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Synthesis of 5-Azacytidine-*6-^{13}C* and -*6-^{14}C*

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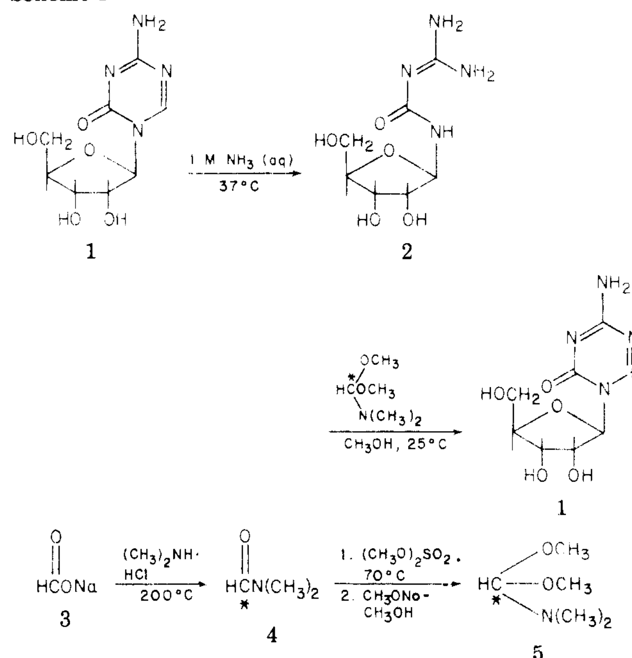
5-Azacytidine (1) labeled with ^{13}C or ^{14}C at the chemically labile C-6 position was synthesized. A method utilizing hydrolytic opening of the triazine ring followed by recyclization with dimethylformamide dimethyl acetal was used. Urinary and biliary excretion was measured in rabbits following intravenous doses of 1-*4-^{14}C* and 1-*6-^{14}C*. Differences in recoveries of the dose from 4-^{14}C and 6-^{14}C demonstrate that ring cleavage of 1 with loss of the C-6 carbon represents a major metabolite route.

The nucleoside antimetabolite 5-azacytidine [4-amino-1-β-D-ribofuranosyl-*s*-triazin-2(1*H*)-one, NSC-102816, 1] is an effective antitumor[1,2] and antileukemic[3-5] agent. Chemically, 1 is known to be relatively unstable in aqueous solution[6,7] as compared to cytidine itself. The facile hydrolytic opening of the triazine ring of 1 at C-6 involves an intermediate which loses C-6 as formate ion and yields the guanylurea derivative 2.[6,7] Metabolically, a scheme involving cleavage of 1 at C-6 was postulated on the basis of the metabolites isolated in 1-treated mouse urine.[8,9] Since both chemical and metabolic evidence indicates that ring cleavage of 1 is a facile process, the in vivo role of this process may contribute to its overall biological activity.

It has been suggested that incorporation of 1 into RNA of mouse liver represents the mechanism of action of this compound,[10] which was demonstrated utilizing 1-*4-^{14}C*. However, the use of 1 labeled with ^{14}C at the C-4 position alone does not allow one to assess the significance of the ring cleavage process. If ring cleavage following RNA incorporation is important in the molecular mechanism of its pharmacological activity, then 1 specifically labeled with ^{13}C and ^{14}C at C-6 should represent a valuable probe for the determination of its biological disposition.

Scheme I



Synthesis. The introduction of the ^{13}C and ^{14}C atoms into 1 is summarized in Scheme I and involves the formation of a triazine ring by condensation of 1-β-D-ribofuranosyl-3-guanylurea (2) with ^{13}C- or ^{14}C-labeled dimethylformamide dimethyl acetal (5). Ring closure occurred under mild conditions with 1 precipitating as formed. Compound 2 was obtained by mild alkaline hydrolysis of 1,[6] and ^{13}C- or ^{14}C-labeled acetal 5 was synthesized by a three-step sequence from sodium formate-*^{13}C* or -*^{14}C* (3). First the ^{13}C- or ^{14}C-labeled sodium formate was allowed to react with dimethylamine hydrochloride. The resulting dimethylformamide (4) was distilled as a hydrate[11] which had to be chemically dried or formation of its dimethyl sulfate addition complex was inhibited. The complex, once formed, however, was immediately converted to 5 upon addition to sodium methoxide in methanol.[12] The identity of the synthetic 1 was substantiated by comparison of the carbon-13 NMR spectrum, mass spectrum, IR spectrum, melting point, and GC properties with those of an authentic sample.

Biological Results. Studies of the difference in the in vivo excretion pattern in urine and in the bile of 1-*4-^{14}C* and 1-*6-^{14}C* were carried out in six male New Zealand white rabbits. Doses of 15 mg/kg of 1-*4-^{14}C* or 1-*6-^{14}C* were

Table I. Percentage of 1 Equivalent Excreted in Rabbits in 8 h after iv Administration of 15 mg/kg of 1-*4*-^{14}C and 1-*6*-^{14}C

	After 1-*4*-^{14}C				After 1-*6*-^{14}C			
	Rabbit no.				Rabbit no.			
Fluid	1	2	3	Av ± SD	4	5	6	Av ± SD
Bile	1.16	0.71	1.14	1.00 ± 0.25	0.84	0.93	0.87	0.88 ± 0.46
Urine	36.90	41.30	25.54	35.30 ± 7.24	3.53	2.45	1.60	2.53 ± 0.97

Table II. Percentage of Unchanged 1 Excreted in Rabbits in 8 h after iv Administration of 15 mg/kg of 1-*4*-^{14}C and 1-*6*-^{14}C

	After 1-*4*-^{14}C				After 1-*6*-^{14}C			
	Rabbit no.				Rabbit no.			
Fluid	1	2	3	Av ± SD	4	5	6	Av ± SD
Bile	0.24	0.26	0.36	0.29 ± 0.06	0.14	0.13	0.06	0.11 ± 0.04
Urine	4.25	1.81	3.84	3.30 ± 1.30	2.11	2.28	2.19	2.19 ± 0.08

administered intravenously in these animals. Total bile and urine were collected over 8 h (animals died) via surgically placed catheters. Total radioactivity in the bile and urine specimens was measured by a liquid scintillation counter. The excretion of radioactivity via these two routes was expressed as percentage equivalent of administered dose and is shown in Table I. Unchanged 1 expressed as percentage administered dose in these specimens, as analyzed by thin-layer chromatography and liquid scintillation counting, is shown on Table II. As shown, much higher percentages of the radioactivity were excreted into the rabbit urine when 1-*4*-^{14}C was used as compared to the same using 1-*6*-^{14}C. However, comparable amounts were excreted into the bile at the same interval using these two drugs. These results indicate the metabolic involvement of the labile C-6, probably losing a formate ion which enters into the endogenous formate pool. Many of the urinary metabolites appear to retain the C-4 moiety. The lower percentage of unchanged 1 found in urine (Table II) after 1-*4*-^{14}C as assayed by TLC–liquid scintillation counting supports this contention. Renal is probably the major excretion route for the intact drug and metabolites, as reflected by the higher radioactivity excreted in urine compared to that excreted in the bile. The amount of unchanged 1 found in urine and in the bile after administration of 1-*4*-^{14}C was essentially the same in the corresponding routes after administration of 1-*6*-^{14}C as expected. Interestingly, after being given 1-*6*-^{14}C, the amount of 1 equivalent found in urine as assayed by total radioactivity was similar in magnitude as the amount of unchanged 1 as assayed by TLC–radioactivity, indicating the potential use of total radioactivity to follow the renal excretion pattern of unchanged drug employing 1-*6*-^{14}C. On the other hand, substantial excretion of ^{14}C-labeled metabolites in addition to unchanged 1 occurred via the biliary route following administration of 1-*6*-^{14}C (Tables I and II). These studies clearly demonstrate ring cleavage of 1 to be a major metabolic pathway in rabbits.

Experimental Section

Compound 1 was obtained from the Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Compound 1-*4*-^{14}C was supplied by the same agency via Monsanto Research Corp. (Dayton, Ohio). Sodium formate-^{13}C (3-^{13}C) (90 atom % ^{13}C) was supplied by Merck and Co., Rahway, N.J., while 3-^{14}C (specific activity = 57.4 mCi/mmol) was purchased from ICN Inc., Irvine, Calif.

Gas–liquid chromatography (GC) was carried out on a Varian Aerograph 1800 equipped with dual flame ionization detectors and two 1.8 m × 2 mm (i.d.) glass columns packed with either 3% OV-1 on Gas Chrom Q (column a) or 3% OV-225 on Gas Chrom Q (column b). GC–mass spectrometry was performed on a Varian Aerograph 2700 equipped with a 1 m × 4 mm (i.d.) glass column packed with OV-1 on Gas Chrom Q and interfaced with a Varian MAT CH-7 mass spectrometer by a Biemann-Watson separator. The ionizing voltage was 70 eV. Infrared spectra were recorded on a Beckman Model 4240 spectrophotometer. A Varian XL-100 spectrometer was used to record the ^{13}C NMR spectra at 25.2 MHz. Liquid scintillation counting was performed on a Unilux II (Nuclear Chicago; Searle, Chicago).

1-*β*-D-**Ribofuranosyl-3-guanylurea (2).** The synthesis of the picrate salt of 2 has been described.[6] The free base was liberated by passing an aqueous solution of the salt through a short glass column containing a fivefold molar excess of Amberlite IR-45 anion-exchange resin (hydroxide form) and then evaporating the effluent to dryness on a rotary evaporator (bath temperature 30 °C). The title compound is extremely hygroscopic and was dried in vacuo over P_2O_5 prior to reaction with acetal **5**.

Dimethylformamide-^{13}C **and** -^{14}C (4-^{13}C **and** 4-^{14}C). The literature method[11] was modified as follows. To 1520 mg (18.6 mmol) of thoroughly dried dimethylamine hydrochloride in a 5-mL round-bottom flask preheated to 190 °C was added 632 mg (9.30 mmol) of **3**-^{13}C (90 atom %) and the mixture was magnetically stirred. A short-path distillation head was immediately attached and the temperature of the bath raised to 260 °C over 90 min. During this period 670 mg of **4**-^{13}C hydrate was collected in an ice-chilled receiver.

The product was dried and purified by vacuum distillation (15 Torr) from P_2O_5 (700 mg), producing 306 mg (45%) of **4**-^{13}C. GC analysis on column b at 75 °C showed a single peak at the same retention time as authentic unlabeled 4.

For the synthesis of 4-^{14}C, 565 mg (8.31 mmol) of unlabeled 3 and 5.8 mg (0.083 mmol, 4.59 mCi) of **3**-^{14}C were condensed with 1360 mg (16.7 mmol) of dimethylamine hydrochloride by an identical procedure. After drying 201 mg (33%) of 4-^{14}C was obtained. Purity was again confirmed by GC.

Dimethylformamide-^{13}C **and** -^{14}C **Dimethyl Acetal (5-**^{13}C **and 5-**^{14}C). The literature method[12] was modified as follows. A solution of 292 mg (3.94 mmol) of dry 4-^{13}C and 497 mg (3.94 mmol) of dimethyl sulfate was heated at 70 °C for 3 h in a small reaction vial. The resulting viscous liquid was cooled to room temperature and added dropwise to an ice-cold stirred solution of 240 mg (4.44 mmol) of sodium methoxide in 2 mL of dry methanol. The reaction flask was then fitted with a short-path distillation head and an ice-chilled receiver. All volatile material was then distilled up to a pot temperature of 200 °C on a heated sand bath. The weight of the total distillate was 1980 mg.

GC analysis of the distillate on column b at 40 °C showed, by peak height comparison, that 300 mg (63%) of **5**-^{13}C was present in the methanolic solution.

By an identical procedure 175 mg (2.39 mmol) of **4**-^{14}C was converted to 200 mg (70%) of **5**-^{14}C isolated as a methanolic solution.

5-Azacytidine-6-^{13}C **and** -6-^{14}C (1-6-^{13}C **and** 1-6-^{14}C). A solution of 379 mg (1.62 mmol) of **2** and 210 mg (1.76 mmol) of **5**-^{13}C in 1.75 mL of methanol was stirred in a stoppered flask for 6 h at room temperature. The white, crystalline product was collected by suction filtration, washed with ethanol, and dried in a stream of air. The crude material (227 mg) was recrystallized

from methanol and dried in vacuo to give 77 mg (19%) of 1-^{13}C, mp 225–227 °C dec. Authentic unlabeled 1 had mp 226–227 °C dec.

In a similar manner 366 mg (1.56 mmol) of **2** and 199 mg (1.67 mmol) of **5**-^{14}C in 1.70 mL of methanol gave, after recrystallization, 194 mg (51%) of 1-^{14}C, mg 224–226 °C dec.

The title compounds were characterized by their infrared spectra [(KBr) 3400, 1685, 1495, and 1100 cm^{-1}] which were identical with that of authentic, unlabeled 1, except minor differences in 1-6-^{13}C due to the high percentage of isotope composition. These compounds were also characterized by the GC retention time of their Me_3Si derivatives from reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide in acetonitrile on column a at 250 °C, which were identical with that of Me_3Si-1, and by the mass spectra of their Me_3Si derivatives. The latter gave major fragments at *m/e* (rel intensity) 245 (36), 217 (98), 147 (49), and 73 (100), as were the major ions of Me_3Si-1. No major ion contained the ^{13}C label. The ^{13}C NMR spectrum of 1 using natural ^{13}C abundance has been published.[13] Synthetic 1-^{13}C (0.15 M in H_2O–D_2O) showed a single chemical shift of 157.5 ppm for C-6 relative to Me_4Si (lit.[13] 157.5 ppm).

Drug Analysis. Thin-layer chromatographic separation of 1 from metabolites was performed by a modified published procedure.[6] Known volumes of biological fluids from radiolabeled 1-treated rabbits along with standards of 1 spiked into blank bile and urine were applied onto Eastman cellulose sheets (Rochester, N.Y.) which were subsequently developed in a solvent system consisting of isobutyric acid–triethylamine–water, 66:2:32. Visible spots under UV lamp (UV SL-25, San Gabriel, Calif.) corresponding to the R_f of 1 (0.72) were sectioned and transferred into liquid scintillation vials. In the case of low concentrations of 1 in the biological samples, a small amount of unlabeled 1 was added to increase visibility, and the spots were processed as before. These TLC materials were assayed for radioactivity using a standard liquid scintillation counting procedure with a Beckman 300 (Beckman, Fullerton, Calif.) liquid scintillation counter. The UV visible spots corresponding to 1 without isotopic dilution were pooled and the specific activity was examined via absorption at 254 nm and liquid scintillation counting. Comparable specific activity to the administered dose of 1 validated the TLC analysis.

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Synthesis of Some Conformationally Restricted Analogues of Fentanyl[1]

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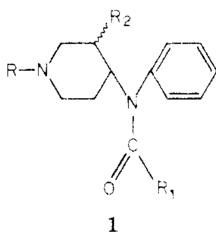
The preparation of *cis*- and *trans*-2,3,4,4a,5,9b-hexahydro-2-phenethyl-5-propionyl-1*H*-pyrido[4,3-*b*]indole is described. These compounds, which are conformationally restricted analogues of the potent analgesic fentanyl, were devoid of analgesic or CNS activity.

The 4-anilidopiperidines 1 as archetypified by fentanyl ($R = CH_2CH_2C_6H_5$; $R_1 = C_2H_5$; $R_2 = H$) have been shown to constitute a class of extremely potent short-acting morphine-like analgesics.[2] Previous efforts in delineating



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the structure–activity relationships in this series have focused on varying the nature and stereochemical relationships of the substituents R, R_1,[2,3] and R_2.[4] Most recently,[5] R_1 has been tied back to the ortho position of the aromatic ring.

The 4-anilidopiperidines are conformationally mobile, although recent ^1H NMR data suggest that the preferred conformers are piperidine chairs with the anilido group equatorially oriented.[3] In view of the nature of the previous studies, it was felt to be of interest to prepare some analogues of fentanyl where conformational mobility is restricted and to assess the effect of this upon biological activity.

Treatment of **2** with sodium cyanoborohydride in trifluoroacetic acid[6] gave **3** (Scheme I). Reaction of **2** with BH_3–THF followed by treatment with 37% HCl and