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Identification of the principal circulating metabolite of a synthetic 5,4′-diaminoflavone (NSC 686288), an antitumor agent, in the rat

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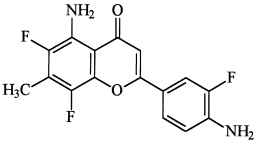
Abstract

During the course of our study to develop analytical methodology for quantitating the investigative antitumor agent 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (DAF; NSC 686288) in plasma, a significant concentration of a metabolite was observed in a post-dosed rat. The results of electron-ionization (EI) mass spectrometric analysis of the metabolite suggested that *N*-acetylation had occurred, but, interestingly, that only one of the compound's two primary amino groups had been transformed. Comparing the mass spectra and gas chromatographic retention times of a mono-acetylated sample of DAF and that of the metabolite showed both to be the same. A retro-Diels–Alder (RDA) fragmentation of the B ring of DAF results in formation of two abundant product ions, each retaining one of the amino groups. The EI mass spectrum of mono-*N*-acetamido-d_3 DAF shows loss of ketene-d_2, leading to formation of an –NHD group. The ensuing RDA fragmentation easily identifies which of the two product ions contains the deuterium, thereby allowing us to assign the site of *N*-acetylation as the amino group on ring C (the 4′ position) of DAF.

Keywords: Retro-Diels–Alder fragmentation; 5,4′-Diaminoflavone; NSC 686288

1. Introduction

Flavonoids are ubiquitous plant pigments, many of which possess potent and diverse biological activities. 5-Amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (DAF; NSC 686288; **1**) [1] (Fig. 1) is a highly function-



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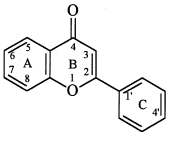

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Fig. 1. Chemical structures of 5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (DAF; NSC 686288) (**1**) and flavone (**2**).

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alized synthetic analog of flavone (**2**). DAF has been shown to have significant activity against the human breast cancer cell line MCF-7 [1], thus prompting the National Cancer Institute to pursue its preclinical development. Thus, our efforts focused on characterizing the disposition of DAF.

In the course of our study to develop analytical methodology for quantitating DAF in plasma, a significant, time-dependent concentration of a metabolite was observed in the post-dosed rat. The results of electron-ionization (EI) mass spectrometric analysis of the metabolite suggested that *N*-acetylation had occurred, but, interestingly, that only one of the two primary amino groups of DAF had been transformed. Using simple spectroscopic techniques we were able to distinguish between two primary amino groups that are situated in dissimilar molecular environments. In this paper we describe our efforts to determine the site of *N*-acetylation of DAF by analyzing and comparing EI-induced fragmentation of the metabolite and of a deuterated analog of the metabolite.

2. Experimental

2.1. Chemicals

5-Amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (DAF; NSC 686288) was obtained from the Pharmaceutical Resources Branch, DTP, DCTDC, NCI (Bethesda, MD, USA). Unless noted to the contrary, all additional solvents and reagents were obtained from commercial sources in grades appropriate for direct use.

2.2. Gas chromatography–mass spectrometry

Analyses were performed using a 5890 Series II gas chromatograph equipped with a capillary inlet and a 5971A mass selective detector, controlled through a DOS-series MS CHEMSTATION (Hewlett-Packard, Palo Alto, CA, USA). Plasma samples were prepared by addition of acetonitrile to denature and precipitate proteins, followed by centrifugation, separation and concentration of the supernatant, followed by reconstitution of the residue in *tert*.-butylmethyl ether. Separations were performed on a 15 m×0.25 mm fused-silica capillary column wall-coated with 0.25 μm DB-1 cross-linked dimethylpolysiloxane (J&W Scientific, Folsom, CA, USA). Helium was employed as the carrier gas at a linear velocity of 27.5 cm/s (pentane, 60°C). Temperatures were 250°C at the injection port and 280°C at the transfer line to the detector. Injections were made at an initial oven temperature of 150°C. The oven temperature was held isothermally at 150°C for 2 min, then increased linearly to 280°C at 10°C/min. The final temperature was maintained for 5 min to desorb less volatile components of the sample. Mass spectral detection (EI, 70 eV) was performed by scanning ions between m/z 50 and 500 at the rate of about 0.77 scan/s. Data were collected between 5 and 20 min post-injection. Nominal resolution and accurate mass measurements were obtained using a VG 70–70 mass spectrometer and data system (Vacuum Generators, Manchester, UK). Samples were introduced into the ion source via the solids' direct exposure (high temperature) probe. Accurate mass measurements (EI, 70 eV) were performed on selected m/z values of samples at a static resolution of ~5000, using perfluorokerosene (PFK) as an internal standard.

2.3. Synthesis of the metabolite, 5-amino-2-(4-acetamido-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one

To 50.0 mg (0.156 mmol) of DAF was added a mixture of acetic anhydride in pyridine (1:1, v/v). The resulting mixture was briefly stirred and then left at ambient temperature for 2 h. Excess reagents were evaporated under a stream of nitrogen gas. Methanol was added to the residue and the resulting mixture was sonicated for 30 min. The methanol was evaporated under a stream of nitrogen gas. The addition, sonication, and evaporation of methanol was repeated and the resulting residue was dried under vacuum (20 mmHg) at ambient temperature. The residue was purified by collecting, concentrating, and combining fractions from silica-gel column chromatography using a mobile phase of hexane–ethyl acetate (25:75, v/v). Following analysis by GC–MS to ensure product purity, collected and concentrated fractions were combined to give 42.0 mg of crys-

talline 5-amino-2-(4-acetamido-3-fluorophenyl)-6,8-difluoro-7-methyl-4H-1-benzopyran-4-one (0.116 mmol, 74%) m.p. 259.0–259.2°C. ^{1}H-NMR (DMSO-d_6): δ 10.01 (bs,1H (exchangeable)), 8.24 (t,1H), 7.88 (dd,1H), 7.81 (dd,1H), 7.01 (bs,2H (exchangeable)), 6.89 (s,1H), 2.28 (s,3H), 2.14 (s,3H). Mass spectrum (EI, 70 eV) *m*/*z* (% relative abundance): 362 ($M^{+\cdot}$, 46), 320 (100), 291 (21), 185 (43), 135 (17), 129 (35). HRMS: *m*/*z* 362.0869 (calculated for $C_{18}H_{13}N_2O_3F_3$: 362.0878).

3. Results and discussion

3.1. Identification of DAF and its principal metabolite in plasma

In a rat, dosed intravenously with DAF, the plasma concentration of DAF, after an initial rapid increase, was seen to decline as a function of time, with a concomitant increase in plasma concentration of an unknown entity which had not been observed in the pre-dosed animal.

The total ion current chromatogram obtained from GC–MS analysis of a plasma sample obtained 2 h after administration of DAF to a rat is shown in Fig. 2. The extracted ion chromatogram for the molecular ion of DAF (*m*/*z* 320) shows that two components in the plasma mixture possess ions at *m*/*z* 320 (at retention times of 13.5 and 15.3 min). The mass spectra of these two components are displayed in Fig. 3. Analyzed separately, the GC retention time and mass spectrum of authentic DAF shows the 13.5-min GC peak to be DAF. The mass spectrum of the second component (GC retention time of 15.3 min) is strikingly similar to the mass spectrum of DAF, with the exception of an abundant *m*/*z* 362 which is found unique to the plasma extract (Fig. 2).

The similarities in spectral features and the apparent addition of 42 a.m.u. to DAF to form the metabolite strongly suggest that the metabolite is the result of *N*-acetylation.

3.2. Isolation and preliminary structure elucidation of the metabolite

The metabolite was isolated by reversed-phase liquid chromatographic fractionation and subjected to analysis by direct probe EI-MS. Accurate mass analysis of the isolated metabolite verified a molecular formula consistent with an acetyl functionality having been added to DAF (calculated for $C_{18}H_{13}N_2O_3F_3$: 362.0878; found: 362.0869). Frequently observed in EI mass spectra is the neutral loss of ketene ($CH_2C{=}O$, 42 a.m.u.) from an acetamido functionality when the amine portion of the amide is aryl [2]. By accurate mass analysis, the ions at *m*/*z* 320 in mass spectrum of the metabolite were shown concordant with the neutral loss of ketene from the molecular species (calculated for

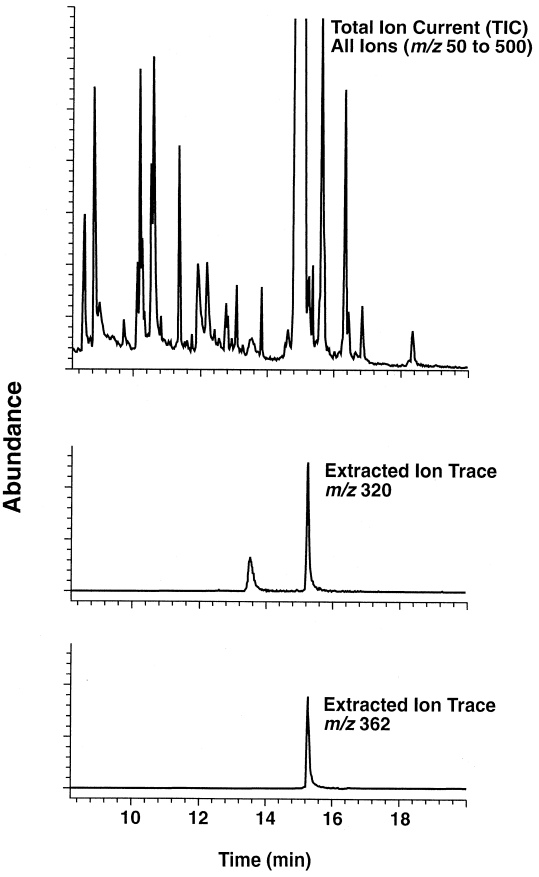

Fig. 2. Total ion current (TIC) chromatogram (A) resulting from GC–MS analysis of a plasma sample obtained from a rat 2 h after i.v. administration of DAF (25 mg/kg). Extracted ion trace of *m*/*z* 320 shows two GC peaks, the 13.5-min peak identified as DAF, and the 15.3-min peak being a metabolite of DAF. The second extracted ion trace shows the uniqueness of *m*/*z* 362 to the metabolite.

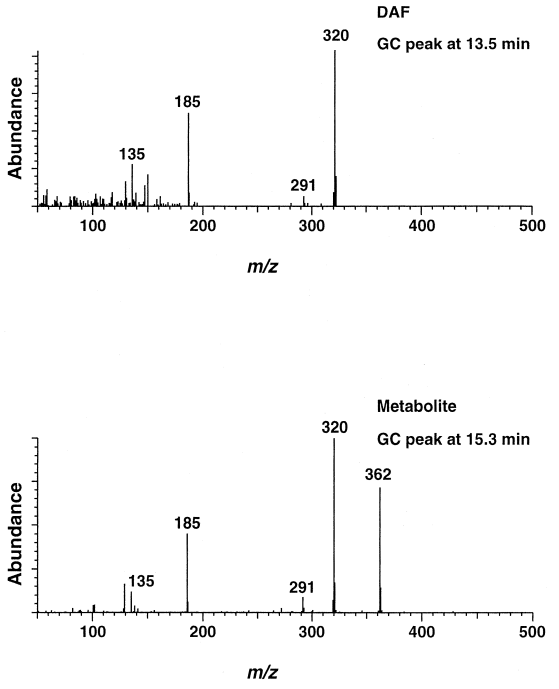

Fig. 3. Electron-ionization (70 eV) mass spectra of the 13.5-min GC peak (DAF) and the 15.3-min GC peak (the metabolite).

$C_{16}H_{11}N_2O_2F_3$ ($M-C_2H_2O$): 320.0773; found: 320.0769).

3.3. N-Acetylation of authentic DAF and GC–MS comparison with the metabolite

Chemical treatment of DAF with acetic anhydride and pyridine produced a simple mixture, the most abundant component of which possessed a GC retention time (15.3 min) and mass spectrum that were identical to those of the isolated metabolite. The remaining structural feature to be determined was assigning one of the two amino groups of DAF as the site of *N*-acetylation.

The synthesized mono-*N*-acetylated DAF was isolated as a high melting, yellow solid (260.4–260.9°C) by silica-gel column chromatography. Unable to grow crystals suitable for X-ray structure analysis and also recognizing the potential complexity of analysis by NMR, the solution to determining the site of *N*-acetylation was sought from MS methods.

3.4. Determining the site of N-acetylation on DAF

3.4.1. Retro-Diels–Alder decomposition

Electron-ionization-induced multibond fragmentation of cyclohexene and cyclohexene derivatives is the reverse or retro direction [3,4] of the well-known Diels–Alder reaction. The retro-Diels–Alder (RDA) decomposition is recognized in the mass spectra of many compounds, including the flavonoids [5]. Not unexpectedly, the molecular ion of DAF (*m*/*z* 320) likewise undergoes an RDA decomposition (Fig. 4). The structural integrity of rings A and C are preserved during the decomposition, whereas ring B fragments to give two ions of masses 135 and 185. Based on structural considerations, only a small energy difference between the ionization potentials of the two fragments is expected. Thus, a distribution of charge between the two fragments is expected instead of preferential retention of charge to one fragment [6]. This is experimentally confirmed, as ions at both *m*/*z* 135 (34%) and *m*/*z* 185 (79%) are present in the mass spectrum of DAF.

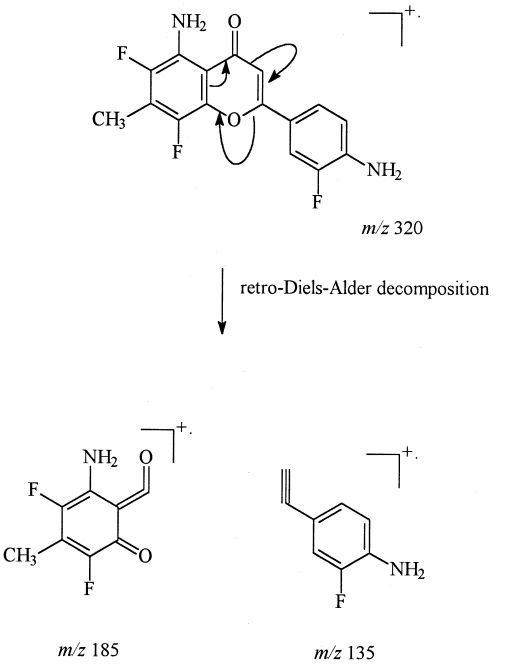

Fig. 4. Electron-ionization induced retro-Diels–Alder decomposition of DAF leads to formation of two fragments, one having a mass of 135 a.m.u., and the other 185 a.m.u.

3.4.2. Mass spectral loss of ketene

Following an initial loss of ketene, the metabolite is then observed to undergo fragmentation by a RDA decomposition, leading to formation of the ions at m/z 135 (11% relative abundance) and m/z 185 (44%). It would therefore seem a simple matter to discern the location of the acetyl group to ring A or ring C if the RDA decomposition occurred directly from the molecular ion, leading to either m/z 177 (135+42) or m/z 227 (185+42). Unfortunately, both ions are of such low abundance as to render them indistinguishable from background, a condition which also serves to indicate that the RDA decomposition is sequential to, not competitive with, the process of losing ketene.

3.4.3. Mass spectral consequence of labelling the acetyl with deuterium

Since the loss of ketene results in the transference of one of the hydrogen atoms of the acetyl group to the participating nitrogen center, acetyl-d_3 would be expected to expel C^2H_2CO, leaving the third deuterium atom on the participating nitrogen center (Figs. 5 and 6). The significance of having substituted a deuterium atom for a hydrogen atom is now observed in the mass spectrum since ion fragments which now possess the deuterium atom appear at an m/z value 1 a.m.u. higher. Therefore, subsequent to the RDA decomposition, observing the presence of ions at m/z 136 and m/z 185 would indicate the deuterium was substituted into the m/z 135 ion fragment, thereby identifying the site of *N*-acetylation as ring C (Fig. 5). Conversely, observing the presence of ions at m/z 135 and m/z 186 would indicate the deuterium was substituted into the m/z 185 ion fragment, thereby identifying the site of *N*-acetylation as ring A (Fig. 6).

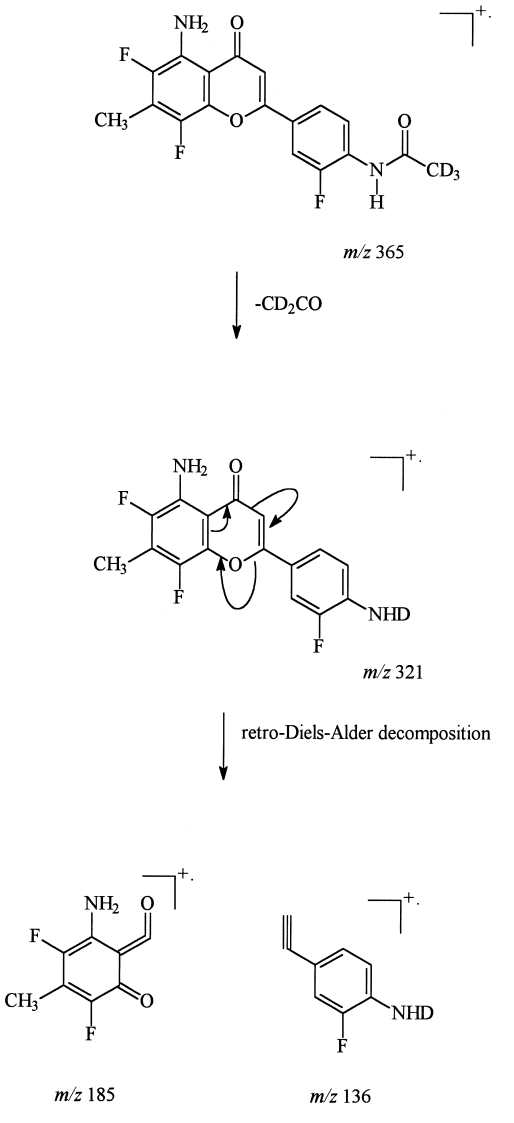

Fig. 5. Loss of perdeuteroketene from DAF having been acetylated on the C ring predicts formation of ions at m/z 136 and 185.

3.4.4. Identification of the site of N-acetylation by deuterium labelling and mass spectrometry

DAF was treated with acetic anhydride-d_6 and pyridine, and the resulting mixture was analysed by GC–MS. The molecular ion appeared at m/z 365 (Fig. 7), consistent with incorporation of three deuterium atoms. Loss of ketene-d_2 resulted in formation of ions at m/z 321, indicative that one deuterium atom had been retained. Following loss of ketene-d_2, the RDA decomposition produced prominent ions at m/z 136 and m/z 185, thereby demonstrating the original site of *N*-acetylation to be on ring C.

4. Conclusions

In vivo *N*-acetylation was recognized by Cohn in 1893 when he was able to isolate and identify 3-

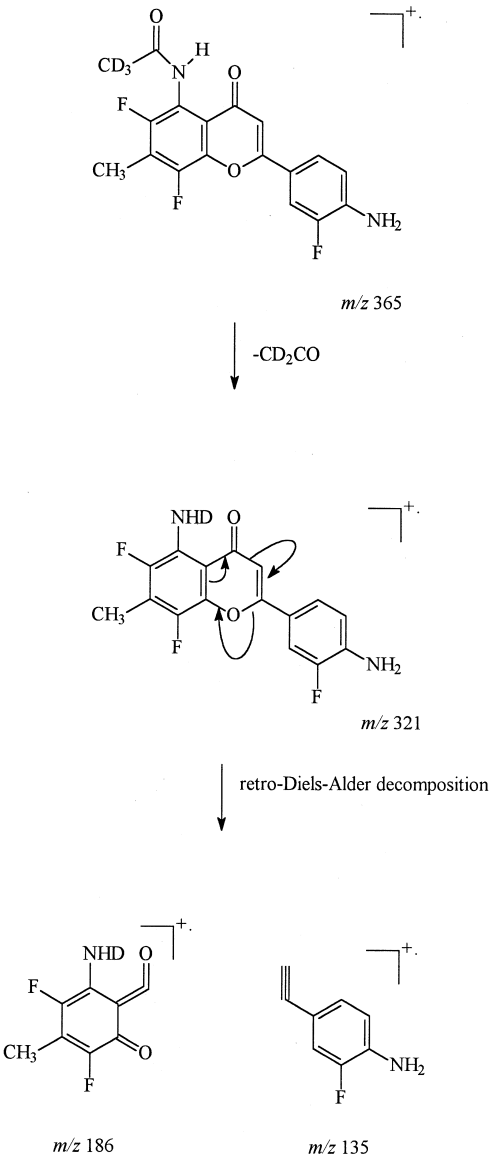

Fig. 6. Loss of perdeuteroketene from DAF having been acetylated on the A ring predicts formation of ions at m/z 135 and 186.

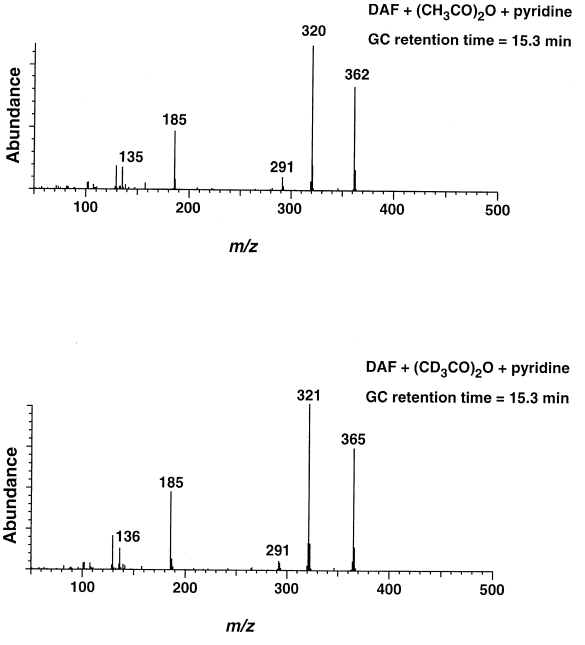

Fig. 7. Electron-ionization (70 eV) mass spectra of DAF treated with acetic anhydride in pyridine (top) and DAF treated with perdeutero-acetic anhydride in pyridine (lower). The molecular ion of the acetylated species is shifted 3 a.m.u. when perdeuterated: loss of perdeuteroketene (and retention of one deuterium atom) is evidenced by observation of ions at m/z 321: the presence of the ions at m/z 136, and not m/z 135 in the deuterated derivative of DAF support the idea of in vivo *N*-acetylation having occurred on the C ring of DAF (see Fig. 5).

acetamidobenzoic acid from the urine of a rabbit subsequent to oral administration of 3-nitrobenzaldehyde [7]. An awareness began to evolve shortly thereafter that significant differences in *N*-acetylation activity exists between species [8]. This has led to the elucidation of the importance of intraspecies polymorphism in genes for drug-metabolizing enzymes (including *N*-acetyltransferases) [9]. *N*-Acetylation is now found to be a common metabolic transformation of xenobiotics and is designated a phase II metabolic pathway [10]. In this paper, such biotransformation has been shown for DAF (**1**) in the rat, and furthermore, that this transformation may demonstrate a high degree of selectivity. Analytical methodology and preliminary pharmacokinetics of DAF will be the subjects of future reports.

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