Heat-Accelerated Degradation of Solid-State Andrographolide

Luelak Lomlim,[a] Noppong Jirayupong,[a] and Anuchit Plubrukarn*,[b]

[a] *Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University; and* [b] *Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University; Hat-Yai, Songkhla 90112, Thailand.* Received July 22, 2002; accepted September 20, 2002

The stability of andrographolide, the major active diterpene lactone from *Andrographis paniculata* (Burm. f.) Wall. ex Nees., was determined to show that, while crystalline andrographolide was highly stable even at 70 °C (75% relative humidity) over a period of 3 months, its amorphous phase degraded promptly. Heat-accelerated conditions revealed second-order kinetics of the decomposition with the rate constant at 25 °C ($k_{25°C}$) predicted from the Arrhenius plot of 3.8×10^{-6} d^{-1}. The major decomposed product under elevated temperature (70 °C, 75% relative humidity) is 14-deoxy-11,12-didehydroandrographolide.

Key words andrographolide; *Andrographis paniculata*; 14-deoxy-11,12-didehydroandrographolide; stability

Andrographis paniculata (Burm. f.) Wall. ex Nees. (Acanthaceae) is a medicinal plant widely used in several Asian countries, including China, India, and Thailand. Andrographis herb, the dried aerial part of *A. paniculata* as specified in the Thai Herbal Pharmacopoeia, has a wide range of activities. For example, as recommended in Thailand's National List of Essential Drugs 1999 (List of Herbal Medicinal Products),[1] the indications for Andrographis herb are for the treatment of noninfectious diarrhea and pharyngotonsillitis. The plant is also used in the treatment of diabetes and hypertension,[2] hepatitis,[3] malarial infection,[4] and also claimed to be an anti-HIV I agent, even though contrary evidence was observed.[5] The active constituents responsible for its activities are labdane-type diterpene lactones, among which the major components are andrographolide (**1**)[6] and some close derivatives such as deoxyandrographolide (**2**), neoandrographolide (**3**),[7,8] and 14-deoxy-11,12-didehydroandrographolide (**4**).[9,10] The isolation of flavonoids from the aerial part was also reported.[11]

Despite its potential for clinical use, the further development of *A. paniculata* has been hampered due to its stability problem. Upon 1-year storage of the dried, powdered aerial part in dry ambient condition, its total lactone contents reportedly decreased by 26%, thus restricting the herb's storage period.[12] However, no thorough investigations of the stability of either Andrographis herb itself or its constituents have been systematically performed to our knowledge. Moreover, the decomposition paths have not been investigated. The determination of andrographolide stability under elevated temperatures is reported and the possible major degradation pathway is discussed here.

Results and Discussion

Stability of Crystalline *versus* Amorphous Phases of Andrographolide (1) Early in this investigation, a preliminary study on the degradation of **1** was initiated in order to optimize sampling schedule for the stability analysis. Crystalline form of **1** was allowed to stand at 70 °C for 3 months. To our surprise, over this period the crystalline sample was perfectly stable, *i.e.*, remaining totally unchanged both physically and chemically. Therefore, another investigation was devised using the amorphous form of **1**, prepared as a 1 : 2 solid dispersion of **1** in PVP-K30. It was found that **1** decomposed promptly during 2-month standing under the same accelerated condition. This result clearly demonstrated the influence of crystallinity on the stability of **1**.

Kinetics of Degradation of 1 The determination of accelerated degradation kinetics was performed at 3 elevated temperatures, *i.e.*, 45, 60, and 70 °C. The degradation of **1** during the sampling periods from 7 to 91 d fitted second-order kinetics, in which the linearity was best met when the reciprocals of the percentages of remaining amounts of **1** at each temperature were plotted against time t (Fig. 1). The rate constant (k) determined for each temperature is $0.05\times$

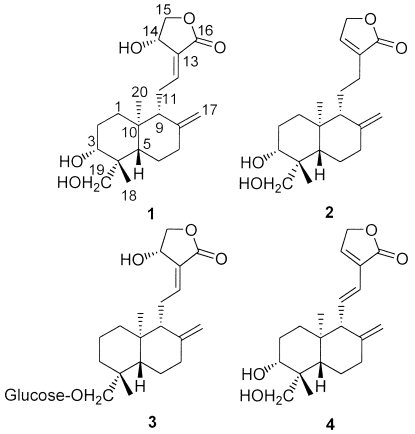

Chart 1. Structures of Andrographolide and Its Derivatives

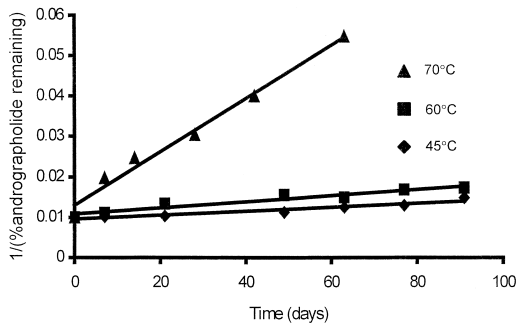

Fig. 1. Second-Order Plots of Degradation of **1** at 45, 60, and 70 °C

The rate equation for each temperature is expressed as $1/c=(0.05\times10^{-3})t+0.0096$, $r^2=0.9128$; $1/c=(0.08\times10^{-3})t+0.0108$, $r^2=0.9248$; and $1/c=(0.70\times10^{-3})t+0.0130$, $r^2=0.9833$, respectively.

* To whom correspondence should be addressed. e-mail: planuchi@ratree.psu.ac.th

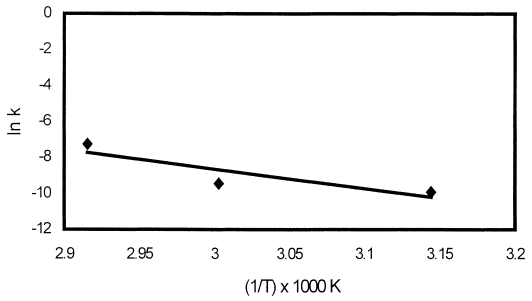

Fig. 2. Arrhenius Plot of **1**

The Arrhenius relation can be expressed as $\ln k = 23.553 - (10.732/T)$; $r^2 = 0.7774$.

10^{-3}, 0.08×10^{-3}, and $0.70\times10^{-3}\,d^{-1}$, respectively. The extrapolation of the Arrhenius plot (Fig. 2) obtained from the relation between $\ln k$ and the reciprocal of thermodynamic temperature (T) leads to the estimation of k at 25 °C ($k_{25\,°C}$) of $3.8\times10^{-6}\,d^{-1}$.

As obligated by second-order kinetics, the half-life ($t_{1/2}$) and shelf-life ($t_{90\%}$) of **1** are dependent on the initial concentration of the sample and thus cannot be generalized. For the experimental conditions used here, predicted $t_{1/2}$ and $t_{90\%}$ at 25 °C are 7.83 and 0.87 years, respectively.

Degraded Products and Degradation Path of 1 under Elevated Temperature Amorphous andrographolide (equivalent to 300 mg of **1**) was allowed to stand at 70 °C. After 2 months, the crude degradation mixture was subjected to isolation using semipreparative RP C-18 HPLC (55% aqueous CH_3CN). Along with the peak of **1** at 5.3 min, the chromatogram also showed one additional detectable peak (t_R 10.6 min). The fraction related to the 10.6-min peak was collected to afford compound **4** as the major degraded product (5.2 mg from an equivalent of 12 mg of **1**; 42% isolated yield).

The ^{1}H-NMR spectrum of **4** (500 MHz; DMSO-d_6) is similar to that of **1**, excepts that some additional signals at δ 7.63 (t, J=1.8 Hz; H-14), 6.73 (dd, J=15.9, 10.0 Hz; H-11), and 6.12 (d, J=15.9 Hz; H-12) are observed. Also, a triplet signal at δ 4.93 and a doublet at δ 5.70, originally assigned to H-14 and 14-OH of **1**, disappear in the spectrum of **4**. These changes in chemical shifts indicate that compound **4** is in fact the known 14-deoxy-11,12-didehydroandrographolide, which was first isolated from the whole plant of *A. paniculata* by Balmain and Connelly.[9] The structure of **4** was confirmed by the comparison with its spectral data later reported by Matsuda *et al.*[10]

The dehydration of **1**, as the major decomposition path under elevated temperatures, is unexplainable by any available elimination mechanisms. Presuming that neither humidity nor PVP are involved directly in the rate equation, this degradation undergoes bimolecular kinetics with only a single species of substrate. Generally, the bimolecular elimination E2 requires a base, whether weak or strong, to abstract the β-proton (or in this case, the δ-proton). On the other hand, the pyrolytic elimination Ei, which can proceed with a stand-alone substrate, is naturally unimolecular. Besides the two contradictory aspects above, 1,4-elimination is generally rare. More often, 1,4-elimination of allylic alcohol is encountered when a more easily abstracted group (*e.g.*, silyl group) is present at the δ position (for example, see ref. 13).

Here, we propose that the mechanism of the bimolecular 1,4-pyrolytic elimination of **1** possibly involve a paired-complex transition state, in which the two molecules of substrate are pairing to help each other in withdrawing the leaving group. This suggestion, nonetheless, needs further and more systematic examination. While the degradation mechanism of the diterpene lactones deposited in dried herb are possibly irrelevant to that of the isolated compound, such study could still lead to the application of andrographolide and its derivatives for more practical clinical use as pure substances.

Conclusions

Upon acceleration by elevated temperatures, the degradation of **1** is significantly influenced by its crystallinity. While crystalline andrographolide was completely stable at 70 °C, its amorphous phase decomposed more promptly. Determination of the degradation of **1** under heat-accelerated conditions showed second-order kinetics, and the extrapolation of the Arrhenius plot revealed the estimated $k_{25\,°C}$ of 3.8×10^{-6} d^{-1}. The major degradation route of **1** under the above accelerating conditions is 1,4-elimination-dehydration at the allylic 14-OH group, yielding 14-deoxy-11,12-didehydroandrographolide (**4**) as the major decomposed product.

Our estimated $k_{25\,°C}$ of $3.8\times10^{-6}\,d^{-1}$ corresponds well with the restricted storage period of Andrographis herb recommended in the Thai Herbal Pharmacopoeia. Nonetheless, the observed degradation route of **1** under these experimental conditions failed to explain the decomposition of lactone derivatives deposited in plant cells. As mentioned earlier, degradation during the storage of Andrographis herb led to the loss in total lactone contents, implying a major transformation at the lactone linkage, not at the allylic moiety as observed here. Further investigations regarding the stability of Andrographis herb itself, therefore, have been already planned. The results of the stability profiles of both Andrographis herb and its active constituents will shed the light on the advanced development of this highly potential medicinal herb for wider clinical use.

Experimental

General Unless stated otherwise, all chromatographic solvents used for HPLC were of analytical grade and were filtered through a membrane filter (230 μm; Millipore). UV spectrum was obtained with a Hewlett-Packard 8452A Diode Array spectrometer. IR spectrum was recorded on a Jasco IR-810 spectrometer. Mass spectrometry was performed in ESI mode on an LCT Module Mass Spectro detector. NMR spectra were obtained using a Varian Unity Inova 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). The signals were reported on the δ-scale and referred to the signals of solvent (DMSO-d_6; 2.49 ppm for ^{1}H and 39.7 ppm for ^{13}C). Optical rotation was measured on a Polax-L polarimeter and was reported as specific rotation. Differential scanning calorimetry (DSC) was performed on a Perkin Elmer DSC7 calorimeter. The elevated temperature experiments were carried out using the Memmert BP600, B50, and Pr402 ovens with the controlled temperatures of 45±2, 60±2, and 70±2 °C, respectively.

Analytical HPLC was performed on a Waters 600 controller-solvent delivery system, equipped with a Waters 717 plus autosampler and a Waters 486 tunable absorbance detector. Preparative HPLC was performed on a Waters 600E solvent delivery system connected to a Rheodyne 7161 injection valve and a Waters 484 tunable absorbance detector.

Chemicals Andrographolide (**1**) was isolated from *A. paniculata* collected in the vicinity of Songkhla district in June 2000. The specimen was identified by an author (A.P.), and a voucher was deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University.

The crude MeOH extract of *A. paniculata* (dried aerial part, 1.5 kg) was subjected to vacuum chromatography over a SiO_2 column using 2% MeOH

in CH_2Cl_2 as eluting solvent. The fractions containing **1** as major component were pooled, and **1** was repeatedly recrystallized from MeOH as white needles (2.9 g, isolated yield 0.2%). The authenticity was confirmed on the basis of NMR spectral analysis, according to data reported by Fujita *et al*.[14]

Solid Dispersion of 1 Compound **1** in the amorphous form was prepared as a 1 : 2 solid dispersion of PVP-K30. An aliquot solution of the 1 : 2 mixture of **1** and PVP-K30 (equivalent to 5.0 mg of **1**) was transferred to a 5-ml vial and then concentrated under reduced pressure to the resulting dried, thin film. The collapse of the crystal structure was detected using DSC, in which the thermograms of the crystalline **1** and the solid dispersion were compared. The signal at 240 °C, which was normally found in the thermogram of standard crystalline **1**, disappeared in the thermogram of the solid dispersion, thus suggesting that **1** in the solid dispersion was no longer in its crystalline form.

Stability Analysis of 1 In all the elevated-temperature systems throughout this study, closed desiccators each containing a jar of saturated NaCl solution were used to achieve the atmosphere of 75±5% relative humidity (adopted from the Handbook of Pharmaceutical Excipients).[15] A series of solid dispersion of **1** in PVP-K30 (equivalent to 5.0 mg of **1** each) were separately allowed to stand at 45±2, 60±2, and 70±2 °C (75% relative humidity) according to the following intervals; 7, 21, 49, 63, 77, and 91 d (45 and 60 °C); or 7, 14, 28, 42, 63, and 84 d (70 °C). After each time, the sample (triplicated) from each temperature was removed and preserved at −80 °C until quantification.

The HPLC condition for the quantification of **1** was: C18 Thermo-Hypersil column (5 μm; 250×4.6 mm) at ambient temperature, eluted with isocratic 30% aqueous CH_3CN (1.5 ml/min), and detected at λ 254 nm. This procedure was validated with a linearity in the range of 10—200 μg/ml (r^2>0.9998). The accuracy was evaluated using a sample at 100 μg/ml to reveal the recovery percentage of 102.7% (%R.S.D.<3.0).

The solutions of decomposed mixtures (in 2.0 ml of CH_3CN each; equivalent to 5.0 mg of the starting amount of **1**) were prepared and then diluted 50-fold with the mobile phase. These were used as sample preparations. Its HPLC chromatogram showed only two major peaks at t_R 5.7 and 24.7 min, belonging to compounds **1** and **4**, respectively. The percentage of the remaining amount of **1** was calculated from its peak area in accordance with the standard curve.

Isolation of Degraded Products of 1 The solid dispersion of **1** (1 : 2 in PVP-K30; equivalent to 300 mg of **1**) was allowed to stand at 70 °C for 2 months. After this period, an aliquot of this mixture was subjected to a semi-preparative HPLC column (C18 Hamilton PRP-1; 10 μm; 305×7.0 mm) using 55% aqueous CH_3CN (1.8 ml/min) as eluting solvent and detected at λ 254 nm. The fraction eluted at t_R 10.6 min was then collected to afford compound **4** as a white solid (5.2 mg from an equivalent of 12 mg of starting **1**; 42% isolated yield).

14-Deoxy-11,12-didehydroandrographolide (**4**): White solid (5.2 mg from an equivalent of 12 mg of starting **1**; 42%). ^{1}H-NMR (DMSO-d_6) δ: 0.74 (3H, s; H-20), 1.08 (3H, s; H-18), 1.11 (1H, ddd, J=13.5, 13.5, 5.0 Hz; H-1ax), 1.17 (1H, m; H-5), 1.31 (1H, ddd, J=13.5, 3.4, 3.4 Hz; H-1eq), 1.38 (1H, dddd, J=13.5, 13.0, 13.0, 4.1 Hz; H-6ax), 1.55 (1H, m; H-2ax), 1.60 (1H, m; H-2eq), 1.71 (1H, dddd, J=13.5, 2.7, 2.5, 2.3 Hz; H-6eq), 1.97 (1H, ddd, J=12.6, 5.0, 5.0 Hz; H-7eq), 2.35 (2H, m; H-7ax and H-9 overlapped), 3.21 (1H, ddd, J=10.3, 5.0, 5.0 Hz; H-3), 3.27 (1H, dd, J=11.0, 2.6 Hz; H-19a), 3.84 (1H, dd, J=11.0, 7.3 Hz; H-19b), 4.13 (1H, dd, J=7.3, 2.6 Hz; OH-19), 4.41 (1H, d, J=1.8 Hz; H-17a), 4.72 (1H, d, J=1.8 Hz; H-17b), 4.87 (2H, d, J=1.8 Hz; H-15), 5.02 (1H, d, J=5.0 Hz; OH-3), 6.12 (1H, d, J=15.9 Hz; H-12), 6.73 (1H, dd, J=15.9, 10.0 Hz; H-11), 7.63 (1H, t, J=1.8 Hz; H-14). ^{13}C-NMR (DMSO-d_6) δ: 13.0 (q; C-20), 22.8 (t, C-6; and q; C-18), 27.0 (t; C-2), 36.0 (t; C-7), 37.0 (t; C-1), 39.0 (s; C-10), 42.0 (s; C-4), 54.0 (d; C-5), 60.0 (d; C-9), 64.0 (t; C-19), 70.0 (t; C-15), 79.0 (d; C-3), 106.6 (t; C-17), 121.0 (d; C-12), 127.0 (s; C-13), 133.0 (d; C-11), 146.5 (d; C-14), 149.0 (s; C-8), 172.0 (s; C-16). IR (thin film) cm^{-1}: 3400, 1750, 1050. UV λ_{max} (CH_3OH) nm (log ε): 208 (3.09), 250 (2.89). ESI-MS *m/z*: 355 $(M+Na)^+$, 333 $(M+H)^+$, 315, 253, 210. $[\alpha]_D^{25}$ +5.33° (c=0.1, CH_3OH).

Acknowledgments This study was financially supported by a research grant from the Faculty of Pharmaceutical Sciences, Prince of Songkla University. We wish to extend our gratitude to Dr. Sirirat Pinsuwan of the Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, for her valuable discussion regarding the reaction kinetics.

References

1) National Drug Committee, "National List of Essential Drugs A.D. 1999 (List of Herbal Medicinal Products)," National Essential Drug List Committee, Ministry of Public Health, Bangkok, 1999.
2) Zhang C., Kuroyangi M., Tan B. K., *Pharmacol. Res.*, **38**, 413—417 (1998).
3) Jain D. C., Gupta M. M., Saxena S., Kumar S., *J. Pharm. Biomed. Anal.*, **22**, 705—709 (2000).
4) A Rahman N. N. N., Furuta T., Kojima S., Takane K., Mohd M. A., *J. Ethnopharmacol.*, **64**, 249—254 (1999).
5) Collins R. A., Ng T. B., Fong W. P., Wan C. C., Yeung H. W., *Life Sci.*, **60**, 345—351 (1997).
6) Cava M. P., Chan W. R., Haynes L. J., Johnson L. F., Weinstein B., *Tetrahedron*, **18**, 397—403 (1962).
7) Cava M. P., Chan W. R., Stein R. P., Willis C. R., *Tetrahedron*, **21**, 2617—2632 (1965).
8) Chan W. R., Taylor D. R., Willis C. R., Bodden R. L., Fehlhaber H.-W., *Tetrahedron*, **27**, 5081—5091 (1972).
9) Balmain A., Connelly J. D., *J. Chem. Soc.*, *Perkin Trans. 1*, **1973**, 1247—1251 (1973).
10) Matsuda T., Kuroyanagi M., Sugiyama S., Umehara K., Ueno A., Nishi K., *Chem. Pharm. Bull.*, **42**, 1216—1255 (1994).
11) Gupta K. K., Taneja S. C., Dhar K. L., Atal C. K., *Phytochemistry*, **22**, 314—315 (1983).
12) Dechatiwonse Na Ayudhya T., Techadamrongsin Y., Jirawattanapong W., "Chemical Specification of Thai Herbal Drugs," Vol. 1, Department of Medical Sciences, Ministry of Public Health, Bangkok, 1993.
13) Maeta H., Suzuki K., *Tetrahedron Lett.*, **33**, 5969—5972 (1992).
14) Fujita T., Fujitani R., Takeda Y., Takaishi T., Kido M., Miura I., *Chem. Pharm. Bull.*, **32**, 2117—2125 (1984).
15) Wade A., Weller P. J., "Handbook of Pharmaceutical Excipients," 2nd ed., American Pharmaceutical Association, Washington, DC, and Royal Pharmaceutical Society of Great Britain, London, 1994.