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August, 2009

Thesis for Master Degree

# Sirt1 Inhibitory Diterpenoids from Vietnamese Medicinal Plant *Croton tonkinensis* Gagnep.

Chosun University Graduate School

College of Pharmacy

Dao Trong Tuan

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# Sirt1 Inhibitory Diterpenoids from Vietnamese Medicinal Plant *Croton tonkinensis* Gagnep.

August 25th, 2009

Chosun University Graduate School

College of Pharmacy

Dao Trong Tuan

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# Sirt1 Inhibitory Diterpenoids from Vietnamese Medicinal Plant *Croton tonkinensis* Gagnep.

지도교수 오원근

이 논문을 약학 석사학위신청 논문으로 제출함

2009년 05월

조선대학교 대학원

약학과

다오트롱투완

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Chosun University Graduate School

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# List of Abbreviations

$[\alpha]^{T}_{D}$: specific rotation
CD: circular dichroism
COSY: H–H correlation spectroscopy
DMSO: dimethyl sulfoxide
HMBC: heteronuclear multiple bond correlation
HMQC: heteronuclear multiple quantum coherence
HPLC: high performance liquid chromatography
HR-EI-MS: high resolution electro impact mass spectroscopy
$IC_{50}$: 50 % inhibition concentration
IR: infrared absorption
*m/z*: mass to charge ratio
NMR: nuclear magnetic resonance
NOESY: nuclear overhauser effect spectroscopy
ppm: parts per million
RP: reverse phase
SIRT1: silent information regulator two ortholog 1
UV: ultraviolet absorption

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# (국문 초록)

## 베트남 약용식물 *Croton tonkinensis* Gagnep. 로부터 SIRT1 억제물인 디터페노이드

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Silent information regulator two ortholog 1 (SIRT1)는 histones 또는 단백질에 있는 lysine 잔류기에서 아세틸기를 제거하는 작용을 갖는 sirtuin deacetylase 계열의 효소로 SIRT1을 저해하는 화합물은 암과 신경퇴행성 질환의 치료제로서 사용가능성이 최근 제시 되었다. 지금까지 SIRT1의 저해물질로 몇몇 저분자 물질이 보고되었으나, SIRT1에 선택적이며 새로운 골격을 갖는 저해 화합물의 발굴은 SIRT1의 기능을 이해하고 치료학적인 적응점을 찾는 과정에서 필요하다.

따라서 본 연구는 SIRT1 효소를 이용하여 탐색한 베트남 식물인 *Croton tonkinensis* Gagnep 잎의 MeOH 추출물로부터 각종 크로마토그래피 방법을 사용하여 7개의 신규 diterpenoid계 화합물과 (1 – 7)와 14개의 diterpenoid계 기지 화합물 (8 – 21)을 분리 하였다. 분리된 화합물들의 구조는 각종 스펙트럼 방법 (1D, 2D-NMR, UV, IR, MS)과 보고된 문헌을 참고하여 동정하였다.

분리된 화합물 중 신규 화합물 1 와 2는 새로운 grayanane-type diterpenoid계 화합물로서 crotokinensin A (1) and crotokinensin B (2)로 명명하였다. 신규 화합물 3 – 7은 새로운 *ent*-kaurane-type diterpenoid계 화합물로서 crotokinensin C (3), crotokinensin D (4), crotokinensin E (5), crotokinensin F (6) 과 crotokinensin G (7)로 각각 명명하였다. 특히 crotokinensin A (1)와 crotokinensin B (2)는 *ent*-kaurane 과 grayanane-type 골격을 갖고 있는 화합물로서 이러한 화합물은 *Croton*

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*tonkinensis* Gagnep로부터 처음으로 분리한 신규 화합물로 대극과에 속한 *Croton*속에서는 최초로 얻어진 것이다.

분리한 화합물 1 - 21의 *in vitro* SIRT1 효소에 대한 저해활성을 측정한 결과, 화합물 14 – 21은 SIRT1 효소에 대하여 16.08 ± 0.11부터 44.34 ± 2.34 μM 농도범위에서 강한 저해작용을 나타내었다. 비록 *ent*-kaurane-type diterpenoids의 구조와 활성 관계가 철저히 연구된 것은 아니지만, 결과적으로 C-16 와 C-17 사이에서의 이중 결합과 ketone group이 C-15에 붙어 있는 것은 *in vitro* SIRT1 저해 활성에서 매우 중요함을 확인하였다. 또한, 수산기가 C-7에 존재하고 acetoxy group이 C-18에 존재하면 저해활성이 증가 됨을 알 수 있었으며, 반대로 acetoxy 또는 수산기가 C-11에 존재하거나, 수산기가 C-18에 존재할 때는 활성이 현저히 감소함을 확인하였다.

본 연구에서는 대극과에 속한 *Croton* 속으로부터 처음으로 분리한 7종의 신규화합물을 (crotokinensin A – G) 포함한 21종의 diterpenoids 계열의 화합물을 분리, 정제하여 구조를 확인하였으며, 화합물 14 – 21이 강한 SIRT1 저해효과를 갖음을 이 논문에서 처음으로 확인하였다.

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# ABSTRACT

## Sirt1 Inhibitory Diterpenoids from Vietnamese Medicinal Plant *Croton tonkinensis* Gagnep.

Dao Trong Tuan

Advisor: Prof. Oh Won-Keun Ph.D

Department of Pharmacy,

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Silent information regulator two ortholog 1 (SIRT1) is a member of the sirtuin deacetylase family of enzymes that removes acetyl groups from lysine residue in histones and other proteins. It has been postulated that inhibitors of SIRT1 might be beneficial in the treatment of cancer and neurodegenerative diseases. To date, though some small molecular inhibitors of SIRT1 have been identified, new inhibitors are still needed to improve the understanding of SIRT1 biological functions and to discover its possible therapeutic indication.

During this screening effort we found that MeOH – soluble extract of the leaves of *Croton tonkinensis* Gagnep. inhibited SIRT1 enzyme by an *in vitro* assay, seven new diterpenoids (**1** – **7**) along with and fourteen known diterpenoids (**8**-**21**) from the leaves of *C. tonkinensis* were isolated by bioassay-guided fractionation using silica gel (63 – 200 µm particle size), C-18 silica gel (40 – 63 µm particle size) column chromatography, and semi-preparative HPLC [ODS-H80 column (150 × 20 mm, 4 µm particle size)]. Their structures were elucidated on the basic of spectral (including 1D, 2D-NMR, UV, IR, and MS) and

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physicochemical analyses. The inhibitory effect of test compounds on SIRT1 enzyme was evaluated.

Compounds **1** and **2** were new grayanane-type diterpenoids, named crotokinensin A (**1**) and crotokinensin B (**2**). Compounds **3** – **7** were new *ent*-kaurane-type diterpenoids, named crotokinensin C (**3**), crotokinensin D (**4**), crotokinensin E (**5**), crotokinensin F (**6**) and crotokinensin G (**7**). Crotokinensin A (**1**) and crotokinensin B (**2**) were two novel grayanane-type diterpenoids from *C. tonkinensis*. This is the first report on the presence of grayanane-type diterpenoids from a species of the *Croton* genus as well as the Euphorbiaceae family.

When compounds **1** – **21** were tested on *in vitro* SIRT1 inhibitory assay, all the isolates, with the exception of **1** – **13**, inhibited strongly SIRT1 activity with $IC_{50}$ values ranging from 16.08 ± 0.11 to 44.34 ± 2.34 μM. Although structure-activity relationships of *ent*-kaurane-type diterpenoids were not thoroughly investigated, our results suggest that the double bond between C-16 and C-17, and ketone group attached at C-15 might be very important for the *in vitro* SIRT1 inhibitory activity. And the presence of a hydroxyl group at C-7 and/or an acetoxy group at C-18 may increase the activity, while the substitution of an acetoxy or a hydroxyl group at C-11, or a hydroxyl group at C-18 may decrease this activity. In addition, SIRT1 inhibitory effects by members of this class have not been previously reported.

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# 1. Introduction

## 1.1. SIRT1 enzyme

Silent information regulator two ortholog 1 (SIRT1) is a member of the sirtuin deacetylase family of enzymes that removes acetyl groups from lysine residues in histones and other proteins. Sirtuins are the mammalian orthologues of the yeast Sir2 protein, which is implicated in chromatin silencing and lifespan regulation.[1] Although there are seven mammalian sirtuins (SIRT1 – 7), deacetylase activity and substrates have only been reported for SIRT1, -2, -3, and -5.[1-3] SIRT1 deacetylates diverse substrate including PGC-1α,[4] p53,[5,6] forkhead transcription factor (FOXO),[7,8-10] NK-κB,[11] Ku70,[12] MyoD[13] and histones.[14] Thus it influences gene silencing, apotopsis, stress resistance, senescence, and fat and glucose metabolism. The combination of these cellular functions might contribute to an anti-aging effect in mammals although SIRT1 could also limit replicative lifespan after chronic genotoxic stress.[15] On the other hand, SIRT1 activity has also been linked to tumorigenesis. SIRT1 is important for tumour cell growth and survival, possibly due to SIRT1's anti-apoptotic effect.[16-18] In addition, SIRT1 participates in the silencing of tumour suppressor genes[19] and SIRT1 overexpression has been observed in cancer cells.[20-23] Therefore, SIRT1 could have important implications in both aging and cancer.

## 1.2. Mechanisms of SIRT1 inhibitors in cancer therapy

SIRT1 can modulate cellular stress response and survival through regulation of p53,[5,6,24] NF-κB signaling,[11] and FOXO transcription factors.[7,8] Studies on SIRT1 function[13,25] and Sirt1 knockout mice[26] suggest its roles in mammalian development and differentiation.

Several reports describe the role of histone deacetylases inhibitors as new anticancer

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agents, and a number of these inhibitors have already entered into clinical trials.[27-30]

Like other histone deacetylases, SIRT1 of sirtuin family is considered to be among the most promising targets in drug development for cancer therapy. Mammalian SIRT1 interacts with a host of factors with known involvement in cancer, and might act in some cases to promote cancer cell survival. SIRT1 binds and deacetylates the tumor suppressor p53,[5,6] thereby inhibiting p53-mediated transactivation and p53-dependent apoptosis in response to DNA damage and oxidative stress.

SIRT1 inhibitors may act in cancer therapy by following mechanisms

**Table 1.** Mechanisms of SIRT1 inhibitors in cancer therapy[31]

| Inhibition of cell proliferation |
|---|
| Augmentation of nuclear receptor response driving terminal cell differentiation |
| Reversal of repression by fusion transcription factors or overexpressed repressors |
| Induction of p21, G1 arrest, and cellular differentiation |
| Reactivation of silenced tumor suppressor genes in combination with DNA methyl transferase inhibitors |
| Suppression of telomerase gene expression |
| Induction of apotopsis |
| Activation of calpain/mitochondria-dependent apotosis |
| Activation and or sensitization of death-receptor mediated killing |
| Mitotic dysfunction, aberrant chromosomal segregation, and DNA damage |
| Induction of topoisomerase II ma alter sensitivity to DNA-damaging agents |
| Other mechanisms |
| Alteration of angiogenic signaling |
| Alteration of microtubule function |
| Induction of MHC antigens on the cell surface to augment immune responses |
| Suppression of IL-2-mediated gene expression |

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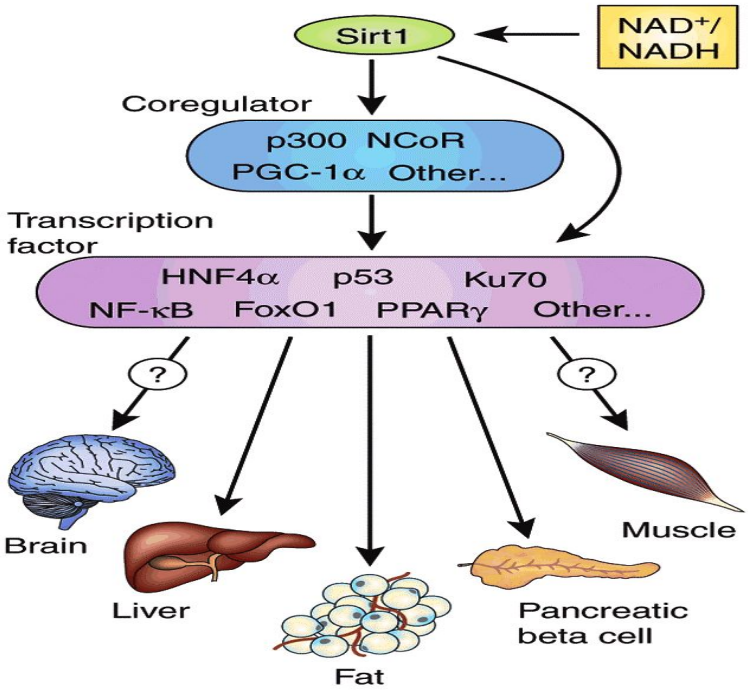


**Fig. 1.** SIRT1 is a metabolic master switch that modulates lifespan

### 1.3. SIRT1 inhibitors

Although recent reports directed at the identification and development of small molecules that affect sirtuin activity, the majority were inhibitors towards human SIRT2 and SIRT1. These investigations described sirtuin inhibition by phloroglucinol,[32] indole,[33] and suramin derivatives[34] as well as adenosine mimetics.[35] Two of the first reported sirtuin inhibitors, sirtinol[36] and splitomicin[37] have been derivatized to more efficacious compounds.[38,39] The phloroglucinol compound aristoforin displayed $IC_{50}$ values of 7 μM with SIRT1[32] (**Fig. 2**). Related natural products guttiferone G and hyperforin yielded similar potency and selectivity for SIRT1. Recently, Trapp *et al.* performed structure – activity studies on suramin analogs as inhibitors of SIRT1 and SIRT2.[34] The various derivatives displayed a wide

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range in potency against SIRT1 and a few yielded marked selectivity for SIRT1 such as compound NF675 (with an $IC_{50}$ of 93 nM) (**Fig. 2**). Known adenosine mimetics that target kinases were also screened for their inhibitory activity against sirtuin,[35]. Most notable compound was a bis(indolyl)maleimide which inhibited SIRT1 with $IC_{50}$ of 3.5 μM. Competition assays with $NAD^+$ and modeling studies indicated that these bis(indolyl)maleimides probably bind to the adenosine pocket. Other high – throughput screening assays, molecular modeling and virtual screens have identified a number of compounds that display low μM to nM potency.[33,40-42] Of particular importance, Napper *et al*. reported that the indole 6-chloro-2,3,4,9-tetrahydro-1-*H*-carbazole-1-carboxamide inhibitited SIRT1 activity with an $IC_{50}$ of 120 nM. Kinetic analysis revealed mixed-type inhibition of deacetylation as well as inhibition of nicotinamide-$NAD^+$ exchange reaction. The authours propose that the compound binds to the nicotinamide-binding site. Some studies have employed acetyl-lysine analogs to probe the mechanistic differences of protein deacetylases, among which, thioacetyl lysine exhibited strong inhibition of human SIRT1, yielding an inhibition constant ($K_{is}$) of 17 nM.[43]

Anyway to date, only a few inhibitors of SIRT1 have been reported. With the exception of nicotinamide,[44] which is released during the reaction with the deacetylase and which serves as an internal agent, almost all inhibitors are synthetically derived compounds and were identified from library screening studies.

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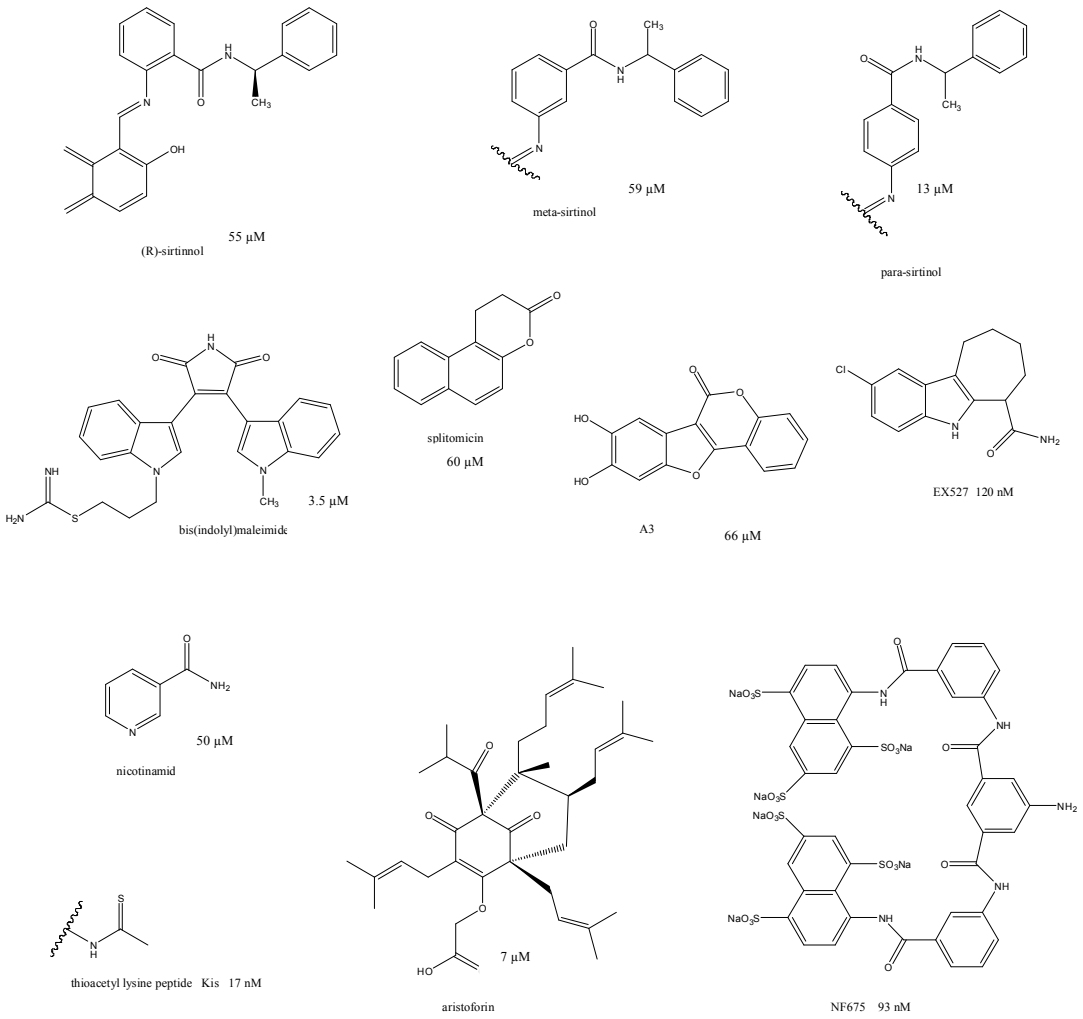


**Fig. 2.** SIRT1 inhibitors derived from natural resources or synthesis

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### 1.4. *Croton tonkinensis* Gagnep.

*Croton* is a genus of Euphorbiaceae comprising around 1,300 species, widely distributed throughout tropical regions. Several species have a long history in the traditional use of medicinal plants in Africa, Asia, and South America. Popular uses include treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers and weight-loss.[45]

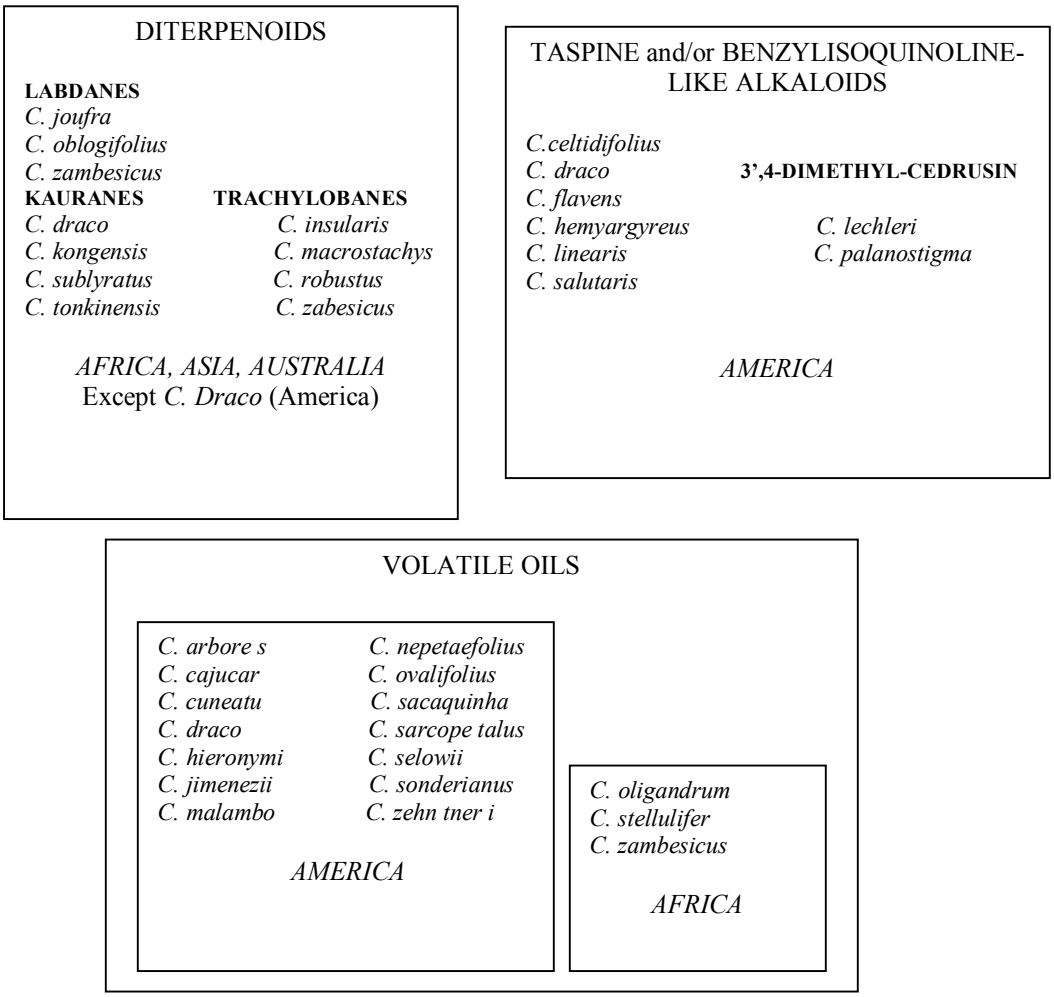


**Fig. 3.** Chemical and geographical affinities among *Croton* species[45]

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*C. tonkinensis* Gagnep. is one out of 31 species that are cultivated at wild in Vietnam.[46] It is a medicinal plant, commonly named in Vietnamese as "Kho sam cho la" or "Kho sam Bac Bo". Its dried leaves (*Folium tonkinensis*) have been used in folklore medicine for treatment of abscesses, impetigo, abdominal pain, dyspepsia, and gastric and duodenal ulcers.

Prior phytochemical investigations have demonstrated that this species contains steroids and diterpenoids, among which some *ent*-kaurane diterpenoids showed significant cytotoxicity against MCF-7, NCI-H460, and SF-268 tumor cell lines, as well as in a brine shrimp lethality assay.[47,48] Recently, the antiinflammatory and cancer chemopreventive activity of *C. tonkinensis* extracts were discovered through its ability to inhibit the activation of the transcription factor nuclear factor kappa B (NF-$\kappa$B), and the activity is assumed to be correlated with the *ent*-kaurane diterpenoids constituents.[49]

Despite a number of studies on the chemical constituents and biological activities of

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the genus *Croton*, there have been few phytochemical investigations on *C. tonkinensis* previously. Moreover, the promising biological activity of *ent*-kaurane diterpenoids encouraged us to continue investigating the constituents as SIRT1 inhibitors from this plant.

In this study, the methanol extract, $CHCl_3$ and $H_2O$-soluble fractions of the leaves of *C. tonkinensis* were tested for inhibitory effects on SIRT1. Bioassay-guided fractionation of a $CHCl_3$-soluble fraction of the leaves of this plant has led to the isolation of a series of diterpenoids, including two novel grayanane-type diterpenoids (**1** – **2**) and other five new *ent*-kaurane-type diterpenoids (**3** – **7**). In this thesis, I would like to describe the isolation and structure elucidation of these compounds and the evaluation of their SIRT1 inhibitory activity.

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# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Plant

The leaves of *Croton tonkinensis* Gagnep. were cultivated at a medicinal garden in Hanoi, Vietnam, and collected in June, 2007. The plant material was identified by Dr. Nguyen Bich Thu, Vietnam Institute of Medicinal Materials. A voucher specimen (VIET–02) has been deposited at the Herbarium of Vietnam Institute of Medicinal Materials.

### 2.1.2. Chemicals, reagents and chromatography.

Column chromatography was conducted on silica gel (Merck, 63 – 200 μm particle size) and C-18 silica gel (Merck, 40 – 63 μm particle size) from Merk. TLC was carried out with silica gel 60 F254 plates from Merck. HPLC was carried out using a Gilson System with UV detector and an ODS-H80 column(20 × 150 mm, 4 μm particle size, YMC Co., Ltd., Japan). Baker analyzed HPLC solvents MeOH and MeCN were purchased from Mallinckrodt Baker, Inc. USA. Deuterated solvent for NMR analysis $CDCl_3$ was purchased from CIL (Cambridge Isotope Lab., USA).

Nicotinamide, tamoxifen were purchased from Sigma Chemical Company (St Louis, MO, USA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). Antibodies against SIRT1, $\beta$-actin, anti-mouse and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTT) was purchased from USB Corporation (Cleveland, OH). Human recombiniant SIRT1, Fluor de Lys SIRT1 deacetylse substrate, Fluor de Lys Developer II, $NAD^+$ and the

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buffer used for assays were purchased from Biomol (Plymouth Meeting, PA)

**2.1.3. General experimental procedures.**

Optical rotations were determined on a JASCO P-1020 polarimeter using a 100 mm glass microcell. IR spectra (KBr) were recorded on a Brucker spectrometer. NMR spectra were obtained on Varian Inova 500 MHz spectrometer with TMS as the internal standard. EIMS and HREIMS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. For column chromatography, silica gel (63–200 μm particle size) and C-18 silica gel (40 – 63 μm particle size), were used. TLC was carried out with silica gel 60 $F_{254}$ and RP-18 $F_{254}$ plates from Merck. Spots were visualized using UV light or 10 % sulfuric acid. HPLC were carried out using a Gilson system with a UV detector, and an ODS-H80 column (20×150 mm, 4 μm particle size, YMC Co., Ltd., Japan).

**2.2. Methods**

**2.2.1. Isolation of compounds from *C. tonkinensis***

The dry leaves of *Croton tonkinensis* (4 kg) were extracted with MeOH (20 L × 2 times) at room temperature for 1 week and concentrated to yield a dry residue (390 g). The extract was suspended in $H_2O$ and partitioned with $CHCl_3$ to afford a $CHCl_3$-soluble fraction (190 g). This fraction, which inhibited SIRT1 enzyme by more than 60 % at 30 μg/mL, was subjected to column chromatography over silica gel (20 × 20 cm), eluted using a step gradient of *n*-hexane/EtOAc (49:1 to 0:1) to obtain nine fractions (F1 – F9) based on TLC profile. The SIRT1 inhibitory activity was concentrated in F6 and F7 by an *in vitro* assay. These active fractions were subjected to additional chromatography. Fraction F6 (3.6 g) was chromatographed over a Sephadex LH-20 column (7 × 30 cm), using MeOH as the eluting

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solvent to give three subfractions (F6.1 – F6.3). Subfraction F6.3 (2.1 g) was continued applying to C-18 silica gel column chromatography (7 × 30 cm) and eluted with a step gradient of MeOH/$H_2O$ (1:1 to 10:1) to afford six subfractions (F6.3.1 – F6.3.6). Further separation of F6.3.1 (150 mg) by preparative HPLC on an ODS-H80 column [(20×150 mm, 4 μm particle size); solvent MeOH in $H_2O$ containing 0.1% formic acid (0 – 65 min: 78% MeOH, 70 min: 100% MeOH); flow rate 3 mL/min; UV detection at 205 and 254nm] resulted in the isolation of compounds **3** ($t_R$ = 51.0 min, 9.0 mg) and **4** ($t_R$ = 54.0 min, 2.0 mg). Compound **5** ($t_R$ = 55.0 min, 1.5 mg) and a mixture of compounds **6** and **7** ($t_R$ = 58.0 - 61 min) were obtained from subfraction F6.3.5 (170 mg). Compounds **6** ($t_R$ = 28.0 min, 1.5 mg) and **7** ($t_R$ = 36.0 min, 2 mg) were separated from this mixture by HPLC (MeCN/$H_2O$, 75:25, 3ml/min). The active fraction F7 (35.5 g) was repeated chromatographed on a silica gel column (7 × 40 cm) eluted with *n*-hexane/EtOAc (9:1 to 0:1) to separate into seven subfractions (F7.1 – F7.7). The fraction F7.5 (5.6 g) was subjected to a C-18 silica gel column (7 × 30 cm) using MeOH/$H_2O$ (3:1 to 10:1) as mobile phase to give six subfractions (F7.5.1–F7.5.6). The fractions F7.5.3 (830 mg) and F.7.5.4 (960 mg) were combined and the major compound in this plant, *ent*-18-acetoxy-7$\alpha$-hydroxykaur-16-en-15-one, was crystallized out from MeOH. The obtained solution after crystallization of the major compound was subjected to HPLC (MeOH/$H_2O$, 62:38, 3 mL/min) to give compound **1** ($t_R$ = 51.0 min, 16.5 mg) and a mixture ($t_R$ = 55 – 57 min). Repeated chromatography of this mixture using HPLC (MeCN/$H_2O$, 55:45, 3 ml/min) resulted in the isolation of compound **2** ($t_R$ = 25.0 min, 28.5 mg) (**Scheme 1**).

Known compounds (**8** – **21**) (**Fig. 4**) were isolated from fractions F6.2, F6.3, F7.4 and F7.5 by bioassay-guieded isolation using silica gel, RP-column chromatography and semi-preparative HPLC.

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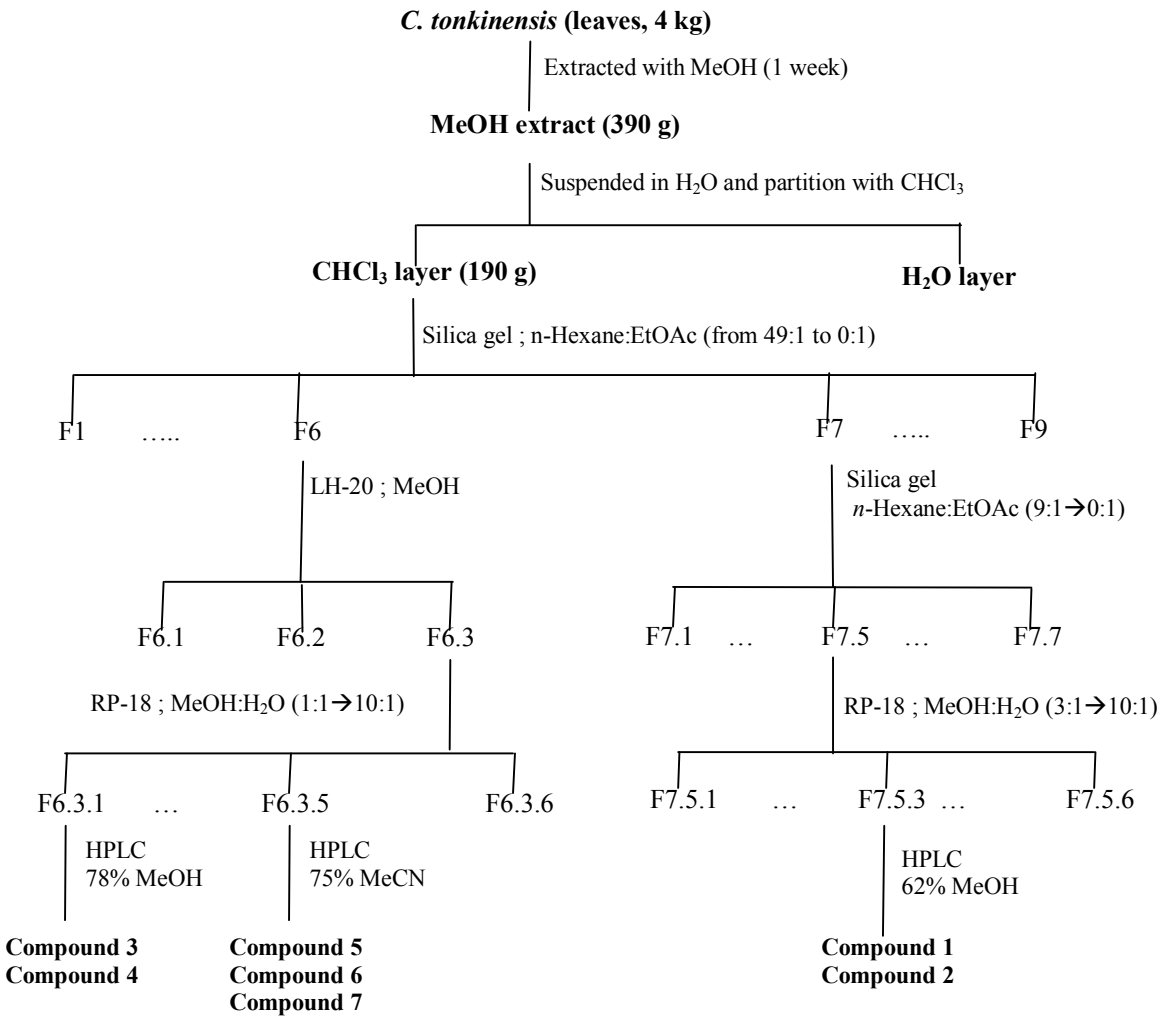


**Scheme 1.** Isolation of compounds (**1** – **7**) from the leaves *C. tonkinensis*

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Compound **1**: Appearance: colorless oil; Mol. Formula: $C_{20}H_{24}O_4$; Exact. Mass: 328.1675; $[\alpha]^{25}_D$: -33° (*c* 0.2, MeOH); IR (film) $\nu_{max}$ 3435, 2960, 2870, 1726, 1691, 1678, 1641, 1631, 1465, 1381, 1341, 1244, 1092, 1063, 912 $cm^{-1}$. EIMS *m/z* 328 $[M]^+$ (43), 310 $[M - H_2O]^+$ (81), 268 (51), 225 (51), 177 (100), 91 (35), 87 (25); HREIMS *m/z* 328.1678 .

$^1H$ NMR (500 MHz) and $^{13}C$ NMR (100 MHz) data, see **Table 2**.

Compound **2**: Appearance: white amorphous powder; Mol. Formula: $C_{20}H_{26}O_3$; Exact. Mass: 314.1882; $[\alpha]^{25}_D$: -36° (*c* 0.2, MeOH); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) 205 (3.88), 230 (3.98) nm; IR (film) $\nu_{max}$ 3426, 2932, 2868, 1737, 1726, 1641, 1631, 1463, 1382, 1241, 1102, 1038 $cm^{-1}$. EIMS *m/z* 314 $[M]^+$ (2), 296 $[M - H_2O]^+$ (100), 281 (18), 253 (17), 198 (19), 177 (16), 149 (26), 131 (31), 91 (25), 87 (42); HRFAB MS *m/z* 315.1962 $[M + 1]^+$, calcd. for $C_{20}H_{27}O_3$: 315.1960.

$^1H$ NMR (500 MHz) and $^{13}C$ NMR (100 MHz) data, see **Table 3**.

Compound **3**: Appearance: white amorphous powder; Mol. Formula: $C_{22}H_{34}O_3$; Exact. Mass: 346.2508; $[\alpha]^{25}_D$: -20° (*c* 0.1, MeOH); CD (MeOH, *c* 0.0002): $\Delta\varepsilon$ 288 (+0.32), 218 (-12.19), 209 (+0.83); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) 210 (3.817) nm; IR (film) $\nu_{max}$ 3498, 2984, 2927, 1710, 1631, 1444, 1378, 1265, 1033 $cm^{-1}$. EIMS *m/z* 284 (29), 269 (23), 255 (100), 239 (35), 213 (14), 199 (19); HREIMS *m/z* 346.2509.

$^1H$ NMR (500 MHz) and $^{13}C$ NMR (100 MHz) data, see **Table 4**.

Compound **4**: Appearance: colorless oil; Mol. Formula: $C_{22}H_{34}O_3$; Exact. Mass: 346.2508; $[\alpha]^{25}_D$: -17° (*c* 0.1, MeOH); CD (MeOH, *c* 0.0003): $\Delta\varepsilon$ 285 (+4.24), 257 (-4.22), 208 (+3.38); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) 206 (3.685) nm; IR (film) $\nu_{max}$ 3482, 2936, 2917, 1725,

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1644, 1471, 1382, 1249, 1043 $cm^{-1}$. EIMS *m/z* 346 $[M]^+$ (47), 328 (15), 273 (24), 255 (35), 123 (30), 109 (100), 107 (30); HREIMS *m/z* 346.2503.

$^1$H NMR (500 MHz) and $^{13}$C NMR (100 MHz) data, see **Table 5**.

Compound **5**: Appearance: colorless oil; Mol. Formula: $C_{22}H_{34}O_3$; Exact. Mass: 346.2508; $[\alpha]^{25}_D$: -25$^o$ (*c* 0.1, MeOH); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) 206 (3.625) nm; IR (film) $\nu_{max}$ 3471, 2929, 2848, 1710, 1631, 1456, 1381, 1271, 1068 $cm^{-1}$. 346 $[M]^+$ (83), 328 (15), 303 (100), 286 (13), 273 (20), 255 (61); HREIMS *m/z* 346.2507.

$^1$H NMR (500 MHz) and $^{13}$C NMR (100 MHz) data, see **Table 6**.

Compound **6**: Appearance: colorless oil; Mol. Formula: $C_{22}H_{34}O_3$; Exact. Mass: 346.2508; $[\alpha]^{25}_D$: -22$^o$ (*c* 0.1, MeOH); CD (MeOH, *c* 0.0003): $\Delta\varepsilon$ 299 (+0.98), 229 (-2.15), 217 (+2.04); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) 208 (3.815) nm; IR (film) $\nu_{max}$ 3437, 2932, 2869, 1726, 1646, 1446, 1382, 1240, 1037 $cm^{-1}$. 346 $[M]^+$ (100), 328 (17), 286 (10), 268 (51), 255 (32); HREIMS *m/z* 346.2509.

$^1$H NMR (500 MHz) and $^{13}$C NMR (100 MHz) data, see **Table 7**.

Compound **7**: Appearance: brownish amorphous powder; Mol. Formula: $C_{22}H_{20}O_5$; Exact. Mass: 364.1111; $[\alpha]^{25}_D$: -27$^o$ (*c* 0.1, MeOH); UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) 230 (3.770) nm; IR (film) $\nu_{max}$ 3384, 2950, 2868, 1711, 1642, 1437, 1369, 1257, 1094 $cm^{-1}$. 300 (91), 272 (9), 176 (100); HREIMS *m/z* 360.2309.

$^1$H NMR (500 MHz) and $^{13}$C NMR (100 MHz) data, see **Table 8**.

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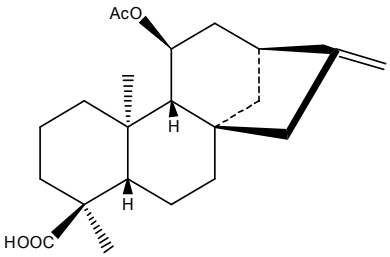
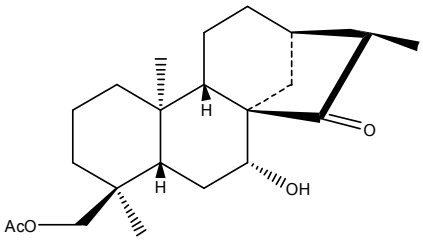
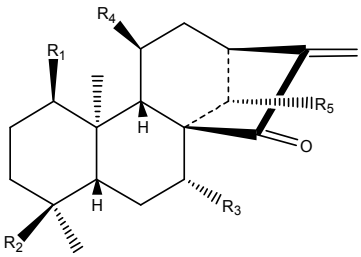
**Fig. 4.** Chemical structures of known compounds (**8** – **21**)

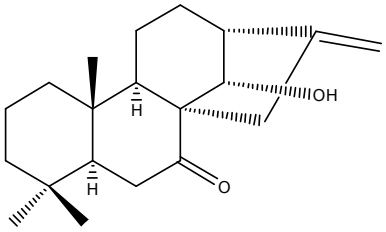



**8** *ent*-11*β*-acetoxy-kaur-16-en-18-oic acid[47]




**9** *ent*-16(S)-18-acetoxy-7*α*-hydroxykauran-15-one[50]




**10** 14*α*-hydroxykaur-16-en-7-one[48]

**11** *ent*-kaur-16-en-15-one-18-oic acid [51] $R_1 = R_3 = R_4 = R_5 = H$ $R_2 = COOH$

**12** *ent*-18-hydroxy-kaur-16-en-15-one [52,53] $R_1 = R_3 = R_4 = R_5 = H$ $R_2 = CH_2OH$

**13** *ent*-18-acetoxy-11*β*-hydroxykaur-16-en-15-one [47] $R_1 = R_3 = R_5 = H$ $R_2 = CH_2OAc$ $R_4 = OH$

**14** *ent*-1*β*,7*α*,14*β*-triacetoxy-kaur-16-en-15-one [54] $R_1 = R_3 = R_5 = OAc$ $R_2 = CH_3$ $R_4 = H$

**15** *ent*-18-acetoxy-7*α*-hydroxykaur-16-en-15-one [55] $R_1 = R_4 = R_5 = H$ $R_2 = CH_2OAc$ $R_3 = OH$

**16** *ent*-7*α*,14*β*-dihydroxy-kaur-16-en-15-one [56] $R_1 = R_4 = H$ $R_2 = CH_3$ $R_3 = R_5 = OH$

**17** *ent*-1*β*-acetoxy-7*α*,14*β*-dihydroxy-kaur-16-en-15-one [49] $R_1 = OAc$ $R_2 = CH_3$ $R_3 = R_5 = OH$ $R_4 = H$

**18** *ent*-1*β*,14*β*-diacetoxy-7*α*-hydroxykaur-16-en-15-one [50] $R_1 = R_5 = OAc$ $R_2 = CH_3$ $R_3 = OH$ $R_4 = H$

**19** *ent*-1*β*,7*α*-diacetoxy-14*β*-hydroxykaur-16-en-15-one [50] $R_1 = R_3 = OAc$ $R_2 = CH_3$ $R_4 = H$ $R_5 = OH$

**20** *ent*-7*α*,18-dihydroxykaur-16-en-15-one [57] $R_1 = R_4 = R_5 = H$ $R_2 = CH_2OH$ $R_3 = OH$

**21** *ent*-18-acetoxy-7*α*,14*β*-dihydroxykaur-16-en-15-one [49] $R_1 = R_4 = H$ $R_2 = CH_2OAc$ $R_3 = R_5 = OH$

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### 2.2.2. *In vitro* SIRT1 assay

***Cell culture***: MCF-7 (human breast adenocarcinoma) cells, the multidrug-resistant subline MCF-7/adriamycin (MCF-7/adr) and MCF-7/tamoxifen-resistant (MCF-7/TAMR) were grown in DMEM containing 10% FBS. Both cell lines were cultured at 37ºC in a humidified $CO_2$ incubator.

***Measurement of cell viability***: Cells were seeded into 96-well plates at a density of 2 x $10^3$/well. Cytotoxicity of the isolates was determined by a MTT assay. After incubation of cells with the compounds at different concentrations for 24 hours, cells were then treated with MTT solution for 40 minutes. The dark blue formazan crystals that formed in intact cells were solubilized with DMSO, and the absorbance at 550 nm was measured with a microplate reader (Varioskan, Thermo Electron Co.). Percent cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the control vehicle.

***SIRT1 deacetylase assay***: The Fluor de Lys fluorescence assay was performed as indicated in the BioMol product sheets.[58] Briefly, assays were performed using Fluor de Lys-SIRT1, $NAD^+$ and SIRT1 in the absence and presence of nicotinamide in SIRT1 assay buffer (25 mM Tris/Cl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1mM $MgCl_2$, 1mg/ml bovine serum albumin, as indicated in the BioMol product sheets). The buffer, dimethyl sulfoxide, nicotinamide, and SIRT1 were preincubated. Reactions were initiated by the addition of 2 x concentrations of the Fluor de Lys peptide and $NAD^+$. Prior to quenching the reaction, 2 mM nicotinamide was added to 1 x Developer II in the histone deacetylase assay buffer (25 mM Tris/Cl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, as indicated in the BioMol product sheets). At each time point, 50 µl of the reaction was removed and mixed with 50 µl of the developer solution. The quenched samples were kept at $37^0$C for 45 min prior to fluorescence reading. Fluorescence readings were obtained using the microplate reader (Varioskan, Thermo

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Electron Co.), with the excitation wavelength set to 360 nm and the emission set to 460 nm (according to BioMol specification).

***Transient transfection and luciferase assay***: To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, cells were plated in 24-well plates at a density of $10^5$ cells/ well, overnight and transiently co-transfected with p53, p53-Luc or SIRT1 and pRL-SV plasmid (Renilla luciferase expression for normalization) (Promega, Madison, WI) using LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA). The cells were then exposed to the compounds for 24 hours. Luciferase activities in cell lysates were measured using a luminometer (TD-20; Turner Designs, Sunnyvale, CA). Relative luciferase activities were calculated by normalizing SIRT1 driven firefly luciferase activity to Renilla luciferase activity (Luminoskan Ascent, Thermo Electron Co.).

***Western blot analysis***: SDS-polyacrylamide gels and transfer to a PVDF (polyvinylidene difluoride) membrane followed standard procedures. After treatment, the cells were collected and washed with phosphate-buffered saline (PBS). The harvested cells were then lysed on ice for 30 min in 100 μl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP-40 (Nonidet P-40)] and centrifuged at 12,000 rpm for 30 min. Supernatants were collected and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Aliquots of the lysates (60 μg of protein) were boiled for 5 min and electrophoresed on 10% SDS-PAGE. Proteins in the gels were transferred onto nitrocellulose membranes, which were then incubated with SIRT1 and mouse monoclonal $\beta$-actin antibodies. The membranes were further incubated with secondary anti-mouse or anti-rabbit antibodies.

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# 3. Results

## 3.1. Structure determination of new compounds isolated from *C. tonkinensis*

### 3.1.1. Structure determination of compound 1

Compound **1** was obtained as an colorless oil with a negative optical rotation $[\alpha]^{25}_{D}$ -33° (*c* 0.2, MeOH). The IR spectrum of **1** showed the presence of hydroxyl (3426 $cm^{-1}$), carbonyl (1737 and 1726 $cm^{-1}$), and conjugated ketone (1642 $cm^{-1}$) groups. The $^{1}H$ NMR spectrum exhibited two singlet olefinic proton resonances at $\delta_H$ 6.05 and 5.41, two oxygenated proton resonances at $\delta_H$ 4.80 (d, 5.5) and 4.48 (br s), ten aliphatic proton resonances (**Table 2**), and three methyl groups at $\delta_H$ 1.50, 1.39, and 1.22 (each single). The $^{13}C$ NMR and DEPT of **1** revealed the presence of twenty carbon resonances, including two ketone carbon ($\delta_C$ 204.0 and 202.3), four olefinic carbons ($\delta_C$ 151.8, 179.1, 142.6, and 118.4), three oxygenated carbons ($\delta_C$ 80.3, 74.0 and 72.3), four aliphatic methylen carbons ($\delta_C$ 51.9, 30.6, 26.1, and 19.4), two methine carbons ($\delta_C$ 57.4 and 42.3), two aliphatic single carbons ($\delta_C$ 65.3 and 41.3), and three methyl groups ($\delta_C$ 26.9, 26.5, 16.1). In the HMBC spectrum (**Fig. 10**), the carbonyl carbon resonance at $\delta_C$ 202.3 showed correlations with two olefinic protons ($\delta_H$ 6.05, 5.41), an oxygenated proton ($\delta_H$ 4.51), and two methine protons ($\delta_H$ 2.99 and 2.13). The COSY spectrum of **1** supported strong $^{1}H$–$^{1}H$ connectivities for proton resonance at $\delta_H$ 2.13 to 1.41 and 1.64, from proton resonances at $\delta_H$ 2.94 to proton at $\delta_H$ 2.45 and 1.61 (**Fig. 9**). In addition, the weak connectivities from proton resonance at $\delta_H$ 2.94 to proton resonances at $\delta_H$ 6.05, 5.41, and 4.48 were also observed. These evidence suggested a diterpene with 14*β*-hydroxykaur-16-en-15-one structure for **1**.[47–50] In the HMBC spectrum, one oxygenated methine was assigned to be at position 7 due to the correlations from proton resonance at $\delta_H$ 4.80 (H-7) to carbon resonances at $\delta_C$ 72.3 (C-14), 66.0 (C-8), 57.4 (C-9), and $\delta_C$ 47.2 (C-5), and the correlation from the proton

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signal at $\delta_H$ 2.75 (H-6) to carbon signal at $\delta_C$ 74.0 (C-7). Two methyl proton resonances at $\delta_H$ 1.22 and 1.39 (H-18 and H-19, respectively) exhibited strong HMBC correlations with two quaternary carbon resonances at $\delta_C$ 39.8 (C-4) and 179.1 (C-5), and a methylene carbon resonance at $\delta_C$ 51.9 (C-3). The strong HMBC correlations from the oxygenated carbon at $\delta_C$ 80.3 to proton at $\delta_H$ 1.36 (H-20) and 1.64 (H-11) indicated that this is C-10. The methyl proton resonance at $\delta_H$ 1.36 (H-20) also showed strong HMBC correlations to carbon resonances at $\delta_C$ 57.4 (C-9) and 142.6 (C-1). Hence, structure of compound **1** seems to be an *ent*-kaurane-type diterpene with a 6/6/6/5 ring system,[47–50] similar to that of *ent*-7*α*,14*β*-dihydroxykaur-16-en-15-one.[56] However, in the 6/6/6/5 ring system, C-10 is a quaternary carbon that could not be oxygenated. Moreover, the HMBC spectrum revealed that the protons H-6 ($\delta_H$ 2.75 and 3.33) exhibited correlations with C-1 ($\delta_C$ 142.6) and C-5 ($\delta_C$ 179.1), but not to C-10 ($\delta_C$ 80.3). These sets of HMBC correlations suggested that the structure of compound **1** is 5/7/6/5 ring system, which is known as grayanane skeleton.[59–63] The strong HMBC correlation between H-7 ($\delta_H$ 4.80) to C-10 ($\delta_C$ 80.3) indicated an 7,10-epoxy group and this was supported by the molecular formula $C_{20}H_{24}O_4$ obtained the molecular ion peak at *m/z* 328.1678 $[M]^+$ (calcd. for $C_{20}H_{24}O_4$, 328.1675) in the HREIMS. In the NOESY spectrum, the observation of correlations between H-20/H-9, H-3/H18 and H-19, H-6*β*/H-19 and H-7, H-6*a*/H-18, and H-13/H-14 (**Fig. 11**) indicated the 7*α*,10*α*-epoxy-14*β*-hydroxy-1(5),16(17)-dien-2,15-dione-grayanane for **1**. The negative optical rotation of **1** recorded in MeOH supported this stereochemistry.[59–63] Hence, compound **1** was determined as 7*α*,10*α*-epoxy-14*β*-hydroxy-1(5),16(17)-dien-2,15-dione-grayanane.

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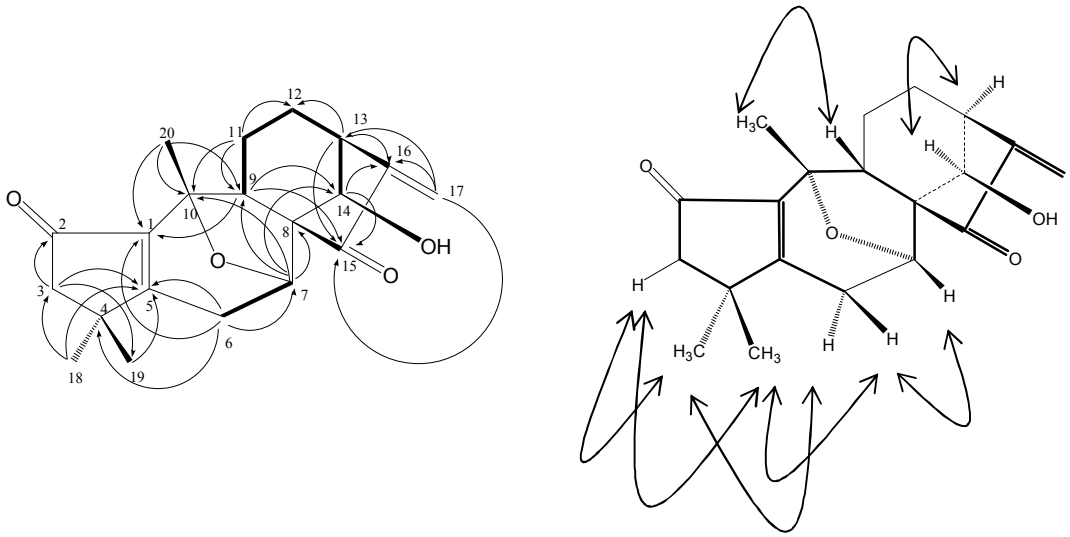


**Fig. 5.** Key HMBC (H→C), COSY ($^1$H—$^1$H) and NOSEY (↔) correlations of compound **1**

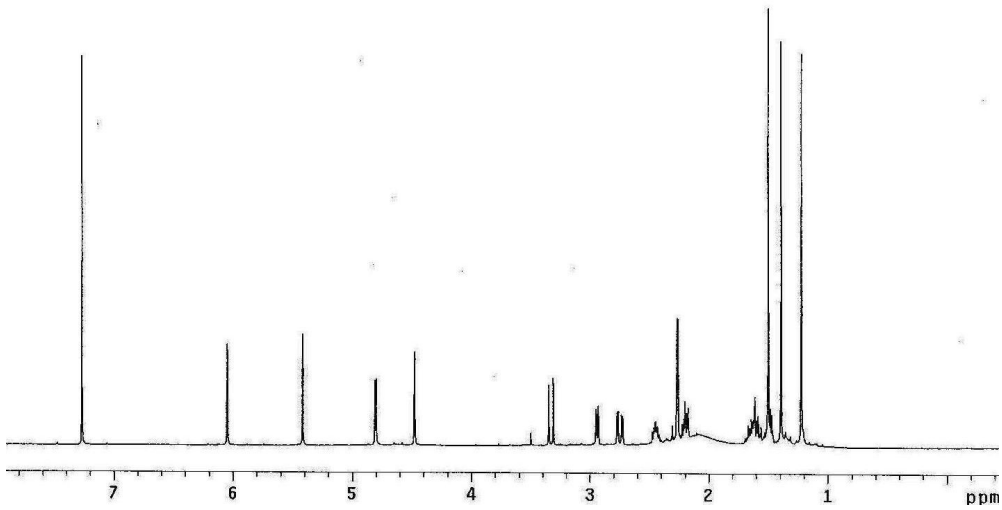


**Fig. 6.** $^1$H-NMR spectrum of compound **1** (500 MHz, $CDCl_3$)

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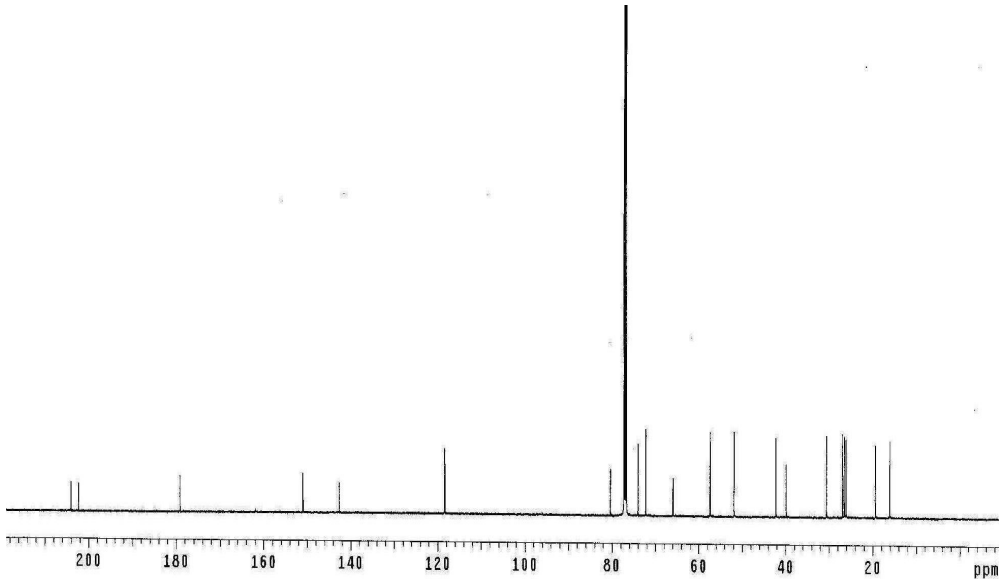


**Fig. 7.** $^{13}$C-NMR spectrum of compound **1** (125 MHz, $CDCl_3$)

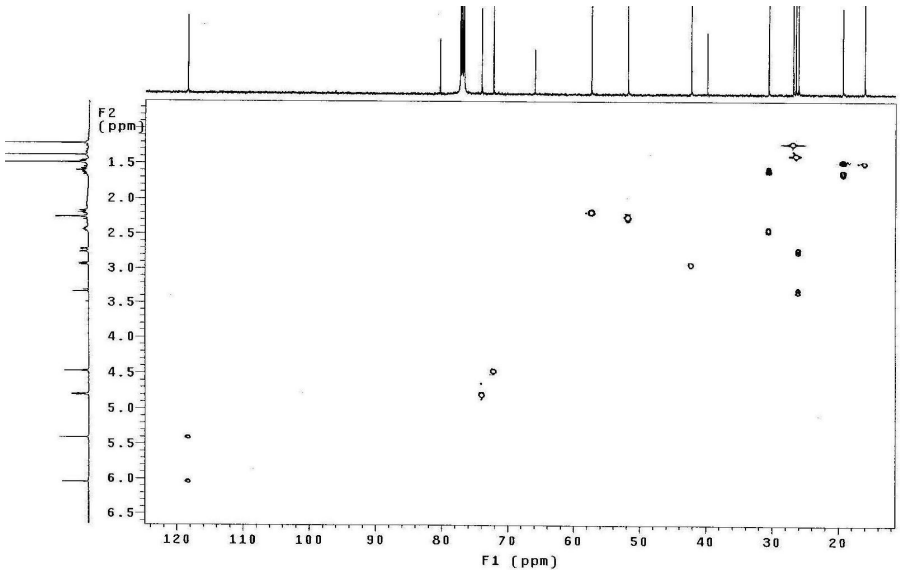


**Fig. 8.** HMQC spectrum of compound **1** (500 MHz, $CDCl_3$)

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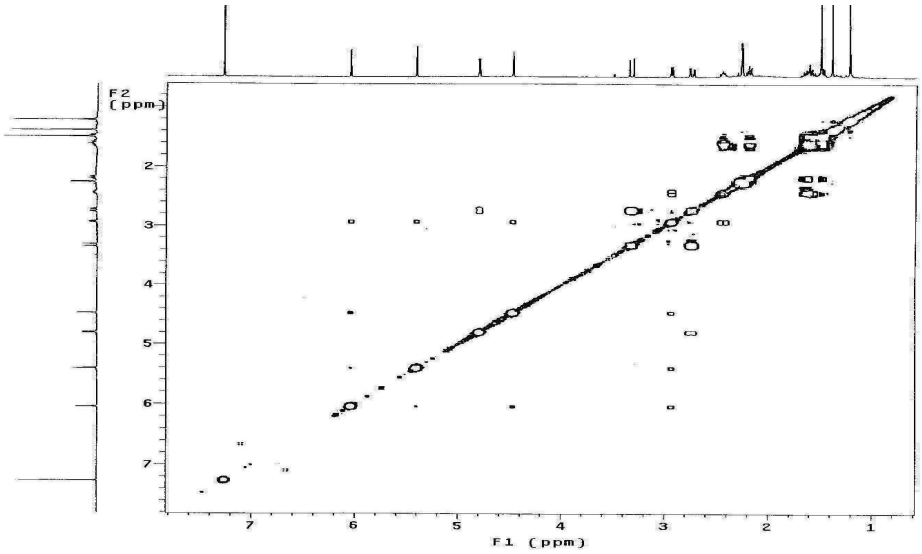


**Fig. 9.** COSY spectrum of compound **1** (500 MHz, $CDCl_3$)

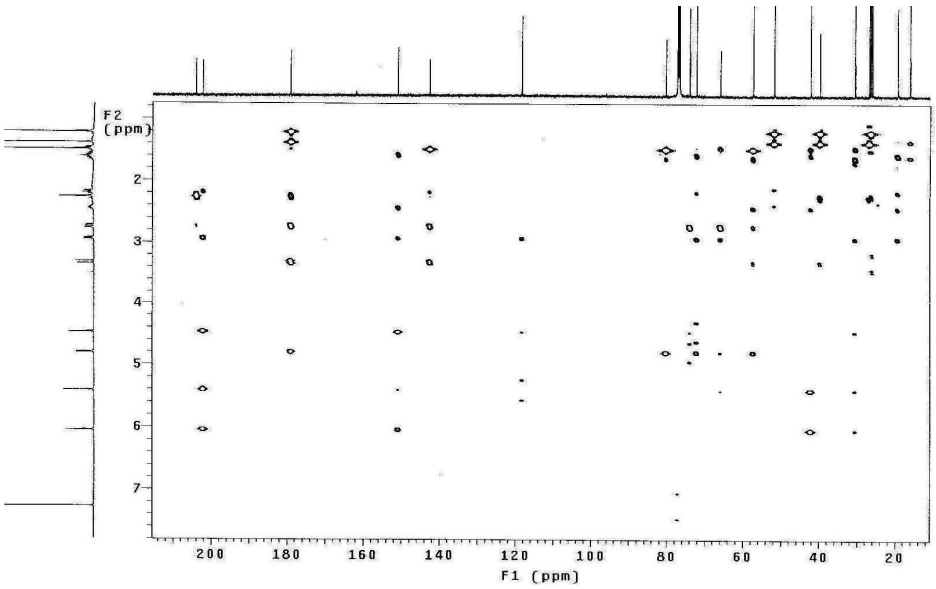


**Fig. 10.** HMBC spectrum of compound **1** (500 MHz, $CDCl_3$)

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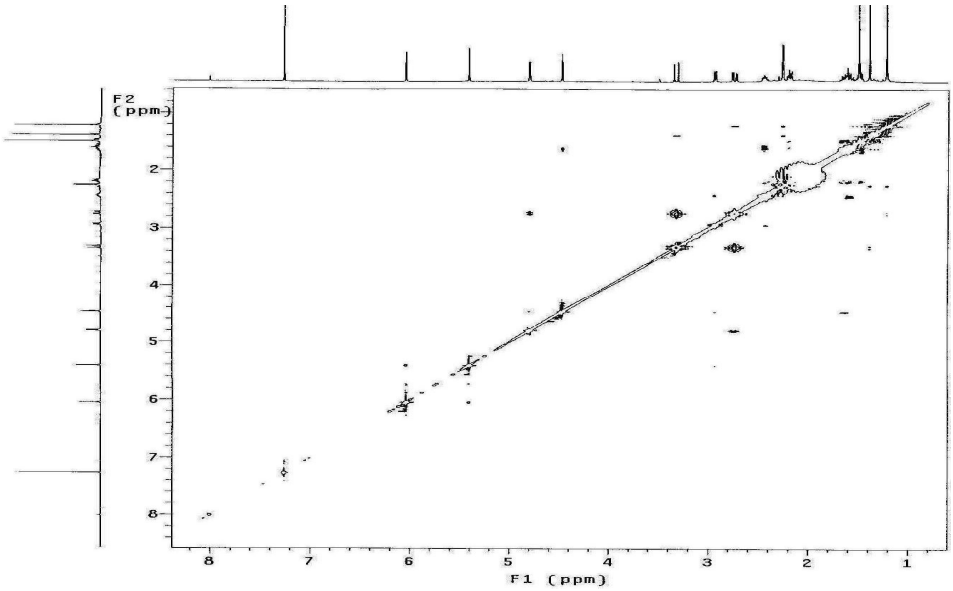


**Fig. 11.** NOESY spectrum of compound **1** (500 MHz, $CDCl_3$)

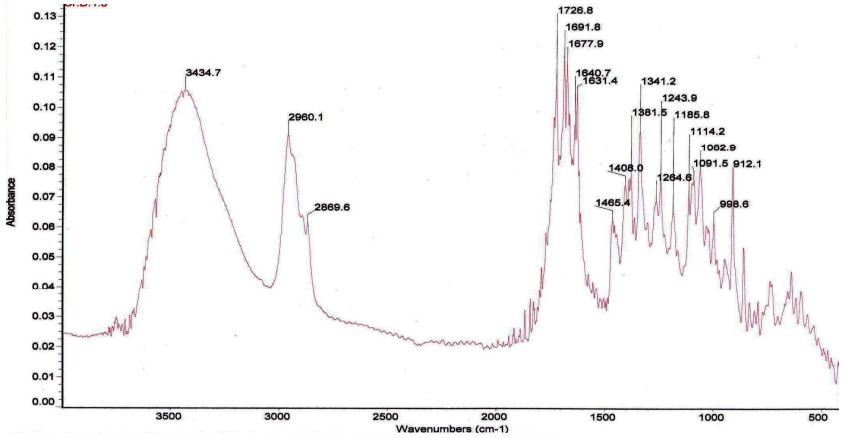


**Fig. 12.** IR spectrum of compound **1**

Collection @ chosun

**Table 2.** $^{1}H$ and $^{13}C$-NMR spectra data of compound **1** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | | 142.6 | |
| 2 | | 204.0 | |
| 3 | 2.26 d (5.0) | 51.9 | C-2, C-5, C-18 |
| 4 | | 39.8 | |
| 5 | | 179.1 | |
| | 2.75 dd (18.5, 5.5) | | |
| 6 | 3.33 d (18.5) | 26.1 | C-1, C-4, C-5, C-7 |
| 7 | 4.80 m | 74.0 | C-8, C-9, C-10, C-14 |
| 8 | | 66.0 | |
| 9 | 2.20 m | 57.4 | C-1 |
| 10 | | 80.3 | |
| | 1.48[a] | | |
| 11 | 1.64[a] | 19.4 | C-9, C-10, C-12 |
| | 1.59[a] | | |
| 12 | 2.45 m | 30.6 | |
| 13 | 2.94 d (8.5) | 42.3 | C-12, C-15, C-16 |
| 14 | 4.48 brs | 72.3 | C-15, C-16 |
| 15 | | 202.3 | |
| 16 | | 151.0 | |
| | 5.41 s | | |
| 17 | 6.05 s | 118.3 | C-13, C-15, C-16 |
| 18 | 1.39 s | 26.5 | C-5 |
| 19 | 1.22 s | 26.9 | C-3, C-5 |
| 20 | 1.50 s | 16.1 | C-1, C-9, C-10 |

*a*: overlapping signals.

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### 3.1.2. Structure determination of compound 2

Compound **2** was also obtained as an amorphous powder with negative optical rotation $[\alpha]^{25}_{D}$ -36° (*c* 0.3, MeOH). The IR, $^{1}$H and $^{13}$C NMR, $^{1}$H–$^{1}$H COSY, HMQC, HMBC, and NOESY (**Table 3**, **Fig. 13**) spectra of **2** were closely similar to those of **1**, supporting a grayanane diterpene with 7*α*,10*α*-epoxy-14*β*-hydroxy-16(17)-en-15-one moiety, except for the resonances of a ketone carbon and a double bond. The $^{1}$H NMR of **2** exhibited three singlet olefinic protons at $\delta_H$ 6.07, 5.37, and 5.21 (each single), and the $^{13}$C NMR revealed the presence of one ketone carbon ($\delta_C$ 202.9) and four olefinic carbons ($\delta_C$ 2151.7, 147.7, 117.0, and 116.3). The HMBC correlations from the olefinic proton resonance at $\delta_H$ 5.21 to carbon resonances at $\delta_C$ 47.3 (C-3) and 41.3 (C-4), and from $\delta_H$ 2.14 and 1.98 (H-3) to carbon resonances at $\delta_C$ 116.3 (C-2) and 147.7 (C-1) indicated one double bond is located at C-1/C-2. This was supported by the molecular formula $C_{20}H_{26}O_3$ by HRFAB MS *m/z* 315.1962 $[M + 1]^+$ (calcd. for $C_{20}H_{27}O_3$: 315.1960). Hence, compound **2** was determined as 7*α*,10*α*-epoxy-14*β*-hydroxy-1(2),16(17)-dien-15-one-grayanane.

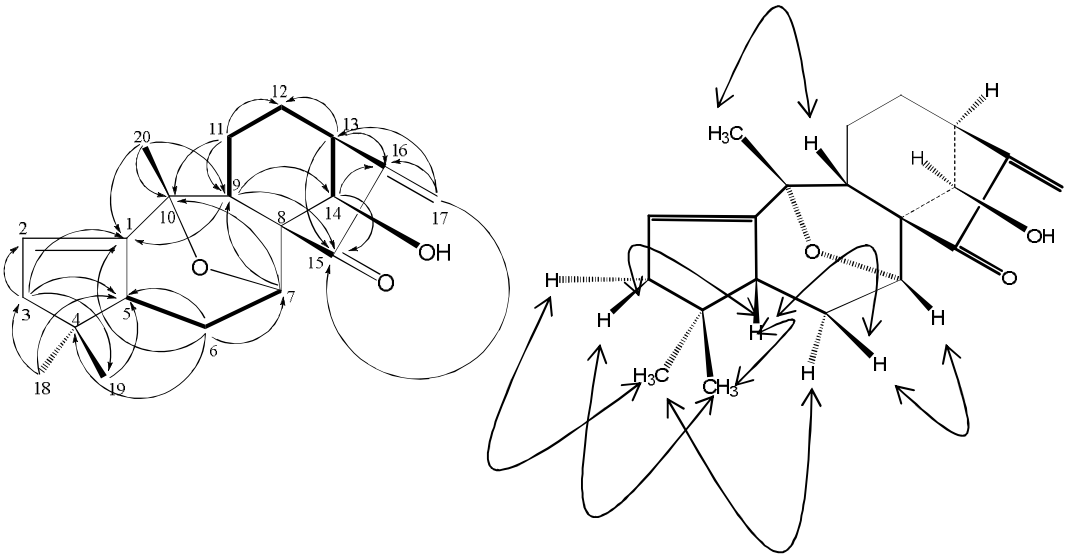


**Fig. 13.** Key HMBC (H→C), COSY ($^{1}$H__ $^{1}$H) and NOSEY (**↔**) correlations of compound **2**

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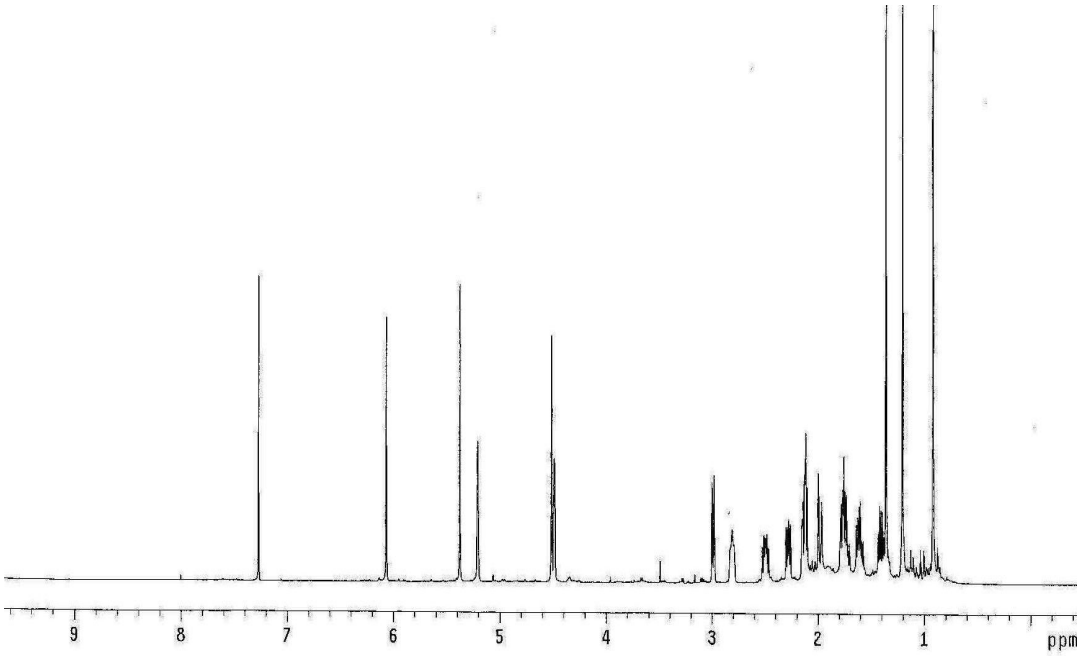


**Fig. 14.** $^{1}$H-NMR spectrum of compound **2** (500 MHz, $CDCl_3$)

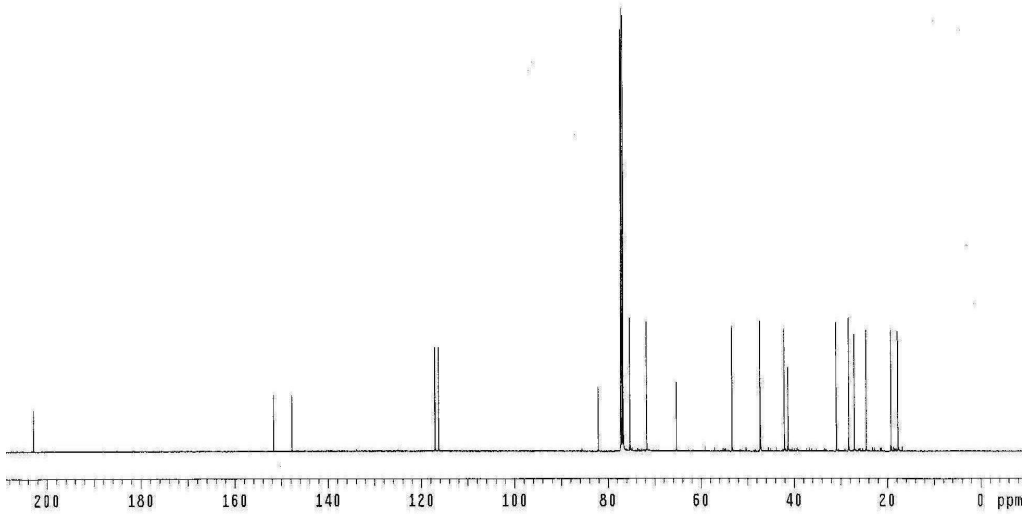


**Fig. 15.** $^{13}$C-NMR spectrum of compound **2** (125 MHz, $CDCl_3$)

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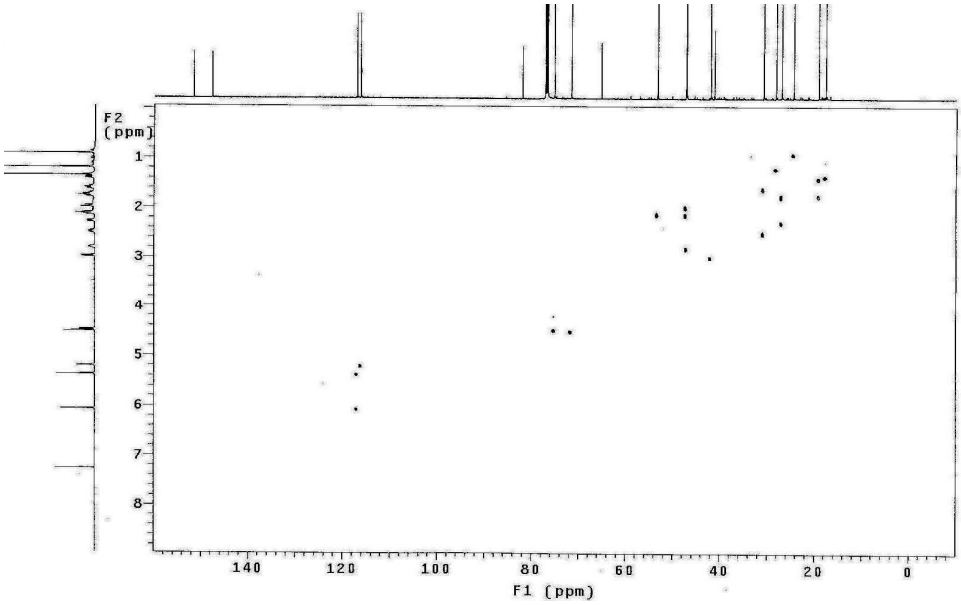


**Fig. 16.** HMQC spectrum of compound **2** (500 MHz, $CDCl_3$)

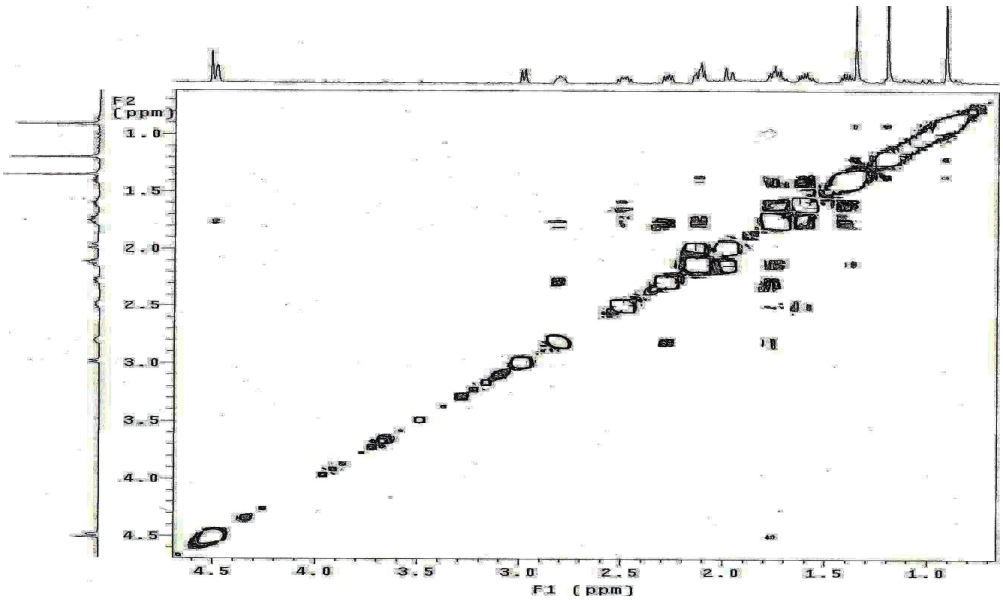


**Fig. 17.** COSY spectrum of compound **2** (500 MHz, $CDCl_3$)

Collection @ chosun

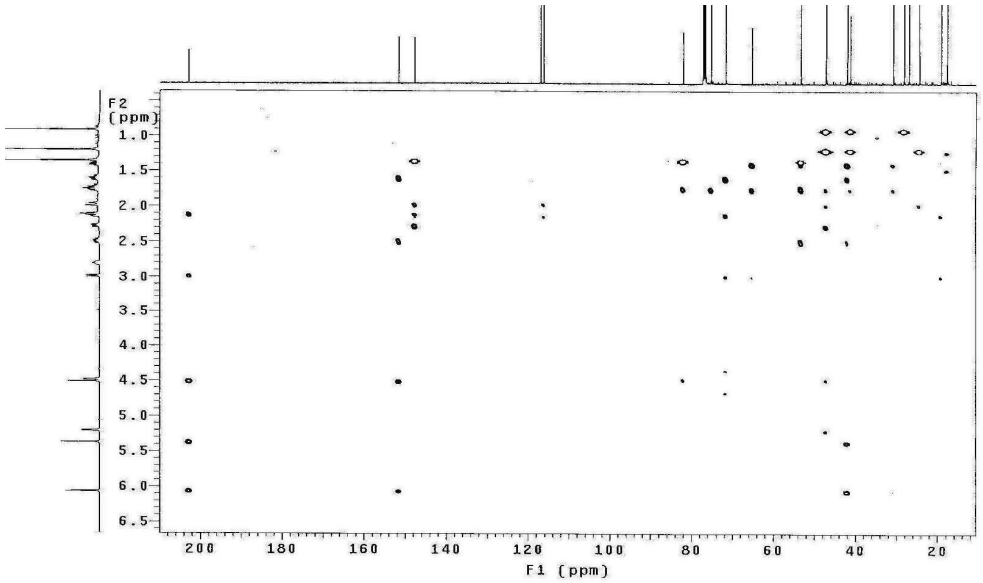


**Fig. 18.** HMBC spectrum of compound **2** (500 MHz, $CDCl_3$)

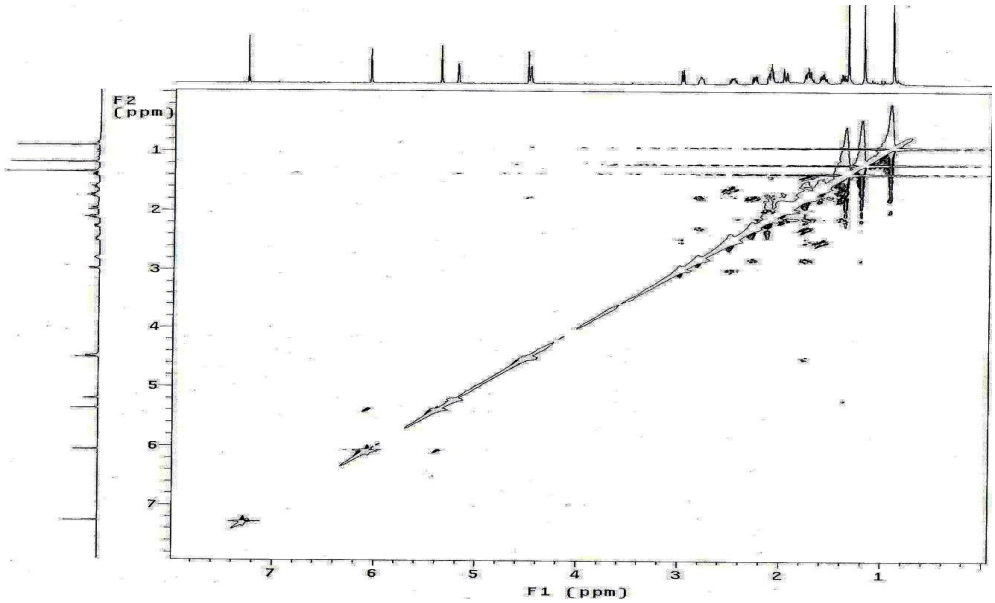


**Fig. 19.** NOESY spectrum of compound **2** (500 MHz, $CDCl_3$)

Collection @ chosun

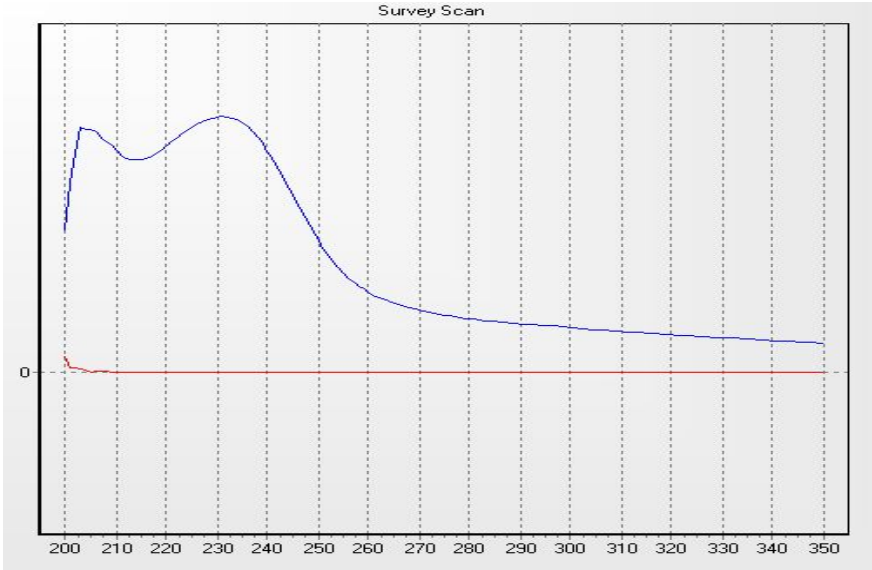


**Fig. 20.** UV spectrum of compound **2**

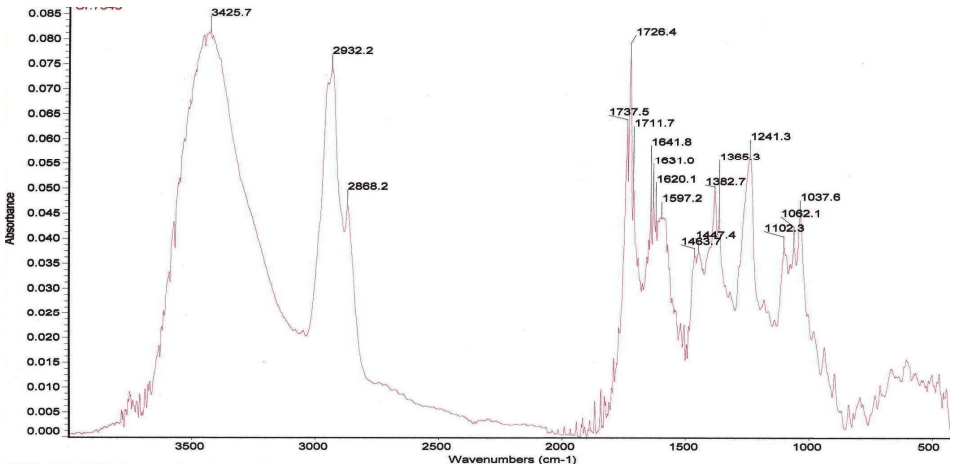


**Fig. 21.** IR spectrum of compound **2**

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**Table 3.** $^{1}H$ and $^{13}C$-NMR spectra data of compound **2** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | | 147.7 | |
| 2 | 5.21 brs | 16.3 | |
| | 1.98 dt (15.5, 2.0) | | |
| 3 | 2.14 m | 47.3 | C-1, C-2, C-5, C-18 |
| 4 | | 41.3 | |
| 5 | 2.81 t (7.5) | 47.2 | |
| | 1.40[a] | | |
| 6 | 1.73[a] | 27.1 | C-1, C-5, C-7 |
| 7 | 4.49 brs | 75.3 | C-8, C-9, C-10 |
| 8 | | 65.3 | |
| 9 | 2.13 m | 53.3 | C-1, C-14 |
| 10 | | 82.1 | |
| | 1.41 m | | |
| 11 | 1.74 m | 19.2 | C-9, C-10, C-12 |
| | 1.61 m | | |
| 12 | 2.50 m | 30.9 | |
| 13 | 2.99 d (10.0) | 42.1 | C-12, C-15, C-16 |
| 14 | 4.51 brs | 71.7 | C-15, C-16 |
| 15 | | 202.9 | |
| 16 | | 151.7 | |
| | 5.37 s | | |
| 17 | 6.01 s | 117.0 | C-13, C-15, C-16 |
| 18 | 1.20 s | 28.2 | C-5 |
| 19 | 0.92 s | 24.5 | C-3, C-5 |
| 20 | 1.36 s | 17.7 | C-1, C-9, C-10 |

*a*: overlapping signals.

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### 3.1.3. Structure determination of compound 3

Compound **3** was isolated as an white amorphous powder, $[\alpha]^{25}_{D}$ -20° (*c* 0.1, MeOH). Its molecular formula was determined to be $C_{22}H_{34}O_3$ by the molecular ion peak at *m/z* 346.2509 $[M]^+$ (calcd. for $C_{22}H_{34}O_3$, 346.2500) in the HREIMS. The IR spectrum of **3** showed the presence of hydroxyl (3498 $cm^{-1}$) and carbonyl (1710 $cm^{-1}$) groups (**Fig. 29**). The $^1H$ and $^{13}C$ NMR (**Table 4**) spectroscopic data suggested that **3** was a kaurane-type diterpenoid with an exocyclic double bond in the ring D [C-17: $\delta_H$ 4.68 (s), 4.82 (s); $\delta_C$ 103.1, 155.2]. These signals were found to be similar to those of *ent*-11α-acetoxykaur-16-en-18-oic acid (**8**),[47] which was isolated from the same extract, except for the presence of oxygenated methylene signals at $\delta_C$ 71.9 (t), $\delta_H$ 3.11 (d, $J$ = 10.5 Hz), and $\delta_H$ 3.42 (d, $J$ = 10.5 Hz) instead of one carboxylic acid carbon in **8**. These data indicated that one of the three tertiary methyl groups in the basic *ent*-kaur-16-en skeleton in compound **3** was oxidized to a hydroxymethyl group and then further carboxylic acid. Analysis of the HMQC spectrum revealed the only signals for Me-19 ($\delta_H$ 0.76, $\delta_C$ 17.5) and Me-20 ($\delta_H$ 1.01, $\delta_C$ 17.9) were observed. Comparing with the previous literature, the location of the hydroxyl group was assigned at C-18 [$\delta_C$ (C-19) *ca.* 17.3][53] rather than C-19.[64] This assignment was confirmed by HMBC spectra, which showed correlations between protons H-18 and carbons C-3, C-4, C-5 and C-19 (**Fig. 26**). The acetoxyl substituent was placed at C-11 as a result of the comparison of the $^1H$ and $^{13}C$ NMR data of **3** with those of **8** and the correlations observed between acetyl methyl ($\delta_H$ 1.93), H-11 ($\delta_H$ 5.07) and the ester carbonyl at $\delta_C$ 170.1 in the HMBC spectrum. (**Fig. 26**). A strong NOE correlation between H-11 and C-20 methyl protons revealed that the acetoxyl group at C-11 had *β*-orientation (**Fig. 27**). In addition, the CD data (**Fig. 30**) and the negative value of optical rotation of **3** indicated the *ent*-configuration.[48,57] Therefore, the structure of compound **3**, named crotokinensin C, was characterized as *ent*-11*β*-acetoxykaur-16-en-18-ol.

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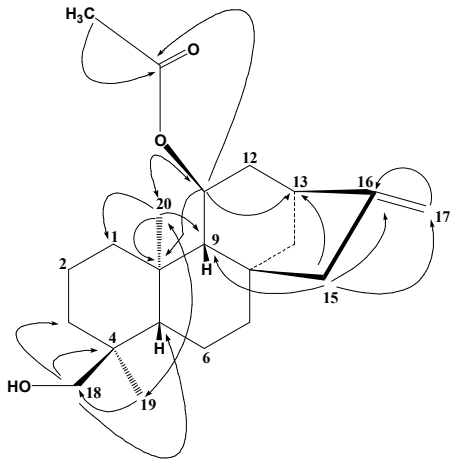


**Fig. 22.** Key HMBC (H→C) and NOSEY (**↔**) correlations of compound **3**

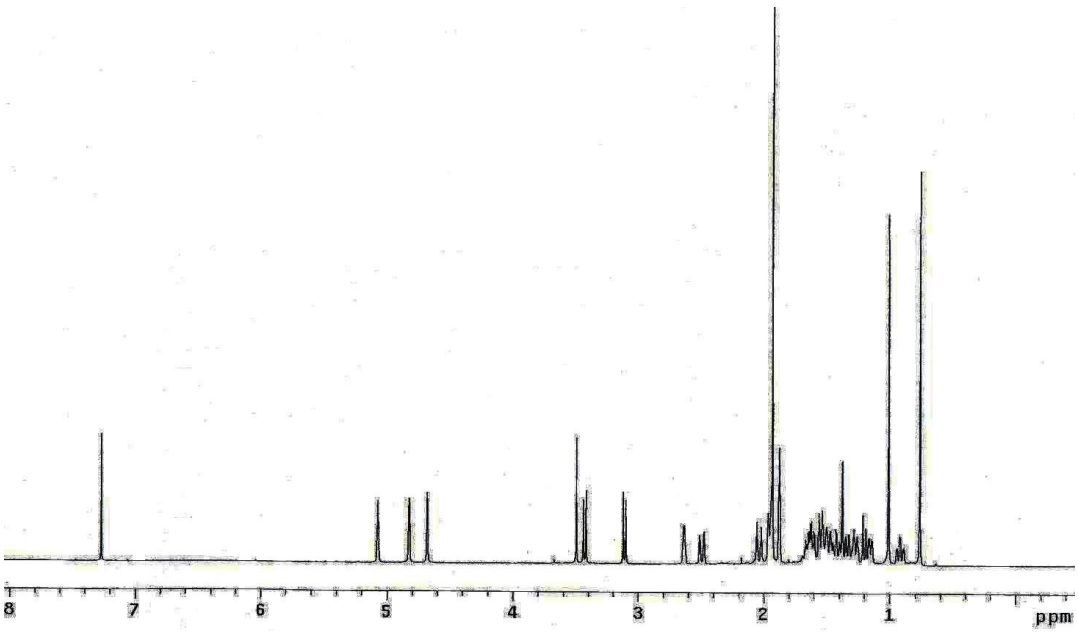


**Fig. 23.** $^{1}$H-NMR spectrum of compound **3** (500 MHz, $CDCl_3$)

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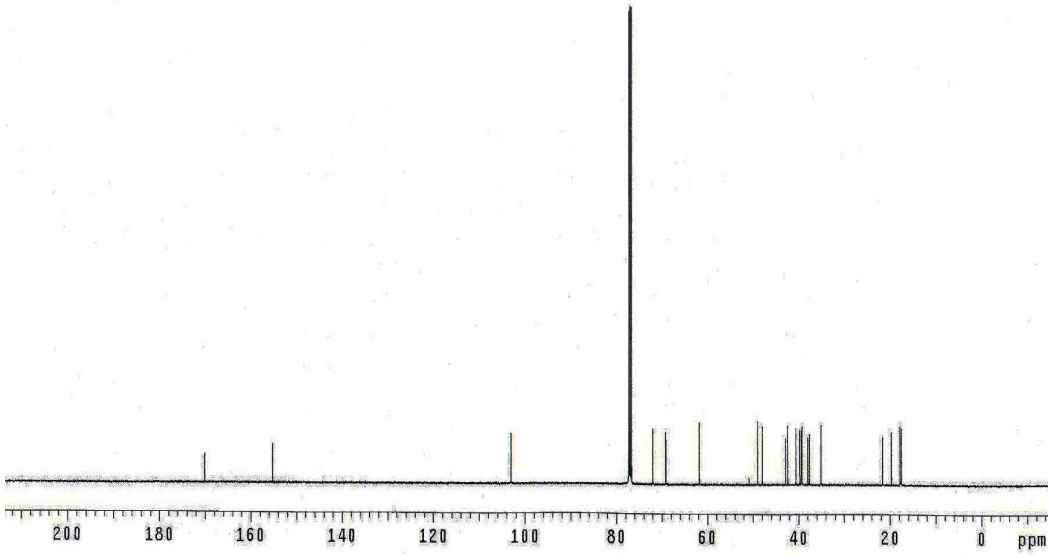


**Fig. 24.** $^{13}$C-NMR spectrum of compound **3** (125 MHz, $CDCl_3$)

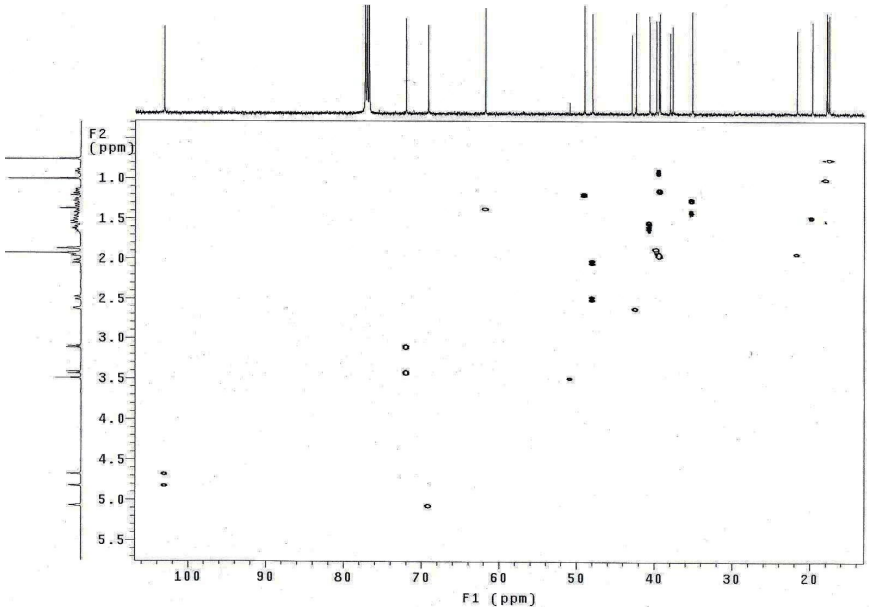


**Fig. 25.** HMQC spectrum of compound **3** (500 MHz, $CDCl_3$)

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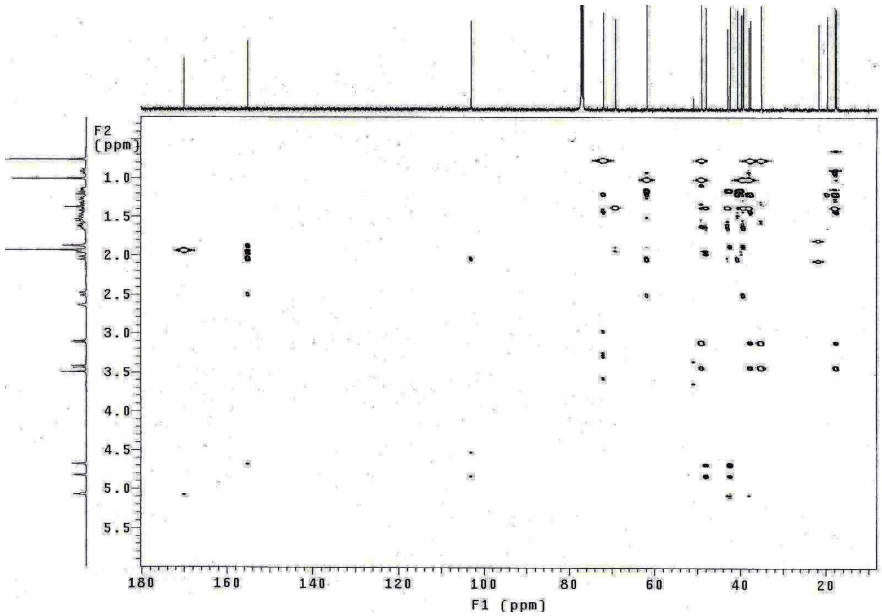


**Fig. 26.** HMBC spectrum of compound **3** (500 MHz, $CDCl_3$)

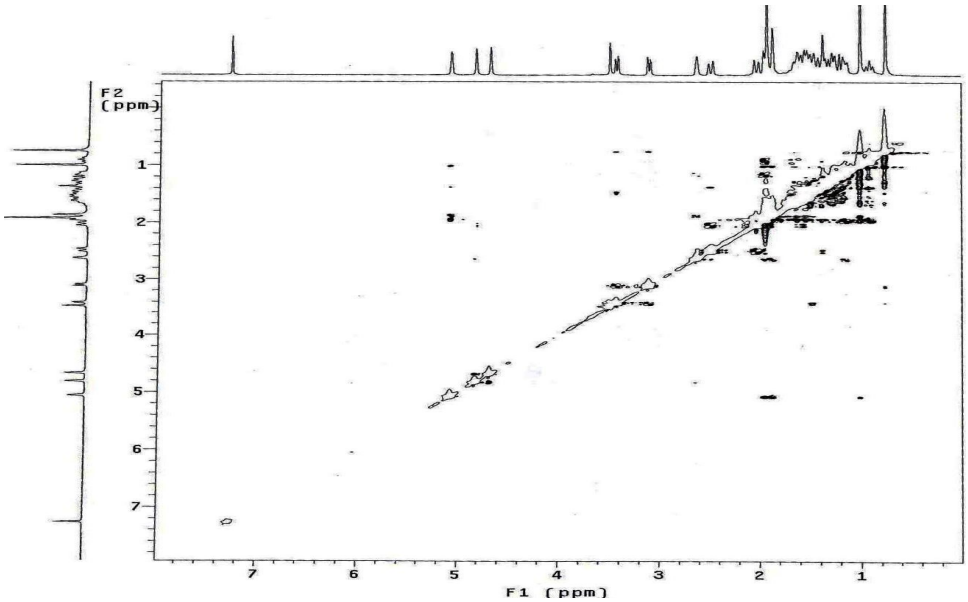


**Fig. 27.** NOESY spectrum of compound **3** (500 MHz, $CDCl_3$)

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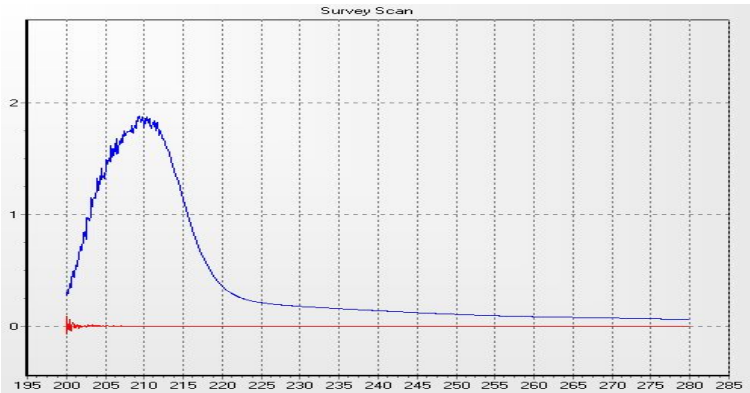


**Fig. 28.** UV spectrum of compound **3**

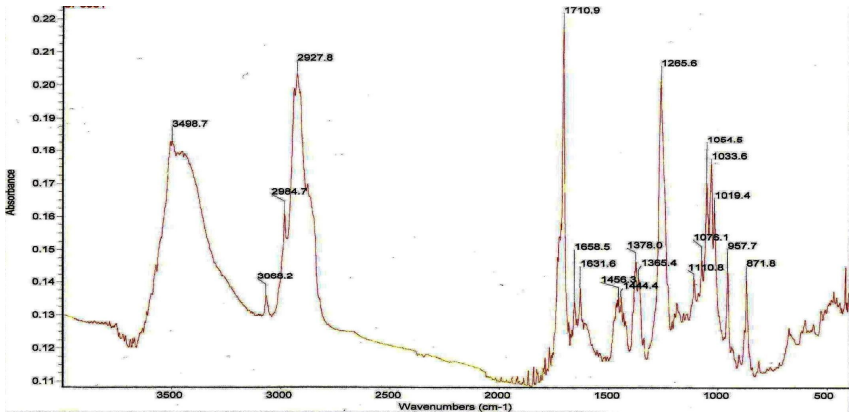


**Fig. 29.** IR spectrum of compound **3**

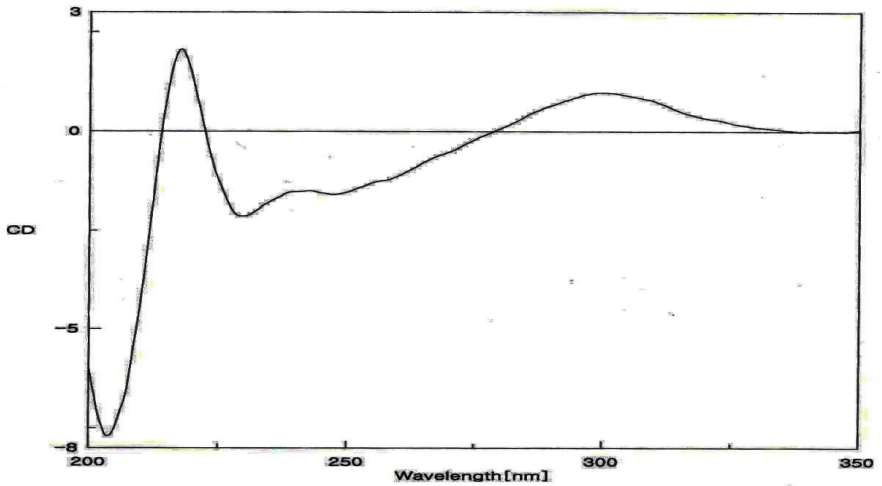


**Fig. 30.** CD spectrum of compound **3**

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**Table 4.** $^{1}$H and $^{13}$C-NMR spectra data of compound **3** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | 0.92 ddd (13.0, 13.0, 3.0)<br>1.96[a] | 39.4 | C-2 |
| 2 | 1.52[a]<br>1.67[a] | 17.7 | C-3 |
| 3 | 1.26[a]<br>1.40[a] | 35.1 | C-2, C-4, C-5 |
| 4 | | 37.6 | |
| 5 | 1.20 dd (12.5, 1.5) | 49.0 | C-4, C-6, C-7 |
| 6 | 1.33 dd (12.5, 2.5)<br>1.49[a] | 19.7 | C-4, C-5 |
| 7 | 1.52[a]<br>1.59[a] | 40.6 | C-8 |
| 8 | | 42.9 | |
| 9 | 1.37[a] | 61.8 | C-11 |
| 10 | | 37.9 | |
| 11 | 5.07 t (3.0) | 69.1 | C-10, C-13, 11-OAc |
| 12 | 1.87[a]<br>1.94[a] | 39.7 | C-11, C-13, C-14 |
| 13 | 2.63 brs | 42.4 | C-8, C-14 |
| 14 | 1.17 dd (11.5, 5.0)<br>1.95[a] | 39.3 | C-12, C-13 |
| 15 | 2.03 dt (16.5, 3.0)<br>2.48 d (16.5) | 47.9 | C-9, C-13, C-16, C-17 |
| 16 | | 155.2 | |
| 17 | 4.68 s<br>4.82 s | 103.1 | C-16 |
| 18 | 3.11 d (10.5)<br>3.42 d (10.5) | 71.9 | C-3, C-4, C-5 |
| 19 | 0.76 s | 17.5 | C-3, C-4, C-5, C-18 |
| 20 | 1.01 s | 17.9 | C-1, C-9, C-10 |
| 11-OAc | 1.93 s | 21.7 | C=O |
| | | 170.1 | |

*a*: overlapping signals.

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### 3.1.4. Structure determination of compound 4

Compound **4** was isolated as an colorless oil, $[\alpha]^{25}_{D}$ -17° (*c* 0.1, MeOH). The IR spectrum indicated the presence of hydroxyl (3471 $cm^{-1}$) and carbonyl (1710 $cm^{-1}$) functional groups. The HREIMS of compound **4** showed a molecular ion peak at *m/z* 346.2503 $[M]^+$, consistent with the molecular weight of **3**. Comparison of the $^1H$ and $^{13}C$ NMR (**Table 5**) data of **4** with those of **3** revealed very close agreement, showing the presence of a 20-carbon skeleton of an *ent*-kaurane diterpenoid together with a hydroxyl and an acetoxyl groups. The $^1H$ and $^{13}C$ NMR signals of oxygenated methylene group at C-18 of **4** ($\delta_H$ 3.86, 3.66; $\delta_C$ 73.1) displayed a downfield shift than those of **3** ($\delta_H$ 3.42, 3.11; $\delta_C$ 71.9). This suggested the location of the acetoxyl group at C-18 position,[47] which was confirmed by HMBC correlations between protons H-18 and carbons C-3. C-5 and C-19 and the ester carbonyl at $\delta_C$ 73.1 (**Fig. 36**). The presence of oxygenated methine at $\delta_H$ 4.15 (brs, 1H) and $\delta_C$ 76.2 assumed that the hydroxyl group attached to the C-14α position.[48] This assignment was confirmed by HMBC spectra, which showed correlations between protons H-14 and carbons C-15 and C-16 (**Fig. 36**). The *α*-orientation of C14-OH remained to be more clarified. However, from the CD data and the negative optical rotation, and the comparison with the other *ent*-kauranes produced from this plant,[47-50,54,55] the structure of **4** was characterized as *ent*-14α-hydroxy-18-acetoxykaur-16-en, named crotokinensin D.

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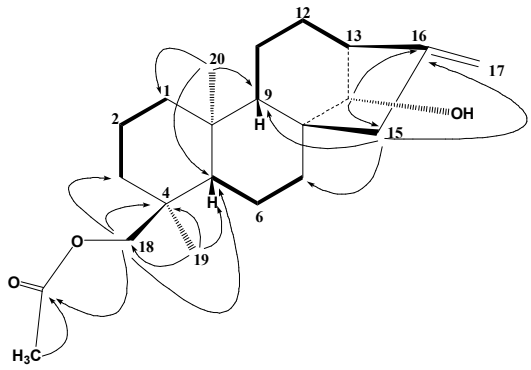


**Fig. 31.** Key HMBC (H→C) and COSY ($^1$H—$^1$H) correlations of compound **4**

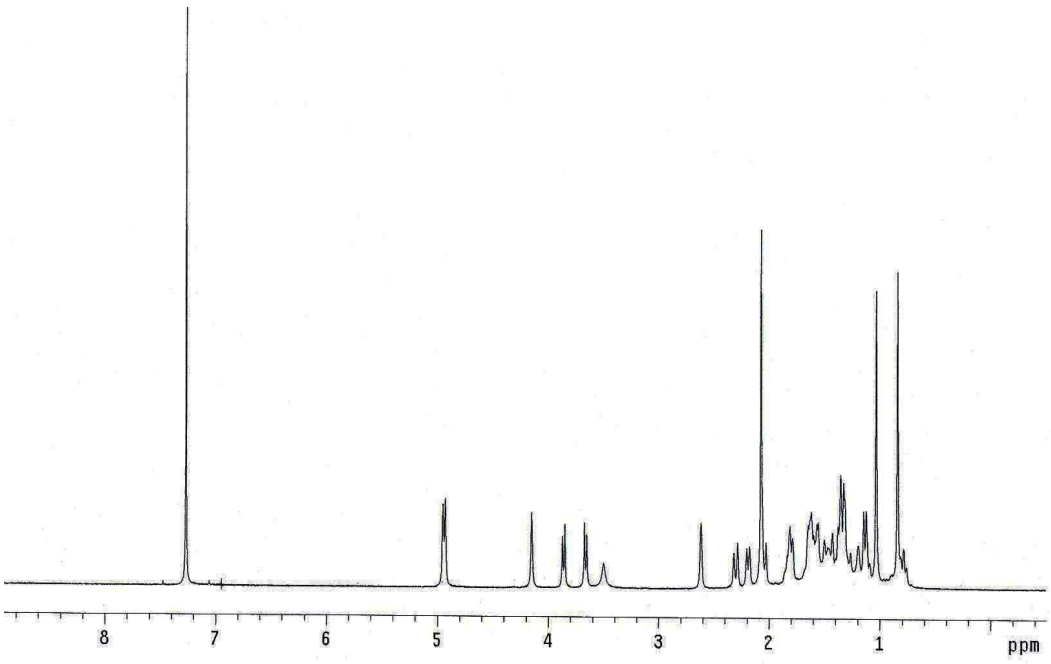


**Fig. 32.** $^1$H-NMR spectrum of compound **4** (500 MHz, $CDCl_3$)

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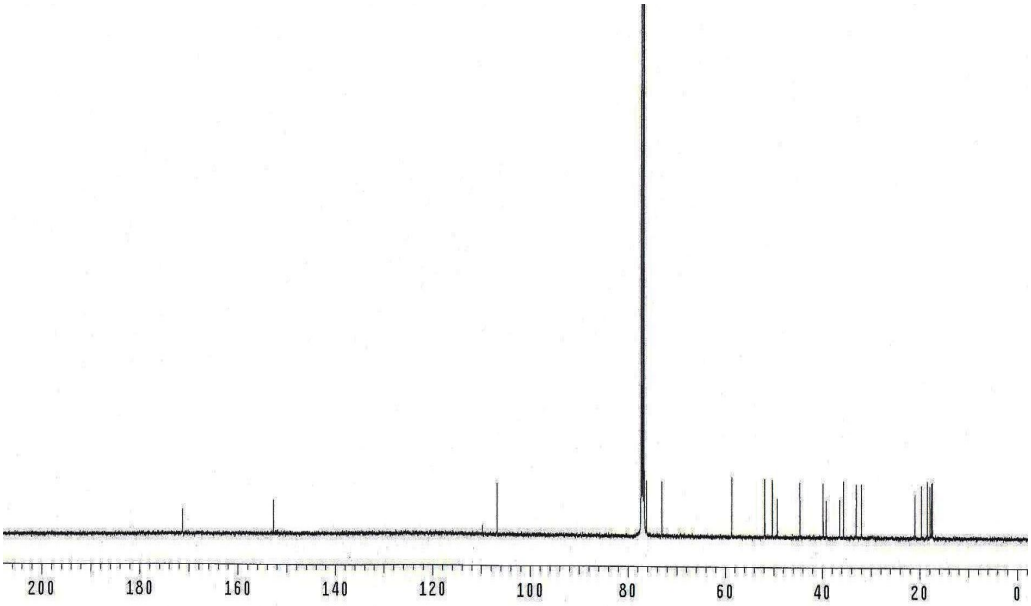


**Fig. 33.** $^{13}$C-NMR spectrum of compound **4** (125 MHz, $CDCl_3$)

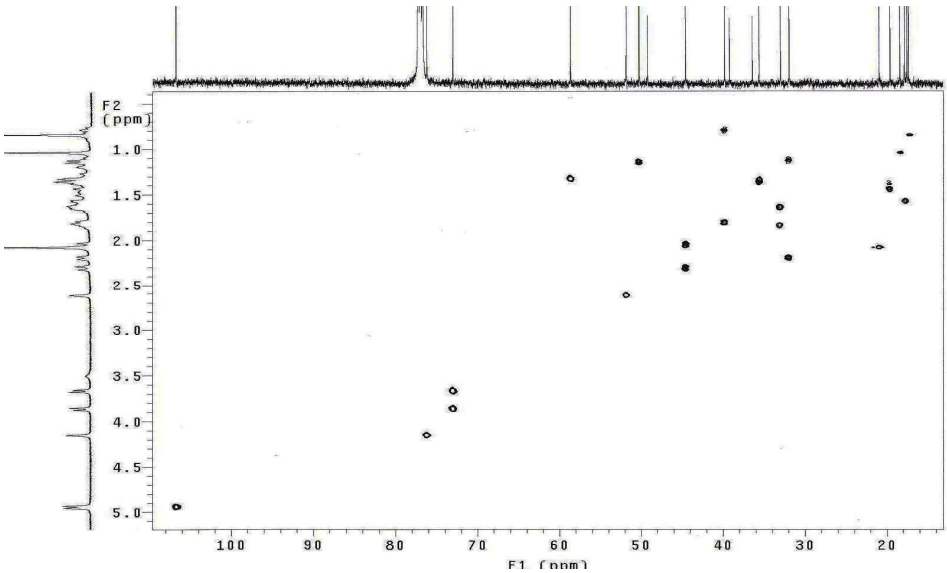


**Fig. 34.** HMQC spectrum of compound **4** (500 MHz, $CDCl_3$)

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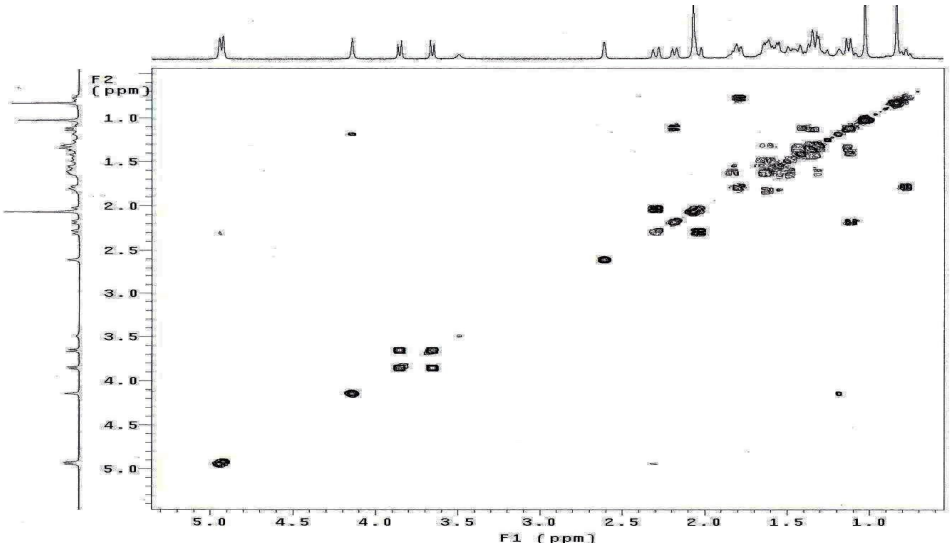


**Fig. 35.** COSY spectrum of compound **4** (500 MHz, $CDCl_3$)

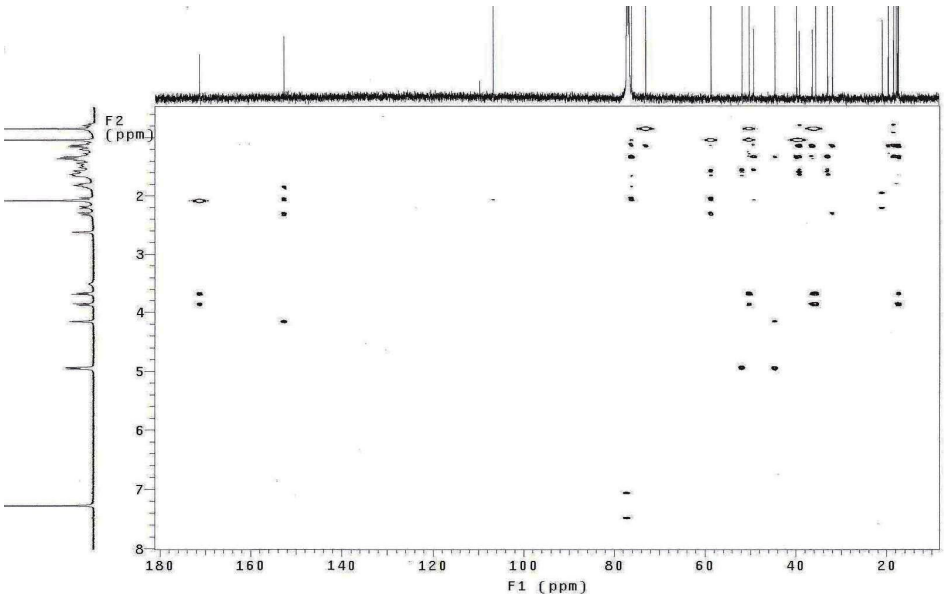


**Fig. 36.** HMBC spectrum of compound **4** (500 MHz, $CDCl_3$)

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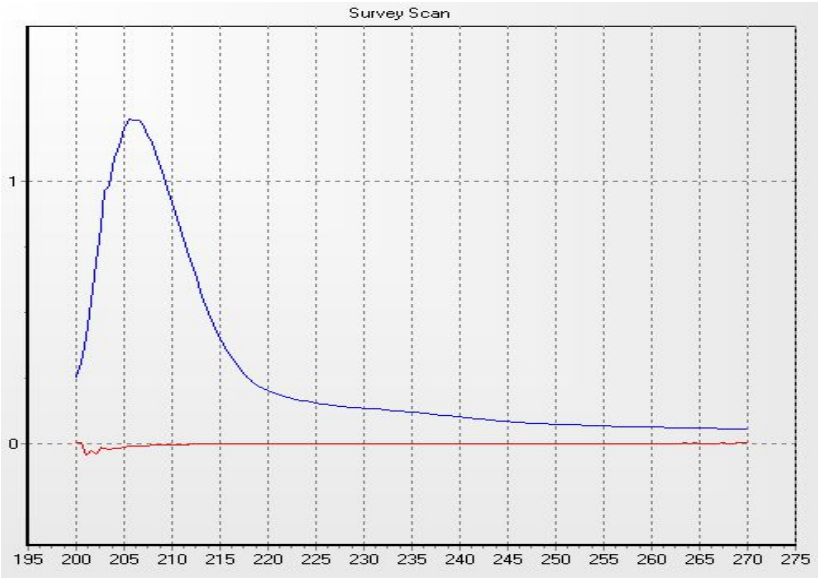


**Fig. 37.** UV spectrum of compound **4**

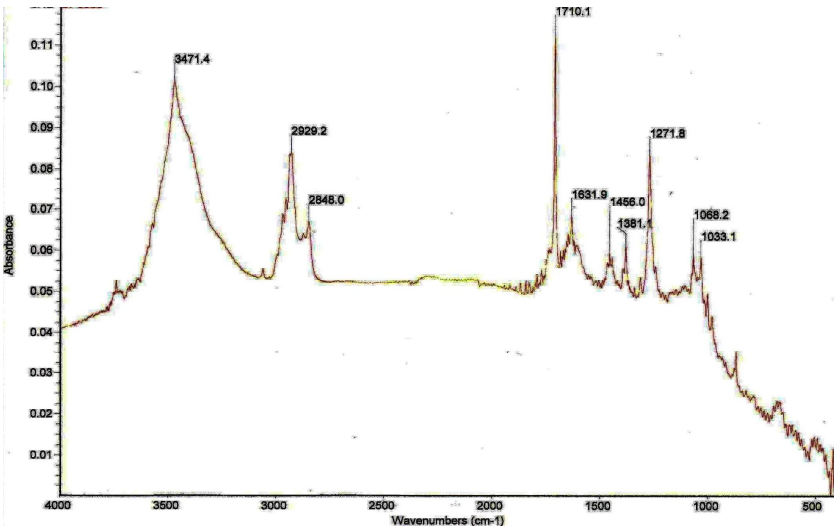


**Fig. 38.** IR spectrum of compound **4**

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**Table 5.** $^{1}$H and $^{13}$C-NMR spectra data of compound **4** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | 0.78[a]<br>1.81[a] | 39.8 | C-10 , C-20 |
| 2 | 1.59[a]<br>1.61[a] | 17.6 | C-10 |
| 3 | 1.38[a]<br>1.40[a] | 35.6 | C-4, C-18 |
| 4 | | 36.4 | |
| 5 | 1.48[a] | 50.3 | |
| 6 | 1.37[a]<br>1.47[a] | 19.7 | C-8 |
| 7 | 1.12[a]<br>2.02[a] | 31.9 | C-6, C-8, C-9 |
| 8 | | 49.3 | |
| 9 | 1.35[a] | 58.7 | C-8, C-14, C-20 |
| 10 | | 39.2 | |
| 11 | 1.32[a]<br>1.56[a] | 17.9 | C-10, C-12, C-13 |
| 12 | 1.64[a]<br>1.81[a] | 33.0 | C-13, C-14 |
| 13 | 2.62 brs | 51.8 | C-8, C-14 |
| 14 | 4.15 brs | 76.2 | C-15, C-16 |
| 15 | 2.18 dt (16.5, 1.5)<br>2.30 d (16.5) | 44.6 | C-7, C-9, C-16 |
| 16 | | 152.7 | |
| 17 | 4.93 s<br>4.95 s | 106.7 | C-13, C-15 |
| 18 | 3.66 d (10.5)<br>3.86 d (10.5) | 73.1 | C-3, C-5, C-19, C=O |
| 19 | 0.84 s | 17.4 | C-4, C-5, C-18 |
| 20 | 1.09 s | 18.5 | C-1, C-5, C-9 |
| 18-OAc | 2.07 s | 21.0 | C=O |
| | | 171.3 | |

*a*: overlapping signals.

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### 3.1.5. Structure determination of compound 5

Compound **5** was isolated as an colorless oil, $[\alpha]^{25}_{D}$ -25° (*c* 0.1, MeOH). The IR spectrum indicated the presence of hydroxyl (3482 $cm^{-1}$) and carbonyl (1725 $cm^{-1}$) functional groups. The HREIMS of compound **5** showed a molecular ion peak at *m/z* 346.2507 $[M]^+$, consistent with the molecular weight of **3** and **4**. The $^1H$ and $^{13}C$ NMR (**Table 6**) data of **5** revealed very close agreement with those of **4**, showing the presence of a 20-carbon skeleton of an *ent*-kaurane diterpenoid together with an acetoxyl and a hydroxyl groups. The main difference was displayed in the upfielded chemical shift of the methine bearing the hydroxyl group ($\delta_H$ 3.86; $\delta_C$ 66.9) compared with 14-OH in **4** ($\delta_H$ 4.15, $\delta_C$ 76.2), while the signals for the acetyl group at C-18 remained unchanged. From the HMBC spectrum the location of the hydroxyl group at C-1 was identified, which showed the correlations between proton H-5 and carbon C-1, and between proton H-1 and carbon C-20 (**Fig. 44**). The $\alpha$-orientation of the hydroxyl group was deduced from the coupling pattern of H-1, which was a doublet-doublet ($\delta_H$ 3.86, dd, $J$ = 5.5, 11.0 Hz). The coupling pattern of H-1 is known to be dependent on the orientation of 1-OH, and H-1$\alpha$ appears as a broad singlet.[49,65] However, H-1β shows a doublet-doublet pattern with a large coupling constant with C-2 methylene protons ($\delta_H$ 3.48, dd, $J$ = 6.0, 10.0 Hz).[66] The *ent*-kaurane configuration of **5** was determined from the negative optical rotation. Therefore, compound **5** was characterized as *ent*-1α-hydroxy-18-acetoxykaur-16-en, named crotokinensin E.

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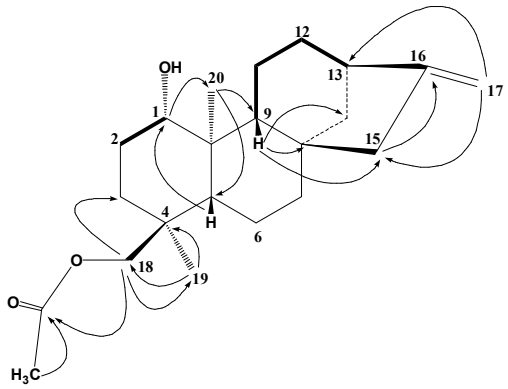


**Fig. 39.** Key HMBC (H→C) and COSY ($^1$H—$^1$H) correlations of compound **5**

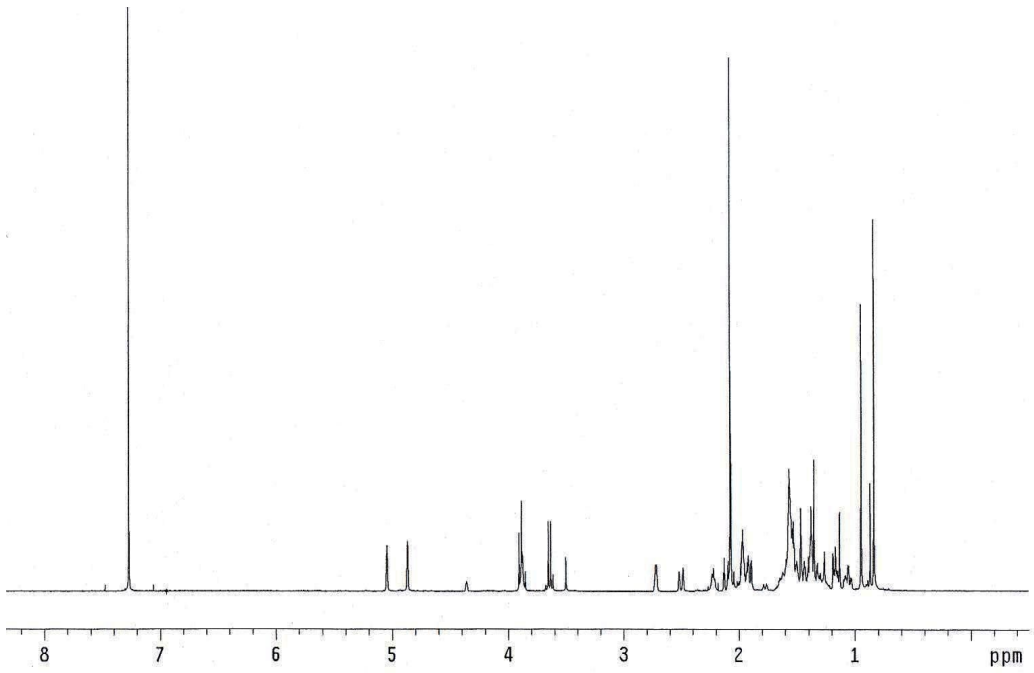


**Fig. 40.** $^1$H-NMR spectrum of compound **5** (500 MHz, $CDCl_3$)

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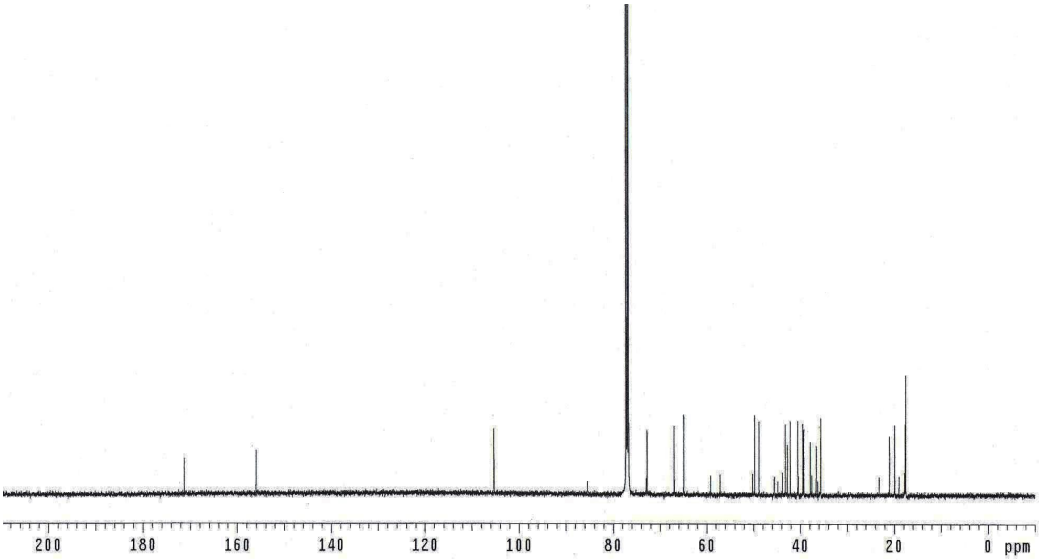


**Fig. 41.** $^{13}$C-NMR spectrum of compound **5** (125 MHz, $CDCl_3$)

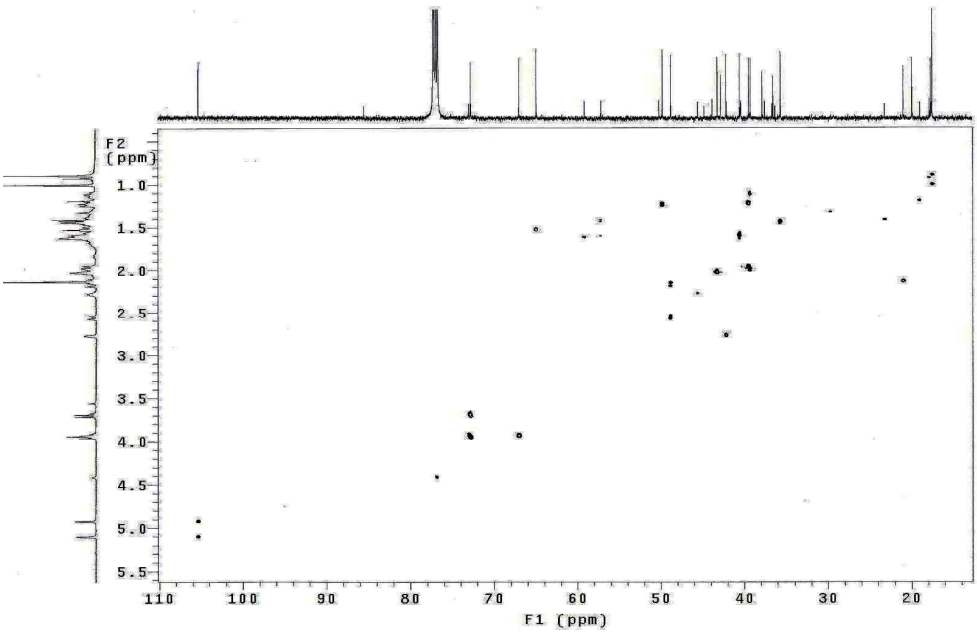


**Fig. 42.** HMQC spectrum of compound **5** (500 MHz, $CDCl_3$)

Collection @ chosun

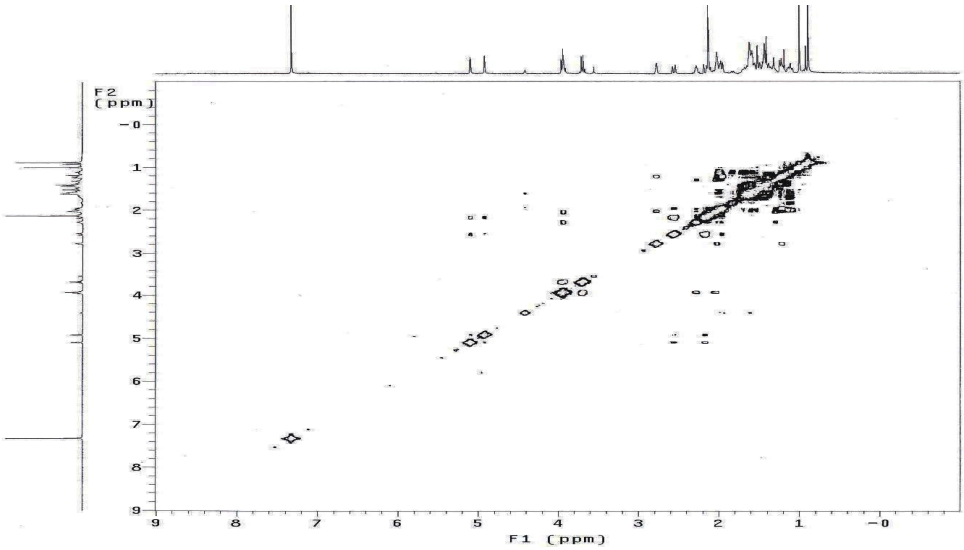


**Fig. 43.** COSY spectrum of compound **5** (500 MHz, $CDCl_3$)

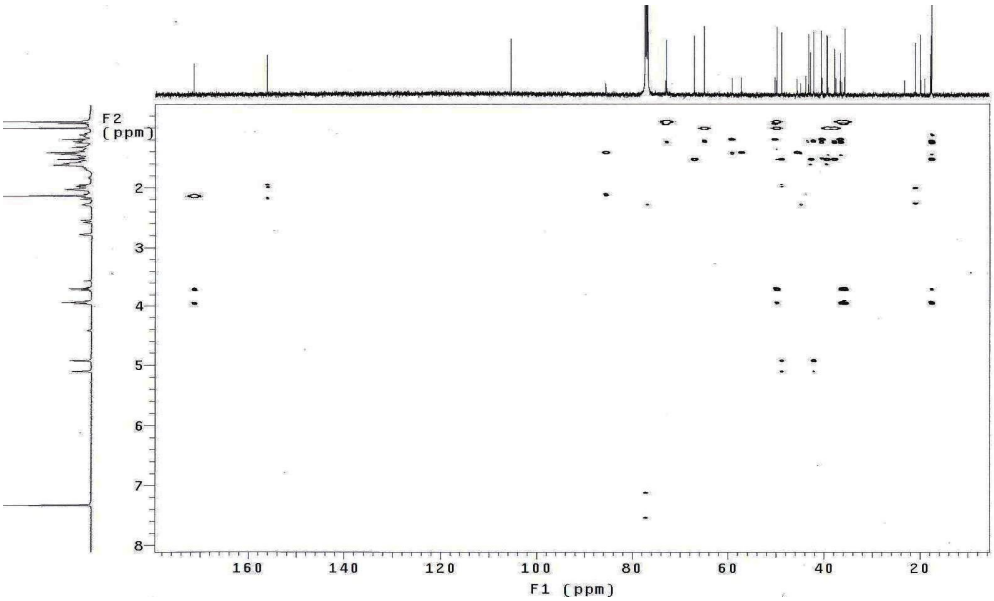


**Fig. 44.** HMBC spectrum of compound **5** (500 MHz, $CDCl_3$)

Collection @ chosun

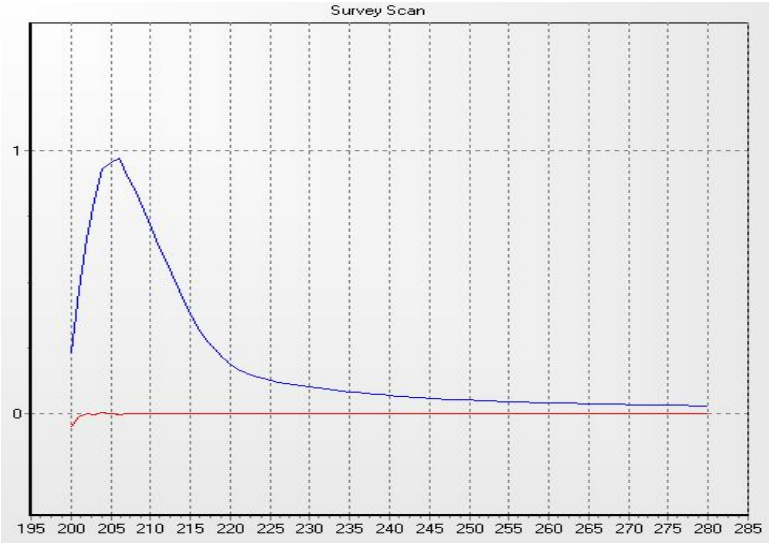


**Fig. 45.** UV spectrum of compound **5**

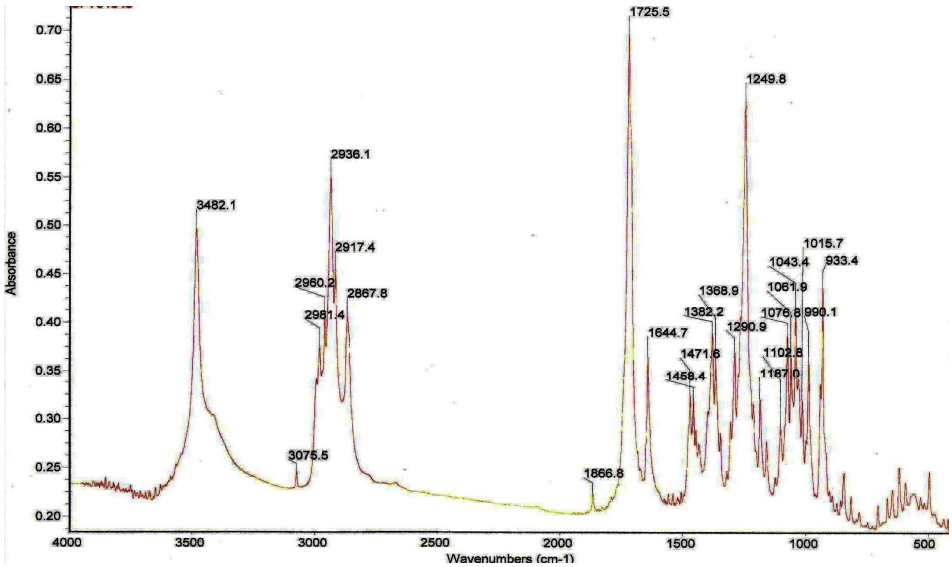


**Fig. 46.** IR spectrum of compound **5**

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**Table 6.** $^{1}H$ and $^{13}C$-NMR spectra data of compound **5** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | 3.86 dd (11.0, 5.5) | 66.9 | C-10, C-20 |
| 2 | 1.52[a]<br>1.98[a] | 37.8 | C-1 |
| 3 | 1.38[a]<br>1.50[a] | 35.6 | C-2, C-5 |
| 4 | | 36.5 | |
| 5 | 1.18[a] | 40.5 | C-3, C-10, C-19 |
| 6 | 1.42[a]<br>1.56[a] | 19.9 | C-5, C-8 |
| 7 | 1.50[a]<br>1.93[a] | 43.2 | C-8, C-14, C-15 |
| 8 | | 42.8 | |
| 9 | 1.46[a] | 64.9 | C-8, C-11, C-14 |
| 10 | | 49.8 | |
| 11 | 1.58[a]<br>2.23[a] | 17.7 | |
| 12 | 1.15[a]<br>1.97[a] | 39.4 | C-11, C-16 |
| 13 | 2.72 brs | 42.1 | |
| 14 | 1.06[a]<br>1.92 dd (11.0, 2.5) | 39.2 | C-9 |
| 15 | 2.11 dt (17.0, 3.0)<br>2.50 d (17.0) | 48.8 | C-16 |
| 16 | | 156.0 | |
| 17 | 4.87 s<br>5.04 s | 105.3 | C-13, C-15 |
| 18 | 3.64 d (11.0)<br>3.89 d (11.0) | 72.8 | C-3, C-19, C=O |
| 19 | 0.84 s | 17.5 | C-3, C-4, C-18 |
| 20 | 0.95 s | 17.6 | C-5, C-9, C-10 |
| 18-OAc | 2.08 s | 20.9 | C=O |
| | | 171.3 | |

*a*: overlapping signals.

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### 3.1.6. Structure determination of compound 6

Compound **6** was isolated as an colorless oil, $[\alpha]^{25}_{D}$ -22° (*c* 0.1, MeOH). The IR spectrum indicated the presence of hydroxyl (3437 $cm^{-1}$) and carbonyl (1737 $cm^{-1}$) functional groups. The HREIMS of compound **4** showed a molecular ion peak at *m/z* 346.2509 $[M]^+$, also consistent with the molecular weight of **3**, **4** and **5**. Comparison of the $^1H$ and $^{13}C$ NMR (**Table 7**) data of **6** with those of **4** revealed very close agreement, showing the presence of a 20-carbon skeleton of an *ent*-kaurane diterpenoid together with a hydroxyl and an acetoxyl groups. Similar to compound **4** and **5** the $^1H$ and $^{13}C$ NMR signals of oxygenated methylene group $\delta_H$ 3.86 (1H, d, $J$ = 11.0 Hz), 3.66 (1H, d, $J$ = 11.0 Hz) and $\delta_C$ 72.7 indicated the location of the acetoxyl group at C-18 position. This was further confirmed by HMBC correlations between protons H-18 and carbons C-3. C-4, C-5 and C-19 and the ester carbonyl at $\delta_C$ 72.7 (**Fig. 52**). The presence of oxygenated methine at $\delta_H$ 3.45 (brs, 1H) and $\delta_C$ 76.2 suggested the hydroxyl group attached to ring A.[67] The location of the hydroxyl group at C-3 was assigned from HMBC spectra, which showed correlations between proton H-3 and carbon C-18 and between protons H-18 and carbon C-3 (**Fig. 52**). The downfield signal of carbon C-2 ($\delta_C$ 29.4) also supported to this assignment.[68] The $\alpha$-orientation of C3-OH of **6** was derived from the coupling constant values of the H-3 equatorial signal at $\delta_H$ 3.45 (dd, $J$ = 11.0 and 4.5 Hz), and by comparison with those reported.[69,70] The negative optical rotation clarified the *ent*-kaurane configuration of **6**. On the basic of above evidences, compound **6**, named crotokinensin F was represented as *ent*-18-acetoxy-3$\alpha$-hydroxykaur-16-en.

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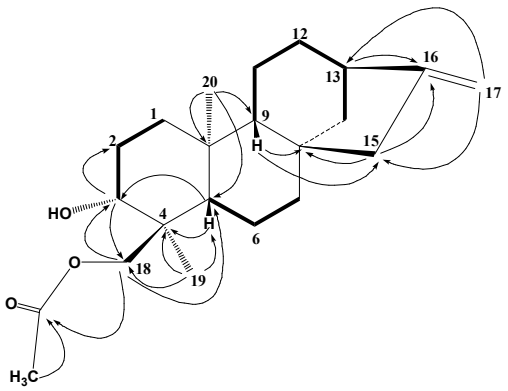


**Fig. 47.** Key HMBC (H→C) and COSY ($^1$H—$^1$H) correlations of compound **6**

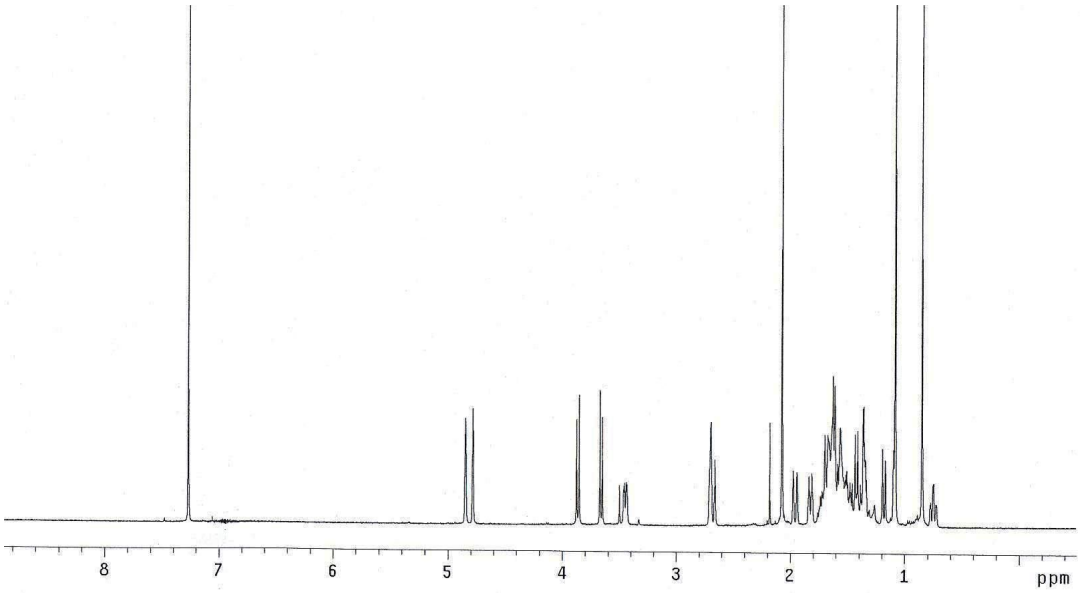


**Fig. 48.** $^1$H-NMR spectrum of compound **6** (500 MHz, $CDCl_3$)

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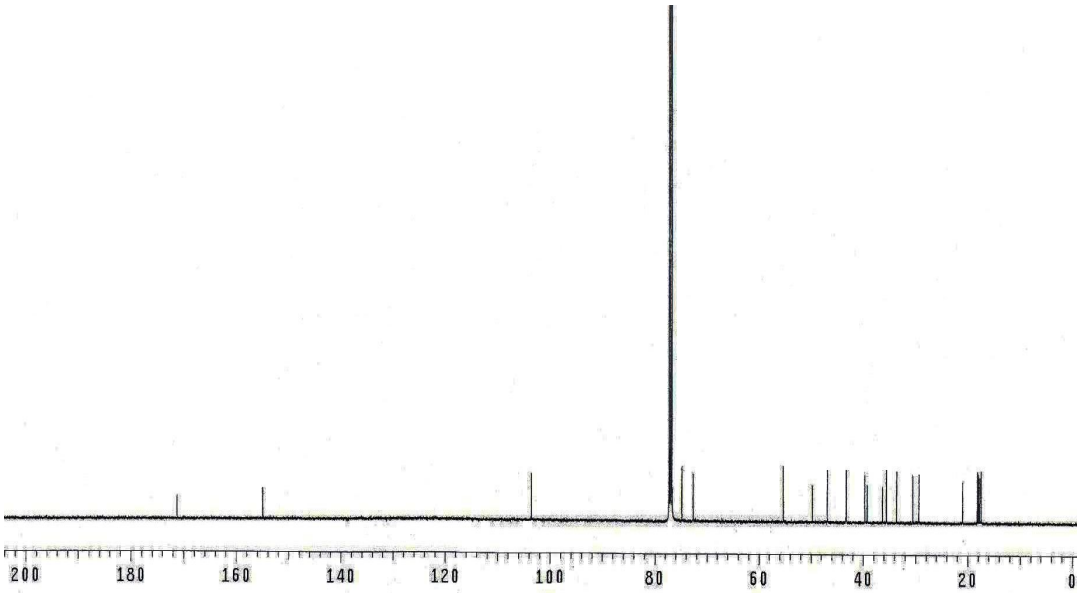


**Fig. 49.** $^{13}$C-NMR spectrum of compound **6** (125 MHz, $CDCl_3$)

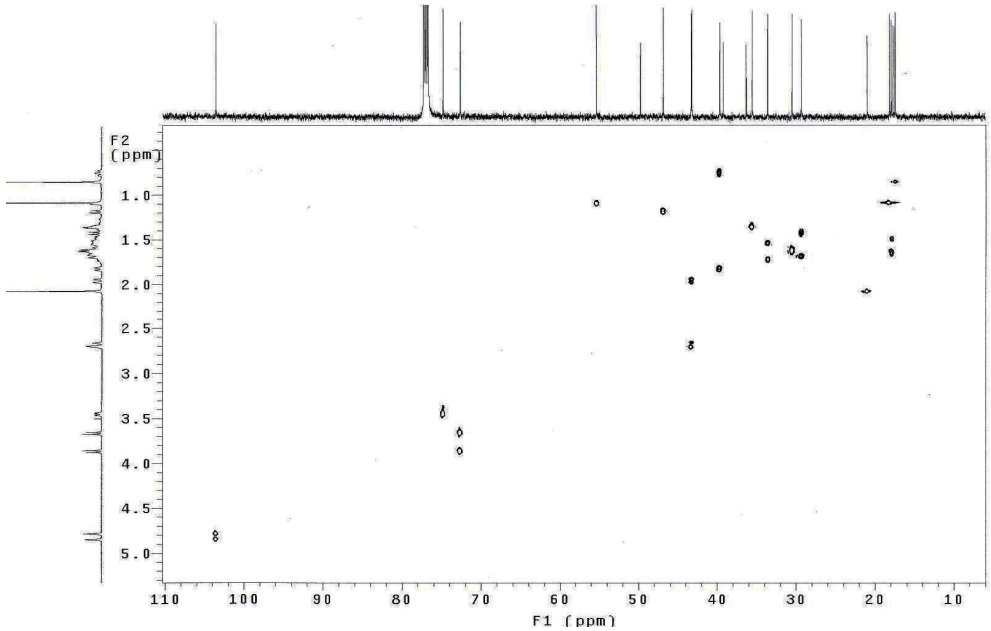


**Fig. 50.** HMQC spectrum of compound **6** (500 MHz, $CDCl_3$)

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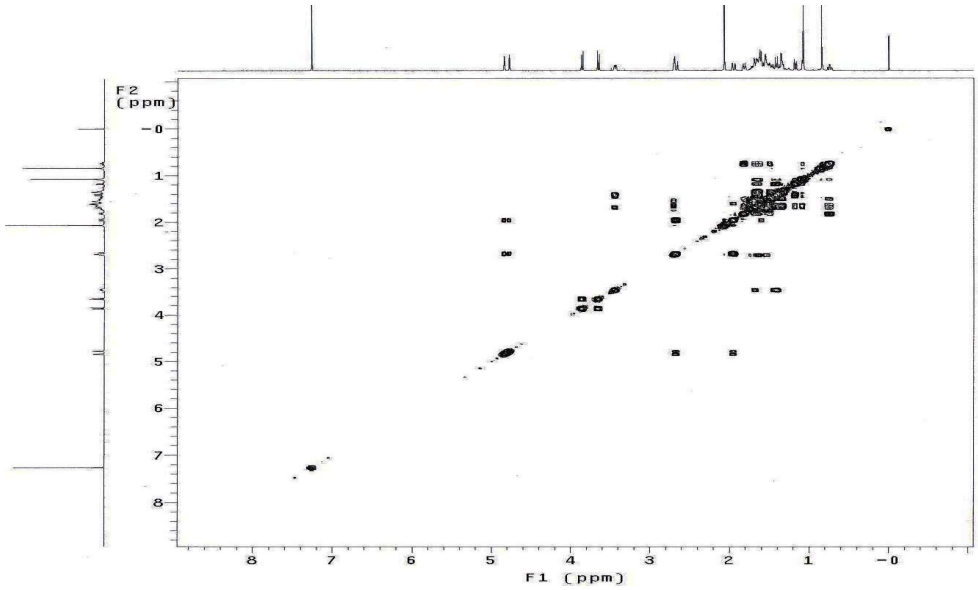


**Fig. 51.** COSY spectrum of compound **6** (500 MHz, $CDCl_3$)

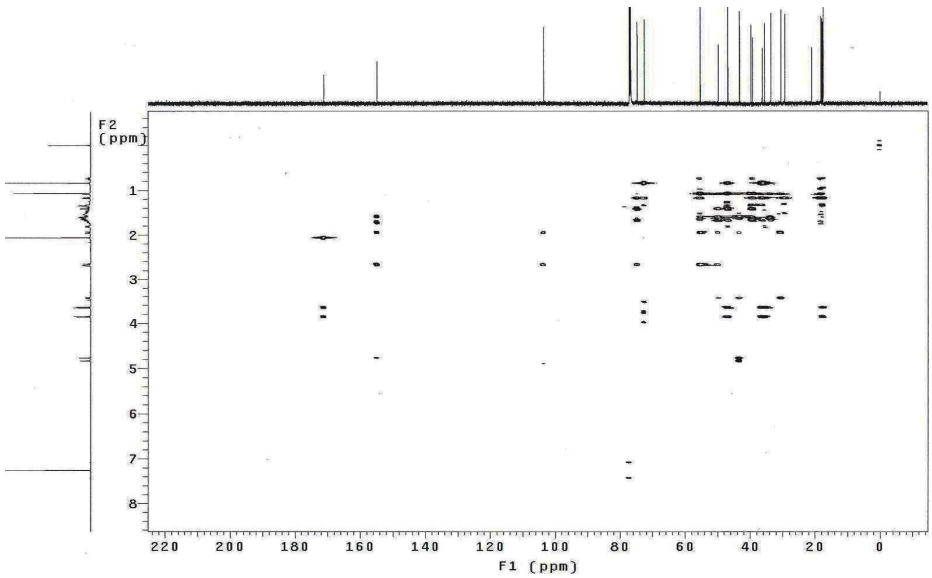


**Fig. 52.** HMBC spectrum of compound **6** (500 MHz, $CDCl_3$)

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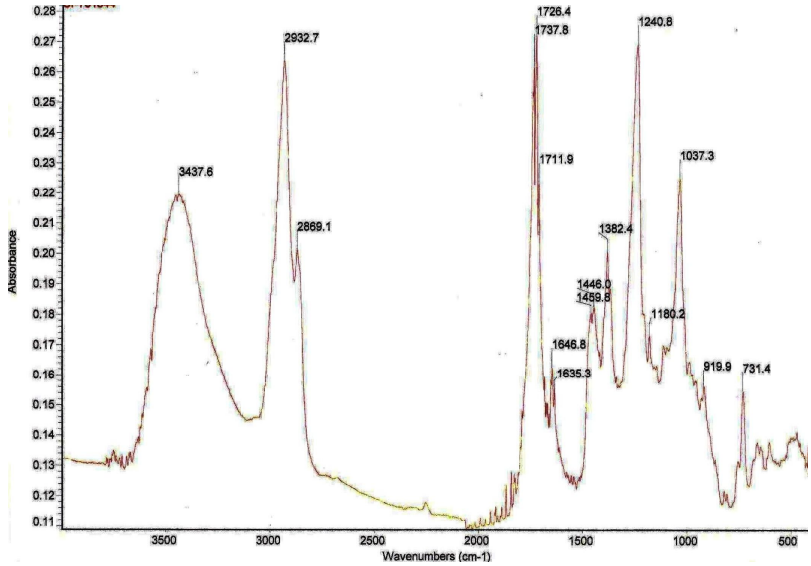


**Fig. 53.** IR spectrum of compound **6**

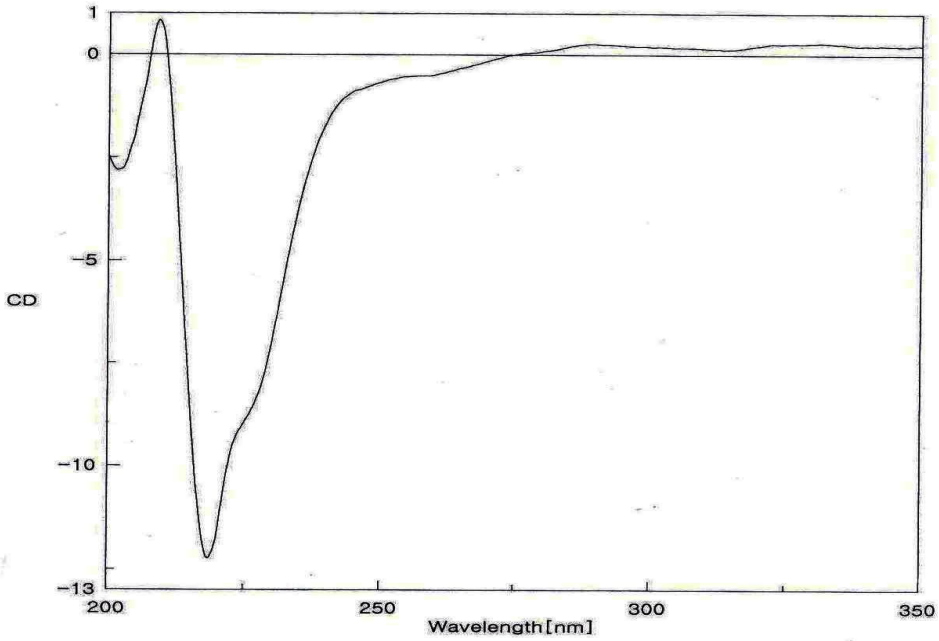


**Fig. 54.** CD spectrum of compound **6**

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**Table 7.** $^{1}H$ and $^{13}C$-NMR spectra data of compound **6** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | 0.75 ddd (13.5, 13.5, 3.5)<br>1.82 dt (13.5, 2.0) | 39.6 | C-20 |
| 2 | 1.45 d (11.0)<br>1.66[a] | 29.4 | C-1, C-3 |
| 3 | 3.45 dd (11.0, 4.5) | 74.8 | C-2, C-5, C-18 |
| 4 | | 39.2 | |
| 5 | 1.18 d (12.5) | 46.8 | C-3, C-4, C-6, C-9, C-10, C-18 |
| 6 | 1.47[a]<br>1.62[a] | 17.9 | C-5, C-8 |
| 7 | 1.17[a]<br>1.61[a] | 30.5 | C-8 |
| 8 | | 49.7 | |
| 9 | 1.09[a] | 55.3 | C-8, C-11, C-20 |
| 10 | | 36.3 | |
| 11 | 1.67[a]<br>1.70[a] | 17.7 | C-9, C-12, C-13 |
| 12 | 1.53[a]<br>1.73[a] | 33.6 | C-13, C-16 |
| 13 | 2.69 brs | 43.3 | C-16 |
| 14 | 1.34[a]<br>1.36[a] | 35.6 | |
| 15 | 1.95 d (16.0)<br>2.68 dt (17.0, 2.0) | 43.2 | C-7, C-8, C-9, C-16, C-17 |
| 16 | | 154.9 | |
| 17 | 4.78 s<br>4.85 s | 103.6 | C-13, C-15, C-16 |
| 18 | 3.66 d (11.0)<br>3.86 d (11.0) | 72.7 | C-3, C-4, C-5, C-19, C=O |
| 19 | 0.85 s | 17.5 | C-5, C-18 |
| 20 | 1.08 s | 18.2 | C-5, C-9, C-10 |
| 18-OAc | 2.07 s | 21.0<br>171.2 | C=O |

*a*: overlapping signals.

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### 3.1.7. Structure determination of compound 7

Compound **7** was isolated as a brownish amorphous powder, $[\alpha]^{25}_{D}$ -27° (*c* 0.1, MeOH). Its molecular formular was determined to be $C_{22}H_{32}O_4$ from a molecular ion peak at *m/z* 360.2309 $[M]^+$ (calcd for 360.2311) in the HREIMS spectrum. The IR spectrum indicated the presence of hydroxyl (3384 $cm^{-1}$) ester (1711 $cm^{-1}$) and conjugated ketone (1642 $cm^{-1}$) functional groups. The $^1H$ and $^{13}C$ NMR (**Table 8**) data of **7** suggested this compound also belonged to the *ent*-kaurane series, including of 22 carbons, which were assignable to three tertiary methyl groups [$\delta_H$ 0.84 (s), 0.91 (s), 1.07 (s); $\delta_C$ 21.5, 33.5, 18.0, respectively], a ketone ($\delta_C$ 208.4), conjugated with an exocyclic methylene [$\delta_H$ 5.25 (s), 5.89 (s); $\delta_C$ 113.4, 149.8], an acetyl group [$\delta_H$ 1.84 (s); $\delta_C$ 169.4, 21.4], six methylene groups, five methine groups, including two oxygenated ($\delta_H$ 4.10, $\delta_C$ 70.9 and $\delta_H$ 5.13, $\delta_C$ 67.9), and three quaternary carbons. These signals were found to be very similar with those of *ent*-11$\beta$,18-diacetoxy-7$\alpha$-hydroxykaur-16-en-15-one[47] with the exception of the lack of the acetoxyl signals at C-18 position. The location of the hydroxyl and acetoxyl groups at C-7 and C-11, respectively, were confirmed by HMBC correlations between proton H-7 and carbons C-6, C-8 and C-15, and between H-11 and carbons C-8, C-9, C-12, C13 and the ester carbonyl at $\delta_C$ 169.4 (**Fig. 59**). The $\alpha$-orientation of the hydroxyl group was deduced from the coupling pattern of H-7, which was a doublet-doublet ($\delta_H$ 4.10, dd, $J$ = 12.0, 4.0 Hz). The coupling pattern of H-7 is known to be dependent on the orientation of 7-OH, and H-7$\alpha$ appears as a doublet with a small coupling constant (d, $J$ = 3.4 Hz).[71] However, H-7$\beta$ shows a doublet-doublet pattern with a large coupling constant with C-6 methylene protons (dd, $J$ = 11.4, 4.4 Hz).[47] The similarity of the pattern of the H-11 proton signal ($\delta_H$ 5.12, d, $J$ = 4.5 Hz) with that in *ent*-11$\beta$,18-diacetoxy-7$\alpha$-hydroxykaur-16-en-15-one[47] suggested the $\alpha$-equatorial orientation of H-11 and thus the $\beta$-axial configuration of the C-11 acetoxyl group. These assignments were further confirmed by the NOE correlations

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between H-11 and H-20, and H-7 and H-5 and H-9 in the NOESY spectrum (**Fig. 60**). The *ent*-kaurane configuration of **7** was determined from the negative optical rotation. Consequently, compound **7**, named crotonkinensin G, was elucidated as *ent*-11$\beta$-acetoxy-7$\alpha$-hydroxykaur-16-en-15-one.

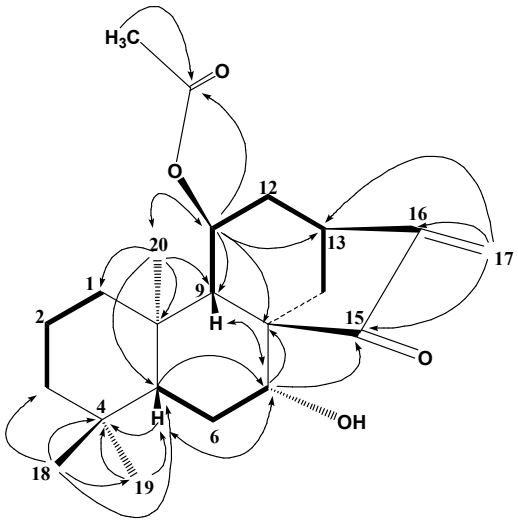


**Fig. 55.** Key HMBC (H→C), COSY ($^1$H—$^1$H) and NOSEY (↔) correlations of compound **7**

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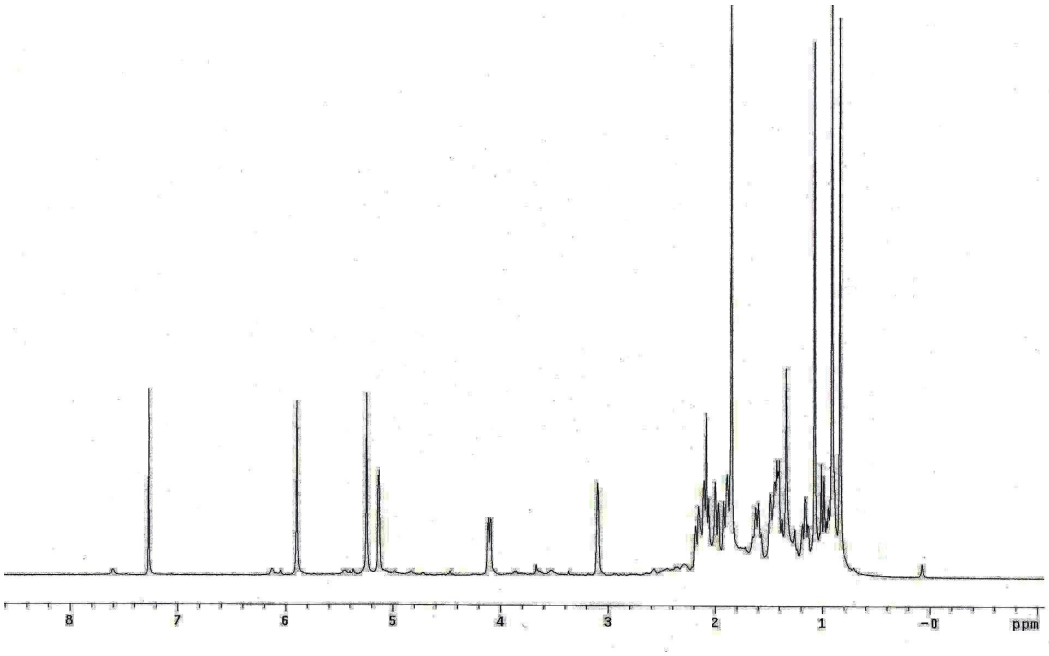


**Fig. 56.** $^{1}$H-NMR spectrum of compound **7** (500 MHz, $CDCl_3$)

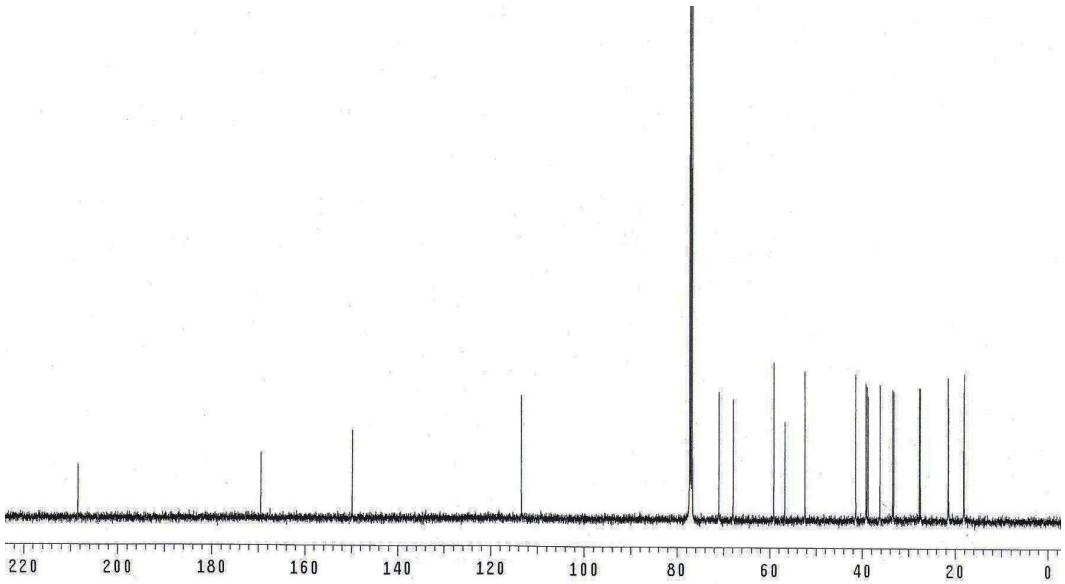


**Fig. 57.** $^{13}$C-NMR spectrum of compound **7** (125 MHz, $CDCl_3$)

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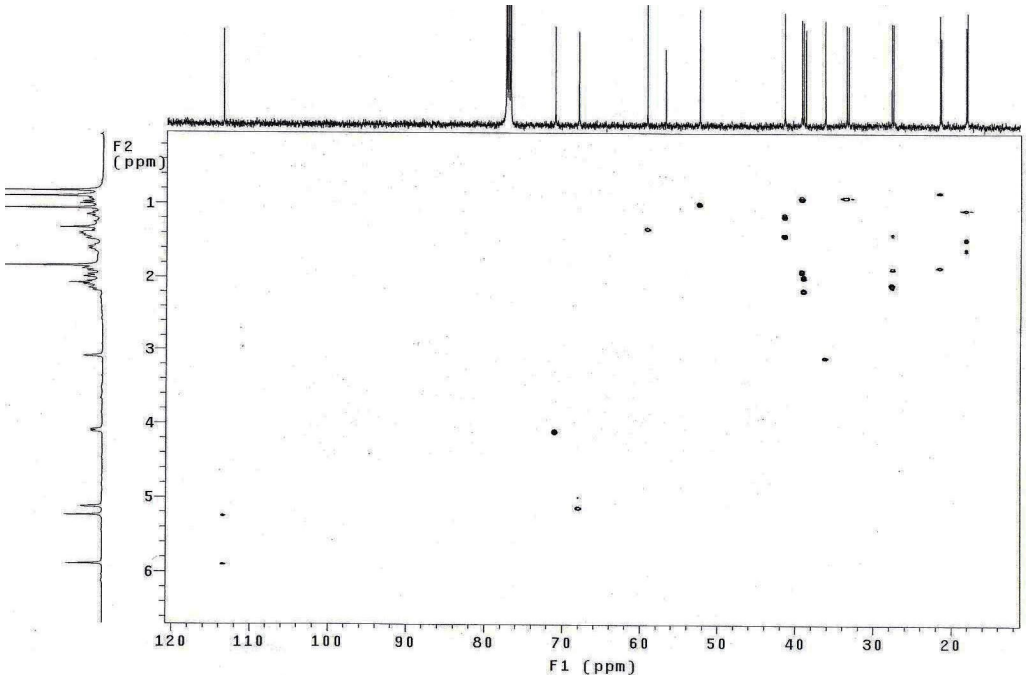


**Fig. 58.** HMQC spectrum of compound **7** (500 MHz, $CDCl_3$)

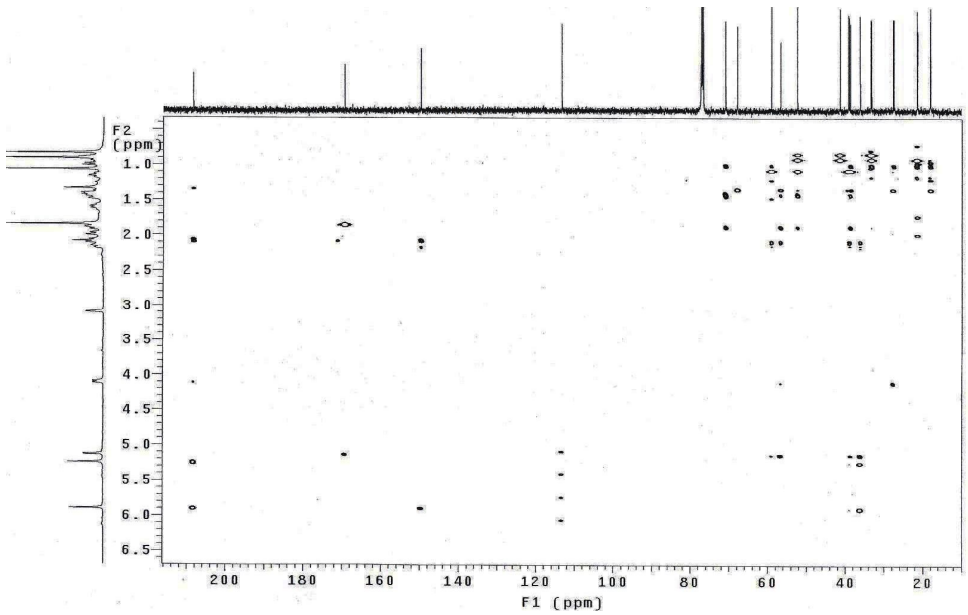


**Fig. 59.** HMBC spectrum of compound **7** (500 MHz, $CDCl_3$)

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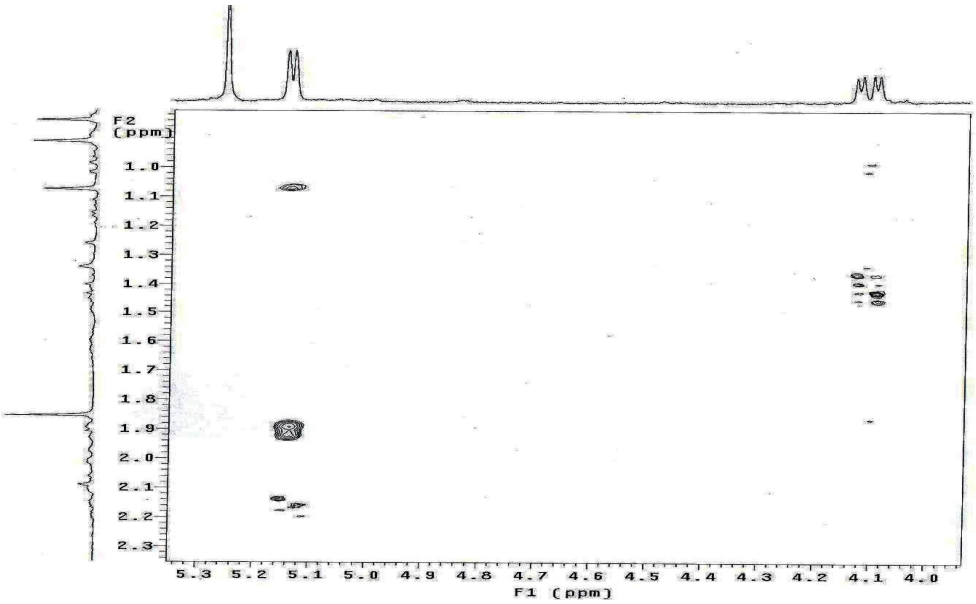


**Fig. 60.** NOESY spectrum of compound **7** (500 MHz, $CDCl_3$)

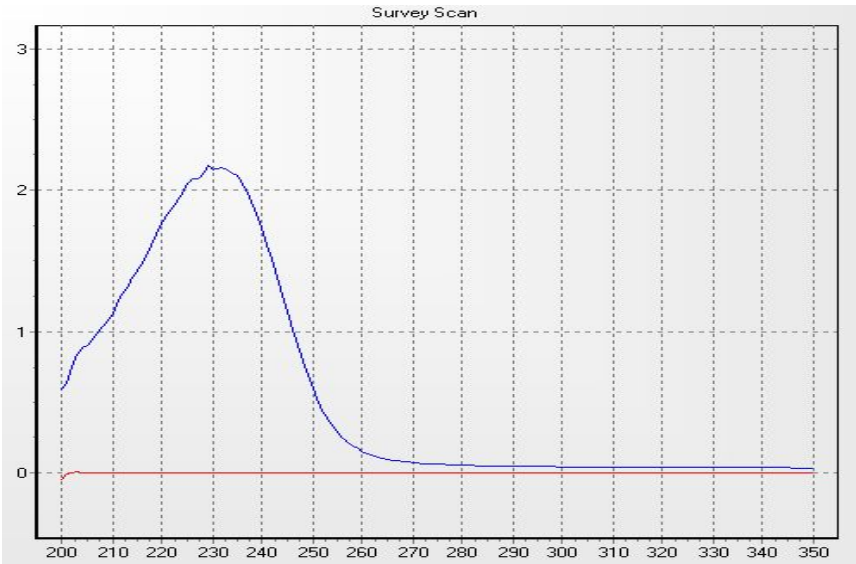


**Fig. 61.** UV spectrum of compound **7**

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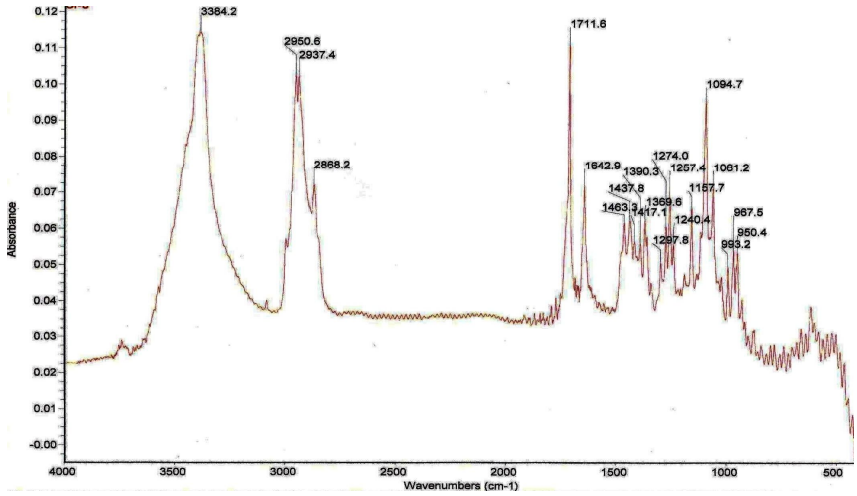


**Fig. 62.** IR spectrum of compound **7**

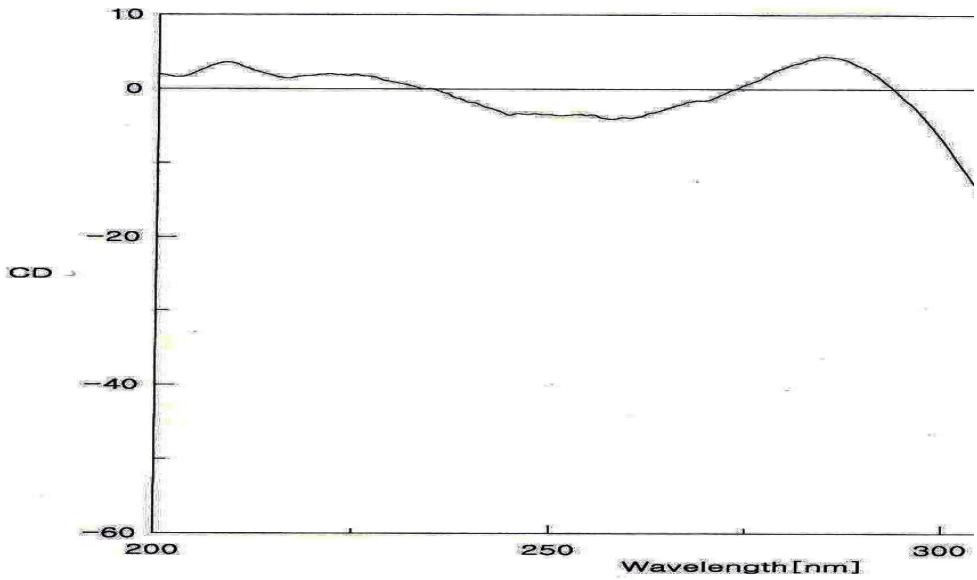


**Fig. 63.** CD spectrum of compound **7**

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**Table 8.** $^{1}H$ and $^{13}C$-NMR spectra data of compound **7** ($CDCl_3$)

| Position | $\delta_H$ mult. (*J* in Hz) | $\delta_C$ | HMBC (H→C) |
|---|---|---|---|
| 1 | 0.91[a]<br>1.88[a] | 39.2 | C-2 |
| 2 | 1.43[a]<br>1.62[a] | 18.1 | |
| 3 | 1.15[a]<br>1.41[a] | 41.4 | C-2, C-4, C-19 |
| 4 | | 33.2 | |
| 5 | 1.00 d (12.5) | 52.4 | C-4, C-6, C-7, C-10, C-19 |
| 6 | 1.42[a]<br>1.90[a] | 27.5 | C-5, C-8 |
| 7 | 4.10 dd (12.0, 4.0) | 70.9 | C-6, C-8, C-15 |
| 8 | | 56.7 | |
| 9 | 1.33[a] | 59.1 | C-7, C-11, C-14, C-15, C-20 |
| 10 | | 38.7 | |
| 11 | 5.12 d (4.5) | 67.9 | C-8, C-9, C-12, C-13, 11-C=O |
| 12 | 1.98[a]<br>2.17 ddd (14.5, 4.0, 3.0) | 39.0 | |
| 13 | 3.09 brs | 36.2 | |
| 14 | 1.84[a]<br>2.10[a] | 27.7 | C-8, C-9, C-13, C-15, C-16 |
| 15 | | 208.4 | |
| 16 | | 149.8 | |
| 17 | 5.25 s<br>5.89 s | 113.4 | C-13, C-15, C-16 |
| 18 | 0.91 s | 33.5 | C-3, C-4, C-5, C-19 |
| 19 | 0.84 s | 21.5 | C-3, C-4, C-5 |
| 20 | 1.07 s | 18.0 | C-1, C-5, C-9, C-10 |
| 11-OAc | 1.84 s | 21.4 | 11-C=O |
| | | 169.4 | |

*a*: overlapping signals.

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### 3.2. Results of SIRT1 inhibitory activity

#### 3.2.1. Effects of the compounds on cell toxicity

MCF-7, MCF-7/TAMR and MCF-7/adr cells were treated by all of the compounds and adriamycin at various concentrations for 24 hours. As shown as in **Table 9**, $IC_{50}$ of these compounds were less than 12 μM. Among them, **15**, **16** and **21** strongly potentiated cytotoxicity in the breast cancer cells.

#### 3.2.2. Effects of the compounds on SIRT1 enzymatic activity

The effects on SIRT1 enzyme of isolated compounds were tested in vitro according to a reported method.[58] The results were shown in **Table 9**. The known SIRT1 inhibitor, nicotinamide ($IC_{50}$ = 52.73 ± 2.14 μM), was used as a negative control in this assay.[44] Compounds **1** – **21** except for **1** – **13** strongly inhibited SIRT1 enzyme in a dose-dependent manner (see **Table 9**). Among the tested compounds, **15**, **16**, **18** – **21** with $IC_{50}$ values ranging from 16.08 ± 0.11 to 27.7 ± 2.13 μM showed double the activity of the negative control, nicotinamide. The *ent*-kaurane-type-diterpenoids (**7**, **11** – **21**) with a ketone group at C-15 and a double bond at C-16 positions exhibited a highly SIRT1 inhibitory activity than the others, compared with the weak or no activity *ent*-kaurane-type-diterpenoids with the absence of ketone group at C-15 (**3** – **6**, **8**, **10**) or double bond at C-16 (**9**) positions, or grayane-type-diterpenoids (**1**, **2**). The presence of a hydroxy group at C-7 position in compounds **15** – **17**, **19** – **21**, exhibited a significantly higher SIRT1 inhibitory property than the others. Remarkably, the most active compound **21** ($IC_{50}$ = 16.08 ± 0.11 μM) with only one more acetoxy group at C-18 showed a higher activity than compound **16** ($IC_{50}$ = 27.36 ± 1.05 μM). In contrast, compound **7** with the acetoxy group attached at C-11 compared to compound **16** and **21** did not showed any activity towards the SIRT1 enzyme ($IC_{50}$ > 60 μM). Another activity relationship on the SIRT1 enzyme was determined for compound **15** and compounds **11**, **12**, **20**, indicating

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that the addition of a hydroxyl group to C-18 might be responsible for the decrease or loss of their activity.

**Table 9.** Cytotoxic and SIRT1 enzyme Inhibitory Activity of Compounds **1–21**

| Compound | Cytotoxic activity $IC_{50}$ (μM)[a] | | | SIRT1 inhibitory activity $IC_{50}$ (μM)[a] |
|---|---|---|---|---|
| | MCF-7 | MCF-7/adr | MCF-7/TAM | |
| **1** | 7.9 ± 0.08 | 8.1 ± 0.09 | 7.9 ± 0.10 | > 60 |
| **2** | 10.8 ± 0.15 | 11.2 ± 0.13 | 13.8 ± 0.14 | > 60 |
| **3** | 8.1 ± 0.06 | 8.6 ± 0.12 | 8.4 ± 0.07 | > 60 |
| **4** | 10.5 ± 0.12 | 12.5 ± 0.16 | 11.3 ± 0.17 | > 60 |
| **5** | 9.4 ± 0.09 | 9.4 ± 0.10 | 9.4 ± 0.11 | > 60 |
| **6** | 8.4 ± 0.13 | 9.7 ± 0.04 | 8.9 ± 0.11 | > 60 |
| **7** | 9.6 ± 0.06 | 10.6 ± 0.10 | 11 ± 0.08 | > 60 |
| **8** | 11.2 ± 0.27 | 12.2 ± 0.25 | 12.4 ± 0.32 | > 60 |
| **9** | 5.7 ± 0.10 | 6.2 ± 0.15 | 5.6 ± 0.13 | > 60 |
| **10** | 7.7 ± 0.11 | 8.2 ± 0.08 | 7.8 ± 0.05 | > 60 |
| **11** | 10.2 ± 0.17 | 11.8 ± 0.15 | 11.4 ± 0.10 | > 60 |
| **12** | 8.5 ± 0.13 | 8.8 ± 0.19 | 8.6 ± 0.05 | > 60 |
| **13** | 9.1 ± 0.08 | 8.7 ± 0.13 | 9.4 ± 0.10 | > 60 |
| **14** | 5.9 ± 0.09 | 5.5 ± 0.10 | 5.5 ± 0.04 | 35.43 ± 1.43 |
| **15** | 2.8 ± 0.08 | 3.1 ± 0.07 | 2.6 ± 0.05 | 20.43 ± 2.63 |
| **16** | 3.1 ± 0.09 | 3.5 ± 0.10 | 3.3 ± 0.07 | 27.36 ± 1.05 |
| **17** | 4.5 ± 0.07 | 4.7 ± 0.18 | 4.9 ± 0.09 | 44.34 ± 2.32 |
| **18** | 4.7 ± 0.05 | 4.9 ± 0.09 | 4.8 ± 0.08 | 24.61 ± 2.27 |
| **19** | 4.8 ± 0.06 | 4.8 ± 0.10 | 4.2 ± 0.06 | 24.87 ± 1.23 |
| **20** | 8.2 ± 0.16 | 8.4 ± 0.06 | 7.8 ± 0.12 | 27.7 ± 2.13 |
| **21** | 3.4 ± 0.14 | 3.4 ± 0.05 | 3.3 ± 0.10 | 16.08 ± 0.11 |
| **Nicotinamide**[b] | | | | 52.73 ± 2.14 |

*a* IC50 values were determined by regression analyses and are expressed as the mean ± SD of three replicates. *b* Negative control.

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SIRT1 inhibitory effects of these compounds in cells were further confirmed by the results of the Luciferase assay and Western blot analysis (see **Fig. 64**, **65**).

### 3.2.3. Effects of the compounds on SIRT1 activity

P53-induced luciferase activity from p53-luc was reduced by SIRT1. To further address the effect of the compounds on SIRT1 activity, MCF-7/TAMR cells were transfected with reporter plasmids containing p53-Luc or SIRT1 and pRL-SV plasmid. Cells were then treated with 1 μM croton compounds and 5 μM nicotinamide (negative control). **Fig. 64**, **65** also depicts that compounds **14** – **21** inhibited SIRT1 activity.

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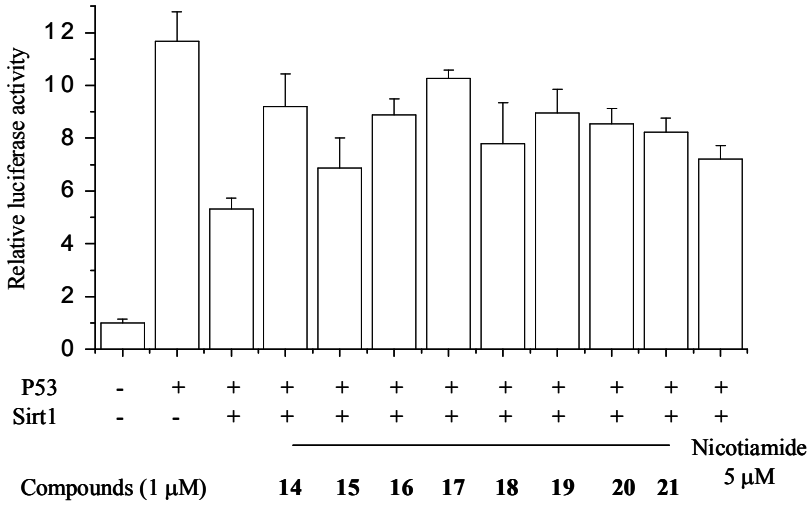


**Fig. 64.** Effects of compounds **14** – **21** on activity of SIRT1 in MCF-7/TAMR cells. Cells were transiently transfected with p53-Luc or SIRT1 plasmids. Following transfection, cells were treated with 1 μM compounds and 5 μM nicotinamide for 24 hours prior to lysis and measurement of luciferase activity.

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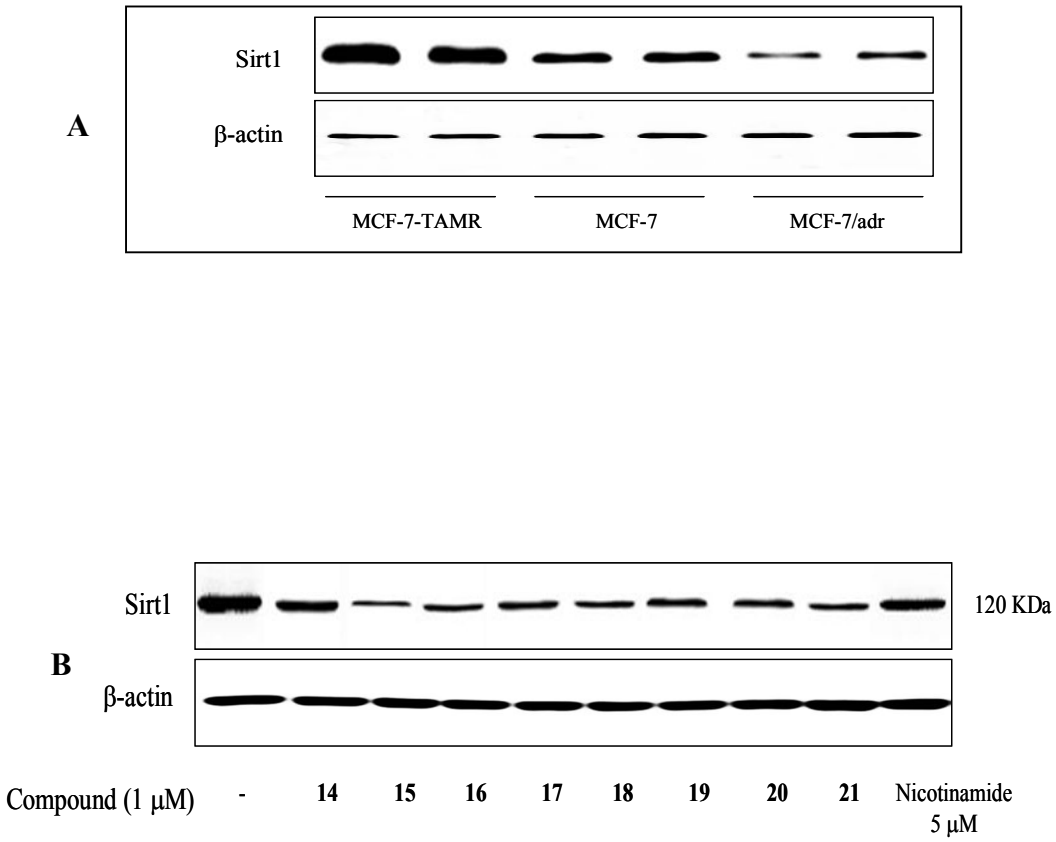


**Fig. 65.** (**A**) SIRT1 protein expression in MCF-7/TAMR, MCF-7 and MCF-7/adr cells cells. (**B**) Effects of compounds **14** – **21** on SIRT1 protein expression in MCF-7/TAMR cells. The cells were treated with the compounds at 1 μM and 5 μM nicotinamide for 24 hours. Lysates of cells were electrophoresed and the expression of SIRT1 was detected with a SIRT1-specific antibody. The *β*-actin band is shown to confirm integrity and equal loading of protein.

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### 3.2.4. Decreased expression of SIRT1 protein in MCF-7/TAMR cells by the compounds

To examine the expression level of SIRT1 protein in the breast cancer cells, MCF-7, MCF-7/TAMR and MCF-7/adr cells, **Fig. 65** detected that SIRT1 protein level in MCF-7/TAMR is higher than that in MCF-7 and MCF-7/adr cells by western blot analysis. To further confirm the inhibition of SIRT1 by compounds **14** – **21**, MCF-7/TAMR, cells were treated with compounds **14** – **21** at 1 μM and 5 μM nicotinamide. **Fig. 65** shown that SIRT1 protein levels were decreased by these compounds. Taken together, these data suggest that compounds **14** – **21** suppressed SIRT1 in MCF-7/TAMR cells.

## 3.3. Discussions

Previous studies have revealed that the *Croton* plants contain diversity of phytochemicals, mainly alkaloids, flavonoids, terpenoids, and volatile oils.[45] Diterpenes in *Croton* plants mostly belong to labdane- and clerodane-type, and kaurane-type diterpenes.[45] Many diterpenes have been isolated from the Vietnamese plant *C. tonkinensis* so far and it is interesting that all of them were identified as kaurane and *ent*-kaurane diterpenoids.[47-50,54,55] In this study, we isolated and identified seven new diterpenoids, including two novel grayanane-type diterpenoids from *C. tonkinensis*. This is the first report on the presence of grayanane-type diterpenoids from a species of the *Croton* genus as well as the Euphorbiaceae family. The grayanane-type diterpenoids have been rarely found in natural source, mainly from the Ericaceae, and are generally occurred as 3□,5□,6□,16□-oxygenated grayanane structure.[59-63] However, it is interesting that grayanane-type diterpenes **1** and **2** have a 7□,10□-epoxy-14□-hydroxy-16-en-15-one moiety, the common structure of *ent*-kaurant type diterpenes in *C. tonkinensis*.[47-50,54,55] In this study, we also isolated some major *ent*-kaurane diterpenes that were previously reported from this plant (see **Fig. 4**). Therefore, it seems that the grayanane-type and

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*ent*-kaurane-type diterpenoids are related to each other and compounds **1** and **2** may be biosynthesized from *ent*-kaurane diterpenes.[72] This observation may well exemplify a hypothesis that the grayanane diterpenes are biosynthesized and from precursors *ent*-kaurene diterpenoids.[73,74]

When testing all the isolated compounds **1** – **21** for SIRT1 inhibitory activity *in vitro* assays, the result showed these diterpenoids possessing a significantly potential activity. Prominently, from the fraction F7.5.3, compound **15** *ent*-18-acetoxy-7$\alpha$-hydroxykaur-16-en-15-one, was crystallized out from MeOH, was the most major compound of the terpenoid fraction of this plant, also revealed a high inhibitory activity on SIRT1 activity, SIRT1 enzymatic activity and SIRT1 protein expression.

Although the structure-activity relationships of *ent*-kaurane-type-diterpenoids were not investigated thoroughly, these results suggest that the double bond between C-16 and C-17, and ketone group attached at C-15 might be very important for the *in vitro* SIRT1 inhibitory activity. And the presence of a hydroxyl group at C-7 and/or an acetoxyl group at C-18 may increase the activity, while the substitution of an acetoxyl or a hydroxyl group at C-11, or a hydroxyl group at C-18 may decrease this activity.

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# 4. Conclusions

It has been postulated that inhibitors of SIRT1 might be beneficial in the treatment of cancer and neurodegenerative diseases.

However, to date, only a few inhibitors of SIRT1 have been reported. With the exception of nicotinamide, which is released during the reaction with the deacetylase and which serves as an internal agent, almost all of the inhibitors are synthetically derived and were identified in library screening studies. So, more inhibitors are still needed to improve the understanding of SIRT1 biological functions and to discover its possible therapeutic indication. Since plants are a promising source for the development of new SIRT1 inhibitors, we have undertaken a screen of hundreds of plant extracts against SIRT1

In the course of *in vitro* SIRT1 inhibitory screening on various extracts from medicinal plants, a methanol-soluble extract of the leaves of *Croton tonkinensis* Gagnep was found to inhitbit SIRT1 activity at a concentration of 30 μg/mL. The MeOH solution was concentrated, suspended in $H_2O$, and sequentially partitioned with $CHCl_3$. The $CHCl_3$-soluble fraction exhibited moderate activity with inhibition value of 60 % on SIRT1 at final concentration 30 μg/mL.

Bioassay-guided fractionation of the MeOH extract of the leaves of *Croton tonkinensis* Gagnep has resulted in the isolation of seven new diterpenoids (**1** – **7**) along with and fourteen known diterpenoids (**8-21**) using chromatography methods (silica gel, C-18, and preparative HPLC). Their structures were elucidated on the basic of spectral (including 1D, 2D-NMR, UV, IR, and MS) and physicochemical analyses. The inhibitory effect test compounds on SIRT1 enzyme were also evaluated on SIRT1 protein expression, SIRT1 activity and SIRT1 enzymatic activity.

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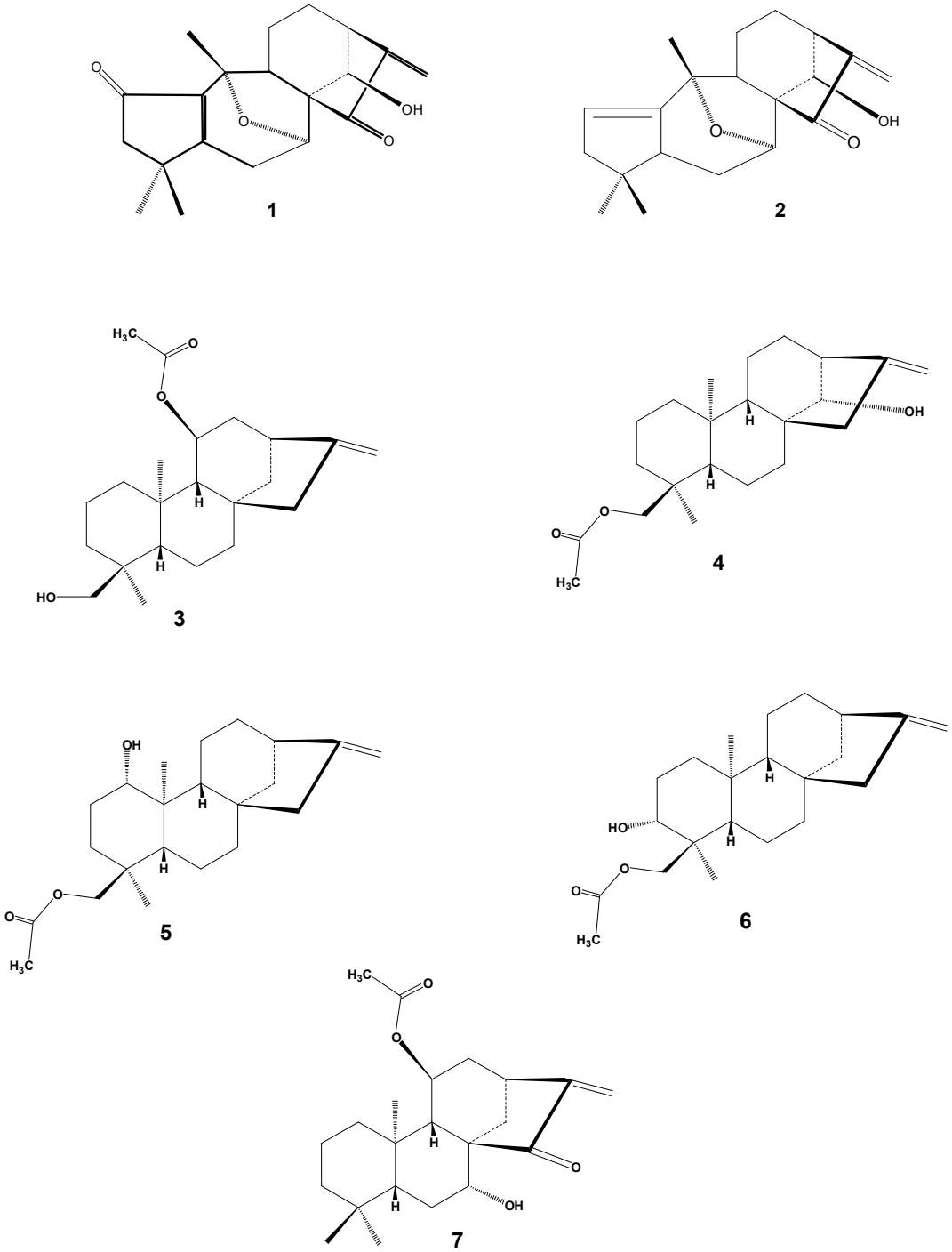
**Fig. 66.** Chemical structures of new compounds from the leaves of *C. tonkinensis*



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Compounds **1** and **2** were new grayanane-type diterpenoids, named crotokinensin A (**1**) and crotokinensin B (**2**). Compounds **3** – **7** were new *ent*-kaurane-type diterpenoids, named crotokinensin C (**3**), crotokinensin D (**4**), crotokinensin E (**5**), crotokinensin F (**6**) and crotokinensin G (**7**).

Crotokinensin A (**1**) and crotokinensin B (**2**) were two novel grayanane-type diterpenoids from *C. tonkinensis*. This is the first report on the presence of grayanane-type diterpenoids from a species of the *Croton* genus as well as the Euphorbiaceae family.

Although structure-activity relationships of *ent*-kaurane-type diterpenoids were not thoroughly investigated, my results suggest that the double bond between C-16 and C-17, and ketone group attached at C-15 might be very important for the *in vitro* SIRT1 inhibitory activity. And the presence of a hydroxyl group at C-7 and/or an acetoxyl group at C-18 may increase the activity, while the substitution of an acetoxyl or a hydroxyl group at C-11, or a hydroxyl group at C-18 may decrease this activity. In addition, SIRT1 inhibitory effects by members of this class have not been previously reported.

Remarkably, among the isolates, compound **15** *ent*-18-acetoxy-7$\alpha$-hydroxykaur-16-en-15-one, the most major diterpenoid in this plant (crystallized out from MeOH), also exhibited a significantly inhibitory activity against SIRT1.

Finally, *ent*-kaur-16-en-15-one diterpenoids can be considered as promising class of SIRT1 inhibitors. Therefore, further investigation and optimization on chemical as well as confirmation of SIRT1 inhibitory effects of these compounds in cell and evaluation of their potential *in vivo* efficacy in a cancer mice model, might enable the discovery of new SIRT1 inhibitors that are potentially useful in the treatment of cancer and neurodegenerative diseases.

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Korea, May, 2009

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# 저작물 이용 허락서

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| 논문제목 | 한글: 베트남 약용식물 *Croton tonkinensis* Gagnep 로부터 Sirt1 억제물인 디터페노이드<br>영문: Sirt1 Inhibitory Diterpenoids from Vietnamese Medicinal Plant *Croton tonkinensis* Gagnep | | | | |

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함.
다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

2009 년 8월 27일

동의 여부: 동의 (0) 조건부 동의( ) 반대 ( )

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