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Isolation and characterization of the acid and base degradation products of andrographolide

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Andrographolide was subjected to degradation under acidic and basic conditions. Three degradation products including two novel compounds, 8,9-didehydroandrographolide (**AG-A**) and 15-*seco*-andrographolide (**AG-B2**), and a known product, 14-deoxy-11,12-didehydroandrographolide (**AG-B1**), were isolated by chromatography and identified on the basis of their NMR and MS data. The degradation product, **AG-A**, may result from the isomerization of andrographolide under acidic conditions whereas the base degradation products, **AG-B1** and **AG-B2**, were formed most likely due to the dehydration of allyl alcohol and the hydrolysis of andrographolide at the lactone ring, respectively.

1. Introduction

Andrographolide is the major bioactive diterpene lactone of *Andrographis paniculata* and accounts for several pharmacological activities of this plant, such as ant-pyretic, anti-inflammatory activity and hepatoprotective effects (Saxena et al. 2000). In order to determine the amount of andrographolide in *A. paniculata* and herbal formulations, several analytical methods for quantitative analysis have been developed (Pawar et al. 2010; Bhope et al. 2009; Akowuah et al. 2006; Du et al. 2003).

Studies on the stability of andrographolide under selected storage conditions as well as heat-accelerated and stress testing conditions according to the ICH guidelines were also reported. The results showed that, over a period of three-month storage, there was no significant reduction of andrographolide content in *A. paniculata* at ambient conditions (30±2 °C, 60±5 % RH) (Ibrahim et al. 2008). The solid-state andrographolide in crystalline form was stable under heat-accelerated conditions (70 °C, 75% RH), while a degradation of its amorphous form to 14-deoxy-11,12-didehydroandrographolide was observed (Lomlim et al. 2003). From our previous forced-degradation studies, andrographolide was quite stable under mild acid, oxidation, dry heat and photochemical degradations (Phattanawasin et al. 2016). No major degradation product of andrographolide was observed in these treatments. However, under mild basic and strong acidic conditions, andrographolide was easily degraded. The degradation products were neither isolated nor structurally characterized.

Due to little information known about the acid and base degradation products of andrographolide, the purpose of this work was to isolate them and to determine their chemical structures. The structural characterization of the degradation products and their possible degradation pathways of andrographolide under acidic and basic conditions are described herein.

2. Investigations, results and discussion

In this study, the degradation conditions of andrographolide were carried out under strong acidic and mild basic stress conditions. The chromatographic separation of the acidic-degraded sample resulted in the isolation of **AG-A**. Under basic conditions, two degradation compounds, **AG-B1** and **AG-B2**, were isolated. The acid and base degradation products were well-resolved from andrographolide on the HPLC chromatogram (Fig. 1). The structures of **AG-A**, **AG-B1** and **AG-B2** were identified on the basis of their NMR and MS data and by comparing with those of andrographolide (Tables 1 and 2).

AG-A was purified as a white amorphous powder and the molecular formula was $C_{20}H_{30}O_5$, as supported by the mass peak of protonated molecule in the HRESI-MS at *m/z* 373.1995 $[M+Na]^+$. Interestingly, the molecular formula of **AG-A** and andrographolide appeared to be the same. From the UV analysis, **AG-A** exhibited a maximum absorption at 225 nm, indicating the presence of an *exo*-cyclic α,β-unstaurated-γ-lactone ring (Arpini et al. 2008) as shown in andrographolide. The ^1H and ^{13}C NMR spectrum of **AG-A** were similar to those of andrographolide, except that a signal at δ 1.92 and two singlet signals at δ 4.67 and 4.89, originally assigned to H-9 and *exo*-methylene protons, H-17α and H-17β, of andrographolide, respectively, disappeared from the ^1H NMR spectrum of **AG-A**. The ^1H NMR spectrum of **AG-A** showed an additional signal of a methyl group at δ 1.55. The ^{13}C NMR spectrum of **AG-A** also showed three methyl carbon signals at δ 18.32, 19.52 and 20.70 instead of two signals at δ 15.53 and 23.36 as seen in that of andrographolide. An HMBC experiment of **AG-A** further revealed that the methyl protons at δ 1.55 showed long range couplings to carbons at δ 34.05 (C-7), 129.44 (C-8) and 135.87 (C-9), suggesting the presence of this methyl group at C-17. Thus, the structure of **AG-A** was identified to be 8,9-didehydroandrographolide. The transformation of andrographolide under acidic conditions into **AG-A** was likely due to the isomerization of double bond, involving the protonation of *exo*-methylene and the abstract of proton at C-9, causing the shifting the *exo*-methylene to the endocyclic position (Fig. 2) (Fujita et al. 1984).

AG-B1 was obtained as a white amorphous powder and exhibited a mass peak in the HRESI-MS at *m/z* 355.1874 $[M+Na]^+$, supporting the molecular formula of $C_{20}H_{28}O_4$. The UV spectra showed no absorption at 220 nm but a maximum at aprroximately 250 nm, indicating the presence of an *endo*-cylcic α,β-unstaurated-γ-lactone ring with conjugated double bonds (Arpini et al. 2008). As compared to the ^1H-spectrum of andrographolide, the methine proton at δ 5.01 (H-14) and the methylene protons at δ 2.60 (H_2-11) shown in andrographolide were disappeared from the spectrum of **AG-B1** whereas the presence of three downfield methine signals at δ 6.11 (H-12), 6.87 (H-11) and 7.17 (H-14) were observed. Therefore, the structure of **AG-B1** was determined to be 14-deoxy-11,12-didehydroandrographolide and its spectral data were in agreement with those

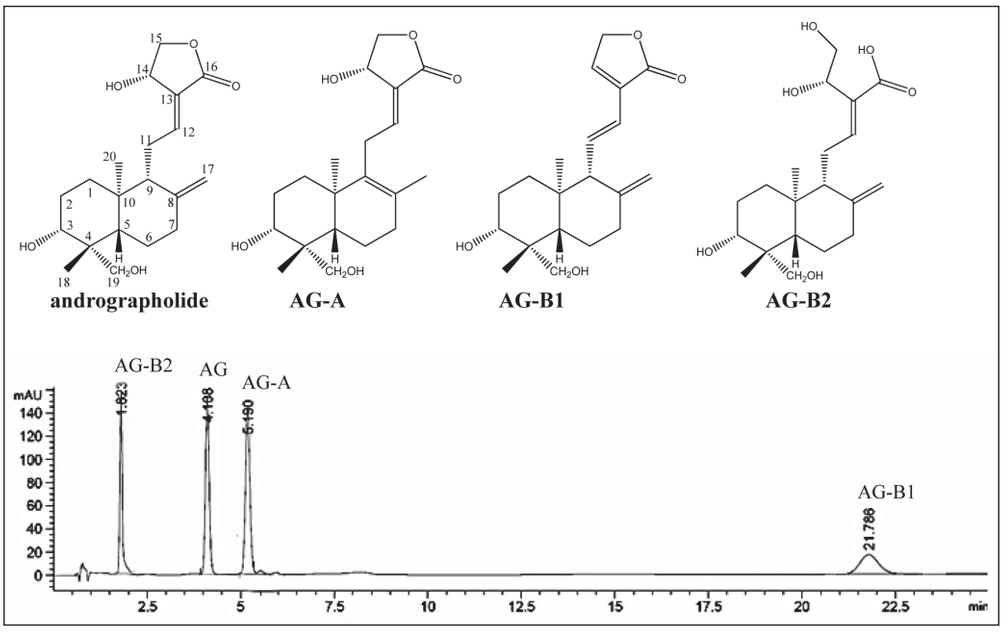

Fig: 1: HPLC chromatogram of andrographolide (**AG**) and its acid (**AG-A**) and base degradation products (**AG-B1** and **AG-B2**)

Table 1: Comparative ^{1}H NMR assignments of andrographolide and the acid and base degradation products, AG-A, AG-B1 and AG-B2

No	Andrographolide	AG-A	AG-B1	AG-B2
1	α 1.83, m β 1.32, m	1.6-1.7, m	α 1.52, dt (13.9, 3.5 Hz) β 1.14, m	α 1.82, m β 1.29, m
2	1.80, m	1.7-1.8, m	α 1.80, m β 1.72, m	1.79, m
3	3.41, t (8.2 Hz)	3.41, td, (12.1, 5.1 Hz)	3.49, dd (11.4, 7.0 Hz)	3.40, t (8.0 Hz)
4	-		-	-
5	1.35, m	1.18, m	1.21, m	1.32, m
6	α 1.39, td (12.7, 3.4 Hz) β 1.88, m	1.7-1.8, m	α 1.35, qd (13.5, 4.2 Hz) β 1.80, m	α 1.37, td (14.1, 4.4 Hz) β 1.82, m
7	α 2.43, dt (12.7, 3.4 Hz) β 2.04, td (12.7, 4.6 Hz)	2.0-2.1, m	α 2.45, dt (13.5, 3.1 Hz) β 2.04, td, (13.5, 4.7 Hz)	α 2.42, br.d (14.1 Hz) β 2.03, td, (14.1, 5.0 Hz)
8	-	-	-	-
9	1.92, dd, (10.0, 3.4 Hz)	-	2.31, d (10.1 Hz)	1.86, m
10	-	-	-	-
11	2.60, m	α 2.99, dd (17.5, 8.4 Hz) β 3.21, dd (17.6, 5.0 Hz)	6.87, dd (15.8, 10.1 Hz)	α 2.37, td (9.8, 6.3 Hz) β 2.58, br.d (9.8 Hz)
12	6.85, td (6.7, 1.3 Hz)	6.78, t (6.3 hz)	6.11, d (15.8 Hz)	6.80, t (6.3 Hz)
13	-	-	-	-
14	5.01, d (6.0 Hz)	5.03, d (5.2)	7.17, s	4.69, br.t (5.2 Hz)
15	α 4.16, dd (10.2, 1.9 Hz) β 4.46, dd (10.2, 6.0 Hz)	α 4.23, d (10.4 Hz) β 4.47, dd (10.3, 6.4 Hz)	4.82, s	α 3.75, dd (11.0, 6.7 Hz) β 3.61, dd (11.0, 5.6 Hz)
16	-		-	-
17	α 4.67, s β 4.89, s	1.55, s	α 4.52, s β 4.78, s	α 4.50, s β 4.90, s
18	1.22, s	1.22, s	1.26, s	1.21, s
19	α 4.12, d (11.2 Hz) β 3.37, d (11.2 Hz)	α 4.18, d (10.9 Hz) β 3.29, d (11.1 Hz)	α 4.21, d (11.1 Hz) β 3.35, d (11.1 Hz)	α 4.12, d (11.1 Hz) β 3.36, d (11.1 Hz)
20	0.75, s	0.91, s	0.81, s	0.75, s

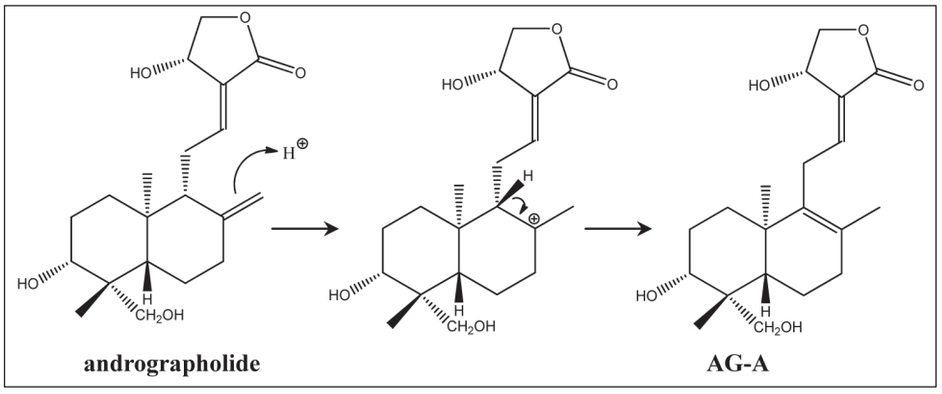

Fig. 2: Proposed structural transformation of andrographolide into **AG-A** under acidic conditions.

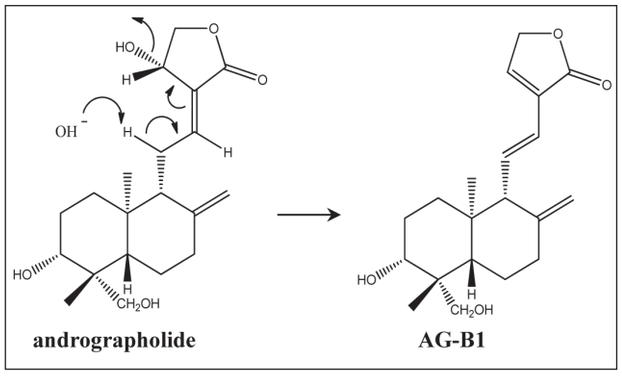

Fig. 3: Proposed structural transformation of andrographolide into **AG-B1** under basic conditions.

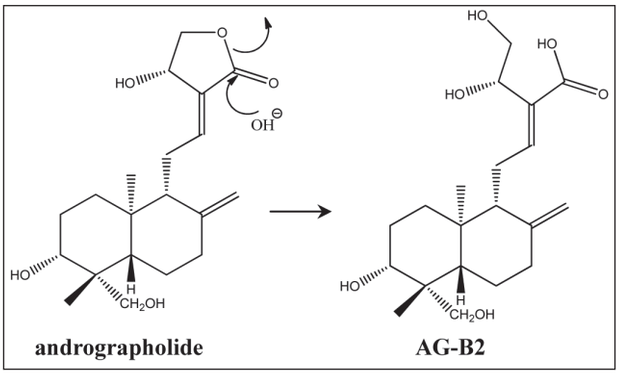

Fig. 4: Proposed structural transformation of andrographolide into **AG-B2** under basic conditions

Table 2: Comparative ^{13}C NMR assignments of andrographolide and the acid and base degradation products, AG-A, AG-B1 and AG-B2

No.	Andrographolide	AG-A	AG-B1	AG-B2
1	38.23	34.73	38.22	38.07
2	29.07	27.86	28.10	29.04
3	81.02	80.19	80.86	80.98
4	43.75	42.46	42.98	43.68
5	56.46	51.55	54.66	56.42
6	25.23	18.66	22.95	25.22
7	39.00	34.05	36.55	39.07
8	148.82	129.44	148.06	148.91
9	57.48	135.87	61.65	57.86
10	40.02	38.27	38.55	40.08
11	25.77	28.13	135.98	24.72
12	149.29	149.16	121.08	148.03
13	129.81	127.18	129.26	131.99
14	66.72	66.06	142.90	71.32
15	76.08	74.45	69.61	66.25
16	172.60	170.56	172.0	171.07
17	109.14	19.52	109.18	108.94
18	23.36	18.32	22.60	23.38
19	64.99	64.10	64.16	65.01
20	15.53	20.70	15.87	15.64

previously reported (Lomlim et al. 2003). The formation of **AG-B1** under basic condition possibly involved the bimolecular elimination E2 by the use of a base to abstract the δ-proton in **AG**, leading to 1,4-elimination of allylic alcohol (Fig. 3).

AG-B2 was isolated as a white amorphous precipitate. The HRESI-MS analysis at *m/z* 391.2092 $[M+Na]^+$ supported the molecular formula of $C_{20}H_{32}O_6$. The ^{1}H and ^{13}C NMR spectrum of **AG-B2** showed a very close resemblance to those of andrographolide. The main differences were the observed significant upfield shifts in the methine proton at H-14 from 5.01 ppm and the methylene protons at H_2-15 from 4.16 and 4.46 ppm in andrographolide to 4.69 ppm and to 3.61 and 3.75 ppm, respectively, in **AG-B2**. The structure of **AG-B2** was then determined to be 15-*seco*-andrographolide, arising from the hydrolysis of the lactone ring under basic condition (Fig. 4). The presence of 15-OH in the opened lactone ring caused shielding effects on H-14 and H_2-15 and thus shifted their signals upfield as compared to those in andrographolide.

Upon the degradation of andrographolide under acidic and basic conditions, 8,9-didehydroandrographolide (**AG-A**), 14-deoxy-11,12-didehydroandrographolide (**AG-B1**) and 15-*seco*-andrographolide (**AG-B2**) were isolated and structurally determined. Two new degradation products, 8,9-didehydroandrographolide and 15-*seco*-andrographolide were reported here for the first time. Furthermore, the identification of the degradation products could be useful in helping establish degradation pathways and in providing an integral part of the information to be assessed in manufacturing and in the analytical method development and validation.

3. Experimental

3.1. Chemicals and reagents

Andographolide was obtained from Sigma (USA). Hydrochloric acid and sodium hydroxide pellets were from RCI Labscan (Thailand). All chemicals and solvents used were of analytical grade.

3.2. Instrumentation

High resolution mass spectrometry was performed in ESI mode on a BRUKER micrOTOF mass spectrometer. NMR spectra were recorded on a Bruker Avance-III 400 and 500 spectrometers, using $CDCl_3$ as solvent for **AG-A** and **AG-B1**, and CD_3OD as solvent for andrographolide and **AG-B2**. UV spectra in the range of 200-400

nm were recorded using a HPLC-DAD system consisted of an Agilent 1260 series pump, a diode-array detector (DAD) and a Chemstation software version A.08.01 (Agilent Technologies, USA). A reversed-phase column, 75 mm x 4.6 mm packed with 3.5 µm, Zorbax Eclipes XDB-C18 modified silica (Agilent Technologies, USA) and a guard column, 20 mm x 3.9 mm packed with 5 µm, C18 (Agilent Technologies, USA) were used at ambient temperature and an isocratic elution was carried out with acetonitrile-water (25:75, *v/v*) at a flow rate of 1 mL min^{-1} and detection at 254 nm. All solutions were filtered through a 0.45 µm Chrom Tech Nylon-66 filter before injection. 20 µL of each solution was subjected to the HPLC analysis.

3.3. Degradation procedure

For acidic degradation, 100 mg of andrographolide was dissolved in 10 mL of ethanol and 10 mL of 4 N HCl was added. The solution was refluxed at 100 °C for 2 h. Excess water (500 mL) was added to the reaction mixture and extracted with ethyl acetate (100 mL x 3). The ethyl acetate extracts were combined and evaporated under reduced pressure to dryness. For basic degradation, 100 mg of andrographolide was dissolved in 10 mL of ethanol and 10 mL of 0.05 N NaOH was added and stirred at room temperature for 10 min. The reaction mixture was worked up as before. The aqueous and ethyl acetate extracts were evaporated under reduced pressure to give dried residues for further purification.

3.4. Isolation and characterization of degradation products

The ethyl acetate extract from acidic condition (90 mg) was subjected to a preparative silica gel TLC plate (2.0-mm thickness, Merck, Germany) using dichloromethane-toluene-methanol (6:3:1, *v/v/v*) as eluent to yield **AG-A** (16 mg). The ethyl acetate extract from basic condition (40 mg) was also purified by a preparative TLC using the same eluent as above to afford **AG-B1** (7 mg). The aqueous residue (90 mg) from basic degradation was chromatographed over a silica gel (15-40 µm, Merck, Germany) column using dichloromethane-toluene-methanol-formic acid (6:2:1:0.1, *v/v/v/v*) as eluent and **AG-B2** (4 mg) was obtained as white amorphous precipitate. The structural characterization of isolated degradation products was carried out by NMR and MS spectroscopy.

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Conflicts of interest: None reported.

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