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Original article

Synthesis and anti-inflammatory activity of derivatives of coumarino-lignoid, cleomiscosin A and its methyl ether

Shelly Sharma [a], S.K. Chattopadhyay [a,*], Priyanka Trivedi [b], D.U. Bawankule [b]

[a] Process Chemistry and Chemical Engineering Department, Central Institute of Medicinal and Aromatic Plants (CIMAP), (Council of Scientific and Industrial Research, CSIR), P.O. CIMAP, Lucknow-226015, Uttar Pradesh, India
[b] Molecular Bioprospection Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), (Council of Scientific and Industrial Research, CSIR), P.O. CIMAP, Lucknow-226015, Uttar Pradesh, India

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ABSTRACT

Six novel cleomiscosin A (a coumarino-lignoid), derivatives have been synthesized for the first time by using electrophilic substitution reaction to give nuclear nitrated and halogenated derivatives of cleomiscosin A in good yields. Structures of these compounds were established on the basis of IR, ^{1}H NMR, ^{13}C NMR and Mass spectral data. Some of the synthesized derivatives were tested for *in-vitro* target based anti-inflammatory study using primary macrophages cell culture bioassay system. The results showed that the compounds **1a**, **3a** and **4a** (1 and 10 μg/mL) exhibited potent anti-inflammatory activity.

1. Introduction

The plant *Cleome viscosa* (General name; Hurhur, Wild mustard) is used in fever, inflammations, liver diseases, bronchitis, diarrhoea and infantile convulsions. The seeds are small, dark brown or black and glandular. The seeds are reported to have rubefacient, vesicant and anthelminthic properties [1]. The seeds of the plant *C. viscosa* have been chemically investigated and they were found to be the first source of an interesting class of compounds known as coumarino-lignoids. Four coumarino-lignoids cleomiscosin A, B, C and D were isolated from the seeds of the plant and cleomiscosin A (cliv A) was the major compound followed by cleomiscosin B and C. We have determined that a combination of three coumarino-lignoids cleomiscosin A, B and C (cliv A, cliv B and cliv C) possessed significant liver protective activity [2]. It has also been reported by us that the combination of cliv A, cliv B and cliv C which we have termed as cliv 92 enhanced the body immune function by significantly increasing the white blood cell counts, hem agglutination antibody titer responses and reducing delayed type hypersensitivity responses towards rabbit red blood cells [3]. Also, it has been established the antioxidant activity of cliv A and cliv C and therefore they could be beneficial in preventing oxidation of low density lipoprotein in atherosclerotic lesions. Cliv C inhibited lipid peroxidation comparable to vitamin E [4].

In a previous publication, Ray et al. have reported the preparation of seven derivatives of cleomiscosin A, which included, cleomiscosin A methyl ether, cleomiscosin A ethyl ether, cleomiscosin A diacetate, cleomiscosin A ethyl ether monoacetate, o-methoxy-trans cinnamic acid derivative from cleomiscosin A ethyl ether; potassium permanganate oxidation product of cleomiscosin A methyl ether of cleomiscosin A and substituted phenyl propionate derivative from cleomiscosin A methyl ether [5]. On the basis of the pharmacological results previously obtained by our group [6], it was reported that the oral administration of coumarino-lignoid mixture containing clemiscosin A, B and C isolated from the plant *C. viscosa* inhibits the pro-inflammatory cytokines in dose dependent manner in Swiss albino mice. The molecular manipulation of promising lead compounds is still a major line of approach to develop newer drugs. In order, to study further the chemistry and pharmacological activity of coumarino-lignoids, we have tried some electrophilic aromatic substitution reactions on the major compound, cleomiscosin A. In this communication, we would like to report the nitration and bromination reaction on cleomiscosin A and its methyl ether derivative. The anti-inflammatory activity of

* Corresponding author. Tel.: +91 9648029167; fax: +91 522 2342666.
E-mail address: chattsk@yahoo.com (S.K. Chattopadhyay).

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all the new molecules was evaluated and included in this publication (Tables 1 and 2).

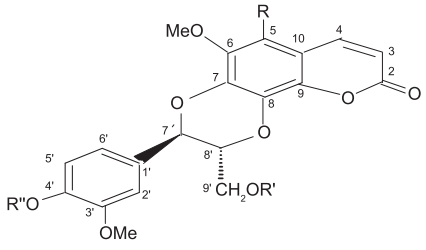
2. Chemistry

The new coumarino-lignoid derivatives **1a**–**5b** were prepared through the synthetic route reported in Schemes 1 and 2. Thus, the reaction of bromination of cleomiscosin A (cliv A) with acetic acid at 100 °C converted it into a monobromo derivative **1a**, ($C_{22}H_{19}O_9Br$, m.p. 70–72 °C), in which the primary alcoholic group was also converted into an ester (Scheme 1). IR spectrum of the derivative **1a**, showed the characteristic absorptions at 1735 cm^{-1} of the carbonyl group of the primary alcohol, in addition to the lactone ring carbonyl at 1722 cm^{-1}. The ^1H NMR spectrum showed the two coumarin protons at δ 6.37 and 8.02 (1H, d each, $J = 9.9$ Hz) for C-3H and C-4H; the downfield shift of C-4H in the ^1H NMR spectrum of the molecule indicated that a bromine group was at C-5 position. This was also confirmed from the ^1H NMR of the compound in which C-5 H was absent. The ^1H NMR spectrum also showed the presence of an alcoholic acetate at δ 2.10 (s). The ESI-MS (positive mode) spectrum showed $[M + H]^+$ and $[M^{+2} + H]^+$ at m/z 507 and 509 in the ratio of almost 1:1. Moreover, the MS/MS spectrum of the molecule showed two diagnostic peaks at m/z 283 and 285 and at m/z 222, the appearance of which can be explained due to retro Diels–Alder cleavage of the dioxane bridge of the molecule. The DEPT 135° spectrum of **1a** showed the presence of two coumarin and three aromatic protons. From the ^1H NMR and Mass spectrum of compound it was confirmed that the Br group was introduced at C-5 position and also the primary alcoholic group was converted into acetate. Compound **1b**, $C_{24}H_{24}O_9$, m.p. 160 °C, was found to be the monoacetate of cleomiscosin A and was confirmed from the reported NMR data (Scheme 1) [5].

Treatment of cleomiscosin A in acetic anhydride with conc. HNO_3 and a catalytic amount for H_2SO_4 converted it into two derivatives, **2a** and **2b**, (Scheme 1). The ESI-MS (positive mode) of **2a**, $C_{24}H_{21}O_{12}N$, m.p. 180–182 °C; showed its $[M + H]^+$ at m/z 516. In the ^1H NMR spectrum of compound **2a**, the C-5H was absent and the C-4H underwent downfield shift at δ 7.64 (d, 1H, $J = 9.9$ Hz) confirming that the nitration took place at C-5H position. The ^1H NMR of **2a** also showed two acetate groups at δ 2.01 and 2.27 (alcohol acetate and phenol acetate). Thus nitration of cliv A yielded a nitro derivative in which both the phenolic and alcoholic group were esterified. Compound **2b**, $C_{24}H_{22}O_{10}$, m.p. 175 °C, was found to be the diacetate of cleomiscosin A and was confirmed from the reported NMR data (Scheme 1) [5].

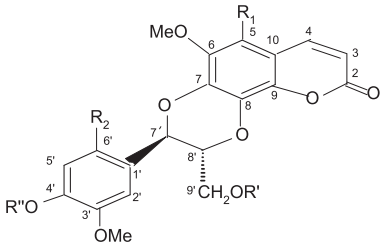
We have also studied the reactivity of methyl ether of cleomiscosin A towards electrophilic aromatic substitution reactions (Scheme 2). Reaction of the methyl ether of cleomiscosin A with *N*-chlorosuccinimide in refluxing $CHCl_3$ yielded a monochloro derivative **3a**, $C_{21}H_{19}O_8Cl$, m.p. 172–174 °C. The ESI-MS (positive mode) spectrum of **3a** showed $[M + H]^+$ and $[M^{+2} + H]^+$ peaks at m/z 435 and 437. In the ^1H NMR spectrum of **3a**, the C-3 and C-4 protons appeared at δ 6.27 and 7.90 (d each, 1H, $J = 9.6$ Hz). The downfield shift of the C-4H and the absence of the C-5H in the ^1H NMR spectrum of **3a** indicated that chlorination took place in the aromatic ring of coumarin at C-5. The same trend was also found in the bromination reaction of the methyl ether of cleomiscosin A with *N*-bromosuccinimide when refluxed with $CHCl_3$ to yield the

Table 1
% Inhibition of pro-inflammatory cytokines of derivatives **1a**, **1b**, **2a**, **3a** and **4a**–**5b**.



1a– 2a

1. R, R', R'' = H, Cleomiscosin A



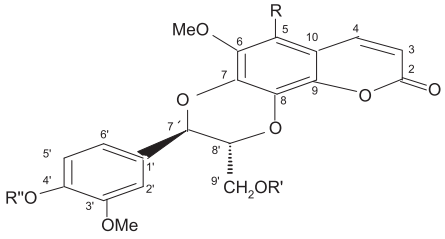
3a - 5b

2. R', R_1, R_2 = H, R'' = Me , Cleomiscosin A methyl ether

Compd.	R	R′	R″	R_1	R_2	Dose (μg/mL)	Pro-inflammatory cytokines		
							TNF-α% inhibition	IL-6% inhibition	IL-1β % inhibition
Vehicle (DMSO)	–	–	–	–	–	–	100.00	100.00**	100.00*
1a	–Br	–OAc	–H	–	–	1	54.49	60.17**	55.27*
						10	54.23	52.48	58.81
1b	–H	–OAc	–H	–	–	1	73.30	81.88	109.32
						10	76.25	78.30	74.84
2a	$-NO_2$	–OAc	–OAc	–	–	1	70.47	87.16	70.38
						10	67.81*	82.03**	65.26*
3a	–	–H	–Me	–Cl	–H	1	50.20*	57.45**	59.19**
						10	49.58**	56.21**	40.12**
4a	–	–H	–Me	–Br	–H	1	44.77**	59.80**	50.08**
						10	41.99	60.03	44.69
5a	–	$-NO_2$	–Me	$-NO_2$	$-NO_2$	1	64.84	81.36	59.08
						10	60.61	79.04	53.28
5b	–	–H	–Me	$-NO_2$	$-NO_2$	1	76.60	86.44	72.02
						10	77.98	80.18	70.20
Diclofenac Sodium	–	–	–	–	–	1	46.46**	51.15**	38.51**

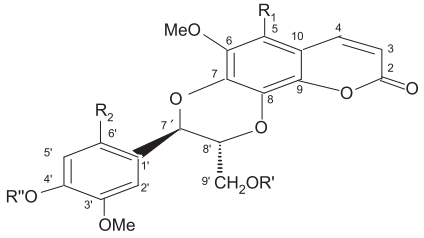
$n = 04$, $^*P < 0.05$, $^{**}P < 0.01$ significance as compared to vehicle control (Student *t*-test).

Table 2
In-vitro target based anti-inflammatory activity of **1a**, **1b**, **2a**, **3a**–**5b** in murine macrophage cell culture system, Quantification of Pro-inflammatory cytokines using sandwich ELISA.



1. R, R', R'' = H, Cleomiscosin A

1a – 2a



2. R', R_1, R_2 = H, R'' = Me, Cleomiscosin A methyl ether

3a - 5b

Compd.	R	R′	R″	R_1	R_2	Dose (μg/mL)	Pro-inflammatory cytokines		
							TNF-α Pg/mL	IL-6 Pg/mL	IL-1β Pg/mL
Vehicle (DMSO)	–	–	–	–	–	–	334.33 ± 23.40	543.00 ± 23.11	103.44 ± 11.79
1a	–Br	–OAc	–H	–	–	1	182.19 ± 45.36^{NS}	326.75 ± 21.13**	57.17 ± 8.75*
						10	181.29 ± 44.91^{NS}	284.98 ± 16.34**	60.83 ± 9.51*
1b	–H	–OAc	–H	–	–	1	245.08 ± 28.11^{NS}	444.60 ± 47.97^{NS}	113.08 ± 33.93^{NS}
						10	254.91 ± 33.35^{NS}	414.77 ± 31.21^{NS}	77.42 ± 5.34^{NS}
2a	$-NO_2$	–OAc	–OAc	–	–	1	235.59 ± 40.25^{NS}	473.27 ± 16.69^{NS}	72.81 ± 5.36^{NS}
						10	226.72 ± 40.28^{NS}	445.44 ± 11.28^{NS}	67.50 ± 4.79^{NS}
3a	–	–H	–Me	–Cl	–H	1	180.67 ± 26.52*	311.98 ± 23.80**	61.22 ± 7.17*
						10	165.77 ± 19.71*	305.21 ± 17.74**	41.50 ± 11.20**
4a	–	–H	–Me	–Br	–H	1	149.68 ± 16.81**	324.69 ± 14.18**	51.81 ± 7.05**
						10	140.38 ± 17.12**	325.95 ± 34.46**	46.22 ± 4.10**
5a	–	$-NO_2$	–Me	$-NO_2$	$-NO_2$	1	216.77 ± 39.67^{NS}	441.81 ± 27.20^{NS}	61.11 ± 15.52^{NS}
						10	202.64 ± 39.23^{NS}	429.17 ± 30.71^{NS}	55.11 ± 9.39^{NS}
5b	–	–H	–Me	$-NO_2$	$-NO_2$	1	256.10 ± 22.93^{NS}	469.36 ± 16.01^{NS}	74.50 ± 2.58^{NS}
						10	260.72 ± 8.54^{NS}	435.38 ± 25.99^{NS}	72.61 ± 8.94^{NS}
Diclofenac Sodium	–	–	–	–	–	1	155.33 ± 8.39**	277.73 ± 20.61**	39.83 ± 5.00**

$n = 04$.
NS: Not Significance.
$^{*}P < 0.05$, $^{**}P < 0.01$ significance as compared to vehicle control (Student *t*-test).

C-5 bromo derivative **4a**, $C_{21}H_{19}O_8Br$, m.p. 105–107 °C, $[M + H]^+$ and $[M^{+2} + H]^+$ (positive mode) at *m/z* 479 and 481.

Treatment of methyl ether of cleomiscosin A with conc. HNO_3 at −5 °C yielded two products; **5a** and **5b** (Scheme 2). In the ^1H NMR spectrum of trinitro derivative **5a**, $C_{21}H_{17}N_3O_{14}$, m.p. 145–147 °C, C-4H, underwent a downfield shift and appeared at δ 7.70 (d, $J = 10.0$ Hz) indicating the introduction of one nitro group at C-5 position. The C-7′ H showed a downfield shift and appeared at δ 5.96 (d, $J = 6.4$ Hz). Also, the ^1H NMR spectrum showed two singlets at δ 7.67 (C-5′) and 7.14 (C-2′) indicating the placement of the second nitro group at C-6′ position. Moreover, the primary alcoholic group was found to be nitrated and its two protons appeared as a double doublet at δ 4.76 and 4.72 ($J = 5.6$ Hz and 14.8 Hz) and as a broad doublet merges with the C-8′ H signal at δ 4.87. The C-9′ H carbon showed a downfield shift in its ^{13}C NMR spectrum and appeared at δ 69.7. The structure of the second minor dinitroderivative, $C_{21}H_{18}N_2O_{12}$, m.p. 200–202 °C; was confirmed as **5b**, as it showed the downfield shift of the C-4H which appeared at δ 7.73 (d, $J = 9.6$ Hz) and also showed the absence of C-5H in its ^1H NMR spectrum. The other nitro group was put at C-6′ as C-5′ H and C-2′ H appeared as singlets at δ 7.57 and 7.12, respectively.

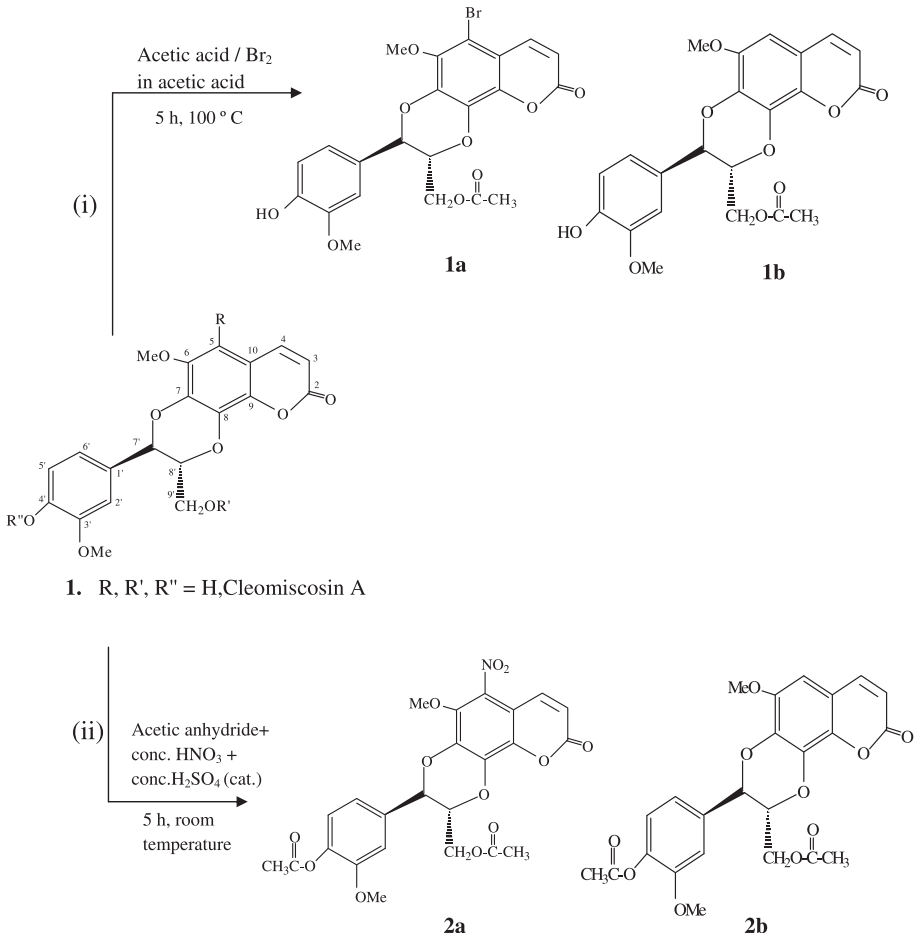
3. Result and discussion

Six new coumarino-lignoid derivatives of both cleomiscosin A and cleomiscosin A methyl ether **1a**–**5b** were reported for the first time. Structures of the synthesized compounds were established on the basis of IR, ^1H NMR, ^{13}C NMR and Mass spectral data.

The target based anti-inflammatory activities of compounds **1a**–**5b** (Tables 1 and 2) have been evaluated in *in-vitro* condition at the initial dose of 1 μg/mL and 10 μg/mL. The compounds **1a**, **3a** and **4a** showed the significant inhibition of pro-inflammatory targets in lipo-polysaccharide (LPS)-induced inflammation in primary macrophages cell culture model. The pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) were quantified from cell culture supernatant using enzyme-linked immunosorbent assay (ELISA). The compounds **1a**, **3a** and **4a** showed the significant inhibition of the pro-inflammatory targets which are the mediators of various chronic inflammatory disorders.

4. Conclusion

In summary, we report that nuclear bromination of cleomiscosin A in acetic acid produced the monobromo derivative in which the primary alcoholic group of cleomiscosin A was also esterified with acetic acid. Furthermore, we have also studied the chlorination and bromination reactions using *N*-chlorosuccinimide (NCS) and *N*-bromosuccinimide (NBS) on the methyl ether of cleomiscosin A. We have found that the reactions of the methyl ether of cliv A with NBS and NCS produced a monobromo and monochloro derivative only. Nitration of cleomiscosin A with conc. HNO_3 and a catalytic amount of conc. H_2SO_4 in presence of acetic anhydride produced the C-5 nitro derivative in which both the alcoholic and the phenolic groups were esterified and also the diesterified cleomiscosin A were obtained. It is very interesting to see that the nitration of cleomiscosin A methyl ether with conc. HNO_3, two



Scheme 1. Synthesis of cleomiscosin A derivatives (**1a**–**2b**). Reagents and reaction conditions: (i) Cleomiscosin A, Acetic acid, Br_2 in acetic acid, Reflux, 5 h. (ii) Cleomiscosin A, Acetic anhydride, Conc. H_2SO_4, Conc. HNO_3, 5 h, room temperature.

nitrated products were obtained. In both the products, nitration took place at C-5 and C-6′ positions and in one product the primary alcoholic group was also nitrated. All the above derivatives **1a**–**5b** were made for the first time from cleomiscosin A and its methyl ether derivative.

The preliminary *in-vitro* anti-inflammatory activity of these novel series of derivatives has evidenced that coumarino-lignoid derivative **1a**, **3a** and **4a** showed significant inhibition of the pro-inflammatory targets which are the mediators of various chronic inflammatory disorders.

5. Experimental protocols

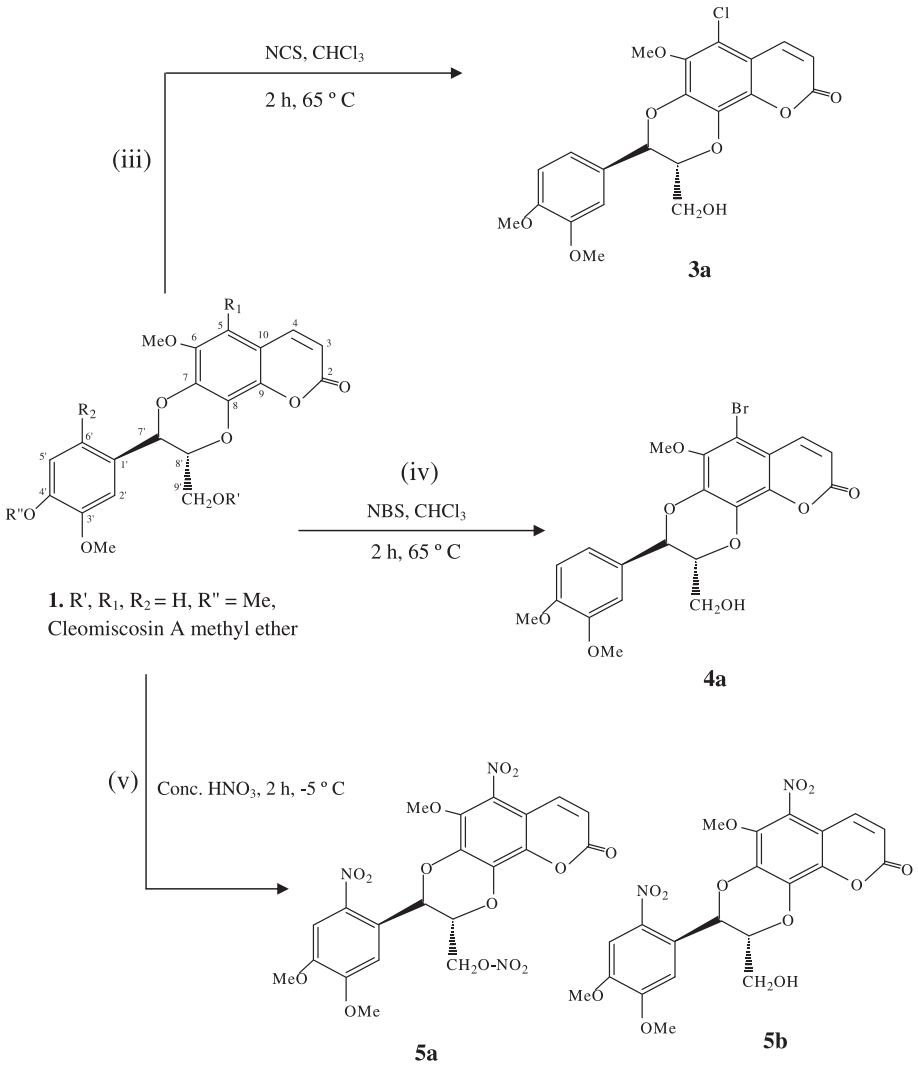
5.1. Chemistry

All the chemicals and solvents were obtained from Lobachemie, Qualigens, Merck and used as such. Melting points were determined in an open capillary tube on a JSGW melting point apparatus and are uncorrected. The NMR spectra of synthesized derivatives **1a**–**4a** were recorded by using Bruker AVANCE 300 MHz whereas the NMR spectra of derivatives **5a** and **5b** were recorded by using Bruker AVANCE 400 MHz FT NMR. IR spectra, KBr pellets, 600–4000 cm^{-1}, were recorded on Perkin–Elmer FTIR, BX Spectrophotometer. Mass spectra (Electrospray ionization in positive mode, ESI^+) were recorded on an API 3000 (Applied Biosystem) mass spectrometer; chromatographic purifications were performed by silica gel 60–120 mesh (MERCK) column chromatography.

5.1.1. General procedure for the synthesis of (5-bromo-9′-acetate) cleomiscosin A (*1a*) and 9′ acetate of cleomiscosin A (*1b*)

A mixture of cleomiscosin A (0.25 mmol) and Br_2 in acetic acid (0.5 mL, 19.74 mmol Br_2 in 2.5 mL, 43.67 mmol acetic acid) in a 100 mL round bottom flask was refluxed at 100 °C for 5 h. After completion of the reaction, reaction mixture was diluted with water and 5% Na_2SO_3 solution and stirred for 30 min at room temperature. It was then extracted with $CHCl_3$. The residue obtained after drying and concentrating the $CHCl_3$ extract was purified by column chromatography (silica gel, 60–120 mesh; petroleum ether/ethyl acetate, 70:30) to afford the desired bromoacetate and a monoacetate derivative of cleomiscosin A.

*5.1.1.1. (5-Bromo-9′-acetate) cleomiscosin A (**1a**).* White amorphous solid, m.p. 70–72 °C, % yield: 38; Mass (ESI^+) $(M + H)^+$/$(M^{+2} + H)^+$: m/z = 507/509. IR (KBr): ν_{max} = 3448, 2940, 1735, 1722, 1602, 1562, 1435, 1125, 1056, 825 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.37 (d, J = 9.9 Hz, 1H, 3H), 8.02 (d, J = 9.9 Hz, 1H, 4H), 7.16 (d, J = 1.8 Hz, 1H, 2′H), 6.97 (d, J = 8.1 Hz, 1H, 5′H), 6.90 (dd, J = 8.1, 1.8 Hz, 1H, 6′H), 5.01 (d, J = 7.5 Hz, 1H, 7′H), 4.09 (m, 1H, 8′H), 4.37 (m, 2H, 9′H), 3.87, 3.94 (2s, 2 × 3H, 2× $-OCH_3$), 2.10 (s, 3H, 9′-$OCOCH_3$) ppm. ^{13}C NMR ($CDCl_3$, 25 °C): δ = 159.9 (C-2), 115.6 (C-3), 143.1 (C-4), 107.9 (C-5), 143.7 (C-6), 131.7 (C-7), 129.7 (C-8), 141.2 (C-9), 112.6 (C-10), 127.5 (C-1′), 109.4 (C-2′), 148.1 (C-3′), 144.9 (C-4′), 124.5 (C-5′), 123.9 (C-6′), 75.8 (C-7′), 76.9 (C-8′), 62.8 (C-9′), 20.9 (CH_3), 170.6 (CO, $-OCOCH_3$), 61.6, 56.9 (2× $-OCH_3$) ppm.



Scheme 2. Synthesis of cleomiscosin A methyl ether derivatives (**3a**–**5b**). Reagents and reaction conditions: (iii) Cleomiscosin A methyl ether, *N*-Chlorosuccinimide (NCS), $CHCl_3$, Reflux, 2 h. (iv) Cleomiscosin A methyl ether, *N*-Bromosuccinimide (NBS), $CHCl_3$, Reflux, 2 h. (v) Cleomiscosin A methyl ether, Conc. HNO_3, 2 h, −5° C.

*5.1.1.2. 9′ Acetate of cleomiscosin A (**1b**).* White amorphous solid, m.p. 160 °C, % yield: 30; Mass (ESI^+) $(M + H)^+$: m/z = 428.9, $[M + Na]^+$ = 450.9. IR (KBr): ν_{max} = 3427, 2925, 1727, 1615, 1574, 1448, 1138, 1058, 841 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.31 (d, J = 9.6 Hz, 1H, 3H), 7.60 (d, J = 9.6 Hz, 1H, 4H), 6.86 (d, J = 1.8 Hz, 1H, 2′H), 6.92 (d, J = 7.8 Hz, 1H, 5′H), 6.90 (dd, J = 8.5 Hz, 1H, 6′H), 4.99 (d, J = 8.1 Hz, 1H, 7′H), 4.37 (m, 1H, 8′H), 4.32, 4.05 (m, 2H, 9′H), 3.89, 3.88 (2s, 2 × 3H, 2× −OCH_3), 2.07 (s, 3H, 9′-$OCOCH_3$) ppm. ^{13}C NMR ($CDCl_3$, 25 °C): δ = 161.1 (C-2), 114.7 (C-3), 144.1 (C-4), 100.8 (C-5), 146.3 (C-6), 137.7 (C-7), 132.3 (C-8), 139.2 (C-9), 112.2 (C-10), 126.9 (C-1′), 110.1 (C-2′), 147.4 (C-3′), 145.3 (C-4′), 115.3 (C-5′), 121.5 (C-6′), 75.8 (C-7′), 77.3 (C-8′), 63.2 (C-9′), 21.1 (CH_3), 170.9 (CO, −$OCOCH_3$), 56.7, 56.4 (2× −OCH_3) ppm.

*5.1.2. General procedure for the synthesis of (5-nitro-4′,9′-diacetate) cleomiscosin A (**2a**)*

A mixture of cleomiscosin A (0.25 mmol) in acetic anhydride (2 mL, 22.39 mmol), conc. HNO_3 (1 mL) and conc. H_2SO_4 (0.1 mL) in a 100 mL round bottom flask was kept for 5 h at room temperature. After completion of the reaction, the reaction mixture was diluted with water and extracted with $CHCl_3$ .The residue obtained after drying and concentrating the $CHCl_3$ extract was purified by column chromatography (silica gel, 60–120 mesh; petroleum ether/ethyl acetate, 80:20) to afford the desired nitro diacetate and diacetate product of cleomiscosin A. Yellow amorphous solid, m.p. 180–182 °C, % yield: 45. Mass (ESI^+) $(M + H)^+$: m/z = 516.3, $[M + Na]^+$: 538.2, $[M + K]^+$: m/z = 554.2. IR (KBR) ν_{max} = 3447, 1773.17, 1749.6, 1718.84, 1653, 1637, 1625, 1572.81, 1541.89, 1524.95, 1364.41, 1288.05, 1133.12 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.41 (d, J = 9.9 Hz, 1H, 3H), 7.64 (d, J = 9.9 Hz, 1H, 4H), 6.90 (d, J = 2.1 Hz, 1H, 2′H), 7.07 (d, J = 8.1 Hz, 1H, 5′H), 6.92 (dd, J = 8.1 Hz, 2.1 Hz; 1H, 6′H), 5.05 (d, J = 7.8 Hz, 1H, 7′H), 4.09 (m, 1H, 8′H), 4.36 (m, 2H, 9′H), 3.79, 3.92 (2s, 2 × 3H, 2× −OCH_3), 2.01 (s, 3H, 9′-OAc), 2.27 (s, 3H, 4′-OAc) ppm. ^{13}C NMR ($CDCl_3$, 25 °C): δ = 158.9 (C-2), 111.9 (C-3), 137.9 (C-4), 106.6 (C-5), 141.3 (C-6), 134.4 (C-7), 129.8 (C-8), 140.0 (C-9), 111.6 (C-10), 134.3 (C-1′), 110.4 (C-2′), 156.4 (C-3′), 139.8 (C-4′), 121.7 (C-5′), 117.6 (C-6′), 72.7 (C-7′), 76.5 (C-8′), 62.3 (C-9′), 20.8 (2 × CH_3), 170.9 (−CO−, 4′-OAc), 168.5 (−CO−, 9′-OAc), 57.2, 63.4 (2× −OCH_3) ppm.

*5.1.3. General procedure for the synthesis of 5-chloro-cleomiscosin A methyl ether (**3a**)*

To a 50 mL round bottom flask was added the cleomiscosin A methyl ether (50 mg, 0.125 mmol), *N*-chlorosuccinimide (NCS) (100 mg, 0.374 mmol) and $CHCl_3$ (5 mL). The reaction mixture was refluxed at 65 °C for 2 h. After completion of the reaction, the

reaction mixture was diluted with water and 5% Na_2SO_3 was added and stirred at room temperature for 30 min. The reaction mixture was then extracted with $CHCl_3$ .The residue obtained after drying and concentrating the $CHCl_3$ extract was purified by column chromatography (silica gel, 60–120 mesh; chloroform/acetone, 98:2) to afford the desired chloro product of cleomiscosin A methyl ether. White amorphous solid, m.p. 172–174 °C, % yield: 55 (30 mg). Mass (ESI^+) $(M + H)^+/(M^{+2} + H)^+$: m/z = 435/437, $[M + Na]^+$: m/z = 457/459, $[M + K]^+$: m/z = 473/475. IR (KBr): ν_{max} = 3450, 1716, 1707, 1605, 1352, 819, 790 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.27 (d, J = 9.6 Hz, 1H, 3H), 7.90 (d, J = 9.6 Hz, 1H, 4H), 6.92 (br s, 1H, 2′H), 6.97 (d, J = 8.1 Hz, 1H, 5′H), 6.85 (d, J = 8.1 Hz, 1H, 6′H), 5.11 (d, J = 8.1 Hz, 1H, 7′H), 3.92 (m, 1H, 8′H), 3.52, 4.01 (m, 2H, 9′H), 3.89 (s, 1H, 9′OH), 3.84 (s, 3H, $-OCH_3$), 3.82 (2s, 2 × 3H, 2× $-OCH_3$) ppm. ^{13}C NMR ($CDCl_3$, 25 °C): δ = 160.4 (C-2), 111.7 (C-3), 140.8 (C-4), 110.8 (C-5), 142.6 (C-6), 142.0 (C-7), 131.5 (C-8), 135.0 (C-9), 117.1 (C-10), 127.8 (C-1′), 110.7 (C-2′), 150.3 (C-3′), 149.8 (C-4′), 114.9 (C-5′), 120.6 (C-6′), 77.5 (C-7′), 79.0 (C-8′), 61.4 (C-9′), 61.7, 56.4, 56.37 (3× $-OCH_3$) ppm.

5.1.4. General procedure for the synthesis of 5-bromo-cleomiscosin A methyl ether (**4a**)

To a 50 mL round bottom flask was added the cleomiscosin A methyl ether (25 mg, 0.063 mmol), *N*-bromosuccinimide (NBS) (50 mg, 0.279 mmol) and $CHCl_3$ (5 mL). The reaction mixture was refluxed at 65 °C for 2 h. After completion of the reaction, the reaction mixture was diluted with water and 5% Na_2SO_3 was added and stirred at room temperature for 30 min. It was then extracted with $CHCl_3$ .The residue obtained after drying and concentrating the $CHCl_3$ extract was purified by column chromatography (silica gel, 60–120 mesh; chloroform/acetone, 98:2) to afford the desired bromo product of cleomiscosin A methyl ether. White amorphous solid, m.p. 105–107 °C, % yield: 67 (30 mg). Mass (ESI^+) $(M + H)^+/(M^{+2} + H)^+$: m/z = 479/481, $[M + Na]^+$: m/z = 501/503, $[M + K]^+$: m/z = 517/519. IR (KBr): ν_{max} = 3448, 2930, 2854, 1734, 1719, 1600, 1458, 1402, 1264, 1124, 1057, 1025, 812 cm^{-1} ^1H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.27 (d, J = 9.9 Hz, 1H, 3H), 7.92 (d, J = 9.9 Hz, 1H, 4H), 6.91 (br s, 1H, 2′H), 6.97 (d, J = 8.1 Hz, 1H, 5′H), 6.85 (d, J = 8.1 Hz, 1H, 6′H), 5.10 (d, J = 8.1 Hz, 1H, 7′H), 4.06 (m, 1H, 8′H), 3.55, 3.84 (m, 2H, 9′H), 3.91 (s, 1H, 9′OH), 3.84 (s, 3H, $-OCH_3$), 3.82 (2s, 2 × 3H, 2× $-OCH_3$) ppm. ^{13}C NMR ($CDCl_3$, 25 °C): δ = 160.4 (C-2), 111.7 (C-3), 143.4 (C-4), 107.6 (C-5), 143.8 (C-6), 141.1 (C-7), 132.1 (C-8), 141.9 (C-9), 112.1 (C-10), 127.8 (C-1′), 110.7 (C-2′), 150.3 (C-3′), 149.8 (C-4′), 115.1(C-5′), 120.6 (C-6′), 77.5 (C-7′), 79.1 (C-8′), 61.4 (C-9′), 61.5, 56.8, 56.4 (3× $-OCH_3$) ppm.

5.1.5. General procedure for the nitration reaction of cleomiscosin A methyl ether, (**5a** and **5b**)

To a 50 mL round bottom flask was added the cleomiscosin A methyl ether (70 mg, 0.175 mmol), and conc. HNO_3 (3 mL). The reaction mixture was kept at 0° to −5 °C for 2 h. After completion of the reaction, crushed ice was added to the reaction mixture and yellow precipitate of nitro derivative was obtained. The reaction mixture was then extracted with $CHCl_3$ .The residue obtained after drying and concentrating the $CHCl_3$ extract was purified by column chromatography (silica gel, 60–120 mesh; petroleum ether/ethyl acetate, 80:20; and petroleum ether/ethyl acetate, 60:40) to afford the desired trinitro and dinitro product of cleomiscosin A methyl ether. The prepared compound was confirmed by ^1H NMR, ^{13}C NMR, ESI-MS (positive mode) and FTIR analysis.

*5.1.5.1. 5,6′,9′-Trinitro-cleomiscosin A methyl ether (**5a**).* Light yellow solid, m.p. 145–147 °C, % yield: 42 (40 mg). Mass (ESI^+) $(M + H)^+$: m/z = 536, $[M + Na]^+$: m/z = 559. IR (KBr): ν_{max} = 3448, 2945, 1737, 1638, 1571, 1525, 1460, 1440, 1281, 1220, 1071 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$ + DMSO, a few drops, 25 °C): δ = 6.49 (d, J = 10.0 Hz, 1H, 3H), 7.70 (d, J = 10.0 Hz, 1H, 4H), 7.14 (s, 1H, 2′H), 7.67 (br s, 1H, 5′H), 5.96 (d, J = 6.4 Hz, 1H, 7′H), 4.87 (br d, 1H, 8′H), 4.76, 4.72 (dd, J = 5.6, 14.8 Hz, 2H, 9′H), 4.02(s, 3H, $-OCH_3$), 3.98, 3.96 (2s, 2 × 3H, 2× $-OCH_3$) ppm. ^{13}C NMR ($CDCl_3$ + DMSO, a few drops, 25 °C): δ = 157.7 (C-2), 116.8 (C-3), 136.9 (C-4), 105.5 (C-5), 140.9 (C-6), 138.7 (C-7), 133.0 (C-8), 139.3 (C-9), 122.2 (C-10), 134.6 (C-1′), 109.8 (C-2′), 153.4 (C-3′), 149.4 (C-4′), 107.8 (C-5′), 138.3 (C-6′), 71.7 (C-7′), 74.8 (C-8′), 69.8 (C-9′), 62.8, 56.0, 56.3 (3× $-OCH_3$) ppm.

*5.1.5.2. 5,6′-Dinitro-cleomiscosin A methyl ether (**5b**).* Dark yellow solid, m.p. 200–202 °C, % yield: 32 (30 mg). Mass (ESI^+) $(M + H)^+$: m/z = 491. IR (KBr): ν_{max} = 3448, 2942, 1733, 1622, 1572, 1571, 1524, 1460, 1440, 1280, 1222, 1072 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$ + DMSO, a few drops, 25 °C): δ = 6.48 (d, J = 9.6 Hz, 1H, 3H), 7.73 (d, J = 9.6 Hz, 1H, 4H), 7.12 (s, 1H, 2′H), 7.57 (s, 1H, 5′H), 5.96 (d, J = 7.2 Hz, 1H, 7′H), 4.55 (distorted triplet, 1H, 8′H), 3.72 (dd, J = 12.8, 4.4 Hz, 1H, 9′H), 3.91 (br d, J = 13.2 Hz, 1H, 9′H), 3.99 (s, 3H, $-OCH_3$), 3.92 (2s, 2 × 3H, 2× $-OCH_3$) ppm. ^{13}C NMR ($CDCl_3$ + DMSO, a few drops, 25 °C): δ = 157.6 (C-2), 116.0 (C-3), 136.9 (C-4), 104.8 (C-5), 141.0 (C-6), 138.5 (C-7), 133.7 (C-8), 139.2 (C-9), 122.3 (C-10), 134.1 (C-1′), 110.1 (C-2′), 152.2 (C-3′), 148.5 (C-4′), 107.5 (C-5′), 138.1 (C-6′), 72.7 (C-7′), 78.4 (C-8′), 59.5 (C-9′), 61.9, 55.8, 55.7 (3× $-OCH_3$) ppm.

5.2. Pharmacology

Inflammation is a multi-step process that is mediated by activated inflammatory cells, including macrophages/monocytes [7]. In the presence of stimuli such as lipo-polysaccharide (LPS), activated macrophages induce the overproduction of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 [8]. In this study, we used macrophage cells stimulated with LPS for target based *in-vitro* anti-inflammatory evaluation of compound **1a**–**5b**.

5.2.1. Primary cell culture and treatment

Primary macrophage cells were collected from the peritoneal cavities of mice (8-week-old female Swiss albino mice) after an intra-peritoneal (i.p.) injection of 1 mL of 1% peptone (BD USA) 3 days before harvesting. Mice were euthanised by cervical dislocation under ether anesthesia and peritoneal macrophages were obtained by intra-peritoneal (i.p.) injection of Phosphate Buffer Saline (PBS), pH-7.4. The peritoneal macrophages at the concentration of 2 × 10^6 live cells/mL were used for the experimentation.

5.2.2. Quantification of pro-inflammatory mediators using enzyme-linked immunosorbent assay (ELISA)

The cells were suspended in RPMI 1640 medium (sigma chemicals co. USA) containing 10% heat-inactivated fetal calf serum (Gibco, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin and incubated in a culture plate (Nunc, Germany) at 37 °C in 5% CO_2 in an incubator. Non-adherent cells were removed after 2 h and the adherent cells were resuspended in RPMI 1640 medium. Cells were pretreated with 1 and 10 μg/mL of test compounds for 30 min and then stimulated with lipo-polysaccharide (LPS, 0.5 μg/mL). After incubation with LPS for 24 h, supernatants were collected and immediately frozen at −80 °C. Harvested supernatants were tested for quantification of pro-inflammatory mediators (IL-1β, IL-6 and TNF-α) by ELISA using commercial kits for mouse IL-1β, IL-6 and TNF-α (BD USA). All the synthesized derivatives were dissolved in Dimethyl sulphoxide (DMSO) and cells treated with 10 μL of DMSO were considered as a vehicle control. *In-vitro* anti-inflammatory activity of the compounds was compared with vehicle control [6].

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