JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Characterization of Water-Soluble Intermediates of Blue Pigments Transformed from Geniposide of *Gardenia jasminoides*

JEE-EUN PARK,† JAE-YOUN LEE,† HONG-GYU KIM,† TAE-RYONG HAHN,‡ AND YOUNG-SOOK PAIK*,†

College of Environment and Applied Chemistry and Plant Metabolism Research Center, Kyung Hee University, Suwon 449-701, Korea

Gardenia blue dye was obtained through the reaction of methylamine with genipin, the aglycone of geniposide isolated from the fruits of *Gardenia jasminoides*. The resulting blue pigments were passed through Bio-Gel P-2 resin yielding five fractions, GM1−GM5. Four fractions (GM1−GM4) were all blue pigments, and the first eluted higher molecular weight fraction GM1 had a higher tinctorial strength than the later eluted lower molecular weight fractions, GM2−GM4. The last eluted GM5 fraction with λ_{max} of 292 nm was colorless and was confirmed as the true intermediate of the blue pigments on the basis of UV−vis spectrophotometric evidence. The GM5 fraction was composed of two epimeric isomers, and their structures were characterized by ^{1}H NMR, ^{1}H−^{1}H COSY, ^{13}C NMR, and HMQC and HMBC spectral measurements.

KEYWORDS: Gardenia jasminoides; genipine; methylamine; intermediates; gardenia blue dye

INTRODUCTION

The fruit of *Gardenia jasminoides* is a unique plant source that provides a series of yellow, blue, and red colorants in the food industry (*1*). Yellow pigments in gardenia fruits, crocin and related carotenoids, have been used traditionally for dyeing foods and fabrics in East Asia (*2*, *3*). Edible blue pigments can be obtained by a simple modification of colorless geniposide **1** (**Figure 1**), the major component of iridoid glycosides in gardenia fruits (*4*−*7*). Enzymatic hydrolysis of **1** yields genipin **2** (**Figure 1**). The reaction of **2** with primary amines such as amino acids or proteins produces polymeric, water-soluble blue pigments. Manipulation of the reaction conditions enables the production of blue, green, violet, and red colorants (*1*). This simple process for the production of edible blue pigments from genipin and primary amines is currently used by the food industry of East Asia, including Korea and Japan.

Although the process to prepare the blue pigments from genipin with primary amines is simple, the detailed mechanism of blue pigment transformation is unclear. Reaction of genipin with the simplest primary amine, methylamine, can provide information regarding the transformation mechanism of the blue pigments. Touyama et al. (*8*) reported that methylamine-reacted genipin in aqueous ethanol gave a brownish-red reaction mixture, which was supposed to consist of intermediates leading to the blue pigments. They reported that the intermediates were organic solvent soluble while the resulting blue pigments were polymeric and water soluble. There have been no reports about water-soluble intermediates during transformation into the blue pigments.

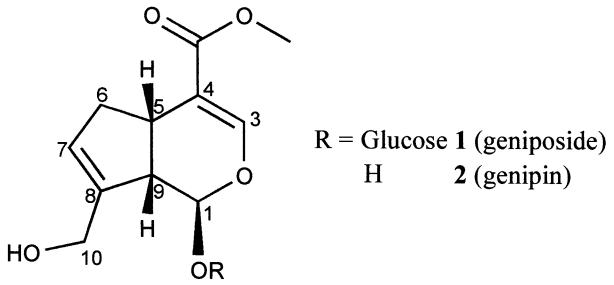

Figure 1. Chemical structures of geniposide **1** and genipin **2**.

In this research, we isolated geniposide **1** from gardenia fruits and hydrolyzed the compound with β-glucosidase. The resulting colorless genipin **2** was transformed into the blue pigments through the reaction with the simplest amine, methylamine, to elucidate the structural characterization of the blue pigments.

EXPERIMENTAL PROCEDURES

Materials. Dried fruits of *G. jasminoides* were obtained from Kyungnam province, Korea, and stored in the refrigerator. The silica gel for column chromatography (Kiesel gel, 230−400 mesh) and TLC plates (Kiesel gel 60 F254) were purchased from Merck (Darmstadt, Germany) in order to isolate geniposide from *G. jasminoides*. Bio-Gel P-2 (MW fractionation range, 200−2600) resin was purchased from Pharmacia Fine Chemicals (Hercules, CA). β-Glucosidase, NMR solvents, and other chemicals including methylamine were obtained from Sigma (Steinheim, Germany) and Aldrich (Milwaukee, WI).

* To whom correspondence should be addressed. Tel: 82-31-201-2422. Fax: 82-31-202-7337. E-mail: paikys@khu.ac.kr.
† College of Environment and Applied Chemistry.
‡ Plant Metabolism Research Center.

10.1021/jf020499b CCC: $22.00
Published on Web 09/27/2002

Spectroscopic Analysis. The UV−vis spectra were recorded on a Milton Roy Spectronics 3000 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra in $CDCl_3$ or D_2O were measured on a 400 MHz FT NMR (JEOL) at 400 and 100 MHz, respectively. EIMS spectra were obtained with a JEOL JMS-AX505 WA mass spectrometer.

Isolation of Geniposide 1 and Genipin 2. Geniposide **1** from *G. jasminoides* was isolated using the methods of Endo and Taguchi (*9*) with minor modifications. Geniposide **1** in acetate buffer (pH 5.0, 37 °C) was treated with β-glucosidase for 5 h to yield genipin **2**. Detailed procedures for the preparation of **1** and **2** were reported previously (*6*). The NMR spectrum of genipin **2** in $CDCl_3$ showed two epimeric isomers with the ratio of about 5:1. ^{1}H NMR (400 MHz, $CDCl_3$) major component **2a** δ: 7.53 (s, H-3), 5.88 (s, H-7), 4.82 (d, J = 8.5 Hz, H-1), 4.35 (d, J = 13.2 Hz, H-10a), 4.29 (d, J = 13.2 Hz, H-10b), 3.74 (s, OMe), 3.22 (qd, J = 8.5, 1.5 Hz, H-5), 2.89 (ddt, J = 16.8, 8.5, 1.5 Hz, H-6a), 2.54 (td, J = 8.5, 1.5 Hz, H-9), 2.07 (ddt, J = 16.8, 9.5, 1.8 Hz, H-6b). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 167.88 (CO), 152.44 (C-3), 142.06 (C-8), 130.86 (C-7), 110.78 (C-4), 96.34 (C-1), 61.34 (C-10), 51.29 (OMe), 48.23 (C-9), 39.04 (C-6), 36.69 (C-5). ^{1}H NMR (400 MHz, $CDCl_3$) minor component **2b** δ: 7.49 (s, H-3), 5.81 (s, H-7), 5.29 (s, H-1), 4.42 (d, J = 13.0 Hz, H-10a), 4.21 (d, J = 12.7 Hz, H-10b), 3.72 (s, OMe), 3.35 (td, J = 8.0, 2.0 Hz, H-5), 2.74 (dd, J = 16.5, 8.0 Hz, H-6a), 2.30 (br d, J = 16.5 Hz, H-6b), 2.16 (m, H-9). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 167.80 (CO), 153.40 (C-3), 139.50 (C-8), 132.70 (C-7), 111.20 (C-4), 95.10 (C-1), 61.40 (C-10), 51.20 (OMe), 49.80 (C-9), 38.40 (C-6), 35.50 (C-5).

Transformation and Separation of Gardenia Blue Pigments. Genipin **2** (50 mg, 0.22 mmol) in 2 mL of 100 mM phosphate buffer (pH 7.0, 70 °C) was treated with methylamine (2.2 mmol) for 5 h. The blue pigments were passed through Bio-Gel P-2 resin (MW fractionation range, 200−2600) eluted with water. The column (25 × 2.2 cm) was first washed with one bed volume of water and then eluted with 150 mL of water. Seventy fractions (2 mL/tube) of the blue pigments were collected and measured at 290 and 585 nm with a UV−vis spectrophotometer. Four types of blue pigments, GM1 (fraction nos. 5−9), GM2 (fraction nos. 21−26), GM3 (fraction nos. 31−34), and GM4 (fraction nos. 37−39), and a colorless GM5 (fraction nos. 46−57) were collected and measured using UV−vis and NMR spectroscopy to clarify their structures.

RESULTS AND DISCUSSION

Geniposide **1** obtained from gardenia fruits was hydrolyzed with β-glucosidase, and the resulting genipin **2** was transformed into blue pigments through reaction with methylamine. UV−vis spectra during the transformation of the blue pigments from genipin with methylamine at 70 °C with a 1-min scanning interval for 30 min showed that the λ_{max} of genipin at 240 nm decreased, while an intermediate peak at about 290 nm started rapidly to appear and then gradually decrease, and finally the λ_{max} of blue pigment polymers at about 580 nm was produced (**Figure 2**). The transformed blue pigments were passed through a Bio-Gel P-2 resin chromatography column eluted with water, and five fractions of GM1−GM5 were collected (**Figure 3**). **Figure 4** shows the UV−vis spectra of GM1−GM5 in H_2O (12.8 μg/mL each). Four fractions, GM1−GM4, were blue pigments. The first eluted, higher molecular weight polymeric fraction GM1 had a much higher tinctorial strength than the later eluted, lower molecular weight fractions GM2−GM4. Absorption maxima in the blue region (ca. 580 nm) of GM1−GM4 were 0.225, 0.024, 0.089, and 0.086, respectively (**Figure 4**). The last eluted GM5 fraction was not a blue pigment. However, the colorless GM5 with an absorption maximum of 292 nm was very unstable and easily converted into blue pigments. **Figure 5** shows the transformation of GM5 to the blue pigments at 70 °C with a 1-min scanning interval for 30 min, indicating that the absorption maximum of GM5 at 292 nm decreased, accompanied by an increase in the blue-absorbing region at about 580 nm, confirming GM5 as a true intermediate of the blue pigments. There were sharp isosbestic points at 268 and 315 nm. Further treatment of GM5 with methylamine under the same conditions elicited no spectroscopic changes, indicating that GM5 is already fully substituted with the methylamine portion. Treatment of each GM2−GM4 fraction at 70 °C with a 1-min scanning interval for 30 min or addition of methylamine at the same condition did not induce any spectroscopic changes,

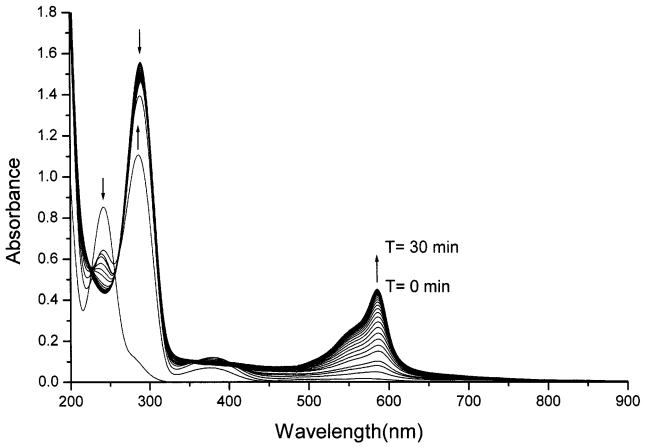

Figure 2. UV–vis spectra for the formation of blue pigments from methylamine-reacted genipin in 100 mM phosphate buffer (pH 7.0; 70 °C; scanning interval, 1 min).

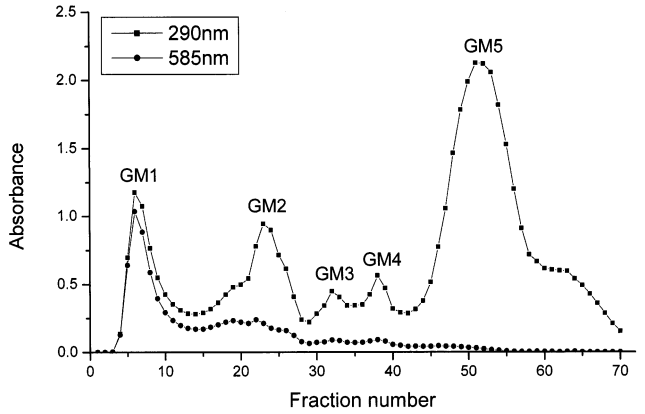

Figure 3. Elution profile of Bio-Gel P-2 resin chromatography for the separation of blue pigments transformed from methylamine-reacted genipin in 100 mM phosphate buffer (pH 7.0) at 70 °C for 5 h: GM1 (fraction nos. 5–9), GM2 (fraction nos. 21–26), GM3 (fraction nos. 31–34), GM4 (fraction nos. 37–39), and GM5 (fraction nos. 46–57).

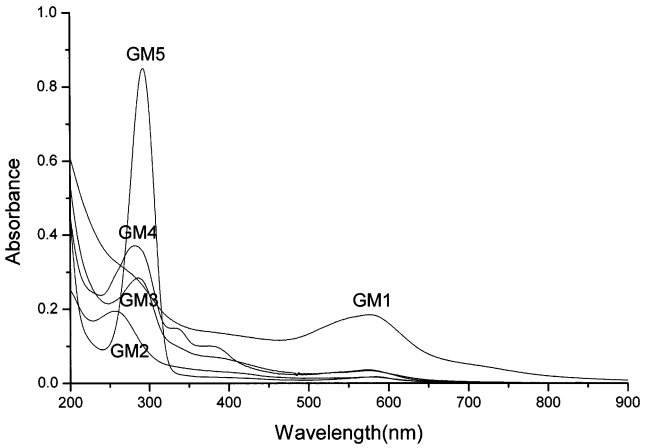

Figure 4. UV–vis spectra of GM1–GM5 in H_2O (12.8 μg/mL each).

Table 1. NMR Data for Compounds **3a** and **3b** in D_2O

	3a				3b			
position	^{13}C	^{1}H (mult, *J* Hz)	COSY	HMBC (H → C)	^{13}C	^{1}H (mult, *J* Hz)	COSY	HMBC (H → C)
1	80.03	4.62 (d 4.4)	9	3, 5	79.42	4.76 (d 3.9)	9	3, 5
3	148.47	7.28 (s)		1, 5, COO, NMe	147.15	7.29 (s)		1, 5, COO, NMe
4	101.07				100.15			
5	33.68	3.07 (td 7.3, 3.4)	6a, 6b, 9	4, 9	34.32	2.94 (m)	6a, 6b, 9	4, 9
6a	39.31	2.59 (ddt 16.3, 7.3, 2.2)	5, 6b, 9	4, 7, 8	40.15	2.67 (br dd 12.0, 8.0)	5, 6b, 7	7, 8, 9
6b		2.08 (br d 16.3)	5, 6a, 7, 10a, 10b	8		1.93 (br dd 12.0, 10.0)	5, 6a	7, 8
7	129.63	5.65 (s)	6b, 10a, 10b	5, 6, 8, 9, 10	131.41	5.80 (s)	6a	5, 6, 8, 9, 10
8	142.60				142.37			
9	50.65	2.92 (m)	1, 5		46.53	2.79 (br dd 8.6, 3.9)	1, 5	7, 8
10a	60.04	4.08 (d 13.7)	6b, 7	7, 8, 9	60.37	4.13 (d 13.7)		7, 8, 9
10b		4.03 (d 13.7)	6b, 7	7, 8		4.10 (d 13.7)		7, 8
COO	172.12				172.72			
OMe	51.96	3.55 (s)		COO	52.01	3.57 (s)		COO
NMe	39.72	2.86 (s)		1, 3	41.01	2.95 (s)		1, 3

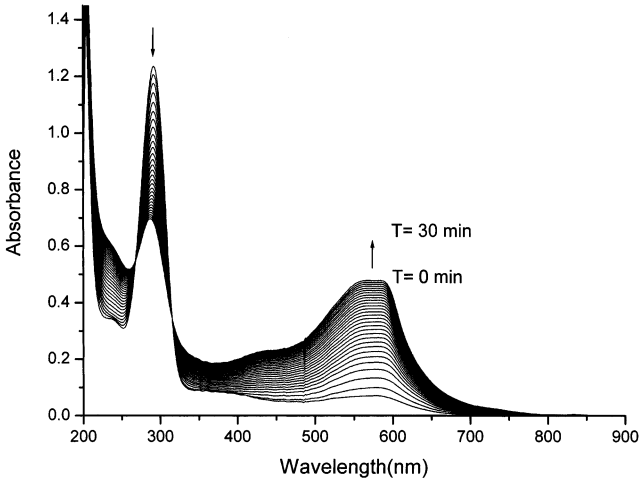

Figure 5. UV–vis spectra for the transformation of GM5 into blue pigments in 100 mM phosphate buffer (pH 7.0; 70 °C; scanning interval, 1 min).

suggesting that each GM2−GM4 fraction is not any intermediate able to transform into the blue pigments.

The IR spectrum of GM5 disclosed a hydroxyl band at 3424 cm^{-1} and a conjugated carbonyl band at 1676 cm^{-1}. The molecular formula of GM5, $C_{12}H_{17}NO_4$, was deduced from the molecular weight of 239.1158 based on HREIMS, 1D and 2D NMR data, and EIMS and spectroscopic data of genipin **2** ($C_{11}H_{14}O_5$), a typical iridoid compound containing a hemiacetal group. The only difference between GM5 and **2** was that GM5 had a $-NCH_3$ group instead of one oxygen atom in **2**. According to ^{1}H and ^{13}C NMR spectra, GM5 was not a single compound. GM5 was composed of two isomers (12 carbons each) with the ratio of about 3:2 (**Table 1**). A similar situation also occurred in genipin **2**. In our experiments, **2** in $CDCl_3$ showed two epimeric isomers (11 carbons each) with the ratio of about 5:1, although Djerassi et al. (*10*), Endo and Taguchi (*9*), and Drewes and Kayonga (*11*) did not describe epimers of this compound in $CDCl_3$. They reported only the major component of **2** in $CDCl_3$. We found that the epimeric position of genipin **2** was the hemiacetal carbon with values of δ 96.34 and 95.10 and with hydrogen atoms at δ 4.82 and 5.29, respectively. The presence of hemiacetal type carbon was also apparent in GM5. The ^{1}H NMR spectrum of GM5 showed signals of two sets of 15 hydrogens (approximate area ratio of 3:2) including two olefinic hydrogens. The ^{13}C NMR spectrum contained signals for two sets of 12 carbons (approximate area ratio of 3:2) including 1 ester carbon at δ 172.12 and 172.72 and 4 olefinic carbons. HMQC data revealed that two sets of 9 carbons, 2 aromatic and 7 aliphatic carbons, were attached to hydrogens.

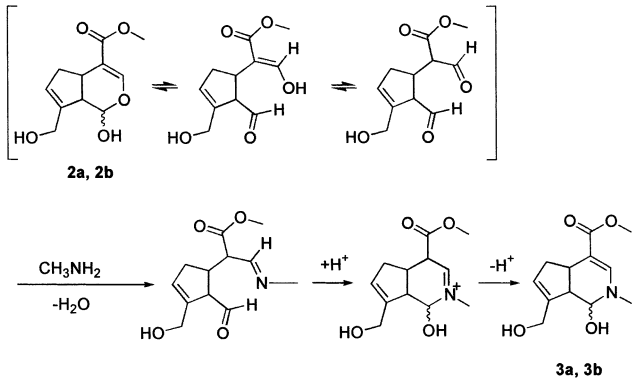

Figure 6. Proposed formation mechanism of GM5 from genipin with methylamine.

The HMQC spectrum of GM5 clearly showed two sets of nitrogen-containing hemiacetal type carbon atoms at δ 80.03 and 79.42 with hydrogen atoms at δ 4.62 and 4.76, respectively. Two sets of methoxy group carbon atoms (OCH_3) at δ 51.96 and 52.01 with hydrogens at δ 3.55 and 3.57 and an *N*-methyl group carbon (NCH_3) at δ 39.72 and 41.01 with hydrogens at δ 2.86 and 2.95, respectively, were also shown by the HMQC spectrum. Two sets of one carbon (C-10) at δ 60.04 and 60.37 connected with two hydrogens at δ 4.08, 4.03 and δ 4.13, 4.10 and another carbon (C-6) at δ 39.31 and 40.15 connected with two hydrogens at δ 2.59, 2.08 and δ 2.67, 1.93, respectively, indicated that GM5 had two methylene groups. The COSY spectrum data in **Table 1** show an obvious coupling between hydrogens of positions 1, 9, 5, and 6 at δ 4.62, 2.92, 3.07, 2.59, and 2.08 (major) and 4.76, 2.79, 2.94, 2.67, and 1.93 (minor). Two sets of HMBC correlations between hydrogens of $N-CH_3$ and C-1 (^{3}J) and C-3 (^{3}J) confirmed the location of the *N*-methyl group (**Table 1**). The $^{1}H-^{13}C$ long-range correlations (^{3}J) were also observed between H-3 and C-1, C-5, COO, and NMe. The structure of GM5 (**Figure 6**) was further ascertained by comparison with ^{1}H and ^{13}C NMR spectra of genipin **2** and three monomeric, organic-solvent-soluble components described previously (*8*, *12*) and our unpublished data. **Figure 6** shows a proposed mechanism for the conversion of **2** with methylamine into **3a** and **3b**.

LITERATURE CITED

(1) Hendry, G. A. F.; Houghton, J. D. *Natural Food Colorants*, 2nd ed.; Chapman & Hall: London, 1996; pp 40−79, 310−341.

(2) Pfister, P.; Meyer, A.; Pfander, H. Isolation and structure elucidation of carotenoid-glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne). *J. Agric. Food Chem.* **1996**, *44*, 2612−2615.

(3) Choi, H.-J.; Park, Y.-S.; Kim, M.-G.; Kim, T.-K.; Yoon, N.-S.; Lim, Y.-J. Isolation and characterization of the major colorant in gardenia fruits. *Dyes Pigm.* **2001**, *49*, 15−20.
(4) Fujikawa, S.; Fukui, Y.; Koga, K.; Kumada, J.-I. Brilliant skyblue pigment formation from gardenia fruits. *J. Ferment. Technol*. **1987**, *65*, 419−424.
(5) Lee, J.-Y.; Hahn, T.-R.; Paik, Y.-S. Physicochemical characteristics for the transformation of blue pigments from genipin of *Gardenia jasminoides* with amino acids. *Agric. Chem. Biotechnol*. **1998**, *41*, 399−404.
(6) Paik, Y.-S.; Lee, C.-M.; Cho, M.-H.; Hahn, T.-R. Physical stability of the blue pigments formed from geniposide of gardenia fruits: effects of pH, temperature, and light. *J. Agric. Food Chem.* **2001**, *49*, 430−432.
(7) Park, J.-E.; Hahn, T.-R.; Paik, Y.-S. Separation and characterization of water soluble blue pigments formed from geniposide of gardenia fruits. *Agric. Chem. Biotechnol.* **2001**, *44*, 190−193.
(8) Touyama, R.; Takeda, Y.; Inoue, K.; Kawamura, I.; Yatsuzuka, M.; Ikumoto, T.; Shingu, T.; Yokoi, T.; Inouye, H. Studies on the blue pigments produced from genipin and methlylamine. I. Structures of the brownish-red pigments, intermediates leading to the blue pigments. *Chem. Pharm. Bull*. **1994**, *42*, 668−673.
(9) Endo, T.; Taguchi, H. The constituents of *Gardenia jasminoides* geniposide and genipin-gentiobioside. *Chem. Pharm. Bull*. **1973**, *21*, 2684−2688.
(10) Djerassi, C.; Nakano, T.; James, A. N.; Zalkow, L. H.; Eisenbraun, E. J.; Shoolery, J. N. Terpenoids. XLVII. The structure of genipin. *J. Org. Chem*. **1961**, *26*, 1192−1206.
(11) Drewes, S. E.; Kayonga, L. Iridoid molluscicidal compounds from *Apodytes* dimidiata. *J. Nat. Prod*. **1996**, *59*, 1169−1170.
(12) Touyama, R.; Inoue, K.; Takeda, Y.; Yatsuzuka, M.; Ikumoto, T.; Moritome, N.; Shingu, T.; Yokoi, T.; Inouye, H. Studies on the blue pigments produced from genipin and methylamine. II. On the formation mechanisms of brownish-red intermediates leading to the blue pigment formation. *Chem. Pharm. Bull*. **1994**, *42*, 1571−1578.

Received for review April 29, 2002. Revised manuscript received July 29, 2002. Accepted July 30, 2002. This work was supported by a grant from the National Science and Engineering Foundation (2001) through the Plant Metabolism Research Center. Y.-S.P. thanks Kyung Hee University for financial support during sabbatical leave (2002).

JF020499B