Note

Structure of the Precursor of a Brown Compound Produced by Heating (–)-Epicatechin with L-Alanine

Toyomasa ANAN, Kanzo SAKATA,*
Akihito YAGI* and Hiromichi KATO**

Department of Tea Processing Technology,
National Research Institute of Vegetables,
Ornamental Plants and Tea,
Haibara, Shizuoka 428, Japan

*Department of Agricultural Chemistry,
Shizuoka University, Ohya, Shizuoka 422, Japan

**Department of Agricultural Chemistry,
The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

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Japanese green tea is made by applying steaming, rolling and drying processes, and a subsequent firing process at a temperature of 100°C or above, which produces the characteristic roast flavor. Catechins are major constituents of tea leaves and are susceptible to oxidation during the heating and storage of tea. It has been reported that the reaction of catechins with amino acids affords unknown brown products.^{1~3)} Brown products are also formed during tea processing and impair the quality of tea liquor. The reaction of catechins with amino acids must occur during the firing process and afford brown compounds, although the reaction mechanism has not yet been clarified. This paper describes the isolation and structural elucidation of the precursor of a brown compound produced by heating a dried mixture of (–)-epicatechin (EC) and L-alanine (Ala).

An aqueous solution of EC and Ala (1:1 molar ratio) was freeze-dried and heated at 140°C for 40 min, before the reaction mixture was dissolved in 50% acetonitrile. TLC of the reaction mixture on a silica gel plate [silica gel GF₂₅₄ (Merck), 0.25 mm; ethyl acetate–diisobutyl ketone–formic acid–water (15:9:1:1, v/v)] showed several colored spots. In addition, a pinkish brown spot appeared at *R_f* 0.4 after standing the plate in the air overnight. The pigment may have been formed by oxidation from a colorless product on the plate. When a methanol extract of the pinkish brown spot at *R_f* 0.4 was developed again by TLC under the same conditions, a pinkish brown spot, which was one of the several colored spots just described, was detected this time at *R_f* 0.9, with nothing at *R_f* 0.4. This spot (*R_f* 0.9) was, therefore, concluded to have originated from the colorless substance (**1**) with *R_f* 0.4, which was a precursor of the pinkish brown pigment. Compound **1** decomposed so easily that enough could not

1 : R = H

2 : R = –COCH₃

be isolated to elucidate by NMR spectrometry. So the reaction mixture of EC and Ala was directly acetylated, and the acetyl derivative of **1** (**2**) was isolated and subjected to structural elucidation by ¹H-, ¹³C-NMR and mass spectrometries. The NMR spectra were measured on a JEOL FX-90Q FT-NMR spectrometer using TMS as an internal standard. MS spectra were obtained with Hitachi M-80A and M-003 mass spectrometers. MS spectra were measured at 20 eV for EI- and CI-MS, and at 70 eV for HR-MS.

A mixture of EC and Ala (2 mmol each) in water (1 l) was freeze-dried and heated at 140°C for 40 min. The reaction mixture was dissolved in 150 ml of acetic anhydride–pyridine (1:1, v/v) and left at room temperature for 24 hr. After adding water (200 ml), the aqueous solution was extracted with ethyl acetate. The extract was evaporated to dryness and dissolved in 5 ml of hexane–ethyl acetate (1:5, v/v). The solution was applied to a column of silica gel (Kieselgel 60, 4 i.d. × 30 cm) equilibrated in hexane–ethyl acetate (1:5, v/v), and was eluted with the same solvent. The eluate gave three peaks when monitored by UV absorbance at 280 nm. It was proved by TLC [silica gel GF₂₅₄, 0.25 mm; benzene–methyl isobutyl ketone (1:1, v/v)] that **2** was contained in the second peak fraction. So the combined second peak fraction was concentrated and applied to preparative TLC [silica gel GF₂₅₄, 0.5 mm; benzene–methyl isobutyl ketone (1:1, v/v)]. The UV-absorbing band of **2** with *R_f* 0.5 was scraped off and extracted with acetone. The extract was concentrated and further purified by preparative HPLC [ODS-120A (Toyosoda), 7.2 i.d. × 250 mm; water–acetonitrile (65:35, v/v)]. The fraction containing **2** was combined, concentrated and freeze-dried to give chromatographically homogeneous **2** (18.8 mg) as an off-white, amorphous powder: IR $\nu_{\max}$ (CHCl₃) cm^{–1}: 1760, 1735, 1640, 1580, 1360, 1100, 1045; UV $\lambda_{\max}$ (CHCl₃) nm: 280, 272, 240; ¹H-NMR (90 MHz, CDCl₃) δ : 7.38~7.24 (3H, m, H-2', 5', 6'), 6.72 (1H, d, *J*_{6,8} = 1.8 Hz, H-8), 6.58 (1H, d, *J*_{6,8} = 1.8 Hz, H-6), 5.42 (1H, m, H-3), 5.12 (1H, s, H-2), 3.73 (2H, q, *J*_{12,13} = 7.2 Hz, H-12), 3.02~2.92 (2H, m, H-4), 2.31, 2.29 and 1.94 (15H, s, 4 × –OCOCH₃, –NCOCH₃), 1.14 (3H, t, *J*_{12,13} = 7.2 Hz, H-13); ¹³C-NMR (22.5 MHz, CDCl₃) δ : 170.3, 169.8, 168.3 and 168.0 (4 × –OCOCH₃, –NCOCH₃), 155.3 and 150.1 (C-5, 7, 9), 142.4 and 142.1 (C-3' or 4'), 135.6 (C-1'), 124.2, 123.3 and 122.0 (C-2', 5', 6'), 115.0 and 114.2 (C-6 or 8), 111.9 (C-10), 76.9

FIG. 1. Mass Fragmentation of Compound 2.

(C-2), 66.6 (C-3), 44.0 (C-12), 26.2 (C-4), 22.8, 20.7 and 20.6 ($4 \times -\text{OCOCH}_3$, $-\text{NCOCH}_3$), 13.2 (C-13). An assignment of the NMR signals was accomplished based on those of the acetyl derivatives of EC (EC-Ac)^{4,5} and its related compounds.⁶⁻⁹

The ^{13}C -NMR spectrum of **2** was very similar to that of EC-Ac and showed the presence of an additional functionality, $-\text{N}-\text{CH}_2-\text{CH}_3$ [δ 44.0 (t) and 13.2 (q)]. Signals ascribable to C-6 and C-8 of **2** were shifted down field to δ 114.2 and 115.0, respectively, compared with those (δ 108.0 and 108.7) of EC-Ac, and no significant shift of other signals was observed except for C-5, C-7 and C-9. These data indicate that the ethylamino group was attached to the A ring of **2**.

This was also supported by the ^1H -NMR spectrum of **2**. Signals assigned to an ethylamide group were observed at δ 1.14 (3H, t) and δ 3.73 (2H, q). Three acetyl groups appeared at δ 2.29~2.31, and a singlet corresponding to two methyls at δ 1.94. In the spectrum of EC-Ac, the acetoxyl group at C-3 was observed at δ 1.96. One of the two methyls at δ 1.94 was, therefore, assigned to an acetoxyl at C-3, and the other to an acetamide methyl group.

The results indicate that the *N*-ethylacetoamide group was attached to either C-5 or C-7. The latter is favored because the chemical shift (δ 111.9) of C-10 in the ^{13}C -NMR spectrum of **2** was very similar to that of EC-Ac (δ 109.6), and those of C-6 and C-8 shifted down field as already described.

In the ^1H -NMR spectrum of the acetyl derivative of (+)-catechin, H-2 was observed at δ 5.38 (d, $J=8.5$ Hz), and H-4a and H-4b at δ 2.90 (dd, $J_{3,4a}=5.5$ Hz) and δ 2.48 (dd, $J_{3,4b}=5.5$ Hz), respectively. In the spectrum of EC-Ac, H-2 appeared at δ 5.08 (s), and H-4a and H-4b were observed at δ 3.00~2.88 (m).⁴ In the case of **2**, H-2 was observed at δ 5.12 (s), and H-4a and H-4b at δ 3.02~2.92 (m). These results indicate that **2** had 2,3-*cis*-stereochemistry, like EC-Ac.

Further support for the structure of **2** was obtained from its MS spectrum: m/z 527 (M^+ , 21.8), 485 (4.0), 467 (100), 443 (44.3), 425 (54.0), 383 (96.6), 341 (64.2), 263 (31.9), 236 (9.1), 208 (20.0), 194 (17.2), 166 (25.0), 152 (48.1). The major and important MS fragmentation pattern of **2** (Fig. 1) was formulated in accordance with earlier works.^{10,11} The normal Retro-Diels-Alder reaction type of fragmentation supports the proposed structure of **2** as shown in Fig. 1. The series of peaks from m/z 467 to m/z 341 is attributable to the loss of one molecule of acetic acid and three molecules of ketene from the peak at m/z 527.

The CI-MS spectrum of **2** showed a protonated molecular ion peak at m/z 528 ($\text{M}+1$, 40.8). HR-MS analysis (EI-MS, m/z 527.1666) gave the molecular formula $\text{C}_{27}\text{H}_{29}\text{O}_{10}\text{N}$ (calcd. 527.1783).

Based on this data, it is concluded that the structure of **2** was tetra-*O*-acetyl-7-*C*-(*N*-ethylacetamido)-(-)-epicatechin, and that of **1** was, therefore, 7-*C*-ethylamino-(-)-epicatechin.

The authors have reported that the presence of amino acids accelerated the formation of colored products from catechins by heating with a low moisture content,² but the involved reaction is still unknown. Compound **1** is the first compound isolated as a precursor of the brown products from the catechin-amino acid system. This experimental result suggests that any similar precursor formed when heating or firing green tea may gradually be changed to a brown compound, which is unfavorable to the quality of tea liquor and green tea itself.

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REFERENCES

- 1) M. Nakagawa, *Study of Tea*, **38**, 26 (1969).

- 2) T. Anan and H. Kato, *Nippon Shokuhin Kogyo Gakkaishi*, **31**, 321 (1984).
 - 3) T. Hara and E. Kubota, *Study of Tea*, **64**, 32 (1983).
 - 4) R. S. Thompson, D. Jacques, E. Haslam and R. J. N. Tanner, *J. Chem. Soc., Perkin Trans. 1*, **1972**, 1387.
 - 5) A. C. Fletcher, L. J. Porter, E. Haslam and R. K. Gupta, *J. Chem. Soc., Perkin Trans. 1*, **1977**, 1628.
 - 6) M. J. Brandon, L. Y. Foo, L. J. Porter and P. Meredith, *Phytochemistry*, **21**, 2953 (1982).
 - 7) G. Nonaka, O. Kawahara and I. Nishioka, *Chem. Pharm. Bull.*, **31**, 3906 (1983).
 - 8) T. Tanaka, G. Nonaka and I. Nishioka, *Phytochemistry*, **22**, 2575 (1983).
 - 9) G. Weeratunga, L. Bohlin, R. Verpoorte and V. Kumar, *Phytochemistry*, **24**, 2093 (1985).
 - 10) M. V. Piretti, G. P. Serrazanetti and G. Paglione, *Annali di Chimica*, **67**, 395 (1977).
 - 11) H. Kim and P. G. Keeney, *J. Food Sci.*, **48**, 548 (1983).
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