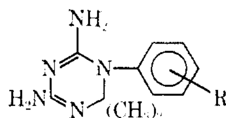
TABLE X

PHYSICAL PROPERTIES OF



No.[a]	R	Yield,[b] %	Mp, °C dec	Formula[c]
15	4-$(CH_2)_2CONHC_6H_4CH_3$-*m*	49[d]	210–211	$C_{23}H_{32}N_6O_4S$
16[e]	3-Cl-4-$O(CH_2)_3OC_6H_5$	20[d]	200–204	$C_{22}H_{30}ClN_5O_5S$
17	3-Cl-4-$OCH_2CONHC_6H_5$	28[d]	200–203	$C_{21}H_{27}ClN_6O_5S$
18	3-Cl-4-$(CH_2)_2C_6H_5$	52[d]	205–207	$C_{21}H_{28}ClN_5O_3S$
19[f]	4-$(CH_2)_4C_6H_5$	44[g]	199–200	$C_{23}H_{33}N_5O_3S$

[a] Synthesized by method D.[23b] [b] Yield of anal. pure material from nitro intermediate. [c] Analyzed for C, H, N. [d] Recrystd from *i*-PrOH–H_2O. [e] See ref 23b for NO_2 intermediate. [f] The synthesis of the HCl salt was previously described by B. R. Baker, B. T. Ho, and G. J. Lourens, *J. Pharm. Sci.*, **56**, 737 (1967). [g] Recrystd from *i*-PrOH.

TABLE XI

PHYSICAL PROPERTIES OF INTERMEDIATES

No.	Compound	Yield,[a] %	Mp, °C	Formula[b]
21[c]	*p*-$NO_2C_6H_4CH{=}CHCONHC_6H_4CH_3$-*m*	61	170–173[d]	$C_{16}H_{14}N_2O_3$
22	4-NO_2-2-$ClC_6H_3OCH_2CONHC_6H_5$	43[e,f]	157–158	$C_{14}H_{11}ClN_2O_4$
23	4-NO_2-2-$ClC_6H_3CH{=}CHC_6H_5$	72[g,h]	105–108[i]	

[a] Yield of anal. pure material. [b] *Anal.* C, H, N. [c] See method A in B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 672 (1968). [d] Recrystd from EtOH–THF. [e] Recrystd from $CHCl_3$–EtOH. [f] Prepd from α-chloroacetanilide according to method A in ref 23b. [g] Prepd from 2-chloro-4-nitrobenzaldehyde according to method A in ref 12c. [h] Recrystd from EtOH–H_2O. [i] Lit. mp 111–112° prepared by an alternate route; see L. Chardonnens and P. Heinrich, *Helv. Chim. Acta*, **23**, 292 (1940).

inhibitors;[12,23] properties are listed in Table X and intermediates in Table XI. Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had proper uv and ir spectra and moved as a single spot on tlc with Brinkman silica gel GF; each gave combustion values for C, H, and N within 0.4% of theoretical.

(23) (a) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 677 (1968); (b) B. R. Baker and G. J. Lourens, *ibid.*, **12**, 95 (1969).

Irreversible Enzyme Inhibitors. CLXXIV.[1,2] Metabolism of 4-[*p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)hydrocinnamido]-*o*-toluenesulfonyl Fluoride (NSC-113423), an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

ADRIAN J. RYAN,[3] NICOLAAS M. J. VERMEULEN,[4a] AND B. R. BAKER[4b]

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

Received June 5, 1970

The ^{14}C-labeled title compound (**1**) was synthesized from [^{14}C]cyanoguanidine, acetone, and *p*-(*p*-aminohydrocinnamido)-*o*-toluenesulfonyl fluoride (**4**). The sulfonic acid **2** corresponding to hydrolysis of the SO_2F group was the only radioactive excretion product, 20% of the radioactivity appearing in the urine and 58% in the feces. Investigation of serum and liver extract of the rat showed that the liver contained a "sulfonyl fluoridase" that rapidly converted **1** into **2**; hydrolysis by serum was much slower, but complete in 20 hr at 37°.

In the previous paper of this series,[2] 9 active-site-directed irreversible inhibitors of dihydrofolic reductase of the SO_2F type were tested against Walker 256 ascites and Dunning leukemia ascites in the rat; although cures could be achieved, no correlation between tissue specificity of irreversible inhibition of the enzyme and *in vivo* effectiveness was apparent. Furthermore, 5 reversible inhibitors—where the SO_2F group had been replaced by H—were as effective *in vivo* as the irreversible inhibitors. These results indicated that other factors such as transport and metabolism might be playing a role which negated correlation. Therefore one of the compounds (**1**) was labeled with ^{14}C in the triazine ring and subjected to a metabolic study in the rat. The results are the subject of this paper.

Chemistry.—The ^{14}C-labeled **1** was synthesized by condensation of [^{14}C]cyanoguanidine, the arylamine precursor **4**,[5] and acetone according to the general method of Modest.[6] Two obvious possibilities for metabolic change of the side chain of **1** were hydrolysis of

(1) This work was generously supported by Grant No. CA-08695 from the National Cancer Institute, U. S. Public Health Service.

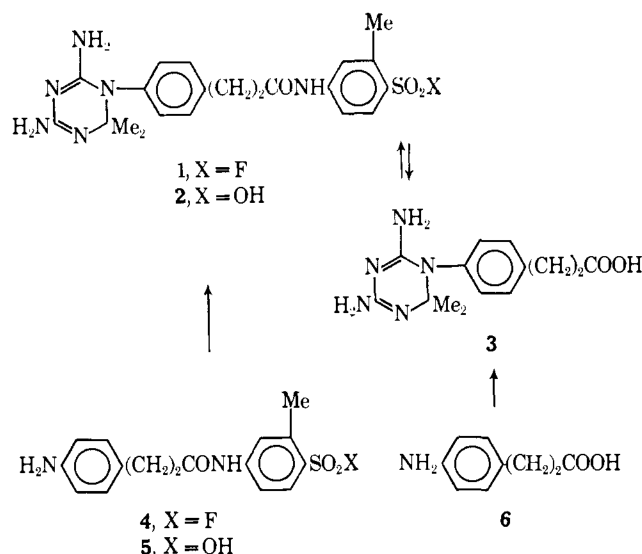
(2) For the previous paper in this series see B. R. Baker, N. M. J. Vermeulen, W. A. Ashton, and A. J. Ryan, *J. Med. Chem.*, **13**, 1130 (1970).

(3) On sabbatical leave from the University of Sydney, Australia.

(4) (a) N. M. J. Vermeulen wishes to thank the Council of Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship. (b) To whom correspondence should be addressed.

(5) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 677 (1968), paper CXXIX.

(6) E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).



the SO_2F group to the sulfonic acid **2** or amide cleavage to **3**; of the two, hydrolysis to the sulfonic acid **2** was considered a major possibility since Folsch and Bertino[7] have previously observed that mouse serum can rapidly destroy **1** with formation of a product that was presumed to be **2** on kinetic and mechanistic grounds.

The propionic acid derivative **3** was readily synthesized from *p*-aminohydrocinnamic acid (**6**) and cyanoguanidine in acetone. Attempts to synthesize **2** by condensation of **5** with acetone and cyanoguanidine were hampered by insolubility; a product containing **2** was obtained, but it was grossly contaminated with what appeared to be the intermediate biguanide.[6] However, **2** could be synthesized from **3** by conversion into the mixed anhydride with ethyl chloroformate, then coupling with 2-methylsulfanilic acid.

In Vivo **Metabolism.**—The excretion results after ip injection of a 5-mg dose of ^{14}C-**1** in the rat are summarized in Table I. Urinary excretion of radioactivity was relatively low (about 20%) and was essentially complete after 24 hr. Fecal excretion was higher, 43% of the radioactivity being excreted in 24 hr and another 14% in 96 hr. The combined excreted radioactivity was 78%.

TABLE I

EXCRETION OF RADIOACTIVITY IN URINE AND FECES OF THE RAT AFTER A 5-MG DOSE OF ^{14}C-1

Time, hr	% dose excreted[a] Urine	Feces
0–24	19	43
24–48	1	10
48–72	1	3
72–96	<1	1

[a] Average for 6 rats each dosed with 5 mg of ^{14}C-**1**; see Experimental Section.

Examination of the urine by tlc showed the presence of only one radioactive spot that moved identically with the sulfonic acid **2**; no sulfonyl fluoride (**1**) or propionic acid could be detected although synthetic **1–3** were readily separated by the tlc system. The radioactivity could be completely solubilized from the dried feces by Soxhlet extraction with aq acetone; tlc again showed only one radioactive spot and this spot moved identically with the sulfonic acid **2**. When the urinary and fecal metabolite were cochromatographed, no separation occurred.

In Vitro **Metabolism.**—The radioactive triazine (**1**) was incubated with (a) fresh rat serum, (b) a 0–45% $(NH_4)_2SO_4$ fraction of rat liver, (c) a 45–90% $(NH_4)_2SO_4$ fraction of rat liver, or (d) phosphate buffer (pH 7.4); the results are summarized in Table II. In serum the proportion of radioactivity fell with time, only 60 and 30% of radioactivity being soluble after 3 and 20 hr, respectively. In contrast, the rat liver fractions showed little binding and >90% of the radioactivity was still soluble after 3 hr.

TABLE II

INCUBATION OF ^{14}C-1 WITH TISSUE PREPARATIONS

Time, hr[a]	% radioactivity in supernatent and ratio of 1:2 — Rat serum	Rat liver, 0–45% $(NH_4)_2SO_4$	Rat liver, 45–90% $(NH_4)_2SO_4$
0.5	92 (100:0)	97 (1:2)	100 (1:5)
1	88 (7:3)	96 (1:7)	97 (1:6)
2	70 (5:4)	98	94
3	61 (1:2)	97	91
20	30 (0:100)		

[a] See Experimental Section for procedures; a control with phosphate buffer (pH 7.4) showed no conversion of **1** into **2**.

The nature of the radioactivity was examined by tlc (Table II). After a lag time of 0.5 hr, **2** began to appear in rat serum, the ratio of **1** to **2** being approximately 7:3 in 1 hr and only **2** present after 20 hr. Hydrolysis by liver extract proceeded much more rapidly than by serum. Of the 2 liver fractions the 45–90% $(NH_4)_2SO_4$ fraction was more active in hydrolyzing **1** to the sulfonic acid (**2**); within 30 min the latter fraction had hydrolyzed about 80% of **1** to **2**.

Discussion

The results obtained by ip dosage of rats with **1** are quite clear cut. There is rapid absorption from the peritoneal cavity, metabolism of **1**, then excretion. Nearly 80% of the radioactivity can be accounted for in urine and feces after 96 hr.

The pattern of excretion is unusual for the metabolic product, the sulfonic acid **2**. Urinary excretion is only about 20% of the dose and is essentially complete after 24 hr. In contrast, fecal excretion is very high, especially in view of ip dosage; more than 40% of the dose is excreted in the feces in the first 24 hr, increasing to nearly 60% after 96 hr. These results can be rationalized as follows.

The sulfonyl fluoride **1** is readily absorbed from the peritoneal cavity, then rapidly hydrolyzed to **2**; most of this hydrolysis appears to take place in the liver, although tissues other than liver and serum were not examined. In the liver, the sulfonic acid **2** is partitioned between blood and bile with the latter predominating. The metabolite in blood is excreted in the urine and the metabolite in the bile is excreted in the feces *via* the gut. Biliary excretion must be very rapid because of the high 24-hr fecal excretion; the rate of fecal excretion approaches what would be expected of a compound poorly absorbed after oral dosage.[8] Furthermore, rats are

(7) E. Folsch and J. R. Bertino, *Mol. Pharmacol.*, **6**, 93 (1970).

(8) A. J. Ryan and P. G. Welling, *Food Cosmet. Toxicol.*, **5**, 755 (1967).

known to have an efficient biliary excretion mechanism for foreign compounds.[9] It is noteworthy that **2** is a relatively high molecular weight sulfonic acid and several of these are readily excreted in the bile of rats.[10]

The *in vitro* experiments support this rationalization. Thus, hydrolysis of **1** in serum was slow compared to hydrolysis in liver extracts. Liver is rich in esterases,[11] including an enzyme that can hydrolyze a fluorophosphate such as DFP to diisopropyl hydrogen phosphate.[12] The enzyme that hydrolyzes **1** to **2** can be called a sulfonyl fluoridase, even though this relatively nonspecific enzyme may also be an esterase.

Active-site-directed irreversible enzyme inhibitors of the SO_2F type can react with an enzyme in two ways; the enzyme becomes inactivated by covalent bond formation but the active enzyme can also catalyze conversion of the SO_2F group into sulfonic acid.[13] It should be noted that the 45–90% $(NH_4)_2SO_4$ fraction of rat liver contains dihydrofolic reductase which presumably can hydrolyze the sulfonyl fluoride **1**. That the dihydrofolic reductase from rat liver is not the major enzyme catalyzing the hydrolysis can be concluded from inactivation data in the paper that follows.[14] If dihydrofolic reductase were the enzyme catalyzing all the hydrolysis, then the ratio of the rate of inactivation of dihydrofolic reductase enzyme to the rate of reductase-catalyzed hydrolysis should remain constant as the enzyme is purified; this is not the case. The ratio increases with purification showing that a second enzyme catalyzes the hydrolysis of **1** to **2**; this enzyme will be called a sulfonyl fluoridase.[15]

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had proper uv and ir spectra and moved as a single spot on tlc with Brinkman silica gel GF; each gave combustion values for C, H, and N within 0.4% of theoretical unless otherwise indicated.

[^{14}C]-4-[*p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)hydrocinnamido]-*o*-toluenesulfonyl Fluoride (1) Ethanesulfonate.—A mixture of 3.2 mg of [^{14}C]cyanoguanidine (10 μCi), 13.6 mg of unlabeled cyanoguanidine (total 0.2 mmole), 67.2 mg (0.2 mmole) of **4**,[5] 2 ml of Me_2CO, and 22 mg (0.2 mmole) of $EtSO_3H$ was refluxed with stirring for about 18 hr. The cryst product was collected on a filter. Two recrystns from MeOH–Et_2O gave 95 mg (83%) of crystals, mp 221–223° dec (lit.[5] mp 219–220° dec), with a constant specific activity of 134,000 dpm/mg (69% incorporation). The product was homogeneous on tlc, the radioactivity coinciding with the uv detectable spot.

(9) R. L. Smith, *Fortschr. Arzneimittelforsch.*, **9**, 299 (1966).
(10) A. J. Ryan and S. E. Wright, *J. Pharm. Pharmacol.*, **13**, 492 (1961).
(11) K. Iwatsuba, *Jap. J. Pharmacol.*, **15**, 244 (1956).
(12) A. Mazur, *J. Biol. Chem.*, **164**, 271 (1946).
(13) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968), paper CXIII.
(14) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **13**, 1143 (1970), paper CLXXV.
(15) For discussion of the possible utilization of this sulfonyl fluoridase in cancer chemotherapy see ref 2.

***p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)hydrocinnamic Acid (3) Ethanesulfonate.**—Reduction of 1.93 g (10 mmoles) of *p*-nitrocinnamic acid with H_2–PtO_2 in DMF gave crude *p*-aminohydrocinnamic acid, mp 130–132°. To this were added 25 ml of Me_2CO, 0.84 g (10 mmoles) of cyanoguanidine, and 1.1 g (10 mmoles) of $EtSO_3H$. After being refluxed with stirring for 18 hr, the mixture was filtered. Recrystallization from MeOH–Me_2CO gave 2.5 g (62%) of white crystals, mp 191–193°. *Anal.* ($C_{16}H_{25}N_5O_5S$) C, H, N.

4-[*p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)hydrocinnamido]-*o*-toluenesulfonic Acid (2).—To a stirred mixture of 400 mg of **3** (1 mmole) and 100 mg (1 mmole) of Et_3N in 5 ml of DMF at −5° protected from moisture was added 108 mg (1 mmole) of ethyl chloroformate. After being stirred for 20 min, the mixture was treated with a soln of 223 mg (1 mmole) of 2-methylsulfanilic acid in 5 ml of DMF cooled to −5°. The mixture was stirred 30 min at −5° and 2 hr at ambient temperature, then evapd *in vacuo*. Trituration of the residue with 10 ml of H_2O gave a white solid which was twice recrystd in the following way. The solid was dissolved in the min vol (~200 ml) of boiling 5% aq AcOH and, then concd *in vacuo* until cloudy and allowed to stand; yield, 340 mg (70%) of white crystals that gradually decompd over 220°. *Anal.* ($C_{21}H_{26}O_4S \cdot 1.5\ H_2O$) C, H, N; calcd 0, 18.1. Found: 0, 18.7.

Metabolism *in Vivo*.—Six male albino rats (about 200 g) were given 5 mg of ^{14}C-**1** dissolved in 0.5 ml of 60% aq DMSO ip. The animals were housed individually in stainless steel metabolism cages. H_2O was available *ad lib* and feeding was permitted once daily. Individual urine samples were collected every 24 hr for 96 hr. Combined feces were collected at the same time.

Urine samples were dild to a known vol for counting and 1-ml aliquots assayed for radioactivity. Feces were dried *in vacuo* over P_2O_5, weighed, mixed with 20 g of pure sand, and finely ground in a mortar. Accurately weighed samples were assayed for radioactivity.

Tlc Chromatography.—Chromatography of excreta and pure compounds was performed with Brinkman silica gel using 1:1 or 1:2 H_2O–Me_2CO as solvent. The order of decreasing movement was **2** > **3** > **1**.

Radioactive Counting.—All counting was done with a Packard liquid scintillation spectrometer. Urine was counted directly by adding a 1-ml dild aliquot to 10 ml of Bray's solution.[16] Ground feces were counted by weighing out aliquots and mixing with 1.00 ml of 1.0 *M* KOH. The mixture was warmed to 50° for 1 hr, dild with 10 ml of Bray's soln, then decanted from the sand, and counted; this procedure gave only faintly colored prepns which counted with good efficiency. A soln of **1** in MeOH was counted by adding 1.0 ml of soln to 10 ml of Bray's soln. Counting efficiency was estimated by means of an external standard, then appropriate corrections for quenching were applied.

Tlc's were counted by scraping off the silica gel in portions 1-cm long along the direction of solvent flow. The gel portion was suspended in 10 ml of Bray's soln, then assayed for radioactivity.

***In Vitro* Incubations.**—^{14}C-**1** was incubated at 1 mg/ml at 37° with (a) fresh rat serum, (b) 0–45% $(NH_4)_2SO_4$ fraction of rat liver, (c) 45–90% $(NH_4)_2SO_4$ fraction of rat liver, and (d) phosphate buffer (pH 7.4). The $(NH_4)_2SO_4$ fractions were reconstituted with Tris buffer (pH 7.4) at 1 ml/g of original liver. Aliquots were removed at appropriate time intervals and added to 4 vol of EtOH. The solns were clarified by centrifugation, then 1 ml of the supernatant was assayed for radioactivity. Some of the supernatent was spotted on a tlc plate, developed with H_2O–Me_2CO, then the radioactive zones counted as described above. The inhibitor (1 mg) was dissd in 20 μl of DMSO, then dild with 1 ml of the appropriate soln for incubation.

(16) G. A. Bray, *Anal. Biochem.*, **1**, 279 (1960).