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Metabolic Fate of Tolazamide in Man and in the Rat

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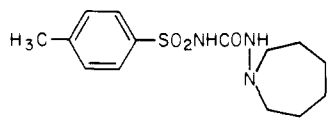
The metabolic fate of tolazamide, 1-(hexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (1), was studied in man and in the rat using tritium-labeled 1. The metabolites were isolated in crystalline form from urine for structure determination. The crystal structure and final molecular structure of one of these, 1-(4-hydroxyhexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**5**), were determined using single-crystal X-ray techniques. Following oral administration of tritiated tolazamide to male humans, 85% of the radioactivity was excreted in urine during a 5-day period. In addition to being excreted in urine unchanged, tolazamide was metabolized to 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)sulfonylurea (**2**), *p*-toluenesulfonamide (**3**), 1-(hexahydroazepin-1-yl)-3-*p*-(hydroxymethylphenyl)sulfonylurea (**4**), 1-(4-hydroxyhexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**5**), and a labile, unidentified metabolite **6** by man. The relative amounts of these materials excreted in 0–24-h urine collections from eight subjects averaged 7, 17, 26, 10, 25, and 15% for 1–**6**, respectively. In the female rat, 79% of an orally administered dose of tritiated tolazamide was excreted in urine during a 5-day period as 1–**4**. The relative amounts of these materials excreted during the 24-h period following administration of tolazamide were 10, 5, 5, and 80% for 1–4, respectively.

The biotransformation of tolazamide,[1] 1-(hexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (1), an orally active,



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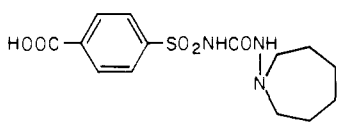
hypoglycemic agent, has not been studied extensively. Forist and Judy[2] reported the isolation and identification of 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)sulfonylurea (**2**) as a urinary metabolite of tolazamide in the



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human; this is the only prior report concerning biotransformation of the drug.

The present work, employing tritium-labeled tolazamide, was undertaken to determine both qualitatively and quantitatively the metabolism of this drug in the human and rat.

Experimental Section

Materials. The preparation of tritium-labeled tolazamide by exposure of nonradioactive 1 to tritium gas has been described.[3] Its chemical and radiochemical purities were ascertained immediately prior to its use. Although the intramolecular distribution of tritium in tritium-labeled 1 was not determined, a similar compound (tolbutamide), also prepared by tritium gas exposure, contained 85% of its tritium in the aromatic ring.

Authentic, synthetic samples of tolazamide (1), *p*-toluenesulfonamide (**3**), and *p*-toluenesulfonylurea, as well as an authentic sample of 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)-sulfonylurea (**2**) which had been isolated from human urine by Forist and Judy,[2] were used as chromatographic and spectral standards.

Radioactivity Measurements. All counting was performed with a Packard Tricarb Model 314EX2A liquid scintillation spectrometer at −8 °C under conditions suitable for measuring tritium. Appropriate aliquots of samples were dissolved in 15 mL of scintillation solvent [toluene–dioxane–MeOH (350:350:210 by volume) containing 73 g of naphthalene, 4.6 g of 2,5-diphenyloxazole, and 0.08 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter]. The absolute counting efficiency for each sample was determined by recounting following addition of an internal standard of tritium-labeled toluene, and results were then converted to disintegrations per minute.

Radiochromatography. Paper chromatography was carried out in the *n*-BuOH–piperidine–H_2O (81:2:17 by volume) system on Whatman No. 1 paper. Dried chromatograms were routinely examined under short-wavelength UV light through a fluorescent screen to locate standards and, when possible, metabolites by fluorescence quenching. Zones of radioactivity were located and quantified by cutting the developed paper strip into sequential 1.25-cm segments and counting the segments in individual vials using scintillation solvent containing 3% H_2O.

Thin-layer chromatography was carried out in the $CHCl_3$–MeOH–formic acid (95:4:1 by volume), $CHCl_3$–EtOAc–formic acid (50:48:2 by volume), and $CHCl_3$–EtOH–heptane–formic acid (33:33:33:0.5 by volume) systems on 0.25-mm films of silica gel GF. The UV absorption of standards and, when possible, metabolites was detected by viewing the dried chromatograms under short-wavelength UV light. The zones of radioactivity were located and quantified by transferring sequential 0.5-cm segments of the developed chromatogram into individual vials and counting using scintillation solvent containing 3% H_2O.

Instrumental Analysis. Electron-impact and field-desorption mass spectra were obtained with a Varian MAT CH5 instrument by direct probe techniques. Ultraviolet analyses were conducted with a Cary 15 spectrometer using 95% EtOH as a solvent. A Perkin-Elmer 421 infrared spectrometer was used to analyze samples in the form of Nujol mulls. Nuclear magnetic resonance spectra were obtained with Varian A-60A and XL100 instruments using acetone-d_6 and DMF-d_7 as solvents. Melting points were determined with a Thomas-Hoover capillary melting point apparatus. Optical rotation was measured with a Bellingham and Stanley polarimeter. Single-crystal X-ray diffraction data were obtained with a Syntex P$\bar{1}$ diffractometer controlled by an IBM 1800 computer.

Single-Dose Administration of Tolazamide to Rats and Collection of Samples. Each of three female, Sprague–Dawley rats, weighing approximately 215 g, was given a 3.8-mg (8.6 μCi) dose of tritium-labeled tolazamide, as a solution of its ammonium salt, by stomach tube. The rats were housed in individual metabolism cages, designed for the separation and collection of urine and feces, and allowed food and water ad libitum during the 5-day study. Each daily urine collection was analyzed for total radioactivity and tritiated water. Each daily fecal collection was analyzed for total radioactivity by an oxygen-flask combustion method.

Preliminary Extraction and Characterization of Rat Metabolites. The 0–24-h urine collections from this study were combined, saturated with $(NH_4)_2SO_4$, acidified with H_2SO_4, and

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extracted five times with equal volumes of Et_2O–EtOH (6:1 by volume). This procedure resulted in extraction of more than 98% of the urinary radioactivity. A small aliquot of this extract was analyzed by radiochromatography in the paper and thin-layer systems along with standard samples of **1**, **2**, *p*-toluenesulfonamide (**3**), and *p*-toluenesulfonylurea. The remainder of the extract was chromatographed on a 1.9 × 100 cm column of Woelm acidic alumina using gradient elution from pure methylene chloride to methylene chloride–MeOH (3:1 by volume) while collecting 10-mL fractions. Four major bands of radioactivity emerged from the column. Each band was analyzed by radiochromatography as previously described.

Multiple-Dose Administration of Tolazamide to Rats and Collection of Samples. In a separate study each of eight female, Sprague–Dawley rats weighing approximately 200 g was given a 50-mg dose of nonradioactive tolazamide, as its ammonium salt, by stomach tube. A ninth rat was given a 10-mg (13 μCi) dose of tritium-labeled tolazamide in an identical manner. For the next 8 days, each of the nine rats was given a 50-mg oral dose of nonradioactive tolazamide. On the tenth day, the dosing regimen of the first day was followed, with eight rats receiving 50-mg oral doses of the nonradioactive drug and the ninth rat a 10-mg (13 μCi) dose of tritium-labeled tolazamide. The rats were housed in metabolism cages during the course of the study, and urine was collected daily for 10 days.

Aliquots of the urine collected from the ninth rat during the two 24-h periods following administration of radioactive tolazamide were subjected to analytical radiochromatography in the paper and thin-layer chromatography systems as previously described.

Isolation, Crystallization, and Identification of Rat Metabolite 4. The urine collections, both radioactive and nonradioactive, from all nine rats were combined, evaporated to 500 mL in vacuo, and extracted as previously described. The extract was evaporated in vacuo to a thick syrup. When an attempt was made to dissolve this syrup in pH 4.5, 0.02 M phosphate buffer in preparation for extraction, a precipitate settled out. Nevertheless, the system, including the precipitate, was extracted five times with 100-mL portions of Et_2O. The Et_2O layer contained 13% of the urinary radioactivity. The aqueous phase including the precipitate was chilled and filtered, and the crystalline material was air-dried. The filtrate contained 24%, and the crystalline material accounted for 63%, of the urinary radioactivity. The solid was recrystallized from EtOH to yield 0.925 g of product, mp 164–164.5 °C (capillary, uncorrected). This material was subjected to radiochromatography in the paper and thin-layer systems, both before and following hydrolysis in 0.1 N HCl at 100 °C. The IR, UV, and NMR spectra of the product were obtained. Anal. ($C_{14}H_{21}N_3O_4S$) C, H, N, S; O: calcd, 19.6; found, 20.1.

Single-Dose Administration of Tolazamide to Humans and Collection of Samples. Finely powdered tolazamide (0.200 g) containing 116 μCi of tritium was administered orally to each of eight normal, male subjects. Urine, feces, and blood samples were taken at appropriate intervals over a period of 5 days. Urine and fecal samples were analyzed for tritium as described previously for the rat.

Preliminary Extraction and Characterization of Human Metabolites. The 0–24-h urine collections from the eight subjects were combined and extracted as previously described for the rat. The combined extracts were evaporated to dryness in vacuo, and the residue was dissolved in H_2O, adjusted to pH 2.1, and extracted five times with EtOAc. This procedure resulted in extraction of more than 97% of the urinary radioactivity. A small aliquot of this extract was analyzed by radiochromatography in the paper and thin-layer systems along with standard samples of **1**–**4** and *p*-toluenesulfonylurea. A 10% aliquot of the extract was chromatographed on a Woelm acidic alumina column in a manner similar to that described for the rat urine extract. Three major bands of radioactivity emerged from the column. Each band was analyzed by radiochromatography as previously described.

Approximately 30% of the urinary extract was subjected to partition chromatography on silica gel in the following manner. Silicic acid (500 g), thoroughly mixed with 250 mL of 0.1 M, pH 5, citrate buffer to give a free-flowing powder, was packed as a slurry with buffer-saturated $CHCl_3$ into a 3.0 × 120 cm column. The urine extract was evaporated to dryness in vacuo, and the residue was slurried with 35 mL of buffer and 70 g of silicic acid. This material was transferred to the column as a slurry in buffer-saturated $CHCl_3$. The column was eluted with a gradient of buffer-saturated $CHCl_3$ to buffer-saturated MeOH–$CHCl_3$ (1:4 by volume) while collecting 20-mL fractions. Four major bands of radioactivity emerged from the column. Each band was analyzed by radiochromatography as previously described.

Multiple-Dose Administration of Tolazamide to Humans and Collection of Samples. In a separate study 0.75 g of nonradioactive tolazamide was administered orally to each of ten normal, male subjects on each of three successive days. Complete urine collections were made for the 3-day period.

Extraction and Preliminary Isolation of Human Metabolites. The urine collections were combined (approximately 75 L) and reduced to a dry residue by evaporation in vacuo followed by freeze drying. The residue was taken up in 4 L of H_2O, spiked with approximately 30% of the previously mentioned EtOAc extract of radioactive urine, and extracted five times with equal volumes of acetone. This procedure resulted in extraction of more than 95% of the radioactivity. The combined extract was evaporated to a thick, aqueous solution and cooled, and a nonradioactive solid was removed by filtration. The filtrate was adjusted to pH 5.0 and extracted four times with equal volumes of $CHCl_3$. This procedure was repeated at pH 4.0 with $CHCl_3$, at pH 3.0 with $CHCl_3$, and at pH 3.0 with EtOAc. This resulted in removal of 85% of the radioactivity from the original acetone extract of urine. Each of the extracts was evaporated to a small volume and allowed to crystallize in the freezer overnight. The crystals were filtered, washed with a small volume of the appropriate, cold solvent, and dried in vacuo. The crystalline material obtained by EtOAc extraction at pH 3.0 contained little drug-related material so it was discarded.

Isolation, Crystallization, and Identification of Human Metabolite 2. The crude crystalline material obtained from the pH 3.0, $CHCl_3$ extract, almost entirely metabolite **2** as determined by paper and thin-layer chromatography, was recrystallized twice from EtOH to obtain 0.364 g of material, mp 189–189.5 °C (capillary, uncorrected). Infrared and UV spectra, as well as equivalent weight by nonaqueous titration, of this material were obtained. It also was subjected to paper and thin-layer radiochromatography along with standard compound **2**. Anal. ($C_{14}H_{19}N_2O_5S$) C, H, N, S.

Separation of Human Metabolites 4 and 5. The crystalline material isolated by $CHCl_3$ extraction at pH 5.0, containing metabolites **2**, **4**, and **5** in relative amounts 14, 22, and 64%, respectively, as determined by paper and thin-layer radiochromatography, was subjected to countercurrent distribution. A 500 tube, 10 mL per phase apparatus was used with the system $CHCl_3$–acetone (1:1 by volume) and 0.1 M, pH 6.8 phosphate buffer for 500 transfers. An aliquot of every fifth tube was counted. On this basis, three zones of radioactivity were found; these corresponded to metabolites **2**, **4**, and **5** as determined by paper and thin-layer radiochromatography. The crystalline material isolated by $CHCl_3$ extraction at pH 4.0 contained approximately equal amounts of **2** and a mixture of **4** and **5**. It was not worked up.

Isolation, Crystallization, and Identification of Human Metabolite 4. Countercurrent tube contents corresponding to **4** were combined and adjusted to pH 4, and the layers were separated. The aqueous phase was extracted four additional times with a mixture of equal parts of $CHCl_3$ and acetone. The combined extracts were evaporated in vacuo, and the residue was recrystallized from EtOH to yield 0.216 g of white crystals, mp 168–170 °C (capillary, uncorrected). Infrared, UV, and NMR spectra, as well as equivalent weight by nonaqueous titration, of this material were obtained. It also was subjected to paper and thin-layer radiochromatography. Anal. ($C_{14}H_{21}N_3O_4S$) C, H, N, O, S.

Isolation, Crystallization, and Identification of Human Metabolite 5. Countercurrent tube contents corresponding to human metabolite **5** were combined and worked up as described for human metabolite **4** to yield 0.407 g of white crystals: mp 140–140.5 °C (capillary, uncorrected); specific rotation $[\alpha]^{25}_D$ 0° (*c* 0.77, 95% EtOH). Infrared, UV, NMR, and mass spectra, as well as equivalent weight by nonaqueous titration, of this material

Table I. Urinary and Fecal Excretion of Radioactivity Following Oral Administration of Tritiated Tolazamide to Three Rats

collection period, h	av % of dose ± SD	
	urine[a]	feces
0-24	75.4 ± 1.9	4.4 ± 4.0
24-48	2.7 ± 0.7	8.5 ± 2.4
48-72	0.45 ± 0.15	0.80 ± 0.37
72-96	0.31 ± 0.05	0.14 ± 0.01
96-120	0.23 ± 0.02	0.07 ± 0.04
total	79.0 ± 1.6	13.9 ± 2.1

[a] Less than 1% of the radioactivity excreted in urine was tritiated water or other volatile, radioactive material.

were obtained. It also was subjected to paper and thin-layer radiochromatography both before and following hydrolysis in 0.1 N HCl at 100 °C. Anal. ($C_{14}H_{21}N_3O_4S$) C, H, N, O, S.

Single-Crystal X-ray Diffraction Studies with Human Metabolite 5. Crystals of **5** were grown as small plates from acetone–Skellysolve B (1:1 by volume). The crystals were triclinic with unit cell parameters, as determined at −150 °C from diffractometer measurements, of a = 8.958 (4) Å, b = 6.494 (3) Å, c = 13.368 (3) Å, α = 92.72 (2)°, β = 97.67 (2)°, γ = 86.71 (3)°. The crystal density, as measured at room temperature by flotation in carbon tetrachloride–heptane, was 1.36 g/cm^3. The calculated density assuming two molecules of $C_{14}H_{21}N_3O_4S$ per unit cell was 1.41 g/cm^3, in reasonable agreement considering the difference in temperature. An initial set of data, collected at room temperature on a heavily deteriorating crystal, did not allow unambiguous interpretation of the disordered area (see Results). A second set of intensity data, reported here, was gathered at low temperature, approximately −150 °C, using the UPACS diffractometer system (a Syntex P$\bar{1}$ diffractometer controlled by an IBM 1800 computer) and graphite monochromated Cu Kα radiation. The step scan technique was used[4] with a scan range of approximately 3.6° and a scan rate of 2°/min. Data were collected to the limit of the Cu sphere within the restrictions of the diffractometer and low-temperature apparatus. No appreciable loss of intensity was observed in the ten reflections which were monitored periodically during data collection. Standard deviations of observed intensities were approximated by $\sigma^2(I) = \sigma^2$ counting statistics + $(0.0294I)^2$, where the coefficient of I in the last term was calculated from deviations in the monitored reflections that were not explained by counting statistics. The final data set consisted of 2600 reflections, of which 2087 were observed at values greater than three times standard deviations.

Determination of Hypoglycemic Activities. The materials tested were administered orally to fasted, intact, glucose-primed, male rats weighing 135–140 g. Two hours after dosing blood samples were withdrawn from the vena cava for blood–glucose analysis. The procedures used have been described previously.[5]

Results

Absorption and Excretion of Tritiated Tolazamide by the Rat. As shown in Table I, an average of 79% of the radioactivity of a single, oral dose of tritiated tolazamide was excreted in urine by the rat over a 5-day period. Most of this was excreted during the first 24-h period following drug administration. Less than 1% of the urinary radioactivity was tritiated water or other volatile, radioactive material. An additional 14% of the dose was accounted for in feces. The 0–24 h urine collected in this study was used to initially characterize and to quantify the metabolites of tolazamide in the rat as follows.

Characterization and Quantification of Tolazamide Metabolites in the Rat. Paper and thin-layer radiochromatography of an aliquot of the extract of the single-dose urine revealed four drug-related materials. Three of these had the mobilities of **1**, **2**, and **3** whereas the fourth

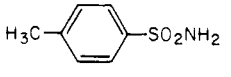

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Table II. Urinary and Fecal Excretion of Radioactivity Following Oral Administration of Tritiated Tolazamide to Eight Humans

collection period, h	av % of dose ± SD	
	urine[a]	feces
0-24	66.4 ± 9.0	0.5 ± 1.1
24-48	15.1 ± 6.6	2.7 ± 3.0
48-72	2.8 ± 2.4	2.7 ± 1.5
72-96	0.3 ± 0.2	0.6 ± 0.5
96-120	0.1 ± 0.1	
total	84.7 ± 3.1	6.6 ± 2.1

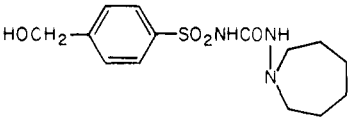
[a] Less than 1% of the radioactivity excreted in urine was tritiated water or other volatile, radioactive material.

was unidentified. They accounted for 10, 5, 5, and 80%, respectively, of the drug-related material excreted in urine.

Attempts to isolate the major, unidentified metabolite by column chromatography of the remainder of the urinary extract on alumina, although successfully separating the four components, and confirming the presence of 1, **2**, and **3**, did not lead to an identification of the major rat metabolite. Therefore, a large supply of urine containing the unidentified metabolite was obtained by chronic, oral dosing of a group of rats with nonradioactive tolazamide.

Identification of the Major Tolazamide Rat Metabolite 4. The major rat metabolite was isolated in crystalline form from the previously mentioned chronic-dose urine. This material showed a single radioactive and UV-absorbing zone, corresponding to the unidentified, major rat metabolite in the paper and three thin-layer chromatography systems. Its elemental analysis corresponded to that of tolazamide with an additional oxygen atom. This was supported, and a hydroxyl group was suggested, by the IR spectrum; it was very similar to that of tolazamide, except for an additional OH/NH absorption band at 3340 cm^{-1}. Acid hydrolysis of the metabolite released a radioactive and UV-absorbing product more polar than *p*-toluenesulfonamide, indicating that the suspected hydroxyl group was on the *p*-tolyl, rather than the hexahydroazepine, moiety of the molecule. The UV spectrum of the metabolite was very similar to that of tolazamide except that the ratio of absorptivities at the 263- and 267-nm peaks was 0.95 compared to 1.10 for tolazamide. This suggested a hydroxymethyl group since previous studies with another *p*-tolylsulfonylurea (tolbutamide) had shown such a shift in the ratio of UV absorptivities when a *p*-hydroxymethyl group replaced a *p*-methyl group. The NMR spectrum of the metabolite was similar to that of tolazamide except that the singlet at δ 2.42 (in DMF-d_7), attributable to the aromatic methyl group of tolazamide, was replaced by a new singlet at δ 4.72, attributable to an aromatic hydroxymethyl group.

It was concluded that this metabolite is 1-(hexahydroazepin-1-yl)-3-*p*-(hydroxymethylphenyl)sulfonylurea (4).



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Absorption and Excretion of Tritiated Tolazamide by the Human. As shown in Table II, an average of 85% of the radioactivity of a single, oral dose of tritiated tolazamide was excreted in urine by the human over a 5-day period. As was the case with the rat, most of this was excreted during the first 24-h period, and less than 1% of the total radioactivity excreted in urine was tritiated water

or other volatile, radioactive material. An additional 7% of the dose was accounted for in feces.

The 0–24-h urine collected in this study was used to initially characterize and to quantify the metabolites of tolazamide in the human as follows.

Characterization and Quantification of Tolazamide Metabolites in the Human. Paper and thin-layer radiochromatography of an aliquot of the extract of the single-dose urine revealed six drug-related materials: 1–4 and two that were unidentified. The relative amounts of these were 7, 17, 10, 25, and 15%, respectively.

Attempts to isolate the two unidentified metabolites by alumina column chromatography of a portion of the urine extract were not successful. Partition chromatography of another portion of the urine extract on a silica gel column was somewhat more successful although it did not lead to a separation or identification of the unidentified metabolites. In this case, four bands of radioactivity emerged from the column. The first was identified as tolazamide (1) by paper and thin-layer radiochromatography; it showed single radioactive and UV-absorbing zones corresponding to standard 1. Attempts to crystallize the material, however, were not successful. The second band was *p*-toluenesulfonamide (**3**). This material was crystallized to yield 2 mg of white crystals, the UV and IR spectra of which were identical with those of standard **3**. It also showed a single radioactive and UV-absorbing zone corresponding to standard **3** when subjected to paper and thin-layer chromatography. The third band from the column contained two radioactive materials, the paper and thin-layer chromatographic mobilities of which were identical with those of the *p*-hydroxymethyl rat metabolite 4 and to one of the unidentified metabolites. The fourth band emerging from the column was identified as the *p*-carboxy metabolite **2**, as judged by paper and thin-layer radiochromatography. Crystalline material was not isolated from either of the latter two radioactive bands from the silica-gel column.

Since two of the human metabolites were not identified, a large supply of urine containing them was obtained by multiple, oral administration of nonradioactive tolazamide to a group of humans.

Identification of Tolazamide Human Metabolites. Metabolites **2**, 4, and one of the previously unidentified, human metabolites of tolazamide were separated and isolated in crystalline form from the previously mentioned multiple-dose urine.

The *p*-carboxy metabolite **2** was crystallized from the pH 3.0, $CHCl_3$ extract of urine. Elemental analysis corresponded to theory for structure **2**. Nonaqueous titration gave an equivalent weight of 173 (calculated, 171) and showed the presence of two acidic groups, one with a pK_a' corresponding to a carboxyl group and the other with a pK_a' corresponding to the $-SO_2NHCO-$ group. The IR and UV spectra, as well as paper and thin-layer chromatographic mobilities, were identical with those of authentic **2**. This metabolite was thereby identified as 1-(hexahydroazepin-1-yl)-3-*p*-(carboxyphenyl)sulfonylurea (**2**).

The *p*-hydroxymethyl metabolite 4 was separated by countercurrent distribution from **2** and an unidentified metabolite in the pH 5.0, $CHCl_3$ extract of urine and crystallized. Elemental analysis corresponded to theory for structure 4. Nonaqueous titration gave an equivalent weight of 319 (calculated, 327) with a pK_a' corresponding to the $-SO_2NHCO-$ group. The IR, UV, and NMR spectra, as well as paper and thin-layer chromatographic mobilities, were identical with those of the previously isolated rat metabolite 4. This metabolite was thereby identified as 1-(hexahydroazepin-1-yl)-3-*p*-(hydroxymethyl)sulfonylurea (4).

The unidentified metabolite **5** was separated by countercurrent distribution from **2** and 4 in the pH 5.0, $CHCl_3$ extract of urine and crystallized. This material showed a single radioactive and UV-absorbing zone, corresponding to the unidentified, human metabolite in the paper and thin-layer chromatography systems. Its elemental analysis corresponded to that of tolazamide with an additional oxygen atom. Nonaqueous titration gave an equivalent weight of 328 (calculated, 327) with a pK_a' corresponding to that of the $-SO_2NHCO-$ group. This was supported, and a hydroxyl group was suggested, by the IR spectrum; it was very similar to that of tolazamide, except for an additional OH/NH absorption band at 3480 cm^{-1}. Mass spectrometry of this material, using a field-desorption technique, revealed a molecular ion at 327 mass units. The UV spectrum of the metabolite, including the ratio of absorptivities at the 263- and 267-nm peaks, was very similar to that of tolazamide, suggesting that the hydroxyl group was not on the *p*-methyl group. Acid hydrolysis of the metabolite released a radioactive and UV-absorbing product having the same paper and thin-layer chromatographic mobilities as *p*-toluenesulfonamide, indicating that the suspected hydroxyl was not on the *p*-tolyl moiety of the molecule. When the metabolite was subjected to mass spectrometry using an electron-impact technique only a weak molecular ion was obtained. However, the fragmentation pattern showed an intense peak at $M^+ - 197$, corresponding to 1-aminohydroxyhexahydroazepine. The NMR spectrum of the metabolite was compared with that of tolazamide. The metabolite spectrum still contained a singlet at δ 2.42 attributable to an aromatic methyl group but revealed a carbinol hydrogen as a broad signal at δ 4.0. The four carbamine hydrogens on carbons two and seven of the hexahydroazepine were still present as judged by absorption at δ 2.95. It therefore was concluded that the metabolite contained a hydroxyl group on carbon three or four of the hexahydroazepine ring.

A single-crystal X-ray investigation was performed to complete the structure determination of metabolite **5**. Since the unit cell is triclinic, two space groups were possible—$P1$ (no symmetry) and $P\bar{1}$ (center of symmetry). Elemental composition and density indicated two molecules per unit cell, compatible with either. However, since the molecule has an asymmetric carbon atom, for $P\bar{1}$ to be correct, both *d* and *l* forms of the molecule would have to be present in approximately equal amounts in the crystal studied; since the observed rotation, $[\alpha]_D$, was zero, a racemate could not be ruled out. Work on the initial room temperature data set was performed in space group $P1$. However, during early refinement, atom positions were analyzed and found to correspond to two molecules related by a center of symmetry, indicating that the racemate was present and space group $P\bar{1}$ is the correct crystal symmetry. All subsequent calculations were performed in the centric space group. The crystal structure was found to be disordered in the hexahydroazepine ring. After many unsuccessful attempts to satisfactorily describe this disorder using the data gathered at room temperature, a low-temperature data set was collected.

A trial structure for the low-temperature data was obtained easily by direct methods using the automatic program DIREC[4] which employs both triplet and quartet[6] relationships. With the lack of crystal deterioration and with thermal effects much reduced, a satisfactory model for the disorder in the hexahydroazepine ring was found. The disorder consists of major and minor conformers

Table III. Final Atomic Coordinates and Their Standard Deviations (in Parentheses) for Metabolite 5 from X-ray Results. All Values Are Multiplied by 10^5

ATOM	X	Y	Z
S	72813 (11)	47945 (15)	21598 (6)
N(1)	78782 (35)	17883 (47)	50134 (20)
N(2)	66922 (35)	32751 (48)	48256 (20)
N(3)	74981 (32)	37823 (45)	32775 (19)
O(1)	54301 (28)	56010 (38)	37760 (16)
O(2)	107137 (42)	-47 (59)	72965 (30)
O(2')	92122 (96)	-22793(119)	66884 (59)
O(3)	72266 (29)	70048 (39)	22746 (19)
O(4)	84566 (29)	37651 (46)	16665 (19)
C(1)	64501 (42)	43117 (54)	39565 (23)
C(2)	90246 (43)	25222 (57)	58253 (26)
C(3)	86046 (43)	26610 (56)	68956 (24)
C(4)	89916 (72)	6817 (69)	74230 (30)
C(5)	81140 (49)	-11516 (58)	69890 (25)
C(6)	81191 (70)	-16415 (85)	58572 (39)
C(6')	68122(116)	-8205(147)	60424 (70)
C(7)	72449 (50)	-2043 (64)	51752 (29)
C(8)	55133 (40)	40495 (54)	15615 (22)
C(9)	43710 (42)	55336 (52)	13306 (25)
C(10)	30023 (42)	49559 (56)	8200 (25)
C(11)	27719 (41)	28861 (56)	5422 (22)
C(12)	39366 (46)	14491 (55)	7897 (25)
C(13)	53175 (44)	19718 (56)	12908 (26)
C(14)	12631 (45)	22572 (60)	119 (26)

occupying the same site, with the hydroxyl oxygen switching from the C(4) position in the major conformer to the C(5) position in the minor one and C(6) changing positions. Coordinates and anisotropic thermal parameters for all atoms, including alternate positions for C(6) and O(2) as defined from the disorder model, a population factor giving the fraction of each of the disordered conformers, and an overall scale factor were refined by full-matrix least squares. Hydrogen atoms were included in the calculations at calculated positions; the methyl hydrogens and the hydroxyl hydrogen of the major conformer were located by difference Fourier and then idealized positions were used, but the hydroxyl hydrogen of the minor conformer could not be found. The final agreement index ($R = \Sigma|F_o - F_c|/\Sigma|F_o|$) was 0.078, and final shifts in all parameters were less than 2% of standard deviations. A final difference map verified that the disorder model had successfully accounted for all electron density. Final atomic coordinates and their standard deviations are given in Table III. Other tables from the X-ray work have been placed in the microfilm edition (see paragraph at end of paper). The final value of the population factor was 0.634 (0.005) indicating a two-thirds to one-third split between major and minor conformers.

Figure 1, drawn from final coordinates, shows the conformations of the major and minor conformers and the bond distances of special interest in the sulfonylurea moiety. Other distances are within expected ranges; in the disordered region some distances show deviations of up to 0.15 Å from expected values, in closer agreement than is usually found in disorder cases. In the crystals studied, the molecule occurs as both the *d* and *l* enantiomers. One sulfonyl oxygen and the adjacent urea nitrogen are hydrogen bonded to the hydroxyl oxygen in a symmetry related molecule. (N–O and O–O distances are 2.98 and 2.88 Å in the major conformer and 3.05 and 2.98 Å in the minor one). The urea oxygen and the other urea nitrogen form a hydrogen bond (2.881 Å) to the corresponding moieties in a symmetry-related molecule. In addition, the hydroxyl oxygen also forms a hydrogen bond (2.75 Å) to the other sulfonyl oxygen in a different symmetry-related molecule in the major, but not the minor, conformer. From

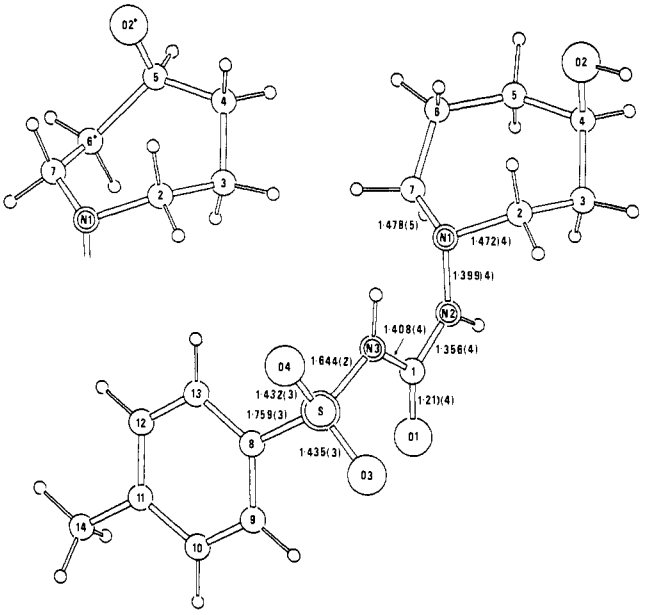
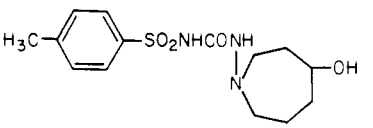


Figure 1. Major and minor (insert) conformers and selected bond distances in angstroms (standard deviation in the last digit is in parentheses) of metabolite 5 from X-ray results.

these results, it was concluded that this metabolite is *dl*-1-(4-hydroxyhexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**5**).



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Studies with Human Unidentified Metabolite 6. The human unidentified metabolite **6**, which had a slightly greater mobility than the carboxy metabolite **2** in the paper chromatography system, appeared to be labile, changing to a material having the same mobility as **2**. Samples of **6** which had been purified by preparative paper chromatography were applied to chromatography paper and allowed to stand in contact with air for various periods up to a month. Fresh samples of **6** when rechromatographed exhibited their initial mobilities whereas samples exposed to air gave two materials, one with the mobility of **6** and the other with that of **2**. After a month's exposure, a single material having the mobility of **2** was observed. Efforts to isolate **6** and further characterize it were not successful.

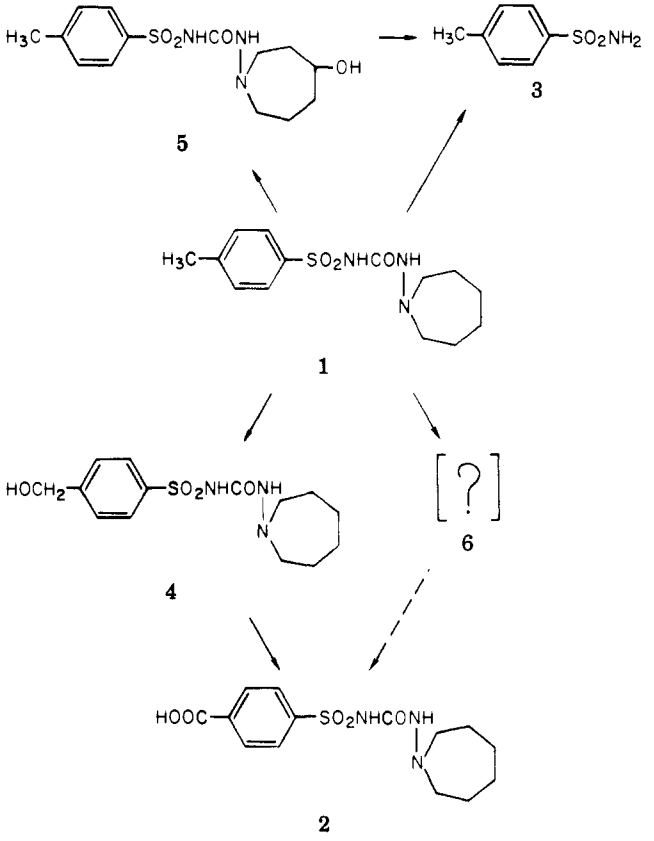
Summary of Biotransformation of Tolazamide by the Human. Tolazamide was extensively metabolized by the human; only 7% of the drug-related material appearing in urine during the 24-h period following drug administration was unchanged tolazamide (**1**). The remainder of the material excreted in urine was accounted for as five metabolites of tolazamide, four of which were isolated in crystalline form and identified. The carboxy metabolite **2**, previously isolated and identified by Forist and Judy,[2] accounted for 17% of the urinary radioactivity. An additional 26% of the drug-related material excreted in urine was found to be *p*-toluenesulfonamide (**3**). A hydroxymethyl metabolite, 1-(hexahydroazepin-1-yl)-3-*p*-(hydroxymethylphenyl)sulfonylurea (**4**), accounted for 10% of the radioactivity excreted in urine. Another 25% of the tolazamide-related material was found to be a ring-hydroxylated metabolite, 1-(4-hydroxyhexahydroazepin-1-yl)-3-*p*-tolylsulfonylurea (**5**). The remainder of the urinary radioactivity, 15%, appeared to be associated with a labile metabolite **6** slightly less polar than **2** but more

Table IV. Relative Amounts of Tolazamide and Its Metabolites in Urine Following Oral Administration of Tritiated Tolazamide to the Human and Rat

compd	rel % in urine[a] human	rat
1	7	10
2	17	5
3	26	5
4	10	80
5	25	<5
6[b]	15	0

[a] During the 24-h period immediately following drug administration. [b] Not identified.

Scheme I. Pathways for Biotransformation of Tolazamide



polar than 1 and 3–5 as judged by paper chromatography. This material was not identified. These results are summarized in Table IV and Scheme I.

Summary of Biotransformation of Tolazamide by the Rat. As was the case with the human, tolazamide was extensively metabolized by the rat; only 10% of the radioactive material appearing in urine during the 24-h period following drug administration was unchanged tolazamide. The remainder was accounted for by three metabolites of the drug. The major metabolite was the hydroxymethyl compound 4 which accounted for 80% of the urinary radioactivity. It is possible that a small amount (less than 5%) of the ring-hydroxylated metabolite 5 also was produced but was not resolved from 4. The other two metabolites of tolazamide appearing in rat urine, each accounting for 5% of the urinary radioactivity, were *p*-toluenesulfonamide (3) and the carboxy metabolite 2. These results are summarized in Table IV and Scheme I.

Hypoglycemic Activities of Metabolites. The oral, hypoglycemic activities of the tolazamide metabolites were compared to that of the parent drug in the rat. These results, summarized in Table V, showed that at least two of the metabolites (compound 4 and 5) had significant hypoglycemic activities relative to tolazamide.

Table V. Hypoglycemic Activities of Tolazamide Metabolites[a]

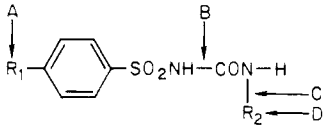
compd	rel hypoglycemic act.
1	1
2[b]	0.05
3[c]	0
4[d]	0.2
5[e]	0.7

[a] Each compound was tested in six rats at each dosage level vs. tolbutamide, which has approximately one-sixth the hypoglycemic activity of tolazamide in this assay. [b] Dosed at 25 and 50 mg/kg body weight. [c] Dosed at 100 mg/kg body weight. [d] Dosed at 9.4 and 18.8 mg/kg body weight. [e] Dosed at 1, 5, 10, and 20 mg/kg body weight.

Discussion

As evidenced by urinary excretion of radioactivity, orally administered, tritiated tolazamide was well absorbed by the human and rat. In this respect, tolazamide is similar to other sulfonylurea hypoglycemic agents, tolbutamide,[7] chlorpropamide,[8] and acetohexamide,[9-11] presently marketed in the United States as well as to the newer sulfonylureas, glisoxepid,[12,13] glipizide,[14,15] glibornuride,[16,17] and glyburide.[18,21]

The biotransformation of para-substituted phenylsulfonylurea hypoglycemic agents 7 is dependent on the



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chemical nature of R_1 and R_2 as well as on the animal species. This biotransformation can involve oxidation or reduction of R_1 at point A, hydrolysis at point B to produce a phenylsulfonamide, dealkylation at point C to produce a phenylsulfonylurea, and hydroxylation of R_2 at point D. It is interesting that neither aromatic-ring hydroxylation nor glucuronide or sulfate conjugation has been observed with the sulfonylureas.

Tolbutamide,[7] in which $R_1 = CH_3$ and $R_2 = n$-butyl, is completely metabolized, primarily to a hydroxymethyl at point A in 7 by the rat and primarily to a carboxy at A by the human. Attack at B and C, although not important in the human and rat, leads to the major metabolites of tolbutamide in the dog. Hydroxylation of tolbutamide at D has not been observed.

Chlorpropamide,[8,22,23] in which R_1 = Cl and $R_2 = n$-propyl, is not completely metabolized by the human, dog, and rat. Attack at point A in 7 has not been observed, but hydrolysis at B and dealkylation at C in the human and dog, and rather extensive dealkylation at C in the rat, are important routes of metabolism. Hydroxylation of chlorpropamide at D is the major metabolic pathway for chlorpropamide in the human and rat.

Acetohexamide,[9-11] in which R_1 = acetyl and R_2 = cyclohexyl, is almost completely metabolized by the human, dog, and rat. Reduction of the acetyl group to hydroxyethyl at point A in 7 and hydroxylation of R_2 at D are major metabolic pathways in each of the three species. Metabolic cleavage of acetohexamide at B and C has not been reported.

Glipizide,[14,15] in which R_1 = 2-(5-methylpyrazine-2-carboxamido)ethyl and R_2 = cyclohexyl, is completely metabolized by the human, dog, and rat. In the dog, the

major metabolite involves hydrolysis of the carboxamide and subsequent acetylation of the resulting amine at point A in 7. Hydroxylation of R_2 at D is a minor metabolic pathway in the dog. In contrast, metabolism involving R_1 is minor and hydroxylation of R_2 at D leads to the major metabolites in man and the rat. No cleavage at B or C was reported for any of the species.

Glibornuride,[17] in which R_1 = CH_3 and R_2 = hydroxybornyl, is completely metabolized by the human. Oxidation of R_1 to hydroxymethyl and carboxy groups at point A in 7 is a minor route of metabolism. The major metabolites are produced by monohydroxylation of two of the methyl groups of R_2 at D. No cleavage at B or C has been reported.

Glyburide,[18-20] in which R_1 = 2-(5-chloro-2-methoxybenzamido)ethyl and R_2 = cyclohexyl, is completely metabolized by the human, dog, rat, and rabbit. Cleavage of R_1 at point A in 7 to give a carboxy group is a minor metabolic pathway in the dog, rat, and rabbit. Monohydroxylation of R_2 at D leads to the major metabolites in all four species. No cleavage at B or C has been reported in any of the species.

Following its oral administration, a small amount of unchanged tolazamide was excreted in urine by both the human and the rat. Both species, however, eliminated most of the drug in the form of metabolites. The structures of these metabolites showed that the biotransformation of tolazamide involved three of the four molecular sites shown in 7. Oxidation of its methyl group at point A in 7 was a major metabolic pathway for tolazamide. In the case of the rat, oxidation practically stopped at the hydroxymethyl stage resulting in the hydroxymethyl metabolite 4 being the principle metabolite of tolazamide. A small amount of the carboxy metabolite 2 was produced by the rat, however. In the case of the human, oxidation was carried further, resulting in excretion of somewhat more carboxy metabolite 2 than hydroxymethyl metabolite 4. This difference in oxidation of the methyl group by the two species was observed for tolbutamide[7] previously.

Cleavage of the sulfonylurea linkage of tolazamide (or the ring-hydroxylated metabolite 5) at point B in structure 7 to produce the sulfonamide 3 was another major metabolic pathway in the human but only a minor pathway in the rat. At least some of the sulfonamide 3 could have arisen by hydrolysis of tolazamide (or its ring-hydroxylated metabolite 5) at point B in structure 7 during storage or workup of urine; this was not investigated. The fate of the 1-aminohexahydroazepine, which likely formed along with *p*-tolylsulfonamide, was not investigated. Little, if any, of the tritium label would have been associated with the hexahydroazepine ring of the tritiated tolazamide.

Metabolic cleavage of tolazamide at point C in 7 did not occur, as judged by the absence of *p*-tolysulfonylurea in urine. Such a cleavage would have involved rupture of a N–N bond in contrast to rupture of a N–C bond, apparently by a dealkylation reaction, as would be the case for the other sulfonylurea hypoglycemic agents.

Hydroxylation of tolazamide at point D of 7 to give the 4-hydroxyhexahydroazepine metabolite 5 was a major metabolic pathway in the human but was not observed in the rat. Metabolic hydroxylation of such a ring system has not previously been reported. However, it has some analogy to hydroxylation of the cyclohexyl ring of acetohexamide (R_1 = acetyl and R_2 = cyclohexyl in 7). In that case both the 3- and 4-hydroxy isomers, each in the cis and trans forms, were produced by the human as well as by the rat.[10] This low degree of specificity with respect to the point of attack on the cyclohexyl ring was attributed to a reaction mechanism in which the substrate is rather loosely associated with enzyme. In the present case, the specificity for hydroxylation in the 4 position of the hexahydroazepine ring may be more apparent than real. The 4-hydroxy metabolite 5 isolated gave no optical rotation, and X-ray diffraction analysis showed, at least for the crystal analyzed, that the metabolite was present as a *dl* pair. Hydroxylation only at ring carbon four, from opposite sides of the hexahydroazepine ring, would require both inversion of the ring nitrogen and reversal of the ring to give such a *dl* pair. However, hydroxylation from the same side of the ring, at carbon four of one molecule and carbon five of another molecule, also would give a mixture of the *d* and *l* forms of the metabolite. Thus, the 4-hydroxy metabolite 5 may well have arisen by relatively nonspecific hydroxylation of tolazamide at carbon atoms four and five of the hexahydroazepine ring.

Whatever the nature of the unidentified metabolite 6, it does not appear to have been a chromatographic artifact. It does, however, appear to be related to the carboxy metabolite 2, since lengthy exposure to air resulted in its conversion to a material having the chromatographic mobility of 2. Furthermore, 6 was detected in the urine of the human, in which 2 is a major metabolite, but not in the urine of the rat, in which 2 is a minor metabolite. Metabolite 6 has not been identified nor characterized beyond its apparent relationship to the carboxy metabolite 2.

Two of the tolazamide metabolites, the ring hydroxylated (5) and the hydroxymethyl (4), had significant oral, hypoglycemic activities. Although neither was as active as tolazamide itself, both were more potent than tolbutamide. A third metabolite, the carboxy (2), had measurable oral, hypoglycermic activity but only about one-third that of tolbutamide. The fourth metabolite, *p*-toluenesulfonamide (3), had no measurable oral activity.

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Supplementary Material Available: Tables of anisotropic temperature parameters, bond distances, bond angles, and torsion angles (4 pages). Ordering information is given on any current masthead page.

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Antitumor Anthracycline Antibiotics. Structure–Activity and Structure–Cardiotoxicity Relationships of Rubidazone Analogues

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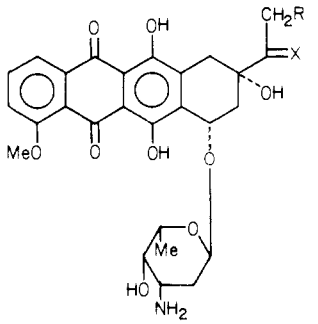
A series of rubidazone analogues (4–14) with varying phenyl group substituents was prepared. The effect of these compounds on inhibition of nucleic acid synthesis in cultured cells, on in vivo antitumor properties, and on cardiotoxicity was examined. Substituent effects on drug–DNA binding as indicated by DNA melting temperature measurements were also investigated. Substituent effects were essentially absent among the rubidazone analogues in in vivo and in vitro test systems which measure cytotoxic characteristics; however, the rubidazone analogues varied substantially in their cardiotoxic effects and this variation was closely correlated with the electronic character of the phenyl substituent.

Daunorubicin (1) and especially adriamycin (**2**) have become important drugs for cancer chemotherapy.[1-3] Not unexpectedly, their usefulness has generated considerable interest in developing analogues with improved properties.[1] Among these analogues, rubidazone (daunorubicin benzhydrazone, **3**) has received considerable attention. After



1, R = H; X = O (daunorubicin)
2, R = OH; X = O (adriamycin)
3, R = H; X = $NNHCOC_6H_5$ (rubidazone)

the first report of its activity in experimental systems,[4] clinical evaluation quickly showed it to be at least equivalent to daunorubicin in efficacy against leukemia and to have potentially advantageous pharmacological properties.[5-8] In particular, rubidazone was described as less toxic and easier to administer than daunorubicin.[8] Biochemical studies have not indicated, however, that rubidazone differs in any fundamental way from the parent antibiotic.[9-11]

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The favorable clinical reports on rubidazone and the very limited published reports on related structure–activity studies[12] led us to consider it as a starting point for our analogue study. We were further encouraged by its decreased cardiotoxicity relative to adriamycin[12,13] because the dose-limiting, cumulative cardiotoxic properties of the latter are well-known.[14] The inclusion of cardiotoxicity evaluation in this study was made possible by development of a reproducible screening system in the rat that is economical in cost and in required amount of drug.[13] Important cardiotoxicity models in the rabbit[15] and rhesus monkey[16] have also been developed, but they are not practical for primary screening. A mouse model using microscopically determined morphological criteria as the end point has also been proposed.[17a] The rat model employs as end point the characteristic electrocardiographic (ECG) changes that follow repeated administration of cardiotoxic anthracycline derivatives. These ECG effects are associated with impairment of heart mitochondrial function.[17b]

Rubidazone is an excellent candidate for a lead on which to base a quantitative structure–activity study because the benzhydrazone moiety is easily incorporated into daunorubicin and the benzoyl group can serve as a readily accessible carrier for systematic variation of substituents. We therefore decided to prepare a series of rubidazone analogues with phenyl group substituents that would provide varying electronic and partition properties. At the beginning of this work we were aware of the problems involved in finding a set of noncollinear aromatic substituents that would describe electronic and partition properties; we therefore chose the initial targets to give low collinearity with reasonable synthetic accessibility.[18,19] A σ vs. π plot of the substituents is shown in Figure 1. This plot is normalized against daunorubicin benzhydrazone (**3**).

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