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B. Poornima, A. Venkanna, B. Swetha, Karthik reddy Kamireddy, Bandi Siva, V.S. Phani, Ramesh Ummanni, K. Suresh Babu

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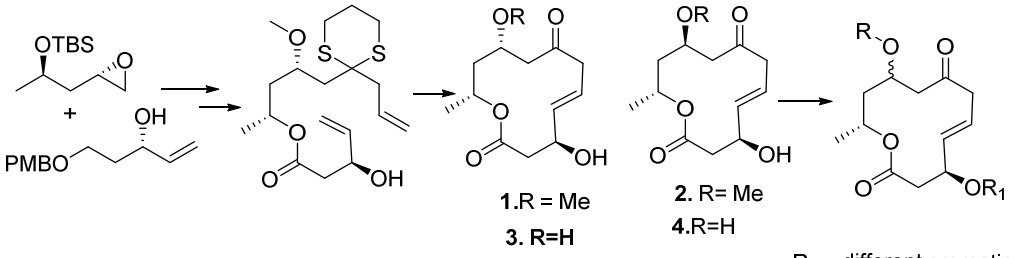
Graphical abstract

Total Synthesis, Biological Evaluation of Dendrodolides A-D and their Analogues.

B. Poornima,[a] A. Venkanna,[a] B. Swetha,[b] Karthik reddy Kamireddy,[b] Bandi Siva,[a] V.S.Phani,[c] Ramesh Ummanni[b]* K. Suresh Babu*[a]



1.R = Me
3. R=H

2. R= Me
4.R=H

Dendrodolide A -D(**1-4**)

R_1 = different aromatic acids
R = Me

Total Synthesis, Biological Evaluation of Dendrodolides A-D and their Analogues†

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[a]*Division of Natural Products Chemistry, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India.*

bCentre for Chemical Biology, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India.

[C] *Centre NMR and Structural Chemistry, CSIR-Indian Institute of Technology, Hyderabad-500 007, India*

E-mail: suresh@iict.res.in; Fax: +91-40-27160512

†*Electronic supplementary information (ESI) available: Copies of* ^{1}*H NMR and* ^{13}*C NMR spectra of all the new compounds.*

* Corresponding author: E-mail: suresh@iict.res.in

Tel: +91-40-27191881, Fax: +91-40-27160512.

Abstract:

A concise total synthesis of dendrodolides A–D (**1-4**) has been accomplished in10 steps from commercially available (R)-propylene oxide and 3-buten-1-ol as starting materials. The key steps involved in the synthesis are Jacobsen hydrolytic kinetic resolution, epoxide ring opening with 2-allyl-1, 3-dithiane, Yamaguchi esterification and ring-closing metathesis (RCM). In addition, a series of ester derivatives were prepared utilizing Yamaguchi esterification at the C-3 position of the dendrodolide core and screened for their efficacy against cancer cell lines.

Keywords: Macrolides, Yamaguchi esterification, RCM approach, Jacobsen hydrolytic kinetic resolution, Analogues, Cytotoxic activity, SAR studies.

Introduction

Marine microorganisms have been proven to be a rich source of innumerable secondary metabolites with novel structures and wide-range of biologically properties,[1] particularly, half of them were from fungal genera,[2] such as Aspergillus, Penicillum, Alternaria, and Cladosporium, are a significant source of new secondary metabolites from marine-derived fungi.[3-7] Dendrodolides are a group of naturally occurring macrolides isolated from Dendrodochium sp., a fungus associated with the sea cucumber Holothuria nobilis Selenka[8] .The gross structures were established by extensive, IR, mass spectroscopy and NMR (1D and 2D) studies. Structurally, dendrodolides contains three chiral centers (C-3, C-9 and C-11) and unusual 11-membered lactone ring that represent a prominent member of lactone ring family of natural products (Figure 1).

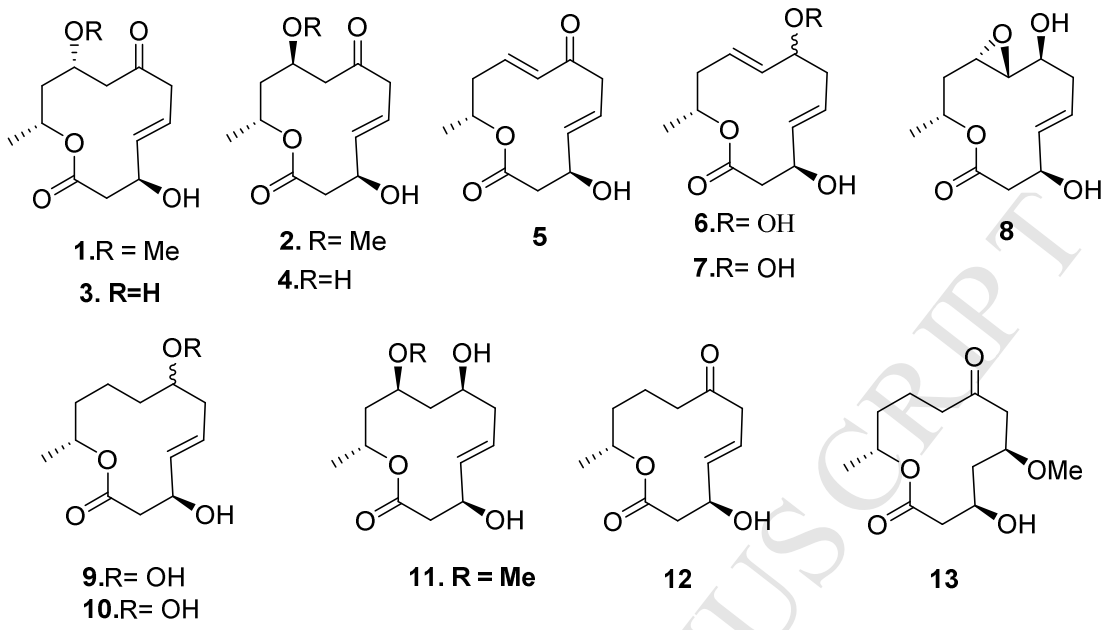
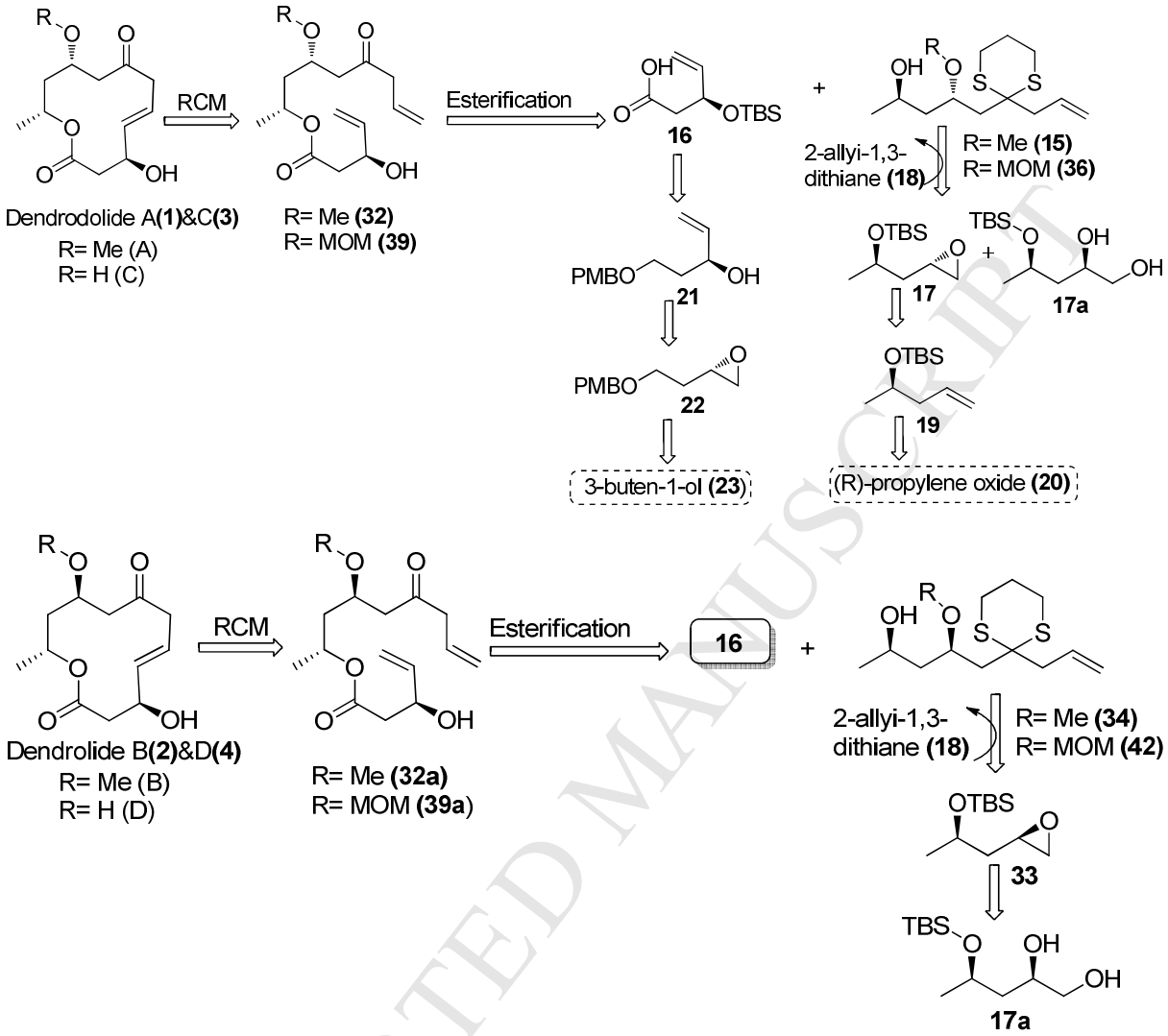


Figure 1.Structures of dendrodolides **A-M** (**1-13**).

It was further shown that dendrodolide A –D (**1-4**) exhibited in vitro cytotoxicity against the tumor cell line SMMC-7721with IC50 value of 19.2 µg/mL [9]. Taken together, these attributes led to the considerable interest in the dendrodolides as target for the total synthesis. As part of our continuing efforts for the synthesis of bio-active macrocyclic natural products,[10] recently, we have reported the first total synthesis of dendrodolide A.[11] Thus, synthetically challenging structures of these macrolides combined with their biological profiles have encouraged us to continue our work for the exploration of new synthetic routes for these dendrodolide series. Herein, we report efficient total synthesis of dendrodolides A–D (**1-4**) by utilizing Jacobsen hydrolytic kinetic resolution, Yamaguchi esterification and ring-closing metathesis (RCM) as key steps. In addition, we also prepared a series of ester derivatives by introducing the various acids and assessed them for their cytotoxic activity. To the best of our knowledge this is the first report on the synthesis of dendrodolide analogues.

Results and discussion

As outlined in scheme-1, we envisioned retro synthetically that all the dendrodolides can be obtained via RCM reaction (Scheme 1). Thus, disconnection of the C-C double bond revealed the bis terminal olefins **32, 39, 32a, 39a** as potential key intermediates. A subsequent disconnection of olefinic intermediates through Yamaguchi esterification converts it into common acid fragment **16** and alcohol fragments **15, 36, 34** and **42**. These alcohol fragments envisaged by regioselective ring opening of epoxide (**17, 33**) with 2-allyl-1, 3-dithiane (**18**), the epoxide (**17**) could, in turn be accessed from ring opening of (R)-propylene oxide (**20**) with vinyl magnesium bromide through epoxidation followed by Jacobsen hydrolytic kinetic resolution. The epoxide (**33**) could, in turn be accessed from **17a**, which is subunit of Jacobsen resolution reaction. On the other hand, common acid fragment (**16**) could be obtained by stepwise transformations from commercially available 3-buten-1-ol (**23**) through epoxidation and Jacobsen resolution reactions. Thus, our present total synthesis will be highlighted by utilization of Jacobsen hydrolytic kinetic resolution which directs the construction of the stereocentres at C-3, C-9, C-11 and Grubb's ring closing metathesis for the formation of 12-membered lactone ring. Furthermore, the simplicity of the precursors of this route makes it attractive for the library synthesis of the dendrodolides for the biological screening.

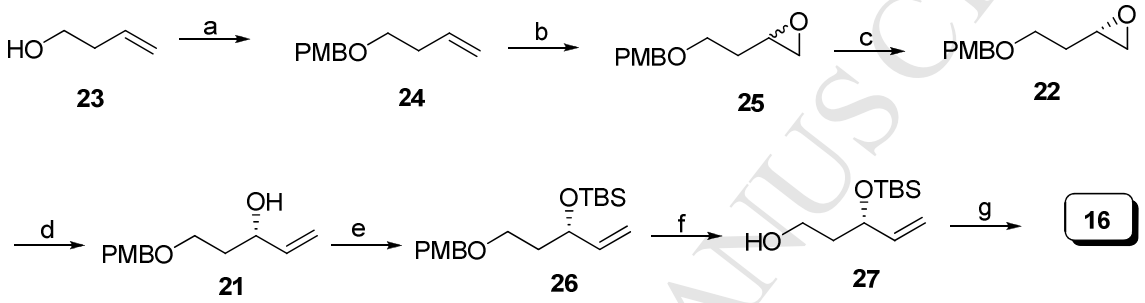


Scheme 1: Retrosynthetic analysis of dendrodolides **A-D (1-4)**.

Synthesis of common acid fragment (16):

Our synthesis commenced with the preparation of acid fragment **16** as a common key intermediate, as iilustrated in scheme-2. The readily available starting material 3-buten-1-ol (**23**) initially protected as PMB ether using PMBBr, NaH and a catalytic amount of TBAI in DMF to afford 24[12] in 96% yield. The compound **24** was subjected to m-CPBA epoxidation to give racemic epoxide **25** [13], which was further subjected to Jacobsen's hydrolytic kinetic resolution using the (S, S)-catalyst to give optically pure epoxide (S)-**22**[14] in 44% yield (determined by

chiral HPLC). The regioselective ring opening of epoxide[15] **22** with trimethylsulfonium methylide ($Me_3S^+I^-$) and n-BuLi in THF afforded the allylic alcohol **21** in 92% yield. Subsequent protection of alcohol functionality in **21** with TBSCl and imidazole in DCM to afford **26**[16] in 96% yield. Finally, deprotection of PMB group with DDQ[15] followed by oxidation with TEMPO/BAIB in CH_2Cl_2:H_2O (1:1) afforded acid **16**[17,18] in 92% yield.

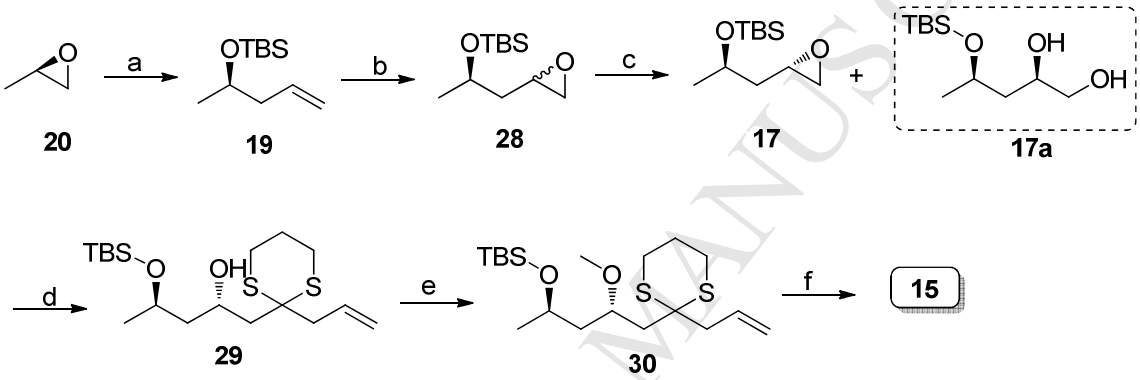


Scheme 2. Reagents and conditions: (a) PMBBr, NaH,TBAI cat., DMF, 0 °C to rt, 12 h, 96%; (b) *m*-CPBA, CH_2Cl_2, 0 °C to rt, 12 h, 89%; (c) (*S*,*S*)-(salen)-Co^{III}·OAc (0.5 mol%), H_2O (0.55 equiv.), 0 °C to rt, 18 h, 44% for 22; (d) $Me_3S^+I^-$, n-BuLi, THF, −20 °C, 4 h, 92%; (e) TBSCl, imidazole, DCM, 0°C to rt, 96%; (f) DDQ, CH_2Cl_2 : P^H 7 buffer (9 : 1), 0 °C to rt, 1 h, 92%; (g) TEMPO, BAIB, CH_2Cl_2 : H_2O (1:1), 0 °C to rt, 2 h, 92%.

Synthesis of alcohol fragment:

We next targeted for synthesis of alcohol fragment **15** as illustrated in scheme-3. The synthesis was intiated with (R)-propylene oxide **20** as a chiral synthon, which was readily prepared from racemic-propylene oxide through Jacobsen hydrolytic kinetic resolution[19] (HKR) using (R, R)-(salen)-CoIII OAc. Thus, treatment of (R)-propylene oxide (**20**) with vinyl magnesium bromide in the presence of CuI afforded homoallylic alcoholin 89% yield[20], in which the secondary hydroxyl group was protected as its TBS ether[19] using TBSCl, imidazole, in DCM to give **19** in 98% yield. Epoxidation of **19** with m-CPBA afforded a mixture of two diastereomers **28** (anti:syn/3:1), which was then subjected to Jacobsen hydrolytic kinetic resolution (HKR)

method with (S, S)-(salen)-CoIII . OAc complex (0.5 mol %) and water (0.55 equiv) in THF (0.55 equiv) to afford the diastereomerically pure epoxide **17** [19] in 45% yield (>94% ee) (determined by chiral HPLC) and diol **17a** in 47% yield. Ring opening of epoxide **17** with 2-allyl-1,3-dithiane (**18**) [21] yielded compound **29**[22] in 87% yield. Subsequent methylation of alcohol **29** using MeI/NaH [22] afforded **30** in 98% yield. Finally, removal of TBS group with TBAF furnished the required alcohol **15**[23] in 96% yield.

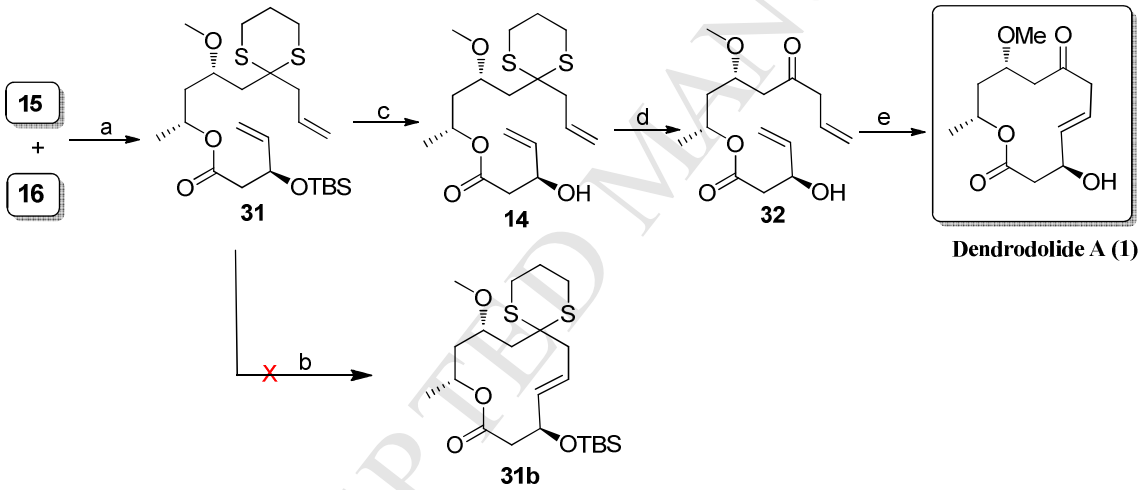


Scheme 3. Reagents and conditions: (a) (i) vinyl magnesium bromide, CuI, THF, -78°C to -20 °C, 12 h, 89%; (ii) TBDMSCl, imidazole, CH_2Cl_2, 0 °C to rt, 4 h, 98%; (b) *m*-CPBA, CH_2Cl_2, 0 °C to rt, 12 h, 92%, (c) (*S,S*)-(salen)-Co^{III}. OAc (0.5 mol %), H_2O (0.55 equiv.), THF, 0°C to rt, 18 h, 45% for **17**, 47% for **17a**; (d) **18**, *n*-BuLi, dry THF,–78 °C to –20 °C, 2 h, 87%; (e) NaH, MeI, THF, 0 °C to rt, 5 h, 98%; (f) TBAF, THF, 0°C to rt, 4 h, 96%.

Synthesis of dendrodolide A (1):

With the two fragments **15** and **16** in our hand, we next targeted for the connection of these fragments (Scheme 4) and subjected the Yamaguchi esterification [24] protocol (2, 4, 6-trichlorobenzoyl chloride, Et_3N, THF, DMAP, Toluene). The highly efficient reaction provided the key intermediate **14** in good yields. We then planned for ring-closing metathesis (RCM) reaction of **31** with the Grubbs second generation catalyst (G-II, 25 mol %) to access the macrolide core. However, the reaction did not progressed[25] and the starting material was completely recovered. At this stage, we investigated the alternative strategy, viz., deprotection of

TBS group and 1, 3 dithiane then followed by the RCM reaction to achieve the target compound 1. Thus, deprotection of TBS group using TBAF gave **14**[26] with 96% yield. Finally, removal of 1, 3-dithiane group[27] in **14** with $CaCO_3$, I_2, in THF:H_2O (4:1) gave **32** with 92% yield. Subsequent RCM reaction using G-II (25 mol %) in dry DCM (deoxygenated) at reflux temperature yielded dendrodolide A (**1**)[25] in 78% yield and with good E-selectivity (>95%). All the intermediate compounds including the dendrodolide A (**1**) were fully characterized by ^{1}H NMR, ^{13}C NMR, and Mass spectral data. The spectral and analytical data of synthetic dendrodolide A (**1**) were in agreement with the reported natural dendrodolide data.[8]

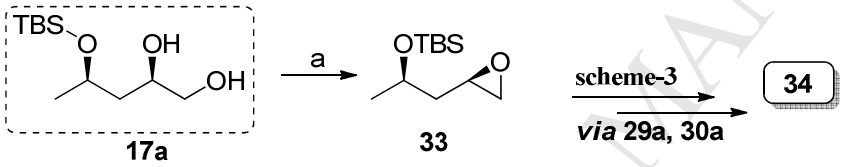


Scheme 4. Reagents and conditions: (a) **16**, 2,4,6-trichlorobenzoylchloride, Et_3N, THF, 0 °C to rt, 2 h, then **15**, DMAP, toluene, 0 °C, 2 h, 98%; (b) G-II (25 mol%), CH_2Cl_2, reflux, 20 h, (c) TBAF, THF, 0^{o}C to rt, 4 h, 96%; (d) $CaCO_3$, I_2, THF:H_2O (4:1), 0 ^{o}C, 20 min, 92%. (e) G-II (25 mol%), CH_2Cl_2, reflux, 12 h, 78%.

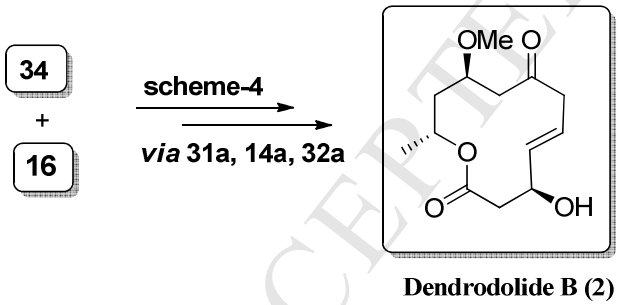
Synthesis of dendrodolide B (2):

The structure of dendrodolide **B (2)** is very similar to the dendrodolide **A (1),** except for the variation of stereo center at C-9. Thus, our strategy for the synthesis dendrodolide-B (**2**) was initiated from diol **17a**, which is the subunit of Jacobsen hydrolytic kinetic resolution (HKR)

reaction [20] of **28** as depicted in scheme 3. The treatment of diol **17a** with tosylimidazole/NaH in THF at 0 °C provided required epoxide (**33**) [28] in 89% yield. The epoxide **33** was further subjected to sequential reactions (opening of epoxide **33** with 2-allyl-1, 3-dithiane (**18**), methylation and TBS ndeprotection) [21-24] as shown in scheme-3, via **29a, 30a**. Finally, the required alcohol **34** was obtained by deprotection of TBS group in **30a** using TBAF in THF afforded **34** in 89% yield. Having both alcohol **34** and acid **16** in hand, construction of the macrocyclic framework was achieved similar to as shown in scheme 6, via **31a**, **14a**, **32a** (which is similar showed in scheme-4). The spectral and analytical data of synthetic dendrodolide **B (2)** were in agreement with the reported natural dendrodolide data.[8]



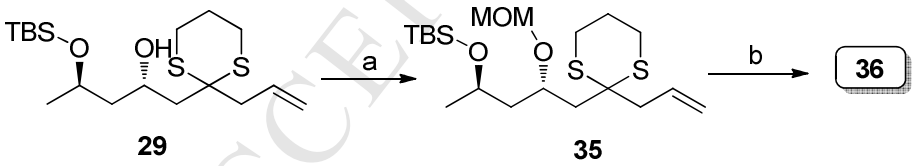
Scheme 5. Reagents and conditions: (a) tosylimidazole, NaH, 0 °C to rt, 1 h, 89%.



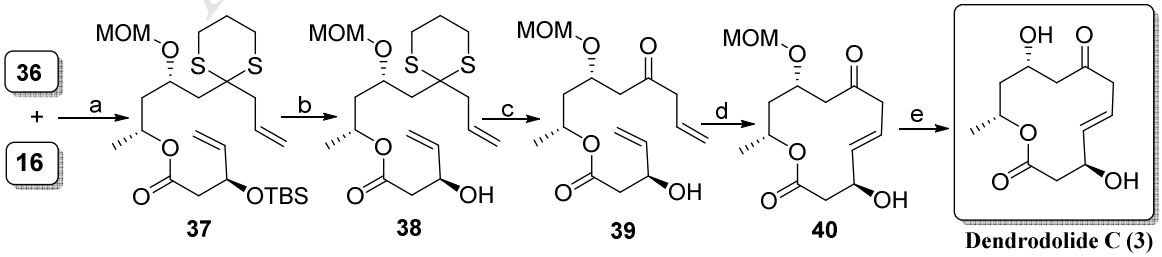
Scheme 6. Reagents and conditions: Reagents and conditions: (a) **16**, 2,4,6-trichlorobenzoylchloride, Et_3N, THF, 0 °C to rt, 2 h, then **34**, DMAP, toluene, 0 °C, 2 h, 95%; (b) G-II (25 mol%), CH_2Cl_2, reflux, 20 h, (c) TBAF, THF, 0^oC to rt, 4 h, 88%; (d) $CaCO_3$, I_2, THF:H_2O (4:1), 0 ^oC, 20 min, 89%. (e) G-II (25 mol%), CH_2Cl_2, reflux, 12 h, 74%.

Synthesis of dendrodolide C (3):

The dendrodolide **C** (**3**) is also similar to the dendrodolide **A** (**1**), with the variation at C-9. Dendrodolide **A** (**1**) having methoxy group at C-9, whereas dendrodolide C (3) has –OH group at C-9. Thus, our strategy for the synthesis of dendrodolide C (**3**) was started from **29**, in which hydroxyl group was protected with MOMCl, in dry DCM to give its corresponding MOM ether **35**[29] in 86% yield. Finally deprotection [26] of TBS group in **35** with TBAF gave alchol **36** in 94% yield (Scheme-7). Having both the alcohol **36** and the acid **16** in hand, construction of the macrocyclic framework was achieved as shown in Scheme 8. The esterification reaction of **36** with acid **16** was carried out under Yamaguchi conditions[24] (2, 4, 6-trichlorobenzoyl chloride, Et3N, THF, DMAP, toluene) to furnish the RCM precursor **37** in 96% yield. The TBS group in compound **37** was deproteced using TBAF in THF to obtain **38** in 97% yield, further removal of 1,3 dithiane group [27] by using CaCO3, I2, THF:H2O (4:1) obtained **39** in 92% yield. The bis olefin **39** was then subjected to the RCM reaction using G-II (25 mol%). in dry DCM (deoxygenated), at reflux temperature yielded macrolide **40** in 68% yield with good E-selectivity (>95%). Finally, removal of MOM[30] in **40** furnished the dendrodolide C (**3**) (Scheme-8).



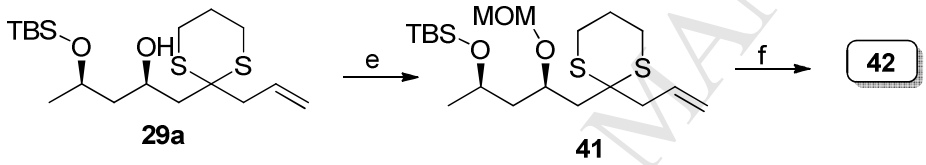
Scheme 7. Reagents and conditions: (a) MOMCl, DIPEA, dry DCM, 0 °C- rt, 12 h, 86%; (b) TBAF, THF, 0^{o}C to rt, 4 h, 94%.



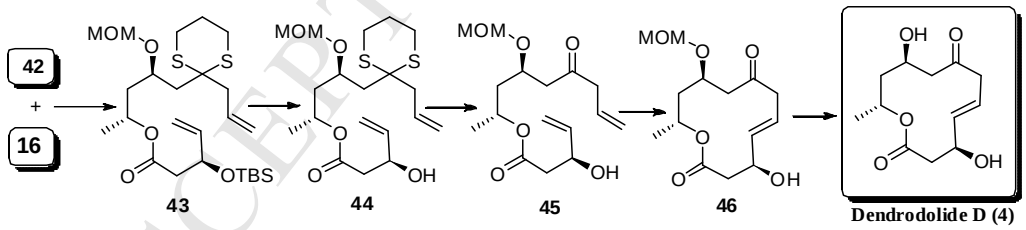
Scheme 8. Reagents and conditions: (a):**16**, 2,4,6-trichlorobenzoylchloride, Et_3N, THF, 0 °C to rt, 2 h, then **36**, DMAP, toluene, 0 °C, 2 h, 96%; (b) TBAF, THF, 0°C to rt, 4 h, 97%; (c) $CaCO_3$, I_2, THF:H_2O (4:1), 0 °C, 20 min, 92%; (d) G-II (25 mol%), CH_2Cl_2, reflux, 20 h, 68%; (e) SMe_2, $BF_3{\cdot}Et_2O$, 0 °C, 1 h, 79%.

Synthesis of dendrodolide D (4):

The synthesis of dendrodolide D (**4**) was achived from **29a**, using similar reactions as mentioned in synthesis of dendrodolide C (**3**), the synthetic route is shown in schemes 9 and 10. All the intermediate compounds including the dendrodolide D (**4**) were fully characterized by ^{1}H NMR, ^{13}C NMR, and Mass spectral data. The spectral and analytical data of synthetic dendrodolide D (**4**) were in agreement with the reported natural dendrodolide data.[8]



Scheme 9. Reagents and conditions: (a) MOMCl, DIPEA, dry DCM, 0 °C- rt, 12 h, 86%; (b) TBAF, THF, 0 °C to rt, 4 h, 94%.

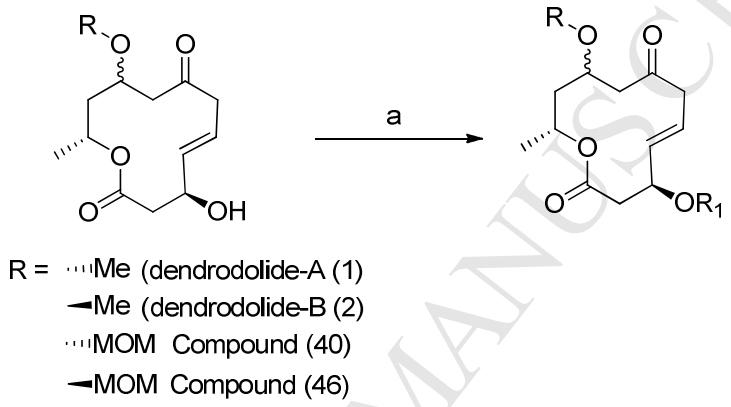


Scheme 10. Reagents and conditions: . Reagents and conditions: (a):**16**, 2,4,6-trichlorobenzoylchloride, Et_3N, THF, 0 °C to rt, 2 h, then **42**, DMAP, toluene, 0 °C, 2 h, 90%; (b) TBAF, THF, 0°C to rt, 4 h, 87%; (c) $CaCO_3$, I_2, THF:H_2O (4:1), 0 °C, 20 min, 82%; (d) G-II (25 mol%), CH_2Cl_2, reflux, 20 h, 68%; (e) SMe_2, $BF_3{\cdot}Et_2O$, 0 °C, 1 h, 72%.

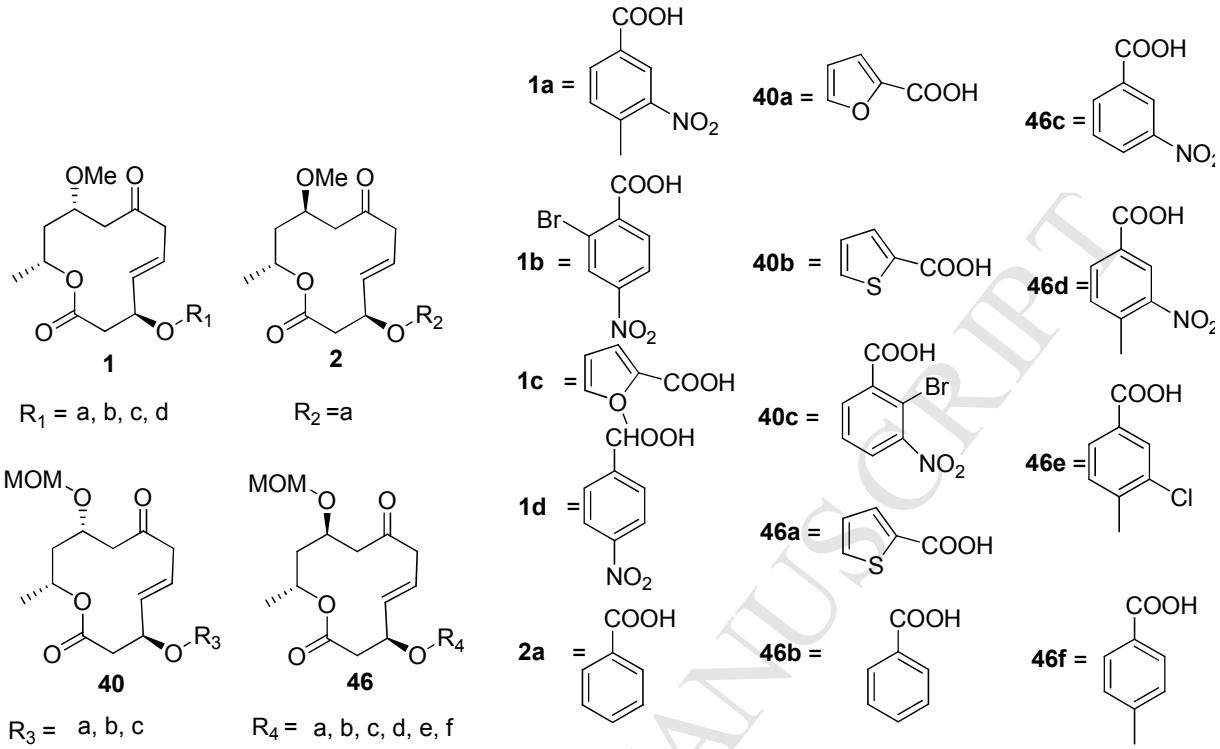
Synthesis of Debdrodolide A-D analogues

Further, in order to improve the activity of Dendrodolides A-D, we planned for the synthesis of derivatives and for an investigation of their structure activity relationships (SARs). It is well

known that introduction of aromatic /aliphatic functionality into organic molecules can often dramatically change their physical and chemical properties.[13] In addition; extension and branching of the hydrocarbon chain may affect their bioactivity. Thus, we synthesized a series of ester derivatives utilizing Yamaguchi esterification[24] (2, 4, 6-trichlorobenzoyl chloride, Et_3N, THF, DMAP, Toluene) at the C-3 position of the dendrodolide core (Scheme-10).



Scheme 11. Reagents and conditions: (a) 2,4,6-trichlorobenzoylchloride, Et_3N, THF, 0 °C-rt 2 h, then acid, DMAP, toluene, 0 °C-rt, 2-4 h, 98%.



Biological activity:

Cytotoxicity evaluation against different cancer cell lines

The ability of Dendrodolide A-D (1-40 and their ester derivatives in inhibiting cancer cell growth were examined against cancer cell lines [prostate cancer cell line (DU-145), Liver carcinoma cell line (HePG2), cervical cancer (HeLa) and Breast cancer cell line (MCF7)] by SRB assay using DMSO and Doxorubicin as negative and positive controls, respectively.[31] Compounds showing showing more than 30% growth inhibition at standard concentration of 10µM were considered as active and those comppounds were further tested to ascertain their IC_{50} values against the cell lines DU145, HeLa, HepG2, MCF7. **Table-1** summarizes their IC_{50} values against panel of human cancer cell lines. Of all compounds analysed, 46b and 46c displayed more cytotoxic effect against DU145 with an IC50 value of 7.7±0.1µM and 7.1±0.4 µM, respectively. The effect of these compounds on DU145 was further tested to study the mode of action. These selected compounds were found to be more cytotoxic against DU145 cell line, the change in morphology

induced by these compounds was also observed. Upon exposure of the cells to the compounds, the inherent morphology of cells was changed and the cells eventually detached from the surface (**Fig 2**). To study the long term effect of the selected compounds on the anchorage-independent proliferative capacity of the DU145, colony formation assay (soft agar assay) mimicking the in vivo environment was performed [32]. From the results of the colony formation assay it was indicated that the selected compounds have an inhibitory effect on proliferating cancer cells. **46b** inhibited proliferation of cells and initiated decrease in the size of individual colonies at 15µM concentration while **46c** showed similar effect at 15µM concentration and at higher concentrations there was significant decrease in the size of the colony (**Fig 3**). A dose dependent inhibition of colony formation ability suggests that these selected compounds affect anchorage independent growth of DU145 cells. As the compounds caused inhibition of proliferation of cells, it was further tested whether the compounds cause any cell death. In order to do so, cell cycle analysis was performed using a FACS flow cytometry. The cells were seeded and treated as above and incubated for 48hrs, upon completion of incubation, FACS for the cells was conducted The resultant histograms from the FACS analysis for the respective compounds show that with increasing concentration of compound, cell population accumulates in the subG1 phase while there is negligible cell population in the G2/M phase. As is shown in the table 3 **46b** and **46c** show a similar effect at higher concentrations of 20µM respectively (**Fig 4**). The results showing increased growth arrest in sub G1 indicates cell death upon exposure to compound in a concentration dependent manner [33](**Table 2**). It has been reported in literature that caspase-3 plays a major role in causing apoptosis and eventual cell death thus taking into consideration the result obtained in the FACS analysis, the activation of caspase-3 was analysed [34]. Caspase-3 is an effective caspase whose activity is influenced by caspase-9[34], thus it was of interest to analyse

the activity of caspase-9. Therefore to determine whether the observed cell death is due to activation of caspases, we have measured activation of key caspases by these selected compounds. This was done using fluorogenic substrate Ac-DEVD-AMC and Ac-DEVD-AFC for caspase -3 and caspase-9, respectively. The results have confirmed the activation of both caspase-3 and caspase-9 in DU145 cells treated with the selected compounds for 48 h continuously. The activation of procaspase-3 is a distinguishing feature of apoptotic cells. A dose-dependent increase in the activity of caspase was observed with different concentrations of the selected compounds as compared to those DU145 cells which were treated with DMSO (**Fig. 5a and 5b**). Activation of caspase-9 is mitochondrial dependant which in turn activates caspase-3 executing apoptotic cell death in cancer (**Fig. 5c and 5d**). Doxorubicin was used as positive control for measurement of caspase activation.

Conclusions

In conclusion, we have accomplished the total synthesis of four dendrodolide A-D (**1-4**) in 10 steps, featuring the Jacobsen catalyst hydrolytic kinetic resolution (HKR), Yamaguchi esterification and ring-closing metathesis reactions (RCM) as key reactions and synthesized their novel analogues. Evaluation for their anticancer activity toward cultured HepG2, DU145, HELA, and MCF7 were carried out. From the series of compounds synthesized in the present study, compounds 46b and 46c affecting prostate cancer cell proliferation by direct targeting cell cycle progression. This from all above experiments it is evident that these two compounds showed more promising anti cancer potential than the other substitutions. We hypothesize that the compounds **46b** and **46c** may target many pro-survival pathways in cancer cells shows aptness to consider this scaffold as basis to develop novel anticancer agent.

Cell Based Assays

Cytotoxicity evaluation against different cancer cell lines

Cellular viability in presence of the compound was tested by performing a Sulforhodamine B Assay (SRB) [31]. Briefly, the cell line of interest was seeded in flat bottom 96- well plate (5000 cells/well) in a medium containing 10% FBS followed by incubating the plate for 20 h in an incubator continuously supplied with 5 % CO2, allowing cells to adhere to the surface of the wells. After 20 hrs the cells were treated with the compound at desired concentration. Working dilutions of 500 µM concentration of the compounds were prepared, of which 2µL aliquot was added to the each well, thereby making the final concentration of compound 0 to 100 µM.DMSO and Doxorubicin (as standard control anti cancer drug) were taken as vehicle and positive controls, respectively. Further, the plates were incubated for another 48 h in the incubator. After 48 h of treatment cells were fixed using 10% TCA for 1 h at 4ºC. The plates were rinsed carefully with MQ water removing TCA and air dried. Then 0.057% SRB solution was added to plates and incubated for 30 mins before washing to remove unbound SRB using 1% acetic acid and the plates were then air dried. 100µL of 10mM Tris Base was added to each well to solubilise the SRB. The absorbance was measured using Tecan Multimode Reader at 510nm. The measure of absorbance is directly proportional to cell growth and is thus used to calculate the IC50 values. In the present study, different types of cancer cell lines i.e., breast cancer (MCF-7), prostate cancer (DU145), Hepatocellular carcinoma (HepG2) and cervical cancer (HeLa) cell lines were tested for the cytotoxic effect of the compounds. Based on the IC50 values obtained, the selected compounds were further assayed to ascertain its effect on prostate cancer cell Line (DU145).

Change in Morphology

As the compounds 46b and 46c were found to be cytotoxic to DU145 cells showing promising IC50 values, its effect on the morphology of cells was further ascertained. Cells were seeded in 24 well plate and incubated for 20 h. Then the cells were treated with increasing concentrations of selected two compounds. After 48 h of incubation, the experiment was terminated and the cells were observed under the microscope and images were captured using Olympus Xi71 microscope.

Colony formation assay

The long term effect of selected compounds on the anchorage-independent growth of DU145 cells was analyzed by soft agar assays as reported previously with minor modifications [32]. In the soft agar assay, base agar was prepared by mixing 1% of agarose (Bacto Agar: Becton, Dickinson, Sparks, MD) with 2 × DMEM with 20% FBS and 2X antibiotics in 6-well plates in order to achieve final concentration of 0.5% of agar in 1X growth medium with 10% serum concentration. After the solidification of the base agar, 2.5 × 104cells were mixed with cultivation medium containing compound at varying concentrations and agar solution to obtain a final concentration of 0.35% agar. This was spread on top of the base agar previously solidified. The plate was incubated for 10 days with periodic replacement of medium and compounds for every 3 days. Over period of time plates were monitored regularly for appearance of colonies. After 10 days of incubation the plates were stained with 0.005% crystal violet solution until colonies turned purple in colour. The excess stain was removed and washed off using MQ water and the colonies were photographed using a Olympus Xi71 microscope

Determination of caspase activity

Caspase-3 and -9 activities were measured by treating the DU145 cells with selected compounds using specific fluorogenic substrates Ac-DEVD-AMC (substrate for caspase-3) and Ac-LEHD-AFC (substrate for caspase-9). DMSO and Doxorubicin were taken as negative and positive

controls, respectively. After 48 h of treatment with selected compounds, harvested cells were lysed directly in caspase lysis buffer (50 mM HEPES, 5mM CHAPS, 5 mM DTT, pH 7.5). The lysates were incubated with the respective substrate (Ac-DEVD-AFC/Ac-LEHD-AMC) in 20mM HEPES (pH 7.5), 0.1% CHAPS, 2mM EDTA and 5 mM DTT at 37 °C for 2 h. The release of AFC and AMC was analyzed by a fluorimeter using an excitation/emission wavelength of 400/505 nm (for AFC) and 380/460 nm (for AMC) which is directly proportional to caspase-9 and -3 activity, respectively. The observed fluorescence values were normalized with total protein concentration estimated by Bradford method

Cell Cycle analysis

DU145 Cells ($1X10^5$ cells per well) were seeded in six-well dishes and incubated for 20 hrs and treated without (Control) or with selected compounds at desired concentrations for 48 hr. The cells of each well were then harvested and fixed with 1 mL 70% ice-cold ethanol at -20^0C overnight. After fixation, the cells were washed in PBS and stained with 500 µL propidium iodide (PI) solution (50µg/mL in PBS) containing 100 µg/mL RNase. The cells were incubated at room temperature for 1 hr in the dark. The resultant PI uptake was analyzed by fluorescence activated cell sorting (FACS Calibur System; BD Bio- science, Erembodegem, Belgium) in a FL-2 fluorescence detector (10000 events were recorded for each condition). Flow cytometry data was analyzed using FCS express 4 software (De Novo Software, Los Angeles, CA).

Table 1: Table shows the IC_{50} values of the selected compounds against the cancer cell lines.

Compound	DU145		MCF7		Hela		HepG2	
	IC_{50}	STD DEV	IC_{50}	STD DEV	IC_{50}	STD DEV	IC_{50}	STD DEV
1b	33.7	0.1	28.0	0.1	27.3	4.6	32.8	1.3
2a	34.2	1.0	43.3	3.3	94.5	3.5	78.9	5.1
40a	11.5	1.1	15.4	2.5	17.4	1.8	41.0	1.0
40c	29.4	1.9	23.1	2.0	46.2	1.9	49.8	1.6
46b	7.7	0.1	9.2	0.6	13.5	1.4	5.8	0.4
46c	7.1	0.4	4.5	0.6	18.0	0.1	27.5	0.3
46d	22.6	3.9	29.7	2.1	63.8	2.0	35.6	1.1
46e	22.5	0.8	40.9	0.4	44.8	4.0	58.9	0.6
46f	16.6	0.9	36.7	0.2	37.8	0.9	38.3	1.1
DOXO	5.6	0.2	4.0	0.4	5.7	0.1	5.7	0.1

Table 2: Selected compounds induced subG1 phase cell cycle arrest in DU145 cells. Cells were treated with varying concentrations of selected compounds (0-20 µM) for 48 h and cell cycle progression was examined by flow cytometry. Table shows the percentage cell fractions in subG1, G0/G1, S and G2/M phases of treated DU145 cells.

		SubG1	G0/G1	S	G2/M
46b	DMSO	2.69	35.74	1.26	6.16
	5µM	19.10	32.62	2.76	4.14
	10 µM	25.85	30.99	1.66	3.29
	15 µM	24.30	26.07	2.51	2.51
	20 µM	32.29	32.45	2.11	3.05
46c	DMSO	2.69	35.74	1.26	6.16
	5µM	23.62	33.08	2.26	3.66
	10 µM	26.16	22.40	1.62	3.23
	15 µM	34.69	23.59	1.60	2.82
	20 µM	39.34	15.82	1.32	2.02

Figure 2: DU145 cell were treated with selected compound at indicated concentration and DMSO. Upon exposure of these compounds to DU145 cells the extent of change in cell morphology is observed with increasing concentration.

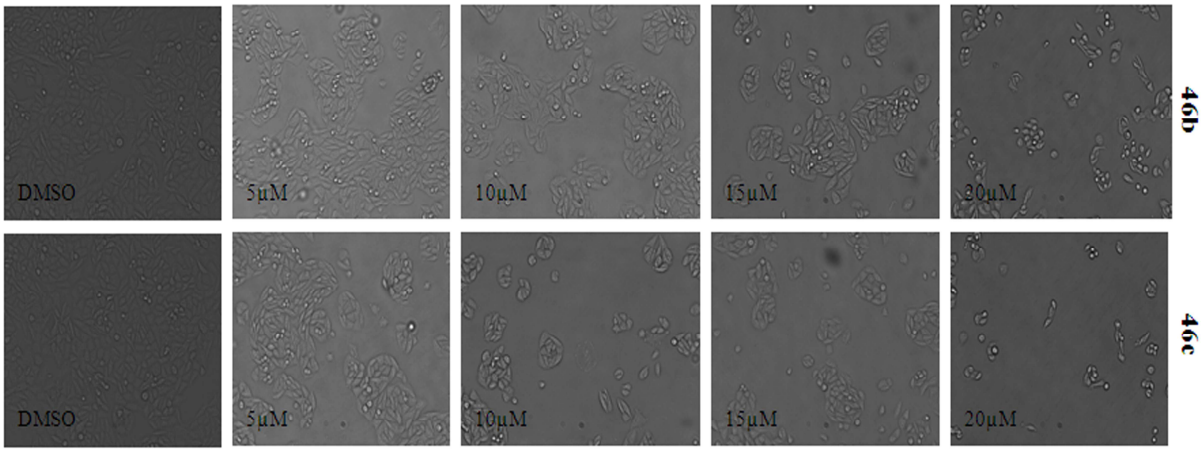


Figure 3: Long term effect of selected compound on the number of colony-forming DU145 cells. DU145 cells were treated with desired concentration of selected compounds(0− 20 μM) and allowed to grow for 10 days to form colonies. Representative images of the colony-forming assay are shown here. Number of colonies and their size formed by DU145 in soft agar is decreased on exposure to these selected compounds.

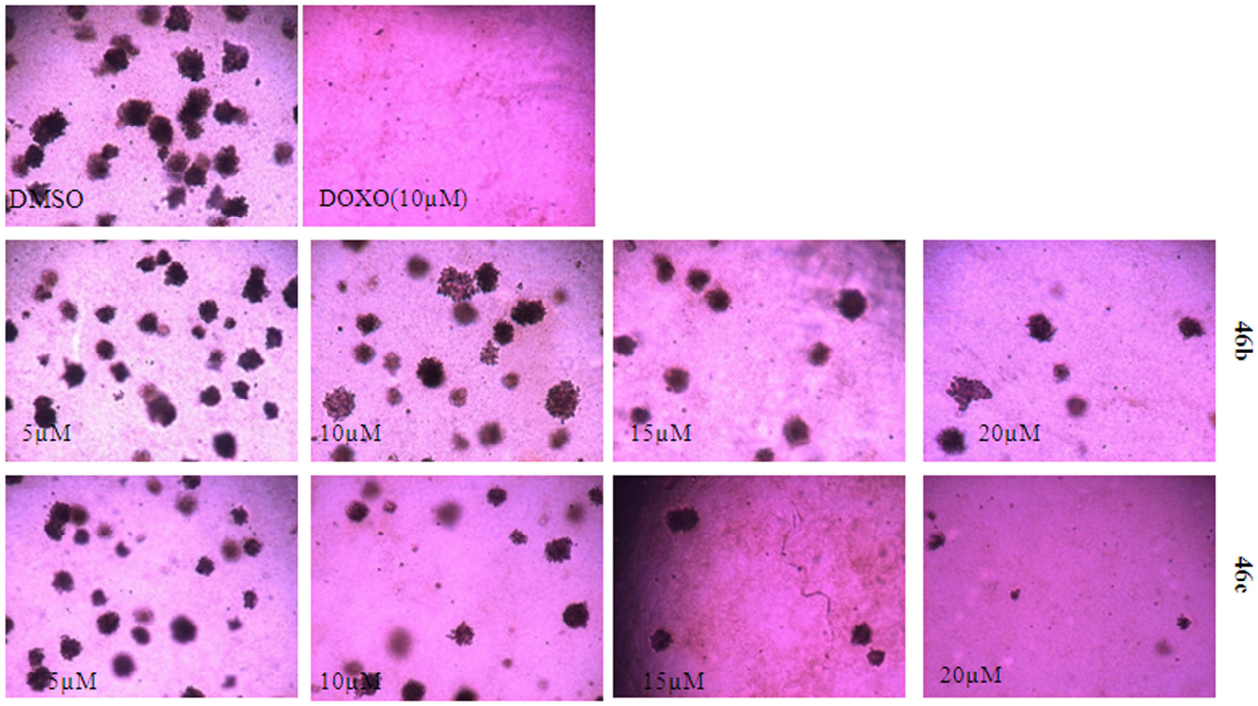


Figure 4: Cell cycle analysis of DU145 cells treated with selected compounds. Cells were treated with compounds and DMSO and the DNA content was measured by propidium iodide staining to determine the distribution of cells in various phases of cell cycle. The percentage of cells increased in subG1 phase up on exposure to these selected compounds suggesting its role in G0/G1 arrest.

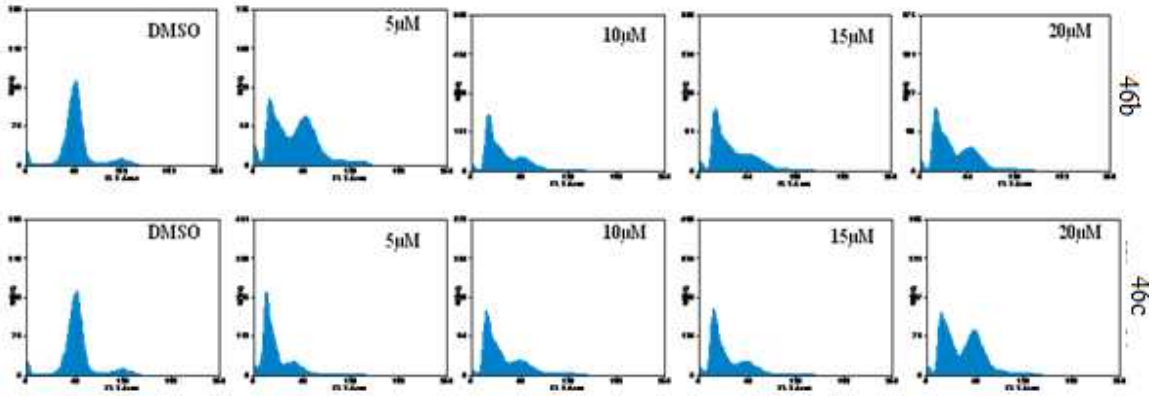
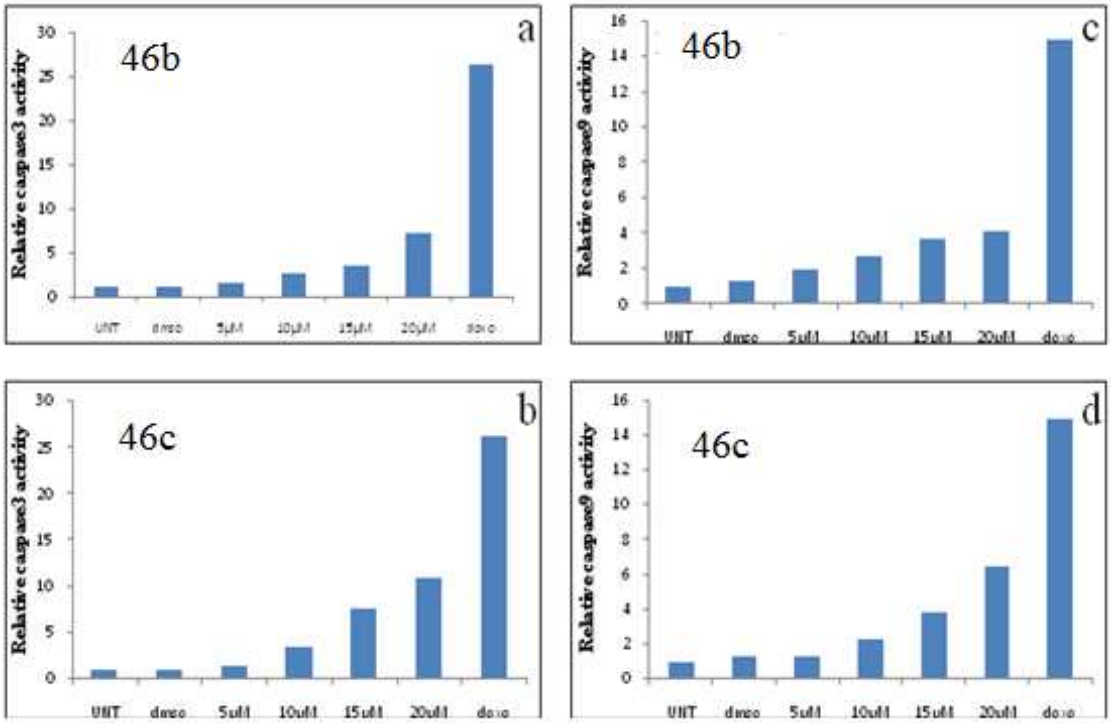


Figure 5: Effect of selected compounds on activation of caspases inducing apoptosis in DU145 cells. Treatment of DU145 cells with different concentration of selected compounds for 48 h induced activation of caspases-3 (a & b) and caspase-9 (c & d) significantly in concentration dependent manner. Doxorubicin was used as control for activation of both caspases.



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Supplementary data:

Supplementary data associated with this article

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