Inhibition of Na^+,K^+-ATPase Activity by (–)-*ent*-Kaur-16-en-19-oic Acid and its Derivatives

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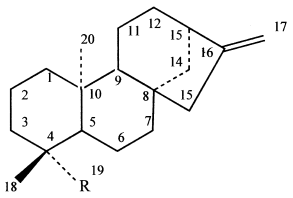
Abstract

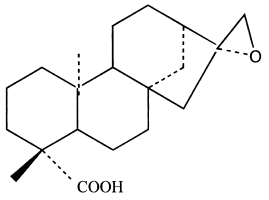
The diterpene, (–)-*ent*-kaur-16-en-19-oic acid, from *Croton oblongifolius* was identified as an Na^+,K^+-ATPase inhibitor. This compound exhibits an IC_{50} of 2.2×10^{-5} M against crude enzyme Na^+,K^+-ATPase from rat brain. The semi-synthetic derivatives, (–)-methyl kaur-16-en-19-oate, (–)-kaur-16-en-19-ol, (–)-16*β*,17-epoxykauran-19-oic acid and (–)-17-hydroxykaur-15-en-19-oic acid were also tested and their IC_{50} were 5.5×10^{-4}, 5.0×10^{-4}, 4.8×10^{-4} and 6.0×10^{-4} M, respectively.

Na^+,K^+-ATPase plays a role in the active transport of Na^+ and K^+ across the cell membrane [1]. Inhibition of Na^+,K^+-ATPase leads to a high level of Na^+ inside the cells. The diminished Na^+ gradient results in slow extrusion of Ca^{2+} by the sodium-calcium exchanger [2]. The subsequent increases in the intracellular level of Ca^{2+} enhance the contractility of muscles such as cardiac muscle and kidney tubular muscle. Thus, brain Na^+,K^+-ATPase is shown to be specifically inhibited by cardiac glycosides such as ouabain and digitoxin [3]. Several kinds of crude drugs used as diuretics are shown to inhibit horse kidney Na^+,K^+-ATPase [4]. Examples of the active substances in these crude drugs are atractylon from *Atractylodes japonica* [5], *β*-eudesmol from Atractylodis Lanceae rhizome [6], and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose from the root cortex of *Paeonia suffuticosa* and *Paeonia lactiflora* [7]. In this report, (–)-*ent*-kaur-16-en-19-oic acid (**1**) and its derivatives (–)-methyl kaur-16-en-19-oate (**2**), (–)-kaur-16-en-19-ol (**3**), (–)-16*β*,17-epoxykauran-19-oic acid (**4**) and (–)-17-hydroxykaur-15-en-19-oic acid (**5**) were tested for their inhibitory activity against crude enzyme Na^+,K^+-ATPase from rat brain.

All parts of *Croton oblongifolius* Roxb. (Euphorbiaceae) have been used as traditional medicine for many applications: the leaves can be used as a tonic, the flowers are used as a teniacide, the fruits are used to treat dysmenorrheal, the seeds are used as a purgative, the barks are used to treat dyspepsia and the roots



1 R = COOH
2 R = $COOCH_3$
3 R = CH_2OH



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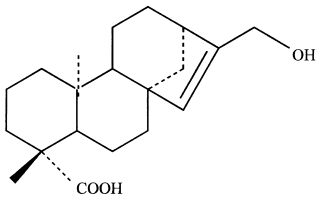

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are used to treat dysentery [8]. Moreover, it is used in conjunction with *C. sublyratus* Kurz. to treat gastric ulcer and gastric cancer. Since *C. oblongifolius* species from different locations are very good sources of diterpenoids with different skeletons [9], it is very interesting to identify the chemical constituents that are responsible for its broad medical applications. The stem bark specimen of *C. oblongifolius* from Ampur Kuiburi, Prachuab Kirikhan province has been screened for rat brain Na^+,K^+-ATPase activity inhibition. Using bioassay guided fractionation, (–)-*ent*-kaur-16-en-19-oic acid **(1)** which is the major constituent in the stem bark was identified as the Na^+,K^+-ATPase activity inhibitor. Moreover, to a lesser extent, its semi-synthetic derivatives **(2 – 5)** also exhibited Na^+,K^+-ATPase activity inhibition as well.

The inhibition of rat brain Na^+,K^+-ATPase activity by compounds **1 – 5** was concentration-dependent. The lowest IC_{50} was obtained from compound **1** (2.2×10^{-5} M). Compounds **2 – 5** were less active with IC_{50} values at 5.5×10^{-4}, 5.0×10^{-4}, 4.8×10^{-4} and 6.0×10^{-4} M, respectively. Thus, this is the first report on kaurane diterpenoids exhibiting Na^+,K^+-ATPase activity inhibition. Compound **1** was reported to exhibit *in vitro* trypanocidal activity against *Trypanosoma cruzi* [10], [11].

Materials and Methods

The stem bark of *C. oblongifolius* was collected from Ampur Kuiburi, Prachuab Kirikhan Province, Thailand, in September 1996. The plant specimen was authenticated by comparison with a voucher specimen (No. BKF 084729) in the herbarium collection of the Royal Forest Department of Thailand.

The powdered, sun-dried stem bark (6.5 kg) of *C. oblongifolius* was extracted with hexane (2 × 10 L). The hexane extract was filtered, and evaporated under vacuum to obtain a dark brown viscous material (100 g). The crude hexane extract (100 g) was fractionated by silica gel column chromatography (1 kg) and eluted with hexane (20 × 500 mL), 5% ethyl acetate in hexane (20 × 500 mL) and 10% ethyl acetate in hexane (20 × 500 mL). Each fraction (500 mL) was evaporated using a rotary evaporator to about 50 mL and left at room temperature. Compound **1** (32.7 g, 0.55% w/w from dry stem bark) was obtained from the 5% ethyl acetate in

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hexane elution (fractions 3 – 12) as colorless crystals; m.p. 171 – 172 °C, $[\alpha]_D^{20}$: –109.6° (*c* 1.0, $CHCl_3$) {lit. [10] m.p. 170.1 °C, $[\alpha]_D^{25}$: –103.5° (*c* 6, $CHCl_3$)} after purification by recrystallization from ethyl acetate in hexane. The structure of compound **1** was also confirmed by 2D-NMR and X-ray crystallographic analysis (data not shown) which are in agreement to those previously reported [12], [13].

Compound **1** (530 mg) was methylated with CH_2N_2 in CH_2Cl_2 to give the methyl ester **2**, quantitatively as a transparent oil; $[\alpha]_D^{20}$: –91.9° (*c* 1.0, $CHCl_3$), {lit. [10] m.p. 72 – 75 °C, $[\alpha]_D^{25}$: –91.9° (*c* 7.93, $CHCl_3$)}. Compound **2** (100 mg) was treated with excess $LiAlH_4$ in dry ether to obtain compound **3** as a white solid; m.p. 133 – 134 °C, $[\alpha]_D^{20}$: –51.6° (*c* 1, $CHCl_3$), {lit. [10] m.p. 134 – 138 °C, $[\alpha]_D^{25}$: –51.64° (*c* 1.46, $CHCl_3$)}.

Compound **1** (500 mg) was treated with *m*-CPBA in CH_2Cl_2 at room temperature for 15 hours to give 16*β*,17-epoxykauran-19-oic acid (**4**) as a white solid; m.p. 154 – 156 °C, $[\alpha]_D^{20}$: –98.4° (*c* 1, $CHCl_3$), {lit. [14] m.p. 157 – 161 °C} and 17-hydroxykaur-15-en-19-oic acid (**5**) as a white solid; m.p. 187 – 188 °C, $[\alpha]_D^{20}$: –101.6° (*c* 1, $CHCl_3$) {lit [15] m.p. 193 – 194 °C}. Compounds **1**, **4** and **5** were previously reported in *Mikania* species and many genera [16], [17]. The physical properties and spectral data of compounds **1 – 5** were in agreement with those reported in the literature. Copies of the original spectra are obtainable from the author of correspondence.

The bioassay of all compounds was carried out using crude rat brain enzyme. The microsome of rat brain enzyme was prepared according to Urayama [3]. Ouabain-sensitive (Na^+,K^+) and ouabain-insensitive (Mg^{2+})ATPase in rat brain microsome were determined with and without 0.5 mM ouabain in a total volume of 420 μL for 30 min according to Satoh [5], [6], [7]. Ouabain, a cardiac glycoside used as positive control, exhibits an IC_{50} value of 2.0×10^{-7} M against this crude enzyme preparation. The standard assay mixture contained 3 mM ATP-tris, 5 mM $MgCl_2$, 0.5 mM EDTA, 140 mM NaCl, 14 mM KCl and 50 mM imidazole (pH 7.2) with and without 0.5 mM ouabain. The stock solution of each compound in DMSO was added to the assay mixture to give final concentrations of 1, 0.1, and 0.01 mM for each compound. The brain microsome sample (5 microgram/mL) was added and incubation was carried out for 30 min at 37 °C. The reaction was stopped by the addition of 50% trichloroacetic acid (TCA). Liberated inorganic phosphate (Pi) was determined by the method of Fiske and Subbarow [18]. One unit of specific activity is defined as the liberation of 1 micromole of inorganic phosphate per mg protein per min. The bioassay was performed in triplicate on a microtitre plate reader (96 wells) which contained 420 μL of mixture/well. Control was the microsomal enzyme containing DMSO in an equivalent amount to that of the tested samples.

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