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In Vitro Metabolism of 10-(3-Chlorophenyl)-6,8,9,10-Tetrahydrobenzo[b][1,8]Naphthyridin-5(7H)-One, a Topical Antipsoriatic Agent. Use of Precision-Cut Rat, Dog, Monkey and Human Liver Slices, and Chemical Synthesis of Metabolites

Shmuel Zbaida[a,*], Yancy Du[a], Daniel Shannon[a], Donald Laudicina[a], C. Mohan Thonoor[a], Kwokei Ng[a], Neil Blumenkrantz[a], James E. Patrick[a], Mitchell N. Cayen[a], Richard Friary[b,*], Vera Seidl[b], Tze-Ming Chan[c], Birendra Pramanik[c], Michael Spangler[c] and Andrew T. McPhail[d]

[a] *Department of Drug Metabolism and Pharmacokinetics, Schering-Plough Research Institute, Mail Stop: K-15-3, 3700, 2015 Galloping Hill Road, Kenilworth, NJ 07033-0539, USA*

[b] *Department of Chemical Research, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033-0539, USA*

[c] *Department of Physical Analytical Chemical Research and Development, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033-0539, USA*

[d] *Department of Chemistry, Paul M. Gross Chemical Laboratory, Duke University, Durham, NC 27706, USA*

ABSTRACT: The metabolism of SCH 40120, which is the clinically effective antipsoriatic drug 10-(3-chlorophenyl)-6,8,9,10-tetrahydrobenzol[b][1,8]naphthyridin-5(7H)-one, was determined *in vitro*. Rat, dog, cynomolgus monkey, and human liver slices hydroxylated the aliphatic, cyclohexenyl ring of the drug and conjugated the resulting carbinol. The identified metabolites comprised the corresponding 6-, 7-, and 9-carbinols, the glucuronide of the 6-carbinol, and the 6-ketone derived from the parent drug.

Although the three carbinols appeared in the liver isolates of all species studied, the relative amounts of these metabolites varied across species. With a high, non-physiological ratio of substrate to liver, the 6-carbinol and its glucuronide were the major metabolites in human and monkey, whereas the 6-ketone was a minor metabolite in dog.

Containing a stereogenic axis and center, the 6-carbinol existed as diastereomeric atropisomers. Its structure was established by ^{13}C and ^{1}H NMR spectroscopy, mass spectrometry, and comparison to an authentic sample. © 1998 John Wiley & Sons, Ltd.

Key words: liver slices; *in vitro* metabolism; antipsoriatic drug; SCH 40120

Introduction

The anti-psoriatic agent SCH 40120 (compound **1** in Figure 1) possesses the potent biological activities and patented chemical structure associated with innovative medicines. In pre-clinical biological assays, SCH 40120 inhibits arachidonic acid 5-lipoxygenation, but not cyclooxygenation. It reduces both leukotriene release and neutrophil-dependent inflammation [1], and potently inhibits production of tumor necrosis factor-α [2]. *In vitro*, 40120 suppresses T-cell but not B-cell proliferation [3]. This pharmacology—especially selective 5-lipoxygenation inhibition and immunosuppression—suggested that it would ameliorate psoriasis and atopic dermatitis.

Clinical trials confirm the expected effects [2,4–6]. Given topically at 0.1 and 1% concentrations, SCH 40120 resembles mid- to high-potency corticosteroids [2] in its anti-psoriatic effect. Freedom from the skin thinning linked to chronic use of such steroids would impart a distinct therapeutic advantage to this non-steroidal experimental drug [7]. The drug also exhibits some efficacy against atopic dermatitis.

In its chemical structure, SCH 40120 comprises three linearly fused rings, namely a pyridine, a 10-(3′-chlorophenyl)-pyridone, and a cyclohexene. Three of its four rings—the pyridine, the electron-rich pyridone, and the 3′-chlorophenyl group—surprisingly proved inert to metabolism, while the remaining cyclohexene ring suffered metabolic attack at two unforeseen sites, namely C(6) and C(7). A partial explanation for the inertness lies in the electron-deficiency of the pyridine and 3′-chlorophenyl rings and in the chemistry of 40120.

* Correspondence to: Shmuel Zbaida, Department of Drug Metabolism and Pharmacokinetics, or Richard Friary, Department of Chemical Research, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033-0539, USA.

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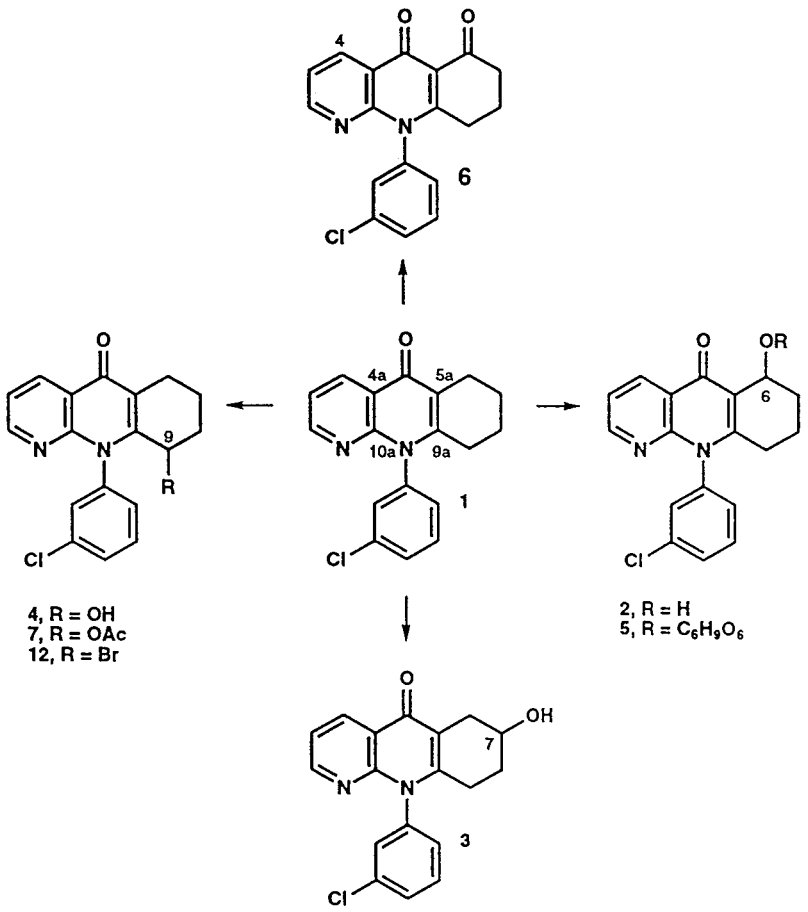

Figure 1. Chemical structures of SCH 40120 and its metabolites

The pyridine nitrogen atom of the drug is chemically neutral and sterically congested. It cannot be titrated with normal hydrochloric acid. Leaving this nitrogen atom unaffected, even *two* equivalents of 3-chloroperbenzoic acid oxidize the drug exclusively at the $\Delta^{5a,9a}$ double bond within the pyridone [8]. The pyridone oxygen atom of 40120, not its pyridine nitrogen atom, forms a hydrogen bond [9]. The 3′-chlorophenyl group like the pyridine ring shows steric hindrance. Deuterium oxide and platinum fail to exchange the hydrogen atoms at the 2′ and 6′ positions, although under forcing conditions (140°, 7 days) they introduce isotopes elsewhere in the 40120 molecule, at C(9) for example [10]. Bromine regiospecifically enters the drug at the 9 position, where enolization occurs normally. This chemistry foreshadows structurally unexpected metabolites if not mechanistically unpredictable biochemistry.

Our plans for drug advancement presented several immediate tasks, one of which was choosing an animal species for toxicological experiments. Such a species would metabolize 40120 as humans do, ideally producing the same metabolite structure(s) and profile(s). Related herein, an *in vitro* method for inducing drug metabolism on a comparatively large scale was adopted because of clinical findings. They suggested that only about two-thirds of the topically administered drug became systemically available for metabolism.

Isolation attempts using dilute clinical samples unsurprisingly afforded no drug-related metabolite specimens sufficient in quantity and purity for identification. No synthetic samples of genuine metabolites were available when the clinical work was done, although samples of certain putative metabolites did aid efforts to exclude structures [11,12]. For example, the samples included 40120 derivatives hydroxylated in the pyridine or chlorophenyl rings. However, the drug did not undergo metabolic hydroxylation in the least electron deficient 3 position, nor suffer attack at the relatively accessible 4′ site. Metabolites from such hydroxylations would have been recognized with the help of our authentic, synthetic samples. These efforts to identify 40120 metabolites in human urine were conclusive but negative. The clinical trial therefore posed the daunting problem of metabolite scarcity.

A solution lay in the possibility of augmenting the available quantity of metabolite(s)—without recourse to larger numbers of human volunteers or higher doses of radioactive drug. It called for decreasing the material losses that accompany repeated purifications, or for increasing the metabolite

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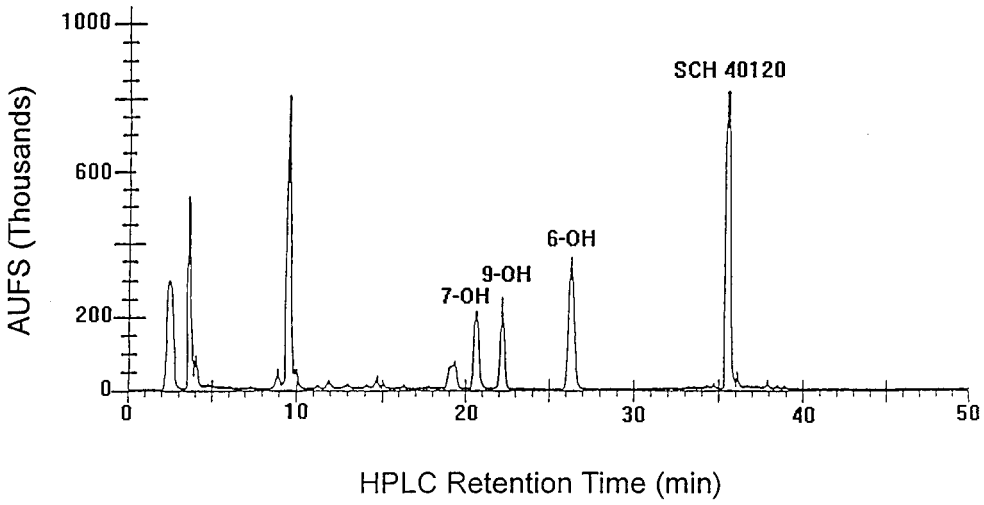

Figure 2. Profile of metabolites from incubation of SCH 40120 with dog liver slices. The *y*-axis represents 'absorption unit full scale' (AUFS)

concentrations. To realize the latter change, we exposed 40120 to precision-cut slices of mammalian livers *in vitro*, expecting the liver to represent a principal site of *in vivo* biotransformation of the drug [13,14]. Primarily we sought to make and isolate identifiably large quantities of liver metabolites from a variety of species, not to ascertain or quantify cross-species metabolic profiles.

Chemical Studies

Instrumentation

High performance liquid chromatography (HPLC) was carried out at room temperature using a Waters system comprising a model 600E gradient pump, a model 712 Wisp Auto-Injector, a model 480 UV detector set at 254 nm, and an Expertease® v 2.3 data collection system. Radioactive samples analysed by method B (*read on*) were detected using a IN/US Radioflow apparatus with a IN/Flow-BD® scintillation cocktail. Radioactivity of samples analysed by method A (*read on*) was monitored using a Radiometric Model A250 Flo-One® Radioactive Flow Detector, which permitted direct analysis of the effluent. The effluent was analysed with a 0.5 mL flow cell through which Packard Flo-Scint V® scintillation fluid was pumped at a flow rate of 4 mL min^{-1}. Samples were dissolved in a mixture of 0.10 mL methanol, 0.08 mL 0.1 M ammonium acetate buffer (pH 6) and 0.02 mL acetonitrile. They were injected onto a DuPont Zorbax® C_8 reversed-phase analytical column (Du Pont; 4.6 mm × 25 cm).

Tissue slices were prepared using a Krumdieck Tissue Slicer, purchased from Alabama Research and Development Corporation (Munford, AL). Incubations of SCH 40120 with liver slices were performed in an Innova 3000 Water Bath Shaker (New Brunswick Scientific, Edison, NJ).

Proton NMR spectra were recorded with a Varian XL-400 (400 MHz) spectrometer, while ^{13}C NMR spectra were acquired with a Varian XL-300 instrument. Samples were dissolved in perdeuteriomethanol unless otherwise noted. Chemical shifts in δ (ppm) are downfield from tetramethylsilane (TMS).

Liquid chromatography-mass spectrometry (LC/MS) and LC/MS/MS analyses were performed on a Finnigan TSQ 70B tandem mass spectrometer interfaced with an HPLC system. The HPLC system consisted of a Waters' 600 multisolvent delivery system, a U6K injector, and a Waters' 600 MS system controller. Samples were injected onto a Zorbax® Rx-C_8, 4.6 mm × 25 cm column, and were chromatographed using HPLC method A.

The thermospray LC/MS method was used to search for protonated molecular ions of drug related metabolites. The vaporizer and ion source temperatures were respectively 109–110°C and 229–230°C. The mass range scanned was m/z 120 to 800. For LC/MS/MS analyses, argon (99.999%) was the collision gas. The collision cell pressure was maintained at 2.2 millitorr and spectra were obtained with the collision energy adjusted to -50 eV.

For LC/MS/MS analyses of the 6-carbinol diastereomers, the protonated molecular ion (m/z 327) was selected as the precursor ion. The third quadrupole was scanned at a mass range of 50–340 amu at 1 s per scan.

LC/MS analyses of the *Cunninghamella echinulata*-derived carbinol **2** were performed by injecting 10 µl of a methanolic solution (1 mg mL^{-1}) of the carbinol **2** onto a reversed-phase column (Waters' Nova-Pak® C_{18} 3.9 × 150 mm). Separation was achieved by elution with an isocratic solvent system composed of 60% methanol and 40% ammonium acetate (pH 6). For LC/MS/MS analyses, 30 µl of the sample was injected onto the same HPLC column.

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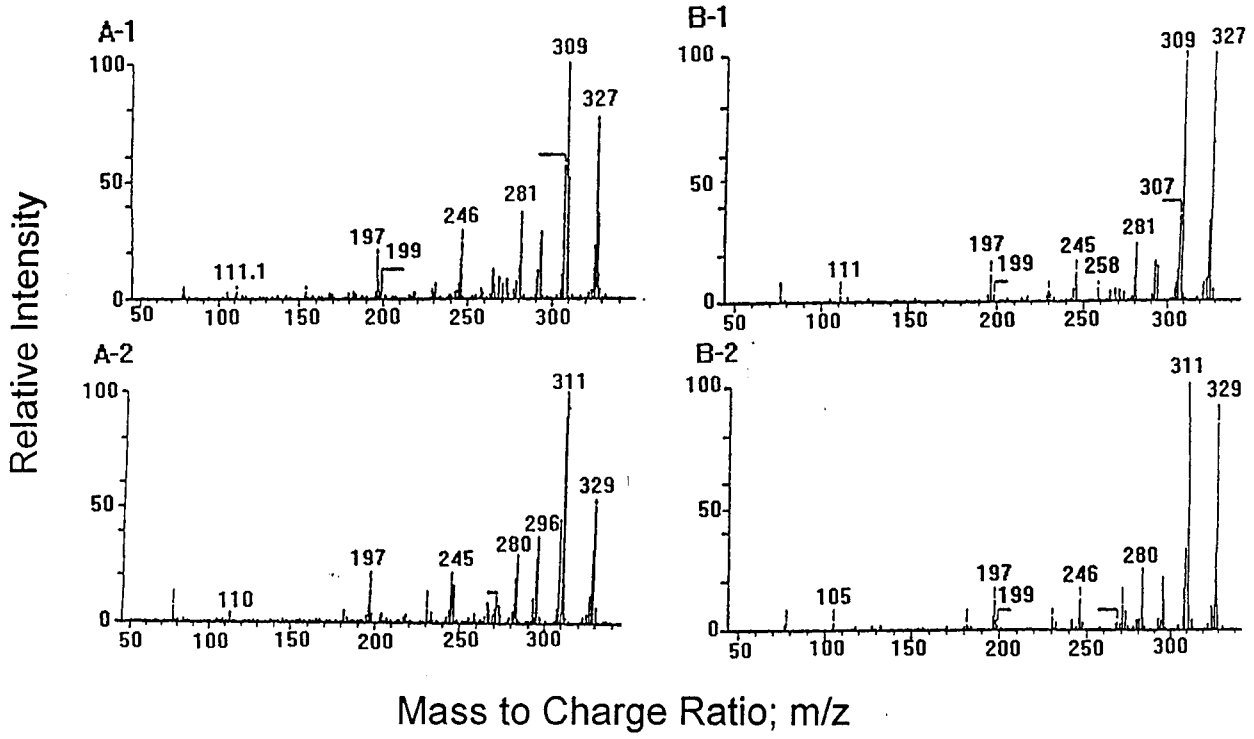

Figure 3. LC/MS/MS spectra of the chlorine isotopomers of metabolite **3**. A-1 and A-2: from incubation of SCH 40120 with cynomolgus monkey liver slices. B-1 and B-2: from the synthetic reference standard

Gas chromatography-mass spectrometry (GC/MS) and GC/MS/MS analyses were performed on a Varian 3400 microprocessor gas chromatograph. The instrument was fitted with a CTC-A200S autosampler and interfaced through a MicroVIP® computer (US Design Corporation, Lanhan, MD) with a Finnigan TSQ 70B tandem mass spectrometer (Finnigan MAT Corporation, San Jose, CA). Samples were derivatized at 75–80°C for 30 min with N,O-bis-(trimethylsilyl)-trifluoroacetamide and injected onto a capillary column (DB-5, 0.53 mm i.d., 30 m, J & W Scientific, Folsom, CA). Methane was used as the carrier gas. Capillary on-column injection was used. The initial temperature of the injector was set at 70°C, the injector was held isothermally for 1 min, and was then rapidly heated to 290°C at 190°C min^{-1}. The initial column temperature upon sample injection was set at 90°C, increased rapidly to 290°C at 40°C min^{-1}, then held at 290°C for 7 min. The transfer line was maintained at 290°C. The Finnigan TSQ tandem mass spectrometer was the detector, wherein the samples were chemically ionized at an ion source temperature of 200°C using methane/ammonia as the reagent gas. The collisionally-induced dissociation mass spectrum was obtained by focusing on the protonated molecular ion, with a collision energy of -43 eV. The collision gas pressure was 2.1–2.5 millitorr, and argon was the collision gas.

HPLC Analysis

Milli Q deionized water with a 19 MΩ resistivity was used for HPLC analysis.

Method A. Linear gradient chromatography was performed at a 1 mL min^{-1} flow rate using 0.1 M ammonium acetate (pH 6) (solvent A) and acetonitrile (solvent B). The percentages of A to B (v/v) were 80:20 (0 min), 65:35 (15 min), 65:35 (25 min) and 20:80 (30 min). Under these conditions, **1** (SCH 40120) eluted at 35.60 min, the carbinol metabolites **2** at 26.20 min, and the compounds **3** and **4** at 20.80 and 22.40 min, respectively (Figure 2). The glucuronide of **2** eluted at $\sim$10 min.

Method B. Linear gradient chromatography was carried out at a 1 mL min^{-1} flow rate using deionized water (solvent A) and acetonitrile (solvent B). The percentages of A to B (v/v) were: 70:30 (0 min), 65:35 (15 min), 65:35 (25 min), 30:70 (30 min), 30:70 (40 min), 20:80 (50 min) and 20:80 (70 min). Under these conditions, SCH 40120 eluted at 35 min, metabolites **2** at $\sim$20 min, and **3** and **4** were eluted at 12 and 16 min, while glucuronide **5** eluted at 4 min, respectively.

Method A was used to determine the number and distribution of metabolites from incubations of SCH 40120 with dog liver tissues (Figure 2). It also served to identify them. Method B was applied in all other cases.

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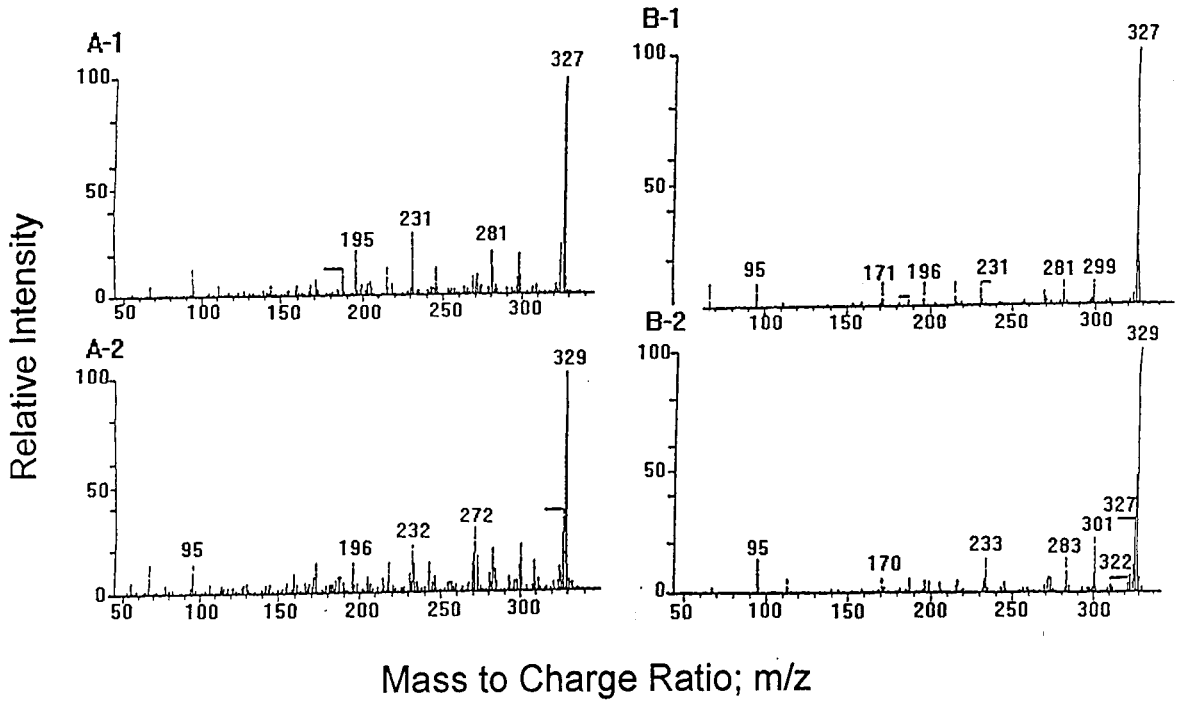

Figure 4. LC/MS/MS spectra of the chlorine isotopmers of metabolite **4**. A-1 and A-2: from incubation of SCH 40120 with cynomolgus monkey liver slices. B-1 and B-2: from the synthetic reference standard

Chemicals

Unlabeled SCH 40120 was obtained from Clinical Manufacturing, Schering-Plough Corporation. ^{14}C-SCH 40120 (349 mCi mg^{-1}; 540 mCi mL^{-1}; 98% radiochemical purity) was obtained from the Radiochemistry Section of Chemical Research, Schering-Plough Research Institute, as a solution in methanol:dichloromethane (3:1, v/v). m-Chloroaniline, uniformly labeled with ^{14}C, was purchased from ChemSyn Science; it was converted to radiolabeled SCH 40120 by the synthesis described in [23]. Acetonitrile (99.9%) and methanol (99.9%) were obtained from Fisher Scientific. Ammonium acetate and β-glucuronidase were obtained from Sigma Chemical Company. β-Glucuronidase from *Helix pomatia* contained 120600 units of β-glucuronidase per millilitre, and up to 2820 units of sulphatase activity per millilitre. N,O-Bis-(trimethylsilyl)-trifluoroacetamide was procured from Pierce. SOS Gases supplied oxygen/carbon dioxide (95%:5%, v/v). Gibco Laboratories Life Technologies, Inc., furnished Williams' Medium E. C_{18} Speed-Pac® solid phase extraction (SPE) cartridges were purchased from Applied Separations.

Biological Studies

Animal Species

Rat. Twelve male Sprague-Dawley rats [Crl:CD^{R}(SD)BR VAF/PLUS™, Charles River Laboratories] 10 weeks of age and acclimated for 1 week, were provided *ad libitum* with Tekland Rodent Diet (W) 8604 and reverse osmosis water treated with ultraviolet light. They ($\sim$250 g) were sacrificed by carbon dioxide asphyxiation, and their livers were removed and stored in Williams' Medium E on ice pending preparation of tissue slices.

Dog. One young adult male beagle dog (Schering-Plough Research Institute Kennels, Lafayette, NJ), acclimated for 2 weeks, was provided *ad libitum* with Purina Mills Lab Canine Diet 5006 and reverse osmosis water treated with ultraviolet light. The animal (17.4 kg) was sacrificed by an overdose of phenobarbital. Pending preparation of liver slices, the excised liver was stored over ice in Williams' Medium E.

Monkey. Two young adult male cynomolgus monkeys (*Macaca fascicularis*, World Wide Primates, Miami, FL) (6.1 and 9.2 kg), acclimated for 2 weeks and provided with Monkey Chow 5038 (Purina Mills) and water, were sacrificed by an overdose of phenobarbital. The livers were removed and kept in Williams' Medium E on ice pending preparation of tissue slices.

Human. A human liver sample was obtained from the International Institute for the Advancement of Medicine (Exton, PA). This sample was from a Caucasian male donor (3 years of age), who had died of anoxia, and was free of HIV and hepatitis A, B, and C. The liver was obtained sliced in 8 mm sections (25–30 mg per slice) in Belzer's solution [15], and was kept at 0–4°C.

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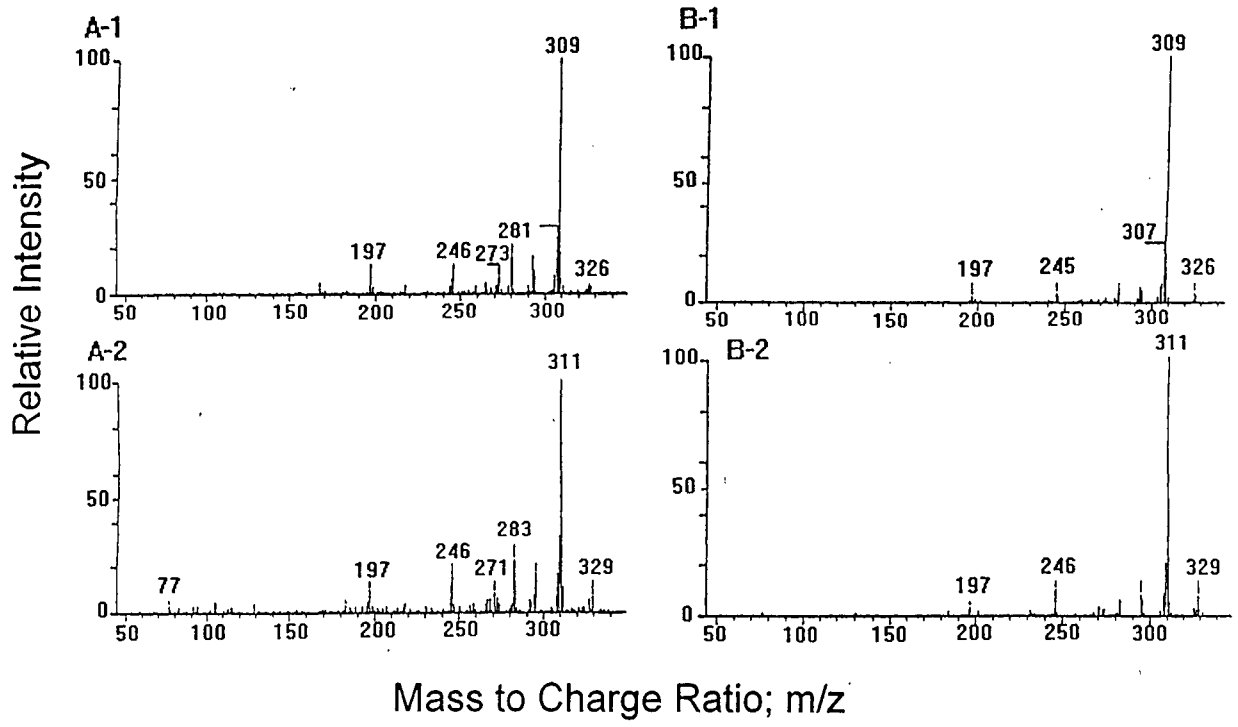

Figure 5. LC/MS/MS spectra of the chlorine isotopomers of metabolite **2**. A-1 and A-2: from incubation of SCH-40120 with cynomolgus monkey liver slices. B-1 and B-2: from the synthetic reference standard

Incubation and Analyses of SCH 40120 with Liver Tissue Slices

Experimental Design. To maximize the quantity of metabolites formed, the initial concentration of 40120 incubated with liver slices was as high as allowed by the solubility of the drug in the incubation buffer (0.096 mM). Constant ratios of drug to liver and of the incubation volume to the number of liver slices ($\sim$0.6 mg SCH 40120 g^{-1} liver and one slice per millilitre of buffer) were maintained to make interspecies comparisons. *In vitro* incubations of human liver slices were also carried out with a 0.080 mM SCH 40120 solution. These experiments increased the ratios of metabolites to the remaining parent drug to facilitate metabolite isolation and identification. A ratio of one slice per millilitre of buffer was also maintained at the drug concentration of 0.080 mM.

The metabolite concentrations were monitored after 4 and 24 h incubations. A 4 h incubation with dog liver slices yielded a sufficient level of metabolites for structure elucidation. However, 24 h incubations were required to produce sufficient quantities of metabolites in rat, monkey and human. Human and monkey samples, which exhibited similar metabolite profiles, were also incubated with ^{14}C-radiolabeled SCH 40120. These incubations exhibited about 40% conversion of the drug to its metabolites after 24 h under the above conditions.

Experiments. Livers (8 mm diameter, 50–60 mg $slice^{-1}$) were sliced as soon as possible after excision in 400 mL Williams' Medium E at 4°C using a Krumdieck Tissue Slicer. They were separated from the medium by decantation, weighed and immediately incubated with SCH 40120 in Williams' Medium E under an atmosphere of oxygen and carbon dioxide (95:5, v:v) at 37°C. SCH 40120 was added to the Williams' Medium E (incubation buffer) from a stock solution. The stock solution contained 10 mg SCH 40120 per millilitre of methanol. In none of the incubations did the proportion of methanol exceed 0.7% (v/v).

In a typical preparative experiment with monkey liver tissues a total of 76.5 mg of SCH 40120 (0.096 mM) in 2.55 L of Williams' Medium E containing 0.3% (v/v) methanol was incubated with 136 g of sliced liver. Ratios of about one slice per millilitre of buffer and $\sim$0.6 mg g^{-1} of liver were maintained; the weight of one slice was 50–60 mg. The monkey liver slices and the incubation buffer were placed in 17 150-mL Erlenmeyer flasks and incubated for 24 h at 37°C under a blanket of 95% O_2:5% CO_2 (v/v). At the end of the incubations, the aqueous medium was decanted from the tissues, which were processed separately. The aqueous medium was filtered using 5.5 cm Whatman Qualitative Filter Paper (# 1), then split in two aliquots. One aliquot was retained for incubation with a mixture of β-glucuronidase and sulphatase and the other was subjected to solid phase extraction.

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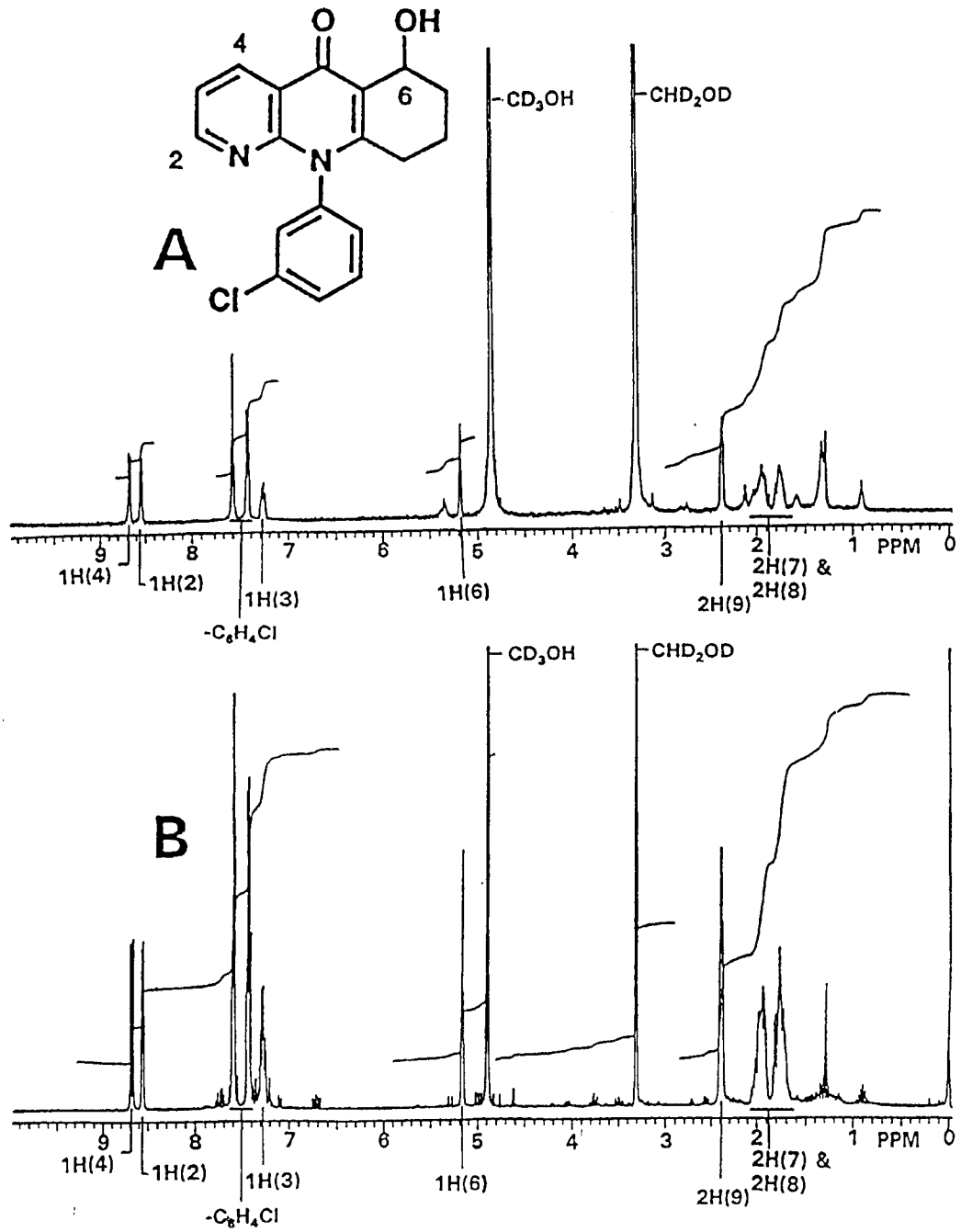

Figure 6. Proton NMR spectra of metabolite **2**. A. From incubation of SCH 40120 with dog liver slices. B. From the synthetic reference standard

A 10 g C_{18} Speed-Pac® cartridge was conditioned by sequential elution with methanol (30 mL) and water (50 mL). The incubation mixture was adsorbed onto the packing material, which was washed with 50 mL of water. The effluent from this wash was discarded. The drug-derived material was then eluted with methanol (50 mL), which was evaporated to dryness under nitrogen at room temperature.

Tissue samples were extracted with methanol (3×40 mL); the extracts were combined and filtered, and were then evaporated to dryness at room temperature under nitrogen. One aliquot of each of the tissue and the aqueous extracts was dissolved in an appropriate organic solvent and subjected to analysis. The other was dissolved and subjected to hydrolysis with a mixture of β-glucuronidase and sulphatase, as described below.

Control Experiments: The stability of SCH 40120 in Williams' Medium E was tested by incubating the parent drug in the aqueous buffer as described above but without the liver tissues. HPLC analyses confirmed that SCH 40120 was stable under these

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Table 1. Identity of ^{13}C chemical shifts[a] in synthetic and metabolic samples of **2**

Carbon no.	Sample			
	Synthetic	Dog liver (*in vitro*)	Monkey liver (*in vitro*)	Monkey urine (*in vivo*) [16]
C(5)	180	[b]	[b]	180
C(9a)	154.4	[b]	154.4	154.4
C(2)	153.9	153.9	153.9	153.9
C(10a)	153	[b]	[b]	153
C(1′)	141	[b]	[b]	141
C(3′)	136.4	[b]	[b]	136.4
C(4)	136.3	136.3	136.4	136.3
C(5′ or 4′)	132	132	132.1	132
C(2′)	130.84	130.85	130.83	130.85
	130.78	130.79	130.77	130.78
C(4′ or 5′)	130.6	130.5	130.54	130.5
C(6′)	129.17	129.17	129.15	129.17
	129.10	129.09	129.08	129.09
C(5a)	121.7	[b]	[b]	121.7
C(3)	121.3	121.3	121.3	121.3
C(4a)	120.6	[b]	[b]	120.3
C(6)	63.29	63.31	63.40	63.24
	63.26	63.28	63.37	63.21
C(7 or 9)	30.70	30.68	30.67	30.68
C(9 or 7)	30.64	30.65	30.62	30.64
C(8)	18	18	18.19	18

[a] δ-values are in ppm downfield from TMS in CD_3OD.
[b] Because of inadequate acquisition of time, the expected resonance of a quaternary carbon atom was not observed.

conditions. HPLC chromatograms of the incubation products formed when tissue was incubated without the drug were used to characterize the impurities extracted from liver tissues. No components from this control incubation were detected at the retention times of the parent drug or its metabolites.

Incubation with β-Glucuronidase: The aliquots retained for enzymatic treatment were dissolved separately in 5 mL of 0.5 M sodium acetate buffer (pH 5.0). One millilitre of a mixture of both β-glucuronidase and sulphatase was added, and the resulting mixtures were incubated in air for 48 h at 37°C in a shaking water bath. The enzymatic hydrolyses were terminated by application of the mixtures to solid-phase extraction cartridges, which were processed as described above.

Isolation and Identification of Metabolites: The crude methanolic extracts from the solid phase extractions were analysed for metabolites by mass spectrometry. The presence of chlorine isotopomers with the natural-abundance ratio of 3:1 distinguished between drug-related compounds and extraneous chemicals. The metabolites were isolated by collecting the appropriate fractions from HPLC, and were identified by NMR and mass spectrometry.

Chemistry

(±)-10-(3-Chlorophenyl)-6,8,9,10-tetrahydro-6-hydroxybenzo[b][1,8]naphthyridin-5(7H)-one (2)

Preparation. Lithium tri-*tert*-butoxy aluminohydride (1 mL of a 1 M solution in tetrahydrofuran) was added to a stirred, cooled (ice bath) solution of 10-(3-chlorophenyl)-8,9-dihydro-benzo[b][1,8]naphthyridin-5,6(7H, 10H)-dione [16], **6** (310 mg, 0.00095 mole) in tetrahydrofuran (6 mL) under nitrogen. The resulting red solution was allowed to stir overnight; ice was not replenished.

For work-up, water (0.3 mL), 15% (w/v) sodium hydroxide solution (0.9 mL) and water (0.3 mL) were added. The mixture was diluted with ether and water, and was extracted with ether. Combined extracts were dried (sodium sulphate), filtered (cotton), and concentrated to give a yellow solid (291 mg), which was chromatographed over silica gel packed in toluene–acetone (95:5, by vol.). The same solvent eluted the desired carbinol (111 mg, 36%) as a light yellow solid, pure to TLC.

This sample was identified by comparison of ^1H NMR spectra and thin-layer chromatograms with those of an authentic specimen prepared by incubation of 10-(3-chlorophenyl)-6,8,9,10-tetrahydroben-

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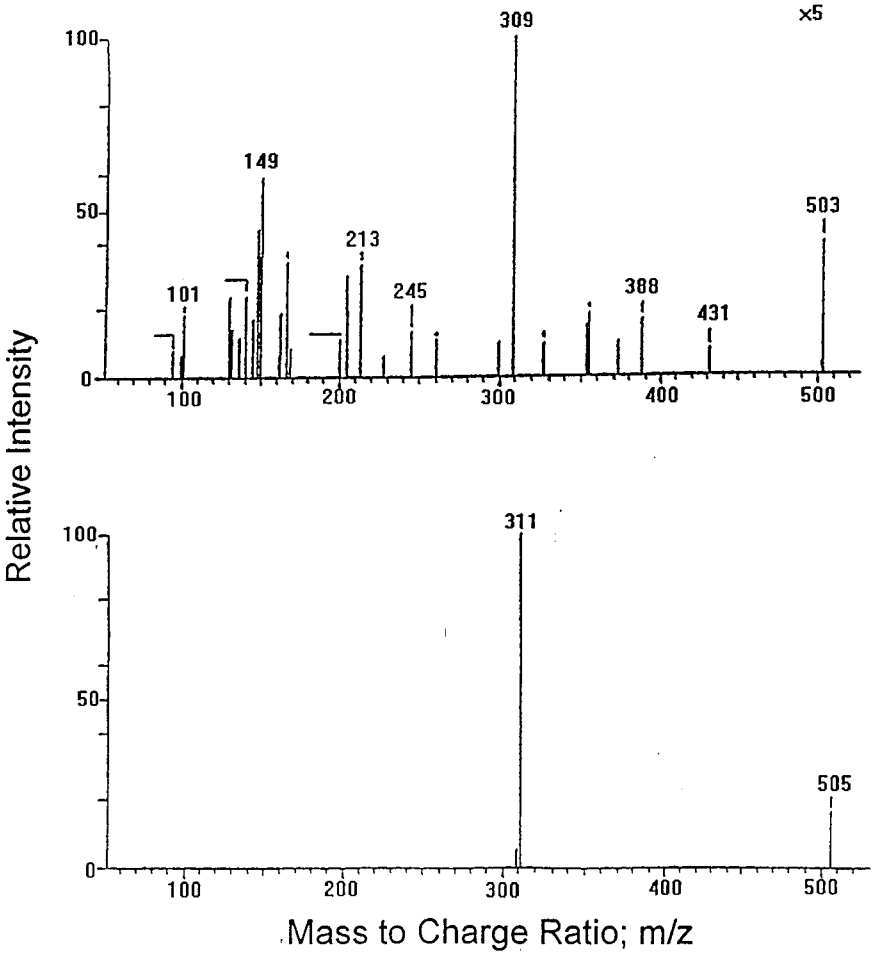

Figure 7. MS/MS spectra of the chlorine isotopomers of the glucuronide **5** from incubation of SCH 40120 with monkey liver slices

zo[b][1,8]naphthyridin-5(7H)-one, **1**, with *Cunninghamella echinulata* (ATCC 8987) [17]. Another sample of the 6-carbinol, prepared by sodium borohydride reduction of the corresponding ketone, was also identified. Its ^{1}H and ^{13}C NMR spectra, as well as its mass spectrum, were identical to those recorded with samples isolated from monkey urine [16] and dog liver.

Tandem Liquid Chromatographic (LC) and Mass Spectrometric (MS) Analyses. A solution (10 mL) of the 6-carbinol (1 mg dissolved in 1 mL methanol) was injected onto a reversed-phase, C18 column with dimensions of 3.9 × 150 mm (Waters Nova-Pak). An isocratic solvent system comprising methanol–0.1 M aqueous ammonium acetate (60:40, by vol.; pH 6) separated the sample into two peaks with retention times of 3 min 10 s and 3 min 58 s at 25°C.

For liquid chromatography experiments in tandem with daughter ion mass spectrometry (LC/MS/MS), 30 mL of the sample solution was injected onto the same column. After HPLC separation, the eluents were analysed by mass spectrometry. The two components gave identical LC/MS and LC/MS/MS spectra; therefore, they were determined to be diastereomers.

The 6-carbinol diastereomers were separated on a 10 μm, 250 mm × 3.9 mm ID, C_{18} analytical HPLC column (Waters, μBondapak) as the stationary phase. The mobile phase was methanol–water (60:40, v/v), and the flow rate was 1.8 mL min^{-1}. A UV detector operating at 254 nm was employed. The stereoisomers showed retention times of 4.8 and 6.0 min at 25°C.

*10-(3-Chlorophenyl)-6,8,9,10-tetrahydro-9-hydroxybenzo[b][1,8]naphthyridin-5(7H)-one (**4**)*

A solution of 10-(3-chlorophenyl)-6,8,9,10-tetrahydrobenzo[b][1,8]naphthyridin-5(7H)-one, **1** (5 g, 16.1 mmol), selenium dioxide (3.33 g, 30 mmol), and dry dioxane (100 mL) was refluxed for 6 h. The reaction mixture was then filtered through a bed of activated carbon combined with diatomaceous earth, and the filtrate was concentrated. The residue was partitioned between water and dichloromethane. The dichloromethane solution was dried, filtered, and concentrated. The residue (5.9 g) was chromatographed over silica gel (130 g) and under medium nitrogen pressure. A solution of 20%

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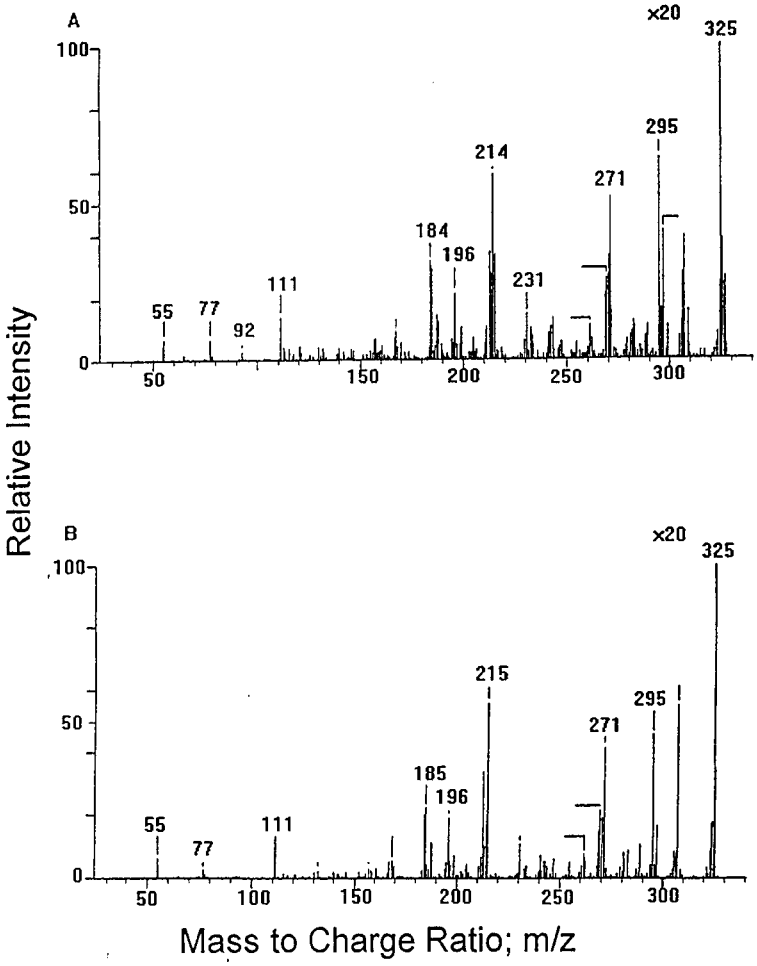

Figure 8. MS/MS spectra of the chlorine-35 isotopomer of metabolite **6**. A. From incubation of SCH 40120 with dog liver slices. B. From the synthetic reference standard

methanol in ethyl acetate eluted the desired carbinol as a diastereomeric mixture of atropisomers (1.55 g, 29.5%). Crystallized from dichloromethane, the product showed m.p. 184–187°C; IR (mineral oil) 3350, 1610, 1590, 1290; EI-MS 329 (7, $[M+1]^+$ for ^{37}Cl), 328 (34, M^+ for ^{37}Cl), 327 (29, $[M+1]^+$ for ^{35}Cl), 326 (100, M^+ for ^{35}Cl), 325 (30, $[M-1]^+$ for ^{35}Cl), 311 (16, $[M-OH]^+$ for ^{37}Cl), 309 (18, $[M-OH]^+$ for ^{35}Cl); ^1H NMR (300 MHz, CD_3OD) 8.67 (dd, $J(4-2)=2$, $J(4-3)=8$, H (4), 8.55 (dd, J $(2-4)=2$, $J(2-3)=4$, H (2)), 7.60–7.52 (m, 2H, Ar), 7.48–7.38 (m, 2H, Ar), 7.23, (dt, $J(3-4)=8$, $J(3-2)=4$, H (3)), 4.45–4.40 (m, H (9)), 2.93 (dd, $J=3$ and 9, 1H), 2.50–2.36 (m, 1H), 2.05–1.67 (m, 4H, 2 CH_2); ^{13}C NMR (300 MHz, CD_3OD) 180 (C(5)), 154 (C(2)), 153 (C(9a)), 150.85 and 150.77 (C(10a)), 140.44 and 140.37 (C(1′)), 136 (C(4)), 135 (C(3′)), 133.14 and 131.49 (C(2′)), 131.23 and 131.17 (C(4′ or 5), 130.60 and 130.32 (C(5′ or 4′)), 130.27 and 128.97 (C(6′)), 121.2 (C(5a)), 121.1 (C(3)), 120 (C(4a)), 63.9 and 63.8 (C(9)), 32.22 and 32.20 (C(8)), 23.41 (C(6)), 16.39 and 16.37 (C(7)) ppm. An attached proton test helped in assignment of peaks to carbons.

A ^{13}C NMR spectrum of another sample of this compound was recorded in the same solvent. Then, the resonances of C(4), C(3′), C(8), and C(7) appeared as two peaks each, respectively at 136.33 and 136.23, 135.45 and 135.41, 32.22 and 32.19, and 16.39 and 16.36 ppm. These observations confirmed that the compound existed as a diastereomeric mixture of atropisomers.

Anal. Calc. for $C_{18}H_{15}ClN_2O_2$: C, 65.25; H, 4.72; Cl, 10.70; N, 8.46. Found: C, 65.27; H, 4.51; Cl, 11.12, N, 8.40.

For further characterization, this carbinol (m.p. 184–187°C) was acetylated, and the resulting acetate compared to an independently synthesized sample.

9-(Acetyloxy)-10-(3-chlorophenyl)-6,8,9,10-tetrahydrobenzo[b][1,8]naphthyridin-5(7H)-one (7)

By Esterification. A solution of 10-(3-chlorophenyl)-6,8,9,10 - tetrahydro - 9 - hydroxybenzo[b][1,8]naphthyridin-5(7H)-one, **4** (1.08 g, 3.3 mmol) in pyridine (5 mL) and acetic anhydride (0.68 g, 6.6 mmol) was stirred for 50 h at ambient temperature under N_2. The reaction mixture was diluted with water (50 mL), and the resulting precipitate (1.2 g) was collected and washed with water. Two crystallizations

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of the precipitate from methanol and dichloromethane gave white crystals, m.p. 243–245.5°C (0.67 g, 55%); IR (dichloromethane) 1740 (ester C=0), 1620 (C=0), 1480, 1420, 1230; EI-MS 370 (8, M^+ for ^{37}Cl), 368 (22, M^+ for ^{35}Cl), 309 (64, $[M-CH_3CO_2H]^+$ for ^{37}Cl), 307 (100, $[M-CH_3CO_2H]^+$ for ^{35}Cl); ^1H NMR (300 MHz, $CDCl_3$) 8.73 (dd, $J(4-2)=2$, $J(4-3)=7$, H (4)), 8.57 (dd, $J(2-4)=2$, $J(2-3)=4$, H (2)), 7.56–7.48 (m, 2H, Ar), 7.32–7.24 (m, 2H, Ar), 7.23–7.14 (m, H (3)), 5.57 (br s, H (9)), 3.50 (d, $J=6$, 1H), 2.55–2.38 (m, 1H), 2.09–2.00 (m, 1H), 1.98 and 1.89 (2s, 3H, CH_3), 1.79–1.65 (m, 3H); ^{13}C NMR (300 MHz, $CDCl_3$) 178 (C(5)), 168.98 ($O=CCH_3$), 168.60 ($O=CCH_3$), 153 (C(2)), 151 (C(10a)), 144 (C(9a)), 138 (C(3′)), 135.7 (C(4)), 135.5 (C(1)), 134.8 (CH_0), 130.98 (C(2′)), 130.81 (C(2′)), 129.78 (C(6′)), 129.70 (CH_1), 129.66 (C(6′)), 128.7 (CH_1), 127.6 (CH_1), 123 (C(5a)), 120 (C(3)), 119 (C(4a)), 65.49 (C(9)), 65.27 (C(9)), 29 (CH_2), 22 (CH_2), 20.96 ($O=CCH_3$), 20.77 ($C(O=CCH_3)$), 16 (CH_2).

According to its ^{13}C NMR spectrum, this compound existed as a diastereomeric mixture of atropisomers. An attached proton test allowed assignment of resonances to carbon atoms.

This acetate was the subject of a single-crystal X-ray analysis, which unequivocally established the ground-state structure and conformation.

Anal. Calc. for $C_{20}H_{17}ClN_2O_3$: C, 65.13; H, 4.65; Cl, 9.61; N, 7.60. Found: C, 65.06; H, 4.63; Cl, 9.57; N, 7.54.

By Bromination and Acetolysis. Bromine (2.1 mL, 0.0322 mole) was added to a stirred solution of 10-(3-chlorophenyl)-6,8,9,10-tetrahydrobenzo[b][1,8]-naphthyridin-5(7H)-one, **1** (10.0 g, 0.0322 mole), sodium acetate (11 g), and acetic acid (50 mL) at 50°C under nitrogen. When the addition was complete, the mixture was stirred for 45 min at 50°C. The solvolyze the resulting bromide, the mixture was refluxed for 14 h.

For work-up, the cooled mixture was poured into water (250 mL) and the resulting precipitate was collected on a filter where it was washed with water. The solid was dissolved in dichloromethane (250 mL) and the solution was washed with 1 M sodium bicarbonate solution, water, and brine. It was dried (sodium sulphate), filtered (cotton), and concentrated to give an oil which solidified on treatment with diisopropyl ether. The collected, washed, and dried solid weighed 9.54 g, and showed the desired acetate as the major component (TLC). Column chromatography over silica gel and elution with dichloromethane–methanol (99:1, by vol.) as well as several recrystallizations were required to remove several minor impurities. They gave an analytical sample (1.87 g, 15.5%), m.p. 242.0–243.5°C, which crystallized from methanol containing a little dichloromethane. From the combined filtrates, another portion of the acetate (6.02 g (51%)) was recovered by chromatography.

The foregoing analytical sample was identified with an authentic specimen prepared as described in the previous section. The two samples showed identical IR, ^1H NMR, and mass spectra. A mixed m.p. (239–243.5°C) was undepressed. The samples were also identical according to side-by-side thin-layer chromatograms.

*6,8,9,10-tetrahydro-5′-5′-dimethyl-10-(3-chlorophenyl)-spiro[benzo[b][1,8]naphthyridin-7-(5H),2′-[1,3]dioxan]-5-one (**8**)*

A solution of the known [18] 3,3-dimethyl-9-(1-pyrrolidinyl)-1,5-dioxaspiro[5,5]undec-8-ene, **9** (14.7 g, 58.6 mmol) and triethylamine (8.1 mL, 59 mmol) in 1,2-dichloroethane (60 mL) was added to a stirred, cooled suspension of 2-[3-chlorophenyl)amino]-3-pyridinecarbonyl chloride (15.6 g, 58.6 mmol), **10**, prepared according to [23], and 1,2-dichloroethane (156 mL). A bath of dry ice and acetone was employed. When addition was complete, the resulting solution was kept in the bath for 1.8 h, and was then refluxed for 6 h. The cooled reaction mixture was washed with water, 1 N hydrochloric acid, half-saturated sodium bicarbonate solution, and saturated brine. The dried, filtered organic solution was concentrated to give an oil (18.7 g). Crystallization of this oil from methanol containing a little dichloromethane gave the desired ketal (4.85 g, pure to TLC), m.p. 212–216°C, in a yield of 20%.

Anal. Calc. for $C_{23}H_{23}ClN_2O_3$: C, 67.23; H, 5.64; Cl, 8.63; N, 6.82. Found: C, 67.23; H, 5.89; Cl, 8.03; N, 6.51.

*10-(3-Chlorophenyl)-6,8,9,10-tetrahydrobenzo[b]-[1,8]naphthyridin-5,7-dione (**11**)*

Ketone (**11**) was prepared by acidic hydrolysis of the corresponding ketal, 6,8,9,10-tetrahydro-5′,5′-dimethyl-10-(3-chlorophenyl)-spiro[benzo[b][1,8]-naphthyridin-7-(5H),2′-[1,3]dioxan]-5-one, **8**, in a yield of 92%. It showed m.p. 216–219°C: IR (dichloromethane) 1720 (ketone C=O), 1620 (pyridone C=O), 1590; FAB-MS: 328 (46, $[M+2]^+$ for ^{37}Cl), 327 (74, $[M+1]^+$ for ^{37}Cl), 326 (69, $[M+2]^+$ for ^{35}Cl), 325 (100, $[M+1]^+$ for ^{35}Cl); ^1H NMR (200 MHz, $CDCl_3$) 8.73 (dd, $J(4-2)=2$, $J(4-3)=8$, H (4)), 8.60 (dd, $J(2-4)=2$, $J(2-3)=4$, H (2)), 7.58–7.56 (m, 2H, Ar), 7.37–7.26 (m, 2H, Ar), 7.23–7.20 (m, H (3)), 3.60 (s, $-C(6)H_2$), 2.80 (t, $J=6.4$, $-C(8)H_2$), 2.60 (t, $J=6.4$, $-C(9)H_2$; ^{13}C NMR ($CDCl_3$, 300 MHz) 207 (C(7 C=O), 176 (C(5) C=O), 152 (C(2)), 151 (C(9a) or C(10a)), 149 (C(10a) or C(9a)), 139 (CH), 136 (C(3′)), 131 (C(1′)), 129.9 (CH), 129.6 (CH), 127 (CH), 120 (CH), 119 (C(4a) or C(5a)), 116 (C(5a) or C(4a)), 37 (CH_2), 36 (CH_2), 28 (CH_2).

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Anal. Calc. for $C_{18}H_{13}ClN_2O_2$: C, 66.57; H, 4.03; Cl, 10.92; N, 8.63. Found: C, 66.56; H, 4.22; Cl, 10.88; N, 8.47.

*10-(3-Chlorophenyl)-6,8,9,10-tetrahydro-7-hydroxy-benzo[b][1,8]naphthyridin-5(7H)-one (**3**)*

To a solution of 10-(3-chlorophenyl)-6,8,9,10-tetrahydrobenzo[b][1,8]naphthyridin-5,7-dione, **11** (1.13 g, 3.48 mmol) in ethanol (200 mL) was added sodium borohydride (0.6 g), and the reaction mixture was stirred under nitrogen overnight. Ethanol was evaporated, and the residue was partitioned between water and dichloromethane. The dried organic solution was filtered and evaporated, and the residue was chromatographed over silica gel (120 g). Ethyl acetate–dichloromethane (1:9, by vol.) eluted the product, which crystallized from dichloromethane in a yield of 44% (0.5 g), m.p. 285–289°C; IR (mineral oil) 3450 (OH), 1610 (pyridone C=O), 1600, 1530, 1280; CI-MS: 327 (100, $[M+1]^+$ for ^{35}Cl); ^1H NMR (300 MHz, CD_3OD), 8.67 (dd, $J(4-2)=2$, $J(4-3)=8$, H(4)), 8.54 (dd, $J(2-4)=2$, $J(2-3)=4$, H(2)), 7.62–7.58 (m, 2H, Ar), 7.46–7.38 (m, 2H, Ar), 7.30–7.25 (m, H(3)), 4.18–4.10 (m, H(7)), 2.95 (dd, $J=3$ and 9, 1H), 2.68 (dd, $J=3$ and 9, 1H), 2.62–2.35 (m, $-C(8)H_2$), 2.00–1.75 (m, $-C(9)H_2$; ^{13}C NMR (300 MHz, CD_3OD) 171.4 and 171.3 (C(5)), 160.43 and 160.38 (C(9a)), 156 (C(2)), 150 (C(10a)), 140 (C(1′)), 137 (C(3′)), 136 (C(4)), 133 (C(5′ or 4′)), 132 (C(4′ or 5′)), 129.5 and 129.4 (C(2′)), 128.0 and 127.8 (C(6′)), 124 (C(3)), 118 (C(5a)), 117 (C(4a)), 63.90 and 63.85 (C(7)), 32 (C(9)), 29.25 and 29.22 (C(6 or 8)), 28.78 and 28.70 (C(8 or 6)) ppm. An attached proton test helped assign the indicated carbons to the listed resonances.

10-(3-chlorophenyl)-6,8,9,10-tetrahydro-7-hydroxybenzo[b][1,8]naphthyridin-5(7H)-one (**3**) was a diastereomeric mixture of atropisomers, according to its ^{13}C NMR spectrum.

Anal. Calc. for $C_{18}H_{15}ClN_2O_2$: C, 66.15; H, 4.63; Cl, 10.85, N, 8.57. Found: C, 66.09, H, 4.70; Cl, 10.65; N, 8.52.

Results

Incubation of SCH 40210 with dog liver slices produced a mixture of eight components (Figure 2), identified in part as the parent drug and four metabolites. Three of these metabolites were the monohydroxylated carbinols **2**–**4** (Figure 1). A minor component of the mixture was the 6-ketone derivative (**6**) of the drug (Figure 1).

Human and cynomolgous monkey liver slices also transformed the drug **1** to the three carbinols **2**–**4**, whereas the major metabolites proved to be the 6-carbinol **2** and its glucuronide **5**. Rat liver slices incubated with the drug also formed a mixture of the three carbinols **2**–**4**. However, additional minor metabolites were obtained exclusively from the rat tissue samples. These incompletely identified metabolites bore two more oxygen atoms than the parent drug. They comprised 40210-like pyridones additionally containing two hydroxyl or carbonyl groups, or one group of each kind. From all other studied species the same metabolites were obtained from the tissue and medium samples.

All three carbinol metabolites **2**–**4** were identified with authentic samples by comparison of HPLC retention times. In addition, the molecular weights of metabolites **2**–**6** were established by mass spectrometry in tandem with liquid or gas chromatography.

No authentic sample of carbinol **2** was available when the incubations were first carried out. Nevertheless, it was possible to identify this abundant carbinol by ^1H and ^{13}C NMR spectra of samples isolated from dog and monkey livers. These and mass spectra established the gross structure of compound **2**, the position and conformations of its hydroxyl group, and the presence of diastereomeric atropisomers of **2**.

Identification of Metabolites

Representative mass spectra of the isolated carbinols **2**–**4** from cynomolgus monkey liver and of the authentic carbinols appear in Figures 3–5. In each case, mass spectra of the chlorine-35 and -37 isotopomers of the metabolites were acquired and favorably compared with the spectra of authentic reference standards, identifying the carbinols **2**–**4**. Furthermore, protonated molecular ($[M+H]^+$, m/z 327) and other ions (m/z 309) appeared in spectra of the carbinols **2**–**4**. These latter ions arose through elimination of water from the protonated molecular ions. The ratios of ion intensities of the peaks with m/z 309 ($[M-H_2O)^+$) to those with m/z 327 ($[M+H]^+$) equalled 8.3 for the 6-carbinol **2** (Figure 5), 1.4 for the 7-carbinol **3** (Figure 3), and 0.04 for the 9-carbinol **4** (Figure 4). Different ratios resulted from variations in resonance stabilization of the ions resulting from dehydration of the parent ion radical. Conjugation of the nitrogen non-bonding electrons with the adjacent double bond stabilized the ion radical derived by water loss from carbinol **2** (Figure 1). In contrast, the 7- and 9-carbinols, **3** and **4**, cannot form such a stabilized ion radical.

The assignment of the 6-carbinol structure to metabolite **2** was based not only on its mass spectra, but also on its NMR spectra. *In vitro* metabolism of SCH 40210 yielded sufficient quantities of the 6-carbinol **2** from dog and monkey liver to record both ^1H and ^{13}C NMR spectra. They sufficed to establish the gross structure of **2**, the position occupied by its hydroxyl group, and the fact that **2** existed as a mixture of diastereomeric atropisomers.

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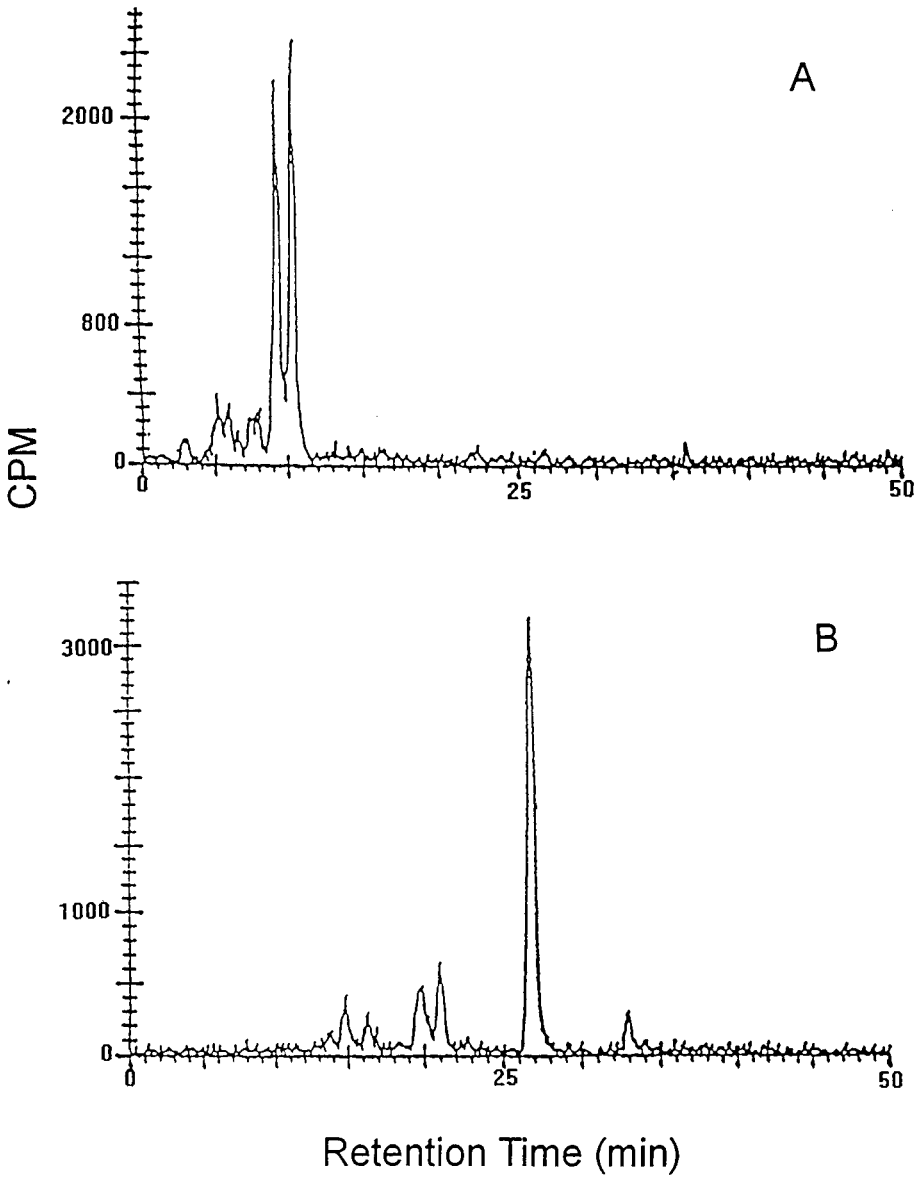

Figure 9. HPLC profiles of monkey urine radioactivity following a 0.05 mg kg^{-1} dose of suspended ^{14}C-SCH 40120. A. Before hydrolysis with β-glucuronidase. B. After hydrolysis with β-glucuronidase [16]. The y-axis represents 'counts per min' (CPM)

When chemical synthesis furnished an authentic sample of the racemic carbinol **2**, comparisons of ^{1}H and ^{13}C NMR spectra confirmed the assignment of structure (Figure 6 and Table 1).

The ^{1}H-NMR spectrum of the carbinol **2** exhibited characteristic features. The proton ($\delta = 5.15$ ppm) at C(6) of this aglycone metabolite was deshielded (Figure 6) in comparison to the 7-hydroxy ($\delta = 4.15$ ppm) and the 9-hydroxy ($\delta = 4.40$ ppm) carbinol protons. Deshielding of the pseudoequatorial proton at C(6) is due to the anisotropy of the proximate carbonyl group at C(5). The structural assignment of the 6-carbinol **2** was also supported by ^{13}C-NMR data (Table 1).

In addition to the monohydroxylated metabolites, the glucuronide **5** of the 6-hydroxy metabolite was identified. The mass spectra of the glucuronide isotopomers derived from carbinol **2** (Figure 7) exhibited protonated molecular ions as well as intense fragment ions. For example, the spectrum of the chlorine-35 isotopomer showed the protonated molecular ion at m/z 503 (40%, $[C_{24}H_{26}{}^{35}ClN_2O_8]^+$) and an intense fragment ion at m/z 309 (100%, $[C_{18}H_{14}{}^{35}ClN_2O]^+$). Glucuronides fragment to aglycones in mass spectrometers [19–21], so the ion at m/z 309 can be attributed either to elimination of the glucuronic acid moiety from the glucuronide ester, or to elimination of water from the protonated 6-hydroxy aglycone fragment (m/z 327).

For the glucuronide **5**, the ratio of ion intensities at m/z 309 and 327 was 7.8 (Figure 7). This value characterized the 6-hydroxy metabolite **2** (Figure 5), and supported the assignment of structure to metabolite **5**.

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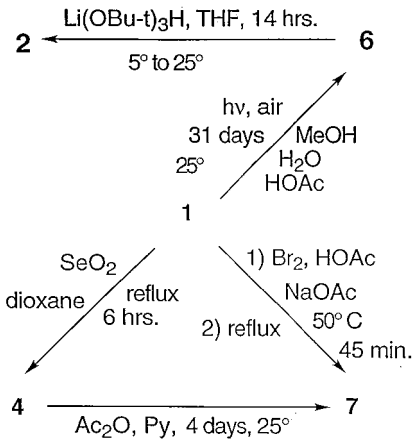

Figure 10. Syntheses of the racemic 6- and 9-carbinol derivatives of SCH 40210

The presence of a stereogenic center and axis in metabolites **2**–**4** and in the aglycone portion of **5** created diastereomers. Ketone **6**, which lacked a stereogenic center, also possessed a stereogenic axis, and formed enantiomers, which a chiral HPLC column resolved [16]. However, the optical purities of the isolated metabolites of **1** remain unknown.

Incubation of dog liver slices with **1** also yielded the 6-keto metabolite **6** as a minor product. The structural assignment was based on comparison of the daughter-ion mass spectra of the chlorine-35 and -37 isotopomers of the metabolite **6** with those of a synthetic reference standard (Figure 8).

Metabolism

Metabolism of SCH 40210 hydroxylated its cyclohexyl ring, not the aromatic chlorophenyl, pyridine, or pyridone rings. *In vivo*, the 6-carbinol **2** was the major, post-hydrolysis urinary metabolite in both monkey (Figure 9) and human [16]. *In vitro*, hydroxylation of SCH 40210 also yielded carbinol **2** as the most abundant metabolite. In both cases, oxygenation occurred where no enolization was possible. By contrast, a synthesis of carbinol **4** established that the drug enolized at C(9) as expected, and that it was oxidized with selenium dioxide at the site of enolization (Figure 10). In another reaction that left C(6) and N(1) of SCH 40210 untouched, C(5a) of the pyridone ring—not C(6)—suffered oxidation with 3-chloroperbenzoic acid [8].

Syntheses of Racemic Aglycones

To confirm assignment of structure to the primary metabolite required a racemic sample of the 6-carbinol derivative. Such a sample was obtained from the corresponding ketone, which was the unexpected product of SCH 40210 photolysis in fluorescent light [16]. The 6-ketone (**6**) served as the starting material for hydride reduction (Figure 10). This ketone proved to be a minor product of dog liver metabolism *in vitro*. It was also the major product of prolonged incubation of the drug with *Cunninghamella echinulata* [17]. The major product of brief incubation of SCH 40210 with this microorganism was the abundant 6-carbinol **2** [17]. A pure sample of **2** from this source became available before the chemical synthesis was complete, and after the metabolic sample was spectroscopically identified. Both microbiological and synthetic samples helped confirm assignment of structure to the metabolic samples.

Mild treatment of the 6-ketone (**6**) with one equivalent of lithium tri-*tert*-butoxy aluminohydride in

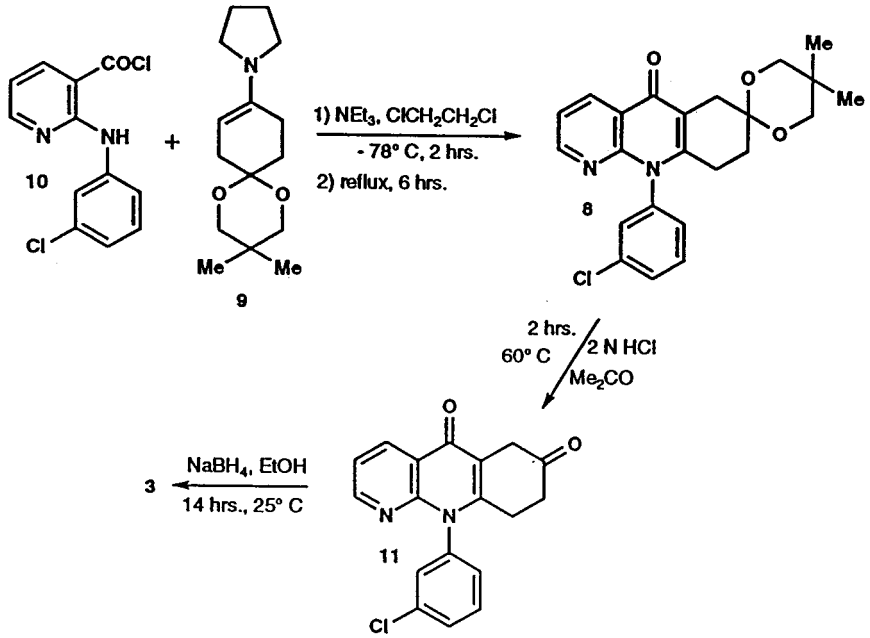

Figure 11. Synthesis of the racemic 7-carbinol derivative of SCH 40210

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Table 2. Selected ^{13}C chemical shifts of diastereomeric carbinol atropisomers[a]

	6-Carbinol	7-Carbinol	9-Carbinol
C(2′)	130.84	129.5	133
	130.78	129.4	131
C(3′)	129.3	128.0	130
	129.1	127.8	129
C(6)	63.29	—	—
	63.26	—	—
C(7)	—	63.9	—
	—	63.8	—
C(9)	—	—	63.9
	—	—	63.8

[a] δ-values are in ppm downfield from TSM in CD_3OD.

tetrahydrofuran gave the desired carbinol in a yield of 36%. Although the reagent was chosen to prevent any intramolecular reduction of the pyridone carbonyl group, several unidentified impurities formed in addition to the desired alcohol. Sodium borohydride in ethanol also reduced the 6-ketone to the 6-carbinol, but in a diminished yield (23%). At least ten unknown by-products accompanied the carbinol when the reductant was borohydride.

SCH 40210 furnished a conjugated enol ($\Delta^{5,5a,9a,9}$) by losing a proton from its 9-position [10]. This enolization formed the basis for a simple synthesis of the 9-carbinol derivative of the drug (Figure 10). Selenium dioxide in hot dioxane changed SCH 40210 into 30% of the desired carbinol, which was characterized as such and as the corresponding 9-acetate.

This acetate was the subject of an independent synthesis and a single-crystal X-ray analysis [22]. Bromination of the SCH 40210 enol under radical or ionic conditions led to the same product, the 9-bromide, **12**. N-Bromosuccinimide in dichloromethane at 25°C, or bromine in acetic acid containing sodium acetate at 50°C, were employed (Figure 10). In the latter case, the bromide solvolyzed to the 9-acetate when the reaction mixture was refluxed. The X-ray analysis directly and unequivocally established the ground-state structure and conformation of the acetate. Indirectly, it confirmed the assignments of structure to the 9-carbinol, the 9-bromide, and to SCH 40210 itself.

To prepare the 7-carbinol derivative called for acylation of enamine **9** with the acid chloride **10** (Figure 11). This C-acylation produced an intermediate enaminone in a standard synthesis of polycyclic 1-aryl-4-pyridones like SCH 40210 [23]. Intramolecular transamination within the intermediate enaminone then expelled pyrrolidine and closed the central ring of the expected tricyclic pyridone ketal. Dilute hydrochloric acid hydrolyzed the ketal to the ketone, which formed the desired 7-carbinol with sodium borohydride.

No chemical synthesis of the unknown 8-hydroxy derivative of SCH 40210 was sought or found.

Each of the 6-, 7-, and 9-substituted carbinols lacked planarity and possessed two elements of asymmetry. These elements were a stereogenic carbinol carbon atom and a stereogenic axis coincident with the bond joining C(1′) to N(8). Steric bulk and buttressing presumably hindered rotation about this axis [24]. Consequently, each carbinol comprised a diastereomeric mixture of atropisomers. The diastereoisomerism was evident on the time scale of ^{13}C NMR spectroscopy. The proton-decoupled spectra of carbinols **2**–**4** showed more resonances than there were carbon atoms in the corresponding molecules. Included were two resonances for each of the aromatic C(2′) and C(6′) atoms (Table 2). The $\Delta\delta$ values for the C(2′) and C(6′) signals, respectively, ranged from 0.06 to 2 ppm for the 9-carbinol. Each of the three carbinol carbons also gave rise to two signals.

The atropisomerism also manifested itself on the time scale of HPLC. At 25°C two different stationary achiral phases resolved the 6-carbinol into two peaks with baseline separation (Figure 12). When the column effluent entered a mass spectrometer, the separate diastereomers provided identical parent and daughter ion mass spectra (Figure 12). Each peak represented one diastereomeric pair of enantiomers (Figure 13). These observations, made for the first time with the carbinol **2**, hinted that the drug itself might comprise two interconverting enantiomers separable on the time scale of liquid chromatography. The drug did so [9].

Discussion

Animals and humans metabolize SCH 40210 via hydroxylation and conjugation. The 6-carbinol metabolite (**2**) and its glucuronide (**5**) were the major metabolites of the parent drug in both human and monkey. The 7- and 9-hydroxy metabolites (**3** and **4**) were also formed by cynomolgus monkeys and humans, albeit in smaller quantities. Incubation of relatively large quantities of substrate in fresh liver slices facilitated the isolation and identification of metabolites. Depending on the mammalian liver source, thousands of slices weighing scores of grams could be incubated with palpable amounts of drug. On two occasions more than 70 mg of SCH 40210 was treated with over 100 g of sliced liver. With such a high, non-physiological ratio of drug to liver, milligram weights of a single pure metabolite were expected to form. Quantities as large as these did result, which permitted assignment of gross structure using both ^1H- and ^{13}C-NMR, not only mass spectrometry.

The remarkable hydroxylation of **1** at carbon (6) occurred at a site where no enolization is possible.

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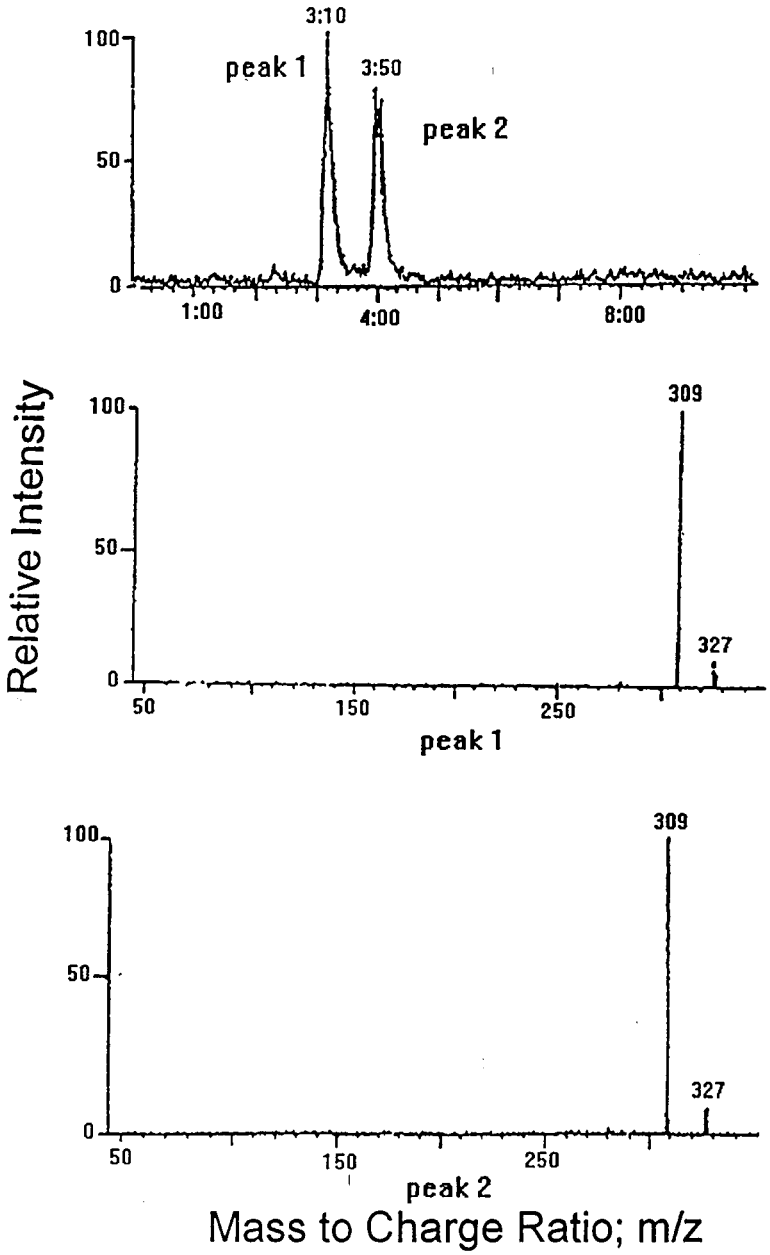

Figure 12. Top: Reconstructed total ion chromatogram from the LC/MS analysis of microbial carbinols **2**. Middle and bottom: LC/MS/MS spectra of the corresponding protonated diastereomeric molecular ions (*m*/*z* 327) from peaks 1 and 2

To explain this observation, we suggest that the transition state for metabolic hydrogen-atom abstraction involves a complexed SCH 40210 carbonyl oxygen atom. Complexation of this electron-rich pyridone oxygen atom to a high-valent, electron-deficient iron-oxo species within hepatic cytochrome P-450 would facilitate hydrogen loss from C(6). Hydrogen atom abstraction would occur through a six membered, cyclic transition state [25,26]. Finally, the loss of a C(6) hydrogen would create a resonance-stabilized radical conjugated through the central double bond ($\Delta^{5a,9a}$) to the lone-pair electrons of the pyridone nitrogen atom.

This conjugation might explain the absence of metabolites hydroxylated at C(4). No comparable stabilization of an intermediate C(4) radical would be possible through resonance. Only orthogonal pyridine sp^2 orbitals would be available to a C(4) radical. Any of several postulated high valent iron-oxo cytochrome P-450 intermediates, e.g. superoxide [P-450(Fe^{+2}).O_2^{-1}] [27], perferryl [P-450(FeO^{+3})] [28], or oxo-ligated iron (IV) porphyrin P-cation radical as found for peroxidase [29], may be responsible for the ensuing oxygen transfer that results in C(6) hydroxylation. There is evidence suggesting involvement of such high valent iron-oxo cytochrome P-450 intermediates in drug metabolism. A number of peroxidative agents can replace NADPH, cytochrome P-450 reductase and oxygen as co-reactants in most oxidative reactions of micro-

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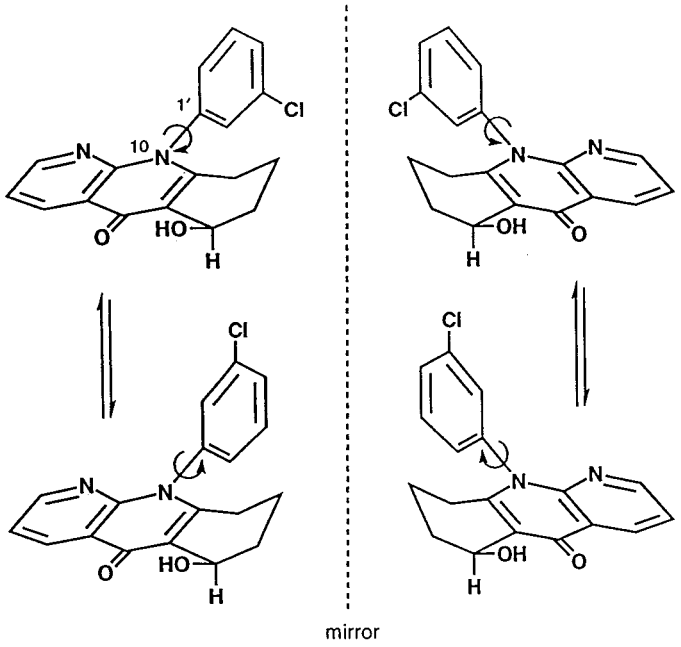

Figure 13. Epimerizing diastereomeric atropisomers of carbinol **2**; half-chair conformations are depicted

somal cytochrome P-450 [30]. Complexation of the pyridone oxygen atom in the transition state might also account for the lack of any metabolites hydroxylated in the chlorophenyl ring. This group would be too remote to suffer hydrogen-atom abstraction. Such a six membered transition state would also account for the lack of metabolites derived from C(5a) oxidation.

Isolation and identification of large quantities of SCH 40210 metabolites from a variety of species accomplished our purpose. Although the 6-, 7-, and 9-hydroxylated metabolites were detected in all species studied, the relative amounts of each of these metabolites varied significantly across these species. In conclusion, the biotransformation products of SCH 40210 in animal and human livers are similar.

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