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FULL-LENGTH PAPER

Chemistry of withaferin-A: chemo, regio, and stereoselective synthesis of novel spiro-pyrrolizidino-oxindole adducts of withaferin-A via one-pot three-component [3+2] azomethine ylide cycloaddition and their cytotoxicity evaluation

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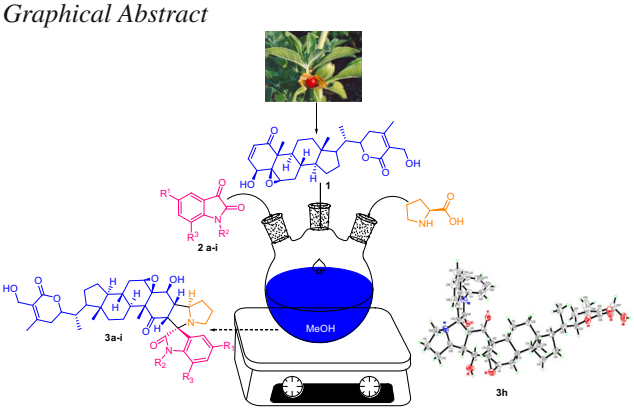
Abstract Withaferin-A (WA) has attracted the attention of chemists as well as biologists due to its interesting structure and various bio-activities. In light of the promising biological importance of WA as well as pyrrolidine-2-spiro-3′-oxindole ring system, we became interested in the synthesis of a combined motif involving both the ring systems via the 1,3-dipolar cycloaddition of WA at Δ^2-bond of the α,β-unsaturated carbonyl system. We now report a facile, atom-economic synthesis of novel spiro-pyrrolizidino-oxindole adducts of withaferin-A (10 compounds) via the intermolecular cycloaddition of azomethine ylides generated in situ from proline and isatins/acenaphthoquinone. The reaction is highly chemo, regio, and stereoselective affording the cis-fused products with β-orienting hydrogen. The structures were determined by 1D/2D NMR spectroscopic data analysis and unequivocally confirmed by X-ray crystallographic analysis in some cases. Bioevaluation of the compounds against six cancer lines (e.g., CHO, HepG2, HeLa, HEK 293, MDCK-II, and Caco-2) identified 4 promising potential anti-cancer compounds.

Graphical Abstract



Keywords Withaferin-A · Spiro-pyrrolizidino oxindole/acenaphthoquinone · Azomethine ylide cycloaddition · 1D/2D NMR · X-ray crystallography

Introduction

Natural products with steroidal framework have opened so many areas for medicinal and pharmacological chemistry. Cholesterol, the essential component of the cell membrane, is a steroid that acts as the precursors of certain vitamins (D). They often play a significant role alongside chemotherapy. Such type of steroidal framework is present in withaferin-A (**1**) (WA; 4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-2,24-dienolide), the first member of the withanolides discovered so far. It is a polyfunctional steroidal lactone based on ergostane framework and was first isolated from winter cherry (*Witha-*

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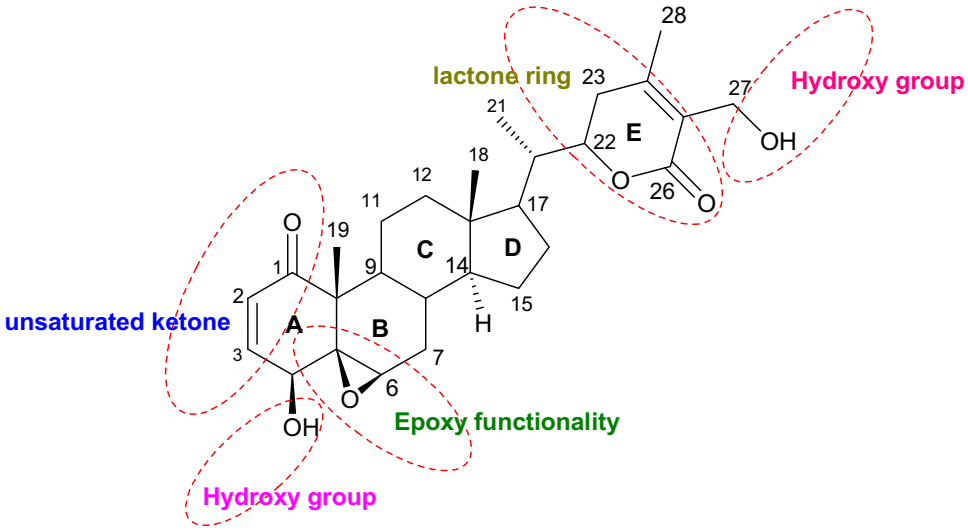


Fig. 1 Functional groups contained in withaferin-A

nia somnifera) [1]. The presence of the steroidal framework has made WA work as an antiangiogenic (formation of new blood vessels) compound by inhibiting both Sp1 and NF-κB transcription factor activity [2–4]. Besides, other WA biological activities of therapeutic importance that are reported include effect in calcium signaling [5], antihypothyroid [6], anabolic (against osteoporosis) [7], anticancer [8–13], anti-inflammatory [14], antistress, anticonvulsant [15,16], cardioprotective [17], immuno-modulatory [18], proteasome inhibitory [19], COX-2 inhibitory [20], and radiosensitizing activity [21].

Incorporation of heteroatoms (N, O & S) has amended the biological activities of new molecules compared to the parent ones [22]. In this way, transforming a parent bioactive natural compound to a more/new bioactive one via semi-synthetic approach has become a modern way of drug development. Chemically, the five major functionalities present in withaferin-A are: (i) α,β-unsaturated ketone group in ring A, (ii) a secondary hydroxyl group at C-4, (iii) a epoxide ring between C-5 and C-6, (iv) a primary hydroxyl group at C-27, and (v) a 6-membered lactone ring (E) with α,β-unsaturated carbonyl group (Fig. 1).

Few reports on synthesis of bioactive semi-synthetic analogs of withaferin-A via transformation of a few of the above 5 functionalities for improved biological activities are available in the literature and these are the introduction of hydroxyl groups using microbial transformation [23–25], functionalization of hydroxyl groups [3,26], 5β, 6β-epoxide ring modification to thiirane, or ring opening [27] to modify the antiproliferative activity of withaferin-A [28]. A few reports that demonstrate analogs synthesized via a Michael addition to the α,β-unsaturated ketone of withaferin-A exhibited cytotoxic activity [29,30] are also available.

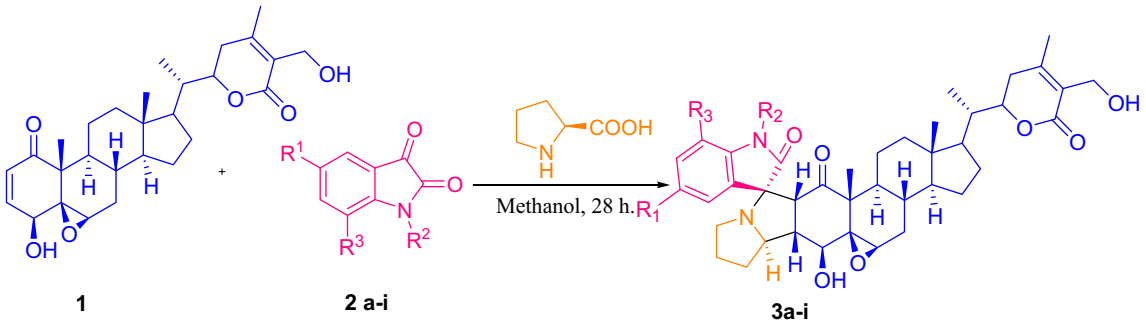
The pyrrolidine-2-spiro-3′-oxindole ring system is found in a variety of oxindole alkaloids, for example, horsfiline [31], spirotryprostatin A and B [32,33], and elacomine [34]. 1,3-Dipolar azomethine ylide cycloaddition is the main way of furnishing this type of ring system. Considering the promising biological importance of withaferin-A and pyrrolidine-2-spiro-3′-oxindole ring system, we became interested in the synthesis of a combined motif involving both the above mentioned ring systems via dipolar cycloaddition reaction. We contemplated that 1,3-dipolar cycloaddition at the Δ^2-bond on the α,β-unsaturated carbonyl system of withaferin-A is the practical approach to generate the combined motif. Addition at both the α and β-position of the α,β-unsaturated carbonyl system has yet to be extensively addressed and may be a practical approach to modify biological activity. It may even be possible to find new activities of the modified compounds. In this endeavor we wish to present the semi-synthesis of several novel-fused pyrrolizidino-2-spiro-3′-oxindole compounds with the well-known bioactive natural core withaferin-A and their cytotoxicity evaluation.

Results and discussion

The cycloaddition reactions of withaferin-A (**1**) were accomplished using isatin analogs and proline (secondary α-amino acid) in refluxing methanol (65 °C) via the in situ generation of azomethine ylides (Scheme 1).

Around 26–28 hours were required for the reaction to produce products **3a**–**i** in the modest (60–70 %) yields (Table 1). With increasing the temperature and time of reflux, the yield is not increased to a good one (∼85 %). The identity of all the products of the cycloaddition reaction was confirmed by mass, NMR spectrometric analysis.

The careful analysis of the NMR spectral data of the products revealed that the signals relating to B/C/D ring system and δ lactone ring remained unaltered. However, the chem-



Scheme 1 Synthesis of novel spiro-pyrrolizidino-oxindolo withaferin-A adducts

Table 1 Yields of **3a–i** derived from withaferin-A

Entry	R^1	R^2	R^3	Product	Yield (%)[a]
1	H	H	H	**3a**	65
2	Me	H	H	**3b**	66
3	Cl	H	H	**3c**	65
4	I	H	H	**3d**	64
5	F	H	H	**3e**	68
6	OMe	H	H	**3f**	70
7	H	Me	H	**3g**	68
8	H	Ph	H	**3h**	69
9	Me	H	Me	**3i**	67

[a] Isolated yield

ical shifts for the nuclei belonging to the α,β-unsaturated ketone containing A ring were distinctly shifted. The shift was observed with C_2 and C_3, suffering profound alteration in the resonance position from δ 131.3 and 141.9 to δ 56.0 and 52.2, respectively. It must be pointed out that withaferin-A numbering has been maintained for the basic skeleton for ease in correlation. The crucial evidence in support of this addition came from the observed HMBC correlation (Fig. 2) in the spectrum of **3a** between signals of C-1 (δ 210.0) and H-2 (δ 4.02), C-2′ (i.e., the oxindole carbonyl, δ 178.9) and H-2 (δ 4.02), C-3′ (i.e., the spiro carbon, δ 72.1) and H-2 (δ 4.02) as also H-3 (δ 2.78), and C-2″ (δ 68.5) and H-3 (δ 2.78). Further the COSY relationship between H-2″ of proline (δ 4.36) and H-3 (δ 2.78), and between H-2 (δ 4.02) and H-3 (δ 2.74) strongly support the mode of addition. Strong NOESY relationship of H-2 (δ 4.02) with both H-3 (δ 2.78) and β-oriented C-19 methyl proton (δ 1.33) suggests that both H-2 and H-3 are β oriented which was also deduced from the coupling constant value of the ring juncture protons ($J_{H2,H3} = 12$ Hz).

The structure of **3h** was confirmed by single-crystal X-ray diffraction (Fig. 3), where the β-orientation of H-2 and H-3 was clearly visible (indicated with blue font).

Following the successful synthesis of spiro-pyrrolizidino-oxindole adducts of withaferin-A using isatin derivatives, we performed the reaction using proline and acenaphthoquinone as the 1,2-diketone compound. In this case we also obtained the spiro-pyrrolizidino-oxindole adduct **5** in around 62 % yield (Scheme 2) and its structure confirmed by 2D NMR analysis.

A critical observation of the HMBC correlation in the spectrum of **5** revealed the crucial evidence in support of the cycloaddition (Fig. 4) in the spectrum of **5** between signals of C-1 (δ 211.9) and H-2 (δ 4.35), C-10′ (i.e., the acenaphthoquinone carbonyl, δ 203.8) and H-2 (δ 4.35), C-9′ (i.e., the spiro carbon, δ 76.9) and H-2 (δ 4.35) as also H-3 (δ 2.93), and C-2″ of proline (δ 70.2) and H-3 (δ 2.93). Also, the observed HMBC correlation between signals of aromatic quaternary carbon C-8″ (δ 138.9) and H-2 (δ 4.35) supports the mode of addition. Further the COSY relationship between H-2″ of proline (δ 4.35) and H-3 (δ 2.93), and between H-2 (δ 4.35) and H-3 (δ 2.93) strongly support the mode of addition. Strong NOESY relationship of H-2 (δ 4.35) with both H-3 (δ 2.93) and β-oriented C-19 methyl proton (δ 1.41) suggests that both H-2 and H-3 are β oriented. The β orientation of H-2 and H-3 was also confirmed from the NOESY correlation of both the proton with aromatic proton H-7″ (δ 7.58). Also, a strong NOESY relationship of H-4 (δ 4.62) with H-6 (δ 3.38) and H-2″ of proline (δ 4.35) confirmly suggests the later to be α oriented as H-4 and H-6 is already in α position.

Again, from a mechanistic point of view, it is expected that the oxindole/acenaphthoquinone carbonyl does prefer to be α-oriented to avoid dipole–dipole repulsion with C-1 carbonyl group of withaferin-A. The reaction proceeds in a highly stereoselective manner as it furnished β-orienting cis-fused ring junction hydrogens due to the presence of the C-4 β-orienting –OH group. Moreover, the reaction also proceeds through a regioselective as well as chemoselective (one of the two double bonds took part in reaction) pathway. The mechanism involves the formation of oxazolidinone intermediate via the loss of H_2O followed by CO_2 loss in a stereospecific

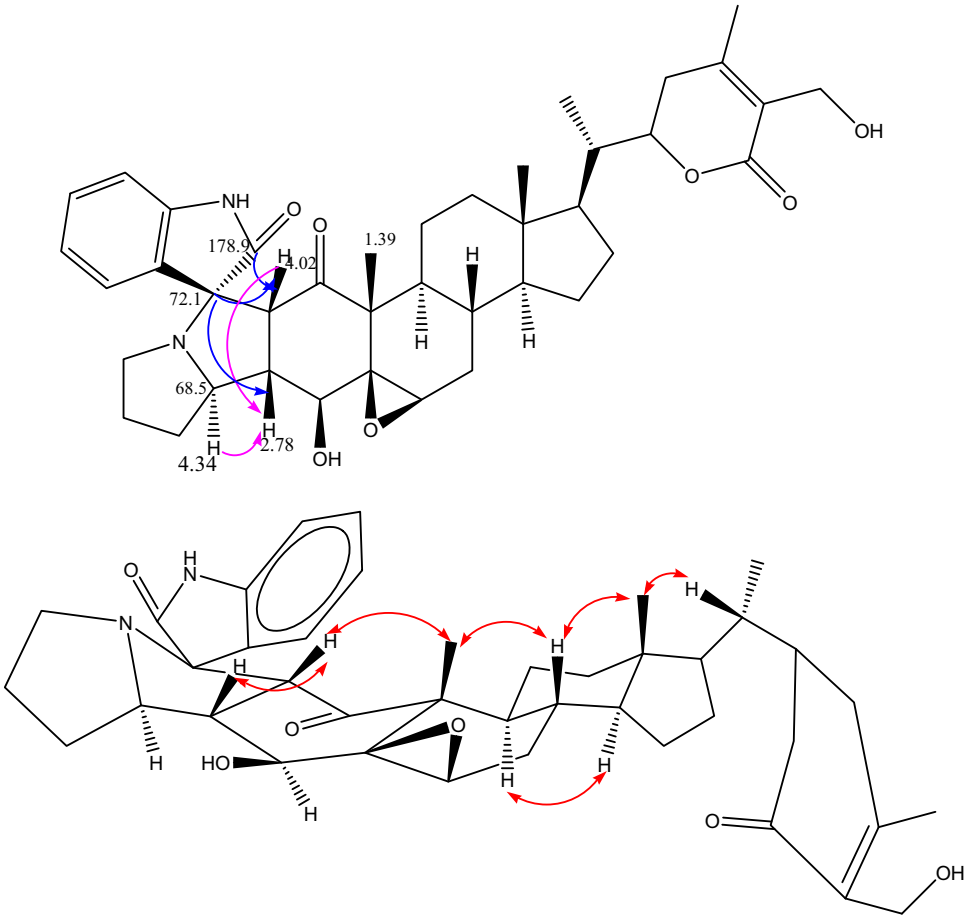


Fig. 2 Important correlations of **3a** [HMBC (C ⟶ H), COSY ( ), NOESY ( )]. (Color figure online)

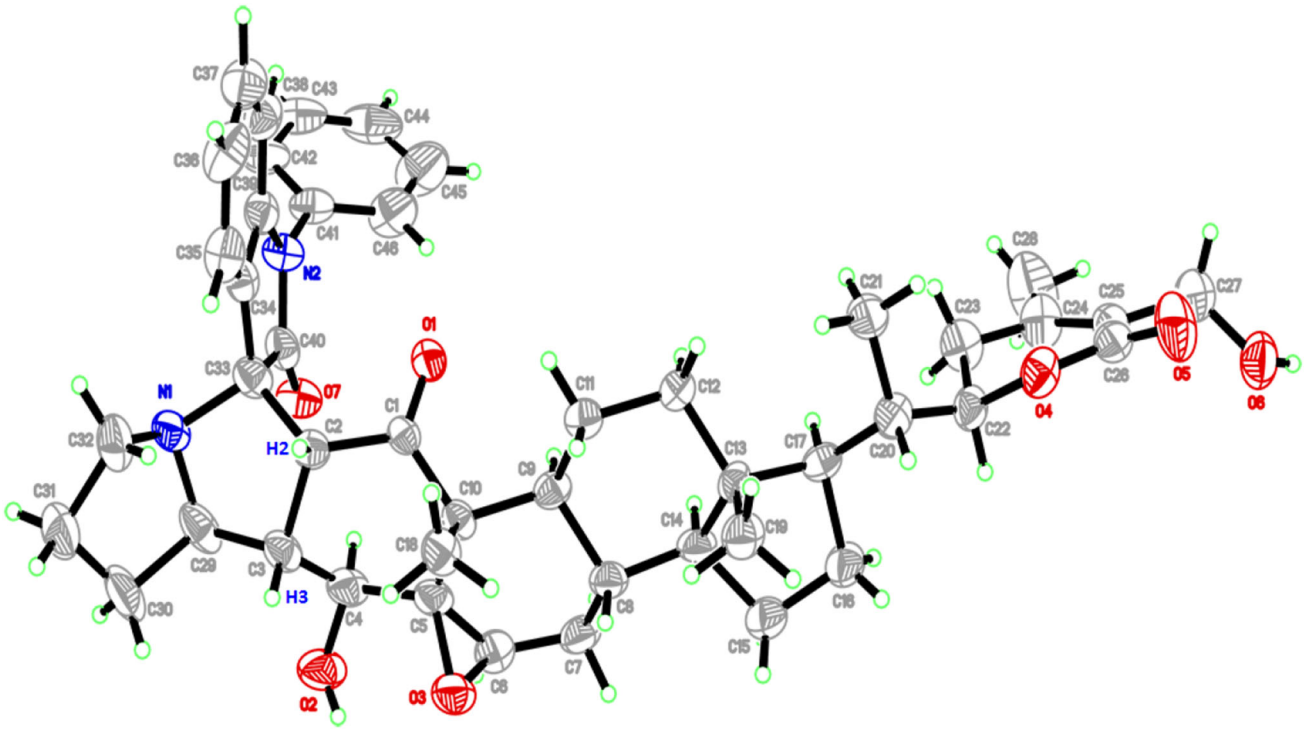
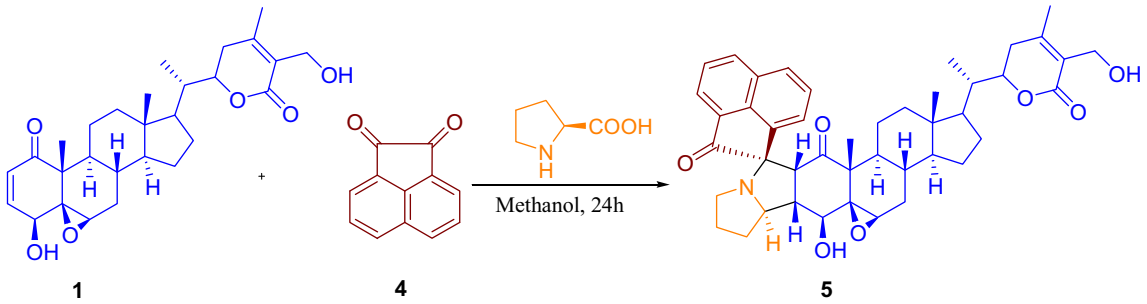


Fig. 3 ORTEP representation of **3h**



Scheme 2 Synthesis of novel spiro-pyrrolizidino-acenaphthoquino withaferin-A

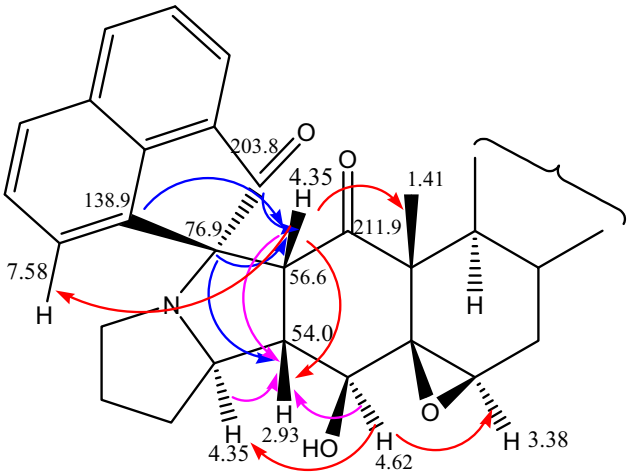


Fig. 4 Important correlations of**5** [HMBC (C⟶H), COSY (⌒), NOESY (⌒)]. (Color figure online)

1,3-cycloreversion pathway to form the azomethine ylide (**I**, **II** Scheme 3) [35,36].

The parent withaferin-A (**1**) is more or less cytotoxic against several types of cancer cell lines [8–13]. Inspired by these reported results, the cytotoxic activity of these 10 compounds compared to **1** and doxorubicin was evaluated against six cancer cell lines using MTT assay (Table 2). The data presented in Table 2 summarize the effect of all types of ring system along with their substituent effect. Cytotoxicity evaluation shows promising potential for compounds **3c**, **3d, 3h**, and **5** with IC50 value in the range of 2–6 μg/mL compared to the parent compound **1** of 7–9 μg/mL. Although very much preliminary but the incremental effect suggests that the semi-synthetic combination motif is a fruitful one.

Moreover, inclusion of the oxindole ring with a halogen substitution as well as the acenaphthoquinone ring system is quite beneficial by increasing the cytotoxicity as well as polarity which in terms increases its percentage of water solubility. However, to reach definite conclusion about the cytotoxicity effect of this semi-synthetic combination motif, a large library of compounds with study in mechanism of actions along with toxicity evaluation is very much needed, which is in progress in our lab.

Conclusion

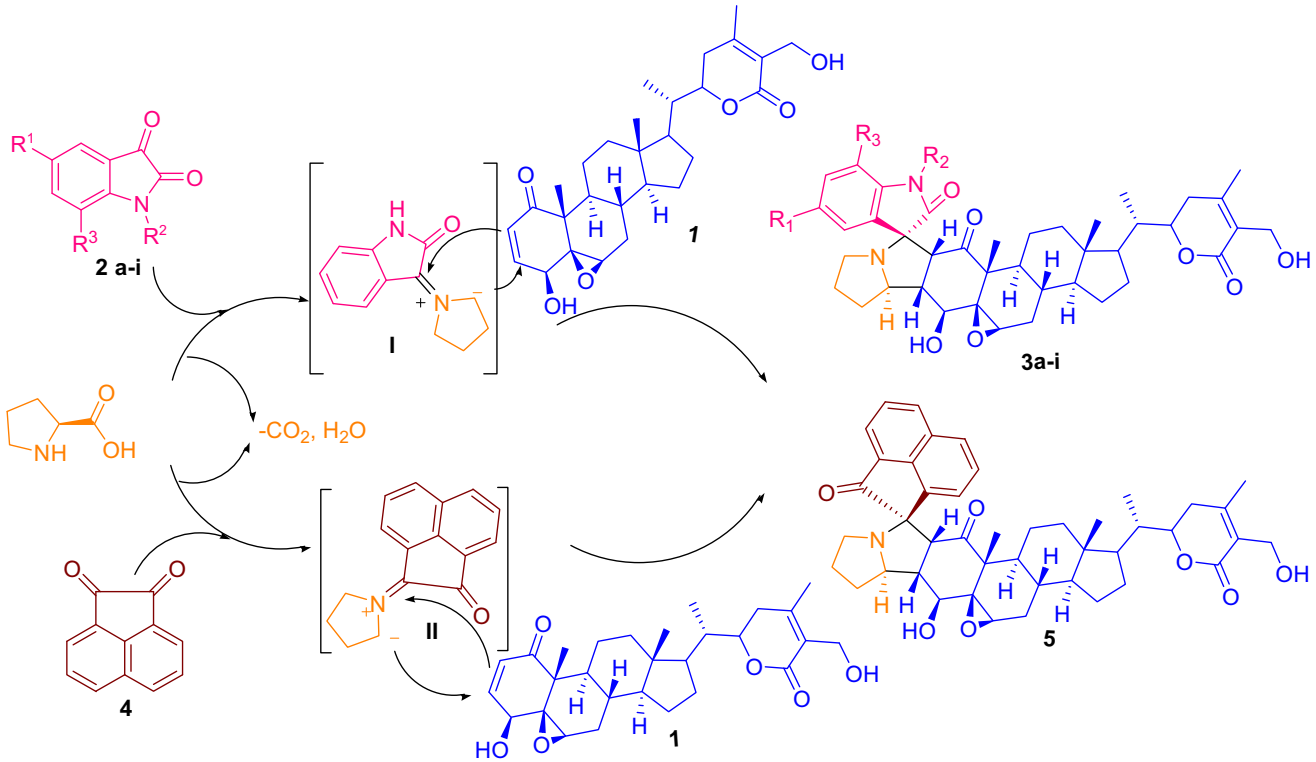
In conclusion, a facile, atom-economic synthesis of novel spiro-pyrrolizidino-oxindole adducts of withaferin-A has been achieved via an intermolecular 1,3-dipolar azomethine ylide cycloaddition generated in situ from proline and isatins/acenaphthoquinone. The reaction is highly chemo, regio, and stereoselective affording exclusively cis-fused products. The structure of the products was determined by 1D/2D NMR analysis and unequivocally confirmed by X-ray crystallographic analysis. Bioevaluation of the compounds against six cancer lines (e.g., CHO, HepG2, HeLa, HEK 293, MDCK-II, and Caco-2) was identified **3c**, **3d, 3h**, and **5** as promising potential anticancer compounds.

Experimental

General

Chemistry

Melting points were determined in capillary melting point apparatus and are uncorrected. IR spectra were recorded as KBr pellets using a JASCO 410 FTIR spectrometer. The NMR spectra were recorded using a Bruker 600 DPX spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C in $CDCl_3$ using TMS as internal standard reporting chemical shifts in δ units and splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; br, broad; and m, multiplet. Mass spectra (positive mode) were obtained on a LC-ESI-Q-TOF micro mass spectrometer manufactured by Waters in the electrospray ionization mode. Withaferin-A was isolated from the chloroform extract of *Withania somnifera* via column chromatography followed by crystallization [37]. Isatins, acenaphthoquinone, and α-amino



Scheme 3 Plausible reaction mechanism for the synthesis of spiro-pyrrolizidino oxindolo/ acenaphthoquino withaferin-A

acids were purchased from Alfa-Aesar Company and used as it was. All other solvents and chromatographic absorbents were procured from E. Merck (Germany) and SRL (India) Ltd. unless otherwise indicated. Thin-layer chromatography was performed on pre-coated silica gel 60 F_{254} aluminum sheets (E. Merck, Germany) using 5 % MeOH in $CHCl_3$ and spots were developed using UV irradiation, iodine, and Liebermann-Burchard reagent.

Typical experimental procedure for the synthesis of spiro-pyrrolizidino-oxindole products **3a–i**, **5**

A mixture of **1** (0.63 mmol, 300 mg), isatin (0.6 mmol, 90 mg) or acenaphthoquinone (0.6 mmol, 110 mg), and proline (0.87 mmol, 100 mg) was taken in a round bottom flask, dissolved in 15–20 mL of methanol, and heated to reflux for 24 h. After completion of the reaction as evident from TLC, the solvent was removed and the residue was subjected to column chromatography using (1 % methanol in chloroform) as eluant. The desired fractions were combined and crystallized from chloroform-methanol mixture to provide the pure solid compounds.

Table 2 Cytotoxicity data for compounds **3a–3i** and **5**

Comp ID	IC_{50} (μg/mL)[a]					
	CHO	HepG2	HeLa	HEK 293	MDCK-II	Caco-2
3a	27.1	40.5	32.8	25.8	28.0	56.5
3b	13.4	14.0	16.4	14.9	11.3	25.5
3c	5.9	8.2	7.9	4.0	5.6	12.5
3d	4.4	5.9	6.7	6.6	6.1	6.9
3e	20.7	24.9	26.2	18.7	15.9	46.6
3f	27.6	39.2	33.2	25.5	19.7	37.9
3g	28.4	38.9	33.1	19.5	25.4	34.7
3h	6.0	11.7	10.0	6.9	9.8	13.2
3i	10.3	16.4	12.4	14.0	8.7	19.1
5	2.4	3.4	4.4	2.8	5.0	6.4
1	9.3	7.2	7.0	10.2	6.9	7.8
Doxorubicin	0.24	0.27	0.19	5.4	0.38	0.27

[a] Incubation: 72 h; values are average of three days' assay, $n = 2$ each day

Spectroscopic data 4β,27-dihydroxy-1-oxo-5β,6β -epoxywitha-24-enolido[2,3-c]pyrrolizidine[2,3′-spiro-oxindole] (**3a**)

Color: White solid; Yield: 65 %; Mp: 210–212 °C; R_f 0.33 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3421, 2944,

1707, 1622. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.56 (1H, s), 7.24 (2H, m), 7.00 (1H, t, J = 7.8 Hz), 6.87 (1H, d, J = 7.8 Hz), 4.36 (5H, m), 4.02 (1H, d, J = 12.0 Hz), 3.31 (1H, s), 2.78 (2H, m), 2.70 (1H, m), 2.47 (1H, m), 2.38 (1H, m), 2.02 (1H, m), 2.00 (3H, s), 1.95 (3H, m), 1.82 (2H, m), 1.72 (5H, m), 1.63 (2H, m), 1.40 (2H, m), 1.33 (3H, s), 1.26 (3H, m), 1.10 (3H, m), 0 .93 (3H, d, J = 6.6 Hz), 0.62 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 210.6 (C=O), 178.9 (C=O), 167.0 (C=O), 153.0 (C), 141.9 (C), 129.5 (CH), 127.7 (C), 125.6 (C), 124.9 (CH), 121.7 (CH), 110.1 (CH), 78.7 (CH), 72.1 (C), 69.5 (CH), 68.5 (CH), 65.5 (C), 57.4 (CH_2), 56.4 (CH), 56.0 (CH), 55.8 (CH), 52.2 (CH), 51.9 (CH), 50.5 (C), 49.7 (CH_2), 42.6 (C), 40.3 (CH), 39.0 (CH_2), 38.7 (CH), 31.8 (CH_2), 30.2 (CH_2), 29.7 (CH_2), 28.3 (CH), 27.1 (CH_2), 25.4 (CH_2), 24.2 (CH_2), 21.5 (CH_2), 20.0 (CH_3), 17.3 (CH_3), 13.3 (CH_3), 11.6 (CH_3).MS [ESI-MS, positive mode]: found m/z 671 [M+H] $^{+}$, 693 [M+Na]$^{+}$. HRMS [ESI-MS, positive mode]: MF: $C_{40}H_{50}N_2O_7$; found m/z 693.3499 [M+Na]$^{+}$ [calcd. 693.3516].

4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(5-methyl) oxindole] (**3b**)

Color: White solid; Yield: 66 %; Mp: 234–236 °C; R_f 0.33 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3409, 2945, 1706, 1494. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.74 (1H, s), 7.09 (2H, m), 6.80 (1H, d, J = 7.8 Hz), 4.43 (4H, m), 4.33 (2H, m), 4.05 (1H, d, J = 12.0 Hz), 3.35 (1H, s), 2.82 (2H, m), 2.75 (1H, m), 2.51 (1H, m), 2.39 (1H, m), 2.36 (3H, s), 2.17 (1H, m), 2.08 (3H, s), 2.00 (3H, m), 1.84 (5H, m), 1.68 (2H, m), 1.43 (2H, m), 1.39 (3H, s), 1.32 (3H, m), 1.13 (4H, m), 0 .97 (3H, d, J = 6.6 Hz), 0.66 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 211.6 (–C=O), 180.1 (–C=O), 168.1 (–C=O), 154.1 (–C), 140.5 (–C), 132.1 (–C), 130.9 (–CH), 128.7 (–C), 126.7 (–CH), 126.6 (–C), 110.8 (–CH), 79.7 (–CH), 73.2 (–C), 70.6 (–CH), 69.5 (–CH), 66.5 (–C), 58.4 (–CH_2), 57.4 (–CH), 57.0 (–CH), 56.9 (–CH), 53.1 (–CH), 52.9 (–CH), 51.5 (–C), 50.7 (–CH_2), 43.6 (–C), 41.3 (–CH), 40.1 (–CH_2), 39.7 (–CH), 32.8 (–CH_2), 31.2 (–CH_2), 30.7 (–CH_2), 29.4 (–CH), 28.1 (–CH_2), 26.4 (–CH_2), 25.2 (–CH_2), 22.5 (–xCH_2), 22.3 (–CH_3), 21.0 (–CH_3), 18.4 (–CH_3), 14.3 (–CH_3), 12.7 (–CH_3); MS [ESI-MS, positive mode]: found m/z 685 [M+H]$^{+}$, 707 [M+Na]$^{+}$. HRMS [ESI-MS, positive mode]: MF: $C_{41}H_{52}N_2O_7$; found m/z 707.3741 [M+Na]$^{+}$[calcd. 707.3672].

4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(5-chloro) oxindole] (**3c**)

Color: White solid; Yield: 65 %; Mp: 228–230 °C; R_f 0.33 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3420, 2926, 1708, 1478. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.66 (1H, s), 7.29 (1H, dd, J = 1.8, 8.4 Hz), 7.26 (1H, d, J = 1.8 Hz), 6.85 (1H, d, J = 8.4 Hz), 4.41 (4H, m), 4.32 (1H, m), 4.03 (1H, d, J = 12.0 Hz), 3.35 (1H, s), 2.81 (1H, td, J = 5.4, 12 Hz), 2.77 (2H, m), 2.52 (1H, m), 2.42 (1H, m), 2.17 (2H, m), 2.08 (3H, s), 2.01 (3H, m), 1.85 (3H, m), 1.69 (4H, m), 1.44 (2H, m), 1.40 (3H, s), 1.35 (1H, m), 1.29 (3H, m), 1.14 (3H, m), 0 .99 (3H, d, J = 6.6 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 211.7 (C=O), 179.5 (C=O), 168.1 (C=O), 154.0 (C), 141.6 (C), 133.0 (C), 130.5 (CH), 128.0 (C), 126.6 (C), 126.4 (CH), 112.1 (CH), 79.7 (CH), 73.1 (C), 70.5 (CH), 69.7 (CH), 66.4 (C), 58.4 (CH_2), 57.44 (CH), 57.40 (CH), 56.9 (CH), 53.1 (CH), 52.9 (CH), 51.4 (C), 50.7 (CH_2), 43.7 (C), 41.4 (CH), 40.1 (CH_2), 39.7 (CH), 32.7 (CH_2), 31.2 (CH_2), 30.8 (CH_2), 29.3 (CH), 28.1 (CH_2), 26.5 (CH_2), 25.2 (CH_2), 22.6 (CH_2), 21.0 (CH_3), 18.3 (CH_3), 14.3 (CH_3), 12.7 (CH_3). MS [ESI-MS, positive mode]: found m/z 705 [M+H] $^{+}$, 727 [M+Na for Cl^{35}] $^{+}$, 729 [M+Na for Cl^{37}] $^{+}$. HRMS [ESI-MS, positive mode]: MF: $C_{40}H_{49}ClN_2O_7$; found m/z 727.3151 [M+Na] $^{+}$ [calcd. 727.3126].

4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(5-iodo) oxindole] (**3d**)

Color: White solid; Yield: 64 %; Mp: 212–214 °C; R_f 0.31 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3419, 2923, 1708, 1473. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.72 (1H, brs), 7.62 (1H, dd, J=1.8, 8.4 Hz), 7.56 (1H, d, J = 1.8 Hz), 6.72 (1H, d, J = 7.8 Hz), 4.42 (4H, m), 4.33 (1H, m), 4.02 (1H, d, J = 12.0 Hz), 3.35 (1H, s), 2.80 (1H, td, J=12, 6 Hz), 2.76 (2H, m), 2.52 (1H, m), 2.41 (1H, m), 2.19 (2H, d, J = 10.2 Hz), 2.08 (3H, s), 2.02 (2H, m), 1.85 (2H, m), 1.70 (5H, m), 1.47 (2H, m), 1.40 (3H, s), 1.38 (1H, m), 1.34 (1H, m), 1.29 (4H, m), 1.14 (2H, m), 1.00 (3H, d, J = 7.8 Hz), 0.68 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 211.1 (C=O), 178.6 (C=O), 167.6 (C=O), 153.6 (C), 142.2 (C), 138.9 (CH), 134.2 (CH), 130.6 (C), 126.2 (C), 112.7 (CH), 84.5 (C), 79.3 (CH), 72.4 (C), 70.1 (CH), 69.3 (CH), 66.0 (C), 58.0 (CH_2), 57.0 (CH), 56.9 (CH), 56.4 (CH), 52.6 (CH), 52.5 (CH), 51.0 (C), 50.3 (CH_2), 43.2 (C), 40.9 (CH), 39.6 (CH_2), 39.3 (CH), 32.3 (CH_2), 30.7 (CH_2), 30.2 (CH_2), 28.9 (CH), 27.7 (CH_2), 26.0 (CH_2), 24.8 (CH_2), 22.1 (CH_2), 20.6 (CH_3), 17.9 (CH_3), 13.9 (CH_3), 12.2 (CH_3). MS [ESI-MS, positive mode]: found m/z 797 [M+H] $^{+}$. HRMS [ESI-MS, positive mode]: MF: $C_{40}H_{49}IN_2O_7$; found m/z 819.2467 [M+Na] $^{+}$ [calcd. 819.2482].

4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(5-fluoro) oxindole] (**3e**)

Color: White solid; Yield: 68 %; Mp: 196–198 °C; R_f 0.32 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3422, 2926, 1707, 1487. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.53 (1H, s), 7.03 (2H, m), 6.86 (1H, m), 4.43 (4H, m), 4.34 (1H, m), 4.02 (1H, d, J = 12.0 Hz), 3.35 (1H, s), 2.82 (1H, td, J=12, 6 Hz), 2.77

(1H, m), 2.52 (1H, m), 2.41 (1H, m), 2.18 (1H, m), 2.08 (3H, s), 2.05 (1H, m), 2.01 (3H, m), 1.85 (4H, m), 1.70 (5H, m), 1.44 (2H, m), 1.39 (3H, s), 1.33 (3H, m), 1.14 (3H, m), 0.98 (3H, d, J = 6.6 Hz), 0.67 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 211.2 (C=O), 179.4 (C=O), 167.6 (C=O), 159.0 (C, $^1J_{C-F}$ = 248 Hz), 153.6 (C), 138.4 (C), 126.2 (2×C), 116.5 (CH, $^2J_{C-F}$ = 24 Hz), 113.6 (CH, $^2J_{C-F}$ =24 Hz), 111.2 (CH, $^3J_{C-F}$ = 7.5 Hz), 79.3 (CH), 72.9 (C), 70.1 (CH), 69.2 (CH), 66.0 (C), 58.0 (CH_2), 57.0 (CH), 56.9 (CH), 56.4 (CH), 52.7 (CH), 52.5 (CH), 50.9 (C), 50.3 (CH_2), 43.2 (C), 40.9 (CH), 39.6 (CH_2), 39.3 (CH), 32.3 (CH_2), 30.7 (CH_2), 30.2 (CH_2), 28.9 (CH), 27.7 (CH_2), 26.0 (CH_2), 24.8 (CH_2), 22.1 (CH_2), 20.6 (CH_3), 17.9 (CH_3), 13.9 (CH_3), 12.2 (CH_3). MS [ESI-MS, positive mode]: found m/z 689 $[M+H]^+$. HRMS [ESI-MS, positive mode]: MF: $C_{40}H_{49}FN_2O_7$; found m/z 711.3375 $[M+Na]^+$ [calcd. 711.3421].

*4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(5-methoxy) oxindole] (***3f***)*

Color: White solid; Yield: 70 %; Mp: 224–226 °C; R_f 0.33 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3409, 2945, 1705, 1492. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.74 (1H, brs), 6.88 (1H, s), 6.83 (2H, m), 4.40 (5H, m), 4.02 (1H, d, J = 12.0 Hz), 3.83 (3H, s), 3.35 (1H, s), 2.81 (3H, m), 2.51 (1H, m), 2.40 (1H, m), 2.17 (1H, m), 2.08 (3H, s), 1.99 (4H, m), 1.84 (5H, m), 1.68 (2H, m), 1.44 (2H, m), 1.38 (3H, s), 1.31 (2H, m), 1.13 (4H, m), 0 .98 (3H, d, J = 6.6 Hz), 0.67 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 210.7 (C=O), 179.1 (C=O), 167.3 (C=O), 155.3 (C), 153.3 (C), 135.6 (C), 129.3 (C), 125.8 (C), 113.4 (CH), 113.2 (CH), 110.5 (CH), 78.9 (CH), 72.6 (C), 69.7 (CH), 68.7 (CH), 65.7 (C), 57.6 (CH_2), 56.6 (CH), 56.4 (CH), 56.1 (CH), 56.0 (OMe), 52.3 (CH), 52.1 (CH), 50.6 (C), 49.9 (CH_2), 42.9 (C), 40.5 (CH), 39.3 (CH_2), 38.9 (CH), 32.0 (CH_2), 30.4 (CH_2), 30.0 (CH_2), 28.5 (CH), 27.3 (CH_2), 25.7 (CH_2), 24.4 (CH_2), 21.7 (CH_2), 20.2 (CH_3), 17.5 (CH_3), 13.5 (CH_3), 11.9 (CH_3). MS [ESI-MS, positive mode]: found m/z 723 $[M+Na]^+$. HRMS [ESI-MS, positive mode]: MF: $C_{41}H_{52}N_2O_8$; found m/z 723.3644 $[M+Na]^+$ [calcd. 693.3621].

*4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(1-methyl) oxindole] (***3g***)*

Color: White solid; Yield: 68 %; Mp: 180–182 °C; R_f 0.58 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3438, 2923, 1708, 1612. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.38 (1H, td, J = 1.2, 7.8 Hz), 7.30 (1H, m), 7.06 (1H, td, J = 0.6, 7.2 Hz), 6.90 (1H, d,J=7.8 Hz), 4.42 (4H, m), 4.07 (1H, d, J = 12.6 Hz), 3.34 (1H, s), 3.27 (3H, s), 2.83 (2H, m), 2.74 (1H, m), 2.51 (1H, m), 2.42 (1H, m), 2.18 (1H, m), 2.08 (3H, s), 2.03 (4H, m), 1.84 (4H, m), 1.69 (4H, m), 1.47 (3H, m), 1.38 (3H, s), 1.34 (3H, m), 1.12 (3H, m), 0.97 (3H, d, J = 6.6 Hz), 0.66 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 210.4 (–C=O), 177.8 (–C=O), 167.2 (–C=O), 153.2 (–C), 145.1 (–C), 129.8 (–CH), 127.4 (–C), 125.8 (–C), 124.7 (–CH), 121.9 (–CH), 108.7 (–CH), 78.9 (–CH), 72.2 (–C), 69.9 (–CH), 68.7 (–CH), 65.7 (–C), 57.6 ($–CH_2$), 56.7 (–CH), 56.0 (–CH), 55.9 (–CH), 52.7 (–CH), 52.1 (–CH), 51.0 ($–CH_2$), 49.9 (–C), 42.9 (–C), 40.3 (–CH), 39.3 ($–CH_2$), 38.9 (–CH), 32.0 ($–CH_2$), 30.4 ($–CH_2$), 29.9 ($–CH_2$), 28.5 (–CH), 27.3 ($–CH_2$), 26.4 (CH_3), 25.5 ($–CH_2$), 24.4 ($–CH_2$), 21.7 ($–CH_2$), 20.2 ($–CH_3$), 17.6 ($–CH_3$), 13.5 ($–CH_3$), 11.9 ($–CH_3$). MS [ESI-MS, positive mode]: found m/z 685 $[M+H]^+$. HRMS [ESI-MS, positive mode]: MF: $C_{41}H_{52}N_2O_7$; found m/z 707.3641 $[M+Na]^+$ [calcd. 707.3672].

*4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(1-phenyl) oxindole] (***3h***)*

Color: White solid; Yield: 69 %; Mp: 240–242 °C; R_f 0.70 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3491, 2942, 1708, 1612. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.52 (4H, m), 7.40 (1H, m), 7.33 (1H, m), 7.25 (1H, dd, J = 1.2, 7.8 Hz), 7.05 (1H, td, J = 0.6, 7.2 Hz), 6.80 (1H, d, J = 7.8 Hz), 4.41 (4H, m), 4.09 (1H, d, J = 12.0 Hz), 3.25 (1H, s), 2.83 (3H, m), 2.45 (1H, m), 2.40 (1H, m), 2.12 (1H, m), 2.03 (3H, s), 2.00 (1H, m), 1.96 (2H, m), 1.81 (3H, m), 1.64 (2H, m), 1.60 (3H, m), 1.41 (2H, m), 1.34 (3H, s), 1.26 (3H, m), 1.10 (4H, m), 0.94 (3H, d, J = 6.6 Hz), 0.63 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 210.9 (–C=O), 178.4 (–C=O), 168.0 (–C=O), 153.9 (–C), 146.2 (–C), 135.7 (–C), 130.5 (2×-CH), 130.4 (–CH), 129.0 (–CH), 128.1 (2×–CH), 126.6 (2×–C), 125.9 (–CH), 123.2 (–CH), 110.7 (–CH), 79.7 (–CH), 73.0 (–C), 70.6 (–CH), 69.9 (–CH), 66.5 (-C), 58.5 ($–CH_2$), 57.8 (–CH), 57.5 (–CH), 56.9 (–CH), 53.3 (–CH), 52.9 (–CH), 51.5 (–C), 50.6 ($–CH_2$), 43.7 (–C), 41.2 (-CH), 40.2 ($–CH_2$), 39.7
(–CH), 32.8 ($–CH_2$), 31.1 ($–CH_2$), 30.7 ($–CH_2$), 29.3 (–CH), 28.1 ($–CH_2$), 26.4 ($–CH_2$), 25.2 ($–CH_2$), 22.5 ($–CH_2$), 21.0 ($–CH_3$), 18.4 ($–CH_3$), 14.3 ($–CH_3$), 12.7 ($–CH_3$). MS [ESI-MS, positive mode]: found m/z 747 $[M+H]^+$. HRMS [ESI-MS, positive mode]: MF: $C_{46}H_{54}N_2O_7$; found m/z 769.3862 $[M+Na]^+$ [calcd. 769.3829].

*4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido [2,3-c]pyrrolizidine[2,3′-spiro-(5,7-dimethyl) oxindole] (***3i***)*

Color: White solid; Yield: 67 %; Mp: 220–222 °C; R_f 0.35 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3416, 2943, 1707, 1480. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.84 (1H, s), 6.93 (1H, s), 6.90 (1H, s), 4.44 (4H, m), 4.32 (1H, m), 4.04 (1H, d, J = 12.0 Hz), 3.35 (1H, s), 2.80 (2H, m), 2.71 (1H, m), 2.51 (1H, m), 2.39 (1H, m), 2.33 (3H, s), 2.25 (3H, s), 2.18 (1H, m), 2.08 (3H, s), 2.02 (3H, m), 1.95 (2H, m), 1.83 (5H, m), 1.69 (2H, m), 1.45 (2H, m), 1.39 (3H, s), 1.35 (2H,

m), 1.14 (4H, m), 0 .97 (3H, d, J = 6.6 Hz), 0.67 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 211.5 (–C=O), 180.3 (–C=O), 168.1 (–C=O), 154.1 (–C), 139.1 (–C), 132.4 (–CH), 131.9 (–C), 128.3 (–C), 126.6 (–C), 124.1 (–CH), 119.1 (–C), 79.7 (–CH), 73.7 (–C), 70.6 (–CH), 69.4 (–CH), 66.5 (–C), 58.4 ($–CH_2$), 57.4 (–CH), 56.8 (–CH), 56.7 (–CH), 53.3 (–CH), 52.9 (–CH), 51.5 (–C), 50.7 ($–CH_2$), 43.7 (–C), 41.3 (–CH), 40.1 ($–CH_2$), 39.7 (–CH), 32.8 ($–CH_2$), 31.2 ($–CH_2$), 30.7 ($–CH_2$), 29.4 (–CH), 28.1 ($–CH_2$), 26.4 ($–CH_2$), 25.2 ($–CH_2$), 22.6 ($–CH_2$), 22.2 ($–CH_3$), 21.0 ($–CH_3$), 18.4 ($–CH_3$), 17.4 ($–CH_3$), 14.3 ($–CH_3$), 12.7 ($–CH_3$); MS [ESI-MS, positive mode]: found m/z 699 [M+H] $^+$, 721 [M+Na]$^+$. HRMS [ESI-MS, positive mode]: MF: $C_{42}H_{54}N_2O_7$; found m/z 721.3802 [M+Na] $^+$ [calcd. 721.3829].

4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-24-enolido[2,3-c]pyrrolizidine[2,1′(2H)-spiro-(acenaphthylene-2-one)] (**5**)

Color: White solid; Yield: 62 %; Mp: 197–199 °C; R_f 0.40 (5 % MeOH–$CHCl_3$); IR (KBr, ν_{max} Cm^{-1}): 3440, 2943, 1705, 1395. ^{1}H NMR ($CDCl_3$, 600 MHz): δ 8.14 (1H, d, J = 7.8 Hz), 8.02 (1H, d, J = 6.6 Hz), 7.95 (1H, d, J = 8.4 Hz), 7.79 (1H, t, J = 7.8 Hz), 7.67 (1H, t, J = 7.2 Hz), 7.58 (1H, d, J = 7.2 Hz), 4.61 (1H, bt, J = 9.6 Hz), 4.42 (2H, m), 4.35 (4H, m), 3.38 (1H, s), 2.93 (1H, m), 2.88 (1H, m), 2.63 (1H, t, J = 7.2 Hz), 2.47 (2H, m), 2.18 (1H, m), 2.06 (3H, s), 1.97 (4H, m), 1.78 (1H, m), 1.67 (3H, m), 1.46 (1H, m), 1.41 (3H, s), 1.34 (2H, m), 1.30 (2H, m), 1.25 (2H, m), 1.15 (1H, m), 1.04 (3H, m), 0 .92 (3H, d, J = 6.6 Hz), 0.63 (3H, s). ^{13}C NMR ($CDCl_3$, 150 MHz): δ 211.9 (–C=O), 203.8 (–C=O), 168.1 (–C=O), 154.1 (–C), 142.8 (–C), 138.9 (–C), 133.4 (–C), 132.1 (–CH), 131.9 (–C), 129.4 (–CH), 128.7 (–CH), 126.6 (–CH), 126.5 (–C), 123.3 (–CH), 121.8 (–CH), 79.7 (–CH), 76.9 (–C), 70.6 (–CH), 70.2 (–CH), 66.6 (–C), 58.4 ($–CH_2$), 57.5 (–CH), 56.8 (–CH), 56.6 (–CH), 54.0 (–CH), 52.9 (–CH), 52.3 ($–CH_2$), 50.7 (–C), 43.6 (–C), 41.1 (–CH), 40.0 ($–CH_2$), 39.7 (–CH), 33.1 ($–CH_2$), 31.2 ($–CH_2$), 30.7 ($–CH_2$), 29.3 (–CH), 28.1 ($–CH_2$), 26.4 ($–CH_2$), 25.2 ($–CH_2$), 22.4 ($–CH_2$), 21.0 ($–CH_3$), 18.5 ($–CH_3$), 14.2 ($–CH_3$), 12.6 ($–CH_3$). MS [ESI-MS, positive mode]: found m/z 706 [M+H] $^+$, 728 [M+Na]$^+$. HRMS [ESI-MS, positive mode]: MF: $C_{44}H_{51}NO_7$; found m/z 728.3547 [M+Na] $^+$ [calcd. 728.3563].

X-ray experiments, structural determination, and refinements

X-ray data were measured at room temperature with Mo Kα radiation (graphite monochromator λ = 0.7107 A°) on a Bruker APEX-II CCD X-ray diffractometer. Data reduction and absorption correction were carried out using SAINT and SADABS. Structural solution and refinement (programs SHELXS, SHELXL, and Olex2) ran routinely.

Crystal data for **3h**

SADABS-2012/1 (Bruker 2012) was used for absorption correction. wR2 (int) was 0.0925 before and 0.0688 after correction. The Ratio of minimum to maximum transmission is 0.8278. The \l/2 correction factor is 0.0015.

There was large amount of disordered solvent, which could not be modeled successfully. Thus, their contribution was removed by solvent masking facility available in Olex2.

$C_{46}H_{54}N_2O_7$, Mr = 746.91, white square-shaped crystals were grown from chloroform-methanol. Space group 'Monoclinic' 'P 2_1.' Lattice constants (A_): a = 17.9420(14), b = 12.7371(9), c = 30.791(3); α = 90, β = 103.605(5), γ = 90, cell volume V = 6839.2(9) $A^{\circ 3}$, formula units/cell Z = 6, Number of independent reflections (Nref) 29379, after convergence R_1 = 0.0714 (10129), and wR_2 = 0.2042 (29379).

Biology

Cell lines [CHO, HepG2, HeLa, HEK 293, MDCK-II, and Caco-2) were obtained from the National Centre for Cell Sciences, Pune, India. MEM-alpha, FBS (fetal bovine serum), and penicillin—streptomycin were purchased from Gibco, India. Cell culture flasks, plates, and tips were obtained from Tarsons, Kolkata, India. DMEM, other components of media and all other chemicals were purchased from Sigma-Aldrich, St Louis, Missouri, USA.

Cell culture and MTT assay

MDCK-II cells were maintained in growth media containing MEM-alpha (Gibco) supplemented with 10 % FBS (Gibco) and penicillin—streptomycin (100U/mL for each). Other cell lines were maintained in DMEM media containing 10 % FBS and 40 μg/mL gentamycin.

HepG2, HEK 293, and Caco-2 cells were plated (in 96-well plates) at 6000 cells/well/180μL media. For other cell lines, it was 4000 cells/well/180μL media. Cell seeding density was optimized so that the wells without any inhibitor can make up to 90 % confluency at the end of incubation period/experiment. The plates containing cells were placed in 37 °C incubator with 5 % CO_2 and 95 % relative humidity for 24 h. Media was aspirated off and replaced with 180 μL fresh media. Test compounds were dissolved at conc. of 10 mg/mL (DMSO) and further serial half dilutions were made in DMSO to reach to conc. of 0.078 mg/mL. These substocks were further 10-fold diluted in respective growth media. Then 20 μL of growth media containing test compounds was added (n = 2) in 96-well test plate to produce final working conc. of 100–0.78 μg/mL (DMSO: 1 %). Each

plate had cell control, vehicle control, media control, and standard inhibitor Doxorubicin at 10 μM.

The plates were placed back to incubator for 72 h. 20 μL of MTT (5 mg/mL of PBS pH 7.2) was added in each well and the plates further incubated for 4 h. The plates were centrifuged (2500 rpm, 10 min), media flicked off, and formazan crystals dissolved in 150 μL DMSO. Absorbance was measured at 510 nm using Spectramax M5 (Molecular Devices, USA). Cell death at each conc. was determined based on OD difference of the test well from that of wells of vehicle control. If the highest test conc. (100 μg/mL) shows less than 50 % cell death then IC_{50} (concentration that causes 50 % cell death) is reported as >100 μg/mL and if lowest test conc. (0.78 μg/mL) shows more than 50 % cell death then IC_{50} is reported as <0.78 μg/mL or else it was calculated using GraphPad Prism 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The MTT assay experiment was performed in duplicate wells each day and in three separate days [38,39].

Supporting information

^{1}H, ^{13}C NMR, and HRMS data along with spectrometric copy of all compounds associated with this article can be found in the online version. Crystallographic data in CIF format are available free of charge via the Internet at **CCDC 1006850**. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; or deposit@ccdc.cam.ac.uk).

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