Isolation and Identification of a Major Metabolite of PNU-107859, an MMP Inhibitor from the Biliary Fluid of Rats

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Abstract □ PNU-107859, an important representative structure in a novel class of matrix metalloproteinases (MMP) inhibitors known as thiadiazoles, was found to be quickly eliminated from rats. A major metabolite (approximately 10% of total dose) was found to be present in the bile of rats. The metabolite in question was isolated and purified from the bile fluids collected from six cannulated rats. From a total of approximately 75 mg of PNU-107859 administered to rats, 3.3 mg of the metabolite was recovered. The NMR and mass spectrometry results indicated that the metabolite is a glucuronide conjugate (1-deoxy-1β-substituted D-glucopyranosiduronic acid) of the intact drug. Furthermore, the UV, MS, and NMR data established that the conjugate is located at the nitrogen α to the thiocarbonyl of the thiadiazole ring.

Introduction

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases which are capable of degrading the extracellular matrix of connective tissues and basement membranes.[1] Overactivation or increased synthesis of the MMPs has been implicated in several disease pathologies, including arthritis, cancer progression, and related connective tissue disorders[2], periodontal disease,[3] atherosclerotic plaque rupturing,[4] and aortic aneurysms.[5] There are currently at least 18 members of this family of proteinases which can be roughly associated into three groups depending on their native substrates. Thus, the gelatinases are effective proteinases of Type IV collagen, the collagenases degrade interstitial collagen, and the stromelysins are effective as protoglycanases. The discovery and characterization of the MMPs is summarized in several recent reports. Recently several research groups have shown that related metalloproteinases are also involved in the conversion of inactive tumor necrosis factor-I (TNF-I) into active TNF.[6,7] Usually this inflammatory cytokine plays a beneficial role in physiological defense responses; however, overproduction of TNF-I can lead to systemic toxicity. Therefore, MMP inhibitors may be indirectly involved in diseases for which TNF-I has been implicated, such as Crohn's disease, MS, cachexia, sepsis, and rheumatoid arthritis.[8] PNU-107859[9] is a member of a novel class of MMP inhibitors known as thiadiazoles. During a preliminary pharmacokinetic study of PNU-107859, it was observed that this experimental drug has rather short half-life (≅40 min) in rats. To investigate the nature of the unfavorable pharmacokinetics, the bile samples from rats treated with this drug were collected and found to contain the intact drug and a metabolite as the only other major thiadiazole-containing component. It was estimated that this putative metabolite represented about 10% of the total drug 3 h after iv administration. A study was therefore initiated to isolate enough metabolite for spectroscopy experiments to elucidate its structure unambiguously.

Experimental Section

Materials—PNU-107859 was synthesized by Pharmacia and Upjohn. All other chemicals were analytical or HPLC grade.

Dosing and Collection of Bile—The jugular veins of six male Sprague−Dawley rats were cannulated using a variation of the Week's chronic jugular vein cannulation technique. The same animals were anesthetized after eight or 9 days and the bile ducts cannulated using PE-10 polyethylene tubing. The free end of the tubing was placed in a test tube to collect draining bile. Animals (230−290 g) were given a 50 mg/kg dose of PNU-107859 (50 mg/mL in a 50% PEG/50% saline vehicle) iv via the chronic jugular vein cannula. The cannulas were flushed with 100 μL of 50% PEG/50% saline followed by 300 μL of 100% saline. Bile was collected hourly for 3 h postdosing. After pooling samples from all animals, a total of 15 mL of bile was collected.

Cation Exchange Methods—One milliliter of pooled bile was passed over one Varian Bondelut SCX cartridge (1 mL) to remove impurities with a UV absorption maximum at 270 nm. The metabolite was not retained by this resin. The resin was washed with water until HPLC showed no presence of the metabolite in the eluent. Fractions containing the metabolite were collected and used directly for prep-HPLC.

Preparative HPLC Methods—The cation-exchange-purified samples were divided into six samples, and each of these was purified by preparative HPLC employing a Waters μBondapak cartridge (2.5 × 10 cm). Elution at 15 mL/min for 5 min at 10/90 ACN/H_2O plus 0.1% TFA was followed by a 15 min gradient to 25/75. These conditions were maintained for 5 min, followed by an elution at 90/10 to wash the column. Detection was at 220 nm. Fractions were collected with a Foxy collector in the peak detect mode. A second run using the same conditions was performed on the pooled fractions from the six initial runs.

Analytical HPLC Methods—Sample was injected onto a Zorbax Rx-C8 column (4.6 × 250 mm) and eluted isocratically with 25/75 ACN/H_2O plus 0.1% TFA at 1 mL/min with detection performed by a Varian 9065 diode array detector at 311 or 220 nm.

FAB-MS Method—The low resolution positive ion fast atom bombardment (FAB) mass spectrum of the glucuronic acid product isolate was obtained on a VG70-SE Nier-Johnson double-focusing mass spectrometer equipped with an OPUS-2 data system. A cesium ion gun operating at 25 kV was used as the source of the ionizing beam, while the instrument was operated at 8 kV accelerating voltage. The instrument was calibrated at selected masses using a mixture of CsI and NaI. A small portion of the isolate as a methanol solution was mixed with the 2-hydroxyethanol disulfide (2-HED) matrix and introduced into the ion source on a stainless steel tip via a direct insertion probe. The sample

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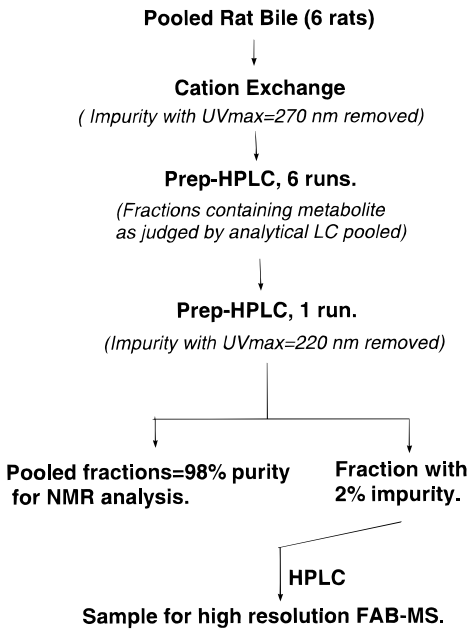


Scheme 1—Purification steps.

spectrum was recorded by scanning the magnetic field from *m*/*z* 2500 to *m*/*z* 15. The results were further analyzed using the mass spectral processing software on the Harris Nighthawk computer system.

The accurate mass of the isolate, as the protonated adduct ([M + H]$^+$), was determined by the peak matching technique relative to the known matrix adduct ion mass of 2-HED at *m*/*z* 463.0445. The mass spectrometer was operating at a mass resolution of approximately 4000 (m/Dm, 10% valley definition).

NMR Methods—^{1}H NMR spectra were recorded at 400.13 MHz using a Bruker ARX-400 spectrometer. Data were processed on an SGI Indy computer using Bruker UXNMR software. Samples were dissolved in 500 μL of DMSO-d_6 in a 5-mm NMR tube. Spectra were recorded at a temperature of 300K (27 °C). One-dimensional proton spectra were recorded as free induction decays of 32K complex points with a spectrum width of 6024 Hz. Four dummy scans were used. The receiver gain and the number of pulses were optimized for each sample. Free induction decays were Fourier transformed with no zero-filling after application of a resolution-enhancing Gaussian window function (LB = −1, GB = 0.2). Two-dimensional homonuclear COSY spectra were recorded in the magnitude mode using 1K × 1K data table (after transformation), with 256 increments in F1 and no zero filling in F2. Unshifted sinebell windows were applied before transformation.

^{13}C NMR spectra were obtained on a Varian XL-300 spectrometer operating at 75.43 MHz. The spectral window was 17391 Hz, and 32K data points were used with an acquisition time of 0.942 s and a pulse delay of 1.00 s. The free induction decays were Fourier transformed using standard sensitivity enhancement parameters.

Heteronuclear Multiple Bond Correlation (HMBC)—NMR spectra were recorded on a Bruker ARX-400 Spectrometer equipped with a 9.4 T magnet, operating at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C. A Bruker 5 mm broadband inverse gradient probe was used for these experiments. HMBC data was acquired using the a gradient pulse sequence. 2D transformations were accomplished using Bruker UXNMR software on an SGI Indy computer. Spectra were recorded in the magnitude mode using a 1K by 512 data table with 160 pulses per 256 increments in F1 domain and no zero-filling in F2 domain. All spectra were acquired at 300K. Relaxation delays typically were 1.1 s.

Results

A brief outline of the purification methods is summarized in Scheme 1. Briefly, cation exchange procedures were used to remove some impurities. The pooled fractions containing the metabolite were then processed by preparatory reverse-phase HPLC. Fractions containing the metabolite were dried and redissolved in perdeuterated methanol or DMSO to obtain structural information by NMR. The HMR spectra also revealed a peptidic impurity partially coeluted with the metabolite. This was confirmed by reanalyzing the fractions by HPLC with detection at 220 nm. This peptide has a slightly longer retention time than the metabolite peak. The fractions containing the metabolite were therefore combined and rechromatographed using the same HPLC conditions. The final preparation was judged to be pure by HPLC analysis employing a diode array detector and by HMR spectroscopy. A total of 3.3 mg of the pure metabolite was obtained. Since approximately 70 mg of PNU-107859 was administered to rats and the average conversion rate was 10%, a recovery of nearly 50% of the metabolite present in bile was obtained.

The purified metabolite was then subjected to NMR, FAB-MS, and UV studies. The 1D NMR chemical shift data are summarized in Table 1. The 2D NMR results are summarized graphically in Figure 2. The FAB-MS results are summarized graphically in Scheme 2.

Table 1—NMR Data of U-107859 and Its Metabolite[a]

	U-107859		metabolite	
position	δ_H	δ_C	δ_H	δ_C
1	7.20	126.8 (d)	7.20	126.6 (d)
2	7.29	128.5 (d)	7.29	128.3 (d)
3	7.12	129.6 (d)	7.12	129.4 (d)
4	—	137.3 (s)	—	136.9 (s)
5	2.83, 3.00	38.8 (t)	2.85, 2.99	38.5 (t)
6	4.42	54.6 (d)	4.42	54.4 (d)
7	—	153.1 (s)	—	153.0 (s)
8	—	154.0 (s)	—	150.8 (s)
9	—	183.6 (s)	—	185.0 (s)
10	—	171.0 (s)	—	170.6 (s)
11	2.62	25.9 (q)	2.60	25.6 (q)
6 N–H	6.81	—	6.89	—
7 N–H	10.71	—	10.95	—
9 N–H	13.8	—	—	—
10 N–H	8.15	—	8.15	—
1′	—	—	5.80	84.3 (d)
2′	—	—	3.70	71.1 (d)
3′	—	—	3.40	76.3 (d)
4′	—	—	3.41	71.4 (d)
5′	—	—	3.80	78.0 (d)
6′	—	—	12.9	169.7 (s)

[a] All spectra were collected in DMSO-d_6 solutions and are reported as chemical shift downfield (ppm) from TMS.

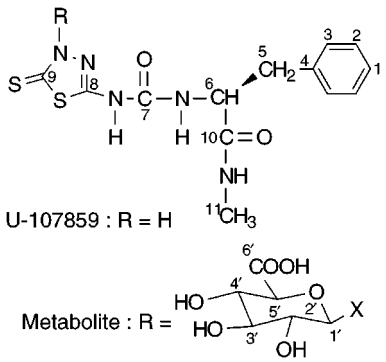


Figure 1—Structure of U-107859 and its metabolite.

Discussion

1. The Nature of the Conjugate—The main features of the HMR spectra of the metabolite collected during the purification stage were the extra resonance signals ob-

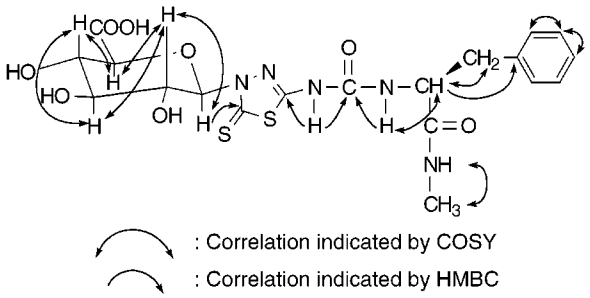
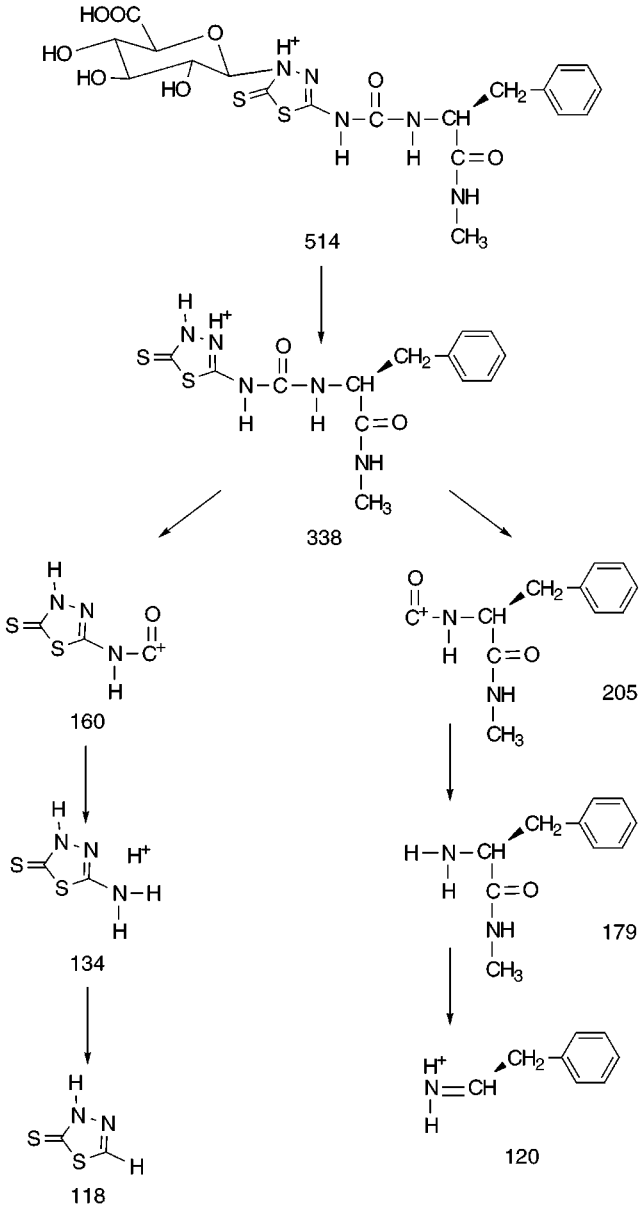


Figure 2—2D NMR results of U-107859 metabolite.



Scheme 2—Major ions observed by FAB-MS.

served between 3 and 6 ppm, suggesting the presence of carbinols, when compared to the parent drug. Integration of these extra signals resulted in a total of five nonexchangeable protons. However, two of the five protons were not resolved in methanol. Resolution of these five signals was attempted with a number of solvents. It was found that DMSO-d_6 provided the best resolution for those five protons. All following experiments, including $^1H-^1H$, $^1H-^{13}C$ COSY, CMR, and $^1H-^{13}C$ HMBC were therefore conducted in DMSO solution. The results are summarized in Table 1 and Figure 2 and discussed below.

The assignments of NMR signals of PNU-107859 are based on various 2D NMR experiments and will not be discussed in this report. Comparison of chemical shift values listed in Table 1 revealed that the major differences were the extra signals (vide infra) obtained for the metabolite when compared to the parent compound. Another important observation was that the conjugate most likely contained a carboxylic acid functional group, as evidenced by the new broad peak at 12.9 ppm and a new carbon signal at 169.7 ppm in the CMR spectrum. The 2D COSY spectrum, the size of $^1H-^1H$ coupling constants (9 Hz), and the HMBC spectrum indicated that the conjugate was a 1-deoxy-1β-aminated-glucopyranuronate. Specifically, the 2D COSY spectrum sequentially linked the five carbinols and connected each of them to an exchangeable hydroxyl proton. The fact that all the carbinols had 9 Hz coupling constants dictated that they adopted a diaxial relationship with their coupled neighbors. Assuming the bulky hydroxyl groups are at the equatorial positions leads to the conclusion that the conjugate is a modified β-D-glucopyranose. Analysis of the HMBC spectrum further connected the 12.9 ppm proton of the pyranoglucoside to the carboxyl carbon, establishing that the conjugate is a glucopyranuronate. Finally, the chemical shift value of the anomeric carbon (84.3 ppm) strongly implied that the conjugate is a 1-deoxy-1β-aminated-D-glucopyranuronate.[10]

2. The Site of Conjugation—There are five possible nitrogens, two on the thiadiazole ring and three on the backbone, that could be linked to the pyranose. Comparison of chemical shift values listed in Table 1 revealed that the 9 N−H signal is missing in the metabolite, suggesting that this position is the site of conjugation. Also, the HMBC spectrum gave a cross-peak between the anomeric proton and the carbon of the thiocarbonyl. Since this experiment will only detect C−H couplings with 2 or 3 bond linkages, the attachment must be on the nitrogen adjacent to the thiocarbonyl. Supporting this conclusion is the observed 3 ppm upfield shift of the C-8 carbon signal caused by the γ gauche effect. Other supporting evidences are the almost identical chemical shift values and the almost identical UV absorption for the metabolite and its parent.

3. Confirmation by Mass Spectrometry—The purified metabolite was subjected to low and high-resolution FAB-mass spectrometry studies. The low resolution FAB-MS results produced the expected molecular ions at 514 $(M + H)^+$ and 536 $(M + Na)^+$ for the glucuronic conjugate. Subsequent high resolution FAB-MS studies unambiguously identified the molecular formula of the metabolite as $C_{19}H_{23}N_5O_8S_2$. The major fragment ions can be rationalized as in Scheme 2. Thus, the FAB-MS results are in total agreement with the conclusions reached by NMR.

Glucuronidation is the most common pathway of conjugation in mammalian metabolism and excretion of xenobiotics.[11] It can be classified as O-, S-, N-, and C-glucuronidation on the basis of the type of atoms to which the glucuronic acid moiety is transferred by a group of inducible UDP-glucuronosyltransferases.[12] We have clearly demonstrated in this study that PNU-107859 is partially cleared by N-glucuronidation. Although N-glucuronidation is a much less commonly observed and a not well understood pathway compared to O-glucuronidation, its participation in the metabolic transformation of xenobiotics has gained increasing recognition in recent years. Examples reported in the literature are of wide structural classes of nitrogen-bearing aglycones, including primary through tertiary amines, and various classes of heterocycles, such as imidazole, hydantoin, pyrimidine, piperidine, tetrahydroiso-

quinoline, triazine, and tetrazole.[13−17] Our result extends this list to include thiadiazole.

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