Co., Easton, Pa., 1975, p. 596.
(5) "Code of Federal Regulations," U.S. Government Printing Office, Washington, D.C., Title 21, 436.204, Apr. 1, 1977 Revision, p. 241.
(6) A. G. Mathews, C. J. Schram, and D. Minty, *Nature*, **211**, 959 (1966).
(7) J. P. Hou and A. Restivo, *J. Pharm. Sci.*, **64**, 710 (1975).
(8) R. Pfeiffer, G. L. Engel, and D. Coleman, *Antimicrob. Agents Chemother.*, **9**, 848 (1976).
(9) M. J. Pikal, A. L. Lukes, and J. E. Lang, *J. Pharm. Sci.*, **66**, 1312 (1977).

Synthesis and Evaluation of Benzylfluorenyl and 1-Arylethyl Quaternary Ammonium Salts for Antimicrobial and Antineoplastic Activities

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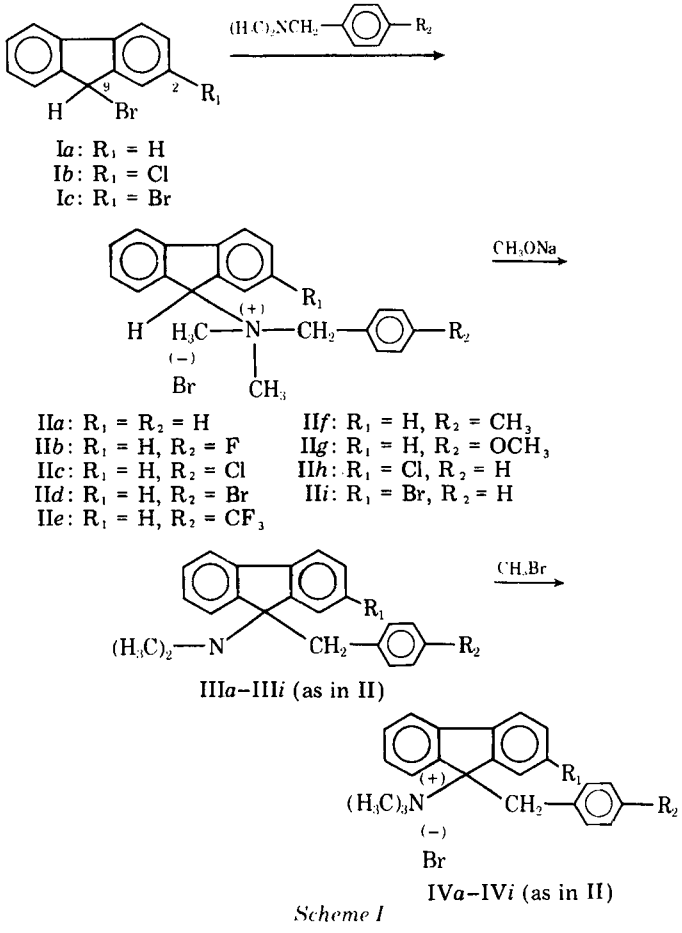
Abstract □ A number of substituted benzyldimethyl-9-fluorenylammonium bromides (II) and 9-benzylfluorenyl-9-trimethylammonium bromides (IV) were synthesized and examined for antimicrobial and anticancer activities. Series IV showed greater antimicrobial activity than Series II while some corresponding acyclic fluorene analogs were bereft of antimicrobial activities. Significant antineoplastic activity was not found in Series II and IV. Representative fluorenes subjected to a preliminary screen for various pharmacological activities revealed marked anti-inflammatory and analgesic properties coupled with some antihistaminic activities. The acyclic quaternary ammonium compounds demonstrated substantial pressor activities.

Keyphrases □ Benzylfluorene—quaternary ammonium derivatives, synthesis, antimicrobial and antineoplastic activity, structure-activity relationships □ Arylethylamines—quaternary ammonium derivatives, synthesis, antimicrobial and antineoplastic activity, structure-activity relationships □ Antimicrobial agents—benzylfluorene, arylethylamines, quaternary ammonium derivatives □ Antineoplastic agents—benzylfluorene, arylethylamines, quaternary ammonium derivatives

As a continuation of studies on new antimicrobial (1–4) and antineoplastic (5–8) agents, it was decided to examine the biological activities of some novel substituted fluorenes, representatives of which have demonstrated antimicrobial (9–11) and anticancer (12–14) activities. Since quaternary ammonium compounds are known to possess antiseptic properties, especially when bulkyl groups are attached to the quadrivalent nitrogen center, which may reduce adsorption onto serum proteins (15), the preparation of fluorenes attached to a quaternary nitrogen atom was contemplated.

The reactivity of benzylic derivatives is documented clearly (16); in the two planned structural isomer series (Scheme I), a benzyl group was attached either to a quaternary nitrogen atom (II) or directly to the fluorene ring (IV). In both cases, facile proton loss from the benzylic methylene group may occur. Furthermore, in Series II, the 9-fluorenyl proton may be considered labile. Thus, both II and IV have the potential for forming carbanions, which would be available for electrophilic attack by biological macromolecules.

In addition, it was proposed to synthesize some fluorene II and IV analogs so that structural requirements for bioactivities might be discerned. In the case of V, which is similar in structure to antifungal 9-fluorenol (9), both the benzyl and quaternary ammonium groups were removed. The quaternary ammonium compound VI was an analog of IV with the benzyl function replaced by a methyl group. Previous investigations compared the antimicrobial activities of flexible acyclic analogs of more rigid cyclic structures with the corresponding cyclic compounds (1–4). For this reason, the synthesis of several quaternary ammonium compounds derived from 1-arylethylamines (VII and VIII) was contemplated.



Scheme I

RESULTS AND DISCUSSION

The synthetic pathway for the prepared fluorenes is illustrated in Scheme I. The appropriate *N,N*-dimethylated benzylamines, prepared by the Eschwieler-Clark procedure, were quaternized with 9-bro-

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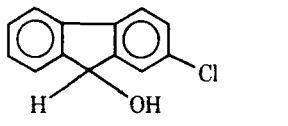
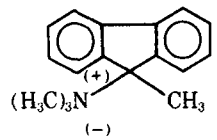
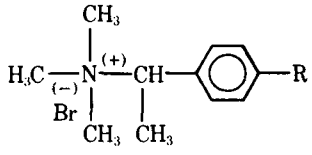


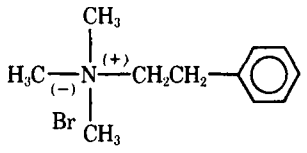
V



VI



VII*a*: R = H
VII*b*: R = Cl
VII*c*: R = CF_3
VII*d*: R = OCH_3



VIII

mofluorene (I), which was prepared by reduction of the appropriate 9-fluorenone followed by bromination. The quaternary ammonium salts II, having acidic hydrogen atoms alpha to the positively charged nitrogen atoms, underwent the Stevens rearrangement to the *N,N*-dimethyl-9-benzylfluorenylamines (III). The amines III were quaternized readily with bromomethane to produce the required salts IV.

Compound VI was prepared by a synthetic route in which methyl magnesium iodide was reacted with 9-fluorenone, and the resultant 9-fluorenol was brominated and treated with trimethylamine. The acyclic quaternary ammonium compounds VII were prepared by the action of methyl magnesium iodide on the substituted benzaldehyde, and the resultant carbinol was brominated and treated with trimethylamine.

The antimicrobial screening results are summarized in Table I. The quaternary ammonium compounds in Series II and IV showed the best activities; the tertiary amines III*a* and III*i*, the fluorenol V, and the 9-methylfluorene VI were either bereft of antimicrobial activity or possessed only marginal potencies. In Series II, with the exception of II*h*, the compounds had approximately the same activity. In Series IV, the halogenated compounds had higher potencies than the unsubstituted compound, which, in turn, was more active than IV*f* and IV*g*, which have electron-repelling groups in the phenyl ring. The average antimicrobial activity in Series IV was 175 compared to 120 in Series II.

The open chain quaternary ammonium compounds VII, which like Series II have one carbon atom separating the substituted phenyl ring from the quaternary nitrogen, were devoid of antimicrobial activity at the highest concentration examined (500 μg/ml). Furthermore, VIII, which may be regarded as an open chain IV*a* analog, was similarly devoid of antimicrobial activity.

The most susceptible bacteria to the compounds were species of *Staphylococci* and *Streptococci* as well as *Bacillus subtilis*. Approximately half of the compounds demonstrated low antifungal activity against *Microsporum gypseum* and *Saccharomyces uvarum*. With the exception of II*c*, II*d*, and IV*d*, all compounds in Table I were inactive at 10 μg/ml against the protozoan *Entamoeba histolytica*. Against *Trichomonas foetus*, the compounds listed in Table I, except II*c*, IV*e*–IV*g*, and VI, were devoid of antiprotozoal activity at the maximum concentration employed, 50 μg/ml.

All compounds listed in Table I plus III*h* were assessed for anticancer activity, except IV*e*–IV*g* and VI. The animal tumor model was the L-1210 lymphoid leukemia system in mice except for II*d* and IV*h*. In those two cases, the screen was conducted *versus* murine P-388 lymphocytic leukemia. No compounds achieved the criterion for activity, namely a 25% increase in mean survival time. The quaternary ammonium compounds II and IV showed murine toxicity at 100 mg/kg (either zero or one survivor out of six after 5 days of injections), but there were no mortalities on Day 5 when the dose was reduced to 50 mg/kg. The only exception to this observation was II*a;* in a screen *versus* Sarcoma 180 in mice, II*a* showed murine toxicity at 2 mg/kg, and there were no mortalities when the dose was reduced to 0.50 mg/kg.

The tertiary amines III*h* and III*i* showed no toxicity at 400 mg/kg, while III*a* showed marginal toxicity at 225 mg/kg (four out of six survivors on Day 5), which disappeared at 200 mg/kg. The *in vitro* KB screen conducted on II*h*, II*c*, III*h*, IV*a*, and IV*i* showed 50% inhibition of human nasopharyngeal epidermoid carcinoma at 25, 44, 100, 9.7, and 15 ppm, respectively, and thus fell short of the 4-ppm established criterion for activity.

The primary screen permitted the assessment of representative compounds for various pharmacological activities (Table II). Since the

Table I—Antimicrobial Evaluation of the Benzylfluorenyl Quaternary Ammonium Bromides [a] (II–VI)

Organism	II*a*	II*b*	II*c*	II*d*	II*h*	II*i*	III*a*	III*i*	IV*a*	IV*b*	IV*c*	IV*d*	IV*e*	IV*f*	IV*g*	IV*h*	IV*i*	V	VI
Escherichia coli (ATCC 8739)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	500	>500	>500	>500	>500	>500	>500	>500
Pseudomonas aeruginosa (ATCC 10145)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
Klebsiella pneumoniae (ATCC 4352)	500	250	>500	>500	250	250	>500	>500	>500	>500	250	500	250	>500	>500	>500	500	>500	>500
Salmonella typhimurium (ATCC 13311)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	500	500	>500	>500	>500	>500	>500	>500	>500
Haemophilus influenzae (ATCC 19418)	500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	500	>500	>500	>500	>500	500	>500	>500
Staphylococcus aureus (ATCC 6538)	250	100	100	100	250	250	>500	>500	100	50	100	50	50	100	100	50	50	>500	500
Streptococcus pyogenes (hospital isolate)	250	250	100	100	250	100	>500	>500	250	250	100	50	100	500	500	250	50	>500	>500
Streptococcus pneumoniae (ATCC 6303)	250	250	250	250	250	250	>500	>500	>500	250	100	100	100	500	500	250	50	>500	>500
Streptococcus faecalis (ATCC 8030)	250	250	500	250	500	250	>500	>500	>500	500	100	250	250	>500	>500	250	100	>500	>500
Bacillus subtilis (ATCC 6633)	500	250	250	250	500	250	>500	>500	500	250	50	50	50	250	250	250	50	>500	>500
Trichophyton mentagrophytes (ATCC 9533)	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	250	>500	>500	>500	>500	>500	>500	>500
Microsporum gypseum (ATCC 14683)	>500	>500	250	250	>500	250	500	>500	500	>500	500	100	>500	>500	>500	250	>500	>500	>500
Aspergillus niger (ATCC 10535)	>500	>500	500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
Candida albicans (ATCC 10231)	>500	>500	500	>500	>500	500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
Saccharomyces uvarum (ATCC 9080)	100	>500	100	500	>500	250	100	>500	500	>500	250	>500	>500	>500	>500	>500	250	>500	>500
Average antimicrobial activity[b]	107	100	160	127	67	160	40	0	67	113	267	320	227	60	60	133	327	0	7

[a] The figures in the table are the minimum inhibitory concentrations of the compounds in micrograms per milliliter. [b] Figures were calculated from the following expression: (combined antimicrobial activity × 100)/number of microorganisms in the screen. The combined antimicrobial activity was determined by giving the following scores at the lowest concentration of the compound required to inhibit microbial growth: 500: 1; 250: 2; 100: 5; and 50: 10.

Table II—Screening Data on Benzylfluorenyl and 1-Arylethyl Quaternary Ammonium Bromides [a] (IV, VI, VII, and VIII)

Compound	Analgesic Activity		Anti-Inflammatory Activity			Antianaphylactic Activity		Antihistaminic Activity		Antidepressant Activity			Cardiovascular Screen [b]		
		Reduction in Writhes,		Effect, %			Inhibition,		Change in Contractile		Protection, %			Change in Blood Pressure,	Change in Heart Rate,
	Dose	%	Dose	3 hr	5 hr	Dose	%	Dose	Force, %	Dose	1.5 hr	3 hr	Dose	%	%
IV*a*	64	78	64	67	83	2[d]	AN	0.1	38	64	67	50	0.1	−7	+7
	32	100											1.0	−31	+10
	16	22											2.0	−42	+10
	8[c]	Algesic													
IV*b*	64	13	64	17	83	2	0	0.1	44	64	21	0	0.1	−7	+2
													1.0	−31	0
													2.0	−52	+4
IV*e*	64	66	64	67	100	4	0	0.1	38	64	38	17	0.1	−22	+3
	32	82											1.0	−12	+1
													2.0	−5	−3
IV*f*	32[c]	Algesic	64	67	83	1	28	0.1	20	32	29	0	0.1	−1	+6
													1.0	−13	+18
													2.0	−20	+15
IV*g*	64	87	64	50	100	2[d]	AN	0.1	24	64	29	4	0.1	−5	+3
													1.0	+8	+8
													2.0	0	+12
VI	8	27	16	0	0	0.5	0	0.1	48	16	21	0	0.1	−21	−3
													1.0	−26	+4
													2.0	−17	+7
VII*a*	16	66	16	0	0	0.5	20	0.1	38	16	0	0	0.1	+14	+4
													1.0	+44	+9
													2.0	+64	+16
VII*b*	16	18	16	0	0	1	20	0.1	22	16	13	4	0.1	+32	+5
													1.0	+50	+6
													2.0	+80	+19
VII*c*	32	69	32	0	17	2	67	0.1	16	16	17	0	0.1	+49	+5
													1.0	+79	+21
													2.0	Respiratory arrest	
VII*d*	16	43	16	0	0	1	20	0.1[e]	Stimulatory effect	16	4	0	0.1	+31	+16
													1.0	+8	+12
													2.0	Respiratory arrest	
VIII	16	5	16	0	50	1	0	0.1	11	16	17	0	0.1	+14	+4
						2	0						1.0	+44	+9
													2.0	+86	+5
Reference compound[f]	50	50	32	17	83	100	91	0.01	100	8	100	100		+30[g]	+30[g]

[a] The doses in all screens are in milligrams per kilogram except for the antihistaminic screen in which the dose is in milligrams per milliliter. [b] The + indicates an increase in blood pressure or heart rate, and the − indicates a fall in either blood pressure or heart rate. [c] At these dose levels, IV*a* and IV*f* increased the number of writhes by 22 and 19%, respectively. [d] Compounds IV*a* and IV*g* increased the wheal formation by 20% in both compounds at the dose levels indicated. [e] Compound VII*d* increased the contractile force of the isolated guinea pig ileum preparation by 6%. [f] The reference compounds for the analgesic, anti-inflammatory, antianaphylactic, antihistaminic, and antidepressant screens were aspirin, indomethacin, disodium chromoglycate, diphenhydramine, and tranylcypromine sulfate, respectively. The criteria for activity in the analgesic, anti-inflammatory, antianaphylactic, and antihistaminic screens were changes of 50, 50, 50, and 20%, respectively; in the antidepressant screen, a compound had to show 30% protection at the end of 1.5 hr and 10% protection after 3 hr for it to be considered active. [g] In addition to increasing the blood pressure and heart rate by 30%, a compound should exert its action considerably in excess of 5 min.

compounds were screened at various dose levels and ED_{50} values were not obtained, a potency comparison was not possible. However, the fluorenes that demonstrated analgesia had marked anti-inflammatory activities, with the exception of VI. Antihistaminic potency as well as a small, but perceptible, heart rate elevation coupled with a decrease in blood pressure was found with the fluorenes. The cardiovascular effects were generally of less than 5-min duration, and in no case was action over 10 min observed. No noteworthy effects were seen by the fluorenes in the antianaphylactic and antidepressant screens.

The acyclic quaternary ammonium compounds VII and VIII were subjected to the primary screens. Analgesic activity was found with VII*a* and VII*c* but, in contrast to the fluorenes, there was an absence of anti-inflammatory activity. The cardiovascular screen showed that all compounds had marked pressor activity; VII*c* showed the maximum activity at the 1.0-mg/kg dose level. The duration of action was generally less than 5 min, and none of the compounds displayed an effect on blood pressure or heart rate for greater than 10 min. No compounds in Table II displayed hypoglycemic activity at 100 mg/kg.

In conclusion, the study showed that some fluorenes with a quaternary ammonium group at position 9 have antibacterial activities against three species of *Streptococcus* and also against *Staphylococcus aureus* and *B. subtilis*, coupled with moderate potency against two fungi. Some related open chain analogs displayed a complete absence of antimicrobial activity. While several useful anticancer drugs were chemically reactive, the quaternary ammonium compounds II and IV displayed murine toxicity but did not produce noteworthy increased mean survival times in mice with leukemia. Preliminary pharmacological tests showed anti-inflammatory analgesia as well as some effect in the cardiovascular screen to be present with a number of the fluorenes in contrast to the open chain analogs VII and VIII, which were devoid of anti-inflammatory and analgesic properties but had potent pressor activities.

EXPERIMENTAL[1]

Melting points and boiling points are uncorrected. Organic extracts were washed with water and dried over anhydrous sodium sulfate. The solvent was removed using a water aspirator. The 60-Hz NMR spectra[2] were determined in deuterochloroform with tetramethylsilane as the internal standard.

Substituted 9-Bromofluorenes (I) and 2-Chloro-9-fluorenol (V)—9-Bromofluorene[3] (I*a*) was used without further purification. 9-Bromo-2-chlorofluorene (I*b*) was prepared as follows. Reaction of 9-fluorenone[3] with *N*-chlorosuccinimide according to a literature method (17) gave 2-chloro-9-fluorenone in a 44% yield, mp 121–122° [lit. (17) mp 122°], which was reduced with lithium aluminum hydride by a reported procedure (18) to give 2-chloro-9-fluorenol (V) in a 50% yield, mp 140–141° [lit. (18) mp 140–141°]. Treatment of V with acetyl bromide using a literature method (19) gave I*b* in a 57% yield, mp 125–127° [lit. (19) mp 126–128°].

[1] Elemental analyses were carried out by Mr. R. E. Teed, Department of Chemistry and Chemical Engineering, University of Saskatchewan, and by Galbraith Laboratories Inc., Knoxville, Tenn.

[2] Varian T 60 spectrophotometer, Varian Associates of Canada Ltd., Georgetown, Ontario, L7G 2J4, Canada.

[3] Aldrich Chemical Co., Milwaukee, WI 53233.

2,9-Dibromofluorene (Ic) was prepared in an 80% yield by the reaction of 2-bromofluorene[3] with *N*-bromosuccinimide using a reported procedure (20). It had a melting point of 127–128° [lit. (20) mp 127°].

Benzyldimethyl-9-fluorenyl Ammonium Bromides (II)—The benzyldimethylamines were prepared as follows. Four of the required substituted benzylamines were treated with formic acid and formalin using a literature method (21) to give *p*-fluorobenzyldimethylamine in an 80% yield, bp 50–51°/2 mm [lit. (22) bp 61–62°/6.5 mm], *p*-chlorobenzyldimethylamine in a 79% yield, bp 100–105°/12 mm [lit. (23) bp 90–95°/9–11 mm], *p*-methylbenzyldimethylamine in an 82% yield, bp 197–198°/750 mm [lit. (23) bp 196–197°/760 mm], and *p*-methoxybenzyldimethylamine in an 80% yield, bp 104–106°/12 mm [lit. (23) bp 105–119°/10 mm].

p-Trifluoromethylbenzyl bromide, bp 93–94°/20 mm [lit. (24) bp 65–66°/5 mm], was prepared in a 61% yield from *p*-trifluoromethyl benzoic acid[3] using a literature synthetic sequence (24). A solution of *p*-trifluoromethylbenzyl bromide (0.05 mole) and anhydrous dimethylamine (0.10 mole) in nitromethane (50 ml) stood at room temperature for 4 hr and then was poured into water (100 ml). The reaction mixture was extracted with ether (2 × 50 ml), and removal of the solvent *in vacuo* gave an oil. On distillation, this oil afforded *p*-trifluoromethylbenzyldimethylamine, bp 107–110°/61 mm, in a 68% yield; the NMR spectrum was in accordance with the proposed structure. *p*-Bromobenzyldimethylamine, bp 130–132°/22 mm [lit. (25) bp 121–122°/20 mm], was prepared similarly from *p*-bromobenzyl bromide[3]. Benzyldimethylamine was obtained commercially[3].

A 0.1 *M* solution of Ia or of the 2-substituted bromofluorenes (Ib or Ic) in nitromethane (10 ml) was added to the appropriate benzyldimethylamine (0.2 mole), and the mixture was stirred at room temperature overnight. The product was precipitated by ether and purified by recrystallization from ether–ethanol. The melting points and yields of the benzyldimethyl-9-fluorenyl ammonium bromides were: II*a*, 154–156° [lit. (20) mp 155–156°], 88%; II*b*, 148–150°, 82%; II*c*, 153°, 84%; II*d*, 148°, 80%; II*e*, 153–154°, 85%; II*f*, 158°, 60%; II*h*, 149°, 50%; and II*i*, 155–156°, 85%. NMR spectra of the benzyldimethyl-9-fluorenyl ammonium bromides were consistent with the structures proposed. In the case of II*g*, ether failed to precipitate the compound from the reaction mixture and it was extracted with water (20 ml). The water was removed to give the crude product, which was not purified further.

9-Benzylfluorenyl-9-dimethylamines (III)—The appropriate benzyldimethyl-9-fluorenyl ammonium bromide (2.0 g) was dissolved in sodium methoxide solution, which was prepared by dissolving sodium (0.5 g) in methanol (7 ml). The reaction mixture stood at room temperature overnight with occasional shaking, and then water (100 ml) was added. The reaction mixture was extracted with ether (60 ml), and removal of the ether gave the crude amine III. With III*a*, III*b*, III*i*, and III*h*, the compounds were recrystallized from nitromethane unless otherwise stated.

9-Benzylfluorenyl-9-dimethylamine (III*a*) was prepared in an 81% yield, mp 98–99° [lit. (26) mp 98.5–99°]. The NMR spectrum of III*a* was consistent with the proposed structure. 9-(*p*-Fluorobenzyl)-fluorenyl-9-dimethylamine (III*b*), mp 55°, was recrystallized from petroleum ether (bp 100°).

Anal.—Calc. for $C_{22}H_{20}FN$: C, 83.24; H, 6.35; N, 4.41. Found: C, 84.00; H, 6.43; N, 4.38.

2-Chloro-9-benzylfluorenyl-9-dimethylamine (III*h*) melted at 108–109°.

Anal.—Calc. for $C_{22}H_{20}ClN$: C, 79.15; H, 6.04; N, 4.20. Found: C, 78.48; H, 6.00; N, 4.19.

2-Bromo-9-benzylfluorenyl-9-dimethylamine (III*i*) melted at 129°.

Anal.—Calc. for $C_{22}H_{20}BrN$: C, 69.84; H, 5.33; N, 3.70. Found: C, 69.71; H, 5.17; N, 3.63.

The remaining derivatives (III*c*–III*g*) were isolated as oils that could not be induced to crystallize. TLC of the products on silica gel with benzene as the solvent showed a single spot, and further purification was not undertaken.

9-Benzylfluorenyl-9-trimethylammonium Bromides (IV)—The 9-benzylfluorenyl-9-dimethylamine (0.01 mole) was dissolved in nitromethane (10 ml) containing bromomethane (0.065 mole), and the resultant solution stood at room temperature overnight. The product, obtained from the reaction mixture by the addition of ether, was recrystallized from ether–ethanol. The physical data for the salts (IV) are summarized in Table III.

9-Methylfluorenyl-9-trimethylammonium Bromide (VI)—A solution of 9-fluorenone[3] (0.035 mole) in anhydrous ether (100 ml) was added slowly to methyl magnesium iodide (0.035 mole), prepared from magnesium (0.85 g) and methyl iodide (5.0 g) in anhydrous ether (50 ml).

Table III—9-Benzylfluorenyl-9-trimethylammonium Bromides (IV)

Compound	Yield, %	Melting Point	Formula		Analysis, % Calc.	Found
IV*a*	80	178°[a]	$C_{23}H_{24}BrN$	C	70.05	70.21
				H	6.13	6.05
				N	3.55	3.55
IV*b*	80	179°	$C_{23}H_{23}BrFN$	C	66.99	66.61
				H	5.62	5.77
				N	3.40	3.38
IV*c*	80	172°	$C_{23}H_{23}BrClN$	C	64.42	64.36
				H	5.40	5.32
				N	3.27	3.25
IV*d*	81	192°	$C_{23}H_{23}Br_2N$	C	58.37	58.05
				H	4.90	4.70
				N	2.96	2.84
IV*e*	85	174°	$C_{24}H_{23}BrF_3N$	C	62.34	62.34
				H	5.01	5.00
				N	3.03	3.00
IV*f*	77	178°	$C_{24}H_{26}BrN$	C	70.58	70.63
				H	6.42	6.35
				N	3.43	3.25
IV*g*	72	175°	$C_{24}H_{26}BrNO$	C	67.92	67.88
				H	6.18	6.01
				N	3.30	3.25
IV*h*	61	180°	$C_{23}H_{23}BrClO$	C	64.42	64.19
				H	5.40	5.63
				N	3.27	3.25
IV*i*	66	190°	$C_{23}H_{23}Br_2N$	C	58.37	58.13
				H	4.90	5.03
				N	2.96	2.94

[a] Lit. (26) mp 175°.

The resultant mixture stood at room temperature overnight and then was poured onto a mixture of crushed ice (100 g) and 10% HCl (100 ml). The aqueous phase was extracted with ether. Solvent evaporation gave the crude product, which on recrystallization from benzene gave 9-methyl-9-fluorenol in a 58% yield, mp 176° [lit. (27) mp 176–177°].

Acetyl bromide (0.035 mole) was added to a solution of 9-methyl-9-fluorenol (0.025 mole) in chloroform (20 ml), and the reaction mixture was shaken. After the vigorous reaction had subsided, acetic acid and chloroform were removed under reduced pressure. The crude bromide was dissolved in nitromethane (20 ml) and added to a solution of trimethylamine in nitromethane (10%). The reaction mixture was kept at 0° for 2 days, and the separated product was recrystallized from absolute ethanol to give VI in a 20% yield, mp 170°.

Anal.—Calc. for $C_{17}H_{20}BrN$: C, 64.15; H, 6.33; N, 4.40. Found: C, 63.63; H, 6.55; N, 4.40.

1-Phenylethyltrimethylammonium Bromides (VII and VIII)—The following three-step synthesis was used for VII. A solution of the appropriate *para*-substituted benzaldehyde (0.03 mole) in anhydrous ether (25 ml) was added slowly to methyl magnesium iodide (0.035 mole), prepared from magnesium (1.0 g) and methyl iodide (5.0 g) in anhydrous ether (50 ml). The reaction mixture stood at room temperature overnight and, after being poured onto a mixture of crushed ice (100 g) and hydrochloric acid (10% v/v, 100 ml), was then extracted with ether. The solvent was removed and the required 1-phenylethanols were purified by distillation to give the *p*-chloro derivative in an 88% yield, bp 130–132°/15 mm [lit. (28) bp 110–120°/9 mm], the *p*-trifluoromethyl derivative in a 90% yield, bp 116–118°/20 mm [lit. (29) bp 106–107°/18 mm], and the *p*-methoxy derivative in an 85% yield, bp 130–132°/20 mm [lit. (30) bp 104°/3 mm].

Acetyl bromide (0.034 mole) was added quickly to the appropriate 1-phenylethanol (0.025 mole). After the vigorous reaction had subsided, acetic acid and acetyl bromide excess were removed under reduced pressure. The residue was dissolved in benzene (50 ml) and shaken with aqueous sodium bicarbonate solution (10% w/v). Solvent removal gave the crude 1-phenylethyl bromide as a pale-red oil. A crude bromide solution (0.016 mole) in nitromethane (10 ml) was added to a trimethylamine solution in nitromethane (10% w/v, 20 ml) at room temperature, and the reaction mixture was left overnight. Ether addition gave the required 1-phenylethyltrimethylammonium bromides, which were recrystallized from ether–ethanol. 1-Phenylethyltrimethylammonium bromide (VII*a*), mp 197–198° [lit. (31) mp 198–200°], was obtained in a 60% yield. 1-(*p*-Chlorophenyl)ethyltrimethylammonium bromide (VII*b*), mp 199°, was obtained in an 80% yield.

Anal.—Calc. for $C_{11}H_{17}BrClN$: C, 47.41; H, 6.15; N, 5.03. Found: C, 47.78; H, 6.53; N, 4.91.

1-(*p*-Methoxyphenyl)ethyltrimethylammonium bromide (VII*d*), mp 165–166°, was prepared in a 60% yield.

Anal.—Calc. for $C_{12}H_{20}BrNO$: C, 52.56; H, 7.35; N, 5.11. Found: C, 52.80; H, 7.58; N, 5.10.

1-(*p*-Trifluoromethylphenyl)ethyltrimethylammonium bromide (VII*c*) was prepared as follows. 1-(*p*-Trifluoromethylphenyl)ethanol (0.067 mole) was added to phosphorus bromide (0.067 mole). After standing at room temperature overnight, the reaction mixture was poured onto ice and extracted with benzene. The organic extracts were washed with aqueous sodium bicarbonate solution (10% w/v), and solvent removal gave the crude bromide. A solution of the crude bromide (3.2 g) in methanol (10 ml) was added to a triethylamine solution in absolute alcohol (10% v/v, 20 ml) to yield VII*c*, mp 218–219°, in a 58% yield.

Anal.—Calc. for $C_{12}H_{17}BrF_3N$: C, 46.17; H, 5.49; N, 4.49. Found: C, 45.47; H, 5.48; N, 4.48.

2-Phenylethyltrimethylammonium bromide (VIII) was prepared by the method described previously (32).

Screening[4] of Compounds—In the antimicrobial screen, the compounds were dissolved in water or dimethyl sulfoxide and diluted serially to various concentrations. The stock solutions were prepared in such a way that when 0.5 ml was added to 15 ml of agar, the desired final concentrations were obtained. The bacterial growth medium was trypticase soy agar with 5% defibrinated rabbit blood added for the growth of *Haemophilus influenzae*, *Streptococcus pyogenes*, and *Streptococcus pneumonia*. Modified Sabouraud agar was employed for the fungi.

The test organisms were grown previously for 2 days at 35° for bacteria and yeasts and for 1 week at 24° for fungi on slants of the media mentioned above. The agar plates were streaked with a loopful of cell suspension, which had been washed off from the slants and diluted to approximately 10^5 organisms/ml. The plates were incubated for 2–14 days at 24° for fungi and at 35° for bacteria. The results (Table I) indicated the minimal inhibitory concentration of the compound that prevented visible growth in the media.

The antiprotozoal screen utilized the tube dilution technique previously described (33). The test organisms were *E. histolytica* (ATCC 30015) grown in a TP-S-1 monophasic medium devised by Diamond (34) and *T. foetus* (ATCC 30003) grown in modified TYM basal media (34). Activity was defined as the minimal concentration that inhibited 90% of the protozoal growth at 10 and 50 μg/ml for *E. histolytica* and *T. foetus*, respectively.

Unless otherwise stated, the same numbers of animals were used as controls and for the evaluation of various pharmacological activities. The screen for examining analgesic activity was the phenylquinone writhing test (35). Five male Swiss albino mice, 18–22 g, were used for each compound except IV*g*, IV*e* (at the 64-mg/kg dose level), VII*a*, and VII*c*, in which cases 10 animals were used.

The anti-inflammatory screen measured the antagonism of the test compound to carrageenan-induced rat paw edema (36). Six female Sprague–Dawley rats, 120–160 g, were used for each compound, and the edema volume was recorded at the end of 3 and 5 hr.

The antianaphylactic screen measured passive cutaneous anaphylaxis inhibition in rats (37). Four female Sprague–Dawley rats were used for each compound except IV*f*, VII*c*, and disodium chromoglycate, in which cases eight rats were employed. Two rats were used for VIII at both dose levels.

The compounds were examined for antihistaminic activity using two guinea pig ileum preparations (38), except in the cases of IV*a*, IV*b*, and IV*f* where three preparations were used and VII*d* where one preparation was used.

The antidepressant screen measured the antagonism of tetrabenazine-induced ptosis in mice (39, 40). Six male Swiss albino mice were used for each compound.

In the cardiovascular screen, a Sprague–Dawley rat of either sex, 250–400 g, was anesthetized by intraperitoneal urethan (1.9 g/kg). After femoral vein cannulation, the blood pressure was monitored *via* a pressure transducer[5] connected to a cannula in the left carotid artery. The arterial blood pressure and heart rate were recorded on a polygraph[6]. After the preparation had stabilized, the test compound was dissolved in saline and/or polysorbate 80[7] (1%) and administered in a volume of 0.01–0.05 ml. The changes recorded in blood pressure and heart rate were expressed as percentage differences between the pre- and postdrug values. Two determinations at each dose level using one rat were made for each compound.

The screen for hypoglycemic activity involved the measurement of blood glucose in rats (41). Four male Sprague–Dawley rats were used for each compound at a dose of 100 mg/kg. A 20% decrease in blood glucose concentration was required for a compound to be considered active.

The anticancer screening was carried out by the Drug Research and Development Division of the National Cancer Institute according to their protocols (42). The compounds were administered to $B_6D_2F_1$ or CD_2F_1 mice by intraperitoneal injection either daily or every 4 days, except in the case of II*a versus* Sarcoma 180 in Swiss mice where injections were made twice daily for a total of 14 injections. The compound showed no activity.

[4] All screens, except for the assessment for anticancer activities, were carried out by Bio-Research Laboratories Ltd., Montreal, Quebec, Canada.

[5] Statham.

[6] Gass.

[7] Tween 80, Atlas Chemical Industries, Wilmington, DE 19899.

REFERENCES

(1) J. R. Dimmock, D. K. Kowal, W. A. Turner, P. J. Smith, L. M. Noble, and W. J. Pannekoek, *J. Pharm. Sci.*, **67**, 401 (1978).

(2) J. R. Dimmock, A. M. Qureshi, L. M. Noble, P. J. Smith, and H. A. Baker, *ibid.*, **65**, 38 (1976).

(3) J. R. Dimmock, W. A. Turner, and H. A. Baker, *ibid.*, **64**, 995 (1975).

(4) J. R. Dimmock and W. A. Turner, *Can. J. Pharm. Sci.*, **9**, 33 (1974).

(5) J. R. Dimmock, P. J. Smith, L. M. Noble, and W. J. Pannekoek, *J. Pharm. Sci.*, **67**, 1536 (1978).

(6) A. J. Repta, J. R. Dimmock, B. Kreilgard, and J. J. Kaminski, *ibid.*, **66**, 1501 (1977).

(7) J. R. Dimmock, N. W. Hamon, K. W. Hindmarsh, A. P. Sellar, W. A. Turner, G. H. Rank, and A. J. Robertson, *ibid.*, **65**, 538 (1976).

(8) J. R. Dimmock, G. B. Baker, and R. G. Sutherland, *Can. J. Pharm. Sci.*, **10**, 53 (1975).

(9) F. Gorssmann, H. Buchenauer, and B. Jank, *Tagungsber, Dtsch. Akad. Landwirtschafswiss. Berlin*, **1971**, 173; through *Chem. Abstr.*, **77**, 44161x (1972).

(10) S. Horiguchi, I. Takeda, M. Mizuno, and T. Sugawara, Japan. Kokai 7,461,332, (June 14, 1974); through *Chem. Abstr.*, **82**, 120059r (1975).

(11) H. S. Lowrie, U.S. pat. 3,843,657 (Oct. 22, 1974); through *Chem. Abstr.*, **82**, 125178p (1975).

(12) S. T. Yancey and W. A. Bleyer, *Cancer Res.*, **34**, 1866 (1974).

(13) A. R. Rhoads, W. L. West, and H. P. Morris, *Res. Commun. Chem. Pathol. Pharmacol.*, **6**, 741 (1973).

(14) M. W. Rana, H. Pinkerton, and A. Rankin, *Proc. Soc. Exp. Biol. Med.*, **150**, 32 (1975).

(15) A. Albert, "Selective Toxicity," 5th ed., Chapman and Hall, London, England, 1973, p. 314.

(16) J. Packer and J. Vaughan, "A Modern Approach to Organic Chemistry," Oxford, London, England, 1958, p. 594.

(17) F. Dewhurst and P. K. J. Shah, *J. Chem. Soc., C*, **1970**, 1737.

(18) G. G. Smith and R. P. Bayer, *Tetrahedron*, **18**, 323 (1962).

(19) D. Bethell, A. F. Cockerill, and O. B. Frankham, *J. Chem. Soc., B*, **1967**, 1287.

(20) J. D. Dickenson and C. Eaborn, *J. Chem. Soc.*, **1959**, 2337.

(21) R. N. Inke, B. B. Wisegarver, and G. A. Alles, in "Organic Syntheses," coll. vol. III, E. C. Horning, Ed., Wiley, New York, N.Y., 1955, p. 723.

(22) F. N. Jones and C. R. Hauser, *J. Org. Chem.*, **27**, 4020 (1962).

(23) E. L. Eliel, T. N. Ferdinand, and M. E. Herrman, *ibid.*, **19**, 1693 (1954).

(24) H. B. Haas and M. L. Bender, *J. Am. Chem. Soc.*, **71**, 1767 (1949).

(25) K. C. Dewkirst and D. J. Cram, *ibid.*, **80**, 3115 (1958).

(26) G. Wittig and G. Felletschin, *Ann.*, **555**, 133 (1944).

(27) V. V. Korshak and N. G. Matveeva, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, **1953**, 542; through *Chem. Abstr.*, **48**, 9918h (1954).

(28) E. Matsui, *J. Soc. Chem. Ind. Jpn.*, **45**, 412 (1942).

(29) E. T. McBee, S. Resconich, L. R. Belohlov, and H. P. Braendlin, *J. Org. Chem.*, **28**, 3579 (1963).

(30) J. Grundy, *J. Chem. Soc.*, **1957**, 5087.

(31) G. S. Dyson, Ph.D. thesis, University of Saskatchewan, Saskatoon, Canada, 1975, p. 127.

(32) P. J. Smith and A. N. Bourns, *Can. J. Chem.*, **52**, 749 (1974).

(33) L. S. Diamond and I. L. Burtgis, *Arch. Invest. Med., Suppl.*, **2** (1971).

(34) L. S. Diamond, *J. Parisitol.*, **54**, 1047 (1968).

(35) H. O. Collier, L. C. Dinneen, C. A. Johnson, and C. Schneider, *Br.*

J. Pharmacol. Chemother., **32**, 295 (1968).
(36) C. A. Winter, in "International Symposium on Non-Steroidal Anti-Inflammatory Drugs," S. Garattini and M. N. G. Dukes, Eds., Excerpta Medica Foundation, Amsterdam, The Netherlands, 1965, pp. 190–202.
(37) J. Goose and A. M. J. N. Blair, *Immunology*, **16**, 749 (1969).
(38) W. L. M. Perry, "Pharmacological Experiments on Isolated Preparations," Livingstone, Edinburgh, Scotland, 1968, pp. 58–68.
(39) B. Rubin, H. H. Malone, M. H. Waugh, and J. C. Burke, *J. Pharmacol. Exp. Ther.*, **120**, 125 (1957).
(40) A. Barnett and R. T. Taber, in "Screening Methods in Pharmacology," vol. II, P. A. Turner and P. Hebborn, Eds., Academic, New York, N.Y., 1971, pp. 203–208.
(41) G. F. Holland, D. A. Jaeger, R. L. Wagner, G. D. Laubach, W. M. McLamore, and S. Y. P'an, *J. Med. Pharm. Chem.*, **3**, 99 (1961).
(42) R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacker, and B. J. Abbott, *Cancer Chemother. Rep. (Part 3)*, **3** (2) (Sept. 1972).

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Separation and Determination of Testosterone and Testosterone Esters in Selected Pharmaceutical Formulations

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Abstract □ A rapid quantitative procedure is presented for the separation of testosterone esters from their hydrolysis products through the use of the acetonitrile–infusorial earth column. The method was applied to testosterone cypionate, testosterone enanthate, and testosterone propionate. Recovery and replication of reference standard testosterone and its three esters through the proposed method ranged from 99.1 to 100.3%, and the percent relative standard deviation ranged from 0.6 to 1.0%. Two samples can be separated into testosterone and testosterone ester fractions in about 1.5 hr. The analyses of 20 injectable and one buccal tablet formulations made by 12 different manufacturers are reported.

Keyphrases □ Chromatography, column—analysis, testosterone and testosterone esters, various pharmaceuticals, acetonitrile–infusorial earth column □ Testosterone—analysis, acetonitrile–infusorial earth column chromatography, separation from testosterone esters, various pharmaceuticals □ Testosterone esters—analysis, acetonitrile–infusorial earth column chromatography, separation from testosterone esters, various pharmaceuticals

The "American Drug Index" (1) lists 57 different drug products that contain testosterone esters; they are mostly injectables and are made by 27 different manufacturers. The active ingredients are testosterone cypionate in 11 formulations, testosterone enanthate in 34, testosterone ketolaurate in one, and testosterone propionate in 11.

The official assay procedure in the USP (2) for testosterone drug substance and injection requires GLC analysis. Cleanup of testosterone propionate oil injectables by reversed-phase chromatography has been described (3), and this method is the basis for the modified cleanup procedure in USP XIX (4) for testosterone cypionate and testosterone propionate injectables. These USP procedures measure total testosterone plus esters. The final determinative step for these two compounds is spectrophotometric measurement of their isoniazid hydrazones, a procedure that is slightly altered from that originally described (5).

The USP–NF (6) presents a high-pressure liquid chromatographic (HPLC) method for testosterone propionate tablets. Other steroid separation methods include column adsorption chromatography (7), GLC (8, 9), TLC (10), paper chromatography (11), HPLC (4), column partition chromatography (12), and use of an acetonitrile–infusorial earth column (13).

This paper reports a relatively rapid quantitative analytical procedure for the separation of testosterone from its cypionate, enanthate, and propionate esters using an acetonitrile–infusorial earth column. The final determinative step compares the standard and sample isoniazid hydrazones. The proposed procedure was applied to the analysis of 21 pharmaceutical formulations made by 12 different manufacturers.

EXPERIMENTAL

Apparatus—The following were used: UV–visible recording spectrophotometers[1] with 1-cm stoppered quartz cells, a high-pressure liquid chromatograph[2] (sensitivity of 0.005 absorbance unit full scale with a 254-nm UV detector[3] and a reversed phase column[4]), glass chromatographic columns for partition chromatography (2.2 × 25 cm, constricted at one end to 0.4 × 5 cm), an aluminum tamping rod, an electrobalance[5], and TLC[6] equipment. Volumetric flasks and pipets were either NBS calibrated or Class A glassware.

Materials—Alcohol USP, distilled-in-glass grade[7] acetonitrile, chloroform, *n*-heptane, absolute methanol, and reagent grade acetic acid were used along with acid-washed infusorial earth[8]. Also used were USP reference standard testosterone cypionate, testosterone enanthate, and testosterone propionate and NF reference standard testosterone.

Reagents—Mutually saturated acetonitrile–*n*-heptane was prepared as follows. Acetonitrile, 30 ml, was mixed with 500 ml of *n*-heptane (sufficient for two determinations) in a separator, agitated vigorously for 2 min, and allowed to stand until both layers were clear. These mutually saturated solutions were used whenever acetonitrile or *n*-heptane was called for in these directions.

Sample Preparation—*Tablets*—Twenty tablets were weighed,

[1] Cary models 15 and 17.
[2] Waters model 6000 pump.
[3] Waters 440 detector.
[4] Separations Group, Vidac TP.
[5] Cahn models G-2 and 25.
[6] TLC plates 6060, Eastman Organic Chemicals.
[7] Burdick & Jackson.
[8] Celite 545, Johns-Manville Product Corp.

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