Screening of natural products for new leads as inhibitors of β-amyloid production: 2-Hydroxy-4-methoxy-3-prenyl-6-styrylbenzoic acid from Cajanus cajan

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2-Hydroxy-4-methoxy-3-prenyl-6-styrylbenzoic acid I has been isolated from the methylene chloride extract of the twigs of Cajanus cajan, and found to inhibit β-amyloid synthesis with an IC50 of 70 μM.

Alzheimer’s Disease (AD) is a neurodegenerative disorder of the central nervous system, mainly affecting people over 65 years of age. The disease begins with loss of short-term memory, which progresses to physical incapacitation and finally to death in two-to-twelve years of time. Increased production and deposition of β-amyloid peptides (Aβ) in certain areas of brain is a hallmark of the disease. Current evidence indicates that β-amyloid deposition may play a central role in the pathogenesis of this disease. Therefore, inhibition of β-amyloid production offers a good strategy for therapeutic intervention in the treatment of AD.

Aβ is derived from the faulty proteolytic processing of β-amyloid precursor protein (β-APP), a transmembrane protein expressed in most mammalian cells. We have employed a cell culture-based assay to identify inhibitors of this proteolytic processing. During our natural product screening program to identify therapeutic leads to inhibit the abnormal processing of β-APP, we found that the methylene chloride extract of the twigs of the plant Cajanus cajan inhibited the production of Aβ from CHO (Chinese Hamster Ovary) cells stably transfected with human APP gene. Herein, we report the bioactivity guided isolation, characterization and biological activity of the compound 2-hydroxy-4-methoxy-3-prenyl-6-styryl-

benzoic acid I isolated from the methylene chloride extract of the twigs of the plant Cajanus cajan.

Cajan cajan, belonging to Papilionaceae family, is cultivated all over India as a pulse crop and is reported to have various medicinal properties. The methylene chloride extract of the twigs of Cajanus cajan, which exhibited the inhibition of β-amyloid (IC50: 1.2 mg/mL), was passed through a column of polyamide to remove tannins, followed by repeated silica gel column chromatography to get pure compound I. Purification was monitored both by TLC and bioactivity testing for inhibition of β-amyloid production in transfected CHO cells. Compound I was identified as 2-hydroxy-4-methoxy-3-prenyl-6-styrylbenzoic acid by analysis of IR, 1H NMR, COSY, 13C NMR and DEPT spectral data and comparison with the data reported in literature. The isolated compound I inhibited β-amyloid production with an IC50 of 70 μM.

Experimental Section

Melting point was determined on a Bristoline apparatus and is uncorrected. IR spectrum was recorded on a Perkin-Elmer 782 spectrophotometer; mass spectra on a VG Quattro II mass spectrometer; and NMR spectra on a Bruker ACP 300 spectrometer. TLC was carried out using precoated silica plate (Article No. 5554, E. Merck) using 5% methanol in chloroform as developing solvent and spots were visualized under UV.

Biological screening assay

Materials. CHO cells, clone 7W.D10, stably transfected with APPΔ671 were obtained from DG Neurosciences, Hoechst Marion Roussel Inc., Bridgewater, N.J., USA. DMEM, fetal bovine serum, geneticin, MTT, β-amyloid peptide fragment 1-40, p-nitro-
phenyl phosphate and antibiotin antibody conjugated to alkaline phosphatase were obtained from Sigma Chemical Co., USA. Anti-αβ monoclonal antibodies 6E10 and 4G8 were obtained from Senetek, Maryland Heights, MO, USA.

**Assay.** Cells were cultured in DMEM with 10% fetal bovine serum and 400 µg/mL of geneticin. 96-well tissue culture plates (Flat bottom, Nunclon) were seeded with 100,000 cells per well and incubated at 37°C overnight. Cells were then washed twice with DMEM without serum, dosed with suitable dilutions of test compounds and incubated at 37 °C for 4 hr. Supernatant medium from these plates was then transferred to ELISA plates (Nunclon, Maxisorp) and incubated at 4°C overnight. Tissue culture plates were then used to determine cytoxicity of the test compound, if any, using the tetrazolium derivative MTT. Following day ELISA was carried out using the monoclonal antibodies, conjugate and the substrate mentioned above. Absorbance of the colour developed in the plates was recorded at 410 nm using a microplate reader (Dyametch, USA). Inhibition of β-amyloid was calculated as percent of cell control.

**Extraction.** Plant material was collected at Dahana, Maharashtra, India. Shade dried plant material (1.0 Kg) was coarsely pulverised and extracted sequentially by stirring for 24 hr at 40°C with 5 liters each of petroleum ether, methylene chloride and methanol. The extracts were concentrated under reduced pressure at 40°C. The crude (16 g) obtained from the methylene chloride extract exhibited β-amyloid inhibitory activity.

**Isolation.** 1 g of crude extract in methanol was passed through a column (0.5 x 20 cm) of polyamide and eluted with methanol. The combined methanol eluates were concentrated under reduced pressure to dryness to obtain enriched crude material (0.8 g). This material was subjected to a silica gel (200-300 mesh) flash column (3.5 x 25 cm) and eluted with 5% methanol in chloroform at a flow rate of 10 ml/min. The fractions were collected in 12 ml size. All the fractions were monitored both by TLC and bioactivity.

The fractions containing 1 were pooled and concentrated under reduced pressure to get semi-pure 1 (0.035 g). The semi-pure material, thus obtained, was finally purified by a second silica gel (200-300 mesh) column (0.5 x 25 cm) using 50% pet. ether in chloroform for elution. The fractions containing 1 were combined and concentrated to dryness under reduced pressure to get pure 1 (0.04 g).

**Compound 1.** White solid, mp 166-68°C; Mol wt. 338; IR (KBr): 3600-3300, 1640, 1600, 1450, 1280, 120, 970, 830 and 750 cm⁻¹; EI-MS: ml/z 338 (M⁺); H NMR (acetone-d₆): δ 10.62 (bs, COOH), 8.03 (d, 16 Hz, 7-H), 7.82 (bs, OH), 7.56 (d, 7.3 Hz, 10-H, 14-H), 7.37 (7.3 Hz, 1-H, 13-H), 7.27 (d, 7.3 Hz, 12-H), 7.00 (d, 16 Hz, 8-H), 6.86 (s, 6-H), 5.20 (t, 7.3 Hz, 17-H), 3.97 (s, OCH₃), 3.34 (d, 7.3 Hz, 16-H), 1.76 (s, CH₃) and 1.63 (s, CH₃); 13C NMR (acetone-d₆): δ 19.2, 25.7, 31.2, 54.3, 104.2, 107.5, 108.9, 117.2, 124.8, 125.6, 126.7, 127.2, 133.3, 133.9, 135.8, 159.5, 168.2, 176; Anal. Found: C, 74.78; H, 6.71. Caled for C₂₂H₂₆O₈: C, 74.55; H, 6.50%. The compound is identical in all respects to 2-hydroxy-4-methoxy-3-prenyl-6-styrylbenzoic acid 1.

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