ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 327–334

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

The isolation and identification of a toxic impurity in XP315 drug substance

Kenneth W. Sigvardson [a,*], Stephen P. Adams [b], T. Bradford Barnes [b,1], Karl F. Blom [c], Joseph M. Fortunak [d,2], Michael J. Haas [c], Kathleen L. Reilly [a], Arnold J. Repta [a], Gregory A. Nemeth [c]

[a] *DuPont Pharmaceuticals Company, Experimental Station, PO Box 80353, Wilmington, DE 19880-0353, USA*
[b] *DuPont Pharmaceuticals Company, 1094 Elkton Road, Newark, DE 19714, USA*
[c] *DuPont Pharmaceuticals Company, Experimental Station, PO Box 80500, Wilmington, DE 19880-0500, USA*
[d] *DuPont Pharmaceuticals Company, Process Research Facility, S1, Deepwater, NJ 08023-0999, USA*

Received 3 January 2001; received in revised form 21 June 2001; accepted 26 June 2001

Abstract

In early safety assessment studies with the experimental anti-neoplastic drug XP315, a toxic reaction was observed in dogs immediately after intravenous (iv) infusion. The reaction was characterized by severe erythema around the ears, eyes, face and body; ocular hyperemia; head shaking; swelling around the eyes, face, paws, head, neck and legs; scratching; and reddened gums, which lasted several hours after dosing. By fractionating the drug substance using preparative HPLC and then infusing the residues into dogs by iv, this reaction was traced to an impurity in the drug substance. Following the preparative isolation of the toxic impurity, characterization was performed using a combination of NMR and mass spectral methods. The proposed impurity was found to be structurally related and nearly twice the molecular weight of XP315, resulting from a dimerization by ring fusion of two 3-aminonaphthalene fragments during the synthetic process. This paper details the steps taken to isolate the toxic impurity and characterize its structure using off-line methods. © 2002 Published by Elsevier Science B.V.

Keywords: Preparative chromatography; Reversed-phase liquid chromatography; Drug substance; Impurity characterization; Nuclear magnetic resonance spectroscopy; Mass spectrometry; XP315

1. Introduction

In the early development of drug candidates, the drug substance is administered to animals to identify any potential toxicity of the drug and any impurities present. Guidance for controlling impurity levels in drug substances has been developed by the International Conference on Harmonization (ICH) [1]. In the early stages of

* Corresponding author. Fax: +1-302-695-1204.
E-mail address: ken.w.sigvardson@dupontpharma.com (K.W. Sigvardson).
[1] Present address: Cephalon, Inc., 145 Brandywine Parkway, West Chester, PA 19380, USA.
[2] Present address: Abbott Laboratories, Inc., Pharmaceutical Products Division, North Chicago, IL 60064, USA.

0731-7085/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0731-7085(01)00550-7

research and development, the synthesis of the drug substance is not well defined and impurity profiles may change significantly from lot to lot. Since a synthetic impurity may result in adverse findings that might be attributed to the drug itself, one approach is to use drug substance of high purity in initial animal toxicity studies to minimize this risk. Later, during the drug development process, more extensive animal toxicity studies are performed with drug substance that is representative of the final synthetic process.

Normally, synthetic impurities are discovered during routine HPLC analysis of the drug substance [2]. An impurity profile of a synthetic drug may require the use of complementary chromatographic methods such as HPLC/UV and LC/MS to permit the observation of non-UV absorbing synthetic impurities. Impurities greater than 0.1% (w/w) are typically subjected to structural elucidation and specification limits are adopted to control their concentrations. Structural elucidation is usually performed using combination techniques such LC-MS and LC-NMR or by using preparative HPLC to first isolate the impurity for spectroscopic characterization [3–5].

The experimental anti-cancer drug, XP315, (R,R) - 1 - [2 - (6 - azaphenanthrene - 1,10 - dicarboximido)propylamino] - 2 - [2 - (3 - nitronaphthalene-1,8-dicarboximido)-propylamino]ethane dihydromethanesulfonate Fig. 1, is an unsymmetrical bisimide [6]. In the initial stage of XP315 development, preliminary genetic toxicity and dose ranging studies in dogs were completed using a purified small-scale sample of XP315 (Lot XP315-000). In more extensive toxicity studies in dogs using a new batch of XP315 (Lot XP315-001), a toxic reaction characterized by erythema and edema was observed following iv infusion. Due to the presence of several additional impurities in XP315-001 not observed in XP315-000, an investigation was undertaken which immediately focused on impurities in the drug substance. Considering that any new impurity might have been responsible for the observed toxicity, an approach was developed to minimize the number of steps required to identify the toxic impurity. This step-by-step approach required the use of limited iv dosing in dogs to identify the presence of the causative agent(s) in the drug substance. Therefore, a combination of preparative HPLC, limited dosing in dogs, NMR and mass spectrometry led to the assignment of a structure for the main toxic impurity.

The preparative HPLC strategy used reversed-phase conditions, similar to the analytical scale separations for characterizing the purity of XP315. The chromatogram was divided into logical fractions and each eluted fraction was collected for injection into a dog to permit identification of the fraction containing the toxic impurity. This approach minimized the number of animals required to establish the retention time of the toxic impurity in both preparative and analytical scale chromatograms. Once this was accomplished, additional drug substance was processed using preparative HPLC until a sufficient quantity of the impurity was isolated for analysis by NMR and mass spectrometry.

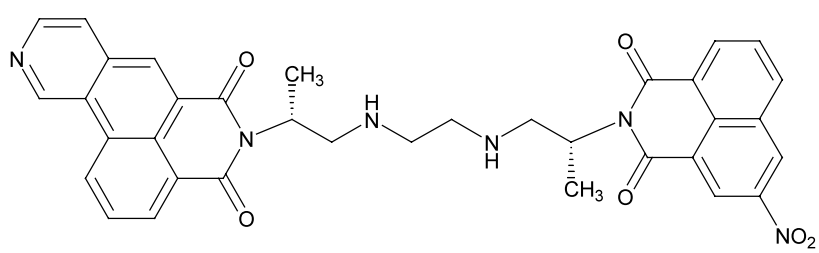


Fig. 1. The chemical structure of XP315.

2. Experimental

2.1. Materials

HPLC grade acetonitrile and methanol (EM Science, Gibbstown, NJ), HPLC grade trifluoroacetic acid (Burdick & Jackson, Muskengen, MI), and deionized water from a Milli-Q Plus ultra-pure water system (Millipore, Molsheim, France) were used in the preparation of the various mobile phases use in analytical and preparative chromatography.

Samples of XP 315-000 and XP 315-001 drug substance were provided by the Chemical and Physical Sciences and Chemical Process Research and Development Departments, respectively, of the DuPont Pharmaceuticals Company.

2.2. Preparative chromatography

Preparative-scale experiments were performed on a HPLC system consisting of a three pump Rabbit solvent delivery system (Varian Inc. Walnut Creek, CA), a Gilson Model 201 fraction collector (Gilson Medical Electronics, Middleton, WI), and a Knauer variable wavelength monitor (Varian Inc. Walnut Creek, CA) controlled by the Dynamax HPLC Method Manager, version 1.4.6 software (Varian Inc. Walnut Creek, CA).

The preparative HPLC method used a Dynamax-60A, C18, 21.4 mm ID × 25 cm, preparative HPLC column with a guard column (Varian Inc. Walnut Creek, CA) using a binary reversed-phase gradient. Mobile Phase A was water:methanol: trifluoroacetic acid (90:10:0.1). Mobile Phase B was methanol:trifluoroacetic acid (100:0.1). A linear gradient from 30% B to 100% B in 50 min was used for the separation. The detector wavelength was 246 nm, flow rate was 15.0 ml/min, the column temperature was ambient and a maximum of 8 ml of a 15 mg/ml XP315 solution was injected at one time. Mobile Phase A was used to dissolve XP315 drug substance for preparative use. Sample solutions were pumped directly on the preparative column using one of the solvent delivery modules with a flow rate of 10 ml/min. During injection, the mobile phase flow was stopped to minimize band broadening during the injection cycle. Mobile Phases and XP315 solutions were filtered before use with disposable 0.45 μm nylon filters (Whatman Autovial).

Fractions were isolated from the mobile phase by allowing the collected fraction to evaporate in a large evaporating dish or a large polyethylene pan placed in a laboratory hood. Laboratory room lights were kept off to minimize any potential photodegradation. Evaporation was complete between 12 and 24 h, depending on the fraction size. These residues were then reconstituted in small volumes of methanol (5–20 ml) and transferred to 50-ml volumetric flasks (for toxicity testing) or 4-ml vials (for NMR and mass spectrometry). The residues were evaporated to dryness in these containers using a stream of nitrogen gas.

2.3. Analytical chromatography

Analytical-scale experiments were performed on a Waters (Waters, Milford, MA) HPLC system consisting of a 600E multisolvent delivery system, an HPLC column oven, a model 486 tunable absorbance detector, and a model 717 autosampler. The chromatographic data was acquired and analyzed on a Multichrom™ data system (Thermal Labsystems, Cheshire, UK). The analytical HPLC method used a Waters (Waters, Milford, MA), Symmetry, C18, 3.9 × 150 mm HPLC column and a binary reversed-phase gradient. Mobile phase A was acetonitrile, water and trifluoroacetic acid: (5:95:0.05). Mobile phase B was acetonitrile, water and trifluoroacetic acid: (65:35:0.05). A linear gradient from 0% B to 100% B in 30 min was used for the separation. Detector wavelength was 246 nm, flow rate was 1.5 ml/min, injection volume was 20 μl, and an oven temperature of 40 °C.

2.4. In-vivo testing

The drug substance, XP315, was given to dogs by iv infusion. In a single-dose study, dogs were given 0.75, 2.25 or 3.37 mg base/kg in 0.213 M lactic acid buffer (pH 3.3) containing 4.5% mannitol. Dosing solutions were prepared by sonicating the drug in the buffer followed by filtration through 0.2-μm cellulose acetate membranes. Solutions were infused using syringe pumps (Harvard Apparatus) over a period of 15 min. Two

lots (XP315-000 and XP315-001) were administered at the above dose levels.

To investigate the toxicity of isolated preparative fractions, residues for toxicity testing were all obtained by collecting the fractions corresponding to 200 mg of drug substance. Residues were prepared in the previously stated manner and were then reconstituted by adding 15 ml of 0.213 M lactic acid buffer (pH 3.3), sonicating if necessary and filtering using 0.2-μm cellulose acetate membranes and then given to dogs iv as described previously. Administration of the residues was terminated immediately upon the onset of any adverse clinical signs.

2.5. Mass spectrometry

Low-resolution mass spectra were acquired on a Micromass Platform II (Beverly, MA) equipped with a Hewlett-Packard 1050 HPLC pump and autosampler system. A 10-μl aliquot of a 5 μg/ml solution of XP315 impurity was introduced by the HP1050 into the mass spectrometer via flow-injection in a mobile phase of water:acetonitrile (50:50) at 0.25 ml/min. The sample was ionized by positive-ion electrospray ionization (ES+) under the following source conditions: source temperature, 150 °C; capillary potential, 3.2 kV; sampling cone potential, 15 V. Mass spectra were obtained over the scan ranges 150–1000 and 400–1400 Da, at a rate of 1 scan per s and a resolution of 1500 (10% valley definition).

Exact mass measurements were obtained on a Finnigan MAT95S sector mass spectrometer (Bremen, Germany) under high-resolution ES+ conditions in automated mode. Since this instrument and procedure have been described in detail elsewhere [7], only the specifics of the analysis of XP315 impurity are discussed here.

A 20-μl aliquot of a 20 μg/ml solution of the XP315 impurity in 50:50 water:acetonitrile was prepared and injected onto the system. Since the $(M+H)^+$ ions at m/z 1195 were so weak under high-resolution ES+ conditions, exact mass measurement was performed on the doubly-charged molecular ion, $(M+2H)^{2+}$, at m/z 598. Five replicate measurements were made at a resolution of 6800 (10% valley definition). The individual exact masses were calculated by interpolation between known exact masses of two $(M+H)^+$ peaks for polypropylene glycol (PPG), and an average of the five results taken as the final value for the exact mass of the XP315 impurity.

2.6. Nuclear magnetic resonance spectroscopy

Dimethylsulfoxide-d_6 (DMSO-d_6) was used as supplied from Isotec, Inc (Miamisburg, OH). NMR data were acquired on either a Varian (Palo Alto, CA) VXR-400 or a Varian (Palo Alto, CA) Unity-400. Carbon-13 NMR data were acquired using a 3-mm broadband probe from Nalorac, Inc (Martinez, CA) and the proton and 2-dimensional NMR spectra were acquired using a 3-mm indirect NMR probe with a Z-axis pulse field gradient, also from Nalorac. Samples were dissolved in 160 μl of DMSO-d_6, and placed in 3 mm NMR tubes. Data were referenced to the solvent at 2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C.

3. Results and discussion

The higher molecular weight and complex structure of the impurity required a variety of NMR techniques combined with mass spectrometry to determine the chemical structure. Due to the variety of impurities and their low levels present in the suspect lot of drug substance, a more selective means than normal HPLC detection was required to detect the impurity in samples. For XP315, the only specific method of identifying the presence of the toxic impurity was using the dog model. The experimental procedure used was developed to minimize the number of animal dosages required to identify the toxic impurity. The use of preparative HPLC and offline spectroscopic methods allowed for more sensitive and extensive structure data to be obtained compared with online hyphenated methods such as LC-MS and LC-NMR.

3.1. Toxicity of XP315-001

The first lot of XP315 examined by Safety Assessment (XP315-000) showed no immediate

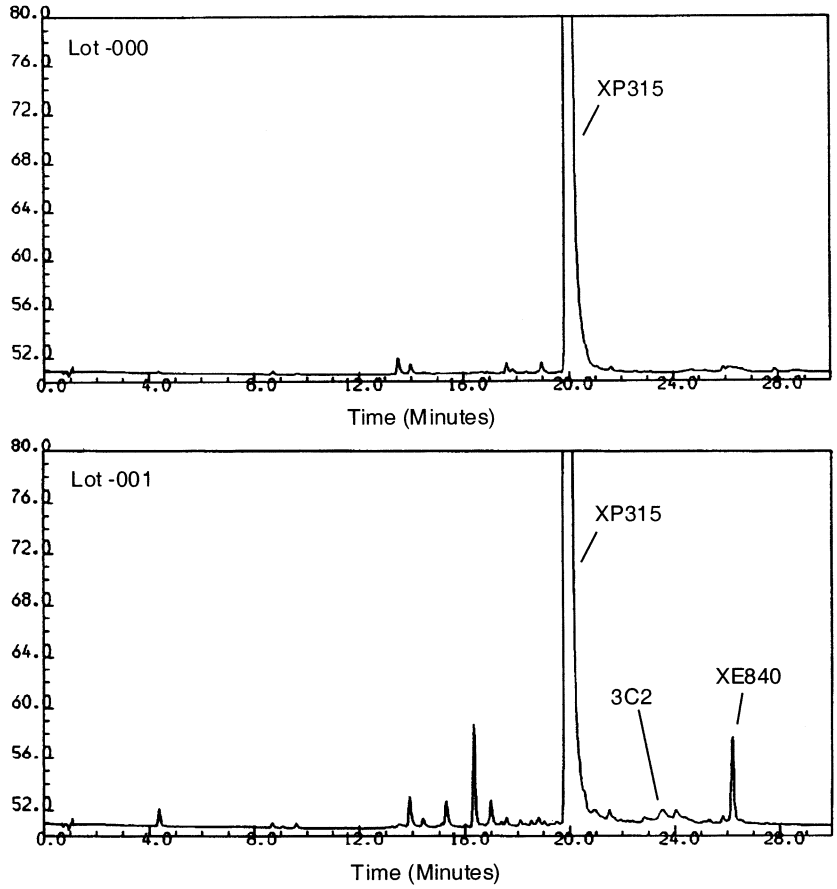


Fig. 2. Analytical HPLC chromatograms of XP315-000 and XP315-001.

toxicity following iv administration to dogs at all dosages tested. A second lot of drug (XP315-001) produced an immediate toxic response at all dosages following iv infusion. Clinical signs associated with the toxic response were erythma around the ears, eyes, face, and body; ocular hyperemia; head shaking; swelling around the eyes, face, paws, head, neck and legs; scratching; and reddened gums. The clinical signs were first observed during the 15-min infusion and then they continued for approximately 2–3 h after dosing.

Examination of the analytical HPLC chromatograms from these two lots showed additional impurities in the second lot, XP315-001. A comparison of these analytical chromatograms is shown in Fig. 2. Since XP315-000 did not elicit the 'allergic-like' reaction, the reaction was thought to be due to impurities in the second lot of drug substance. Since there were many low-level impurities in XP315-001, it was not possible to determine the impurity or impurities responsible by comparing the analytical chromatograms alone.

3.2. Isolation of the toxic impurity

To locate the impurities responsible for the toxic reaction, XP315-001 was fractionated chromatographically into three elution volumes, collecting the peaks eluting before, during, and after the main XP315 peak. A representative preparative chromatogram is shown in Fig. 3 with the three fractions (1–3) identified. The three recon-

stituted residues from the collected fractions were then administered intravenously to dogs. The residue administered to each dog corresponded to approximately 14.1 mg/kg of XP315-001 drug substance. This experiment showed that the toxicity was confined to Fraction 3. Fraction 3 was then resolved further into three subfractions (3A, 3B and 3C) by using the same preparative chromatography conditions. Fraction 3C exhibited the most toxicity in the dog model and was further fractionated by the same preparative separation and quantities of XP315-001 to resolve two peaks isolated as Fractions 3C1 and 3C2. Additional testing in dogs showed that Fraction 3C1 was inactive and Fraction 3C2 was highly active, producing the toxic reaction almost immediately after starting the infusion.

3.3. Characterization of the toxic impurity

3.3.1. Analytical HPLC

The analytical HPLC analysis of the drug substance was performed using a gradient reversed phase separation to improve the resolution of the synthetic impurities. The principal impurity responsible for the toxic response in dogs was present at a low concentration ($< 0.1\%$ by peak area) and thus was considered a minor impurity in the drug substance. Analytical chromatograms show the presence of several impurity peaks eluting before and after XP315, with XE840 being the largest single synthetic impurity. XE840 is a symmetrical analog of XP315 having nitronaphthalene groups on each end of the molecule. This impurity is the last eluting impurity in the analytical chromatograms (Fig. 2). In the preparative separations, it has an earlier retention time, eluting immediately after the XP315 peak in Fraction 1. The isolated Fraction 3C2 was spiked into a sample of XP315-001 to determine where this impurity eluted. The small unspiked peak is labeled Fraction 3C2 in the chromatogram in Fig. 2. Based on area comparisons to the parent drug, the concentration of the impurity was estimated at 0.01% (w/w), based on analytical HPLC area percent measurements.

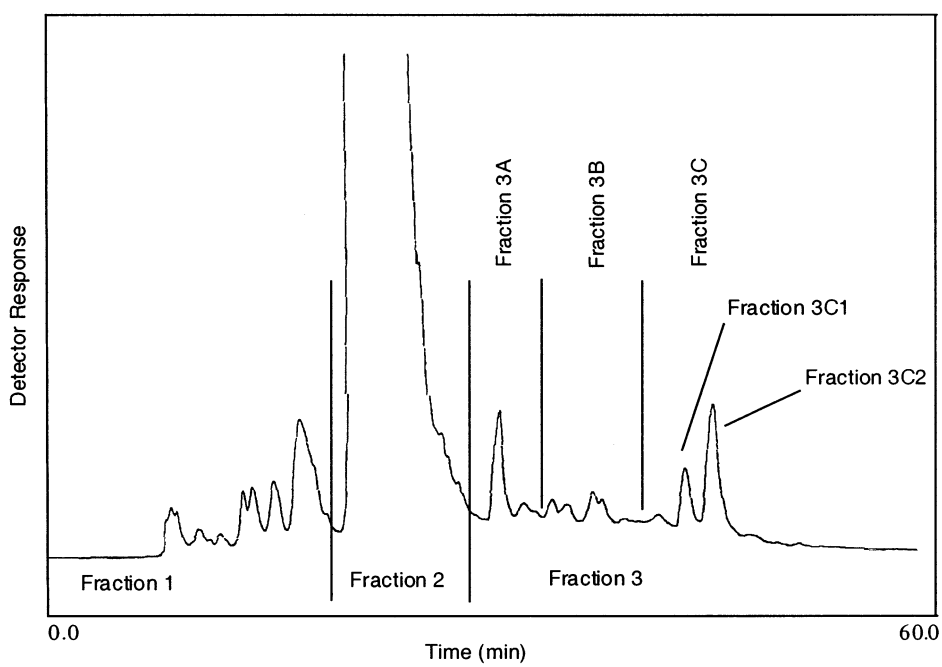


Fig. 3. A representative preparative HPLC chromatogram of XP315-001 with fractions identified.

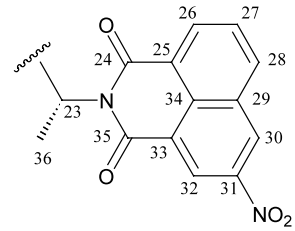
3.3.2. Mass spectrometry

The base peak in the low-resolution ES+ spectra (150–1000 Da and 400–1400 Da ranges) was observed at m/z 598. The low intensity of the M + 1 peak at m/z 599 (8% of base peak) indicated that m/z 598 was a multiply-charged ion. In the high-resolution ES+ spectrum, it was apparent that m/z 598 was doubly-charged from the one- and two-^{13}C isotope peaks observed at m/z 598.7 and 599.2, respectively, i.e. the three peaks were 0.5 Da apart, characteristic of doubly-charged ions, instead of 1.0 Da as for singly-charged ions. Closer inspection of the low-resolution ES+ spectrum (400–1400 Da) revealed the presence of low-intensity ions at m/z 1195–1199. It was determined that the low-resolution mass spectrum was consistent with a molecular weight of 1194 Da, where m/z 1195 and 598 were $(M + H)^+$ and $(M + 2H)^{2+}$, respectively.

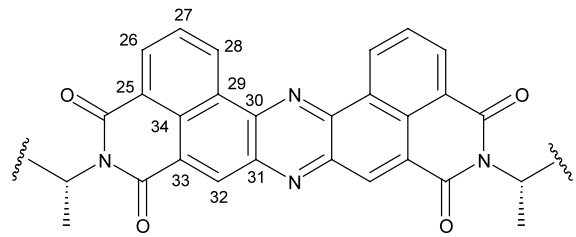
The measured exact mass of m/z 598 was 598.2341 ± 0.0013 ($n = 5$); thus the measured exact mass for M + 2H was twice this value, or 1196.2382 ± 0.0026 Da. Elemental analysis of this exact mass yielded a molecular formula of $C_{70}H_{58}N_{12}O_8$ for the impurity (MW 1194 Da). The calculated exact mass for the M + 2H of this formula, 1196.2357 Da differs from the measured exact mass by −2.5 mDa (−2.2 ppm), well within the accepted limits of error for such an experiment on this instrument [8]. In light of this and other analytical data, $C_{70}H_{58}N_{12}O_8$ was judged to be the 'best fit' molecular formula for the XP315 impurity.

3.3.3. Nuclear magnetic resonance spectroscopy

The proton and carbon assignments for XP315 and the isolated impurity were determined from the gradient enhanced homonuclear chemical shift correlated spectrum (gCOSY) and both the one bond and multiple bond gradient enhanced heteronuclear chemical shift correlated spectra (gHSQC and gHMBC). The most striking feature of the NMR spectrum of the impurity is that it was a relatively simple spectrum obtained from a compound with a mass of 1194 Da. From the mass and the number of proton resonances, it was quickly concluded that the impurity was a symmetrical dimer of XP315 and that the challenge



XP315



XP315 Impurity (Fraction 3C2)

Fig. 4. The numbering system used for the polyaromatic rings in XP315 and the toxic impurity.

was determining the form of the dimer. Of particular interest is the assignment of the carbon C30 (see Fig. 4 for numbering scheme). This carbon was assigned by the long-range correlations from protons H32 and H28 to C30. In XP315, there is also a one bond H–C correlation from H30 to C30 in the gHSQC spectrum. In the XP315 impurity, again C30 was assigned from the long-range correlations from the H32 and H28 protons to C30, but in this case no one bond correlation into C30 was observed, indicating that C30 no longer bears a proton. In addition, there was a 14 ppm downfield shift in the chemical shift of C30 in the impurity compared with the chemical shift of C30 in XP315.

Taking into consideration the molecular formula, $C_{70}H_{58}N_{12}O_8$, obtained from the high resolution/exact mass spectroscopy and the results from the NMR experiments it becomes apparent that the dimer formed via a condensation of two nitro-napthylimides groups resulting in the polyaromatic heterocycle shown in Fig. 5. This structure for the dimer is consistent both with the observed loss of the proton on C30 and with the attachment of nitrogen at C30 to account for observed 14 ppm downfield shift for the C30 carbon.

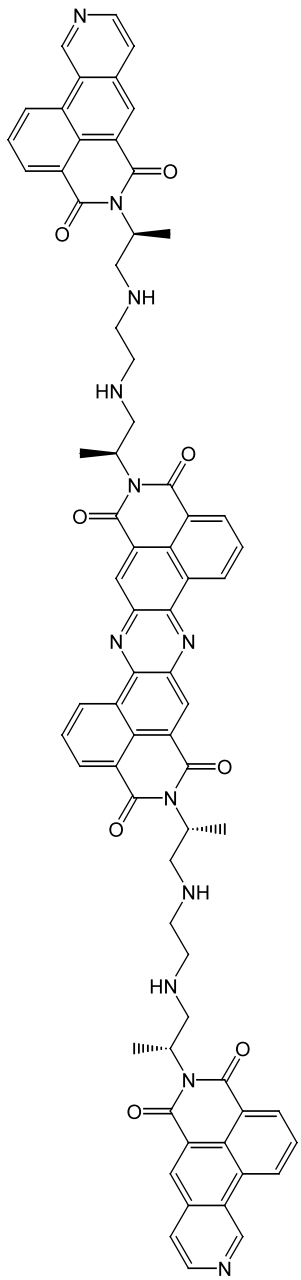


Fig. 5. The chemical structure of the toxic impurity isolated from XP315-001.

4. Conclusions

Following iv administration of XP315, the toxic reaction in the dog was caused by a drug substance impurity present at a very low concentration. This represents an example where an impurity, occurring in a drug substance below ICH threshold levels (e.g., 0.1% for XP315), required specific action. For development activities to continue into Phase I clinical studies, the toxic impurity would have to be identified and eliminated from the drug substance synthetic process. Additional work should also involve synthesis of the toxic impurity for structure confirmation and for use as a standard to permit accurate quantitation.

References

[1] International Conference on Harmonization; Guidelines Availability: Impurities in New Drug Substances; Notice, Fed. Reg., 61 (3), (1996) 371–376.
[2] S.V. Prabhu, J.M. Ballard, R.A. Reamer, D.K. Ellison, Talanta 40 (7) (1993) 989–994.
[3] R.M. Ladd, A. Taylor, LC-GC 7 (1989) 584 pp.586–589.
[4] B.C.M. Potts, K.F. Albizati, M.O. Johnson, J.P. James, Magn. Reson. Chem. 37 (6) (1999) 393–400.
[5] J. Ermer, J. Pharm, Biomed. Anal. 18 (1998) 707–714.
[6] Robert J. Cherney, Stephen G. Swartz, Arthur D. Patten, Emeka Akamike, Jung-Hui Sun, Robert F. Kaltenbach Jr, Steven P. Seitz, Carl H. Behrens, Zelleka Getahun, et al., Bioorg. Med. Chem. Lett. 7 (2) (1997) 163–168.
[7] M.J. Haas, Rapid Commun. Mass Spectrom. 13 (1999) 381–383.
[8] M.L. Gross, J. Am. Soc. Mass Spectrom. 5 (1994) 57.