3-[(Phenylacetyl)amino]-2,6-piperidinedione Hydrolysis Studies with Improved Synthesis and Characterization of Hydrolysates

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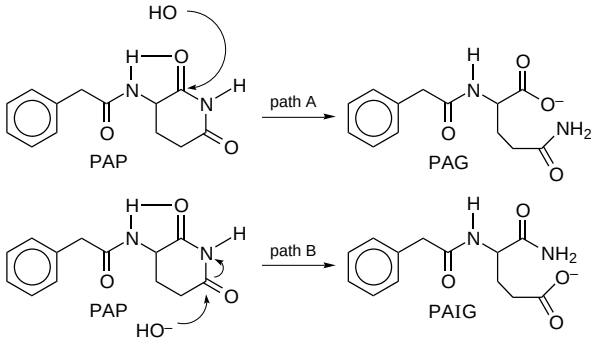
Abstract □ In an attempt to clarify ambiguities in earlier reports on the preclinical chemistry of Antineoplaston A10 (3-[(phenylacetyl)amino]-2,6-piperidinedione; PAP), we detail herein hydrolysis studies with improved synthesis and characterization of PAP hydrolysis products, (phenylacetyl)-glutamine (PAG), (phenylacetyl)isoglutamine (PAIG), and (phenylacetyl)-glutamic acid (PAGA). Flash chromatography proved superior to extraction in the isolation of synthetic standards and hydrolysates. Synthesis of PAIG directly from commercial isoglutamine showed consistently better yields than the previously reported method. The ^{1}H and ^{13}C NMR and HPLC–MS data from the synthesized standards matched the data from the isolated PAP hydrolysates formed under acid and alkaline degradation conditions. Multiple quantum coherence NMR methods (HMQC and HMBC) and HPLC–MS–MS methods were applied to provide unambiguous structural assignments for key isomers PAG and PAIG. Previous PAP hydrolysis studies are shown to be reproducible, and the structures of hydrolysis products are elucidated in detail.

Introduction

With controversy,[1,2] 3-[(phenylacetyl)amino]-2,6-piperidinedione (PAP) (Scheme 1) has been reported to be effective in the treatment of cancer.[3,4] Originally isolated from urine,[5] PAP is generally prepared synthetically.[5,6] Although the synthetic, sparingly-soluble PAP has been administered to patients orally,[3] many postulate that the drug is degraded into hydrolysates by alkaline and acidic digestive juices.[7] More commonly, patients are administered the soluble sodium salts of hydrolysates (phenylacetyl)glutamine (PAG) and (phenylacetyl)isoglutamine (PAIG) manufactured by the alkaline hydrolysis of PAP.[8] Synthetic PAP has been characterized by Raman, IR,[9] UV, and mass spectroscopy, by binding studies with DNA,[10] and by model studies involving DNA interactions.[11] In contrast, the previously reported[12–14] PAP hydrolysates were not characterized by mass spectroscopy and were characterized only briefly by ^{1}H NMR. Therefore, we provide additional spectroscopic and chromatographic characterizations of the key hydrolysates along with improved synthetic and isolation methods.

Results and Discussion

Alkaline and Acidic Hydrolysis—As a way of testing the reproducibility of reported studies[13] and in order to identify and characterize the hydrolysates more completely, PAP was reacted with aqueous NaOH to a neutral end point. The resulting sodium salts of PAG and PAIG (Scheme 1) were analyzed by HPLC–UV and shown to exist in a 3.8:1 integrated area ratio similar to the previously reported ratio of 4:1.[13] The NMR and mass spectroscopic data of the isolated hydrolysates matched the data of the synthetic standards and were consistent with proposed structures. We present a possible explanation for the dominance of the PAG isomer over the PAIG isomer in the PAP base hydrolysis (Scheme 1). Since one of the two carbonyl functions in the piperidinedione ring system is positioned to hydrogen bond intramolecularly with the neighboring amide hydrogen, possible selective hydrolysis at the hydrogen-bonded carbonyl may account for the dominance of PAG.



Scheme 1— Alkaline hydrolysis mechanisms of PAP. Hydrolysis path A is favored over path B by a 3.8:1 ratio.

To test the stability of PAP to acid, PAP was dissolved in concentrated HCl and heated. The graph in Figure 1 shows the relative abundance of hydrolysates PAG, PAIG, (phenylacetyl)glutamic acid (PAGA), and phenylacetic acid (PAA) at 15 min intervals over a 1 h reaction. Initial hydrolysis products PAG and PAIG disappeared after 45 min along with the last trace of starting material PAP. Ensuing hydrolysis products PAGA and PAA accumulated with reaction time. After 60 min, the concentrations of PAP, PAG, and PAIG were essentially zero while concentrations of PAGA and PAA were significant. The hydrolysis pathway shown in Scheme 2 is consistent with the above observations and verifies that the previously reported PAP acid hydrolysis studies are reproducible.[12] The hydrolysates corresponded to synthetic standards in HPLC–UV spiking experiments conducted to verify structural assignments. In addition, the NMR and MS data of the isolated hydrolysates PAGA and PAA matched the data of the corresponding standards and were consistent with proposed structures.

Synthesis—Hydrolysate standards PAG, PAIG, and PAGA were synthesized as shown in Scheme 3. In each reaction the reagent phenylacetyl chloride was coupled with the appropriate glutamic acid derivative in aqueous bicarbonate medium to afford the corresponding PAGA derivative. The reported PAIG synthesis method, which featured formaldehyde addition to PAGA,[13] was difficult to reproduce. Therefore, we employed the more direct method as shown in Scheme 3 starting with isoglutamine. Isolation of the standards by flash chromatography was more efficient than previously described extraction and recrystallization methods.[13] Each hydrolysate standard was characterized by ^{1}H and ^{13}C NMR (Figure 2) and by HPLC–MS–MS (Figure 3).

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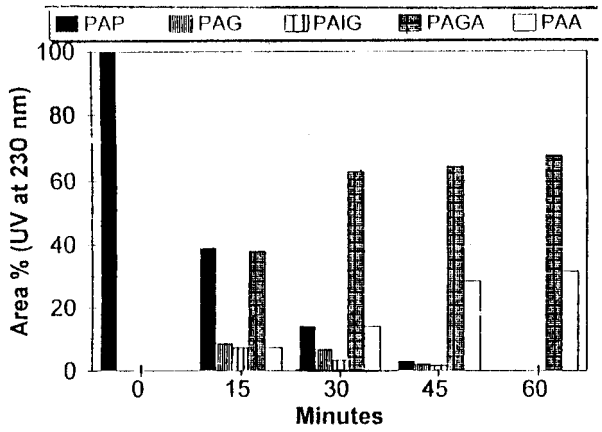
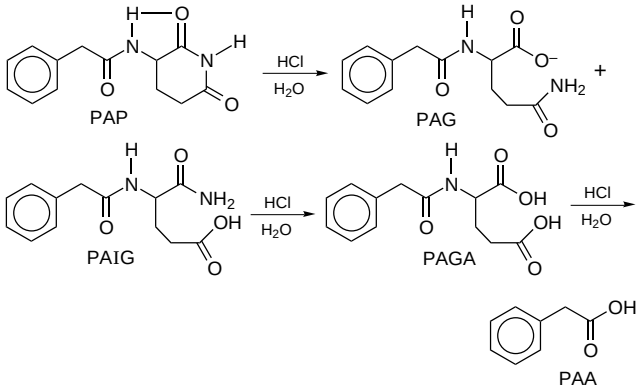
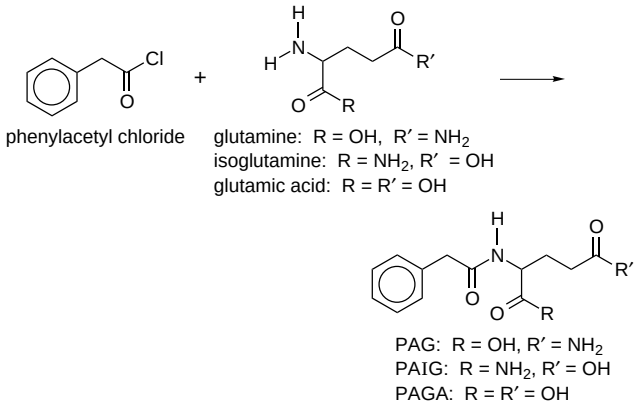


Figure 1—Acid hydrolysis of PAP.



Scheme 2— Acid hydrolysis mechanism of PAP.



Scheme 3— Synthesis of hydrolysate standards.

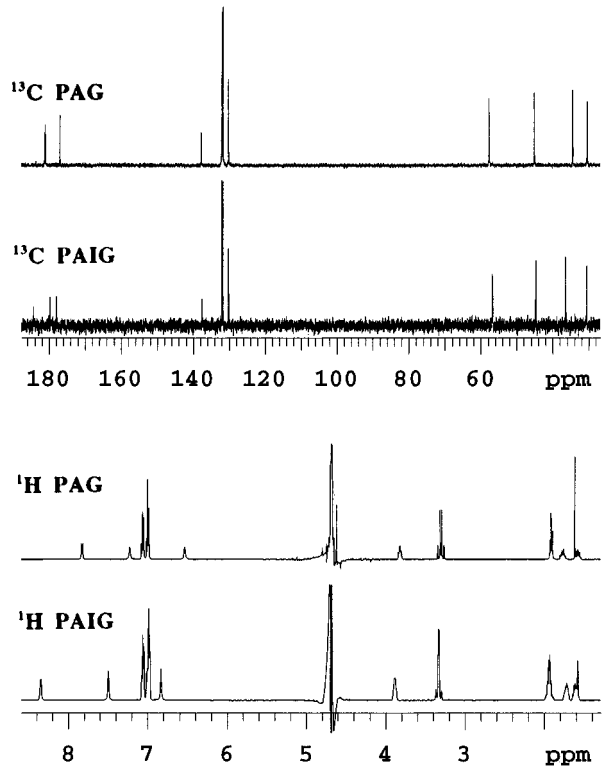


Figure 2—^{1}H and ^{13}C NMR spectra of hydrolysates.

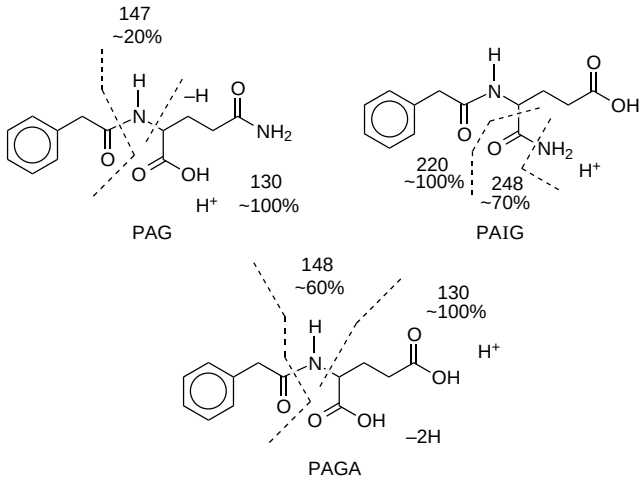
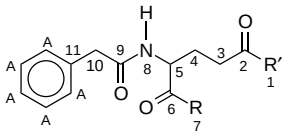


Figure 3—HPLC–MS–MS of hydrolysates.

NMR—PAG and PAIG, key hydrolysates thought to be clinically active,[7] show very similar ^{1}H and ^{13}C NMR spectra (Figure 2). The few differences that did exist (amino proton and carbonyl carbon shifts) did not provide a direct means for definitive isomer assignment. We were able, however, to make unambiguous assignments through the use of two-dimensional heteronuclear correlation methods of HMQC and HMBC (single and multiple bond respectively, for H−C or H−N correlations). Results from these experiments are summarized in Tables 1 and 2. We first confirmed the assignment of the amino protons through a single-bond correlation experiment. Although this assignment may be apparent from inspection of the spectra shown in Figure 2, the N−H HMQC confirmed the assignment unambiguously. Secondly, all single-bond H−C correlations were established also through the HMQC method. On the basis of proton chemical shift and integrated intensities, the aromatic, benzylic, and methine resonances were all identified. Finally, the key experiment, HMBC, yielded multiple-bond connectivities which provided ample information to assign all proton and carbon resonances, and in particular, the carbonyls of both isomers were assigned. Connectivities from the amido proton to a single carbonyl established the separate isomer identities. All NMR measurements were carried out at 2 °C to minimize exchange between the amido protons and aqueous solvent. HMBC experiments at 25 °C proved unsuccessful for PAG as the amido proton exchange was sufficient to compete with the development of multiple-bond coherence between proton and nearby carbonyl carbons. Tables 1 and 2 report final proton and carbon assignments for PAG and PAIG, respectively.

HPLC−MS−MS—Analysis of the fragmentation patterns of the two alkaline hydrolysates, PAG and PAIG, by HPLC−MS thermospray indicated that each isolate showed the same molecular ion (MH^{+}, 265 amu) and similar fragment ions. In contrast, the isolates showed very different ion intensities

Table 1—Single- and Multiple-Bond Correlations Observed for PAG



^{1}H Chemical Shift, ppm (No. of Protons)	^{1}H Position Scheme	Single-Bond Connectivities, ppm		Carbon Atom Positions (ppm) to Which Multiple-Bond Correlations Were Obsd
		^{13}C	^{15}N	
1.59 (1)	4a	30.42		3 (34.47), 4 (47.64), 2 (181.19)
1.77 (1)	4b	30.42		3 (34.47), 5 (57.65), 2 (181.29)
1.93 (2)	3	34.47		4 (30.42), 5 (57.65), 2 (181.29)
3.34 (2)	10	45.11		A (131.75), 11 (137.78), 9 (177.04)
3.85 (1)	5	57.65		4 (30.42), 3 (34.47), 9 (177.04), 6 (181.04)
6.56 (1)	1a		0.35	3 (34.47), 2 (181.29)
7.01 (3)	A[a]	130.14 132.04		10 (45.11), A (130.14), A(131.75), A (132.04), 11 (137.78)
7.08 (2)	A	131.75		A (131.75), 11 (137.78)
7.25 (1)	1b		0.35	2 (181.29)
7.85 (1)	7		2.31	4 (30.42), 5 (57.65), 8 (177.04), 6 (181.04)

[a] Aromatic protons.

Table 2—Single- and Multiple-Bond Correlations Observed for PAIG

^{1}H Chemical Shift, ppm (No. of Protons)	^{1}H Position Scheme	Single-Bond Connectivities, ppm		Carbon Atom Positions (ppm) to Which Multiple-Bond Correlations Were Obsd
		^{13}C	^{15}N	
1.60 (1)	4a	30.58		3 (36.05), 5 (56.71), 6 (179.70), 2 (184.29)
1.71 (1)	4b	30.58		3 (36.05), 5 (56.71), 6 (179.79), 2 (184.29)
1.93 (2)	3	36.05		4 (30.58), 5 (56.71), 2 (184.45)
3.33 (2)	10	44.71		A (131.94), 11 (137.55), 9 (177.88)
3.89 (1)	5	56.71		4 (30.58), 3 (36.41), 9 (177.88), 6 (179.70)
6.83 (1)	7a		0	5 (56.71), 6 (179.70)
6.98 (3)	A[a]	130.11 131.94		10 (44.71), A (130.11), A (131.94)
7.08 (2)	A	131.71		A (131.71), 11 (137.55)
7.49 (1)	7b		0	6 (179.70)
8.34 (1)	8		1.93	4 (30.58), 5 (56.41), 9 (177.88)

[a] Aromatic protons.

under thermospray MS/MS conditions. Therefore, the isolates must be isomers. In Figure 3 the major daughter ions of each hydrolysate are summarized.

Conclusions

The alkaline hydrolysis products of PAP were shown to be PAG and PAIG in a 3.8:1.0 integrated area ratio, while the acidic hydrolysis products were PAG, PAIG, PAGA, and PAA. Previous reports have been shown to be reproducible.[12–14] The synthesis of the hydrolysis standards features improvements over existing methods.[13] The isolation of the standards by flash chromatography proved superior to extraction methods.[13] In order to supplement previously reported data,[13] the structures of the clinically active[7] hydrolysis products, PAG and PAIG, were defined in detail by (1) independent synthesis, (2) HPLC–UV, (3) HPLC–MS–MS, (4) ^{1}H and ^{13}C NMR, and (5) unambiguous assignment with multiple quantum coherence NMR methods (HMQC and HMBC).

Experimental Section

Materials—Isoglutamine, PAA, and PAP were purchased from Sigma Chemical Company, St. Louis, MO.

Alkaline Hydrolysis—PAP (303 mg, 1.2 mmol) was slurried into 3 mL of water. Sodium hydroxide pellets (48 mg, 1.2 mmol) were dissolved in 2 mL of water. The alkaline solution was added dropwise into the slurry over a period of 1 h with stirring until all solids were dissolved and the solution pH was approximately 8. The resulting solution was analyzed by HPLC–UV at 230 nm. The volume ratio of the phosphate buffer and acetonitrile in the mobile phase was 90:10. The remaining hydrolysates were separated by a pass through a flash chromatography column followed by preparative HPLC chromatography described below.

Preparative Chromatography—As an example, the hydrolysates resulting from alkaline hydrolysis (see above) of PAP (140 mg) were separated by passage through a flash chromatography column (19 mm × 45 cm) containing 50 g of LiChroprep (EM Science), 25–40 mesh, C-18 packing. The eluting solvent consisted of 50 mM dihydrogen phosphate buffer (pH 5) and methanol (90:10 v/v). Fractions were concentrated under reduced pressure and set aside for preparative HPLC. Concentrated solutions of each enriched hydrolysate were injected through a 2 mL loop onto a Spherisorb 5 ODS column (250 × 10 mm) under HPLC–UV conditions with a flow rate of 3.5 mL/min. The eluting solvent was aqueous acetic acid (pH 3) and methanol (80:20 v/v). Pure fractions were combined to give 80 mg of PAG and 10 mg of PAIG (each isolate showed 98–99 area % purity by HPLC–UV analysis).

Acidic Hydrolysis—PAP (160 mg) was dissolved in 3 mL of 12 M HCl and transferred to a conical-shaped vial fitted with a screw top lid with a Teflon-faced septum. The vial was placed into an oven at 100 °C. Aliquots (50 μL) were set aside at 15 min intervals over a period of 1 h. Solvents were removed from each aliquot under reduced pressure. The residues were placed into a vacuum desiccator with sodium hydroxide pellets overnight. Each aliquot residue was dissolved in 0.5 mL of water and analyzed by HPLC–UV at 230 nm. The mobile phase volume ratio of phosphate buffer and acetonitrile was 80:20 (v/v). The relative area percent of each hydrolysate as a function of time interval was calculated. The HPLC–UV spiking experiments with standards verified the identity of each hydrolysate PAG, PAIG, PAGA, and PAA. The remaining hydrolysate solution

was reduced to a residue as described above. The hydrolysate residue was subjected to flash chromatographic separations as previously described. Hydrolysates PAGA and PAA were isolated. The chromatographic and spectroscopic data of the isolates matched the data of corresponding standards.

Synthesis—PAG, PAIG, and PAGA were synthesized as shown in Scheme 3 by coupling of phenylacetyl chloride with the appropriate glutamic acid derivative. As an example, the synthesis of PAIG is given: Into 50 mL of water were added isoglutamine (1 g, 6.8 mmol) and sodium bicarbonate (2.8 g) with vigorous stirring until a clear solution developed. Phenylacetyl chloride (1.24 g, 8 mmol) was added slowly with stirring. The solution was stirred for 2 h. The pH of the solution was adjusted to 2.5 with 12 M HCl. The solution was extracted twice with 25 mL portions of dichloromethane. The aqueous layer was concentrated under reduced pressure to a white precipitate, which was dissolved in 10 mL of eluting solvent and purified by two passes through a flash chromatography column (described above). The eluting solvent consisted of aqueous acetic acid (pH 3) and methanol (85:15 v/v). The product PAIG resulted as a white solid (600 mg, 33% yield). The spectroscopic data of the synthetic standards are reported in Figures 2 and 3.

NMR—Solid isolates of PAG (60 mg) and PAIG (8 mg) were dissolved in water in separate NMR tubes, and the pH was adjusted to 6.4 by addition of potassium hydroxide solution. D_2O was added (approximately 10% v/v) to the solutions for a field frequency lock. HMQC, HMBC, ^1H, and ^{13}C experiments were carried out at 2 °C on a Unity-Plus NMR spectrometer (Varian) operating at a proton resonance frequency of 500 MHz. Collection conditions were as follows: proton pulse length = 10.5 μs, sweep width = 4287 Hz, acquisition time = 1.9 s, number of transients = 8; carbon pulse length = 12 μs, sweep width = 21 231 Hz, acquisition time = 1.35 s, number of transients = 300; HMQC sweep width in f_2 = 4287 Hz, sweep width in f_1 = 21 231, acquisition time in t_2 = 0.2455 s, acquisition time in t_1 = 0.006 55 s, number of increments in t_1 = 256, number of transients = 8, number of complex data points in t_2 = 2048, Gaussian apodization employed in both dimensions prior to Fourier transformation; HMBC conditions were identical to those for HMQC except 64 (PAG) and 128 (PAIG) transients were collected at each t_1 increment. Additionally, for HMBC, sine-bell apodization was employed in the t_2 dimension using absolute-value processing and Gaussian filtering was used in t_1 in the phase-sensitive mode. Suppression of the intense H_2O signal by transmitter preirradiation was carried out for all except the ^{13}C NMR experiments.

HPLC-UV—Solution aliquots of synthetic standards, commercial standards, and hydrolysates were injected manually onto a 3 μm, Ultracarb, C-18 column (150 × 4.6 mm) attached to a Hewlett-Packard 1090M HPLC system equipped with a diode array detector. The flow rate was 1 mL/min. The mobile phase consisted of a mixture of 50 mM dihydrogen potassium phosphate buffer (pH 3) and acetonitrile. The volume ratio of the eluting solvent and the selected wavelength varied with the application.

HPLC–MS–MS—Solution aliquots of synthetic standards and isolated hydrolysates were injected into a Finnigan TSQ-70 mass spectrometer with a Waters 590 HPLC pump at a flow rate of 0.8 mL/min without a column. The mobile phase consisted of 50 mM ammonium acetate buffer (pH 5) and methanol (75:25 v/v). The thermospray vaporizer temperature was 80 °C, the jet temperature was 220 °C, and the block temperature reading was 175 °C.

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