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Norisoprenoids and Hepatoprotective Flavone Glycosides from the Aerial Parts of *Beta vulgaris* var. *cicla*

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(+)-Dehydrovomifoliol (**1**), 3-hydroxy-5α,6α-epoxy-β-ionone (**2**), vitexin 7-*O*-β-D-glucopyranoside (**3**), and vitexin 2"-*O*-β-D-glucopyranoside (**4**) were isolated as new constituents from the aerial parts of *Beta vulgaris* var. *cicla*. Compounds **3** and **4** demonstrated hepatoprotective activity with values of 65.8 and 56.1%, respectively, in primary cultured rat hepatocytes with CCl_4-induced cell toxicity, compared to controls. This was comparable to that of silibinin (69.8 %) which was used as a positive control.

Key words: *Beta vulgaris* var. *cicla*, Hepatoprotective activity, Flavonoid, (+)-Dehydrovomifoliol, 3-Hydroxy-5α,6α-epoxy-β-ionone, Vitexin 7-*O*-β-D-glucopyranoside, Vitexin 2"-*O*-β-D-glucopyranoside

INTRODUCTION

The esculent plant, *Beta vulgaris* L. var. *cicla* L. (Chenopodiaceae), is an annual or biannual cultivar. The dark green leaves are not only eaten, but also used in Korea as an anti-inflammatory and haemastatic herb (Kim *et al.*, 2003). In the course of a preliminary screening test for *in vitro* hepatoprotective activity of vegetables cultivated in Korea, the *n*-BuOH soluble fraction of this plant markedly blocked the release of GPT from CCl_4-injured hepatocytes at a concentration of 50 μg/mL. These results led to the separation of the biologically active materials from this plant, which contains several flavonoids and phenolic compounds (Kim *et al.*, 2003; Gil *et al.*, 1998). This paper describes the isolation and structure elucidation of norisoprenoids and flavonoids as well as *in vitro* hepatoprotective activity of flavonoids.

MATERIALS AND METHODS

General experimental procedures

Optical rotation was measured with a JASCO DIP-1000 digital polarimeter (Tokyo, Japan). CD spectra were recorded on a JASCO J-715 spectrometer. ESI-MS spectra were obtained on an Agilent 1100 series LC/MSD. UV and IR spectra were recorded on a Shimadzu UV-2101 and JASCO FT/IR-300E, respectively. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker spectrometer at 400 MHz and 100 MHz, respectively, with tetramethylsilane as an internal standard. Column chromatography was performed using a Sephadex LH-20 (Pharmacia) and Kiesegel 60 (Art. 7734; Merck, Darmstadt, Germany). HPLC was performed on a column of YMC (J'sphere ODS-H80, S-4 μm, 250 × 10 mm i.d., Japan). TLC was conducted on pre-coated Kiesegel 60 F_{254} plates (Art. 5715; Merck, Darmstadt, Germany). Spots on the TLC were detected under UV light (CN-6, Vilber Lourmat, France).

Plant materials

Aerial parts of *B. vulgaris* var. *cicla* were collected from the Hanaro Mart of the Agricultural Cooperatives Federation in Seoul, Korea and identified by one of the authors. A voucher specimen (SNUPH-0030) was deposited at Seoul National University.

Extraction and Isolation

MeOH (10 L) was used to make an extraction from dried whole herb (5.5 kg) and the MeOH extract was concentrated *in vacuo* into a residue (603 g). This residue was suspended with water and then subsequently partitioned with *n*-hexane, CH_2Cl_2 and *n*-BuOH, successively. The

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CH_2Cl_2 soluble fraction (17.2 g) was fractionated into nine fractions, using silica gel column chromatography (*n*-hexane-EtOAc = 10:1→1:1, 400 mL each). The second fraction (1.5 g) was subjected to reversed-phase C_{18} column chromatography which produced six sub-fractions. The first sub-fraction was purified using HPLC (AeCN-H_2O = 23:77, 2 mL/min) to yield (+)-dehydrovomifoliol (**1**) (3.8 mg) and 3-hydroxy-5α,6α-epoxy-β-ionone (**2**) (4.3 mg). The *n*-BuOH soluble fraction (63.5 g) was subjected to silica gel column chromatography ($CHCl_3$-MeOH = 5:1 →0:1, 1000 mL each) providing nine fractions. The seventh fraction (8.7 g) was further applied to silica gel column chromatography ($CHCl_3$-MeOH-H_2O = 5:5:1) resulting in seven sub-fractions. Purification of the fourth sub-fraction was achieved using a Sephadex LH-20 (MeOH) followed by HPLC (AeCN-H_2O = 17:83, 2 mL/min), which finally afforded vitexin 7-*O*-β-D-glucopyranoside (**3**) (81.1 mg) and vitexin 2"-*O*-β-D-glucopyranoside (**4**) (5.3 mg).

(+)-Dehydrovomifoliol (1)

$C_{13}H_{18}O_3$, a light yellow oil, $[\alpha]_D^{20}$ +134.4° (*c* = 0.11, MeOH); UV λ_{max} (MeOH) nm (log ε) 237 (4.15); CD (*c*, 0.07 mg/mL, MeOH): $[\theta]_{209.5}$ -284853, $[\theta]_{225.0}$ 0, $[\theta]_{244.0}$ +335800, $[\theta]_{282.5}$ 0, $[\theta]_{323.0}$ -20745; ESI-MS (negative mode) *m/z* 221 $[M-H]^-$, IR ν_{max} (KBr) 3441 (OH), 1667 (C=O), 1127 (C-O), 987 (C=C-H) cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.99 (1H, d, *J* = 15.8 Hz, H-7), 6.42 (1H, d, *J* = 15.8 Hz, H-8), 5.93 (1H, s, H-4), 2.60 (1H, d, *J* = 17 Hz, H-2b), 2.30 (3H, s, H-10), 2.27 (1H, d, *J* = 17 Hz, H-2a), 1.89 (3H, s, H-13), 1.05 (3H, s, H-12), 1.01 (3H, s, H-11); ^{13}C-NMR (100 MHz, CD_3OD) δ: 201.5 (C-3), 201.2 (C-9), 165.5 (C-5), 149.1 (C-7), 132.5 (C-8), 128.8 (C-4), 80.8 (C-6), 51.3 (C-2), 43.5 (C-1), 28.4 (C-10), 25.5 (C-12), 24.3 (C-11), 20.0 (C-13).

3-Hydroxy-5α,6α-epoxy-β-ionone (2)

$C_{13}H_{20}O_3$, a light yellow oil, $[\alpha]_D^{20}$ -55.1° (*c* = 0.01, MeOH); UV λ_{max} (MeOH) nm (log ε) 230 (3.27); CD (*c*, 0.06 mg/mL, MeOH) $[\theta]_{225.0}$ 0, $[\theta]_{232.5}$ -3974, $[\theta]_{268.5}$ 0, ESI-MS (positive mode) *m/z* 247 $[M+Na]^+$; IR ν_{max} (KBr) 3396 (OH), 1675 (C=O), 1627 (C=C), 1180 (C-O), 987 (C=C-H) cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.16 (1H, d, *J* = 15.8 Hz, H-7), 6.17 (1H, d, *J* = 15.8 Hz, H-8), 3.75 (1H, m, H-3), 2.32 (1H, dd, *J* = 14.1, 1.7 Hz, H-4), 1.64 (1H, dd, *J* = 14.1, 9.0 Hz, H-4), 1.57 (1H, dd, *J* = 12.7, 1.7 Hz, H-2), 1.24 (1H, dd, *J* = 12.7, 10.7 Hz H-2), 2.28 (3H, s, H-10), 1.18 (3H, s, H-13), 1.17 (3H, s, H-12), 0.95 (3H, s, H-11); ^{13}C-NMR (100 MHz, CD_3OD) δ: 201.0 (C-9), 146.2 (C-7), 134.6 (C-8), 71.6 (C-3), 69.6 (C-6), 65.2 (C-5), 48.4 (C-2), 42.1 (C-4), 35.9 (C-1), 30.6 (C-11), 28.2 (C-10), 25.9 (C-12), 20.8 (C-13).

Vitexin 7-*O*-β-D-glucopyranoside (3)

$C_{27}H_{30}O_{15}$, a yellow powder, $[\alpha]_D^{20}$ -70.6° (*c* = 0.29, MeOH); UV λ_{max} (MeOH) nm (log ε) 268 (4.16), 332 (4.21); ESI-MS (negative mode) *m/z* 593 $[M-H]^-$; IR ν_{max} (KBr) 3367 (OH), 2921, 1654 (C=O), 1605, 1363, 1247, 1180 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.23 (1H, s, 5-OH), 8.04 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.88 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.85 (1H, s, H-3), 6.60 (1H, s, H-6), 4.94 (1H, d, *J* = 7.1 Hz, H-1'''), 4.87 (1H, d, *J* = 10.0 Hz, H-1''). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 182.3 (C-4), 164.6 (C-2), 162.0 (C-4'), 161.1 (C-7), 160.8 (C-5), 154.9 (C-9), 129.2 (C-2', 6'), 120.9 (C-1'), 116.0 (C-3', 5'), 107.2 (C-8), 105.7 (C-10), 102.4 (C-3), 101.2 (C-1'''), 98.5 (C-6), 81.9 (C-5''), 78.5 (C-3''), 77.1 (C-5'''), 75.9 (C-3'''), 73.4 (C-1''), 73.3 (C-2'''), 71.6 (C-2''), 70.7 (C-4''), 69.4 (C-4'''), 61.3 (C-6''), 60.6 (C-6''').

Vitexin 2"-*O*-β-D-glucopyranoside (4)

$C_{27}H_{30}O_{15}$, a yellow powder, $[\alpha]_D^{20}$ -27.2° (*c* = 0.20, MeOH); UV λ_{max} (MeOH) nm (log ε) 272 (4.04), 332 (4.06); ESI-MS (negative mode) *m/z* 593 $[M-H]^-$; IR ν_{max} (KBr) 3342 (OH), 1654 (C=O), 1576 (C=C), 1511, 1364, 1285, 1178 (C-O), 1079, 1026 (C=C-H), 840 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 13.11 (1H, s, 5-OH), 7.97 (2H, d, *J* = 8.7 Hz, H-2', 6'), 6.88 (2H, d, *J* = 8.7 Hz, H-3', 5'), 6.66 (1H, s, H-3), 6.13 (1H, s, H-6) 4.83 (1H, d, *J* = 9.8 Hz, H-1'') , 3.94 (1H, d, *J* = 7.8 Hz, H-1'''); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 182.0 (C-4), 163.7 (C-2), 162.6 (C-7), 161.5 (C-4'), 160.9 (C-5), 156.7 (C-9), 129.1 (C-2',C-6'), 122.2 (C-1'), 116.2 (C-3', C-5'), 105.3 (C-8), 104.2 (C-1'''), 103.8 (C-10), 102.8 (C-3), 100.0 (C-6) 82.1 (C-5'') 81.6 (C-2''), 78.6 (C-3''), 76.7 (C-3'''), 76.4 (C-5'''), 74.7 (C-2'''), 71.9 (C-1''), 70.6 (C-4'') , 69.8 (C-4''') , 61.4 (C-6'') , 60.8 (C-6''').

***In vitro* hepatoprotective activity**

Isolated rat hepatocytes from male Wistar rats were prepared by the collagenase perfusion technique of Berry and Friend with minor modifications. The cell suspension was diluted to 5×10^5 cells/mL in the culture medium consisting of Waymouth's MB 75211 medium supplemented with 10% BSA (fraction V), 10^{-6} M dexamethasone, 10^{-7} M insulin, 53.2 mM L-serine, 40.9 mM L-alanine, 26.7 mM $NaHCO_3$, 100 IU/mL penicillin and 100 mg/mL streptomycin. Primary cultured rat hepatocytes were plated onto collagen-precoated culture dishes and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After the cultured cells were exposed to a medium containing 6.5 mM CCl_4/water for 1.3 h, one-day incubation was used to induce hepatotoxicity (Chin *et al.*, 2003). Glutamic pyruvic transaminase (GPT) was released into the culture medium and its activity was determined by the kits (Yeongdong Pharmaceutical Co., Korea) using the Reitman and Frankel method (Reitman and Frankel *et al.*, 1957).

RESULTS AND DISCUSSION

The methanolic extract of the aerial parts of this plant

(5.5 kg) was suspended in H_2O and successively partitioned with *n*-hexane, CH_2Cl_2 and *n*-BuOH. Repeated column chromatographic analyses of the CH_2Cl_2 and *n*-BuOH soluble fractions led to the identification of two norisoprenoids **1-2** and two flavonoids **3-4**, respectively.

Compound **1** was obtained as a light yellow oil and its UV spectrum was maximal at 237 nm. The molecular formula of $C_{13}H_{18}O_3$ was derived from the pseudomolecular ion at *m/z* 221 $[M-H]^-$ by the ESI-MS. The ^{1}H-NMR spectrum of **1** revealed signals assignable to a vinyl proton at δ 5.93 (1H, s, H-4), two *trans* olefinic protons at δ 6.42 (1H, d, *J* = 15.8, Hz, H-8) and 6.99 (1H, d, *J* = 15.8 Hz, H-7), in addition to two *gem*-dimethyl protons at δ 1.01 (H-11) and 1.05 (H-12), a methyl proton at δ 1.89 (H-13) connected to a double bond, and a methyl group at δ 2.30 adjacent to a carbonyl group. Geminally coupled signals at δ 2.27 (d, *J* = 17.0 Hz, H-3) and 2.60 (d, *J* = 17.0 Hz, H-3) suggested that the carbonyl group was connected to this methylene and that *gem*-dimethyl was substituted to C-1. The ^{13}C-NMR spectrum of **1** exhibited two carbonyl signals at δ 201.2 (C-9) and 201.5 (C-3), four olefinic carbon signals at δ 128.8 (C-4), 132.5 (C-8), 149.1 (C-7), and 165.5 (C-5), and an oxygenated carbon signal at δ 80.8 (C-6). Thus, this compound was predicted to be a 3-oxo-6-hydroxy-ionone (Kato *et al.*, 1977). The absolute configuration of C-6 proved to be *S* by the positive value ($[\theta]_{244.0}$ +335800) in the CD spectrum as described in the literature (Ito *et al.*, 2001). Based on this data, **1** was established to be (+)-dehydrovomifoliol (Kisiel *et al.*, 2004; Achenbach *et al.*, 1995).

The quasimolecular ion of compound **2** was observed at *m/z* 247 $[M+Na]^+$, with the molecular formula of $C_{13}H_{20}O_3$. The ^{1}H-NMR spectrum of **2** lacked a vinyl signal in comparison with **1**. Instead, there was one more methylene group at δ 1.64 and 2.32 and a carbinol proton at δ 3.75. The splitting patterns of two methylene groups showed that there was a proton (H-3) at δ 3.75 between them, causing different couplings to the vicinal protons. In the ^{13}C-NMR spectrum, three oxygenated carbon signals were observed at δ 65.2 (C-5) and 69.6 (C-6) due to an epoxy moiety, and at δ 71.6 (C-3) due to a hydroxy group. This spectral data suggested that **2** was a homologue of 3-hydroxy-5,6-epoxy-ionone. The absolute configuration of C-6 was determined as *R* by the negative cotton effect ($[\theta]_{233.5}$ -3974) in the CD spectrum. Based on the above data, the structure of **2** was confirmed as 3-hydroxy-5α, 6α-epoxy-β-ionone, in good agreement with the literature (Miyase *et al.*, 1987).

Compound **3**, a yellow powder, exhibited a pseudo-molecular ion peak at *m/z* 593 $[M-H]^-$ corresponding to the molecular formula, $C_{27}H_{30}O_{15}$ in the ESI-MS. The maxima of the UV absorption bands at 268 and 332 nm suggested **3** to have a flavonoid moiety and the IR spectrum revealed the presence of a carbonyl group conjugated (1654 cm^{-1}) with double bonds. The ^{1}H-NMR spectrum of **3**, by observation of signals at δ 6.60 (1H, s, H-6), 6.85 (1H, s, H-3), 6.88 (2H, d, *J* = 8.7 Hz, H-3', 5'), and 8.04 (2H, d, *J* = 8.7 Hz, H-2', 6'), showed characteristics of an apigenin moiety missing a peak of H-8. Two anomeric protons appeared at δ 4.87 (1H, d, *J* = 10.0 Hz, H-1") and 4.94 (1H, d, *J* = 7.1 Hz, H-1'"), which implied an anomeric proton of a *C*-glycoside and an anomeric proton of an *O*-glycoside with a β-linkage, respectively, in the ^{1}H-NMR spectrum (Markham and Geiger, 1993). By means of ^{13}C-NMR and HMQC, the sugars attached to **3** were proved to be glucopyranosides, when compared to the reference data (Agrawal and Bansal, 1987). The long-range correlations between the signals at δ 4.94 (H-1'") and δ 161.1 (C-7) as well as δ 4.87 (H-1") and δ 107.2 (C-8) in the HMBC spectrum confirmed that two glucopyranosides were *O*-linked at C-7 and *C*-linked at C-8 of **3**, respectively. Therefore, the structure of **3** was identified as vitexin 7-*O*-β-D-glucopyranoside (Chopin *et al.*, 1984).

The spectral data of **4** were almost identical to those of **3**, except that the chemical shift for C-2" (δ 81.6) was shifted downfield by 10 ppm compared with that of **3**, which suggested that a glucose was attached to C-2". Thus, compound **4** was elucidated as vitexin 2"-*O*-β-D-glucopyranoside (Agrawal and Bansal, 1987).

These isolates, (+)-dehydrovomifoliol (**1**), 3-hydroxy-5α, 6α-epoxy-α-ionone (**2**), vitexin 7-*O*-β-D-glucopyranoside (**3**), and vitexin 2"-*O*-β-D-glucopyranoside (**4**) were isolated from this plant for the first time.

The hepatoprotective activities of compounds **3** and **4**

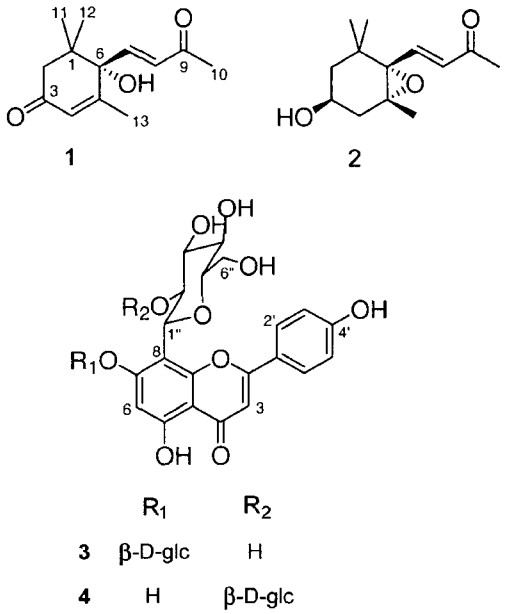

Fig. 1. Structures of compounds **1-4**.

Table 1. Effects of compounds **3** and **4** on CCl_4-induced toxicity in primary cultures of rat hepatocytes

Compound	GPT (IU/L) relative protection[a](%)	
	50 μM	100 μM
Control	11.5 ± 0.4 (100)*[b]	11.5 ± 0.4 (100)*[b]
CCl_4-treated	82.4 ± 0.6 (0.00)*	82.4 ± 0.6 (0.00)*
3	64.5 ± 0.6 (21.8)*	28.8 ± 0.5 (65.8)*
4	n.d[d]	36.2 ± 0.2 (56.1)*
Silibinin[c]	23.8 ± 1.0 (69.8)*	-

[a]Primary cultures of rat hepatocytes were exposed to 6.5 mM CCl_4 with or without each compound.

[b]The values of parenthesis is a relative percent. The % of protection is calculated as 100 × (values of CCl_4 - value of sample)/(value of CCl_4 - value of control).

[c]Positive control.

[d]Negative value.

*Significant difference from positive control at $P<0.01$. The each value represents the mean ± SD (n=3).

were assessed by measuring their effects on the release of glutamic pyruvic transaminase (GPT) from the primary cultures of rat hepatocytes injured by CCl_4. The compounds **3** and **4** exhibited hepatoprotective activities with values of 65.8 and 56.1%, respectively, at a concentration of 100 μM, comparable to that of silibinin which was used as a positive control (69.8% at 50 μM) (Table I). In addition, according to previous literature, the flavone *C*-glycoside, vitexin, is known to possess an inhibitory activity on TNF-α induced cell death in primary cultured mouse hepatocytes (Banskota *et al.*, 2000). Therefore, the flavone glycosides **3** and **4** are considered to be the hepatoprotective principles in this plant.

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REFERENCES

Achenbach, H., Lottes, M., Waibel, R., Karikas, G. A., Correa, M. D., and Gupta, M. P., Alkaloids and other compounds from *Psychotria correae*. *Phytochemistry*, 38, 1537-1545 (1995).

Agrawal, P. K. and Bansal, M. C., Flavonoid glycosides: In Agrawal P. K. (Eds). Carbon-13 NMR of Flavonoids. Elsevier, Amsterdam, pp. 283-364, (1989).

Banskota, A. H., Tezuka, Y., Adnyana, I. K., Xiong, Q., Hase, K., Tran, K. Q., Tanaka, K., Saiki, I., and Kadota, S., Hepatoprotective effect of *Combretum quadrangulare* and its constituents. *Biol. Pharm. Bull.*, 23, 456-460 (2000).

Chin, Y.-W., Lim, S. W., Kim, S.-H., Sin, D.-Y., Sur, Y.-G., Kim, Y. B., Kim, Y. C., and Kim, J., Hepatoprotective pyrrole derivatives of *Lycium chinense* fruits. *Bioorg. Med. Chem. Lett.*, 13, 79-81 (2003).

Chopin, J., Dellamonica, G., Markham, K. R., Ramachandran Nair, A. G., and Gunasegaran, R., 2"-*p*-Coumaroylvitexin 7-glucoside from *Mollugo oppositifolia*. *Phytochemistry*, 23, 2106-2108 (1984).

Ito, H., Kobayashi, E., Li, S.-H., Hatano, T., Sugita, D., Kubo, N., Shimura, S., Itoh, Y., and Yoshida, T., Megastigmane gycosides and an acylated triteterpenoid from *Eriobotrya japonica*. *J. Nat. Prod.*, 64, 737-747 (2001).

Gil, M. I., Ferreres, F., and Tomas-Barberan, F. A., Effect of modified atmosphere packaging on the flavonoids and vitamin C content of minimally processed Swiss chard (*Beta vulgaris* subspecies *cycla*). *J. Agri. Food. Chem.*, 46, 2007-2012 (1998).

Kato, T., Tsunakawa, M., Sasaki, N., and Aizawa, H., Growth and germination inhibitors in rice husks. *Phytochemistry*, 16, 45-48 (1977).

Kim, Y., Han, M. S., Lee, J. S., Kim, J., and Kim, Y. C., Inhibitory phenolic amides on lipopolysaccharide-induced nitric oxide production in RAW 264.7 cells from *Beta vulgaris* var. *cicla* seeds. *Phytother. Res.*, 17, 983-985 (2003).

Kisiel, W., Michalska, K., and Szneler, E., Norisoprenoids from aerial parts of *Cichorium pumilum*. *Biochem. Syst. Ecol.*, 32, 343-346 (2004).

Markham, K. R. and Geiger, H., "The Flavonoids," ed. by Harbone J.B., Chapman and Hall, London, 1993, pp. 441-497.

Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H., and Karasawa, H., Studies on the glycosides of *Epimedium grandiflorum* Morr. var. *thunbergianum* (Miq.) Nakai. *Chem. Pharm. Bull.*, 35, 1109-1117 (1987).

Reitman, S. and Frankel, S. A., A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.*, 28, 56-63 (1957).