Evaluation of 2-Benzylidenecyclohexanones and 2,6-Bis(benzylidene)cyclohexanones for Antitumor and Cytotoxic Activity and as Inhibitors of Mitochondrial Function in Yeast: Metabolism Studies of (*E*)-2-Benzylidenecyclohexanone

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Abstract □ Some 2-benzylidenecyclohexanones, 2,6-bis(benzylidene)cyclohexanones, and related compounds were evaluated for antitumor and cytotoxic activities; (*E*)-2-benzylidenecyclohexanone (I*a*) was shown to have significant cytotoxic properties and a potent inhibitory effect on yeast mitochondria. After intraperitoneal injection of I*a*, unchanged drug and a metabolite, tentatively identified as 2-(*p*-hydroxybenzyl)cyclohexanol, were found in the urine. No metabolites were found in the feces. Oral administration of I*a* afforded three unidentified metabolites in the urine and three unidentified metabolites in the feces.

Keyphrases □ Benzylidenecyclohexanones, substituted—evaluated for antitumor and cytotoxic activity, effect on mitochondrial function in yeast, metabolism studies □ Antitumor agents, potential—substituted benzylidenecyclohexanones evaluated □ Cytotoxic agents, potential—substituted benzylidenecyclohexanones evaluated □ Mitochondrial activity—substituted benzylidenecyclohexanones evaluated, yeast □ Structure–activity relationships—substituted benzylidenecyclohexanones evaluated for antitumor and cytotoxic activity, effect on mitochondrial function in yeast

Recently, some substituted 2-benzylidenecyclohexylamines were required as intermediates in the preparation of novel antineoplastic agents. The synthesis envisaged involved the preparation of the substituted 2-benzylidenecyclohexanones (I) followed by oximation to give the derivatives (V) to be reduced with lithium aluminum hydride to the desired substituted 2-benzylidenecyclohexylamines. The intermediate 2-benzylidenecyclohexanones are α,β-unsaturated ketones, a class of compounds known as alkylating agents (1–4) and possessing activity in various tumor systems (5, 6).

Recently, the preparation (7) and the antitumor and cytotoxic properties (5) of some substituted 1-phenyl-1-nonen-3-ones, which may be regarded as more flexible analogs of the substituted 2-benzylidenecyclohexanones, were described. In fact, the 1-nonen-3-ones are capable of adopting both (*S*)-*cis*- and (*S*)-*trans*-conformations whereas the compounds in Series I are locked in the (*S*)-*cis*-conformation. A comparison of the antitumor and cytotoxic properties of the two series of compounds may indicate some stereochemical requirements for activity in this group. It is well documented that in certain series the rigid analogs have higher pharmacological activity than the related acyclic derivatives (8, 9), although acyclic analogs of rigid cyclic compounds have enhanced biological activity (10–12) in other cases.

RESULTS AND DISCUSSION

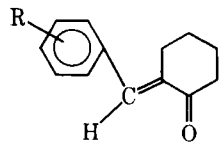
The preparation of the compounds in Series I*a*–I*e*, II, III, and V was described previously (13, 14). Condensation between dichlorinated benzaldehydes and cyclohexanone led invariably to the bis compounds (III*b*–III*d*). In Series I and III, the compounds were shown by spectroscopic means to have the (*E*)- and (*E,E*)-configurations; II, prepared by UV irradiation from I*a*, was shown to have the (*Z*)-configuration. A representative 2-benzylidenecyclohexanone (I*a*) was reduced with lithium aluminum hydride to the corresponding alcohol (IV) with retention of the double bond, and it was considered that the oximes (V) derived from Series I would afford the required substituted 2-benzylidenecyclohexylamines on treatment with lithium aluminum hydride. However, reduction of V led to the formation of the epiminocyclohexanes (VI) (15).

With the exception of I*c* and III*a*, Compounds I–IV were given by intraperitoneal injection against the L-1210 lymphoid leukemia screen in mice, using the standard Q4D and QD1-9 protocols, at doses of 400–100 mg/kg and were found to be inactive. No toxicity was observed with the derivatives at 400 mg/kg with the exception of I*f*; with I*f* the dose had to be reduced to 50 mg/kg to prevent animal mortalities. The bis ketone (III*a*), assessed only using the Q4D protocol, was inactive. The cyclic derivatives thus resemble the related acyclic 1-phenyl-1-nonen-3-ones in showing no significant activity against L-1210 lymphoid leukemia and no murine toxicity except for the *para*-quaternary ammonium iodides.

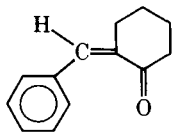
The only difference in toxicity between the cyclic and acyclic series was the absence of toxicity in the unsubstituted compounds I*a* and II at a dose of 400 mg/kg whereas a dose of 100 mg/kg was fatal for the mice with the acyclic series. A marked difference in cytotoxicity existed between the stereoisomeric 2-benzylidenecyclohexanones in the KB screen, whereby the (*E*)-isomer was found to be approximately 17 times more active than the (*Z*)-isomer. The acyclic 1-phenyl-1-nonen-3-ones showed no activity in this screen at a concentration of 100 ppm, although I*b*–I*f* were similar in potency to the acyclic series of derivatives.

A number of biological alkylating agents, such as the nitrogen mustards, have five carbon atoms between the centers at which nucleophilic attack can occur (16); therefore, cross-linking between the N^7-atoms of near-opposite guanine residues of DNA may occur (17). With the 2,6-bis(benzylidene)cyclohexanones (III), the possibility of cross-linking occurring is conceivable if alkylation occurs at the benzylic carbon atom. However, the screening data indicated no significant activity in either the L-1210 or KB screen. Furthermore, high murine toxicity is absent, which may be associated with a failure for cross-linking due to steric impedence at the benzylic carbon atoms.

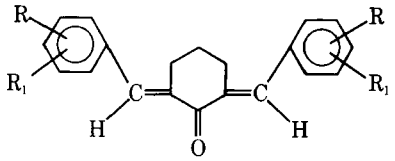
Possible explanations were sought for the wide variations in activity in the KB screen. Earlier work on α,β-unsaturated ketones had showed that polarity of the olefinic double bond (as measured by the τ values of the olefinic protons and IR carbonyl stretching frequencies) was dependent upon the Hammett and Taft values of the nuclear substituents (18). In Series I–III, no correlation between altered polarity of the charge on the benzylic carbon atom and KB activity could be seen. It is well known that in certain series of compounds, correlations exist between the planarity of the molecules and biological activity (19, 20). A clearcut evaluation indicating whether planarity of the molecules favors or retards cyto-



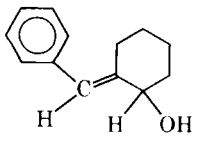
Ia: R = H
Ib: R = 2-Cl
Ic: R = 3-Cl
Id: R = 4-Cl
Ie: R = 4-$N(CH_3)_2$
If: R = 4-$N^+(CH_3)_3I^-$



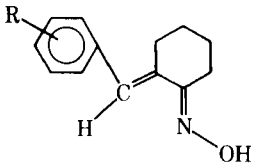
II



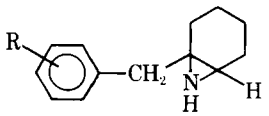
IIIa: R = R_1 = H
IIIb: R = 2-Cl, R_1 = 4-Cl
IIIc: R = 2-Cl, R_1 = 6-Cl
IIId: R = 3-Cl, R_1 = 4-Cl
IIIe: R = 4-$N(CH_3)_2$, R_1 = H



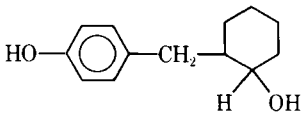
IV



Va: R = H
Vb: R = 2-Cl
Vc: R = 4-Cl
Vd: R = 4-$N(CH_3)_2$



VIa: R = H
VIb: R = 2-Cl
VIc: R = 4-Cl



VII

toxic activity was not found, although the eight compounds that were more active than 100 ppm had, on average, a lower θ value than the four compounds that were inactive at 100 ppm.

Examination of the effect of the α,β-unsaturated ketones on yeast mitochondria suggested an explanation for the high activity of Ia. In the absence of mitochondrial function, the yeast will grow on complex glucose medium, which provides energy for growth by fermentation of glucose. However, the complex ethanol medium containing ethanol, a nonfermentable energy source, does not provide energy for the growth of the yeast if interference with mitochondrial function occurs. Table I shows that Ia is a potent mitochondrial poison, significantly more potent than certain established mitochondrial inhibitors such as chloramphenicol and tetracycline (21).

The selective effect on yeast mitochondria was confirmed by observing the threefold difference in concentration of Ia required to inhibit 50% of the growth of *Saccharomyces cerevisiae* on minimal salts ethanol medium and minimal salts glucose medium. Reduction of the carbonyl group of Ia with lithium aluminum hydride gave IV, shown by NMR evidence to have an equatorial hydroxyl group. The unsaturated alcohol IV had a similar absence of murine toxicity and activity against L-1210 leukemia as Ia but was approximately 150 times less potent in the KB screen. The drastic reduction in activity of IV may be due to facile hydrogen bonding preventing penetration *via* the cancer cell membrane to a site of action. Paradoxically, the compound showed significant activity against yeast mitochondria.

Table I—Assessment of the Cyclohexane Derivatives against the KB Tumor System and *S. cerevisiae*

Compound	KB Cell Culture Screen[a], μg/ml	Mean Area of Inhibition of Growth of *S. cerevisiae*, mm[b]		Interplanar Angle, θ[b]
		In Complex Ethanol Medium	In Complex Glucose Medium	
Ia	0.25	159.9[c]	0.0[d]	0
II	4.3	—	—	52
Ib	20	—	—	37
Ic	3.2	0	0	29
Id	21	—	—	0
Ie	>100	0	0	0
If	49	151.9[e]	85.4[f]	0
IIIa	29	0	0	0
IIIb	>100	0	0	52
IIIc	>100	0	0	63
IIId	16	0	0	33
IIIe	>100	0	0	0
IV	38	91.2[g]	0.0[d]	0

[a] Values indicate the dose inhibiting 50% of the growth of human epidermoid carcinoma of the nasopharynx in Eagle's medium. A compound should have an activity of 4 μg/ml or less to be considered as having significant cytotoxic activity. [b] Calculated using the absorption maxima described in Ref. 13, with the exception of If and IV which are described in this work. [c] *SE* 4.5. [d] All five replicates gave the same value. [e] *SE* 11.1. [f] *SE* 6.33. [g] *SE* 13.2.

Earlier work had shown the antitumor effect of oximes in the presence or absence of metals (22, 23), and aziridines have been used in the treatment of tumor systems (24). However, the cyclohexane derivatives (Va–Vd and VIa–VIc) were inactive in the intraperitoneal L-1210 system using the standard Q4D protocols. In the QD1-9 protocol, Va–Vd, VIa, and VIb were shown to be inactive at doses of 100–400 mg/kg. Toxicity was noted with all of the aziridines at 400 mg/kg.

The interesting level of cytotoxic activity of Ia and its efficacy as a mitochondrial inhibitor suggested its study for metabolism *in vivo*. Reduction of the olefinic double bond (25) and carbonyl group (26) of α,β-unsaturated ketones has taken place *in vivo*, as has the formation of the cysteine conjugates (27). The urine of rats, previously injected intraperitoneally with Ia, was extracted using a modification of the method of Curry (28) and shown by TLC to contain two components not present in the urine of control rats.

Mass spectrometry showed one component to be unreacted Ia and the other to be a metabolite possessing a molecular formula of $C_{13}H_{18}O_2$ (molecular ion: 206.1311; calculated for $C_{13}H_{18}O_2$: 206.1307). A loss of water from the parent peak gave an ion at *m/e* 188 with an accurate mass of 188.1194 (calculated for $C_{13}H_{16}O$: 188.1201), confirmed by the appropriate metastable peak at *m/e* 171.5; this finding suggested that a substituted cyclohexanol was present, loss of water being a major mass spectral pathway in the case of cyclohexanol (29). The mass spectrometry of a sample of 2-benzylcyclohexan-1-ol prepared by an unambiguous route showed loss of water as the major fragmentation pathway. A prominent peak at *m/e* 107 was attributed to a nuclear-hydroxylated benzyl group; since aromatic hydroxylation is favored in the *para*-position, evidence from mass spectrometry suggests the possibility of the metabolite being 2-(*p*-hydroxybenzyl)cyclohexan-1-ol (VII).

The acyclic α,β-unsaturated ketone, 1-phenyl-1-penten-3-one, was metabolized to the corresponding saturated alcohol, 1-phenylpentan-3-ol, and to 1-(*p*-hydroxyphenyl)pentan-3-one (26), indicating that complete reduction of the α,β-unsaturated keto group and aromatic hydroxylation occurred. TLC analysis indicated one component in each of the basic and amphoteric fractions, the sample in the amphoteric fraction having the same retention time as Ia. No metabolites were detected in the feces.

An examination of the efficiency of the extraction technique showed that Ia was extracted from control urine using the modified Curry technique with an efficiency of 11.4%. Use of liquid–liquid extraction techniques increased the efficiency to a little greater than 99.9%. Liquid–liquid extraction of urine at pH 9.0, 7.2, and 2.0 revealed only two components, one with an identical retention time to Ia. The quantity of unchanged drug found in urine after

Table II—Quantities of Unchanged Ia Obtained by Liquid–Liquid Continuous Extraction of Urine from Rats Previously Administered the Drug by Intraperitoneal Injection

Days after Injection	Unchanged Ia Found in Urine, %					
	400-mg/kg Dose Level			40-mg/kg Dose Level		
	pH 9.0	pH 7.2	pH 2.0	pH 9.0	pH 7.2	pH 2.0
1	0.50	0.10	—	0.54	0.06	—
2	0.13	—	—	0.07	—	—
3	0.02	—	—	—	—	—
4	—	—	—	—	—	—
	0.65	0.10	—	0.61	0.06	—
Total	0.75			0.67		

intraperitoneal injection was approximately 1%. Since the high dose used (400 mg/kg) could saturate enzyme systems, preventing metabolism of Ia, a lower dose of 40 mg/kg was used, but the quantity of unmetabolized Ia in the urine remained the same (Table II).

After oral administration of Ia, three metabolites were detected in the urine using either the modified Curry technique or liquid–liquid extraction. The GLC characteristics of one component had a retention time of unchanged Ia. The quantity of metabolites represented approximately 8.4% of the administered dose of which Ia was present to an extent of 0.66% (Table III). Examination of the feces after oral administration showed the presence of three metabolites by both Curry's modified method and the liquid–liquid extraction technique. The metabolites accounted for approximately 81% of the administered dose (Table IV). Repeated attempts to isolate the metabolites by preparative GLC proved unsuccessful. These metabolic studies indicated the presence of approximately 1% unconjugated metabolites and unchanged drug after intraperitoneal injection and approximately 89% of unchanged drug and unidentified metabolites after oral administration of Ia.

EXPERIMENTAL[1]

Preparation of Compounds

The preparation of Series Ia–Ie, II, III (13), V (14), and VI (15) was described previously.

(*E*)-2-(*p*-Dimethylaminobenzylidene)cyclohexanone Methiodide (If)—A solution of Ie (10.0 g, 0.04 mole) and methyl iodide (12.4 g, 0.08 mole) in anhydrous benzene (100 ml) was heated under reflux for 16 hr. The hot solution was filtered, and the residue was recrystallized from absolute ethanol to give If as bright-yellow crystals (6.6 g, 40%), mp 148–150°; IR (potassium bromide): 1615 (C=C) and 1682 (C=O) cm^{-1}; UV (alcohol): 280 (ϵ 13,590) and 219 (13,810) nm; NMR (deuterium oxide): δ 8.13–7.80 (m, 2, aromatic C^2H, C^6H), 7.80–7.47 (m, 2, aromatic C^3H, C^5H), 7.27 [t, 1, J = 2 Hz, $(CH_3)_3N^+C_6H_4CH$=], 3.77 [5.9, $(CH_3)_3N^+$], 3.07–2.53 (m, 2, C^3H_2), 2.80–2.33 (m, 2, C^6H_2), 2.17–1.50 (m, 4, C^4H_2, C^5H_2); mass spectrum: *m/e* 299 (parent peak).

Anal.—Calc. for $C_{16}H_{22}INO$: C, 51.76; H, 5.97. Found: C, 51.37; H, 6.01.

***cis*-2-Benzylcyclohexanol**—A solution of (*E*)-2-benzylidenecyclohexanone (Ia, 75.0 g, 0.403 mole) in dry ether (250 ml) was added to a suspension of lithium aluminum hydride (5.74 g, 0.151 mole) in dry ether (50 ml) and stirred at room temperature for 1 hr. After addition of water, the solvent was evaporated to give a pale-yellow syrup (73.0 g) which, on distillation, gave a colorless oil (63.4 g), bp 120°/0.3 mm. Recrystallization of the solidified distillate from petroleum ether[2] gave essentially IV as colorless crystals (52.3 g, 69%), mp 62.5° [lit. (31) mp 63–64°].

GLC analysis indicated the presence of two components (98:2) which could not be changed on repeated recrystallization; IR (potassium bromide): 3390 (OH) cm^{-1}; NMR (deuterochloroform): δ 7.53–7.00 (m, 5, C_6H_5), 6.55 (s, 1, C_6H_5CH=), 4.40–4.07 (m, 1, CH), 3.00–2.40 (m, 1, equatorial C^1H), 2.40–1.17 (m, 7, axial C^3H, C_4H_2, C^5H_2, C^6H_2), and 2.00 (s, 1, OH, exchanges with D_2O); mass spectrum: *m/e* (relative intensity) 188 (M^+, 100) and 170 (9).

Anal.—Calc. for $C_{13}H_{16}O$: C, 82.93; H, 8.57. Found: C, 82.81; H, 8.43.

A solution of IV (0.166 g) in ethanol (200 ml) was hydrogenated over 10% palladium-on-charcoal (0.251 g) at 51 psi for 24 hr. Removal of both catalyst and solvent gave *cis*-2-benzylcyclohexanol as a colorless oil (0.159 g, 95%), shown by GLC to be one component [lit. (32) *cis*-isomer as an oil, *trans*-isomer as a solid, mp 76–77°]; IR (smear): 3405 (OH) cm^{-1}; NMR (deuterochloroform): δ 7.37–7.00 (m, 5, C_6H_5), 3.87–3.63 (m, 1, CH), 3.40–2.97 (m, 1, C_2H), 2.77–2.23 (m, 2, $C_6H_5CH_2$), 2.07–0.63 (m, 8, C_3H_2, C_4H_2, C_5H_2, C_6H_2), and 1.68 (s, 1, OH, exchanges with D_2O); mass spectrum: *m/e* (relative intensity) 190 (M^+, 37) and 172 (100).

Inhibition of Mitochondrial Function

Complex glucose medium containing yeast extract (1%), bactopeptone (2%), glucose (2%), and agar (2%) in water and complex ethanol medium containing ethanol (4%) in place of glucose were employed. The compounds (5.0 mg) were dissolved in 95% ethanol (1 ml) and 0.02 ml of the solution was applied to antibiotic disks (12.7 mm). The disks were placed on the surface of the complex glucose medium and complex ethanol medium plates previously spread with 0.2 ml of a culture of *S. cerevisiae*[3] grown for 48 hr in liquid complex ethanol medium.

The plates were incubated at 28° for 3 days, and the area of inhibition (square millimeters) surrounding the disk was recorded. Five replicates for each compound were undertaken. The area of growth inhibition on complex ethanol medium in excess of growth inhibition on complex glucose medium is a quantitative estimation of the inhibition of mitochondrial function (Table I).

For growth inhibition studies of Ia, a diploid strain of *S. cerevisiae* homozygous for *ade* 2/*ade* 2[3] was grown to an early stationary phase in a liquid medium. The medium, placed in 8 × 150-mm tubes, was inoculated to an optical density (640 nm) of 0.1 unit/ml. The inhibitors were added at concentrations of 1, 2.5, 5, 7.5, 10, 50, 100, 250, 500, 750, and 1000 μg/ml of minimal salts glucose medium or minimal salts ethanol medium. After 24 hr of growth at 30°, the percentage growth was ascertained by the change in optical density and expressed as a percentage of the control.

Minimal salts ethanol medium contains 1% ethanol as a nonfermentable energy source, and minimal salts glucose medium employs 5% glucose as a fermentable energy source. Thus, growth in minimal salts ethanol medium is dependent upon mitochondrial function whereas growth on minimal salts glucose medium can occur by glycolysis. Six replicates were undertaken on minimal salts ethanol medium, and the concentration of Ia required to inhibit 50% of the growth of *S. cerevisiae* was 56.3 μg/ml, *SE* 10.77. The concentration required to inhibit 50% of the growth of the yeast on minimal salts glucose medium was 168 μg/ml.

[1] Boiling points and melting points are uncorrected. IR spectra were recorded on a Unicam SP-200G spectrophotometer calibrated with polystyrene. A Beckman DU spectrophotometer was employed for determining the UV spectra, using 1-cm quartz cells. NMR spectra were determined on a Varian T-60 instrument. Mass spectrometry was undertaken using an AEI MS12 single-focusing instrument operated by Mr. D. R. Bain, Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, and high-resolution mass spectrometry was carried out by Dr. D. Durden of the Department of Psychiatric Research, University Hospital, Saskatoon, Canada. Elemental analyses were carried out on a Coleman model 33 carbon–hydrogen analyzer by Mr. O. Douglas, College of Pharmacy, University of Saskatchewan, Saskatoon, Canada. GLC of the compounds synthesized was undertaken employing a Pye 104 gas chromatograph equipped with a flame-ionization detector and using 1.5-m × 0.6-cm (5-ft × 0.25-in.) o.d. glass columns packed with 4% SE-30 adsorbed onto acid-washed, silanized Chromosorb W (100–120 mesh). Nitrogen was used as the carrier gas at 55–60 ml/min, a detector temperature of 250°, and isothermal runs from 140 to 200°. GLC of the extracts obtained in the metabolism studies employed a Hewlett Packard 5750B instrument equipped with a flame-ionization detector and using 1.2-m × 0.3-cm (4-ft × 0.125-in.) o.d. stainless steel columns packed with 3% OV-101 on Chromosorb W (80–100 mesh H.P.). Nitrogen was the carrier gas at 40 ml/min; a detector and injection port temperature of 245° was maintained. Silica gel 7GF (J. T. Baker Chemical Co.) and silica gel 60HR (Merck) were used for TLC.

Adult male albino rats of the Wistar strain were housed individually in rat metabolism units with urine–feces separators (Econo-cage E110 metabolism unit, Maryland Plastics Inc.). They were allowed free access to food and water in a temperature- and humidity-controlled room having a 12-hr day/night cycle.

Evaluations of the compounds for antitumor and cytotoxic activity were carried out by the Drug Development Division, National Cancer Institute, Bethesda, Md., according to their protocols (30).

[2] Skelly F, Skelly Oil Co., Tulsa, Okla.

[3] Strain GR13 constructed in the Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Table III—Percentage of Ia Excreted Unchanged and/or Converted into a Metabolite after Oral Administration

Days after Administration	Administered Dose Excreted, %								
	Component 1[a,b]			Component 2[c]			Component 3[d]		
	pH 9.0	pH 7.2	pH 2.0	pH 9.0	pH 7.2	pH 2.0	pH 9.0	pH 7.2	pH 2.0
1	0.54	0.12	—	0.29	0.24	4.75	1.72	—	—
2	—	—	—	0.05	0.04	—	0.59	—	—
3	—	—	—	—	—	—	0.02	—	—
Total	0.54	0.12	—	0.34	0.28	4.75	2.33	—	—
	0.66			5.37			2.33		

[a] Unchanged ketone. [b] Compound with a retention time of 2.60–2.63 min. [c] Compound with a retention time of 1.72–1.83 min. [d] Compound with a retention time of 5.21 min.

Examination of Rat Urine and Feces after Intraperitoneal Administration of Ia

Compound Ia (4.0000 g) was administered intraperitoneally in distilled water containing hydroxypropyl methylcellulose[4] to 40 male albino Wistar rats at a dose level of 400 mg/kg. The urine (test urine) and feces (test feces) were collected daily for 5 days and stored at −6°. Urine (control urine) and feces (control feces) were collected daily for 5 days from 10 male albino Wistar rats injected with distilled water containing hydroxypropyl methylcellulose.

Urine—The test urine (25 ml, pH 8.8) was extracted with ether (3 × 25 ml) to give an organic extract containing the basic (D) and neutral (C) fractions of urine. The aqueous phase was then acidified (pH 7.2) and extracted with ether (3 × 25 ml) to give an ethereal extract of the amphoteric (E) fraction. The aqueous phase was then acidified (pH 2.0) and extracted with ether (3 × 25 ml). The organic extract was washed immediately with aqueous sodium bicarbonate solution (5% w/v) (2 × 10 ml), and the two layers were separated into an aqueous phase (1) and an ethereal phase (1).

The aqueous phase (1) was acidified with hydrochloric acid (50% v/v) and extracted with ether (3 × 25 ml) to give an ethereal extract containing the strong acid (A) fraction. The ether layer (1) was washed with sodium hydroxide solution (0.45 *N*, 2 × 10 ml) to give an aqueous phase (2). The aqueous phase (2) was acidified immediately with hydrochloric acid (50% v/v) and extracted with ether (3 × 25 ml) to give an extract of the weakly acidic (B) fraction. Evaporation of the ethereal extracts gave Fractions A, B, E, and C-D. Control urine (25 ml) was extracted in a identical manner.

GLC—The fractions from both test and control urine were dissolved in ethanol, and 0.5–4-μl solutions were injected onto a GLC column at 190° (Fractions A and B) and 170° (Fractions C-D and E). The chromatograms of A and B showed similar constituents in both test and control urine. One extra peak was found in each of the C-D and E test fractions, with retention times of 0.60 and 1.95 min, respectively.

TLC—The test and control extracts of Fractions A and B were dissolved in ethanol and spotted onto silica gel 7GF. Upon development with chloroform–acetone (97:10), no compounds with R_f values differing from the control fractions appeared in the test fractions.

Fraction C-D from both test and normal urine was dissolved in ethanol and placed on silica gel 7GF plates. Development with chloroform–methanol (97:5) indicated a component with an R_f value of 0.70 found only in the test fraction. The test and control extracts of Fraction E were dissolved in ethanol, spotted onto silica gel 7GF plates, and developed with chloroform–methanol (97:5). A spot with an R_f value of 0.97 from the test urine appeared black under shortwave UV light, but the component with an R_f value of 0.97 from control urine appeared fluorescent blue.

A check that no degradation of the extracts from Fractions A, B, C-D, and E occurred on the silica gel was performed. The fractions were dissolved in ethanol and spotted onto silica gel plates. Two-dimensional chromatography in chloroform–acetone (97:10) for A and B and utilization of a developing mixture of chloroform and methanol (97:5) for C-D, E, and pure Ia showed no evidence of degradation.

Mass Spectrometry—The silica gel containing the black spot from the Fraction E test, R_f 0.97, was extracted with distilled acetone for 2–3 min. The solution was filtered through a sintered-glass filter and evaporated to dryness. The residue was then dissolved in ethanol, respotted on a silica gel 7GF plate, and developed in chloroform–methanol (97:5). The silica gel containing the one observable component, R_f 0.97, was removed from the plate, extracted with redistilled acetone for 2–3 min, filtered, evaporated to dryness, and rechromatographed on silica gel HR60.

The silica gel containing the black spot, R_f 0.97, was extracted with redistilled acetone, evaporated to dryness, and submitted for mass spectral analysis; mass spectrum: *m/e* (relative intensity) 186 (M^+, 75), 185 (100), 158 (13), 157 (15), 143 (15), 130 (25), 129 (46), 128 (31), 127 (13), 117 (33), 115 (54), 102 (17), 91 (79), 77 (33), and 67 (71). The mass spectrum for Ia showed *m/e* (relative intensity) 186 (M^+, 61), 185 (100), 158 (7), 157 (8), 143 (10), 130 (20), 129 (37), 128 (21), 127 (8), 117 (26), 115 (43), 102 (13), 91 (21), 77 (12), and 67 (43). A quantity of the extracted component was dissolved in ethanol and had a retention time of 1.96 min when 1.0 μl was injected onto a 3% OV-101 column at 170°.

The black spot, R_f 0.70, of Fraction D was extracted in a similar manner and submitted for mass spectrometry; mass spectrum: *m/e* (relative intensity) 206 (M^+, 11), 188 (30), 181 (9), 170 (10), 165 (7), 159 (10), 152 (7), 149 (16), 147 (9), 146 (7), 145 (14), 142 (7), 141 (9), 136 (7), 135 (34), 134 (25), 133 (11), 131 (14), 129 (14), 128 (11), 127 (7), 124 (10), 123 (98), 121 (21), 120 (100), 119 (25), 117 (11), 116 (7), 115 (18), 111 (7), 110 (7), 109 (13), 108 (24), 107 (99), 105 (14), 104 (7), 103 (9), 101 (7), 98 (7), 97 (21), 96 (9), 95 (14), 94 (16), 93 (9), 92 (18), 91 (62), 89 (9), 86 (9), 85 (9), 84 (9), 83 (16), 82 (11), 81 (25), 80 (9), 79 (28), 78 (15), 77 (43), 76 (7), 73 (10), 71 (14), 70 (10), 69 (24), 68 (7), 67)26), 66 (14), 65 (30), 63 (13), 60 (14), 59 (13), 58 (13), 57 (32), 56 (14), 55 (40), 54 (9), 53 (17), 52 (9), 51 (21), 50 (10), 45 (11), 44 (21), 43 (64), 42 (14), 41 (62), and 40 (9).

Feces—Feces (25.0 g) from the test rats were soaked in ether, ground in a mortar, and placed in a soxhlet extractor with ether (300 ml) for 66 hr. Evaporation of the ether gave a black residue, which was suspended in water (50 ml) and filtered. The aqueous extract (pH 4.2) was acidified (pH 2.0) with 1 *N* sulfuric acid, extracted with ether (3 × 75 ml) to give aqueous *a* and ether *a* layers. The aqueous *a* layer was basified (pH 7.2) with ammonium hydroxide solution and extracted with ether to give the ether extract containing the amphoteric (E) fraction. Basification of the aqueous *a* layer (pH 9.0) with ammonium hydroxide solution followed by extraction with ether gave an organic extract containing the basic (D) fraction.

The ether *a* layer was washed with freshly prepared sodium bi-

Table IV—Percentage of Metabolites of Ia Found in Feces after Oral Administration of Drug

Days after Administration	Administered Dose Excreted, %								
	Component 1			Component 2			Component 3		
	pH 9.0	pH 7.2	pH 2.0	pH 9.0	pH 7.2	pH 2.0	pH 9.0	pH 7.2	pH 2.0
1	—	10.90	—	—	15.68	—	—	24.78	—
2	—	10.14	—	—	11.65	—	—	27.88	—
3	—	—	—	—	—	—	—	—	—
Total	—	1.04	—	—	27.33	—	—	52.66	—
		81.03							

[4] Methocel HG, Dow Chemical of Canada, Ltd.

carbonate solution (5% w/v, 2 × 50 ml), and the two layers were separated, giving aqueous *b* and ether *b* layers. Acidification of the aqueous *b* layer with hydrochloric acid (50%) and extraction with ether gave an ethereal extract of the strong acid (A) fraction. The ether *b* layer was washed with aqueous sodium hydroxide solution (0.45 *N*, 2 × 100 ml) to give aqueous *c* and ether *c* layers. Acidification of the aqueous *c* layer with hydrochloric acid (50%) and extraction with ether gave an ethereal extract containing the weak acid (B) fraction. The ether *c* layer contained the neutral (C) fraction.

After water-soluble extraction, the black residue was dissolved in ether (50 ml). The ether layer was extracted with 1 *N* sulfuric acid, and the procedure was followed as described previously. Feces (26.0 g) from control rats were extracted in exactly the same manner. Evaporation of the ether gave Fractions A–E.

A second method of extraction for both test and control feces was undertaken in a similar manner to that described previously, except that the feces were soaked in methanol and then extracted with methanol for 66 hr. A third method of extraction of both test and control feces (90.0 g) used ether (600 ml) in a soxhlet extraction apparatus for 66 hr. The ether was evaporated to 200 ml and extracted as described previously, but there was no prior extraction of water-soluble materials.

TLC—The test and control fractions, A–E, obtained by the three extraction procedures were purified prior to investigation.

Ether was added to Fraction A and extracted with an equal volume of aqueous sodium hydroxide solution (0.45 *N*). After acidification of the aqueous layer with 1 *N* sulfuric acid, it was extracted exhaustively with ether. Fraction B was purified in a similar manner. Fraction C was added to a mixture of equal parts of hexane and ethanol. An equal amount of water was added, and the ethanol–water layer was removed by separation. The ethanol–water mixture was evaporated to dryness and dissolved in water, and the aqueous phase was extracted with ether. Ether was added to Fraction D, and the ethereal solution was shaken with an equal volume of 1 *N* sulfuric acid. The aqueous layer was then basified with 0.45 *N* sodium hydroxide solution and extracted with ether. Fraction E was purified in a similar manner. Finally, the ethereal extractions of test and control Fractions A–E were evaporated to dryness.

The purified residues obtained after the three extraction procedures were examined separately by TLC on silica gel. In each case, the developing solvent for Fractions A–C was a mixture of chloroform and acetone (97:10). For Fractions D and E, the solvent employed was a chloroform and methanol mixture (97:5). When the fractions of both test and control urine were examined, no additional spots appeared in the test fractions regardless of the method of extraction of the fecal material.

GLC—The purified extracts in ethanol (2.0–3.0 μl) from both test and control feces were injected onto a column at 190° (Fractions A–C) and 170° (Fractions D and E) under the conditions previously described. No additional peaks were evident in the test fractions.

Efficiency of Extraction Procedures for Isolating Ia from Urine—Compound Ia (1.0000 mg) was added to rat urine (25 ml) and extracted as described previously. Fraction E was diluted in ethanol to 0.8 ml. A sample of Ia was dissolved in ethanol and diluted to 0.8 ml. Samples of 0.6 μl of both ethanolic solutions were injected onto a GLC column at 170°, and the peak area of Fraction E was 11.40% that of the standard solution.

In a separate series of experiments, Ia (25.0000 mg) was mixed with control urine (100 ml, pH 8.6) and extracted with ether (200 ml) in a liquid–liquid continuous extraction apparatus for 24 hr. Samples of the extract (0.6 ml) were removed periodically, and 2.0-μl samples were injected onto a GLC column at 170°. The resultant peak area was compared with a peak area resulting from the injection (2.0 μl) of a solution of Ia (0.2500 mg in 1.0 ml of ethanol). The experiment was repeated, except that the urine was acidified to pH 7.2 prior to extraction. After 1, 2, 3, 4, 5, 6, and 24 hr, the percentage recovery of Ia from urine at pH 8.6 was 76.02, 77.11, 95.10, 99.13, 99.43, 99.93, and 99.49, respectively, and the percentage recovery of Ia from urine at pH 7.2 was 65.66, 74.73, 81.02, 88.40, 92.37, 96.68, and 96.38, respectively.

Quantitation of Unchanged Ia Excreted *via* Urine after Intraperitoneal Injection of Drug—Compound Ia (897.5000 mg) was administered to nine rats at a dose of 400 mg/kg. The urine was collected daily and extracted using the following procedure. The urine collected on Day 1 (231 ml, pH 7.3) was basified with ammonium hydroxide solution to pH 9.3 and extracted with ether (3 × 240 ml) to obtain an ethereal solution of the C-D fractions. The aqueous layer was acidified with 1 *N* sulfuric acid to pH 7.2 and extracted with ether (3 × 260 ml) to give an organic extract (742 ml) containing Fraction E.

The daily E fractions were diluted to 500 μl in ethanol. A standard curve of Ia in ethanol was prepared using the absolute calibration technique (33). Injections of 1.0 μl of the daily E fractions were also injected onto a GLC column. The resultant peak area was measured and compared with the standard curve to give the quantity of unchanged drug. The percentage of unchanged drug after 1, 2, 3, 4, 5, and 6 days was 0.50, 0.29, 0.26, 0.03, 0.00, and 0.00, respectively, *i.e.*, a total of 1.08%. The 11.40% extraction efficiency of this procedure and the volume of ether lost on extraction were considered in these calculations.

The quantitation of unchanged drug in urine using a liquid–liquid extraction procedure was undertaken. Compound Ia (404.0000 mg) was administered to five rats at a dose of 400 mg/kg, and the urine was collected daily. A urine sample (32 ml) collected after 1 day was basified to pH 9.0 with ammonium hydroxide and extracted with ether (360 ml) for 6 hr. The ethereal layer (270 ml) was separated, and the aqueous phase was acidified with sulfuric acid to pH 7.2 and extracted with ether (200 ml) for 6 hr. The ether was separated (165 ml), and the aqueous phase was further acidified to pH 2.0; ether (200 ml) was added, the solution was extracted for 6 hr, and the organic extract (165 ml) was separated.

Quantities of each of the ether extracts (2.0 μl) were injected onto a GLC column, and the peak areas were measured. A comparison with the standard curve indicated the quantity of unchanged Ia present. A correction factor for the volume of ether lost on extraction was made. Urine samples on Days 2, 3, and 4 were extracted in a similar manner and the results are in Table II.

In a further experiment, Ia (198.2000 mg) was administered to 25 rats at a dose of 40 mg/kg. The urine was collected daily and extracted using the liquid–liquid continuous extraction procedure at pH values of 9.0, 7.2, and 2.0, as outlined previously. The results are shown in Table II.

Examination of Rat Urine and Feces after Administration of Ia—Compound Ia (148.8000 mg), suspended in hydroxypropyl methylcellulose solution, was administered to 20 male albino Wistar rats at a dose level of 40 mg/kg. The urine and feces were collected for 3 days. Ten control rats were given only hydroxypropyl methylcellulose solution.

Test and control urines (40 ml) from each of the three daily urine samples were extracted in the same manner as for the intraperitoneal route to give Fractions A, B, C-D, and E. These fractions were dissolved in ethanol and injected onto a 3% OV-101 column in volumes of 4.0 μl. Fractions A, B, and E showed similar peaks in both test and control fractions. In Fraction C-D, three extra peaks appeared with retention times of 1.68, 2.61, and 6.11 min. The retention time of Ia under these conditions was 1.67 min.

Liquid–Liquid Continuous Extraction Procedure—Samples of test urine (40 ml) from each of the three daily urine collections were adjusted with alkali or acid to pH 9.0, 7.2, and 2.0 and extracted with ether for 6 hr using the liquid–liquid continuous extraction procedure. Control urine (40 ml) was extracted in a similar manner.

The fractions were dissolved in ethanol and injected onto a GLC 3% OV-101 column at 170°. The test urine of the pH 9.0 fraction contained three additional peaks with retention times of 1.83, 2.60, and 5.21 min. Under these conditions, Ia had retention times of 2.57–2.64 min. There were two extra peaks in the test sample of the pH 7.2 urine with retention times of 1.72 and 2.63 min; the retention time of Ia was 2.66 min under identical conditions. The pH 2.0 test urine had an extra peak with a retention time of 1.76 min.

Quantitation of Unchanged Drug and/or Metabolites—A sample of the evaporated urine fractions dissolved in ethanol (2.0–4.0 μl) was injected onto a GLC column, and the peak areas of the unchanged drug and/or metabolites were compared to a standard curve of Ia constructed by the method described earlier (33). The quantities of these compounds found in the urine are summarized in Table III and account for 8.36% of the administered dose.

A quantity of test urine from the liquid–liquid extraction (pH 9.0) was placed on an inactivated silica gel 7GF plate and developed in chloroform–methanol (97:5). A black spot, R_f 0.97, was seen in the extract from the test urine, and a fluorescent blue spot, R_f 0.96, was found in an extract from the control urine. The R_f

value of Ia was 0.97, appearing as a black spot under identical conditions. The plate was sprayed with an ethanolic solution of 2,4-dinitrophenylhydrazine (34); the spots in the test urine, R_f 0.96, and from Ia turned to an orange color but the spot with an R_f value of 0.96 in the control urine remained unchanged. The plate was sprayed with aqueous ferric chloride solution (5% w/v) (35), but no spots on the chromatogram changed their color.

Feces (30.0000 g) from both test and control rats were ground with a pestle and mortar and then extracted in a soxhlet apparatus for 48 hr with ether (185 ml). The ether was then extracted as described previously and separated into five fractions. GLC analysis of Fractions A, B, and E showed similar components. The test urine of Fractions C and D had one extra peak at 2.12 and 3.78 min, respectively.

Feces (30.0000 g) from each daily collection from the test rats were extracted by the liquid–liquid extraction procedure using ether for 24 hr. The ethereal extracts were evaporated to dryness, and distilled water (75 ml) was added. The pH was adjusted to 9.0, 7.2, and 2.0, and the solution was extracted with ether (200 ml) for 6 hr. The feces from the control rats were treated in a similar manner. GLC analysis (170°) showed similar components in the pH 9.0 and 2.0 fractions. There were three extra peaks in the pH 7.2 test fraction with retention times of 1.76, 10.30, and 23.95 min.

Quantitation of Metabolites in Feces—A sample of the extract (2.0–4.0 μl) was injected onto a GLC column at 170° under the conditions previously described, and the peak areas of the three components were compared to a standard curve of Ia similar to that described previously. The quantities of compounds found are summarized in Table IV.

A quantity of test (pH 7.2) and control feces extract was placed on an inactivated silica gel 7GF plate and developed in chloroform–methanol (97:5). A black spot appeared in the test extract, R_f 0.92, and a fluorescent blue spot, R_f 0.92, was found in the control extract. The plate was sprayed with 2,4-dinitrophenylhydrazine reagent, but no orange spots appeared. The plate was then sprayed with aqueous ferric chloride solution (5% w/v). A dark-green spot appeared in the test fraction at the R_f 0.92 spot but not in the control extract.

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