the reported residues, the lowest value is obtained for LYS 713 (feasible binding, −9.46 kcal/mol for **1**, −8.54 kcal/mol for **2**, −8.13 kcal/mol for **3a** and −8.81 kcal/mol for **4**). However, in docked complexes LYS 713 is not within 3.5 Å from the ligand. Hence, despite good binding energy values, there remain scientific limitations to consider any type of noncovalent interactions (H-bonding or electrostatic interaction) between the ligands and the flexible residues. These observations preclude a similar/common binding for lupane and friedelan derivatives in Topoisomerase IIα (1 bgw, PDB).

To obtain a better understanding of the binding site, amino acids neighbors within 3.5 Å from the ligand were considered for final docking studies. Interestingly, the calculated binding energies of the ligands to the residues that are well within 3.5 Å are acceptable values in every case.

The results of molecular docking of hemisynthetic friedelan analogs on Topo IIα are overlaid in Fig. 3. The compound **1** binds to the hydrophilic region of the enzyme involving ASN 765, ASN 752, GLY 766, TYR 760, ARG 1172, indicating the bulk of intermolecular interactions as non-ionic. In addition, the carbonyl group in ring A of the compound binds via a hydrogen bond to amino group of LEU 791. It also forms a water mediated hydrogen bond with ASN 769 (Fig. 3a). Compound **2** differs from compound **1**, in having an additional beta hydroxyl group at position C-2. The presence of the beta hydroxyl group modifies the binding properties of the molecule to the DNA-binding domain of the Topo II enzyme. Most of the interactions between compound **2** and Topo II are essentially hydrophobic and the molecule interacts with aliphatic side chains of Topo II, including ALA 778, ALA 781, ALA 723, GLY 633 and ALA743 (Fig. 3b). Presence of the carbonyl group and non bonding electron pair in oxygen atom of the lactone ring impart some polar properties to the compound **4**. From the results in Fig. 3d, it is evident that lactone ring in the molecule interacts with a polar region of the enzyme containing ASP 513, GLU 512, TYR 511, ARG 508. Moreover, carbonyl group in lactone ring also forms hydrogen bond with hydroxyl group of TYR 511 and side chain amino group of ARG 508. On the other hand, rest of the nonpolar part of compound **4** binds to an alanine rich hydrophobic pocket of Topo II consisting

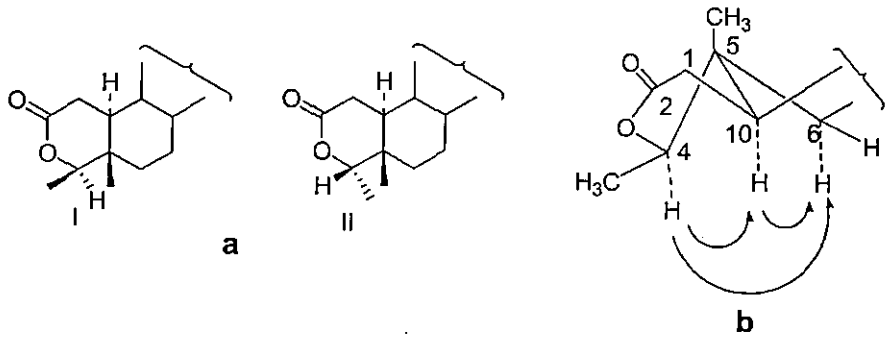

Fig. 2. (a) Partial structures of the two possible lactones; (b) Key NOESY correlation of compound **4**.

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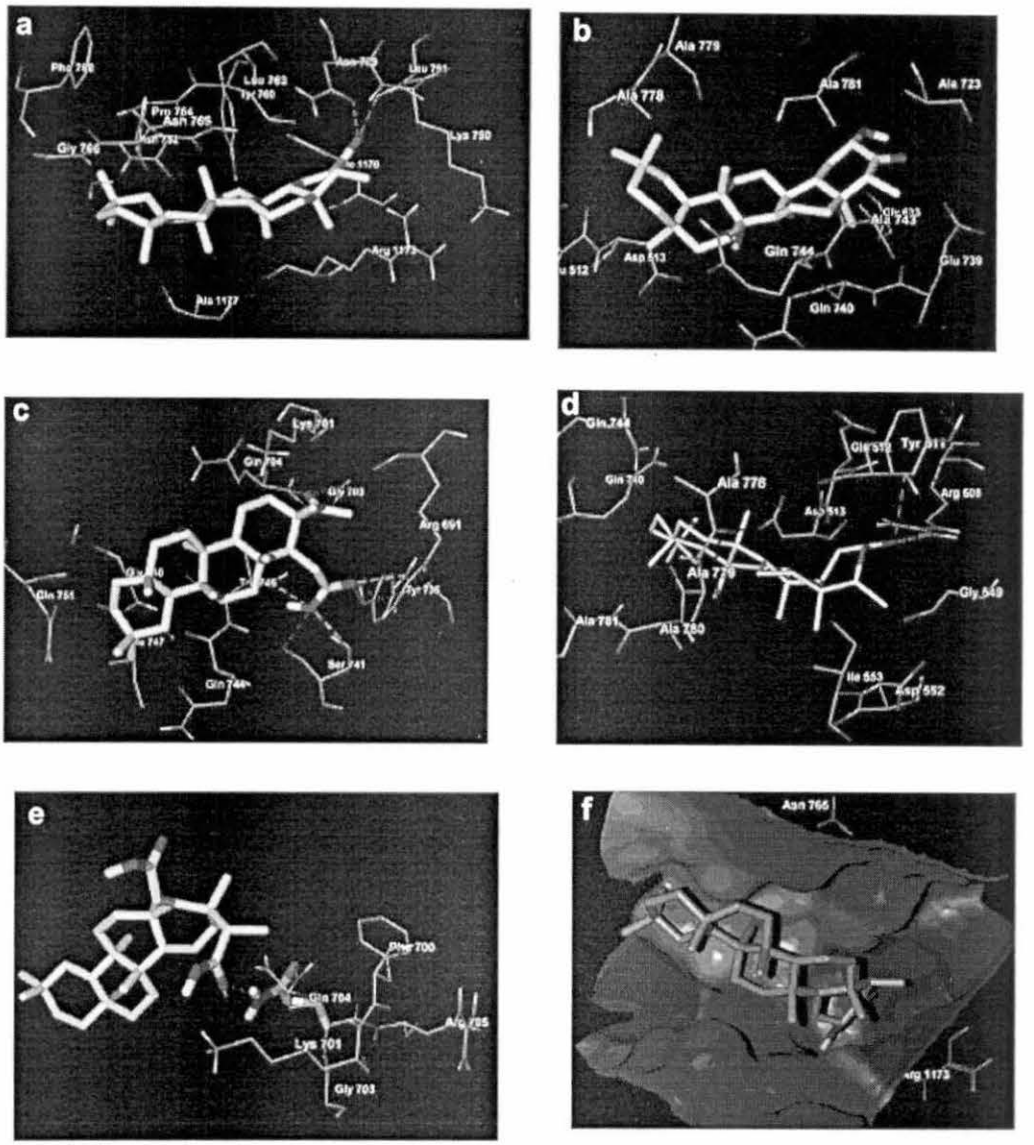

Fig. 3. Detailed docked view of different compounds; (a) for compound **1**, (b) for compound **2**, (c) for compound **3a**, (d) for compound **4**, (e) for compound **5a** and (f) elctrostatic interaction view for compound **2**.

of aliphatic side chains of ALA 778, ALA 779, ALA 780 and ALA 781, and also to ILE 553.

Compounds **3a** and **5a** both are secofriedelan derivatives and they have certain structural similarities. Their skeletons contain carboxylic acids, which on deprotonation will generate the carboxylate ion. Hence, it can be predicted that both **3a** and **5a** will bind at similar sites in the structural space of the enzyme and the binding site should have a condensed positive charge for the best fitting of the ligands. As shown in Fig. 3c and e, compounds **3a** and **5a** bind to a condensed positively charged polar site of the enzyme that permits attractive electrostatic interactions with the neighboring residues. In addition, GLY 703, GLN 704 and LYS 701 residues are in common in their binding domain, thus confirming the prediction. The compound **3a** also showed three H-bond interactions with the neighboring THR 745, SER 741 and TYR 735 residues.

From the present docking studies it can be inferred that some subtle change in molecular structure of the drug molecule alters the ligand binding domain in the drug target. These phenomena is very interesting as well as desirable for drug designing because repeated application of same drug/compound leads to the development of resistance to the action of the drug, due to unavoidable conformational modifications in the drug target. A suitable change in structure or more precisely specific modification in the structural arrangement/connectivity of the drug molecule may enable it to bind with some other nearby residues within the radius of binding domain. As a result, the newer molecule may again act as a good inhibitor to the host molecule and subsequently can show its drug efficacy.

2.3. Topoisomerase inhibitory activity of friedelan derivatives

Inhibition of catalytic activity of topoisomerase constitutes a useful strategy for the identification of potential anti-tumor agents. Topo IIα creates transient breaks in supercoiled DNA resulting in DNA relaxation. The relaxed DNA can be distinguished

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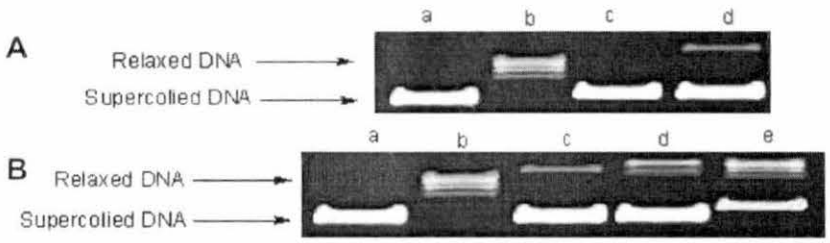

Fig. 4. Effect of compound **3a** (**A**) and **5a** (**B**) on DNA relaxation activity by DNA topoisomerase IIα(**A**), Lane a: supercoiled DNA alone; Lane b: control no drug (2 unit of topo II); Lanes c and d: 100 and 50 μM of **3a** respectively. (**B**) Lane a; supercoiled DNA alone; Lane b: control no drug (2 unit of topo II); Lanes c, d and e: 100, 50 and 25 μM of **5a** respectively.

from supercoiled DNA by gel electrophoresis analysis. The results of molecular docking studies on interaction of hemisynthetic friedelan derivatives with DNA-binding domain of human Topo IIα, was further confirmed by examining DNA relaxation activity of Topo IIα. The ATP dependent relaxation of supercoiled DNA by the enzyme was monitored in absence or presence of hemisynthetic friedelan derivatives **3a** and **5a**. From the results in Fig. 4, it is evident that the inhibitory effects of compounds **3a** and **5a** were dose dependent. They showed complete inhibition of the catalytic activity of Topo IIα at 100 and 50 μM concentrations, whereas partial inhibition of the activity was observed at a concentration of 25 μM. Compounds **3b** and **5b** couldn't be studied due to their partial solubility in DMSO.

3. Conclusion

We have successfully synthesized some oxygenated friedelan derivatives. All the molecules were characterized by spectral data and by comparison to that reported in literature. 3D molecular docking of these derivatives in the central catalytic domain of topoisomerase IIα (1 bgw PDB for topoisomerase IIα) revealed the nature of the binding and the type of interactions between the synthesized compounds and the enzyme. The topoisomerase IIα inhibitory activity was further confirmed by *in vitro* experiments. This is the first report of the antitopoisomerase activities of friedelan derivatives. We do believe our findings will definitely enrich the modern drug designing toward the invention of newer plant based chemotherapeutics to fight against human ailments. In addition the findings may provide a better understanding of the structure activity relationship toward the topoisomerase inhibitory activity of the pentacyclic triterpenoids, whether the carboxylation at ring A is truly the key factor of the defined activity or not.

4. Experimentals

4.1. Material and method

The bark of *Q. suber* (Cork) was collected from commercial source. All the chemicals used were of commercial grade and were purified prior to their use.

Melting points were recorded in open capillary method and are uncorrected. IR spectra were recorded in Shimadzu 800 FT-IR spectrophotometer using both KBr disc as well as nujol. NMR spectra were recorded in Bruker-Avance 300 MHz FT-NMR instrument at ambient temperature with a 5 mm BBO probe. The NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of $CDCl_3$ solvent as the standards. ^1H spectra were recorded in 300 MHz frequencies and ^{13}C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. The mass spectra were taken in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer.

4.2. Extraction and isolation of friedelin (*1*) and cerin (*2*)

Mixture of friedelin and cerin was extracted by petroleum ether (60–80 °C) in a soxhlet apparatus. From the mixture cerin was isolated as a chloroform insoluble part that was then recrystallized from hot chloroform. Friedelin was collected from the chloroform solution, dried and purified by column chromatography.

4.3. Oxidation of *2* with CrO_3 in glacial acetic acid

Cerin, 2 (750 mg, 1.7 mmol) was dissolved in 5 mL of glacial acetic acid in a 50 mL round bottom flask. In another 50 mL round bottom flask 168.3 mg (1.7 mmol) of CrO_3 was dissolved. Both the solutions were made homogeneous and the first solution was added slowly to the second solution with shaking. Shaking was continued for another 2 h. The resulting solution was then poured into ice cold water and worked up with chloroform. It was then purified over a column of silica gel.

4-oxa-3,4-secofriedelan-3-oic acid (**3a**), mp 214–215 °C, IR at 3079, 1734 (H-bonded >C=O), 1696 (carbonyl of COOH group), 1465 (C-O), 1419, 1302, 1074 and 899 cm^{-1}, ^1H NMR eight tertiary methyls at δ_H 0.90 (s, 3H, Me-25), 0.94 (s, 3H, Me-30), 0.99 (s, 3H, Me-26), 1.01 (s, 3H, Me-27), 1.05 (s, 3H, Me-29), 1.13 (s, 3H, Me-24), 1.18 (s, 3H, Me-28) and 2.20 (s, 3H, Me-23), 1.90 (dd, 1H, $J_{1eq10ax} = 4.0$ Hz, $J_{gem} = 15.6$ Hz, H-1), 2.35 (1H, dd, $J_{1ax10ax} = 6.6$ Hz, $J_{gem} = 15.6$ Hz, H-1), 2.30 (dd, 1H, $J_{10ax}1_{eq} = 4.0$ Hz, $J_{10ax1ax} = 6.6$ Hz, H-10) and at δ_H 2.32 (s, 1H, COOH).

4.4. Esterification of *3a* to prepare *3b*

Compound **3a** (500 mg, 1.05 mmol) of was dissolved in diethyl ether (50 mL) at cold (0–5 °C) in a 250 mL conical flask. To this an excess of diazomethane dissolved in cold ether (0–5 °C) was added slowly with constant shaking. The whole reaction sequence was carried out in a fume cupboard. The resultant yellowish solution was kept overnight in dark. To this 2 mL of glacial acetic acid was added to neutralize the traces of diazomethane. The solution was then diluted with cold water and extracted with ether. Evaporation of solvent in vacuum gave a gummy residue that was purified over a column of silica gel (60–120 mesh). 4-oxa-3,4-secofriedelan-3-methanoate (**3b**), mp 167–168 °C, IR at 3079, 1734, 1465 (C-O), 1419, 1302, 1248, 1074 and 899 cm^{-1}. All ^1H NMR peaks are similar to those of **3a**, except 3.66 (s, 3H, $-OCOCH_3$).

4.5. Oxidation of friedelin (*1*) with ammonium vanadate in glacial acetic acid

To the stirred mixture of ammonium vanadate (32 mg, 0.27 mmol) and concentrated HNO_3 (10 mL) maintained at 0 °C was added slowly a solution of **1** (1 g, 2.34 mmol) in glacial acetic acid (15 mL) during the course of 15 min. The reaction mixture was stirred for an additional 1 h at 0 °C and then poured in ice cold

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water whereby a white solid separated out. The solid was extracted out with ether and the ether solution was washed with water and a solution of 10% aqueous NaOH solution (3 × 100 mL) and with water till neutral and dried. The total aqueous layer was acidified with dilute HCl (6 N) and the white precipitated so formed was extracted with chloroform, washed with water till neutral and dried (anhydrous Na_2SO_4) and purified over a column of silica gel (60–120 mesh).

4.6. LTA oxidation of 3a/5a

A mixture of compound **3a** (400 mg, 0.84 mmol), LTA (744.8 mg, 1.6 mmol) and glacial acetic acid (10 mL) was heated with stirring under nitrogen atmosphere for 4 h. Ethane diol (15 mL) was added to neutralize excess LTA and then the mixture was poured into ice cold water. The aqueous portion was extracted with ether, washed with 10% sodium bicarbonate solution again washed with water till neutral and then dried over anhydrous sodium sulfate and purified by column chromatography.

3-oxafriedelan-2-one (**4**), mp 266–267 °C, molecular formula $C_{29}H_{48}O_2$ from ESI (*m/z* 429 M+1), TOF (*m/z* 428 M^+), IR at 2939, 2866, 1730 (six membered lactone), 1459 (C-O), 1388 (CH-CH_3), 1241 and 1082 cm^{-1}, ^1H NMR at δ_H 0.84 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H) and 1.17 (s, 3H, H_3-28), 1.20 (d, 3H, $J = 9$ Hz, H_3-23), 4.05 (1H, q, $J = 6.3$ Hz).

4.7. 3D molecular docking

Three-dimensional molecular docking studies were carried out with AutoDock 4. Structural drawing, 3D structure optimization and energy minization were done by ACD Labs 12.0 and Arguslab respectively. Initially we selected the ligand and by neighbor selection through Arguslab we located amino acid residues within 3.5 Å surrounding the ligand. Molecular viewing was performed by Molegro molecular viewer as well as in AutoDock 4.

4.8. Topoisomerase II assay

Human TOPO-IIα activity was measured by measuring the relaxation of supercoiled pBR 322 plasmid DNA. Reaction mixture contained 10 mM Tris (pH-6.9), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, pBR 322 plasmid DNA (100 ng) and 2 Units of Topo IIα (USB, USA), in a final volume of 20 μL. For inhibition studies, the compounds were preincubated with human TOPO-IIα and DNA for 15 min. Compounds were used at the appropriate concentrations by dissolving in 2% (v/v) DMSO. DMSO didn't show detrimental effect on the enzyme activity at concentration up to 2% v/v. Reaction mixture was incubated at 37 °C for 30 min and stopped by addition of 2 mL of 7 mM EDTA. Reaction product was mixed with DNA loading dye and electrophoresed on 1% TAE-Agarose. The gel was stained with ethidium bromide (0.5 μg/ml) for 20 min, destained twice in TAE buffer and then visualized using a Gel Doc-Imaging system (Spectronics, USA).

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.04.037.

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Synthesis of bioactive 28-hydroxy-3-oxolup-20(29)-en-30-al with antileukemic activity

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An easy and efficient route to partial synthesis of bioactive 28-hydroxy-3-oxolup-20(29)-en-30-al (**1**), starting from betulinic acid (**2**), has been developed (eight steps, 44% overall yield). Structures of all the compounds were determined by spectral studies (IR, ^{1}H, ^{13}C NMR, MS, NOESY, COSY, etc.). Compound **1** and the precursors (**2**, **3**, **5**, and **7**) showed antiproliferative activities against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor.

Keywords: triterpenoid; partial synthesis; betulinic acid; antileukemic activity

1. Introduction

Triterpenes represent a varied and important class of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclasses which have shown to possess several medicinal properties [1,2]. The antitumor properties of plant extracts comprising lupane-derived triterpenoids have been demonstrated during the past 25 years for their cytostatic activity on various *in vitro* and *in vivo* cancer model systems [3]. Betulinic acid, one of the lupane-derived triterpenoids, exerts a selective antitumor activity on cultured human melanoma [3], neuroblastoma [4,5], malignant brain tumor [6], and leukemia cells [7]. Other pharmacological activities of lupane-type triterpenoids include anti-inflammatory activity [8], anti-carcinogenic activity [3], photosynthetic inhibitors [9], anti-HIV [10], antidiabetic [11] and so on. Therefore, it is important to supply those triterpenoids with novel structures in sufficient amounts for further biological testing. And, in this regard, synthesis rather than isolation from natural sources is more efficient and also economical.

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of white blood cells. It is a broad term covering a spectrum of diseases. In turn, it is part of the even broader group of diseases called hematological neoplasms. In 2000, approximately 256,000 children and adults around the world developed some form of leukemia, and 209,000 died from it [12]. About 90% of all leukemias are diagnosed in adults. Most forms of leukemia are treated with pharmaceutical medications, typically combined into a multi-drug chemotherapy

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regimen. Some are also treated with radiation therapy. In some cases, a bone marrow transplant is useful. All these treatments are useful but only to a very limited extent, and the chemotherapeutics used to date are not specific to the affected cells, thus causing severe damage to the body. Despite recent improvements in the treatment of early-stage disease, leukemia blast crisis remains a therapeutic challenge because it is highly refractory to standard induction chemotherapy, with a response rate in myeloid blast crisis of less than 30% [13]. Therefore, development of mild selective chemotherapeutics is a real demand in contemporary medical sciences.

Medicinal plants and their phytoconstituents have always been a better choice for leukemia and nutraceuticals have been proven to have antileukemic activity in experimental studies [14]. Derivatives of betulin, basically triterpenoids of lupane skeleton, are reported to possess significant cytotoxicity against a wide variety of cancer cell lines [15]. In a recent publication, one such naturally occurring compound, 28-hydroxy-3-oxolup-20(29)-en-30-al (**1**), which has been reported [16] for the first time from the bark of *Acacia mellifera*, showed significant cytotoxicity against NSCLC-N6 cell line. Subsequently, Chen et al. [17] reported the presence of **1** in the methanol extract of *Microtropis fokienensis*, which has been reported [17] to induce apoptosis of human leukemia HL60 cells and mediate cleavage of PARP and upregulation of Bax proteins. Compound **1** (28-hydroxy-3-oxolup-20(29)-en-30-al) was among the most cytotoxic substances obtained [22]. They also investigated the potential effects of **1** on growth inhibition of HL60 cells [17]. According to their results [17], this compound induced apoptosis in a dose-dependent manner. The molecular mechanism of compound **1** toward cancer cells is still a subject of continuous investigation and a specific target(s) has yet to be identified. Therefore, suitable derivatives of lupane may be considered as a group of compounds having promising bioactivity which can be used for further chemical as well as biological research. Henceforth, the present demands of this type of rare (scarce) naturally occurring triterpenoids are enormous in the contemporary medicinal research. Thus, it was felt necessary to supply this novel triterpenoid **1** in sufficient amounts for further biological testing. And, in this regard, synthesis rather than isolation from natural sources is more efficient and economical.

In view of the above and in continuation of our studies toward the chemical transformations of pentacyclic triterpenoids [18], we report herein a multistep protocol for the synthesis of 28-hydroxy-3-oxolup-20(29)-en-30-al (**1**) from betulinic acid (**2**; Figure 1). *In vitro*, anticancer activities of all the compounds having C-30 —CHO group (**1**, **2**, **3**, **5**, and **7**) are

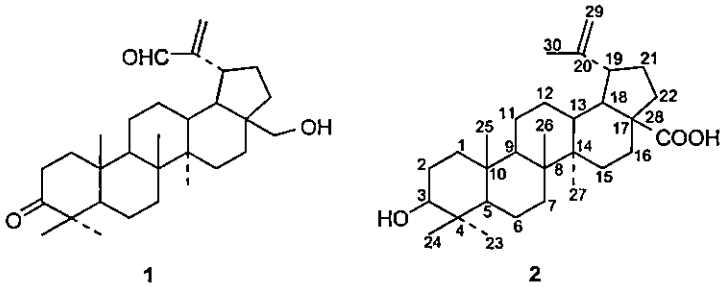


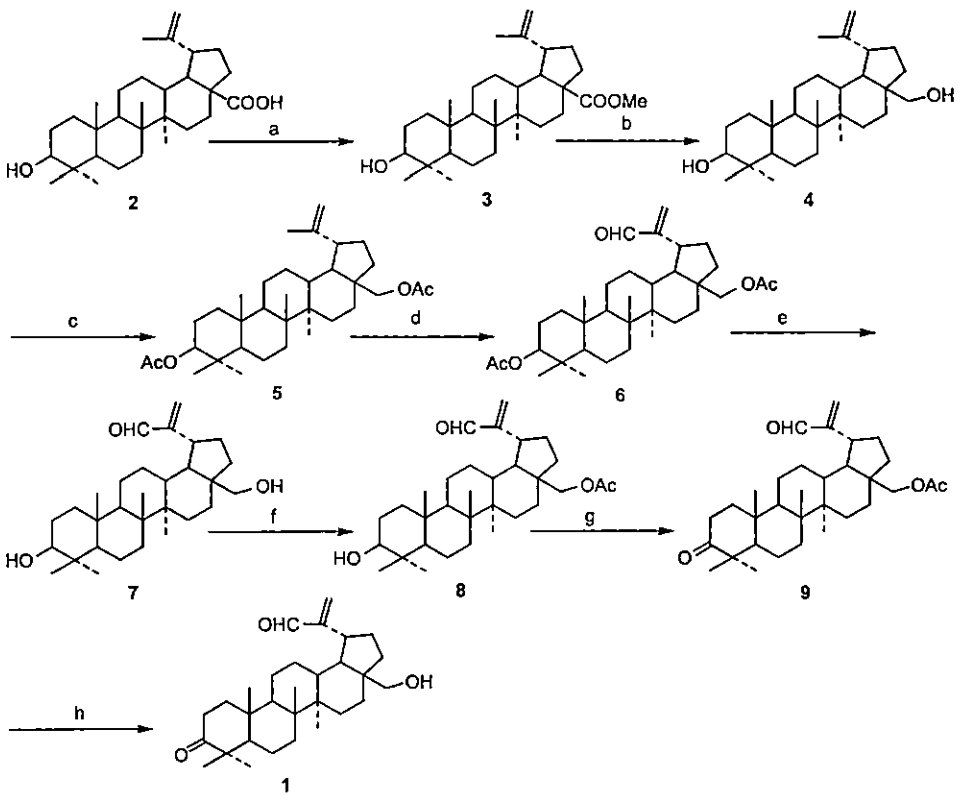
Figure 1. Structures of compounds 1 and 2.

also tested against different cell lines. Derivatization of C-30 methyl group was accomplished in good yield by SeO_2 oxidation in refluxing aqueous dioxan. This is the first report of synthesis of compound **1** from **2** and its potent anticancer activity against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor cell lines.

2. Results and discussion

2.1 Chemical part

The sequential steps involved in the synthesis of compound **1** are illustrated in Scheme 1. Betulinic acid (**2**), isolated from *Bischofia javanica*, was esterified (diazomethane) at C-28 to form 28-carbomethoxy-lup-20(29)-en-3β-ol (**3**) almost quantitatively. Lithium aluminum hydride reduction of **3** in anhydrous THF gave betulin (**4**) in 72% yield. Allylic oxidation of the C-30 methyl was then carried out with SeO_2 in aqueous dioxan under refluxing condition after protecting C-3 and C-28 hydroxyl groups as acetate. The incorporation of formyl group was assigned by IR and NMR (both ^1H and ^{13}C) spectra of compound **6**. The IR spectrum showed peaks at 1732 ($-OCOCH_3$), 1691 (conjugated aldehyde), 1459 and 1369 (gem dimethyl), and 1244, 1028, 978, 936, and 889 ($=CH_2$) cm^{-1}. The molecular formula of



Scheme 1. Partial synthesis of compound **1** from betulinic acid (**2**). Reagents and conditions: (a) CH_2N_2, ether, over night, AcOH (gal.), Na_2SO_4; (b) $LiAlH_4$, dry THF, 2 h, saturated Na_2SO_4 solution, ether, Na_2SO_4; (c) C_5H_5N, Ac_2O, 6 h (100°C) ice-cold H_2O, ether, Na_2SO_4; (d) SeO_2, aq. dioxan, 2 h, ice-cold H_2O, ether, Na_2SO_4; (e) 10% alcoholic KOH, THF, 4 h, ice-cold H_2O, ether, Na_2SO_4; (f) C_5H_5N, Ac_2O, (5–10°C), 8 h, ice-cold H_2O, Na_2SO_4; (g) C_5H_5N, dry CrO_3, overnight, ice-cold H_2O, CH_2Cl_2, $MgSO_4$; (h) 10% alcoholic KOH, THF, 4 h, ice-cold H_2O, ether, Na_2SO_4.

compound **6** was assigned as $C_{34}H_{52}O_5$ (M^+ 540.29, analytical calculation % C 75.42, % H 9.56). The ^1H NMR spectrum of compound **6**, taken in $CDCl_3$, gave a singlet at δ_H 9.56 with an integration of one proton in addition to five methyls at δ_H 0.93 (s, 3H), 0.94 (s, 3H), 1.02 (s, 3H), 1.06 (s, 3H), and 1.39 (s, 3H) for lupane skeleton. Two olefinic protons appeared at δ_H (proton chemical shift position) 6.28 (s, 1H) and at δ_H 5.93 (s, 1H) and two geminal hydrogens of C-28 each gave a doublet at δ_H 4.24 (d, 1H, $J = 10.6$ Hz) and at δ_H 3.68 (d, 1H, $J = 10.6$ Hz). Two acetyl methyls appeared as two sharp singlets at δ_H 2.07 (s, 3H) and at δ_H 2.03 (s, 3H). In the proton-decoupled ^{13}C spectrum of **6**, a singlet at δ_c 194.6 (Table 1) clearly indicated the presence of a formyl group at C-30. Careful hydrolysis of **6** gave a yellowish gummy residue after evaporation of the solvent *in vacuo*. Purification of this gummy material over a column of silica gel gave almost quantitatively a powdered white solid of compound **7** with melting point (mp) 274–276°C. The IR spectrum gave peaks at 3393 (—OH), 1688 (conjugated aldehyde), 1453, and 1375 (gem dimethyl), and 1029, 942, and 890 (=CH_2) cm^{-1}. The molecular formula of compound **7** was assigned as $C_{30}H_{48}O_3$ (M^+ 456.32, analytical calculation % C 78.66, % H 10.52). Disappearance of ^1H NMR signals at δ_H 2.03 (s, 3H) and 2.07 (s, 3H) and the presence of two singlets at δ_c 78.9 (for C-3) and 60.2 (for C-28) in the ^{13}C NMR spectrum of **7** clearly indicated the deprotection of both the hydroxyl groups at C-3 and at C-28 during the formation of **7** from diacetate (**6**).

A number of methods [19] were then applied for the selective oxidation of C-3 secondary hydroxyl group, keeping C-28 primary hydroxyl group intact. None of the existing methods [19], including the one developed by Mckillop et al. [20] in 1979, was found effective in producing the desired selectivity. Thus, once again the protection–deprotection method was applied for the formation of the desired aldehyde **8**.

A solution of **7** (350 mg, 0.76 mmol) in $CHCl_3$ (10 ml) and pyridine (15 ml) was treated with Ac_2O (10 ml, 0.098 mmol) at very low temperature (5–10°C). After the reaction was over (checked by TLC), the mixture was diluted with $CHCl_3$ (20 ml), and then the organic layer was washed with saturated aq. NaCl (10 ml × 3), dried over anhydrous sodium sulfate, and concentrated *in vacuo*. Purification of the reaction mixture gave compound **8** with mp 260–262°C. The IR spectrum gave peaks at 3461 (—OH), 1732 (—$OCOCH_3$), 1691 (>C=C—CHO), 1459, 1369 (gem dimethyl), and 1244, 1028, 978, 936, and 890 (=CH_2) cm^{-1}. The molecular formula of compound **8** was assigned as $C_{32}H_{50}O_4$ (M^+ 498.35, analytical calculation % C 77.02, % H 10.12). The ^1H NMR spectrum of **8** showed a singlet at δ_H 9.56 (s, 1H) for the aldehyde proton. The ^{13}C NMR spectrum of compound **8** (Table 1) accounted for all the carbons. Acetylation of only the C-28 —OH group was confirmed by comparison of ^1H and ^{13}C NMR data of C-3 hydroxy compound and with the acetate as reported in the literature [21]. The assigned ^{13}C NMR data of all the compounds are given in Table 1. Thus, on the basis of the above spectral data, the structure of compound **8** was assigned as 28-acetoxy-lup-20(29)-en-3β-ol-30-al.

In the subsequent step, C-3 —OH group was converted to ketone **9** (175 mg), using anhydrous CrO_3 in dry pyridine (see Experimental section) at ambient temperature. After purification it showed mp 276–278°C. The exact structure of compound **9** was elucidated by spectroscopic studies. The IR spectrum gave peaks at 1730 (>C=O) and at 1706, 1696 (CH_2 =C—CHO) cm^{-1}. The molecular formula of compound **9** was assigned as $C_{32}H_{48}O_4$ (M^+ 496.34, analytical calculation % C 77.28, % H 9.62). In its ^1H NMR spectrum, compound **9** gave a singlet at δ_H 9.56 (s, 1H) for the aldehydic proton. Two olefinic

Table 1. ^{13}C NMR data of compounds 1–9 and betulinic acid taken in $CDCl_3$.

Position	1*	1	2	3	4	5	6	7	8	9	Betulonic acid
1	39.6	39.6	38.8	38.8	38.8	38.4	39.3	39.0	39.0	39.0	38.8
2	34.1	34.1	34.0	34.3	34.2	34.1	35.0	34.3	34.3	34.3	34.0
3	218.0	218.1	78.9	79.0	78.9	80.9	81.8 (172.0[a])	78.9	78.9	218.1	218.1
4	47.4	47.3	47.3	50.5	50.5	50.2	47.6	48.0	48.0	48.0	47.3
5	55.0	54.9	54.8	55.3	55.3	55.3	56.3	55.3	55.3	55.3	54.8
6	19.6	19.6	19.5	19.5	19.3	19.1	19.1	19.3	19.3	19.3	19.5
7	33.5	33.5	33.6	32.1	32.3	34.1	35.0	33.8	33.8	33.8	33.6
8	42.7	42.3	40.6	42.3	42.4	42.6	43.5	42.6	42.6	42.6	40.6
9	49.6	49.6	49.8	49.4	49.4	48.8	48.7	48.0	48.0	48.0	49.8
10	36.9	36.8	36.8	36.9	36.9	37.0	35.5	36.9	36.9	36.9	36.8
11	21.4	21.3	21.3	20.8	20.8	21.0	21.7	20.9	20.9	20.9	21.3
12	27.6	27.1	25.4	25.5	25.4	27.0	27.9	27.6	27.6	27.6	25.4
13	37.1	38.1	38.5	38.2	38.2	37.8	37.9	37.1	37.1	37.1	38.5
14	40.8	40.5	42.4	40.6	40.6	40.8	41.7	40.8	40.8	40.8	42.4
15	26.9	26.5	29.6	29.6	29.6	27.0	28.9	26.9	26.9	26.9	29.6
16	29.1	29.5	32.0	27.9	27.9	27.9	28.4	28.1	28.1	28.1	32.0
17	48.0	47.1	56.3	46.5	47.6	47.7	47.3	47.3	47.3	47.3	56.3
18	52.3	51.4	49.1	51.2	51.3	51.2	51.0	50.2	50.2	50.2	49.1
19	36.5	37.5	46.8	46.9	46.9	37.5	38.7	38.7	38.7	38.7	46.8
20	157.0	156.2	150.2	150.6	150.7	150.2	157.1	157.1	157.1	157.1	150.2
21	32.8	31.8	30.5	30.6	30.5	34.5	31.0	31.8	31.8	31.8	30.5
22	33.9	36.7	36.8	37.2	37.1	37.0	38.1	36.4	36.4	36.4	36.8
23	26.6	26.6	26.6	27.4	27.4	27.0	24.6	26.9	26.9	26.9	26.6
24	21.1	21.0	20.9	19.3	19.3	20.8	22.0	21.4	21.4	21.4	20.9
25	15.9	15.8	15.8	15.3	15.3	16.0	17.1	15.9	15.9	15.9	15.8
26	15.8	15.7	15.9	15.9	15.9	16.1	16.9	15.3	15.3	15.3	15.9
27	14.6	14.8	14.6	14.7	14.7	14.7	15.6	14.6	14.6	14.6	14.6
28	60.2	60.2	181.6	176.6	61.3	62.8	63.4 (172.5[a])	60.2	64.2	60.2	181.6
29	133.2	133.6	109.7	109.5	109.5	109.8	133.7	133.6	133.6	133.6	109.7
30	194.9	194.6	19.3	18.3	18.3	18.1	194.6	194.6	194.6	194.6	19.3

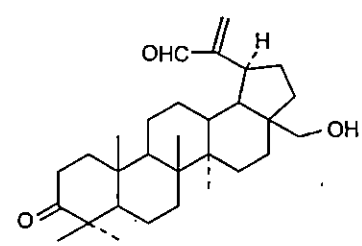
Notes: *Represents that reported in the literature; [a]represents the chemical shift position of diacetate.

protons of C-29 appeared at δ_H 6.23 (s, 1H) and 5.92 (s, 1H). For two geminal protons of $C_{28}-H_2$ group, each appeared as a doublet at δ_H 3.83 (d, 1H, $J = 10.6$ Hz) and 3.38 (d, 1H, $J = 10.6$ Hz). All the five methyls appeared between δ_H 0.75 and 0.95 along with other peaks of lupane skeleton. The ^{13}C NMR spectrum (Table 1) was also in good agreement with the proposed structure of compound **9** (Table 1). Deprotection of C-28 hydroxyl group finally afforded a white amorphous solid **X** (44% overall yield), mp 288–290°C.

The IR spectrum gave peaks at 3424 (—OH), 1725 (>C=O), 1706, 1697 (conjugated aldehyde), 1461, 1380 (gem dimethyl), and 1163, 987 cm^{-1}. The molecular formula was established as $C_{30}H_{46}O_3$ by HR-EI-MS (*m/z* 554.38 $[M]^+$, calcd. 554.34). In the ^1H NMR spectrum, five tertiary methyl groups appeared at δ_H 0.91 (s, 3H), 0.93 (s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), and 1.15 (s, 3H). Two olefinic C-29 protons appeared at δ_H 6.91 (s, 1H) and 6.28 (s, 1H). The singlet at δ_H 9.52 (s, 1H) was due to the aldehydic proton of C-30. Two geminal protons of $C_{28}-H_2$ appeared at δ_H 3.78 (d, 1H, $J = 10.6$ Hz) and 3.36 (d, 1H, $J = 10.6$ Hz). The ^{13}C spectrum revealed the presence of two olefinic carbons at δ_c 133.6 (C-29) and 156.2 (C-20). The aldehydic C-30 carbon appeared at δ_c 194.6. C-3 appeared at δ_c 218.1 and C-28 appeared at δ_c 60.2.

Thus, the presence of C-30 aldehyde and $C_{20}-C_{29}$ double bond is obvious from the above ^{13}C and ^1H NMR spectra of compound **X**. However, the shift in δ values for carbons C-12, C-13, C-17, C-18, C-19, and C-21 (Table 1) in compound X in comparison with that observed for betulinic acid [17] and lupeol [18] may be either because of the isomeric nature of the attached isopropenyl group of the cyclopentane ring at C-19 of lupane skeleton [18] or because of the introduction of C-30 —CHO group during the SeO_2 oxidation of the C-30 methyl [18]. At this juncture, it is relevant to mention that the shift in ^{13}C signals for the above-mentioned carbons started appearing in all the compounds (*viz.* compounds **6**, **7**, **8**, and **9**) once the SeO_2 oxidation step was carried out. The shifts in the NMR spectrum and possible epimerization of the side chain during the SeO_2 oxidation were already reported in the literature from our laboratory [18] and the existence of such a conformational isomer is also documented [23]. However, in the present case, we could not get the isomeric peaks for the relevant carbons in the ^{13}C NMR spectra of **1**, **6**, **7**, **8**, and **9** as observed by the previous workers [18,23]. Therefore, on the basis of the above spectral analysis, the structure of compound **X** has been assigned as 28-hydroxy-3-oxolup-20(29)-en-30-al (**1**) or **10** (Figure 2).

Further confirmation of the stereochemistry at C-19 was settled by carrying out the 2D NMR techniques on compound **6** (first compound after the SeO_2 oxidation) in which the probable stereochemical change would occur. The NOSEY spectrum of compound **6** gave significant information about the stereochemistry at C-19. All nuclear overhauser effect (NOE) cross-peaks have opposite phase to the diagonal, indicating that these arose from positive NOE enhancements as anticipated for a molecule of this size (M^+ 540.4) under ambient conditions. H-19 at δ_H 2.76 (m, 1H) showed strong correlations (Figure 3) with β-H-12 at δ_H 1.03 (m, 1H), β-H-13 at δ_H 1.66 (td, 1H, $J = 12.2$,



10

Figure 2. Epimeric form of compound 1.

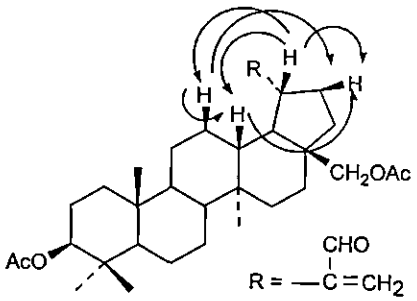


Figure 3. Key NOESY correlations of compound **6**.

3.6 Hz), and β-H-21 at δ_H 2.18 (m, 1H). The NOE observed in the NOESY spectrum of compound **6** indicated the involvement of β-H-12 at δ_H 1.03 (m, 1H) and β-H-13 at δ_H 1.66 (td, 1H, J = 12.2, 3.6 Hz) with β-H-21 at δ_H 2.18 (m, 1H). These observations may be explained by considering the H-19 configuration as β as depicted in Figure 3. Additionally, β-H-13 at δ_H 1.66 (td, 1H, J = 12.2, 3.6 Hz) showed positive NOE with β-H-12 at δ_H 1.03 (m, 1H; Figure 2). From these data, it can be concluded that the original stereochemistry at C-19 was retained during SeO_2 oxidation on **5** and the shift in chemical shift values for C-12, C-13, C-17, C-18, C-19, and C-21 in the ^{13}C NMR spectrum of subsequent oxidized products with respect to that for lupane skeleton is due to the angular dependence through space effects, such as the anisotropic magnetic susceptibility and/or electric field effect [16], offered by the conjugated carbonyl group. The through space distance is obviously more important than the number of intervening bonds since the chemical shift changes are much smaller for C-13 than for C-12, which is one bond further away but closer in space [23]. SeO_2 oxidation of compound **5** to **6** may only be considered as the step where the stereochemical change at C-19 would have occurred, since in all other subsequent steps no reaction was carried out that can alter the stereochemistry at C-19. Therefore, the stereochemistry at C-19 would be the same as **6** for all the subsequent molecules (**7**, **8**, **9**, and **1**) derived from it.

2.2 Antileukemic activity

In this study, compounds were subjected to cytotoxic assay against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor, and the assays were carried out in three independent experiments as per the guidelines of biosafety committee of West Bengal State University (Figures 4–6). To determine whether the compounds had any effect on cell lines, cell cultures were incubated with various concentrations of compounds (dissolved in 0.1% v/v DMSO). DMSO had no effect on the growth of cell lines at a final concentration of 0.1% (v/v). Compounds **1**, **2**, **3**, **5**, and **7** had differential effects on the growth of the cell lines. The effect of **4** and **6** could not be checked due to its poor solubility in DMSO.

All the compounds showed potent activities against the entire cell lines used; although the activity against murine MEL erythroid progenitor was not as good as for other two cell lines (Figure 5). However, **1** was the most cytotoxic against murine MEL erythroid progenitor. The anti-proliferative effect of all the compounds helped us to predict some of the structure–activity relationships. A comparison of bioassay data between **2** and **3** revealed that compound **2** was more active than **3** against human K562 leukemia and murine WEHI3 leukemia. The only difference between their structures was at C-28. Compound **2** had a —COOH group and **3** had a $-COOCH_3$ group, i.e. more polar grouping is necessary (—COOH) to have high activity. Incorporation of aldehyde group at C-30 had increased the activity further. The study also revealed that against human K562 leukemia and murine WEHI3 leukemia cell lines, **7** showed better

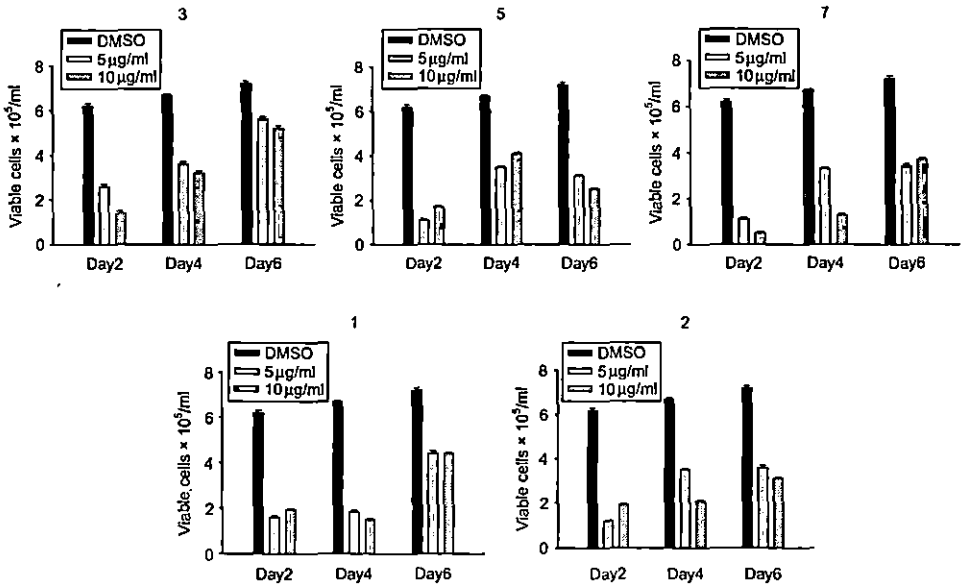


Figure 4. Anti-proliferative effect of **3**, **5**, **7**, **1**, and **2** on WEHI3 cells.

activities than **1**. A closer look at the structures of these two compounds revealed that they had a difference in their structures only at C-3 and the data indicated that —OH group at C-3 was the one which contributed to higher activity.

3. Experimental

3.1 General experimental procedures

$[\alpha]_D$ was measured in Autopol III Automatic Polarimeter. Melting points were recorded by open capillary method and are uncorrected. IR spectra were recorded in

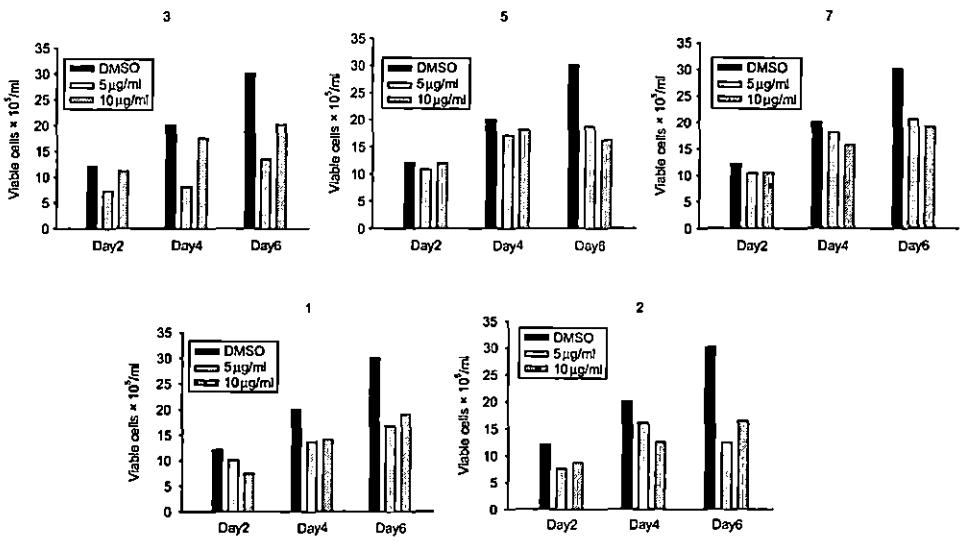


Figure 5. Anti-proliferative effect of **3**, **5**, **7**, **1**, and **2** on MEL cells.

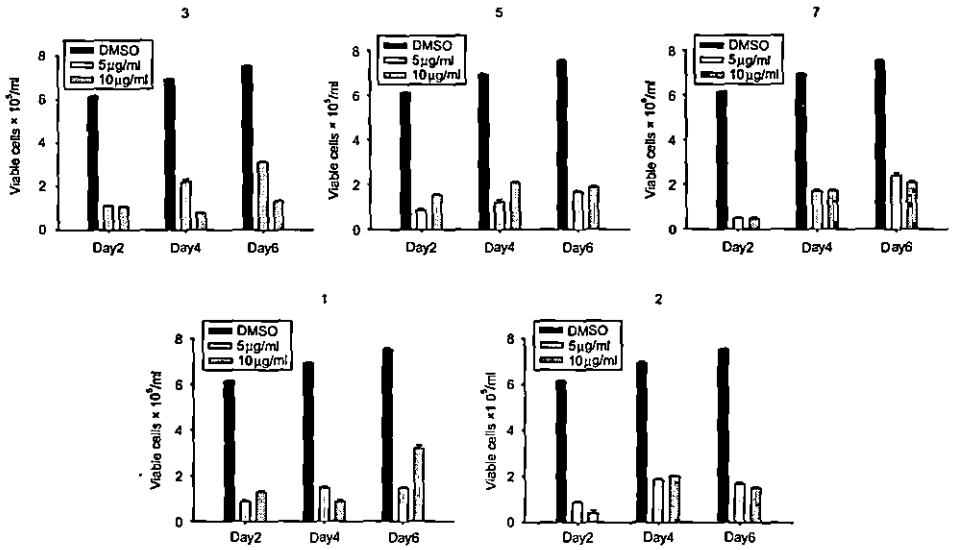


Figure 6. Anti-proliferative effect of **3**, **5**, **7**, **1**, and **2** on K562 cells.

Shimadzu 800 FT-IR spectrophotometer and NMR spectra were recorded in Bruker-Avance 300 MHz FT NMR spectrometer located in the Department of Chemistry, University of North Bengal, India – 734 013. IR spectra were recorded using both the KBr disk and nujol and the NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of $CDCl_3$ solvent as the standards. ^1H spectra were recorded at 300 MHz frequencies and ^{13}C NMR spectra were recorded at 75.4 MHz frequencies. Coupling constant '*J*' was calculated in Hz. Betulinic acid was isolated from the outer bark of *B. javanica* through soxhlet apparatus in toluene. All the chemicals used were of commercial grade and were purified prior to their use. The mass spectra were recorded in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer and elemental analyses were carried out in Vario EL-III from CDRI, Lucknow, India – 226 001.

3.2 *Methyl betulinate (3)*

Three grams (6.58 mmol) of betulinic acid were dissolved in diethyl ether (100 ml) at very low temperature (0–5°C) in a 250 ml conical flask. To this an excess of diazomethane dissolved in ether at very low temperature (0–5°C) was added slowly with constant shaking. The whole reaction sequence was carried out in a fume cupboard. The resultant yellowish solution was kept overnight under darkness. After that 2 ml of glacial acetic acid was added to this to neutralize the traces of diazomethane. The solution was then diluted with cold water and extracted with ether. Evaporation of solvent at vacuum gave a gummy residue that was purified over a column of silica gel (60–120 mesh).

Compound **3** was obtained (2.9 g, 98%) as a white solid with mp 222–224°C and $[\alpha]_D + 5.0$ ($CHCl_3$). The IR spectrum gave peaks at 3540 (—OH), at 1733 (—COOMe), and at 1660 and 890 (=CH_2) cm^{-1}. Elemental analysis: found: C, 78.79%, H, 10.52%; calcd for $C_{31}H_{50}O_3$: C, 79.10%, H, 10.71%. ^1H NMR δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.40 (s, 3H), 1.68 (s, 3H), 2.99 (m, 1H, H-C-3), 3.18 (dd, 1H, $J = 5.1$, 10.8 Hz, H-3), 3.66 (s, 3H, —OMe), 4.59 (s, 1H, H-C-29), and 4.73 (s, 1H, H-C-29). The compound was found

identical to an authentic sample (co-TLC, mixed mp).

3.3 *Lup-20(29)-en-3β,28-diol (4)*

Compound **3** (2.8 g, 5.95 mmol) was added to 50 ml of dry THF in a 150 ml round-bottomed flask. The solution was homogenized and to this LAH (75.28 mg, 1.98 mmol) was added in small lots at low temperature (10–15°C), and the solution was stirred for 15 min. Stirring was continued for another 3 h at room temperature and saturated solution of sodium sulfate was added dropwise till the excess LAH was destroyed. The reaction mixture was poured into 200 ml cold water and extracted with diethyl ether and dried over anhydrous sodium sulfate. Evaporation of solvent gave a residue that was then purified over a column of silica gel (60–120 mesh).

Compound **4** was obtained (76%) as a white solid with mp 256–257°C and $[\alpha]_D + 16$ (10% v/v MeOH in $CHCl_3$; 0.35 g/ml). The IR spectrum gave peaks at 3393 (—OH), 1453 and 1375 (gem dimethyl), and 1229, 1029, 942, and 889 (=CH_2) cm^{-1}. ^1H NMR δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.37 (s, 3H), 1.68 (s, 3H), 2.43 (m, 1H, H-C-18), 3.84 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.24 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.46 (m, 1H, H-C-3), 4.59 (s, 1H), 4.68 (s, 1H). The compound was found identical to an authentic sample (co-TLC, mixed mp, *etc.*).

3.4 *Lup-20(29)-en-3β,28-diyl acetate (5)*

Compound **4** (1.8 g, 4.06 mmol) was dissolved in 50 ml dry pyridine in a 100 ml round-bottomed flask and to this 10 ml acetic anhydride was added. The reaction mixture was warmed under water bath for 6 h. After cooling, it was poured into 150 ml ice-cold water and extracted with diethyl ether. The ether layer was washed with 6 N HCl and again with water till neutral. The residue obtained after evaporation of the solvent at reduced pressure was dried and purified over a column of silica gel (60–120 mesh).

Compound **5** was obtained (1.6 g, 94%) as a white solid with mp 222–223°C and $[\alpha]_D + 22$ ($CHCl_3$; 0.4 g/ml). The IR spectrum gave peaks at 1735 (—$OCOCH_3$), 1639, 1459, and 1370 (gem dimethyl), and 1243, 1027, 979, and 889 (=CH_2) cm^{-1}. Elemental analysis: found: C, 77.46%, H, 10.31%; cacld for $C_{34}H_{54}O_4$: C, 77.52%, H, 10.33%. ^1H NMR δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.91 (s, 3H), 0.96 (s, 3H), 1.59 (s, 3H), 1.65 (s, 3H), 2.43 (m, 1H, H-C-18), 3.84 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.24 (d, 1H, $J = 11.1$ Hz, H-C-28), 4.46 (m, 1H, H-C-3), and 4.59 (s, 1H), 4.68 (s, 1H). Acetate peaks appeared at δ_H 2.04 (s, 6H). The compound was found identical to an authentic sample (co-TLC, mixed mp, *etc.*).

3.5 *Lup-20(29)-en-3β,28-diyl acetate-30-al (6)*

Compound **5** (1.5 g, 2.85 mmol) was dissolved in 10 ml of aqueous dioxan and 20 ml of SeO_2 was added to it. The resultant reaction mixture was refluxed for 2 h and after cooling poured into 100 ml ice-cold water. A curdy white precipitate developed. After usual work up with diethyl ether, it was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The yellow gummy residue obtained was purified over a column of silica gel (60–120 mesh).

Compound **6** was obtained (62%) as a white solid with mp 246–248°C. IR ν_{max}: 1732 (—$OCOCH_3$), 1691 (>C=C—CHO) 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 889 (=CH_2) cm^{-1}. ^1H NMR: δ_H 0.93 (s, 3H), 0.94 (s, 3H), 1.02 (s, 3H), 1.06 (s, 3H), 1.39 (s, 3H), 6.28 (s, 1H, H-C-29), 5.93 (s, 1H, H-C-29), 4.24 (d, 1H,

$J = 10.6$ Hz, H-C-28) and δ_H 3.68 (d, 1H, $J = 10.6$ Hz, H-C-28), 9.56 (s, 1H, aldehyde hydrogen at C-30). Two acetyl methyls appeared at δ_H 2.07 (s, 3H) and at 2.03 (s, 3H). C-3 hydrogen appeared as a broad multiplet centered at δ_H 4.44 (m, 1H). Elemental analysis: found: C, 75.42%, H, 9.56%; cacld for $C_{34}H_{52}O_5$: C, 75.51%, H, 9.69%.

3.6 *Lup-20(29)-en-3β,28-diol-30-al (7)*

Compound **6** (900 mg, 1.66 mmol) was refluxed with 10% (w/v) alcoholic KOH solution for 4 h. After completion of the reaction, it was poured into 100 ml ice-cold water. After usual work up with diethyl ether, the ether layer was washed several times with water, dried over anhydrous sodium sulfate, and the recovered material was purified by column chromatography over silica gel (60–120 mesh).

Compound **7** was obtained (756 mg, 84%) as a white solid, mp 274–276°C, IR ν_{max}: 3393 (—OH), 1688 (>C=C—CHO), 1453, 1375 (gem dimethyl), 1029, 942, 890 (=CH_2) cm^{-1}. ^1H NMR: δ_H 0.75 (s, 3H), 0.81 (s, 3H), 0.95 (s, 3H), 1.00 (s, 3H), 1.38 (s, 3H), 2.62 (m, 1H, hydrogen at C-3), 3.15 (m, 1H, proton of-OH at C-3), 3.38 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.83 (d, 1H, $J = 10.6$ Hz, H-C-28), 5.94 (s, 1H, H-C-29), 6.32 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Elemental analysis: found: C, 78.66%, H, 10.52%; calcd for $C_{30}H_{48}O_3$: C, 78.90%, H, 10.59%.

3.7 *3β-Hydroxy-28-acetoxy-lup-20(29)-en-30-al (8)*

A solution of **7** (350 mg, 0.76 mmol) in $CHCl_3$ (10 ml) and pyridine (15 ml) was treated with Ac_2O (10 ml, 0.098 mmol) at very low temperature (0–5°C) for 12 h. The solution was diluted with $CHCl_3$ (20 ml), and then the organic layer was washed with saturated aq. NaCl (10 ml × 3), dried, concentrated, and purified by column chromatography over silica gel (60–120 mesh).

Compound **8** was obtained (72%) as a white solid, mp 260–262°C, IR ν_{max}: 3461 (—OH), 1732 (—$OCOCH_3$), 1691 (>C=C—CHO), 1459, 1369 (gem dimethyl), 1244, 1028, 978, 936, 890 (=CH_2) cm^{-1}. ^1H NMR δ_H 0.75 (s, 3H), 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.95 (s, 3H), 2.1 (s, 3H, acetate methyl), 3.18 (m, 1H, proton at C-3), 3.38 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.83 (d, 1H, $J = 10.6$ Hz, H-C-28), 5.94 (s, 1H, H-C-29), 6.32 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30). Elemental analysis: found: C, 77.02%, H, 10.12%; calcd for $C_{32}H_{50}O_4$: C, 77.06%, H, 10.10%.

3.8 *28-Acetoxy-3-oxolup-20(29)-en-30-al (9)*

Compound **8** (150 mg, 0.30 mmol) was dissolved in dry pyridine (30 ml). Two hundred and thirty milligrams (2.91 mmol) of dry CrO_3 were added in small lots and the reaction mixture was kept overnight. It was then poured into ice-cold water and the resultant yellowish solid was extracted with ether, washed with 6 N HCl and again with water till neutral, and dried over anhydrous magnesium sulfate to get a gummy residue. Purification of the gum over a column of silica gel (60–120 mesh) yielded compound **9**.

Compound **9** was obtained (84 mg, 56%) as a white solid, mp 276–278°C. IR ν_{max}: 1730 (>C=O), 1706, 1697 (>C=C—CHO), 1461, 1380 (gem dimethyl), 1244, 1163, 987, 890 (=CH_2) cm^{-1}. Elemental analysis: found: C, 77.28%, H, 9.62%; calcd for $C_{32}H_{48}O_4$: C, 77.38%, H, 9.74%. ^1H NMR δ_H 0.75 (s, 3H), 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.95 (s, 3H), 2.17 (s, 3H, acetate methyl at C-28) 3.38 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.83 (d, 1H, $J = 10.6$ Hz, H-C-28), 5.92 (s, 1H, H-C-29), 6.23 (s, 1H, H-C-29), 9.56 (s, 1H, aldehyde hydrogen at C-30).

3.9 28-Hydroxy-3-oxolup-20(29)-en-30-al (1)

Compound **9** (75 mg, 0.15 mmol) was refluxed with 10% alcoholic KOH solution for 4 h. After completion of the reaction, it was poured into 100 ml ice-cold water. After usual work up with ether, the ether layer was washed several times with water, dried over anhydrous magnesium sulfate, and the recovered material was purified by column chromatography (silica gel, 60–120 mesh).

Compound **1** was obtained (80.6 mg, 96%) as a white amorphous solid; $[\alpha]_D + 16.3$ (MeOH), mp 288–290°C. IR ν_{max}: 3424 (—OH), 1725 (>C=O), 1706, 1697 (>C=C—CHO), 1461, 1380 (gem dimethyl), 1163, 987 cm^{-1}. ^1H NMR: δ_H 0.91 (s, 3H), 0.93(s, 3H), 1.01 (s, 3H), 1.06 (s, 3H), and 1.15 (s, 3H), 6.91 (s, 1H, H-C-29), 6.28 (s, 1H, H-C-29), 3.36 (d, 1H, $J = 10.6$ Hz, H-C-28), 3.78 (d, 1H, $J = 10.6$ Hz, H-C-28), 9.52 (s, 1H, aldehyde hydrogen at C-30). ^{13}C NMR spectral data are depicted in Table 1. HR-EI-MS: *m/z* 554.38 $[M]^+$ (calcd for $C_{30}H_{46}O_3$, 554.34). Elemental analysis: found: C, 79.18%, H, 10.21%; calcd for $C_{30}H_{46}O_3$: C, 79.25%, H, 10.20%.

4. Bioassay

The assays against human K562 leukemia, murine WEHI3 leukemia, and murine MEL erythroid progenitor cells were carried out in three independent experiments as per the guidelines of biosafety committee of West Bengal States University. K562 and WEHI3 cells were obtained from cell repository of National Centre for Cell Science, Pune (agreement between two institutes). MEL cells were obtained as gift from Prof. Michael H. Kershaw, Cancer Immunotherapy Research, Peter MacCallum Cancer Centre, Australia (through an agreement with Walter and Eliza Hall Institute, Australia). The cells were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mmol glutamine, supplemented with 1% penicillin–streptomycin, and were incubated at 37°C under 5% CO_2 atmosphere. In these experiments, cells were seeded in quadruplicate in 24-well plates (105 cells/mL) with the compounds (**1**, **2**, **3**, **5**, and **7**) dissolved in DMSO (0.1% v/v) at various concentrations. The effect of **4** and **6** could not be checked due to its poor solubility in DMSO. The cells were incubated for 2, 4, and 6 days. Growth of cells was monitored by counting the number of live cells microscopically using Neubauer hemocytometer by trypan blue exclusion method. Statistical analyses for all experiments were carried out by Student's *t*-test using the program SigmaPlot.

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RESEARCH LETTER

Greener approach toward one pot route to pyrazine synthesis

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A very simple, cost effective, and environmentally benign method has been reported for the preparation of pyrazine derivatives of pentacyclic triterpenoids. The versatility of the method is determined by synthesizing a large number of pyrazine derivatives of smaller molecules.

Keywords: green synthesis; one pot; triterpenoid; pyrazine; cost effective

Introduction

Compounds containing *N*-heterocyclic moieties are a class of privileged compounds that have found numerous applications as pharmaceuticals. Pyrazines are important components of aroma fragrances (*1*), potential pharmacophore of a large number of biologically active substances (*2–6*), and widely used as agrochemicals (*7–9*). For example, methoxy pyrazines are relevant components of aromas of many fruits, vegetables, and wines; methyl phenyl derivatives of dihydropyrazines inhibit the growth of *Echerichia coli* by generating hydroxyl and carbene-centered radicals that cause DNA strand breakage; and alkylpyrazines have been recognized as flavor components in foods, as pheromones in various insect species (*7, 8*), and as versatile synthetic intermediates. Pyrazine derivatives are known for use as relaxing cardiovascular and uterine smooth muscle, anti-thrombotic, anti-aggregation, COX-2 inhibiting, and analgesic effects (*10*). Because of the wide variety of applications associated with the pyrazine moieties, their synthesis has remained the goal of many research groups over the years. Among the various methods developed, pyrazine compounds are synthesized by the reaction of diamines with diols in a vapor phase reaction in presence of granular alumina (*11*). Catalytic systems such as copper-chromium (*12*), copper-zinc-chromium (*13*), zinc-phosphoric acid-manganese (*14*), and silver (*15*) are also patented as catalysts for the preparation of 2-methylpyrazine from ethylene-diamine and propylene glycol. Pyrazines are also obtained from condensation reaction of diamines and epoxides using copper-chromium catalyst (*16*), condensation reaction between alkanolamines (*17*), or cyclodehydrogenation of *N*-(-hydroxyalkyl) alkyldiamine (*18*) using the same catalysts. In the presence of a palladium catalyst, dehydrogenation of piperazines yields corresponding pyrazines in high yield (*19*). Recently, synthesis of pyrazines from α-hydroxy ketones and 1,2-diamines via MnO_2 catalyzed tandem oxidation process under refluxing conditions has been reported, but the yields are not encouraging and the loading of the catalyst was also high (*20*). The method of bubbling oxygen under refluxing condition (*21*) suffers from scientific drawbacks. Strategically, direct condensation reaction of 1,2-diketones with 1,2-diamine (*22*) is the most straightforward as well as the classical route for the preparation of pyrazines via dihydropyrazines (*22*). Although, a number of methods are reported in literature for the synthesis of pyrazine, none of them was found to be effective because of poor yield, harsh reaction condition, and tedious work-up procedures (*23*). Attempts to carry out dehydrogenation under a variety of milder and more convenient laboratory procedures were not successful (*24*). Although, some of them are apparently useful, most of them are limited by long reaction time, low yields, and use of toxic solvents or heavy metals as the catalyst (*24*). Therefore, development of mild, efficient, and environmentally benign method for synthesizing pyrazines has been a major challenge in contemporary organic synthesis.

Results and discussion

Triterpenoids are widely distributed in nature, and recent reports have demonstrated the interesting biological activities of this class of natural products. However, triterpenoids possessing a nitrogen containing

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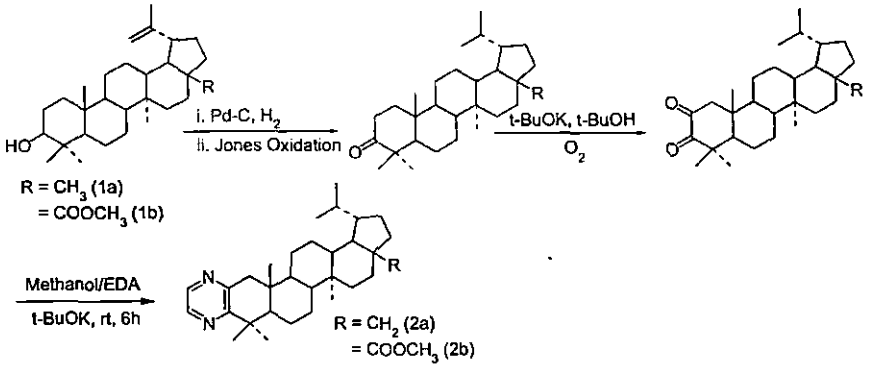
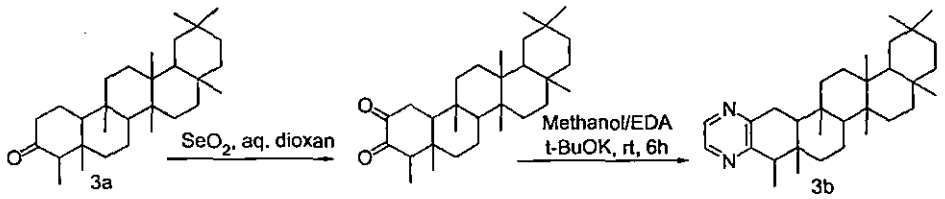
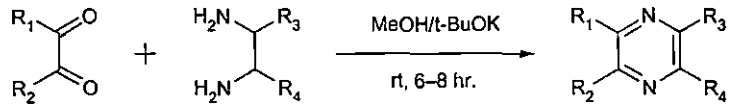


Scheme 1. Preparation of diketo derivatives and synthesis of pyrazine derivative.



Scheme 2. Preparation of diketofriedelin and synthesis of pyrazine derivative.



Scheme 3. Preparation of pyrazines. R_1 may be alkyl, aryl or furyl group; R_2 may be alkyl, aryl or furyl group or hydrogen; R_3 may be hydrogen, alkyl or nitrile group; R_4 may be hydrogen or nitrile group.

heterocycle condensed to an isoprenoid skeleton are rare. Since compounds containing *N*-heterocyclic moieties have found numerous applications as pharmaceuticals as well as in medicines, it is also anticipated that incorporation of a pyrazine ring into the molecule of a pentacyclic triterpenoid may induce or enhance its biological activity. With this view in mind and in continuation of our studies on the transformative reactions of triterpenoids, we report herein the incorporation of pyrazine ring into ring A of the pentacyclic triterpenoids of lupane and friedelan skeleton (Schemes 1 and 2). The protocol comprises a direct condensation between the respective 1,2-diketo compounds with 1,2-diamines in aqueous methanol catalyzed by potassium tert-butoxide (t-BuOK) at room temperature. This high yielding process did not require any added expensive catalyst or bubbling of oxygen (*21*) at higher temperature (Scheme 1). Detection of dihydropyrazine along with pyrazines as well as the starting material at an early stage of the reaction indicated that the developed method involved aromatization following a very simple one pot route via the formation of dihydropyrazine, removing any additional steps as reported in literature.

In order to show the general applicability, we attempted our procedure using a number of both structurally and chemically diversified 1,2-dicarbonyls and 1,2-diamines to synthesize pyrazine derivatives (Table 1) and were able to get identical results in each case. Thus, this cost-effective process may also be considered as an excellent environmentally benign alternative for the preparation of pyrazine derivatives from a host of compounds (Scheme 3).

General experimental detail

All the melting points were determined in an open capillary method; UV spectra were recorded in JASCO V-530 UV/VIS spectrophotometer; IR was recorded in Perkin-Elmer FT-IR spectrophotometer; and NMR was recorded in Bruker-Avance 300 MHz FT-NMR instrument using TMS as the internal standard. NMR spectra were recorded in $CDCl_3$.

Table 1. Selective synthesis of pyrazine.

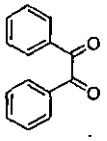
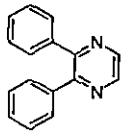
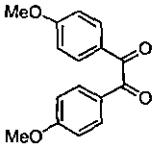
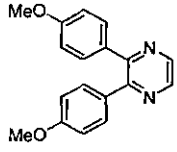
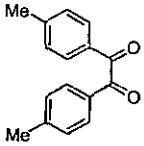
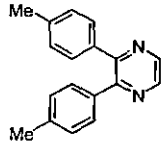
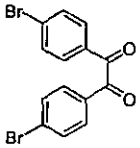
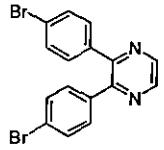
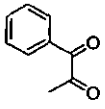
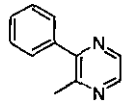
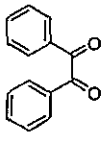
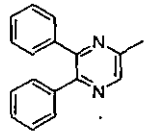
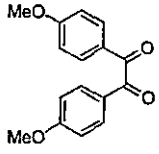
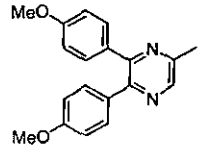
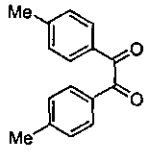
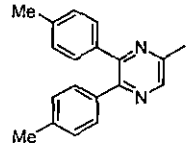
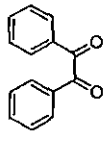
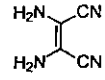
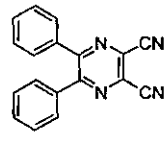
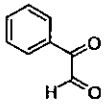
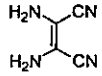
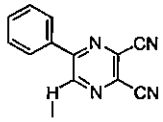
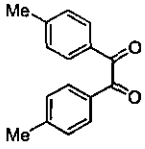
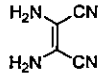
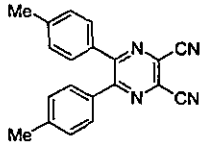
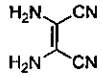
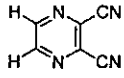
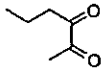
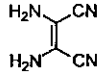
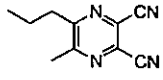
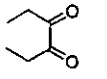
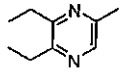
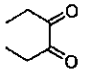
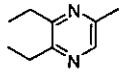
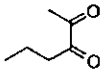
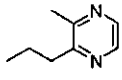
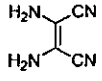
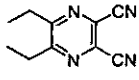
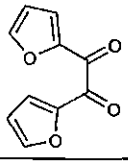
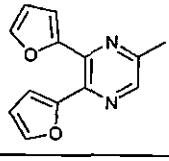
Entry	1,2-Diketone	1,2-Diamine	Time (h)	Pyrazine	% Yield
1			8		82
2			8		78
3			7.5		82
4			10		84
5			8		76
6			8		78
7			8.5		78
8			8		86
9			4		86

Table 1 (*Continued*)

Entry	1,2-Diketone	1,2-Diamine	Time (h)	Pyrazine	% Yield
10			3.5		88
11			5		78
12			3.5		82
13			4		76
14			4.5		74
15			5		76
16			4.5		78
17			6		76
18			7		82

Note: % Yield refers to the isolated yield of all the compounds.

The entire chemicals were purchased from Merck, Fluka, SRL, and S.D. fine chemicals companies. The reagents from Merck and Fluka were used as received and others were purified following the standard methodology prior to their use. Betulinic acid, lupeol, and friedelin were isolated from their natural sources (see supporting information).

Preparation of 1,4-pyrazine derivatives

In a typical reaction procedure, in a 50 ml round bottom flask, 2 mmol of recrystallized benzil was dissolved in 3 ml of aqueous methanol and was made homogeneous by stirring with a magnetic spinning bar. To this 2 mmol of ethylene diamine and catalytic amount of t-BuOK (10 mg or 0.08 mmol) were added. Stirring was continued until the reaction is completed (checked by TLC). Methanol was evaporated under reduced pressure, and the crude product was purified by chromatography using silica gel. Varied proportion of petroleum ether and ethyl acetate was used as eluent.

Conclusion

A mild, efficient, and environmentally benign method has been developed for the synthesis of pyrazines that is superior in every respect than the already reported methods. Introduction of the pyrazine nucleus is expected to induce potent biological activity into the triterpenoids that will be tested with the help of a sister institution having these facilities. The data obtained in the process may be helpful to study the SAR (structure–activity relationship) among this particular class of compounds, especially the triterpenoids of above skeletons.

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SUPPORTING INFORMATION

General experimental detail

All the melting points were determined in an open capillary method, UV spectra were recorded in JASCO V-530 UV/VIS spectrophotometer; IR spectra was recorded in Perkin-Elmer FT-IR spectrophotometer; NMR was recorded in Bruker-Avance 300 MHz FT-NMR instrument using TMS as the internal standard. NMR spectra were recorded in $CDCl_3$. The chemicals/reagents purchased from Merck, Fluka, SRL, S d fine chemicals companies and were used either as received (Merck, Fluka) or after purification prior to use. Triterpenoids, Betulinic acid, lupeol and friedelin were isolated from their natural sources (Please see the supporting information) and were used as starting materials in the present investigation.

Isolation of triterpenoids

Betulinic acid, lupeol and friedelin were isolated from *Biscofia javanica, Xanthozylum budrungs* and *quercus suber* respectively in a soxhlet extractor using petroleum ether (60-80°C) as the solvent. All the triterpenoids were purified by column chromatography followed by crystallization.

Preparation of diketo derivatives

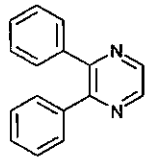
Betulinic acid and lupeol were hydrogenated and oxidized to get the correspobding 3-keto compound. The corresponding diketo compounds were prepared by auto-oxidation of each of them following the method as reported elsewhere (Gangully, A. K.; Govindachari, T. R.; Mohamed, P. A. *Tethedron*, **1966,** *22*, 3597-3599.). Diketo derivative of friedelin was prepared by SeO_2 oxidation of friedelin in aq. Dioxin.

Preparation of 1, 4-pyrazine derivatives

In a typical reaction procedure, in a 50 ml round bottom flask 2 mmol of recrystallized benzil was dissolved in 3 ml of wet methanol and was made homogeneous by stirring with a magnetic spinning bar. To this 2 mmol of ethylene diamine and small amount of ^tBuOK were added. Stirring was continued until the reaction was complete (checked by tlc). Methanol was evaporated under reduced pressure and the crude product was purified by chromatography using silica gel. Varied proportion of petroleum ether and ethyl acetate was used as eluent.

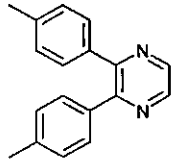
CHARACTERIZATION OF SOME REPRESENTATIVE COMPOUNDS:

2, 3-diphenyl pyrazine



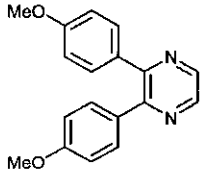
^1H NMR ($CDCl_3$, 300MHz): δ 7.14-7.25 (m, 5H, five aromatic hydrogen); 7.37-7.44 (m, 5H, five aromatic hydrogen); 8.52 (s, 2H, 2 aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR ($CDCl_3$, 75MHz): δ 128.1, 128.2, 128.5, 129.5, 138.5, 141.9 and 152.6.

2, 3-di *p*-tolyl pyrazine



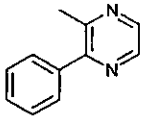
^1H NMR ($CDCl_3$, 300MHz): δ 2.31 (s, 6H, 2-CH_3); 7.04-7.12 (m, 3H, aromatic hydrogen); 7.27-7.46 (m, 5H, five aromatic hydrogen); 8.51 (s, 2H, two aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR ($CDCl_3$, 75MHz): δ 21.3, 129.0, 129.4, 135.8, 138.5, 141.7 and 152.6.

2, 3-bis (4-methoxy phenyl) pyrazine



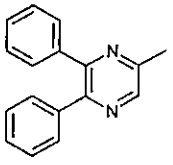
^1H NMR ($CDCl_3$, 300MHz): δ 3.77 (s, 6H, 2-OCH_3); 6.75-6.85 (m, 4H, four aromatic hydrogen); 7.33-7.43 (m, 4H, four aromatic hydrogen); 8.45 (s, 2H, two aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR ($CDCl_3$, 75MHz): δ55.2, 113.7, 130. 9, 131.2, 141.4, 152.1 and 159.9.

2-methyl-3-phenyl pyrazine



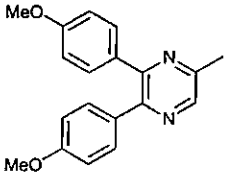
^{1}H NMR ($CDCl_3$, 300MHz): δ2.54 (s, 3H, $-CH_3$); 7.46-7.59 (m, 5H, five aromatic hydrogen); 8.44 (d, 2H, J = 2.4Hz). ^{13}C NMR ($CDCl_3$, 75MHz): δ23.1, 128.4, 128.7, 128.9, 138.5, 141.5, 142.1, 151.8 and 154.4.

2, 3-diphenyl-5-methyl pyrazine



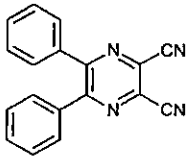
^{1}H NMR ($CDCl_3$, 300MHz): δ 1.86 (s, 3H, $-CH_3$); 6.63 (d, 10H, J = 5.1Hz, ten aromatic hydrogen); 7.68 (s, 1H, one aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR ($CDCl_3$, 75MHz): δ20.5, 127.4, 127.6, 128.7, 128.8, 137.8, 141.0, 148.8, 150.3, 150.7.

2, 3-bis (4-methoxy phenyl)-5-methyl pyrazine



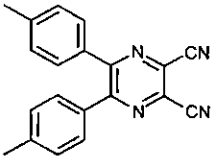
^{1}H NMR ($CDCl_3$, 300MHz): δ 2.62 (s, 3H, $-CH_3$); 3.80 (s, 6H, 2-OCH_3); 6.82 (dd, 4H, J = 1.8 Hz, four aromatic hydrogen); 7.38 (dd, 4H, J = 1.8 Hz, four aromatic hydrogen); 8.39 (s, 1H, one aromatic hydrogen of the heterocyclic moiety). ^{13}C NMR ($CDCl_3$, 75MHz): δ21.2, 53.4, 55.2, 130.8, 130.9, 131.2, 131.4, 141.1, 149.0, 150.5, 150. 9, 159.8, 159.6.

5, 6-diphenyl pyrazine-2, 3-dicarbonitrile



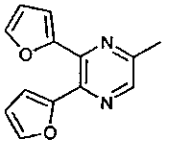
^{1}H NMR ($CDCl_3$, 300MHz): δ7.16-7.30 (m, 5H, five aromatic hydrogen); 7.45 (t, 2H, J = 7.3 Hz, two aromatic hydrogen); 7.57 (t, 1H, J = 7.3 Hz, aromatic hydrogen); 7.78 (d, 2H, J = 7.2 Hz, aromatic hydrogen). ^{13}C NMR ($CDCl_3$, 75MHz): δ126.5, 127.5, 128.2, 128.4, 130.0, 132.4, 137.5, 143.8, 196.8 (carbon of nitrile group).

5, 6-di*p*-tolylpyrazine-2, 3-dicarbonitrile



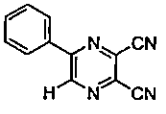
^{1}H NMR ($CDCl_3$, 300MHz): δ 2.32 (m, 6H, 2 $-CH_3$); 6.99 (m, 4H, aromatic protons); 7.43 (m, 1H, aromatic proton); 7.94, (m, 3H, aromatic protons). ^{13}C NMR ($CDCl_3$, 75MHz): δ21.6, 126.8, 128.6, 129.3, 130.0, 139.2, 144.0, 144.3, 193.3.

2, 3 di-(furan-2-yl)-5-methyl pyrazine



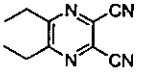
^{1}H NMR ($CDCl_3$, 300MHz): δ 2.59 (s, 3H, $-CH_3$); 6.56 (m, 4H, aromatic protons); 7.52 (m, 2H, aromatic protons), 8.37 (s, 1H, aromatic proton). ^{13}C NMR ($CDCl_3$, 75MHz): δ21.3, 112.1, 112.7, 139.2, 140.8, 141.7, 143.4, 143.7, 150.5, 150.6, 151.2.

5-phenylpyrazine 2, 3-dicarbonitrile



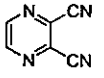
^{1}H NMR ($CDCl_3$, 300MHz): δ7.61 (d, 3H, J = 7.5 Hz); 8.13 (d, 2H, J = 6.6Hz); 8.51 (s, 1H). ^{13}C NMR ($CDCl_3$, 75MHz): δ 128.0, 129.8, 130.8, 132.5, 133.0, 144.1, 154.8.

5, 6-diethylpyrazine-2, 3-dicarbonitrile



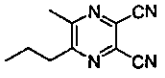
^{1}H NMR ($CDCl_3$, 300MHz): δ 1.39 (m, 6H); 1.97(m, 2H); 2.97 (m, 2H); ^{13}C NMR ($CDCl_3$, 75MHz): δ 11.2, 19.8 (2-CH_3); 25.1, 27.8 (2-CH_2); 113.4, 130.2 (aromatic carbon); 161.3 (-CN).

Pyrazine-2, 3-dicarbonitrile



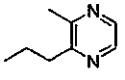
^{1}H NMR ($CDCl_3$, 300MHz): δ9.00 (s, 2H, aromatic protons). ^{13}C NMR ($CDCl_3$, 75MHz): δ 113.1, 133.84 (aromatic carbons); 147.5 (-CN).

5-methyl-6-propiopyrazine-2, 3-dicarbonitrile



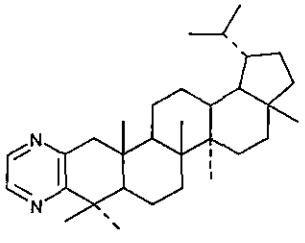
^{1}H NMR ($CDCl_3$, 300MHz): δ1.06 (t, 3H, J = 7.2Hz); 1.78-1.88 (m, 2H); 2.75 (s, 2H, -CH_3); 2.94 (t, 2H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$, 75MHz): δ13.8, 20.4, 22.3, 36.9, 113.3, 113.4, 129.9, 130.4, 157.7, 161.2.

2-methyl-3-propylpyrazine



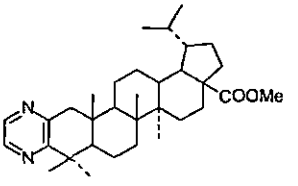
^{1}H NMR ($CDCl_3$, 300MHz): δ 0.96-1.04 (m, 3H, -CH_3); 1.70-1.82 (m, 2H,); 2.57 (s, 3H, -CH_3); 2.79 (t, 2H, J = 7.5Hz). ^{13}C NMR ($CDCl_3$, 75MHz): δ14.0, 21.5, 21.7, 36.9, 141.1, 141.4, 152.3, 156.0.

1, 4-pyrazine derivative of 1, 2-diketo lupane



Crystallization afforded white needle shaped crystals, $C_{32}H_{50}N_2$, m.p. 220 °C, IR at 1650, 1430 and 1120 cm^{-1}. UV absorption maxima at 272nm (ε = 5831) and 278nm (ε = 5792). Anal.Calc.: 83.12% C, 10.82% H; found 83.10% C, 10.81% H. Mass spectrum showed molecular ion peak at m/z 462. ^{1}H NMR ($CDCl_3$, δ ppm^{-1} relative to TMS): 0.78, 0.83, 0.98, 1.11, 1.29, 1.31 (6s, 18H, 6t-CH_3); 0.77, 0.86 (2d, 6H, CH (CH_3)$_2$, J = 7 Hz); 2.47, 3.04 ppm (2d, J = 16 Hz); 8.27(d, J = 3 Hz); 8.41(dd, J = 3 Hz).

1, 4-pyrazine derivative of 1, 2-diketone of methyl dihydrobetulonate



Crystallization from $CHCl_3$-MeOH mixture, $C_{33}H_{50}O_2N_2$, m.p. 220 °C. IR spectrum showed peaks at 1710 cm^{-1}(CO_2Me); 1665, 1430 and 1120 cm^{-1}. UV spectrum showed absorption maximum at 272nm (ε = 5712) and 278 nm (ε = 5603). Anal. Calc.: 78.26% C, 9.88% H, 5.53% N; found 78.25% C, 9.73% H, 5.50% N. Mass spectrum showed molecular ion peak at m/z 506 as base peak. ^{1}H NMR ($CDCl_3$, δ ppm^{-1} relative to TMS): 0.82, 0.985, 0.99, 1.28, 1.305, 0.76 and 0.88 ppm (2d, 6H, j = 7 Hz); 2.48, 3.04 ppm (2d J = 16 Hz); 8.27, 8.41 ppm (2d, J = 3 Hz); 3.66 (s, 3H, ester methyl).

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Sodium dodecyl sulfate in Water: Greener Approach for the Synthesis of Quinoxaline Derivatives

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Abstract

A mild and efficient synthetic method has been developed for the preparation of biologically important quinoxalines in excellent yield from relatively safe precursor α-bromoketones and 1,2-diamines using catalytic amount of miceller sodium dodecylsulfate (SDS) in water at ambient temperature. The method is also found effective for the introduction of quinoxaline moiety into the ring A of pentacyclic triterpenoid, friedelin. Ambient reaction conditions, renewable catalytic condition, inherently safer chemistry, excellent product yields and water as a reaction medium display both economic and environmental advantages.

Key words: Quinoxaline, water, miceller SDS, room temperature, greener approach.

Introduction

Quinoxalines are ubiquitous heterocyclic units in pharmaceuticals and bioactive natural products (*1-4*). They are used as pharmaceuticals and antibiotics such as echinomycin, levomycin and actinoleutin which are known to inhibit the growth of Gram-positive bacteria and are also active against various transplantable tumors (*1-3*). Antitumoral properties of quinoxaline compounds have also been investigated (*4*) Beside these, they are well known for their application in dyes (*5*) as an efficient electroluminescent materials (*6*) in organic semiconductors (*7*) as building blocks for the synthesis of anion receptors (*8*) as cavitands (*9-10*) dehydroannulenes (*11*) and DNA cleaving agents (*12-13*). Conventionally, quinoxalines are synthesized by a double condensation reaction involving a dicarbonyl precursor and *o*-phenylenediamine (*14-15*). Due to the highly reactive nature of the dicarbonyls, alternative routes have been proposed recently (*16*). Antoniotti and Donach have reported one of these methods to synthesize quinoxalines

from epoxides and ene-1,2-diamines (*16*) Active manganese oxide and molecular sieves in combination or manganese oxides in combination with microwaves have also been used in producing quinoxalines (*17-18*). These processes, however, require excessive amounts of corrosive manganese oxide as stoichiometric oxidants and scaling them up for industrial processes can lead to the formation of large amounts of toxic waste leading to environmental issues. In additional studies, Robinson and Taylor reported a homogeneous catalytic process utilizing $Pd(OAc)_2$, $RuCl_2$ $(PPh_3)_2$ to synthesize quinoxalines from hydroxy ketones (*19*) and recently a copper catalyzed oxidative cyclization process has been reported (*20*). An improved ruthenium catalyzed direct approach to synthesize quinoxalines from diols and *o*-diamines has also been reported (*21*). These processes suffer from the major drawback that the catalysts are expensive, toxic and cannot be recovered and reused. In addition to the above catalytic methods, synthesis of quinoxalines using zeolites (*22-25*) microwave (*26-27*) and solid supports (*28-30*) has also been reported. Nevertheless, these methods suffer from unsatisfactory product yields, critical product isolation procedures, expensive and detrimental metal precursors and harsh reaction conditions, which limit their use as environmentally friendly protocol. In addition most of the reported methods are not recommended as a clean protocol.

Although very few of the recent reports have claimed α-bromoketones as an equivalent safe chemical precursor of α-hydroxyketones, epoxides or dicarbonyls as reaction partners of *o*-phenylenediamine to prepare quinoxalines (*31-32*) they involved the use of either $HClO_4$-SiO_2 or TMSCl as catalyst. Although useful, $HClO_4$ has huge hazardous nature than its potential usefulness, whereas those catalyzed by TMSCl needs higher

temperature, with lower yield of the desired products not satisfying the principles of green chemistry protocol in contemporary science as well as their acceptance for industrial applications.

In this context the development of an alternative route to quinoxaline from less reactive α-bromoketones in aqueous medium was felt necessary not only due to the increased regulatory pressure focusing on organic solvents, but also because of the emphasis given towards the development of green protocol for organic synthesis nowadays.

The use of water as a medium for organic synthesis is one of the latest challenges in organic synthesis. Reactions in water emerged as a useful alternative route for several organic reactions owing to many of its potential advantages such as safety, economy and friendly towards catalytic and stereoselective processes and more importantly of environmental concern (*32-33*) and the progress has been dramatic. Additionally, water facilitates ion separation through salvation which often results in altered behavior of reactants in an aqueous environment. Keeping these above facts in mind, we recently have tested water as a solvent in many of our ongoing studies towards organic syntheses and transformative reactions. Very recently we have reported [33] the selective synthesis of 1,2-disubstituted benzimidazole in water catalysed by SDS, and now reporting herein the result of another successful attempt for an efficient synthesis of quinoxaline from α-bromoketones and *o*-phenylenediamine mediated by water and catalysed by SDS at room temperature in excellent yields. This is the first report of synthesizing quinoxaline derivatives in a very mild way in water catalyzed by nucleophilic SDS (*33*) at ambient temperature starting from the less reactive safer precursor α-bromoketones in an efficient manner.

Results and Discussion

Initially, efforts were directed towards the evaluation of catalytic ability of SDS for the synthesis of quinoxalines. Preliminary studies using phenacylbromide (1 mmol) and *o*-phenylenediamine (1 mmol) without SDS in water at room temperature did not afford the desired quinoxaline. Increase of the reaction time, temperature or by changing the molar proportion of the reactants did not make any influence on the course of the reaction. Addition of some common salts like NaCl, NH_4Cl, KBr *etc.* had no positive effect on the reaction. Similar molar ratios of substrates in tap water yielded the desired product (**3**) only in presence of catalytic amounts of SDS. The modified method gave excellent yield of the product within 6 hours at room temperature (Scheme 1). Thus, the catalytic role of SDS in the present transformation is well established.

This excellent catalyzing ability of SDS inspired us to investigate the above transformation in details. In order to evaluate an optimized and general reaction protocol, a couple of experiments were carried out (Table 1) using varying amounts of SDS (0.34 mol%, 0.17 mol%, 0.06 mol%, 0.03 mol%, 0.02 mol% and 0.01 mol%) in combination with different types (both cationic and anionic) and proportions of surfactants *viz.* tetra-n-butylammonium bromide (TBAB), cetyl trimethyl ammoniumbromide (CTAB), cetyl pyridiniumchloride (CPC), sodium dodecylbenzenesulfonate (SDBS) and tetra-n-butylammoniumiodide (TBAI), in different reaction conditions for the above model study (Table 1). It is interesting to note that, although all the surfactants can afford (**3**) as the major product but their combination with SDS showed excellent selectivity not only in forming the desired product (**3**) but also in directing the reaction to proceed in a very cleaner way (Entry 1-7, Table 1). Thus it was established that, α-bromoketone (1 mmol)

and 1,2-diamine (1 mmol) in water (3 mL) gave the best result within 6 hrs in presence of SDS (10 mg, 0.03 mol%) at room temperature.

It was also observed that during the reaction the substrates and reactants do not mix together in water; addition of SDS not only raised the solubility of the components in water but also catalyzed the process tremendously. Addition of catalytic amount of SDS (0.03 mol %) turned the reaction mixture into a clear yellowish colored solution that slowly transferred into reddish yellow as the reaction progressed. After completion of the reaction (checked by tlc), products were purified by simple filtration (and in some cases by column chromatography, silica 60-120 mesh) followed by crystallization to get the products in good to excellent yields.

In order to demonstrate the versatility of SDS as a catalyst for the synthesis of quinoxalines, a series of α-bromoketones and 1,2-diamines were subjected to undergo one pot condensation-aromatization in presence of SDS under the optimized reaction protocol (Table 2). All of the reactions tried showed good selectivity with excellent isolated yields.

While investigating the influence of the substituents present either on ketone part or on 1,2-diamine on the course of the reaction, it was observed that compounds having electron donating or withdrawing groups on the ketone (Entry 2, 3, 5, 6, 7 and 8, 9, of Table 2) both underwent the reactions in almost similar fashion and gave good yields. Although, *p*-bromo phenacylbromide (Entry 5, of Table 2) gave better yield than its meta isomer (Entry 9, of Table 2), the corresponding *p*-nitro and *m*-nitro derivative underwent the reaction in identical fashion (Entry 10, of Table 2). Disubstituted α-bromoketones (Entry 11, 12, of Table 2) also gave excellent yields of the expected quinoxalines.

Sensitive molecules like 1,2-diaminomalionitrile (Entry 15, of Table 2) was also found compatible to the reaction condition and gave 84% yield of the corresponding quinoxaline. All the observed results were summarized in table 2.

Potential of pentacyclic triterenod as bioactive candidate is well described (*34*). In order to see the effect on their bioactivities by the introduction of quinoxaline ring on ring A, we applied our protocol on 2α-bromofriedelin (**5**) (prepared from friedelin) (**4**) and to our delight we have isolated the corresponding quinoxaline derivative (**6**) in 58% yield within 8 hours under identical condition (0.03 mol% of SDS). This is also the very first report of preparing quinoxaline derivative of pentacyclic triterenoids in water at room temperature (Scheme 2).

As was mentioned earlier, a simple filtration or easy work up procedure of the reaction and reuse of the catalyst, SDS directly from the aqueous extract of the reaction mixture for a fresh run, are the great advantages of the developed process. Gratifyingly, it was tested that the recovered water layer can be reused for six consecutive runs (Table 3).

It is well known that under ambient condition surfactant molecules can aggregate in an aqueous phase to micelles with hydrophobic core and a hydrophilic corona (*35-36*). To determine whether micellisation had occurred or not we first measured the CMC (critical micellisation concentration) of SDS (Fig 1) and the value was found to be 8.33 mM. In the present study, under the optimized reaction condition the concentration of SDS was 11.57 mM (10 mg of SDS in 3 ml water). Since the value was far beyond the CMC value of SDS (8.33 mM), micellization was anticipated.

It was reported in the literature (*37*) that the dimensionless packing parameter *P* of the molecular geometry as an index to predict the size and shape of the micelles. *P* was defined as $V/(a_0 l)$, where V is the hydrocarbon chain volume, a_0 is the optimum head group area per molecule, and *l* is the hydrocarbon chain length that is taken to be ca. 80-90% of the fully extended chain length (*37*). The overall prediction was concluded as follows:

Spherical micelles $P < 1/3$

Cylindrical micelles $1/3 < P < 1/2$

Bilayers (or vesicles) $1/2 < P < 1$

Inverted structures $P > 1$

The value of packing parameter *P*, an index to predict the size and shape of the micelles (*37*), of SDS was found to be 0.235 (taking *l* as 90 % of the fully extended chain length) indicating the spherical nature of the developed micelles.

For further confirmation DLS (Dynamic Light Scattering) measurement was carried out of a 11.57 mM aqueous solution of SDS that indicated the presence of micelles (Fig 2) of radius 161 nm (diameter of 322 nm) with the PDI (Polydispersity index) of 0.348.

The role of SDS as a nucleophile is well investigated in our previous communication (*33*). Considering the above caharacteristics of SDS, the most probable mechanism of the miceller SDS in effecting the present transformation may be depicted as shown in scheme 3. In the miceller solution, 1,2-phenylenediamine and phenacyl bromide, both of which are hydrophobic in nature, are entered into the hydrophobic core of the micelles and thus assist the condensation between the phenacyl bromide and *o*-

phenylenediamine to form dihydroquinoxaline derivative (**A**) (Scheme 3). Nucleophilic nature of SDS may have assisted the in situ aromatization of the dihydro derivative (**A**) to afford quinoxalines (Scheme 4).

Conclusion

A simple, energy efficient, one step SDS catalyzed (0.03 mol%) greener method for the synthesis of quinoxaline derivatives under water mediating condition has been developed. Structurally diversified α-bromoketones, commonly regarded as safer chemicals, were used as reaction partners of 1,2-diamines in water at ambient temperature. Effect of the nature and position of the substituents on both the reactants in consideration to the reaction condition was also studied. Disubstituted α-bromoketones and 2α-bromo friedelin (a representative of pentacyclic triterpenoids) also formed the corresponding quinoxalines that may serve as lead compound in near future. Except water, no other organic solvents were used. The ambient reaction conditions, comparatively lower reaction time, excellent product yields and simple work up procedure not only make this methodology an alternative platform to the conventional acid/base catalyzed thermal processes, but also found to be significant under the umbrella of environmentally greener and safer processes that may find its place in industry. Moreover, water as a solvent used with miceller SDS has both economic and environmental advantages. As micelles of diameter of 322 nm were formed, it was anticipated that the entitled reactions were occurring inside the hydrophilic core of the micelles. Scaling up the reaction upto 5 moles scale gave good results. We believe our developed process not only satisfied the principles of green chemistry, can open a new way of synthesizing bioactive molecules

by catalyzing SDS in water. Further explorative studies of this efficient combination to various organic syntheses is undergoing in our laboratory.

Experimental

General

All the chemicals used were reagent grade and purified prior to their use. SDS was purchased from Sigma-Aldrich, India. The NMR chemical shift was reported in ppm relative to 7.20 and 77.0 ppm of $CDCl_3$ solvent as the standards. ^1H spectra were recorded in 300 MHz frequencies and ^{13}C NMR spectra were recorded in 75.4 MHz frequencies. Coupling constant 'J' was calculated in Hz. Conductance was measured in Systronics conductivity meter 304 at 25°C (298 K). DLS measurement was performed in Nano ZS90 (Malvern, UK).

General procedure for quinoxalines

In a typical experimental procedure, *o*-phenylenediamine (1 mmol) and α–bromoketone (1 mmol) in 1:1 molar ratios was taken in a 50 ml round bottom flask. To this water (3 ml) and 10 mg (0.03 mol %) sodium dodecylsulfate was admixed. The reaction mixture was then allowed to stir with magnetic spinning bar at room temperature. After the completion of the reaction (checked by tlc), the residue was filtered, washed with water, dried and finally recrystallized from methanol. The desired pure product was characterized by spectral (IR, ^1H- and ^{13}C-NMR) data and compared to those reported in literature.

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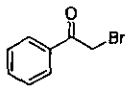
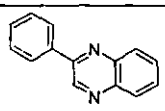
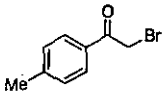
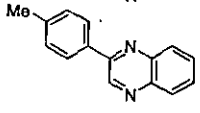
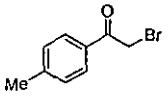
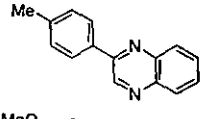
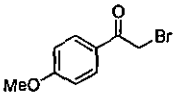
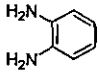
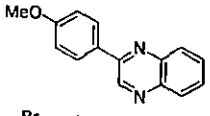
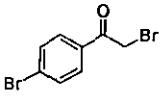
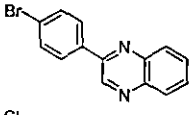
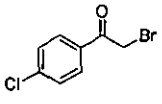
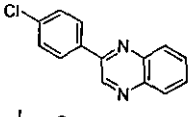
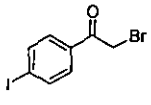
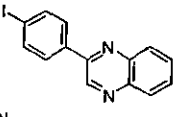
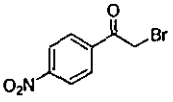
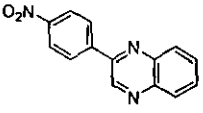
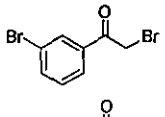
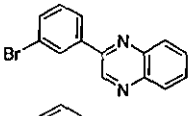
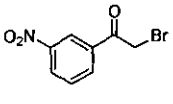
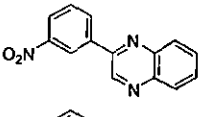
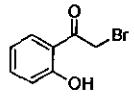
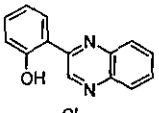
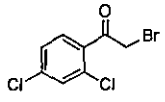
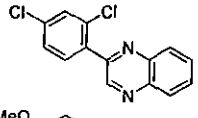
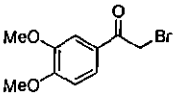
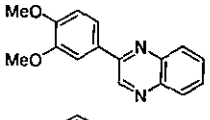
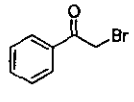
Table 1 Optimisation of quinoxaline synthesis using phenacyl bromide and *o*-phenylenediamine in presence of different surfactants and their amounts.

Entry	Ratio of aldehyde and diamine	Surfactant	Amount of surfactant (mg)	Temp (°C)	Time (hr)	% Yield[a] of 3
1	1:1	SDS	100	RT	6	96
2	1:1	SDS	50	RT	6	94
3	1:1	SDS	20	RT	6	96
4	1:1	SDS	15	RT	6	94
5	1:1	SDS	10	RT	6	95
6	1:1	SDS	7	RT	12	80
7	1:1	SDS	5	RT	15	64
8	1:1	SDS	5	50	8	68
9	1:1	TBAB	100	RT	10	78
10	1:1	TBAB	100	50	10	76
11	1:1	TBAB	200	100	10	80
12	1:1	CTAB	100	RT	10	66
13	1:1	CTAB	200	100	10	68
14	1:1	CPC	100	RT	10	78
15	1:1	CPC	200	100	10	80
16	1:1	TBAH	100	RT	10	76
17	1:1	TBAH	200	100	10	78
18	1:1	TBAI	100	RT	10	68
19	1:1	TBAI	200	100	10	74
20	1:1	SDBS	100	RT	8	82
21	1:1	SDBS	50	RT	8	80
22	1:1	SDBS	30	RT	8	64
23	1:1	SDBS	30	50	10	70

[a] % Yield refers to the isolated yield of all the compounds after chromatographic seperation.

Table2 Preperation of quinoxaline derivatives.

Entry	α-Bromo carbonyl compound	Diamine	Time (h)	Product	%Yield
1			6		94
2			6		92
3			6		92
4			6		86
5			6		92
6			6		89
7			6		87
8			7		84
9			6		87
10			8		85
11			7		83
12			6.5		98
13			7		82
14			6		92
15			5		84

% Yield refers to the isolated yield of all the compounds.

Continuation of Table2

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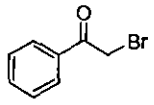
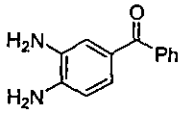
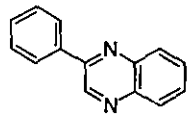
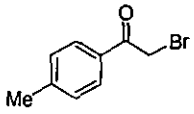
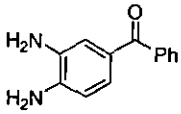
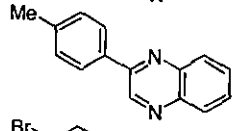
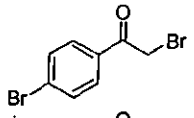
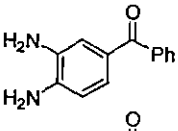
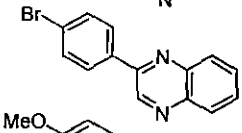
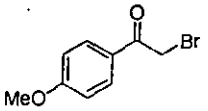
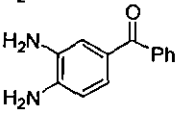
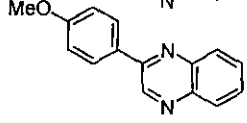
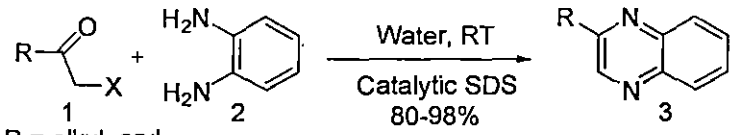
16			6.5		88
17			6		86
18			6		86
19			6		86

% Yield refers to the isolated yield of all the compounds.

Table 3 Recycling experiment using SDS

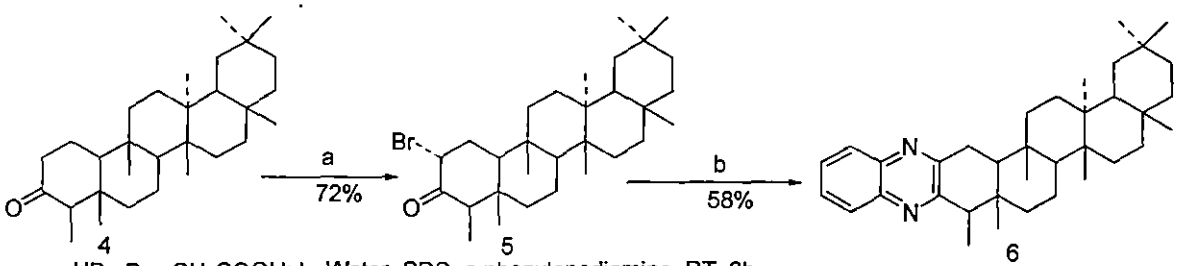
Entry	No. of Cycle	% Yield
1	0	92
2	1	87
3	2	82
4	3	78
5	4	72
6	5	68

% Yield refers to the isolated yield of the c ompound afterchromatography.



R = alkyl, aryl.
X = Br

Scheme 1 SDS catalysed synthesis of quinoxalines in water



a, HBr, Br_2, CH_3COOH; b, Water, SDS, *o*-phenylenediamine, RT, 8h

Scheme 2 Preperation of quinoxaline derivative of friedelin.

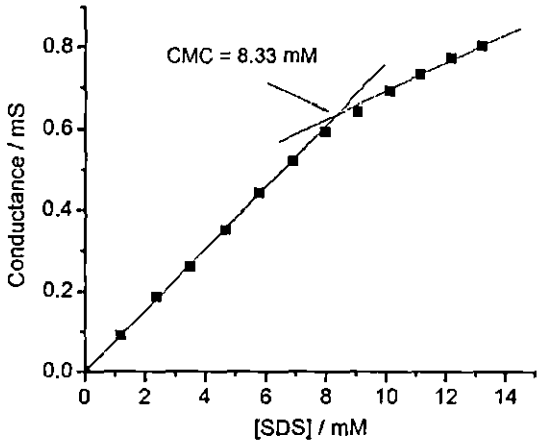


Fig 1 Plot of conductance vs. concentration of SDS for the calculation of CMC value of SDS.

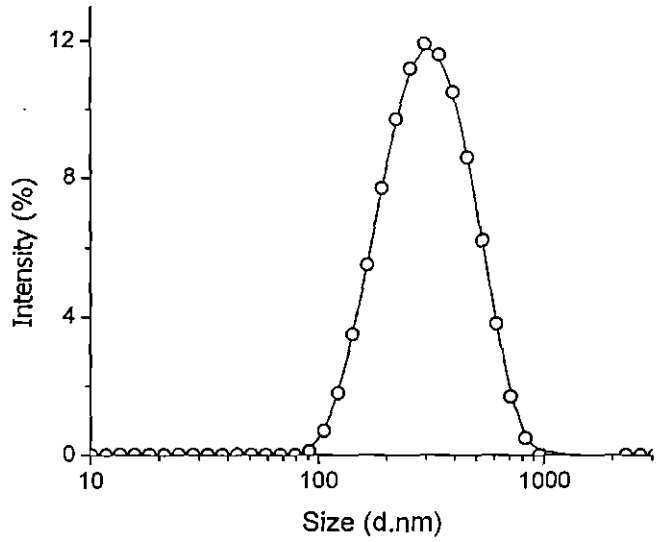
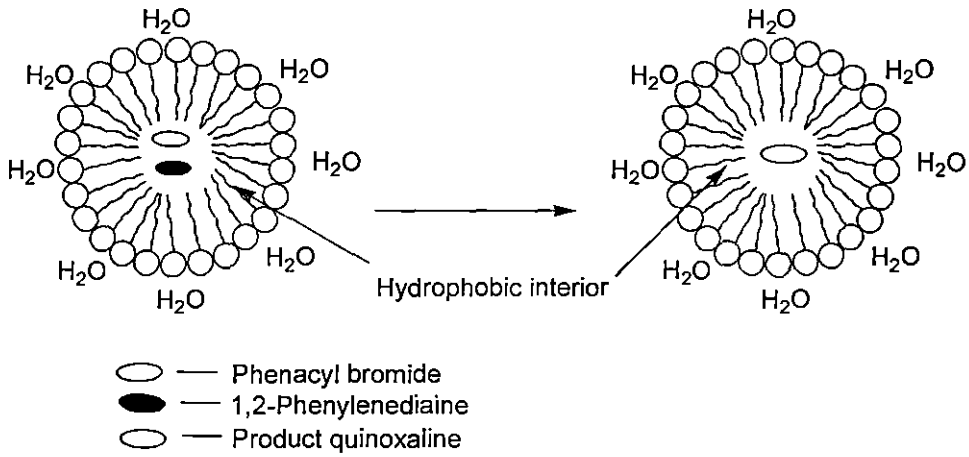
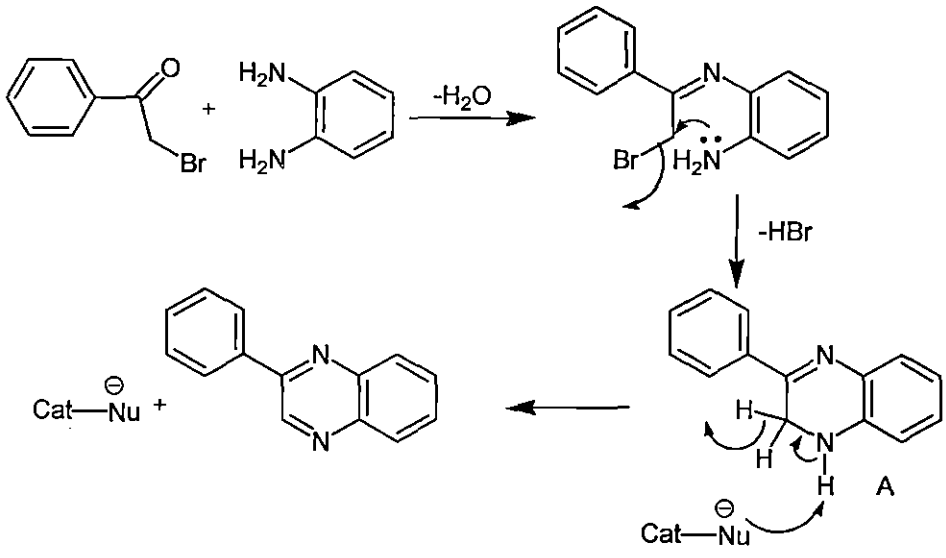


Fig 2 Graph of intensity vs. size (nm) of the micelles based on DLS measurement.



Scheme 3 Proposed model for the synthesis of quinoxaline in water-SDS



Scheme 4 Plausible mechanism of the SDS catalysed quinoxaline formation.

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A New Bioactive Ursane Type Triterpenoid from *Croton bonplandianum* Bail

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Running title: Triterpenoids from croton

ABSTRACT

A new triterpenoid 3α-hydroxy-urs-12,15-dien (**1**) of ursane skeleton along with three others, *viz.* oleanolic acid (**2**) and ursolic acid (**3**) and β-sitosterol (**4**) were isolated from the root of *Croton bonplandianum* Bail. Their chemical structures were established on the basis of spectroscopic analysis (IR, 1D NMR, 2D NMR, Mass *etc.*) and by chemical means. A probable biosynthetic pathway of compound **1** was also proposed. Preliminary investigation with the new compound showed potent activity against a series of fungal pathogens.

Keywords *Croton bonplandianum*, 3α-hydroxy-urs-12,15-dien, antifungal.

1. Introduction

Croton bonplandianum Bail belongs to the family of Euphorbiaceae, is native to southern Bolivia, Paraguay, southwestern Brazil and northern Argentina and was reported from India during late 1890 by Kaul.[1] It now occurs widely along roadsides, railway abandoned field in wide open ravines, and paddy or sugarcane fields and on sandy or sandy clay soils. Due to the resemblance of the leaves and flower cymes to that of Tulsi, this plant is often called Ban Tulsi locally. *C. bonplandianum* was reported to have many medicinal uses including the repellent property against the insects,[2-5] mosquito, *A. aegypti*.[6] Local people in the remote area of West Bengal, India are using its root against snake bite and the leaf extract against high fever. In addition the plant is used both as a fuel and as detergent. The methanol extract of *C. bonplandianum* has been found to exhibit antitumor properties using *Agrobacterium tumefaciens* and has larvicidal activities.

Phytochemically Croton is rich in secondary metabolites including alkaloids and terpenoids.[7,8] Diterpene resins found in many species of croton have been used experimentally in the studies of tumor initiation and conceivably prove to be useful in cancer therapy.[9] Apart from the above limited reports, no systematic study has yet been initiated for the total phytochemical investigation of *C. bonplandianum* Bail.

2. Results and Discussion

During our search towards bioactive natural products in tropical plants,[10] we have investigated the chemical constituents of the MeOH extract of roots of *C. bonplandianum*, which showed potent antifungal activity against a series of fungal and bacterial pathogens. The investigation yielded the isolation of a new triterpene 3α-

hydroxy-urs-12,15-dien (**1**), along with two known triterpenoid oleanolic acid (**2**), ursolic acid (**3**) and most abundant β-sitosterol (**4**). All the compounds (**1**, **2** and **3**) have been screened for their antimicrobial activity.

The MeOH extract of the roots of *C. bonplandianum* was suspended in water, partitioned successively between hexane, EtOAc and n-BuOH to get four fractions (hexane phase, ethylacetate phase, n-butanol phase and water phase). Since results of preliminary screening with the EtOAc soluble fraction was found to be most active against the fungal strain used, it was purified (through repeated column chromatography over silica gel) to isolate the active ingredients *viz.* 3α-hydroxy-urs-12,15-dien (**1**), along with two known triterpenoids, oleanolic acid (**2**) and ursolic acid (**3**) and most abundant β-sitosterol (**4**). The structures of all the compounds were elucidated on the basis of chemical and spectroscopic analysis and by comparison (for compounds **2**, **3** and **4**) with the spectral data of already reported compounds.

The elucidation of the structure of the new compound was accomplished by extensive analysis of its spectral data. Compound **1** was obtained as a white powder of melting point (mp) 244-246 °C, $[\alpha]_D$ +4.0, UV inactive, showed strong absorption band at 3422 (-OH), 2850, 1461, 1376 (gem dimethyl), 1053, 970, 959, 838 cm^{-1} in the IR spectrum indicative of the presence of hydroxyl, olefinic and gem dimethyl groups. ^{13}C NMR spectrum at ambient temperature displayed signals for thirty carbons which were distinguished as eight methyls, seven methylenes, nine methines (five aliphatic, one oxygenated and three olefinic) and six quaternary with the aid of DEPT experiment. The ^1H NMR spectra of compound **1** (Table 1) showed characteristic proton signals due to six tertiary methyl groups at δ_H (proton chemical shift) 0.68 (3H, s, C_{23}-Me), 0.69 (3H, s,

C_{24}-Me), 0.76 (3H, s, C_{25}-Me), 0.84 (3H, s, C_{26}-Me), 1.01 (3H, s, C_{27}-Me), 1.25 (3H, s, C_{28}-Me), and two secondary methyl signals as doublet centered at 0.79 (3H, d, J = 6.3 Hz, C_{29}-Me) and 0.92 (3H, d, J = 6.3 Hz, C_{30}-Me). The assignment of the signals of the methyl groups and the remaining ^{1}H and ^{13}C signals were performed through analysis of the HSQC, HMBC and COSY experiments and the results were found consistent with pentacyclic triterpene skeleton and its molecular formula was suggested to be $C_{30}H_{48}O$ on the basis of ESIMS and TOF MS data [m/z = 424 (M^{+})]. On acetylation it gave crystalline acetate of mp 223-224 °C. The molecular formula of this acetate was suggested to be $C_{32}H_{50}O_2$ [ESIMS data *m/z* = 467 (M+1)]. The difference in mass spectral data of **1** and its acetate indicated the formation of a monoacetate and hence indicated the presence of only one hydroxyl group in compound **1**. IR spectrum of the monoacetate showed peaks at 2850, 1461, 1376 (gem dimethyl), 1248 (-OCOMe), 1053, 959, 970, 838 cm^{-1}. The appearance of a sharp peak at 1376 cm^{-1} in the IR spectra of compound **1** and its monoacetate revealed the presence of a gem dimethyl group in ring A of the pentacyclic triterpenoid skeleton and the concordance of δ_H values in the ^{1}H NMR spectrum for the ring A with that of olean or ursane skeleton clearly rejected the possibility of the presence of friedelan skeleton. Mass fragmentation of the compound also followed the same pattern as was observed for typical pentacyclic triterenoids.[11] A possible mass fragmentation pattern is depicted in scheme 1.

The comparison of the chemical shift positions (δ_H) of the A, B and C rings of **1**, especially the resonances of the methyl groups and two secondary methyl signals on ring E provides the most useful indicator for the presence of ursane type triterpenoid skeleton.[12] A hydroxy methine signal at δ_H 3.5 (1H, m, H-3) and a trisubstituted olefinic

signal at δ_H 5.33 (1H, d, J = 2.7 Hz, H-12). However the splitting nature and small J value may be considered by the consideration of long range coupling involving H-12 and H-19, both are closer in space. The through space distance is obviously more important than the number of intervening bonds, suggesting that **1** is a 3-hydroxy-urs-1,2-en type triterpenoid without possessing angular carboxyl group at C-28.[10] Additionally **1** showed an AB quartet (>CH=CH<) centered at δ_H 5.11 (2H, J = 15.1 Hz and 8.5 Hz, H-15 and H-16) signifying the presence of another olefinic double bond having two olefinic protons that must be attributed to the vinylic protons of a disubstituted double bond in a six membered ring.[13,16]

^{13}C NMR spectrum showed all the 30 carbons and thereby confirmed the presence of a triterpene skeleton (Table 1). Four downfield signals at δ_C 121.7 (C-12), 140.7 (C-13), 129.2 (C-15) and 138.3 (C-16) indicated the presence of four sp^2 hybridized carbons. The signals at δ_C 121.7 and 140.7 are characteristic for a C-12/C-13 double bond in the ursane type structure.[13] The signal at δ_C 140.7 is due to the presence of a trisubstituted olefinic carbon at C-13 and peaks at δ_C 138.3 and 129.2 were indicative of the presence of another double bond having two disubstituted olefinic carbon. The ^{13}C values for these two carbons (δ_C 138.3 and 129.2) clearly removed the possibility of any trisubstituted double bond. C-3 carbon appeared at δ_C 71.8 whereas for compounds **2** and **3** it appeared at δ_C 78.2, i.e. a shielding of seven units that signifying a stereochemical change at C-3. Therefore, for compound **1** the hydroxyl group is alpha instead of beta, i.e. it is an 3-epi-urs-1,2-en type triterpenoid. On the other hand, the chemical shift of C-18 (δ_C 55.9) in compound **1** was very close to that in compound **3** (δ_C 53.6), which indicated that these two compounds have the same configuration at C-18. Eight methyl signals appeared at δ_C

21.1 (C-23), 29.1 (C-24), 18.7 (C-25), 19.8 (C-26), 24.3 (C-27), 19.4 (C-28), 19.0 (C-29) and 23.0 (C-30). Assignments of all the carbons were made by comparison with that reported in literature for ursane skeleton[14] and are tabulated in table 1.

The structure of compound **1** was finally established by 2D NMR experiments. The methyl singlet at δ_H 1.25 was correlated with a carbon signal at δ_C 19.4 in the HMQC spectrum were assigned to H_3-28 from HMBC correlation observed from H_3-28 (δ_H 1.25) to C-17 (δ_C 45.8). Another Methyl singlet at δ_H 0.84 (3H, s, C_{26}-Me), was correlated with carbon singlet at δ_C 19.8 in the HMQC spectrum, were assigned to H_3-26 from the HMBC correlation observed from H_3-26 (δ_H 0.84) to C-8 (δ_C 39.7). The presence of a trisubstituted olefin between C-12 and C-13 was revealed by the HMBC correlations observed for H_2-11/C-12, H_2-11/C-13, H-18/C-13 and H_3-27/C-13. Finally 2D NMR techniques were applied to locate the exact position of another double bond. Both the COSY and HMBC spectra gave significant information regarding the position of the double bond between C-15 and C-16. The COSY spectrum revealed two significant correlations of H-18 at δ_H 2.26 (1H, d, J = 4.3 Hz) with a proton at δ_H 5.13 (1H, AB q, J = 15.1 and 8.5 Hz) and a proton at δ_H 5.01 (1H, AB q, J = 15.1 and 8.5 Hz). In the HMBC spectrum cross peaks were observed due to H_3-27 at δ_H 1.01 (s, 3H)/δ_C 129.2 and H_3-28 at δ_H 1.25 (s, 3H)/δ_C 138.3. Two olefinic protons at δ_H 5.13 (1H, AB q, J = 15.1, 8.5 Hz) and δ_H 5.01 (1H, q, J = 15.1, 8.5 Hz) in effect showed correlations in COSY spectrum. From the above data it was confirmed that compound **1** has a second double bond between C_{15} and C_{16}. The appearance of such an AB quartet for such type of double bond in ring D of pentacyclic triterpenoid, between C_{15} and C_{16}, was reported in literature.[15,16]

Thus on the basis of the above data the structure of compound **1** was established as 3α-hydroxy-urs-12,15-diene.

Spectral analysis (IR, NMR, Mass *etc.*) of the prepared acetate derivative provide further evidence in favor of the proposed structure of compound **1** (3α-hydroxy-urs-12,15-diene). Mass spectrum showed the incorporation of only one acetyl group (at m/z 467) and thus confirming the presence of only one hydroxyl group. IR spectrum of the monoacetate showed peak at 1248 cm^{-1} in addition to other peaks for the acetate group. In the ^1H NMR spectrum acetate methyl appeared as a singlet centered at δ_H 2.33 (3H, s) and the C_3-H shifted downfield (δ_H 4.61, m, 1H) in comparison to the same (δ_H 3.52, m, 1H) for the hydroxyl methine part in compound **1**. The ^{13}C spectrum showed all the carbons for the monoacetate derivative. C-3 appeared at δ_C 74.0 (shifted downfield with respect to that of the original compound, **1**) and the acetate carbonyl signal appeared at δ_C 170.5. The DEPT and COSY spectra of the acetylated derivative were in good agreement to that proposed for the acetylated derivative (**5**) and thus established the structure of compound **1** as 3α-hydroxy-urs-12,15-dien.

Purification of compound **2** yielded a white powder of mp 306-308 °C and its molecular formula was suggested to be $C_{30}H_{50}O_3$ on the basis of FAB MS data [m/z = 458.2 $(M)^+$] and Its structure was elucidated as oleanolic acid by ^1H and ^{13}C NMR data and that already reported in literature.[17]

Compound **3** was also obtained as a white powder of mp 280-282 °C. Its molecular formula was suggested to be $C_{30}H_{50}O_3$ on the basis of FAB MS data [m/z = 458.5 $(M)^+$ and was finally identified as ursolic acid by ^1H and ^{13}C NMR data and that already reported in literature.[17]

Purification of the most polar fraction (8% ethyl acetate in petroleum ether) afforded white crystals of mp 136-137°C, M^+ 414, and was identified as β-sitosterol **4** by spectral analysis and by comparison with an authentic sample of β-sitosterol (mixed m.p., co-IR, and co-tlc etc.).

A probable biosynthetic pathway for the formation of the new triterpenoid **1** has depicted in scheme 2 on the basis of the formation of α-amyrin.[18] This is followed by the formation of a transient carbanion intermediate (S3) and its subsequent rearrangement to S5. Catalytic role of $NADP^+$ present in cells/tissue was then suggested for the formation of **1**.

All the compounds (**1**, **2** and **3**) isolated from the ethyl acetate soluble fraction were tested for their antifungal activity by the disc diffusion method (Table 3) against the microorganisms which are very much native to North Bengal,[19] India; viz. *Calletotricheme camellie*, *Fussarium equisitae*, *Alterneria alternate*, *Curvularia eragrostidies* and *Colletrichum Gleosproides.* These fungal pathogens are responsible to cause wilt disease to tomatoes, pine apple *etc.* cultivated traditionally in this region. The MIC values including that of reference sample Bavistin are tabulated in table 1. The MIC values of Bavistin against these fungal pathogens were also determined. A comparison of antifungal activities of compound **1, 2** and **3** to that of Bavistin showed that although they are less active compare to Bavistin, but all of them exhibited moderately good activity against all the fungal pathogens tested.

3. Conclusion

A new triterpenoid of ursane skeleton has been isolated from the root of *C. bonplandianum* and characterized as 3α-hydroxy-urs-12,15-dien (**1**) along with two

known triterpinic acids, oleanolic acid (**2**) and ursolic acid (**3**). β-sitosterol (**4**) was also obtained as the most polar fraction of the ethylacetate phase. Antifungal potentiality of compound **1** was also detected against five different fungal pathogens. A plausible biosynthetic pathway was also suggested.

4. Experimental

4.1. General experimental procedure

Melting points were determined by open capillary method and were uncorrected. IR spectra were measured on Shimadzu 8300 FT-IR spectrophotometer. NMR spectra were recorded on Bruker-Avance 300 and 400 MHz FT-NMR spectrometer. ESIMS was obtained on Applied Biosystem API 2000 and FAB MS were obtained on Jeol SX 102 mass spectrometer.

4.2. Plant Material

Plants of *C. bonplandianum* used in this experiment were collected from North Bengal, India in May, 2008.

4.3. Extraction and Isolation

The air dried roots of *C. bonplandianum* (2 Kg) was chopped into small pieces and extracted with MeOH (2 L) in a soxhlet extractor for 7 days and MeOH was recovered in *vacuo*. The extracted mass (350 g) was suspended in distilled water (1L) and was partitioned successively between hexane (300 x 3), EtOAc (300 x 3) and n-BuOH (300 x 3) to obtain four fractions (hexane phase, ethyl acetate phase, n-butanol phase and water phase). The ethyl acetate phase (1.5 g) was purified by repeated column chromatography (silica gel) using petroleum ether (PE) and PE:ethyl acetate of varying concentrations as eluent.

4.4. Preparation of acetyl derivative of compound 1

Compound **1** (100 mg, 0.23 mmol) in a 50 mL round bottom flask was warmed over a water bath with 10mL of pyridine and 2 mL of acetic anhydride for 6 hours. The reaction mixture was then poured into 50 mL of ice cold water, worked up with ether, dried over anhydrous sodium sulfate. The crude product was then purified over a column of silica gel.

Purification of the gummy material, gave white crystalline monoacetate of mp 223-224 ° C. IR spectrum showed peaks at 2850, 1461, 1376, 1248, 1053, 959, 970, 838 cm^{-1}. In the mass spectrum it showed a molecular ion peak at 467. The ^1H NMR spectrum of the acylated derivative showed characteristic signals due to five tertiary methyl groups at at δ_H 0.68 (3H, s, C-23), 0.69 (3H, s, C-24), 0.76 (3H, s, C-25), 0.84 (3H, s, C-26), 1.01 (3H, s, C-27), 1.25 (3H, s, C-28), and two secondary methyl signals as doublet centered at 0.79 (3H, d, J = 6.3 Hz) and 0.92 (3H, d, J = 6.3 Hz). The acetate methyl appeared as a singlet centered at 2.33 (3H, s).

4.5. Bioassay

Suitable fungal strains were procured from the microbiology laboratory of our institute. DMSO (dimethyl sulfoxide) was used as solvent to prepare different concentrations of the triterpenoid. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in petridishes and were incubated at 37 °C for 48 hour. Culture media for fungal pathogens were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber.

5. Acknowledgement

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6. References

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Table 1 1H, 13C chemical shift of compound **1** and 13Cchemical shifs of **1**[a], **2** and **3**

	1		**1**[a]	**2**[b]	**3**[b]
C	δC	δH	δC	δC	δC
1	40.5	1.03 (m),1.59 (br. s)	40.5	39.0	39.2
2	29.7	1.65 (m), 1.94 (m)	29.7	28.1	28.2
3	71.8	3.50 (m)	74.0	78.2	78.2
4	33.9		33.9	39.4	39.6
5	56.8	0.86 (br. s)	56.7	55.9	55.9
6	18.7	1.39 (m), 1.48 (m)	18.7	18.8	18.8
7	31.9	1.51 (m), 1.82 (m)	31.8	33.4	33.7
8	39.7		39.7	39.8	40.1
9	50.2	1.55 (m)	50.0	48.2	48.1
10	36.5		36.5	37.4	37.5
11	24.3	1.84 (m), 1.98 (m)	24.3	23.8	23.7
12	121.7	5.33 (s, t-like)	122.6	122.6	125.7
13	140.7		139.6	144.8	139.3
14	42.3		42.3	42.2	42.6
15*	129.2		129.2	28.4	28.8
16*	138.3		138.3	23.8	25.0
17	45.8		45.8	46.7	48.1
18	55.9	2.26 (d, J = 4.3 Hz)	55.9	42.1	53.6
19	56.0	2.21 (m)	56.0	46.6	39.5
20	56.7	1.43 (br. s)	56.6	31.0	39.4
21	31.6	1.52 (d J = 5.6 Hz), 2.26 (d, J = 5.6 Hz)	31.8	34.3	31.1
22	37.2	2.01 (br. s), 2.21 (br. s)	36.9	33.2	37.4
23	21.1	0.68 (s)	21.1	28.8	28.8
24	29.1	0.69 (s)	29.1	16.5	16.5
25	28.2	0.76 (s)	28.2	15.6	15.7
26	19.8	0.84 (s)	19.8	17.5	17.5
27	26.0	1.01 (s)	26.0	26.2	24.0
28	19.4	1.25 (s)	19.3	180.0	179.7
29	19.0	0.79 (d, J = 6.3 Hz)	19.0	33.4	17.5
30	23.0	0.92 (d, J = 6.3 Hz)	23.0	23.8	21.4

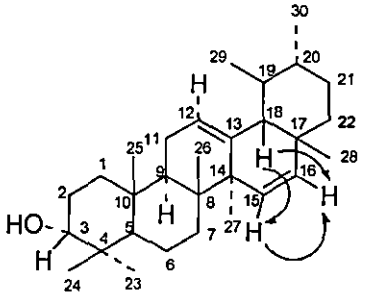
[a] Denotes acetyl derivative of compound **1**; [b] measured in pyridine d_5 * Olefinic hydrogens attached to these carbons appeared as an AB q centered at 5.11 ppm with J = 15.1 and 8.5 Hz.

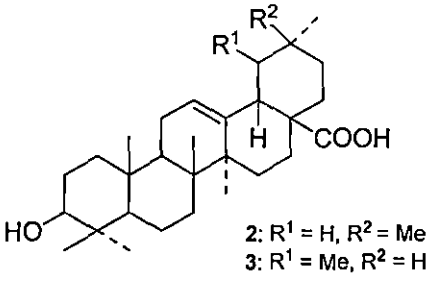
Table 2 1H-13C long range correlation detected in the HMBC spectra of compound **1** (δ relative to TMS in $CDCl_3$)

Proton	Correlated C
1.01 (H-27)	39.7 (C-8), 140.7 (C-13), 42.3 (C-14), 129.2 (C-15), 50.1 (C-9), 128.3 (C-16)
0.84 (H-26)	39.7 (C-8), 42.3 (C-14)
1.25 (H-28)	45.8 (C-17)
0.68 (H-23)	33.9 (C-4), 56.8 (C-5), 71.8 (C-3)
1.55 (H-9)	36.5 (C-10), 39.7 (C-8), 19.8 (C-26), 28.2 (C-25)
5.33 (H-12)	50.1 (C-9), 42.3 (C-14), 55.9 (C-18)
2.26 (H-18)	121.7 (C-12), 140.7 (C-13), 42.3 (C-14), 128.3 (C-16), 45.8 (C-17), 56.0 (C-19), 56.7 (C-20), 19.4 (C-28)

Table 3 MIC of Compound **1** against different fungi

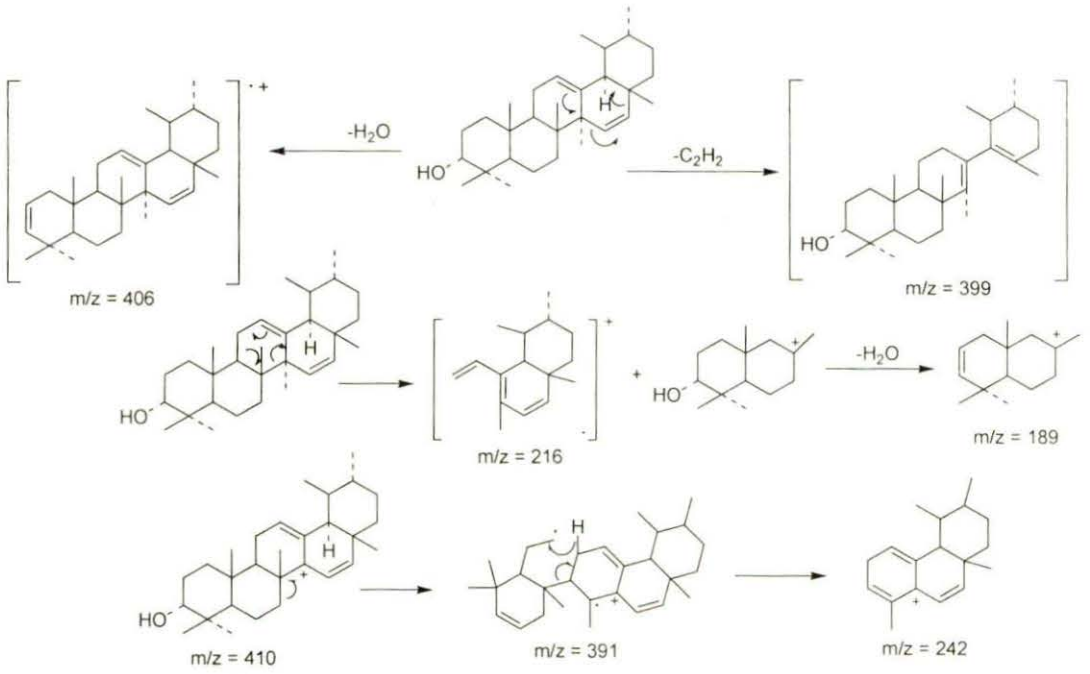
Compound	MIC of **1** in µg/mL against different fungi				
	CC	CG	AA	FE	CE
1	10	<15	10	<10	15
2	<10	20	10	<10	10
3	15	<10	10	<15	<15
Bavistin	2.5	1.25	2.5	2.5	<2.5



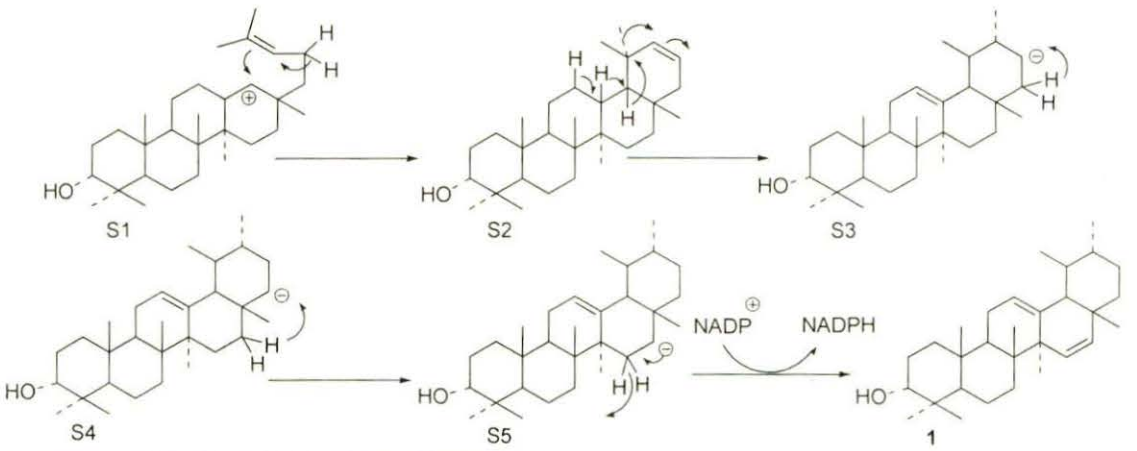


2: R^1 = H, R^2 = Me
3: R^1 = Me, R^2 = H

Key COSY correlation of compound **1**



Scheme 1 Proposed mass fragmentation pattern of compound **1**



Scheme 2 Probable biosynthetic pathway of compound **1**