Biologically Active Substance from Kenyan Plant, *Vernonia hindii* S. Moore (Asteraceae)

Glaston Mwangi Kenji[a], Shuhei Nakajima, Naomichi Baba
and Junkichi Iwasa
(Departmemt of Bioresources Chemistry)

A novel stigmastane-type steroid glucoside has been isolated from the aerial parts of *Vernonia hindii*. The structure was elucidated by spectroscopic methods. The compound exhibited lettuce seedling growth inhibitory activity.

Key words : biologically active substance, Kenyan plant, *Vernonia hindii*, steroid glucoside, lettuce seedling growth inhibitor

Introduction

The tribe Vernonieae is dominated by a single genus *Vernonia* that alone has 800 to 1000 species growing in Brazil and tropical Africa[1]. Many *Vernonia* species have had wide use medicinally in native cultures to cure a variety of diseases. In East Africa, the leaves of *Vernonia amygdalina* are used as a folk medicine to cure fever[2], and are eaten after macerating with water for helping digestion[3]. A possible use of this plant by chimpanzees as a medicine has recently been reported[4]. Jisaka *et al.* isolated some steroid glycosides including vernonioside A_4 and the steroids are suspected to be of medicinal value to chimpanzees[4].

In the course of our investigations of Kenyan plants for biologically active substances, *Vernonia hindii* S. Moore (Asteraceae) exhibited lettuce growth inhibitory activity. We have earlier tentatively reported the isolation of a steroid glucoside (**1**)[5]. This report describes the detailed chemical structure study of compound **1** and its lettuce seedling growth inhibitory activity.

Materials and Methods

General

Melting points were measured with Yanagimoto melting point apparatus and are uncorrected. Infrared spectra were measured on Hitachi EP-G3 spectrometer and Nicolet FT-IR 710 GC. Gas chromatograms were taken on Hitachi G3000 (column, F-WCOT OV-1, 0.25 mm×5 m). Mass spectra were recorded with a JEOL JMS D-300. ^{1}H and ^{13}C NMR were measured with Varian VXR 500 instrument.

Extraction and isolation

The aerial parts of the plant material (2.8 kg) were collected at Jomo Kenyatta University of Agriculture and Technology farm. They were identified by Seki et al[6] and were extracted with methanol over two weeks at room temperature. The extracts were concentrated and successively extracted with hexane (500 ml×3) and ethyl acetate (500 ml×3). The ethyl acetate layer on concentration gave a grayish powder (4.5 g). The powder (500 mg) was chromatographed on an activated alumina column (90 g, Nakalai Chemicals ; 35 mm i.d.). Elution was with mixtures of methanol : water : acetic acid (60 : 40 : 1,200 ml ; fraction A, 60 mg). Fraction A was further chromatographed on reverse phase column (ODS

Received October 1, 1997
a) *Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya*

-Q3, Fuji gel, 170 mm×35 mm i.d.), eluting with 70 % methanol. This yielded the fraction A-2 (10 mg) that was rich in active compound **1**. When the fraction A-2 was subjected to preparative TLC (silica gel 60 F_{254}, Merck) developing with chloroform : methanol (8 : 1) six times, it yielded 4 mg of **1**. Compound **1** ; mp 226-228 °C ; FABMS (glycerin) m/z : 621 (MH^+) ; IR ν_{max} (KBr) cm^{-1} ; 3400 (broad), 2850, 1710, 1450, 1390, 1080, 1035. ^1H NMR (500 MHz, CD_3OD) : δ 0.93 (3H, s), 1.10 (3H, d, J=6.8 Hz), 1.15 (6H, d, J=6.7 Hz), 1.17 (3H, s), 1.38 (3H, d, J=5.7 Hz), 1.5-1.9(m), 2.00 (1H, dd, J=12.6, 12.4 Hz), 2.52 (1H, dd, J=12.8, 12.7 Hz), 2.60 (1H, dd, J =11.6, 11.0 Hz), 2.80 (1H, ddd, J= 13.5, 8.1, 7.1 Hz), 3.18 (1H, q, J=5.7 Hz), 3.68 (1H, dd, J=10.4, 4.8 Hz), 3.77 (1H, m), 3.90 (1H, d, J= 11.0 Hz), 3.95 (1H, d, J=10.4 Hz), 4.42 (1H, ddd, J=8.2, 7.2, 4.6 Hz) 4.42 (1H, d, J=7.8 Hz). ^{13}C NMR (126 MHz, CD_3OD) : δ 12.0, 13.5, 18.5, 19.1, 20.4, 22.5, 28.3, 30.1, 31.2; 33.7, 35.3, 37.0, 37.2, 37.3, 40.1, 40.6, 43.5, 47.0, 48.1, 48.2, 48.3, 50.6, 56.8, 58.0, 62.1, 62.8, 69.5, 71.7, 73.1, 75.1, 77.9, 78.1, 78.6, 102.3, 214.0

Acid hydrolysis of **1**

Compound **1** (2 mg) was dissolved in 0.5 N HCl (0.4 ml) and held at 85 °C for 2 hr. The aqueous layer was made neutral with 0.1 N NaOH. The neutralized solution was extracted with benzene (2 ml×3). It was further extracted with chloroform (2 ml×3). The aqueous layer was subjected to preparative TLC (silica gel 60 F_{254}, methanol : chloroform, 1 : 1) This gave approximately 1 mg of sugar.

Methanolysis and acetylation of the sugar component of **1**

The sugar fraction (1 mg) from acid hydrolysis was treated with 5 % hydrogen chloride methanol solution (Wako Pure Chemical Industries) and kept at 90 °C for 11 hr. The solvent was evaporated to give the methyl glucoside. This methylated sugar was dissolved in a mixture of pyridine (0.5 ml) and acetic anhydride (0.5 ml) and maintained at 30 °C overnight. The reaction mixture was taken in methanol and freed from pyridine by evaporation *in vacuo*. Purification of the reaction mixture was done by preparative TLC (silica gel 60 F_{254}, methanol : chloroform, 1 : 10) to give a tetraacetate (1 mg). Tetraacetate ; ^1H NMR (500 MHz, $CDCl_3$) : δ 3.68 (1H, m), 4.10 (1H, dd, J=5.0, 10.5 Hz), 4.22 (1H, dd, J=5.1, 10.4 Hz), 4.40 (1H, d, J=7.8 Hz), 4.96 (1H, dd, J=7.8, 8.2Hz), 5.07 (1H, t, J=7.8 Hz), 5.18 (1H, t, J=7.8 Hz).

Enzymatic hydrolysis of **1**

The powder (4 mg) of **1** was suspended in water (5 ml) and β-glucosidase (20 mg, from almonds, Sigma) was added. The mixture was kept at 32 °C for 20 hr with stirring. The aglycone was extracted with ethyl acetate (5 ml×3). The extract was purified by preparative TLC (silica gel 60 F_{254}) to give 2.0 mg of the aglycone of **1**. Aglycone of compound **1** ; FABMS (glycerin) m/z : 459 (MH^+) ; EIMS m/z : 458 (M^+), 440, 414, 287 ; IR ν_{max} (KBr) cm^{-1} ; 3350, 1710 ; ^1H NMR (500 MHz, $CDCl_3$) : δ 0.84 (3H, s), 1.01 (3H, d, J= 6.8 Hz), 1.04 (3H, d, J=6.8 Hz), 1.07 (3H, s), 1.08 (3H, d, J=6.8 Hz), 1.15-1.35 (m) 1.32 (3H, d, J=5.7 Hz), 1.40-1.90 (m), 1.95 (1H, m), 2.01 (1H, dd, J= 12.6, 3.0 Hz), 2.35 (1H, dd, J=12.8, 12.7 Hz), 2.40 (1H, dd, J=11.6, 11.0 Hz), 2.79 (1H, ddd, J =13.5, 8.1, 7.1 Hz), 3.14 (1H, q, J =5.7 Hz), 3.60 (1H, m), 3.80 (1H, d, J=11.0 Hz), 4.42 (1H, ddd, J=8.2, 7.2, 4.6 Hz).

Lettuce seedling growth bioassay

The bioassay was performed as described by Kato *et al.*[7] with some modifications.

Results and Discussion

Compound **1** was isolated as a white-gray amorphous solid, m.p. 226-228 °C. FABMS gave MH^+ ion at m/z 621 corresponding to the molecular formula $C_{35}H_{56}O_9$ which was established on the basis of elemental analysis (Found : C, 67.74 %, H, 8.96 %. Calcd. for $C_{35}H_{56}O_9$: C, 67.71 %, H, 9.03 %). The IR spectrum of compound **1** showed the presence of carbonyl and hydroxyl group(s)

(1710 and a broad band at 3400 cm^{-1}, respectively). The ^{13}C NMR spectrum of **1** revealed 35 carbons and confirmed the presence of a ketone group with a signal at δ 214. ^1H NMR of **1** showed a signal at δ 4.42 (1 H, d, J = 7.8 Hz). This suggested that the compound was a β-glucopyranoside. The presence of glucopyranoside was further supported by ^{13}C NMR signals at δ 102.3, 78.6, 78.1, 75.1, 71.7 and 62.8. The formation of a tetraacetate from **1** on acetylation confirmed the presence of four hydroxyl groups. The resultant sugar after hydrolysis was conclusively identified as glucose by GLC experiments. ^1H NMR spectrum of the methylated tetraacetate of the sugar gave signals corresponding to α-and β-methyl D-glucopyranosyl tetraacetate because of mutarotation. FABMS of an aglycone of **1** showed MH^+ ion at m/z 459 and EIMS showed $M^{\cdot+}$ ion at m/z 458 with major fragmentation ions at m/z 440 ($M^{\cdot+}$ - H_2O), 414 ($M^{\cdot+}$ - $OCHCH_3$) and 287 ($M^{\cdot+}$ - H_2O-side chain). ^1H-^1H COSY spectrum of the aglycone indicated the presence of an isopropyl group. Two methyl doublets at δ 1.08 (J = 6.8 Hz) and 1.04 (J = 6.8 Hz) coupled to a proton at δ 1.60. This was further supported by nuclear Overhauser effect (nOe) experiments. Two other methyl singlets were observed at δ 1.07 and 0.84 while two other methyl doublets were observed at δ 1.01 (J = 6.8 Hz) and 1.32 (J = 5.7 Hz). The latter methyl protons coupled to a proton quartet at δ 3.14 (J = 5.7 Hz) and two ^{13}C NMR signals due to two species of carbon carrying oxygen δ 57.0 (-CH-O-) and 62.1 (-O-C-) suggested the presence of an epoxide ring. This ^1H NMR pattern together with the mass fragmentation was in close agreement with the pattern observed for steroids[8~10]. Since ^1H NMR and ^{13}C NMR for compound **1** and its aglycone are comparable to those of vernonioside A_4 and other stigmastanoids, we concluded that the compound **1** was a stigmastane-type steroid glucoside. Therefore, we could assign the isopropyl group at C-25, C-26 and C-27. The signals at δ 3.14 and 1.32 could also be assigned to the protons at C-28 and C-29 respectively. ^1H-^1H COSY experiments revealed that the methyl doublet observed at δ 1.01(H-21) was coupled to the proton at δ 1.95 (m, H-20), which was coupled to the proton at δ 1.05 (H-17). The latter proton was in turn coupled to the proton at δ 4.42 (H-16) which was coupled to the signals at δ 2.83 (βH-15) and 1.20 (αH-15). This confirmed the connectivity of C21-C20-C17-C16-C15. The rest of the protons were assigned as follows. The proton at δ 1.30 (H-14) was found to be coupled to the protons at δ 2.83 (βH-15) , 1.20 (αH-15) and 2.40 (H-8). The downfield shift of the proton at C-8 suggested the presence of a ketone at C-7. Two signals at δ 2.35 and 2.01 ($\alpha\beta$, H-6) were found to be coupled to the proton at δ 1.49 (H-5) which was coupled to the proton at δ 1.58 (H-4) and the signal at δ 3.58 (H-3). The position of the sugar moiety was deduced by comparing the ^1H NMR signals of the parent compound, its acetate and the aglycone of compound **1**. The signal at δ 3.77 in the parent compound was shifted upfield to δ 3.58 in the aglycone. All the other signals due to the aglycone were unchanged. In the acetate of **1** this signal remained at δ 3.77. This suggested **1** did not contain any free hydroxyl group except on a sugar moiety that could be acetylated. The hydroxyl group at C-16 was therefore replaced by an ether. The signal at δ 3.79 (dd, J = 11.0, 1.0 Hz) was found to be coupled to the proton at δ 1.55 (H-22) which was coupled to the signal at δ 1.95 (H-20). This allowed the assignment of the signal at δ 3.79 to the proton at C-23. The stereochemistry at C-5, C-16 and C-20 was assigned on the basis of the coupling constants and nOe experiments. The irradiation at δ 1.07 (H-19) did not have any effect on the proton at C-5 as would be expected for a β-H. The small coupling constant (J = 3.0 Hz) for the proton at δ 2.01 (H-6) is due to the coupling with the α-H at C-5. On the other hand, the irradiation at δ 0.84 (H-18) enhanced the signals due to the protons at C-16 and C-20 and

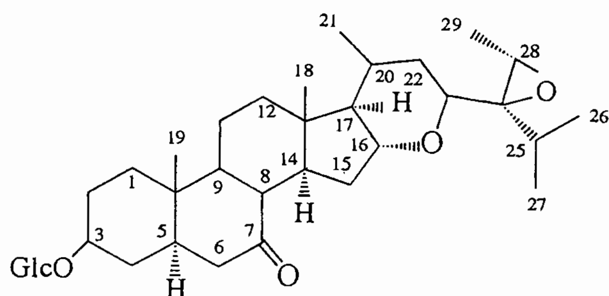

Fig. 1 Structure of compound 1.

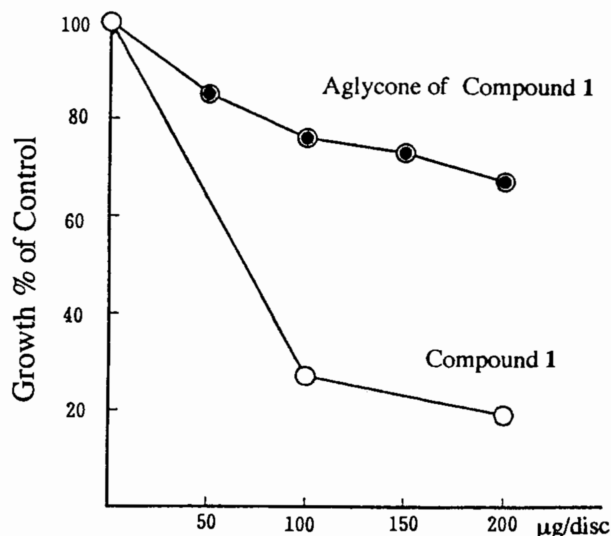

Fig. 2 The lettuce growth bioassay results for compound 1 and its aglycone.

did not have any effect on the signals at C-14 and C-17. This would result when both of the protons at C-16 and C-20 were on the same plane with the methyl group at C-18. Assuming the methyl to be β (as would be expected from biosynthesis) then the two protons at C-16 and C-20 would be β and the other protons at C-14 and C-17 would be α.

All these data are in close agreement with the steroidal structure of compound **1** (Fig. 1) and the same structure could not be found by literature survey.

Lettuce growth inhibitory activity

The lettuce growth bioassay on the isolated compound indicated that it had growth inhibitory effect. The total growth length was reduced to 24 % at a concentration of 100 μg/disc. The aglycone reduced the total growth to 76 % at the same concentration suggesting that the presence of the glucose moiety may be necessary for its activity (Fig. 2).

Acknowledgment

This work was supported in part by a grant for the specific research "Functions of organisms in special environments and their application to substance production" at Okayama University in 1994-1996.

References

1) Harbone, J. B. and C. A. Williams : Vernonieae : Chemical review. in The Biology and Chemistry of the Compositae 1 (Heywood, V. H. *et al.* eds.), pp. 523-537 Academic Press, London (1977)

2) Kokwaro, J. O. : Medicinal Plants of East Africa. pp. 72, East African Literature Bureau, Nairobi (1976)

3) Arene, E. O. : Compositae : 7, 24 (28)-stigmastadien-3β-ol from *Vernonia amygdalina*. Phytochemistry, **11**, 2886-2887 (1972)

4) Jisaka, M., H. Ohigashi, K. Takegawa, M. Hirota, R. Irie, M. A. Huffman and K. Koshimizu : Steroid glucosides from *Vernonia amygdalina*, a possible Chimpanzee medicinal plant. Phytochemistry, **34**, 409-413 (1993)

5) Kenji, G. M., S. Nakajima, N. Baba and J. Iwasa : A novel plant growth inhibitor from *Vernonia hindii* S. Moore (Asteraceae). Chemitry Express, **6**, 233-236 (1991)

6) Seki, T. and W. Ngumi : A flora of plants at Jomo Kenyatta College of Agriculture and Technology, Central Province, Kenya, Hikobia, **9**, 509 (1986)

7) Kato, T. and N. Takahashi : Dormancy Substances : Studies on germination and growth inhibitors in rice husks. in Advances in Natural Products Chemistry (Natori S. et al eds.), pp. 195-209, Kodansha, Tokyo (1981)

8) Tokes, L., G. Jones and C. Djerassi : Mass spectrometry in structural and stereochemical problems. CLXI. Elucidation of the course of the characteristic ring D fragmentation of steroids. J. Am. Chem. Soc., **9**, 5465-5477 (1968)

9) Schnider, H. J., U. Bchheit, N. Becker, G. Schmidt and S. Ultrich : ^{1}H-NMR analysis, shielding mechanisms, coupling constants, and conformations in steroids bearing halogen, hydroxy, oxo groups and double

bonds. J. Am. Chem. Soc., **107**, 7027-7039 (1985)

10) Misra, T. N., R. S. Singh, J. Upadhyay and R. Srivastava : Isolation of natural sterol and aliphatic acid from *Vernonia cinerea*. Phytochemistry, **23**, 415-417 (1984)

ケニヤ産キク科薬用植物 *Vernonia hindii* に含まれる生物活性物質

グラストン ムワンギ ケンジ・中島 修平・馬場 直道・岩佐 順吉
(生物資源開発学講座)

熱帯・砂漠など特殊な環境に自生する植物には，生物活性2次代謝物質を含むものが多く，古くから民間伝承薬や天然殺虫剤として使用されてきた。しかし，まだ十分に調査・研究されていない植物も残されている．本研究では，ケニヤに自生するキク科薬用植物の *Vernonia hindii* に含まれる活性物質の単離と構造解明を行い，その生物活性を調べた．植物生長制御試験を指標として *V. hindii* のメタノール抽出物を分画・精製した結果，新規の化学構造を持つ植物生長抑制物質を単離した。さらに種々の分析機器を用いて化学構造を解析し，stigmastan 型のステロイド配糖体であると決定した。この化合物は100μg/disc でコントロールと比べ，24%までレタスの生長を抑制した．