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Short communication

Isolation and structure elucidation of degradation products in the potential anticancer drug PAC-1

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ABSTRACT

PAC-1 was subjected to acid degradation conditions at 80 °C for 10 h. Four unknown degradation products were isolated from PAC-1 by semi-preparative high performance liquid chromatography using isocratic elution conditions. Based on the ESI-MS and NMR spectral data the structures of these four degradation products were characterized as 2-allyl-6-((E)-((E)-(2-hydroxy-3-(2-hydroxypropyl)benzylidene)-hydrazono)methyl)phenol, 2-hydroxy-3-(2-propenyl)-[[2-hydroxy-3-(2-propenyl)phenyl]methylene]-hydrazone, 6,6′-(1E,1′E)-hydrazine-1,2-diylidenebis(methan-1-yl-1-ylidene)bis(2-(2-hydroxypropyl)-phenol) and 2-hydroxy-3-(2-hydroxypropyl)benzaldehyde.

1. Introduction

A small molecule compound PAC-1, (4-benzyl-piperazin-1-yl)-acetic acid (3-allyl-2-hydroxy-benzylidene)-hydrazine (Fig. 1), was discovered by Hergenrother et al. [1]. In pharmacology studies, PAC-1 can directly activate the conversion of procaspase-3 to caspase-3, which will become a new anticancer strategy [1–3].

Previously, our group evaluated the potential of PAC-1 as a drug, the PK properties of PAC-1 after oral administration and intravenous in rat were studied using HPLC [4]. Also, the metabolic profile of PAC-1 was investigated using liquid chromatography–mass spectrometric techniques, and some metabolites were detected and identified [5]. The degradation studies play an important role in the drug development process [6,7]. This paper describes the isolation and characterization of unknown degradation products of PAC-1.

2. Experimental

2.1. Materials and reagents

PAC-1 (>98% pure) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade hydrochloric acid was obtained from Kermel Chemical Reagent Ltd. (Tianjin, China). HPLC grade methanol was purchased from Fisher Scientific (NJ, USA). DMSO was purchased from Sigma–Aldrich, USA. Other chemicals were of analytical grade. Milli-Q water was used throughout the study.

2.2. Preparation of degraded sample

A solution of PAC-1 (5 mg/ml) was prepared by dissolving known amounts of the compound in methanol. Subsequently, the PAC-1 solution (1 mg/ml) was prepared by diluting fivefold with 5N hydrochloric acid. The solution was subjected to solvent–solvent liquid extraction with ethyl acetate. After exposure to the acid conditions at 80 °C for 10 h, the compounds were extracted into organic layer and the aqueous layer was discarded. The ethyl acetate fractions were pooled together and concentrated under high vacuum on a rotavapor. The residue obtained was reconstituted with methanol to achieve the maximum solubility. 2 g of PAC-1 was used throughout the study.

2.3. Semi-preparative high performance liquid chromatography

Isolation of degradation products was carried out on a semi-preparative HPLC system consisting of a Shimadzu LC-8A pump connected to semi-pack PRO-ODS (250 mm × 20 mm; 15 μm, Shimadzu, Kyoto, Japan), a FRC-10A fraction collection trap and a SPD-10A detector (Kyoto, Japan). Methanol–water (95:5, v/v),

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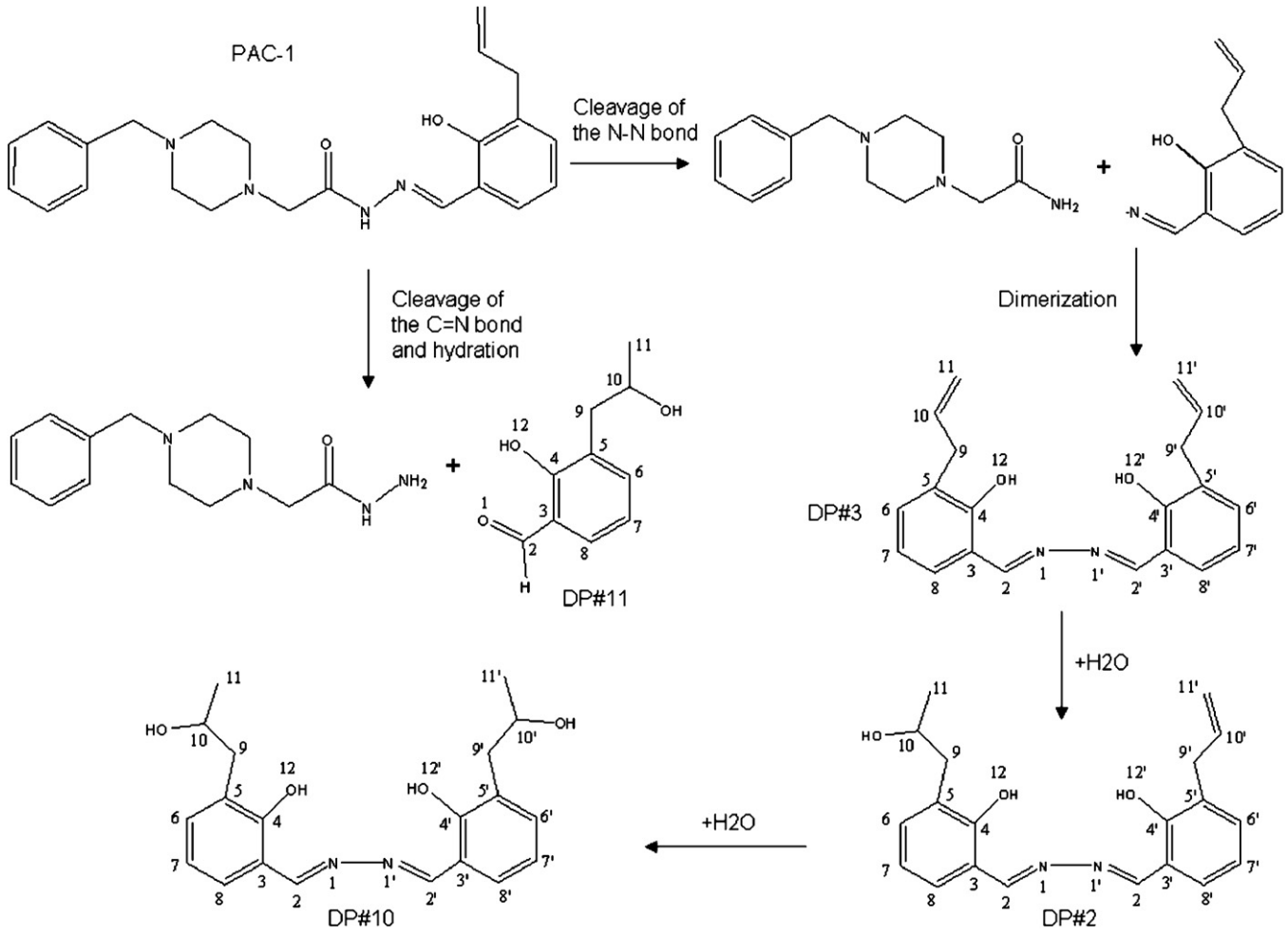

Fig. 1. The proposed degradation pathway of PAC-1 (DP: degradation product).

methanol–water (75:25, v/v) and methanol–water (60:40, v/v) were used as mobile phase at room temperature. The flow rate was 10 ml/min and the wavelength of detection was 281 nm. The injection volume was 1 ml.

2.4. NMR spectroscopy

The ^{1}H and ^{13}C NMR spectroscopy were recorded on Bruker 300 MHz and 600 MHz nuclear magnetic resonance spectrometer respectively, using DMSO-d_6 as solvent and TMS as internal standard. The sample concentration was approximate 8 mg/ml.

2.5. FT-IR spectroscopy

The IR spectra were recorded in the solid state as KBr dispersion using a Bruker IFS-55 spectrophotometer.

2.6. Mass spectrometry

Samples (about 100 ng/ml) were prepared in methanol before injecting into mass spectrometer. Mass data were obtained using a 2010EV Shimadzu quadrupole mass spectrometer (Kyoto, Japan). The analytes were ionized using an ESI source in positive ion mode under the following source conditions: nebulizing gas 1.5 l/min; drying gas 2.0 l/min; CDL temperature 250 °C; heat block temperature 200 °C, and a detector voltage 1.60 kV. The other parameters were fixed as the tuning file.

3. Results and discussion

3.1. Isolation of degradation products by semi-preparative HPLC

It was difficult to separate the degradation products in one semi-preparative HPLC analysis because of the disparity of polarity. The isolation of degradation products was achieved by means of several different isocratic elutions.

Isocratic chromatography was performed firstly using the mobile phase of methanol–water (95:5, v/v) at ambient temperature. In this step, three fractions were collected. A representative chromatogram is shown in Fig. 2A. The fractions were pooled together and concentrated on a rotaevaporator under vacuum respectively. The residues obtained were reconstituted with methanol. The degradation products named #2 and #3 were obtained from purification of fractions 2 and 3. Fraction 1 was subjected to an isocratic elution using a mobile phase of methanol–water (75:25, v/v). As shown in Fig. 2B, four fractions were obtained named from fractions 4 to 7. The treatment procedure for fraction solutions was same as that mentioned above. Fraction 4 was subjected to isocratic chromatography using a mobile phase of methanol–water (60:40, v/v) and two fractions 8 and 9 were collected. The degradation products named #10 and

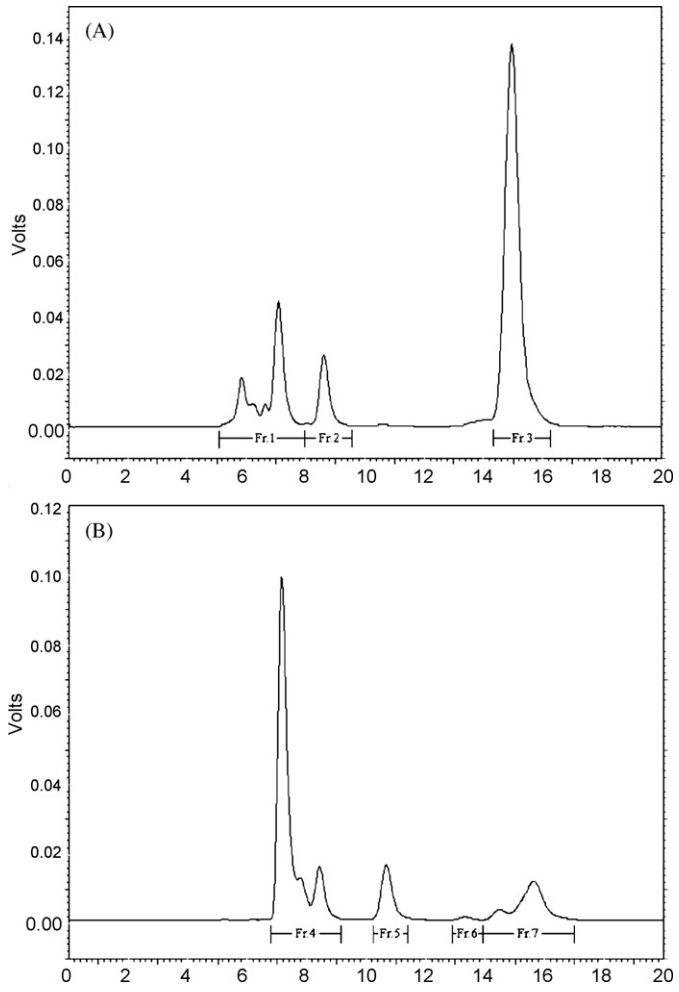

Fig. 2. The chromatograms of degraded sample of PAC-1 isolated by isocratic chromatography using a mobile phase of methanol–water (95:5, v/v) (A) and fraction 1 isolated by isocratic chromatography using a mobile phase of methanol–water (75:25, v/v) (B).

#11 were obtained from purification of fractions 7 and 8 separately.

3.2. Structure elucidation of degradation products #2, #3 and #10

The electrospray ionization (ESI) mass spectrum (Fig. 3A) exhibited a molecular ion peak at 339 $[M+H]^+$ and an ion at 361 $[M+Na]^+$ supporting a molecular weight of 338 for degradation product #2. The ^1H and ^{13}C NMR data are given in Table 1 and the assignments correspond to the numbers in the structure shown in Fig. 1. 10 protons and 10 carbon signals, 11 protons and 10 carbon signals are observed from the ^1H and ^{13}C NMR spectra of degradation products #3 and #10, respectively. The number of protons and carbon signals were not in agreement with the molecular ion peak at *m/z* 321 $[M+H]^+$ and 357 $[M+H]^+$. Based on the MS fragments, it is proposed that the structures of degradation products #3 and #10 are symmetric. The ^1H and ^{13}C NMR spectra only showed the half of their structures. The ^1H and ^{13}C NMR data are given in Tables 1 and 2. The assignments correspond to the numbers in the structure shown in Fig. 1. In IR spectrum of degradation product #3, the characteristic absorption bands are 3427 (O–H stretch), 1600, 1582 (C=C and C=N stretch), 1380 (C–H bend), 919 (N–N stretch). In the MS spectrum (Fig. 3A) of degradation product #2, the ion at *m/z* 321 corresponds to loss of H_2O from the $[M+H]^+$ ion at *m/z* 339. The ion at *m/z* 162 is generated by cleavage of the N–N bond and further lost of vinyl moiety to yield the ion at *m/z* 132. From the MS spectrum of degradation product #3 (Fig. 3B), the ion at *m/z* 187 corresponds to loss of C_9H_9O from the $[M+H]^+$ ion. The formation of ions at *m/z* 162 and 132 is analogous to degradation product #2. For degradation product #10 (Fig. 3C), the ion at *m/z* 339 corresponds to loss of H_2O from the ion *m/z* 357 $[M+H]^+$ and further lost another H_2O to form the ion at *m/z* 321.

From the above spectral information, the structures of degradation products #2, #3 and #10 were confirmed as 2-allyl-6-((E)-((E)-(2-hydroxy-3-(2-hydroxypropyl)benzylidene)hydrazono)-methyl)phenol, 2-hydroxy-3-(2-propenyl)-[[2-hydroxy-3-(2-propenyl)phenyl]methylene]hydrazone and 6,6′-(1E,1′E)-hydrazine-1,2-diylidenebis(methan-1-yl-1-ylidene)bis(2-(2-hydroxypropyl)phenol).

3.3. Structure elucidation of degradation product #11

Degradation product #11 exhibits a molecular ion peak at 181 $[M+H]^+$ and an ion at 203 $[M+Na]^+$ in the MS spectrum (Fig. 3D) supporting a molecular weight of 180. The ion at *m/z* 153 corresponds to loss of CHO from the $[M+H]^+$ ion. The ^1H and ^{13}C NMR data are given in Table 2 and the assignments correspond to the numbers in the structure shown in Fig. 1. The structure of degradation product DP#11 was confirmed as 2-hydroxy-3-(2-hydroxypropyl)benzaldehyde.

3.4. Proposed degradation pathway of PAC-1

The structure of PAC-1 contains an unstable chemical acid hydrazide group. The degradation product #3 is generated by dimerization of the 3-allyl-2-hydroxy-N-benzylidene moiety after the cleavage of the N–N bond of PAC-1. #2 is formed by hydration of #3 at the allyl moiety and further hydrated at the other allyl side

Table 1
^1H and ^{13}C NMR assignments for degradation product (DP) #2 and #3.

Position[a]	DP #2: ^1H (ppm)	DP #2: ^{13}C (ppm)	DP #3: ^1H (ppm)	DP #3: ^{13}C (ppm)
1, 1′	–	–	–	–
2, 2′	9.04/2H, s	166.50	8.84/2H, s	166.55
3, 3′	–	118.55; 118.47	–	118.47
4, 4′	–	159.02; 158.93	–	158.60
5, 5′	–	128.10; 129.16	–	129.16
6, 6′	7.45/2H, m	136.30; 134.74	7.34/2H, d	134.76
7, 7′	6.93/2H, m	120.45; 120.57	6.92/2H, t	120.58
8, 8′	7.30/2H, m	132.18; 132.29	7.26/2H, d	132.19
9, 9′	2.73/2H, m; 3.39/2H, d	40.55; 34.68	3.44/4H, d	34.68
10, 10′	3.94/1H, m; 5.96/1H, m	68.21; 137.73	6.02/2H, m	137.73
11, 11′	1.06/3H, d; 5.05/2H, m	23.13; 115.96	5.05/4H, d	115.94
12, 12′	11.69/1H, s; 11.57/1H, s	–	11.68/2H, s	–

[a] According to Fig. 1: s, singlet; d, doublet; t, triplet; m, multiplet.

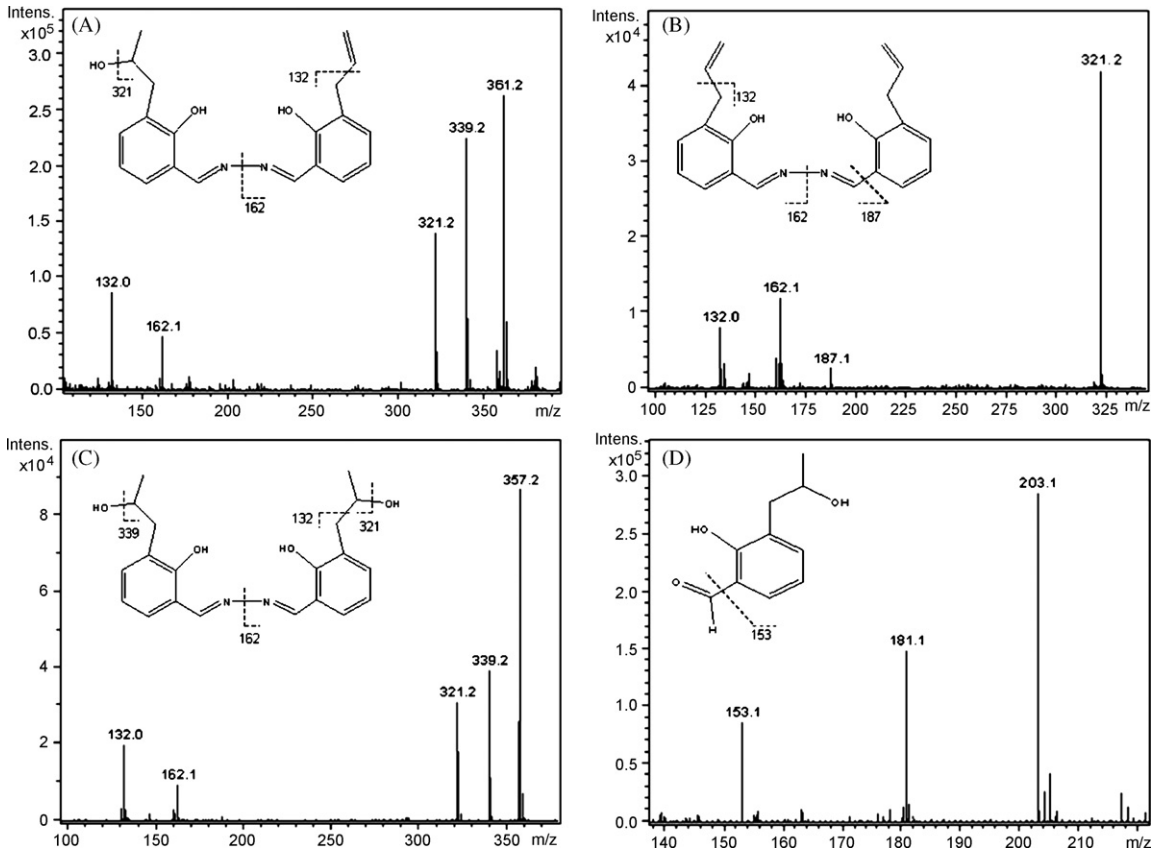

Fig. 3. The MS spectra of degradation products #2 (A), #3 (B), #10 (C) and #11 (D).

Table 2
^{1}H and ^{13}C NMR assignments for degradation product (DP) #10 and #11.

Position[a]	DP #10: ^{1}H (ppm)	DP #10: ^{13}C (ppm)	DP #11: ^{1}H (ppm)	DP #10: ^{13}C (ppm)
1, 1′	–	–	–	–
2, 2′	9.02/2H, s	164.93	10.06/1H, s	196.38
3, 3′	–	114.40	–	121.26
4, 4′	–	156.30	–	159.09
5, 5′	–	127.45	–	127.95
6, 6′	7.42/2H, m	135.07	7.45/1H, dd	138.57
7, 7′	6.88/2H, m	119.02	6.96/1H, t	119.54
8, 8′	7.27/2H, m	130.43	7.59/1H, dd	130.75
9, 9′	2.69/4H, m	29.01	2.68/2H, m	38.91
10, 10′	3.92/2H, m	66.01	3.91/1H, m	65.98
11, 11′	1.04/6H, d	23.48	1.04/3H, d	23.30
12, 12′	11.66/2H, s	–	11.63/1H, s	–

[a] According to Fig. 1: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

chain to form #10. The C=N bond of PAC-1 is also subject to the degradation and cleaved then hydrated to form #11. The proposed degradation pathway of PAC-1 is shown in Fig. 1.

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