Imidazoles V

5(or 4)-(3-Alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides

By Y. FULMER SHEALY, CHARLES A. KRAUTH*, SARAH J. CLAYTON, ANITA T. SHORTNACY, and W. RUSSELL LASTER, Jr.

Several 5(or 4)-(3-alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides (III–X) were prepared from 5-diazoimidazole-4-carboxamide and the appropriate *N*-alkyl methylamine. Most of these derivatives significantly increased the life span of leukemic (L1210) mice. Inhibition of the growth of sarcoma 180, adenocarcinoma 755, and Walker carcinosarcoma 256 was observed, but inhibition of solid tumors was generally accompanied by large, adverse, host-weight changes. Qualitative observations of the sensitivity to light of aqueous alcoholic solutions of the dimethyltriazeno (NSC-45388) and the butylmethyltriazeno derivatives indicate a pronounced variation in stability with the kind of light exposure.

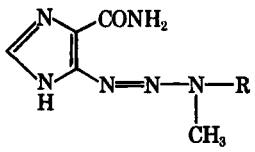
REPRESENTATIVES OF BOTH disubstituted-triazeno and monosubstituted triazeno imidazole-4(or 5)-carboxamides have been previously reported (1, 2), and the modes of dissociation of the simpler members of these two groups have been shown to differ. The substituents on the triazeno group included straight-chain alkyl, cyclic alkyl, aralkyl, aryl, and functionally substituted alkyl and aryl groups, and the number of carbon atoms in the straight-chain groups was doubled in a few representatives in order to perform a preliminary sampling of alkyl groups for antineoplastic effectiveness. Among the disubstituted-triazeno derivatives, 5(or 4)-(3,3-dimethyl-1-triazeno)imidazole-4(or 5)-carboxamide (I, NSC-45388) demonstrated the most interesting activity against mouse neoplasms (3) and is currently undergoing clinical trial (4, 5). Likewise, the monomethyltriazeno derivative (II), despite its instability, was the most effective of the monosubstituted triazeno derivatives in initial evaluations (2). The methyl ester corresponding to I (6) and the *v*-triazole analog of I (7) were also inhibitory to transplanted mouse neoplasms, and among some phenyltriazenes, association of activity with the presence of a methyl group had also been noted (8, 9). For these reasons several 5(or 4)-(3-alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides (III–X) were prepared and are recorded in this report. Relevant to the structure-activity question, however, is the fact that the bis(2-chloroethyl)triazeno derivative (10, 11), prepared subsequently to these compounds, is the most active of the triazenoimidazoles *versus* mouse lymphoid leukemia L1210.

The method of preparation, the reaction of 5-diazoimidazole-4-carboxamide with an appropriate amine, was described earlier (1). Subsequently, the yield of the dimethyltriazene (I) was improved considerably by employing ethyl acetate as the solvent, and this modification was used to prepare the new 3-alkyl-3-methyl-1-triazeno derivatives. However, 2-azahypoxanthine (XI), a potential by-product, is less soluble in this solvent and is more likely to contaminate the initially precipitated product. 2-Azahypoxanthine can be removed by washing the crude products with water and by recrystallizing them from methanol. Contamination of the disubstituted triazenes by 2-azahypoxanthine can be detected by thin-layer chromatography (TLC) if proper precautions are taken to prevent the artifactual formation of 2-azahypoxanthine *via* light-catalyzed decomposition of the triazenes during chromatography.

Previously, the light-catalyzed decomposition of several disubstituted triazeno derivatives, as well as the concomitant formation of 2-azahypoxanthine, was demonstrated by ultraviolet spectroscopy (1). The light wavelengths (or other conditions) responsible for this instability have not been defined precisely, but by TLC it has been shown that 2-azahypoxanthine forms quite rapidly when a 50% aqueous alcohol solution of the dimethyltriazene is exposed to sunlight (filtered through glass) or to an ultraviolet lamp emitting principally at 365 mμ. 2-Azahypoxanthine was not detected by TLC in similar solutions during the first 4 days of exposure to incandescent lighting or during the first day of exposure to fluorescent lighting, but a small amount was detectable after longer periods.

Received May 20, 1968, from Southern Research Institute, Birmingham, AL 35205
Accepted for publication June 18, 1968.
This work was supported by contract PH43-64-51 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md, and by the C. F. Kettering Foundation.
Previous papers in this series: Part III, *Reference 6*; Part IV, *Reference 2*.
* Present address: Taejon Presbyterian College, Taejon, Korea.

Exposure of solutions of the butylmethyltriazene (IV) to sunlight and to incandescent lighting gave similar results.

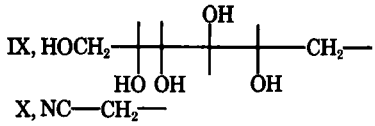


R

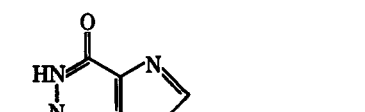
I, CH_3
II, H
III, C_3H_7
IV, *n*-C_4H_9
V, *iso*-C_4H_9

R

VI, *n*-C_5H_{11}
VII,
VIII, $HOCH_2CH_2$—



IX, $HOCH_2$—



X, NC—CH_2—

XI

Antitumor Evaluation—When compounds III–VII were administered daily according to the standard protocol (12), they significantly increased the life span (T/C values of 125–170%) of L1210-bearing mice at doses within the range of 25–75 mg./kg./day (Table I). The hydroxyethyl derivative (VIII) was less effective at 75 mg./kg./day, but it showed significant activity at a considerably higher dose. The optimum dose is presumed to be intermediate (and somewhat higher than the optimum doses of III–VII). Administration of larger single doses of III–VII on the second day after implanting the animals with 10^5 L1210 cells also significantly increased the survival times of the treated animals (T/C values of 125–165%, see Table II). In some of these tests, evidence of toxicity was manifested by a large difference between the treated and control groups in the average host-weight change. When an increase in the survival time of treated mice was accompanied by a large weight loss, it was not possible to decide from these tests whether the enhancing effect on survival time of killing large numbers of leukemic cells was partly cancelled by the depressing effect of toxicity to the host.

The effectiveness of these compounds against solid rodent tumors is not unequivocal because of the adverse host-weight change of treated animals in many of the tests. Most of the previously undescribed compounds (Table III), as well as some of those reported earlier (1), inhibited the growth of sarcoma 180 (S180) and adenocarcinoma 755 (Ca755) to 50% or less of the average weights of untreated tumors without causing host deaths in excess of permissible limits (12). At doses administered in accordance with protocols (12) in effect when S180, Ca755, and L1210 (QD 1–30) constituted the primary screen of the Cancer Chemotherapy National Service Center, the previously described disubstituted triazeno derivatives (1, *cf.* Table IV) lacking a methyl substituent were not active *versus* L1210.[1] Two compounds (V and VII) employed in limited testing *versus* intramuscular Walker carcinosarcoma 256 (W256IM) in rats effectively inhibited tumor growth. However, in most of the solid tumor tests showing inhibition of tumor growth, the treated animals suffered large weight losses, and drug-induced host inanition (13, 14), therefore, may account for much of the observed inhibition of growth of solid tumors. It has been shown (15, 16) that the effectiveness of an anticancer drug may be increased by arriving at an optimum relationship between dose size and frequency of administration, and it is conceivable that further variation in dose size and frequency of administration of these compounds might decrease the chronic toxicity manifested as host weight loss.

EXPERIMENTAL[2]

5(or 4)-(3-Alkyl-3-methyl-1-triazeno)imidazole-4(or 5)-carboxamides—*Preparation*—During all operations involved in the preparation and purification of compounds III–X, they, as well as the starting material, were shielded from light in solution and in the solid state. The following example is typical of the procedures used.

To a solution that was being stirred under a current of nitrogen and that consisted of 60 ml. of *N*-methylpentylamine and 480 ml. of ethyl acetate was added, portionwise during a 1-hr. period, 12 g. of 5-diazoimidazole-4-carboxamide (1, 17). Vigorous stirring was continued for 2 hr.; and the precipitate was then collected by filtration, washed with ethyl acetate, and dried *in vacuo*. The crude product (18.5 g., 88%), shown by TLC to contain some 2-azahypoxanthine (XI), was suspended in 400 ml. of water, the pH was adjusted to 9–10 with 1 *N* NaOH, and the resulting mixture was stirred for 10 min. The solid was collected on a filter, washed well with water, dried *in vacuo;* wt. 16.0 g. (76%). TLC showed that the product was free of XI. Recrystallization of 15.6 g. from methanol with the aid of activated-carbon decolorization afforded 9.9 g. (47% yield) of yellowish white crystals (in two crops) of pure VI.

Compounds III–V and VII–VIII were prepared

[1] The pyrrolidinylazo derivative gave a T/C value of 126% in a single (unconfirmed) test at 250 mg./kg./day.

[2] The authors are indebted to Drs. W. J. Barrett, W. C. Coburn, Jr., and P. D. Sternglanz of the Analytical and Physical Chemistry Division of this Institute for spectral determinations and elemental analyses and to Miss Kathleen Hewson and Mrs. Mary B. Emory for TLC determinations.

TABLE I—DAILY TREATMENT OF L1210 WITH III–X[a]

Compd. No.	Compd. R	Dose, mg./kg./day	Mortality,[a] Deaths/ Total	Av. Wt. Change, g., T/C[a]	Av. Survival Time, Days, T/C	T/C Ratio, %
III	*n*-Propyl	75	0/6	−1.7/+1.1	11.0/8.1	135
		50	0/6	−2.0/+1.1	12.8/8.1	158
		38	0/6	−0.9/+1.1	12.3/8.1	151
IV	*n*-Butyl	120	0/6	−3.1/+1.3	8.8/8.7	101
		80	0/18	−1.6/+1.2	14.0/8.4	167[b]
		40	0/6	−0.9/+1.3	14.3/8.7	164
		20	0/6	−0.1/+1.3	10.8/8.7	124
V	Isobutyl	100	0/6	−3.2/+0.5	9.0/8.3	108
		80[c]	0/6	−2.7/+1.3	10.0/9.1	109
		75	0/12	−1.3/+1.9	13.7/8.7	157[d]
		50	0/12	−0.4/+1.8	12.8/8.6	149[d]
		48[c]	0/6	−0.6/+1.3	12.5/9.1	137
		37	0/6	−0.4/+2.0	15.3/9.0	170
		28[c]	0/6	−1.4/+1.3	11.5/9.1	126
		25	0/6	+0.2/+1.9	12.3/8.5	144
		12.5	0/6	+0.2/+1.9	10.8/8.5	127
VI	*n*-Pentyl	200[c]	0/6	−3.2/+1.3	7.5/9.1	82t[e]
		120[c]	0/6	−3.4/+1.3	8.8/9.1	96
		100	0/6	−2.5/+1.3	13.7/8.6	159
		75	0/6	−2.6/−0.6	10.8/9.2	117
		72[c]	0/6	−2.2/+1.3	11.7/9.1	128
		50	0/6	−3.5/−0.6	11.7/9.2	127
		42[c]	0/6	−2.0/+1.3	11.7/9.1	128
		25	0/6	−1.4/−0.6	12.0/9.2	130
		15	0/6	+0.3/+1.9	10.5/8.8	119
		7.5	0.6	+0.8/+1.9	9.0/8.8	102
VII	Cyclohexyl	153[c]	0/6	−1.9/+1.0	9.2/7.7	83t[e]
		100	0/6	−1.8/+0.7	11.0/8.1	135
		90[c]	0/18	−1.4/+1.2	10.6/8.9	118[b]
		75	0/6	−1.1/+1.3	14.8/8.9	166
		54[c]	2/12	−1.4/+1.4	11.1/8.8	126[b]
		50	0/6	−0.5/+0.7	12.2/8.1	150
		32[c]	0/12	−0.9/+1.4	10.7/8.8	121[b]
		25	0/6	−0.6/+0.7	9.3/8.1	114
VIII	2-Hydroxyethyl	400[c]	0/6	−0.5/+1.2	12.8/9.3	137
		75	0/6	0.0/+1.3	10.7/8.9	120
IX	Pentahydroxyhexyl	200	0/6	+0.4/+1.1	8.2/9.1	90
		100	0/6	+0.9/+1.2	7.8/7.8	100
X	Cyanomethyl	150[f]	0/6	−1.3/+1.3	9.5/9.1	104
		100	0/6	−2.1/+0.5	9.7/8.3	116
		75	0/6	+0.4/+1.5	8.8/8.2	107
		50	0/6	+0.1/+1.5	9.7/8.2	118
		37.5	0/6	+0.5/+1.1	8.5/8.3	102
		25	0/5	+1.8/+1.1	8.4/8.3	101

[a] QD 1–30 days or to death unless otherwise stated. Mice implanted intraperitoneally with 10^6 L1210 cells. T = treated mice, C = control (untreated leukemic) mice. Av. wt. change = average weight change of host animals. Mortality and weight changes determined after the first 4 days of treatment (5 days after implantation). [b] Values in the last 3 columns are averages of 3 experiments. [c] QD 1–15 days or to death. [d] Values in the last 3 columns are averages of 2 experiments. [e] t = toxic. T/C below 85% considered to be caused by toxic dose. [f] QD 1–11 days or to death.

TABLE II—SINGLE-DOSE TREATMENT OF L1210 WITH III–VIII[a]

Compd. No.	Compd. R	Dose mg./kg.	Mortality, Deaths/ Total	Av. Wt. Change, g., T/C	Av. Survival Time, Days, T/C	T/C Ratio, %
III	*n*-Propyl	400	1/4	−2.9/+1.4	7.3/8.7	83t
		300	0/6	−4.1/+1.7	11.0/9.4	117
		200	0/10	−3.4/+1.6	13.0/9.1	143[b]
		133	0/6	−2.4/+1.7	11.7/9.4	124
		100	0/4	−1.1/+1.4	11.0/8.7	126
		89	0/6	−1.3/+1.7	11.0/9.4	117
IV	*n*-Butyl	400	0/6	−4.3/+3.1	11.3/9.2	122
		200	0/6	−2.5/+3.1	13.0/9.2	141
		100	0/6	−0.4/+3.1	11.0/9.2	119
V	Isobutyl	600	6/6			
		400	1/4	−3.9/+4.2	12.7/8.4	151t
		400	3/6	−4.1/+3.1	14.3/9.5	150t
		267	0/6	−2.9/+3.1	12.5/9.5	131
		200	0/4	−0.6/+4.2	10.3/8.4	122
		100	0/4	+1.6/+4.2	9.8/8.4	116

(*Continued on next page.*)

TABLE II (*Continued.*)

Compd. No.	Compd. R	Dose, mg./kg.	Mortality, Deaths/ Total	Av. Wt. Change, g., T/C	Av. Survival Time, Days, T/C	T/C Ratio, %
VI	*n*-Pentyl	600	0/6	−4.1/+3.1	12.2/9.5	128
		400	0/10	−2.8/+3.6	14.9/9.0	165[b]
		267	0/6	−1.9/+3.1	13.3/9.5	140
		200	0/4	−1.0/+4.2	12.8/8.4	152
		178	0/6	−2.1/+3.1	11.2/9.5	117
		100	0/4	+0.9/+4.2	10.0/8.4	119
VII	Cyclohexyl	600	0/12	−1.4/+2.7	12.2/9.4	129[b]
		400	0/16	−1.5/+3.2	11.8/9.1	130[c]
		267	0/12	−1.2/+2.7	11.5/9.4	122[b]
		200	0/4	−0.1/+4.2	10.8/8.4	128
		178	2/12	−1.8/+2.7	11.2/9.4	119[b]
		100	0/4	+0.8/+4.2	10.5/8.4	125
VIII	2-Hydroxyethyl	400	0/4	+1.6/+4.2	9.0/8.4	107
		200	0/4	+2.6/+4.2	9.5/8.4	113
		100	0/4	+3.1/+4.2	9.0/8.4	107

[a] Mice implanted intraperitoneally with 10^5 L1210 cells. T = treated mice, C = control mice. Av. wt. change = average weight change of host animals. Mortality and weight changes determined after the first 4 days of treatment (5 days after implantation). t = toxic (see *Footnote e*, Table II). [b] Values in the last 3 columns are averages of 2 experiments. [c] Values in the last three columns are averages of 3 experiments.

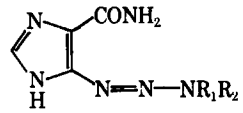
TABLE III—5 (OR 4)-(3-ALKYL-3-METHYL-1-TRIAZENO)IMIDAZOLE-4 (OR 5)-CARBOXAMIDES[a]

Compd. No.	Compd. R	Yield[b]	Formula	Anal. Calcd.	Anal. Found	Ultraviolet Absorption λ_{max}	Ultraviolet Absorption $\epsilon \times 10^{-3}$	Ultraviolet Absorption Solvent[c]
III	C_3H_7	57	$C_8H_{14}N_6O$	C, 45.70	45.63	236	12.4	CH_3OH
				H, 6.71	6.54	327	18.1	
				N, 39.97	40.24	237	11.7	Buffer
						331	18.2	
IV	*n*-C_4H_9	60	$C_9H_{16}N_6O$	C, 48.20	48.44	237	11.6	Buffer
				H, 7.19	7.16	331	18.3	
				N, 37.48	37.73	249	13.4	Base
						345	16.2	
V	*iso*-C_4H_9	54	$C_9H_{16}N_6O$	C, 48.20	48.33	237	11.7	Buffer
				H, 7.19	7.36	331	18.8	
				N, 37.48	37.70	249	13.6	Base
						345	16.8	
VI	*n*-C_5H_{11}	47	$C_{10}H_{18}N_6O$	C, 50.41	50.67	238	11.7	Buffer
				H, 7.61	7.41	331	18.6	
				N, 35.26	35.25	249	13.6	Base
						345	16.5	
VII		43	$C_{11}H_{18}N_6O$	C, 52.78	52.74	238	11.2	Buffer
				H, 7.25	6.98	333	19.4	
				N, 33.58	33.84	249	13.4	Base
						345	17.2	
VIII	$HOCH_2CH_2$—	35	$C_7H_{12}N_6O_2$	C, 39.62	39.56	237	11.7	Buffer
				H, 5.70	5.65	329	17.3	
				N, 39.61	39.45	248	13.3	Base
						344	15.6	
IX	$HOCH_2(CHOH)_4$—CH_2—	25	$C_{11}H_{20}N_6O_6$	C, 39.75	39.84			
				H, 6.07	6.02			
				N, 25.29	24.96			
X	$NCCH_2$—	55	$C_7H_9N_7O$	C, 40.57	40.72			
				H, 4.38	4.25			
				N, 47.32	47.11			
I[d]	CH_3					237	12.2	Buffer
						329	18.0	
						248	13.9	Base
						344	15.9	

[a] When these triazenes are heated in a capillary tube or on a Kofler-Heizbank apparatus, they decompose with intense darkening and evolution of gas. The decomposition temperatures vary markedly with the temperature at which heating is started and with the rate of heating. Decomposition at high temperatures may be explosive. [b] Yield of analytically pure product. [c] Buffer = phosphate buffer, pH 7. Base = 0.1 *N* NaOH. All of these derivatives produced absorption maxima in 0.1 *N* HCl at 223–226, *ca.* 275 (sh), and 323–327 mμ. Solutions for UV spectra were prepared by dissolving the sample in methanol and diluting an aliquot (5 ml. or less) to 50 ml. with the designated solvent. All spectra were recorded with a Cary model 14 recording spectrophotometer. [d] *Reference 1.*

and purified similarly. Compound IX was prepared by adding 5 g. of the diazoimidazolecarboxamide to a solution of 21.4 g. of 1-deoxy-1-(methylamino)-glucitol in 1.5 l. of methanol and stirring the mixture for 24 hr. The precipitate was not purified further. Methylaminoacetonitrile for the preparation of X was extracted with two 250-ml. portions of chloroform from a solution consisting of 50 g. of the amine

TABLE IV—EVALUATION OF III–X AND RELATED COMPOUNDS *versus* SOLID TUMORS[a]



No.	R_1	R_2	Tumor	Dose, mg./kg./day	Mortality, Deaths/Total	Av. Wt. Change, g., T/C	Av. Tumor Wt.,[b] T/C	T/C Ratio, %
III	*n*-C_3H_7	CH_3	S180	91	3/6	−3.7/+1.4	618/1826	t
				62	1.6	−3.4/+1.4	625/1826	34
				46	0/6	−2.2/+1.4	678/1826	37
				31	1/6	−3.3/+1.4	1018/1826	55
			Ca755	50	5/10	−3.4/−1.1	128/1169	t
				40	1/10	−2.5/−1.1	544/1169	37
				30	0/10	−2.7/+1.6	341/1129	30
				20	3/10	−0.9/+1.6	441/1129	39
				15	1/10	−1.3/+1.6	539/1129	47
				10	1/10	+0.2/+1.6	581/1129	51
IV	*n*-C_4H_9	CH_3	S180	187	6/6			t
				125	4/12	−5.8/+1.1	212/1142	19[c]
				62	0/6	−5.0/+2.5	361/1148	31
				31	0/6	−1.3/+2.5	510/1148	44
			Ca755	90	5/10	−4.7/+2.9	94/1392	t
				75	2/10	−3.1/+3.1	77/1476	5
				60	1/10	−4.4/+2.9	112/954	11
				37	0/10	+0.1/+3.1	699/1476	47
				18	0/10	+1.9/+3.1	1264/1476	85
V	*iso*-C_4H_9	CH_3	S180	75	0/7	−6.5/−1.0	509/1774	28
				50	0/7	−3.1/+1.4	544/1323	41
				25	0/7	−3.4/+1.4	1222/1323	92
			W256IM	200	1/12	−13/+16	0.8/8.7	9[c]
VI	*n*-C_5H_{11}	CH_3	S180	150	3/6	−3.7/+0.4	230/1434	t
				75	2/12	−4.7/+0.2	419/1411	30[c]
				50	0/12	−4.2/+0.2	654/1411	46[c]
			Ca755	100	6/10	−3.9/+6.1	173/2499	t
				50	0/10	−2.5/+3.9	1212/2814	43
VII		CH_3	S180	75	0/7	−4.3/+1.2	673/1353	49
			Ca755	200	10/10			t
				100	3/32	−2.9/+4.8	568/2440	24[d]
				50	0/10	+0.3/+3.9	2347/2814	83
			W256IM	400	2/6	−17/+17	0/4.2	0
				200	1/18	−8/+18	0.6/7.1	8[d]
				100	0/6	−4/+17	0.3/4.2	7
				50	0/6	+9/+17	2.2/4.2	52
VIII	$HOCH_2CH_2$—	CH_3	S180	250	1/12	−1.5/+0.1	706/1056	67[c]
				125	0/6	−2.4/0.0	788/1078	73
			Ca755	375	5/10	−4.2/+3.1	10/1523	t
				250	0/10	−1.9/+3.7	72/1146	6
				187	0/10	+0.2/+3.1	396/1523	26
				93	0/10	+2.1/+3.1	871/1523	57
IX	$C_6H_{13}O_5$	CH_3	S180	300	0/6	+1.0/+1.0	1486/1006	147
				200	0/6	+2.4/+2.0	1450/1529	94
X	NC—CH_2—	CH_3	S180	300	3/6	−6.0/−0.3	165/1231	t
				225	1/6	−3.3/−0.3	469/1231	38
				150	1/6	−2.1/+1.1	725/940	77
			Ca755	300	10/10			t
				150	4/10	−1.8/+2.4	578/1502	t
				150	0/10	0.0/+3.0	293/703	41
				75	0/10	+0.9/+0.8	748/1018	73
e	C_6H_5	CH_3	S180	250	1/6	−1.7/−0.3	374/919	40
				125	1/6	−0.7/−0.7	353/753	46
				62	0/6	−1.7/−0.7	749/753	99
			Ca755	200	5/10	−4.1/+1.7	107/779	t
				100	0/10	−2.7/+2.2	406/992	40
e	C_2H_5	C_2H_5	S180	250	4/6	−2.1/−1.1	763/1014	75
				125	0/6	−4.1/−0.1	398/758	52
				62	0/6	−1.2/+2.2	620/603	102
e	C_4H_9	C_4H_9	S180	500	6/6			t
				250	0/7	−2.5/+1.1	525/1227	42
			Ca755	375	6/10	−2.0/+2.5	144/1539	t
				250	0/10	−1.9/+1.2	602/838	71
e	C_8H_{17}	C_8H_{17}	S180	500	0/6	+0.4/−0.1	1115/1066	104

(*Continued on next page.*)

TABLE IV (*Continued.*)

No.	R_1	R_2	Tumor	Dose, mg./kg./day	Mortality, Deaths/Total	Av. Wt. Change, g., T/C	Av. Tumor Wt.,[b] T/C	T/C Ratio, %
e	$C_6H_5CH_2$—	$C_6H_5CH_2$—	S180	250	0/6	−2.5/−0.3	250/879	28
				125	2/6	−1.1/−0.3	565/919	61
				62	1/7	+0.3/+1.1	735/1227	59
				31	1/7	−0.9/+1.1	606/1227	49
			Ca755	500	2/10	+1.6/+1.4	771/614	125
				250	0/10	−0.1/+2.3	896/1253	71
e	R_1R_2N = 2-pyrrolidinyl		S180	250	0/6	+1.0/+1.7	919/857	107
				125	0/6	+2.5/+1.7	632/857	73
			Ca755	500	4/10	−1.0/+0.9	205/654	t
				250	1/10	−1.6/+0.2	611/782	78
e	R_1R_2 = 2-piperidino		S180	500	6/6			t
				250	1/6	+0.2/+1.8	1387/2108	65
			Ca755	375	4/10	−2.4/+0.9	291/654	t
				250	0/10	+3.0/+4.6	991/1838	53
				250	3/10	−0.3/+1.4	885/1055	83
				188	0/10	+1.6/+3.4	743/1660	44

[a] T = treated animals, C = control animals, t = toxic. Avg. wt. change = average weight change of host animals. Mortality and weight changes determined at the end of the tests. [b] Average tumor weights in milligrams for S180 and Ca755 and in grams for W256IM. [c] Values in the last 3 columns are averages of 2 experiments. [d] Values in the last 3 columns are averages of 3 experiments. [e] *Reference 1.*

hydrochloride, 250 ml. of saturated sodium chloride solution, and sufficient 6 *N* NaOH to raise the pH to 8.3. Stirring a mixture of the dried ($MgSO_4$) chloroform solution, 250 ml. of methanol, and 6.0 g. of the diazoimidazolecarboxamide for 48 hr. gave 5 g. of X that was homogeneous according to TLC. Additional data obtained in characterizing compounds III–X are summarized in Table III.

Thin-layer Chromatography—2-Azahypoxanthine was separated from the triazenes by TLC on silica gel in ethyl acetate–methanol (9:1) (Solvent A). In this system, however, the triazenes generally remained near the origin. Chloroform–methanol (3:1) (Solvent B) failed to separate significantly 2-azahypoxanthine from some of the triazenes on silica gel, but this solvent mixture moved the triazenes well and permitted examination of triazene specimens for slower moving impurities, which were sometimes observed in crude samples. The two solvent mixtures were, therefore, used to complement each other in evaluating a triazene sample. Subsequently, a butanol–water–15 *N* ammonia system (86:14:4) (Solvent C) was also employed to examine specimens of I (NSC-45388) for the presence of both XI and 5(or 4)-aminoimidazole-4(or 5)-carboxamide (AIC), a precursor and potential contaminant of I. The order of increasing distance from the origin on TLC plates developed in this solvent system was XI, AIC, I. Hypoxanthine, a potential contaminant of the AIC precursor, was not separated from XI in this solvent system, but in Solvent A it moved more slowly than XI.

Detection was by ultraviolet light (365 mμ), by ultraviolet light (254 mμ) after spraying with an optical whitening agent,[3] and by iodine vapor. The triazenes were protected from light during TLC.

Sensitivity to Light—The solutions of the dimethyltriazene (I) and the butylmethyltriazene (IV) described below were contained in Pyrex volumetric flasks, and, unless otherwise stated, 10-mcg. aliquots were applied to TLC plates and developed in Solvents A and B. Water in the aliquots usually produced weak spots at the origin in these solvent systems. 2-Azahypoxanthine (XI) and AIC (for Solvent C) were spotted as reference compounds at 1 and 0.5 mcg., respectively. Both compounds are easily detectable in these (and smaller) amounts, but no attempt was made to quantitate the results described below by employing minimum amounts of reference compounds and maximum amounts of aliquots.

A 50% aqueous ethanol solution of I protected from light showed no evidence of 2-azahypoxanthine formation during a period of 4 days at room temperature. A similar solution of I exposed [about 21 cm. (6 in.) distant] to an ultraviolet lamp emitting principally at 365 mμ contained a small amount of XI after 1 hr. of exposure, and the conversion of I to XI was near completion after 3 hr.[4] TLC of a 20-mcg. aliquot in Solvent System C did not reveal any AIC after 3 hr. of exposure.

Solutions of I and IV in 50% aqueous methanol (5 mg./ml.) were exposed to light under the conditions described below, and the results determined by TLC of aliquots removed at appropriate time intervals were as follows.

(*a*) 2-Azahypoxanthine (XI) was not detectable within 4 days in the I and IV solutions exposed continuously to incandescent lighting (only). A small amount of XI could be detected in the solution of I after 7 days.

(*b*) 2-Azahypoxanthine was detectable within 0.5 hr. in a solution of I exposed to direct sunlight. Conversion of I to XI was estimated to be near 50% of completion within 1 hr. and to be essentially complete within 3 hr. Aliquots removed from a solution of IV after 1-hr. and 3-hr. periods of exposure in the same way gave similar results. The light reaching these solutions passed through both window glass and Pyrex glass. In order to determine whether AIC might also be formed, a second solution was exposed to direct sunlight passing only through the Pyrex glass of the flask, and 20-mcg. aliquots were chromatographed in Solvent System C. After 3 hr. of exposure, only XI was detectable.

(*c*) A solution of I exposed to fluorescent lighting (15-w., 42-cm. fluorescent lamp, approximately 30 cm. from the solution) showed no XI after 24 hr., a small amount after 48 hr., and an increased amount after 96 hr.

[3] Ultraphor WT by BASF Colors and Chemicals, Inc., Charlotte, N. C.

[4] An assay method based on the photochemical decomposition of I was recently reported, Reference 18.

REFERENCES

(1) Shealy, Y. F., Krauth, C. A., and Montgomery, J. A., *J. Org. Chem.*, **27**, 2150(1962).
(2) Shealy, Y. F., and Krauth, C. A., *J. Med. Chem.*, **9**, 34(1966).
(3) Shealy, Y. F., Montgomery, J. A., and Laster, W. R., Jr., *Biochem. Pharmacol.*, **11**, 674(1962).
(4) MacDonald, C., Wollner, N., Ghavimi, F., and Zweig, J., *Proc. Am. Assoc. Cancer Res.*, **8**, 43(1967).
(5) Loo, T. L., Stasswender, E. A., Jardine, J. H., and Frei, E., III, *ibid.*, **8**, 42(1967).
(6) Shealy, Y. F., Krauth, C. A., Pittillo, R. F., and Hunt, D. E., *J. Pharm. Sci.*, **56**, 147(1967).
(7) Shealy, Y. F., and O'Dell, C. A., *J. Med. Chem.*, **9**, 733(1966).
(8) Clarke, D. A., Barclay, R. K., Stock, C. C., and Rondestvedt, C. S., Jr., *Proc. Soc. Expll. Biol. Med.*, **90**, 484(1955).
(9) Rondestvedt, C. S., Jr., and Davis, J. D., *J. Org. Chem.*, **23**, 200(1957).
(10) Shealy, Y. F., and Krauth, C. A., *Nature*, **210**, 208(1966).
(11) Shealy, Y. F., Krauth, C. A., Holum, L. B., and Fitzgibbon, W. E., *J. Pharm. Sci.*, **57**, 83(1968).
(12) *Cancer Chemotherapy Rept.*, **No. 25**, 1 (1962); **No. 1**, 42 (1959).
(13) Laster, W. R., Jr., Schabel, F. M., Jr., Skipper, H. E., Wilcox, W. S., and Thomson, J. R., *Cancer Res.*, **21**, 895 (1961).
(14) Skipper, H. E., Wilcox, W. S., Schabel, F. M., Jr., Laster, W. R., Jr., and Mattil, L., *Cancer Chemotherapy Rept.*, **29**, 1(1963).
(15) Kline, I., Venditti, J. M., Tyrer, D. D., and Goldin, A., *Cancer Res.*, **26**, 853(1966), and references cited therein.
(16) Skipper, H. E., Schabel, F. M., Jr., and Wilcox, W. S., *Cancer Chemotherapy Rept.*, **51**, 125(1967).
(17) Shealy, Y. F., Struck, R. F., Holum, L. B., and Montgomery, J. A., *J. Org. Chem.*, **26**, 2396(1961).
(18) Loo, T. L., and Stasswender, E. A., *J. Pharm. Sci.*, **56**, 1016(1967).

Keyphrases

Imidazoles—synthesis
TLC—separation, identity
UV light—identity
Antitumor activity—imidazoles
Degradation, imidazoles—light exposure

Specificity of Fluorometry of 5-Hydroxytryptamine by Means of Products with Ninhydrin

By W. B. QUAY

The specificity and relevant technical factors of the fluorometry of 5-hydroxytryptamine (5-HT) by means of its product with ninhydrin (1,2,3-indantrione) were studied in experiments with 121 test compounds and tissues from rats and cockroaches. Aromatic amines producing fluorescence capable of confusion with that from 5-HT were: a number of carboline derivatives, a few hydroxyindole congenors of 5-HT, 4-(aminomethyl)-piperidine, and 2-(2-aminoethyl)-pyridine. Comparisons of results from two different fluorometric procedures showed close correspondence with most mammalian tissues, but not for stomach, nor for cockroach tissues. More significant in the discrimination between 5-HT and the related compounds were the time and temperature-dependent fluorescence development and decay curves of incubations with ninhydrin.

5-HYDROXYTRYPTAMINE (5-HT) occurs widely and is of special interest in its association with neural and neuroeffector mechanisms (1). Its measurements in tissues has been by both bioassay and chemical means. Of the latter, fluorometric procedures are of paramount utility. One of these depends upon the fluorescence of 5-HT (and other 5-hydroxy and 5-methoxy indoles) in strong acid (fluorescence maximum at 540 mμ, excitation maximum at 295 mμ) (2, 3). Another depends on the fluorescence of a reaction product of 5-HT and ninhydrin (1,2,3-indantrione hydrate) (4, 5). This second technique is the more sensitive and has been thought to be more specific (5). In practice this appears to be true for most mammalian and reptilian tissues studied so far (6). But for some groups of animals and kinds of tissues the results by the two methods are divergent, with the ninhydrin technique giving falsely high values (Quay, unpublished results).

This report is a summary of an evaluation of the specificity of the fluorometric technique for 5-HT with ninhydrin, with special attention to amines most apt to produce a reaction product, and to derivatives and relatives of 5-HT. Several types of limitations in specificity are described, and methods for checking specificity are noted.

EXPERIMENTAL

The 121 compounds tested consisted of 72 indoleamines and their derivatives, 11 catecholamines and

Received March 11, 1968, from the Department of Zoology, University of California, Berkeley, CA 94720
Accepted for publication June 14, 1968.
This work was supported in part by a research grant (NB-06296), U. S. Public Health Service, Bethesda, Md.