Indian Journal of Chemistry
Vol. 43B, April 2004, pp. 869-872

Screening of natural products for new leads as inhibitors of IκBα kinase: Coumarin derivatives from plant extracts

N V S Ramakrishna*[†], A S Kulkarni, T S More, R G Bhat, S Krishnamurthy
E P DeSouza, E K S Vijayakumar, R D Gupte & R V S V Vadlamudi

Research Centre, Hoechst Marion Roussel Limited, LBS Marg, Mulund (W), Mumbai 400 080, India

Received 21 November 2002; accepted (revised) 23 July 2003

Mammea A/AB **1**, Mammea A/AD **2**, 1‴-acetoxy-mammea E/BB **3** and Mammea A/BB **4** have been isolated by bioactive purification. Compounds **1** and **2** are isolated from CH_2Cl_2-MeOH (1:1) extract of fruits of *Mammea suriga* and **3** is isolated from the same solvent extraction of twigs. **4** is isolated from methanol extract of leaves of *Mesua ferrea*. All the four compounds **1**, **2**, **3** and **4** exhibit interesting IκBα kinase inhibitory activity.

IPC: Int.Cl.[7] C 07 D 311/10

The role of IκBα kinase enzyme in phosphorylation of IκB, which is an important step in the activation of the transcription factor NFκB and in turn the role of NFκB activation in arthritis have been described earlier[1,2].

Mammea is a small genus of trees and three species have been recorded in India[3]. *Mammea suriga* is a large tree with a cylindrical trunk and found in evergreen forests of western India ascending to an altitude of 600 metres. Several medicinal properties have been reported for this plant in literature[3]. The buds of *Mammea suriga* possess mild stimulant, carminative and astringent properties and are used in dyspepsia and haemorrhoids. The fruit is edible. The wood is occasionally used for building purposes. Wide variety of chemical compounds have been isolated from the plants belonging to *Mammea* genus. Mammea A/AB[4,5] **1**, Mammea A/AD[6] **2** and 1‴-Acetoxy-mammea E/BB[7] **3** have been isolated earlier from extracts of genus *Mammea* or/and *Mesua*.

Mesua is a genus of trees or shrubs and one species is found in India[8]. *Mesua ferrea* is a medium to large evergreen tree and found in Himalayas, Deccan peninsula and Andaman islands in India ascending to an altitude of 1500 metres. The fruit is edible. The pericarp contains tannins. The flowers are astringent and stomachic. The seed oil is used as an embrocation in rheumatism and for skin diseases. *Mesua* timber is used in railway sleepers, bridges, beams, construction work and boat building etc. Mammea A/BB[7] **4** has been isolated earlier from extracts of *Mammea americana*.

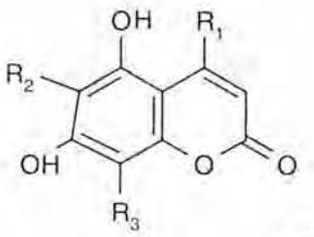
This communication describes the bioactivity guided isolation, characterization and IκBα kinase inhibitory activity of **1**, **2**, **3** and **4** (**Figure 1**).

The CH_2Cl_2-MeOH (1:1) extract of the fruits of *Mammea suriga* was concentrated *in vacuo* and chromatographed on polyamide column using methanol as eluting solvent to remove interfering tannins. The resulting material was chromatographed over Sephadex LH-20 column in methanol, to get semipure concentrate. The compounds **1** and **2** were obtained in pure form by semi-preperative HPLC using the conditions as described in the experimental section. Compounds **1** and **2** were identified as Mammea A/AB[4] and Mammea A/AD[6] by comparison of the spectral data with the data reported in literature.

The CH_2Cl_2-MeOH (1:1) extract of the twigs of *Mammea suriga* was concentrated *in vacuo* and chromatographed twice over Sephadex LH-20 column in MeOH, to get semipure concentrate. Compound **3** was obtained in pure form by analytical HPLC using the conditions as described in the Experimental Section. Compound **3** was identified as 1‴-acetoxy-mammea E/BB[7] by comparison of the spectral data with the data reported in literature.

Methanol extract of the dried leaves of the plant *Mesua ferrea* was concentrated and the resultant crude was triturated twice with CH_2Cl_2. The soluble

[†] Current address: Suven Pharmaceuticals Ltd., Serene Chambers, Road No. 7, Banjara Hills, Hyderabad - 500 034.



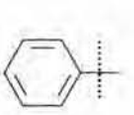
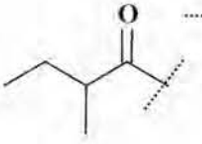
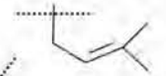
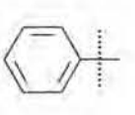
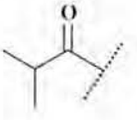
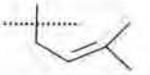
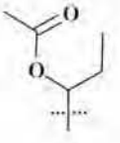
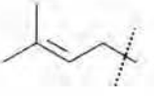
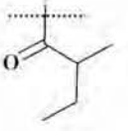
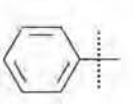
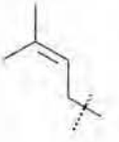
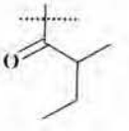
Compd	Name	R_1	R_2	R_3	IC_{50} (μM)
1	Mammea A/AB				37.50
2	Mammea A/AD				23.50
3	1‴-Acetoxy-mammea E/BB				38.00
4	Mammea A/BB				55.50

Figure 1

portion was chromatographed over silica gel using ethyl acetate-petroleum ether gradient as eluting solvent to obtain **4** in pure form which was identified as Mammea A/BB[7] by comparison of the spectral data with the data reported in literature.

Isolation of compounds **1**, **2**, **3** and **4** was monitored by TLC or HPLC and IκBα kinase inhibitory activity.

Experimental Section

Melting points were determined on a Bristoline apparatus and were uncorrected. IR spectra were recorded on a Perkin-Elmer 782 spectrophotometer. UV spectra were recorded on a Chemito 2500 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker ACP 300 spectrometer and mass spectra on VG Quattro II mass spectrometer. TLC was carried out using precoated silica plates (Article No. 5544, E. Merck) and spots were visualized under UV.

Biological screening assay

Materials. IkB kinase 96 well plate ELISA assay was specially developed by ProScript Inc., 38 Sidney Street, Cambridge, MA 02139, U.S.A. All special reagents required for this assay like blocking peptide MX-116 (Ac-DRHDSGLDSMKD-CONH2), substrate PX-0203 (Biotin-$[CH_2]_6$-DRHDSGLDSMKD-CONH2), standard phosphopeptide PX-0225 (Biotin-$[CH_2]_6$-DRHDS$[PO_3]$GLDSMKD-$CONH_2$), IkB kinase were also tailor made by ProScript. Antibody was obtained from New England Biolabs. Reacti-bind Protein-A coated 96 well plates were obtained from Pierce, Rocford, IL., U.S.A. ½ - area polystyrene 96-well plates were obtained from Costar. Streptavidin conjugated Horseradish Peroxidase was obtained from Pierce, Rocford, IL, U.S.A. 3,3′,5,5′- tetramethylbenzidine (TMB) Peroxidase 1-component reagent and TMB 1-component stop solution were obtained from Kirkegaard & Perry Lab, Gaithersburg, MD.

Microcystin used as phosphatase inhibitor was obtained from Calbiochem, La Jolla, CA., U.S.A.

Assay. Reacti-bind Protein-A coated plates and ½ - area Costar plates were blocked for 1 hour by addition of 5% non fat milk in phosphate buffer saline (*p*H 7.4), containing MX-116 at 10 mM final concentration. After washing 3 times with phosphate buffer saline, antibody was added to only protein-A coated plate and left at room temperature for 2 hr. Blocked Costar plate was used to carry out kinase-substrate reaction, wherever necessary in presence of test substances. IkB kinase was incubated with its biotinylated substrate (PX-0203) at 37°C for 30 min and the reaction mixture was then transferred from Costar plate to Protein-A coated blocked plate, coated with antibody. Phosphopeptide formed during the reaction got attached to antibody, since specific antibody was used, and the amount of phosphopeptide was assayed by measuring biotin label by standard ELISA technique i.e. treatment with streptavidin HRP at room temperature for 1 hour followed by colour development with TMB reagent and finally stopping the reaction with a stop reagent. The readings were taken on Dynatech microplate reader at 450 nm.

Extraction and isolation of 1 and 2

The fruits of *Mammea suriga* were collected in Mumbai, India. Shade dried and pulverised (in grinding mill) 1.1 kg of this material was extracted with 1:1 mixture of CH_2Cl_2-MeOH (5 lts, 48 hr) at 45°C. Extract was filtered and concentrated *in vacuo* to get crude plant extract (155 g).

Crude plant extract (600 mg) was loaded on a polyamide column (1.6 × 16 cm) and was eluted with 450 mL of MeOH and the eluate was concentrated to dryness (545 mg). This material was triturated with 60 mL of CH_3CN and filtered to get the clear solution containing the compounds **1** and **2**, which was concentrated to remove the solvent. This material was chromatographed over Sephadex LH-20 (6.8 × 62.5 cm) column using MeOH as eluting solvent. Fractions were collected in 30 mL volumes each and were monitored by bioactivity. Fractions containing compounds **1** and **2** were mixed and concentrated to dryness to get enriched material. The semi-pure material (260 mg) was finally purified by semi-preparative HPLC on a (16×250 mm) Knauer Eurosphere RP-18 (10 μ) column using gradient of 0.1% aqueous orthophosphoric acid (*p*H 2.5) to CH_3CN in 20 min and then in 100 % CH_3CN for 10 min at a flow rate of 8 mL/min and UV detection at 210 nm. Fractions containing the required compounds (tested by bioactivity and HPLC) which were concentrated *in vacuo* at 35°C and lyophilised to get 75 mg of Mammea A/AB **1** and 56 mg of Mammea A/AD **2**.

Extraction and isolation of 3

The twigs of *Mammea suriga* were collected in Mumbai, India. Shade dried and pulverised (in grinding mill) material (1.1 kg) was extracted with 1:1 mixture of CH_2Cl_2-MeOH (10 ltrs, 48 hr) at 45 °C. The extract was filtered and concentrated *in vacuo* to get crude plant extract (171g).

The crude (200 mg) was chromatographed twice over Sephadex LH-20 column (1.6 × 60 cm) in MeOH and eluted with the same solvent at a flow rate of 0.7 mL/min. Eluates were collected in 5 mL volume each. All the fractions were monitored by bioactivity. The active fractions were pooled and concentrated under vacuo at 40 °C to obtain enriched material (60 mg). The semi-pure material was finally purified on an analytical HPLC, LiChrocart (250 × 4 mm) RP Select B (5μ) column using a gradient of 0.1 % aqueous trifluoroacetic acid (*p*H 2.5) to CH_3CN in 30 min and then in 100 % CH_3CN for 5 min at a flow rate of 1 mL/min and UV detection at 220 nm. Fractions containing active peak were concentrated *in vacuo* at 35 °C and lyophilised to get 35 mg of 1‴-acetoxy-mammea E/BB **3**.

Extraction and isolation of 4

The leaves of *Mesua ferrea* were collected in Coorg, Karnataka, India. Shade dried and pulverised (in grinding mill) material (0.7 kg) was extracted with MeOH (10 lts, 48 hr) at 45 °C. The extract was filtered and concentrated *in vacuo* to get crude plant extract (26 g).

Crude plant extract (18 g) was triturated with CH_2Cl_2 (2 × 250 mL). The soluble portion was chromatographed over silica gel (200-300 mesh, 3.5 × 30 cm) using ethyl acetate-petroleum ether gradient as eluting solvent. Fractions were collected in 15 mL volume each. Fractions were monitored by analytical HPLC and bioactivity. Fractions containing the compound **4** were mixed and concentrated to dryness to get pure compound (20 mg).

Purity of the compounds **1**, **2**, **3** and **4** was checked by HPLC on a LiChrocart (4 × 250 mm) RP Select B (5μ) column using a gradient of 0.1 % aqueous orthophospharic acid (*p*H 2.5) to CH_3CN in 20 min and

then in 100% CH_3CN for 4 min at a flow rate of 1 mL/min and UV detection at 220 nm.

Mammea A/AB 1 : Yellow crystalline solid; m.p. 106-07 ^{0}C; soluble in MeOH, CH_3CN and DMSO; EI-MS: 406(M^+); ^{1}H NMR (300 MHz, $CDCl_3$) : δ 0.85 (t, 6.1 Hz, CH_3), 1.10 (d, 7.3 Hz, CH_3), 1.37 (m, CH_2), 1.78 (s, CH_3), 1.90 (s, CH_3), 3.58 (m, CH), 3.57 (d, 7.3 Hz, CH_2), 5.28 (bt, =CH), 5.97 (s, Ar-H), 7.42-7.54 (m, 5 × Ar-H), 9.84 (bs, OH, D_2O exchangeable), 10.89 (bs, OH, D_2O exchangeable); ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.7 (CO), 162.1 (CO), 159.2 (=C), 158.7 (=C), 158.1 (=C), 154.1(=C), 134.9 (=C), 134.7 (=C), 128.4 (2×=CH), 127.7 (=CH), 126.2 (2 × =CH), 118.1 (=CH), 110.4 (=C), 108.4 (=C), 107.5 (=C), 106.1 (=CH), 47.3 (CH), 25.3 (CH_3), 24.1 (CH_2), 19.3 (CH_3), 13.9 (CH_3), 12.2 (CH_2), 11.0 (CH_3). Anal. Found (%): C, 74.01; H, 6.52. Calcd. for $C_{25}H_{26}O_5$: C, 73.89; H, 6.40. It is identical in all respects to Mammea A/AB.

Mammea A/AD 2 : Yellow crystalline solid; m.p. 130-31 ^{0}C; soluble in MeOH, CH_3CN and DMSO; EI-MS: 392(M^+); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.11 (d, 6.1 Hz, 2 × CH_3), 1.77 (s, CH_3), 1.89 (s, CH_3), 3.57 (d, 6.1 Hz, CH_2), 3.72 (m, CH), 5.28 (dd, 6.1, 7.3 Hz, =CH), 5.97 (s, Ar-H), 7.41-7.55 (m, 5 × Ar-H), 9.76 (bs, OH, D_2O exchangeable), 10.97 (bs, OH, D_2O exchangeable); ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.7 (CO), 162.1 (CO), 159.2 (=C), 158.7 (=C), 158.1 (=C), 154.1(=C), 134.9 (=C), 134.7 (=C), 128.4 (2 × =CH), 127.7 (=CH), 126.2 (2 × =CH), 118.1 (=CH), 110.4 (=C), 108.4 (=C), 107.5 (=C), 106.1 (=CH), 41.6 (CH), 25.3 (CH_3), 16.4 (2 × CH_3), 19.3 (CH_3), 12.2 (CH_2). Anal. Found (%): C, 73.68; H, 6.31. Calcd. for $C_{24}H_{24}O_5$: C, 73.46 %; H, 6.12. It is identical in all respects to Mammea A/AD.

1‴-Acetoxy-mammea E/BB 3 : Semi-solid; soluble in MeOH, CH_3CN and DMSO; EI-MS: 430(M^+); ^{1}H NMR (300 MHz, $CDCl_3$) : δ 0.99 (t, 6.1 Hz, CH_3), 1.02 (t, 6.1 Hz, CH_3), 1.26 (d, 7.6 Hz, CH_3), 1.60 (m, CH_2), 1.84 (s, CH_3), 1.88 (s, CH_3), 1.92 (m, CH_2), 2.19 (s, $COCH_3$), 3.50 (m, CH_2), 3.91 (m, CH), 5.24 (t, 6 Hz, -OCH), 6.28 (bdd, =CH), 7.12 (bs, OH, D_2O exchangeable), 13.67 (s, OH, D_2O exchangeable); ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.7 (CO), 171.0 (CO), 162 (CO), 161.9 (=C), 160.3 (=C), 159.2 (=C), 150.7 (=C), 134.7 (=C), 117.9 (=CH), 109.9 (=C), 109.2 (=CH), 108.7 (=C), 107.4 (=C), 84.7 (CH), 47.0 (CH), 41.6 (CH_3), 25.3 (CH_3), 24.5 (CH_2), 24.1 (CH_2), 19.3 (CH_3), 13.9 (CH_3), 12.0 (CH_2), 11.0 (CH_3), 10.6 (CH_3). Anal. Found (%): C, 67.13; H, 6.86. Calcd. for $C_{24}H_{30}O_7$: C, 66.97; H, 6.86. It is identical in all respects to 1‴-Acetoxy-mammea E/BB.

Mammea A/BB 4 : White solid; m.p. 124-25 ^{0}C; soluble in MeOH, CH_3CN and DMSO; EI-MS: 406(M^+); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.05 (t, 7.3 Hz, CH_3), 1.28 (d, 6.1 Hz, CH_3), 1.51 (m, CH_2), 1.67 (s, CH_3), 1.74 (s, CH_3), 1.96 (m, CH_2), 3.31 (d, 6.1 Hz, CH_2), 3.98 (m, CH), 5.10 (t, 7.3 Hz, =CH), 5.97 (bs, OH, D_2O exchangeable), 6.01 (bt, =CH), 7.45 (m, 2 × Ar-H), 7.56 (m, 3 × Ar-H) and 11.1 (bs, OH, D_2O exchangeable); ^{13}C NMR (75 MHz, $CDCl_3$): δ 200.7 (CO), 162.0 (CO), 161.8 (=C), 159.1(=C), 158.1 (=C), 150.7 (=C), 134.9 (=C), 134.6 (=C), 128.4 (2 × =CH), 127.7 (=CH), 126.2 (2 × =CH), 117.9 (=CH), 108.7 (=C), 107.4 (=C), 105.9 (=CH), 47.2 (CH), 25.3 (CH_3), 24.1 (CH_2), 19.3 (CH_3), 13.9 (CH_3), 12.2 (CH_2), 11.0 (CH_3). Anal. Found (%): C, 74.27; H, 6.56. Calcd. for $C_{25}H_{26}O_5$: C, 73.89; H, 6.40. It is identical in all respects to Mammea A/BB.

Acknowledgement

Authors wish to thank Dr (Mrs) Bhavana Jothwani, Mr R. D. Yeole for HPLC analysis and mass spectral data. Authors are grateful to Dr Bansi Lal for providing research facilities and encouragement. Authors also wish to acknowledge the support received from Dr A. Batzer, Dr B. Kirschbaum, Dr A. Enhsen and Dr J. Wink of HMR, Frankfurt and Dr L. Dang, Dr V. Palombella and Dr R. Stein of ProScript, Boston.

References

1 Ramakrishna N V S, Vijayakumar E K S, Jain A K, Kalakoti B S, Gupte R D, Panicker R B & Vadlamudi R V S V, *Indian J Chem*, 39B, **2000**, 801.

2 Ramakrishna N V S, Jain A K, Kota S, Vijayakumar E K S, Kalakoti B S, Gupte R D, Panicker R B & Vadlamudi R V S V, *Indian J Chem*, 40B, **2001**, 345.

3 *The Wealth of India*, Vol. VI, [PID (CSIR), New Delhi], p 252.

4 Carpenter I, McGarry E J & Scheinmann F, *J Chem Soc*, **1971**, 3783.

5 Crombie L, Jones R C F & Palmes C J, *J Chem Soc Perkin Trans*, **1987**, 317.

6 Chakraborty D P & Das B C, *Tetrahedron Lett*, **1966**, 5727.

7 Crombie L, Games D E, Haskins N J & Reed G F, *J Chem Soc Perkin Trans I*, **1972**, 2255.

8 *The Wealth of India*, Vol. VI, [PID (CSIR), New Delhi], p 349-352.