(49) W. H. Carnes, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **30**, 995 (1971).
(50) S. F. Townsend, *Am. J. Anat.*, **109**, 133 (1961).
(51) S. F. Townsend, personal communication.
(52) M. H. McAdoo, A. M. Dannenberg, Jr., C. J. Hayes, S. P. James, and J. H. Sanner, *Infect. Immunol.*, **7**, 655 (1973).
(53) A. L. Willis, personal communication.
(54) R. E. Lee and W. E. M. Lands, *Biochim. Biophys. Acta*, **260**, 203 (1972).
(55) I. S. Maddox, *Biochim. Biophys. Acta*, **306**, 74 (1973).

Podophyllotoxin Analogs. 1. Synthesis and Biological Evaluation of Certain *trans*-2-Aryl-*trans*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactones as Antimitotic Agents†,1

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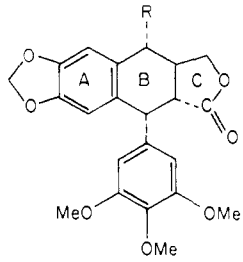
A series of cis and trans bicyclic lactones was prepared as congeners of podophyllotoxin (1) and evaluated as antimitotic agents both in cell cultures grown in vitro and in an in vitro protein binding assay. All compounds displayed insignificant activity—a result which may reflect insufficient structural similarity to podophyllotoxin or which may be interpreted as in agreement with previous observations of the stereochemical requirements for antimitotic activity defined for 1.

Podophyllotoxin (1) is a naturally occurring lignan lactone which is found in several species of *Podophyllum*, more commonly known as American mandrake or May apple. In the late 1940's and early 1950's the anticancer action of 1 was demonstrated.[2-4]



1, R = OH
2, R = H

Clinical testing, however, has revealed the inadequacies of 1 in the treatment of most cancers.[5] Moreover, the severe damage to rapidly proliferating tissues, consistent with a mechanism of cellular destruction based upon arrest of dividing cells at mitosis,[6] as well as its toxic effect on the CNS has limited its usefulness in cancer chemotherapy. Consequently a variety of semisynthetic and synthetic analogs has been prepared[7-19] in attempts to exploit the antitumor activity of podophyllotoxin in a molecule presenting more defined and narrow pharmacological properties. The most therapeutically useful substances arising from those studies to date appear to be several of the semisynthetic derivatives, notably podophyllic acid ethyl hydrazide[20-22] and 4'-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene)-β-D-glucopyranoside,[23-26] both of which have been used clinically with some success.

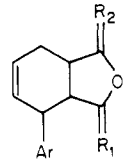
† Dedicated to Edward E. Smissman who was Chairman of this Department from 1960 until his death, July 1974.

The present study was initiated with an aim toward determining the minimum structural (and stereochemical) requirements necessary for the antimitotic activity of podophyllotoxin. In this regard our efforts have initially been directed toward the synthesis of B–C ring analogs of 1. In this first report we wish to relate the synthesis and biological evaluation of certain *trans*-2-aryl-*trans*-6-hydroxymethyl-3-cyclohexenecarboxylic acid γ-lactones (**12**), compounds which perhaps more appropriately could be termed analogs of deoxypodophyllotoxin (**2**), a known cytotoxic derivative present in several plants.[27-30]

Chemistry. The synthetic route employed for the preparation of the all-cis bicyclic lactones **7** is outlined in Scheme I. Treatment of the arylallylcarbinol **3**, prepared from the benzaldehyde and allyl chloride,[31] with TsOH in benzene at reflux for 2 hr generated the arylbutadiene, which was not isolated. Subsequent reaction of the preformed diene with maleic anhydride in benzene as solvent at reflux for 3 (**3b**) or 24 hr (**3a**) or alternatively in a steel autoclave at 120° for 24 hr (**3c**) afforded the Diels–Alder adducts **4a–c** in good yield. Reduction of anhydrides **4** with either $NaBH_4$–THF, Red-Al–C_6H_6, $LiAlH_4$–THF at −55°, or Li(*O*-*t*-Bu)$_3$AlH–C_6H_6 gave, in all cases, two products **5** and **6**, in roughly equal proportions, easily separable by column chromatography or fractional crystallization. One of the products (**5**) was found to be lactonic in nature (ir, C=O, 1770 cm^{-1}) while the other was determined to be a hydroxy acid (ir, broad OH, C=O, 1700 cm^{-1}) (Table I). Treatment of **6** with DCC yielded a different lactone **7**. These results differ from the metal hydride reduction of anhydrides reported by others[32,33] wherein only the lactone resulting from reduction of the most hindered carbonyl was formed.

That lactones **5** and **7** were indeed positional isomers as depicted and not geometrical isomers was shown by dehydrogenation experiments. When **5** and **7** were heated

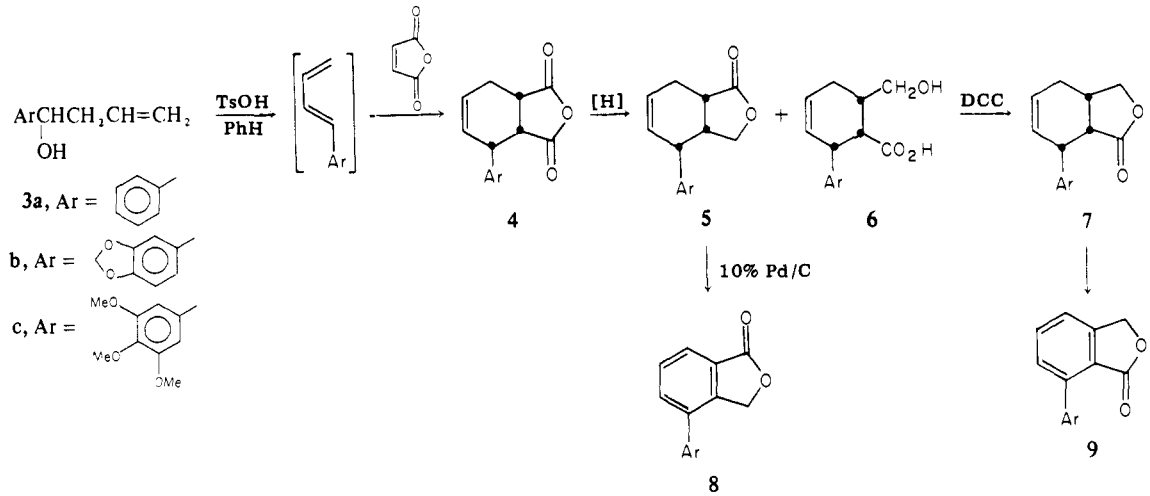
Table I



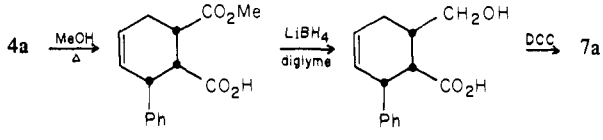
Compd	R_1	R_2	$\nu_{C=O}$, cm^{-1}	Yield,[a] %	Mp, °C	Formula[b]
4a	O	O	1840, 1770	47.3	120-121[c]	
4b	O	O	1840, 1770	70.6	155-157[d]	
4c	O	O	1840, 1770	54.5	131-133	$C_{17}H_{18}O_6$
5a	H_2	O	1770	26.6	76-78	$C_{14}H_{14}O_2$
5b	H_2	O	1770	16.8	123-126	$C_{15}H_{14}O_4$
5c	H_2	O	1775	25.6	136-138	$C_{17}H_{20}O_5$
7a	O	H_2	1770	63.6[f]	47-49	$C_{14}H_{14}O_2$[e]
7b	O	H_2	1770	58.8[f]	101-103	$C_{15}H_{14}O_2$
7c	O	H_2	1765	10.1[g]	128-130	$C_{17}H_{20}O_5$
11a	H_2	O	1780	33.3	120-122	
11b	H_2	O	1779	22.0	164-166	$C_{15}H_{14}O$
11c	H_2	O	1775	44.4	151-152	
12a	O	H_2	1780	55.8	114-116	$C_{14}H_{14}O_2$
12b	O	H_2	1780	52.5	148-149	$C_{15}H_{14}O_4$
12c	O	H_2	1775	46.9	137-138	$C_{17}H_{20}O_5$

[a] The yield of analytically pure compounds is given, and in most cases no attempt was made to optimize the yields. [b] The compounds were analyzed for C and H. Analytical results are within 0.4% of the theoretical value unless otherwise noted. [c] Lit. mp 120-121°: O. Diels, K. Alder, and P. Pries, *Ber.*, 62, 2081 (1929). [d] Lit.[31] mp 156-157°. [e] C: calcd, 78.48; found, 77.92. [f] Yield based on pure hydroxy acid. [g] Yield based on crude hydroxy acid.

Scheme I



Scheme II



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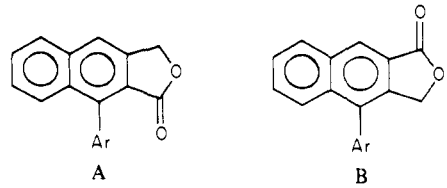
with 10% Pd/C in mesitylene for several hours, two clearly different fully aromatic compounds **8** and **9** were formed; had **5** been merely a cis-trans isomer of **7** only one phthalide would have been produced.

The assignment of the two lactone structures given in Scheme I rests upon the unambiguous synthesis of **7a** (Scheme II). Reduction of the known[34] half-acid ester **10**, formed by reaction of anhydride **4a** with methanol at reflux, with $LiBH_4$ followed by DCC ring closure yielded a compound identical in all respects with cis lactone **7a**.

Additional supportive evidence for the structure assignments made was found in the ^1H NMR of the phthalides **8** and **9**. The NMR spectra of compounds **8** displayed a one-proton multiplet downfield from the aromatic envelope assignable to the peri hydrogen deshielded by the carbonyl group. No such multiplet was present in biphenyls **9**.

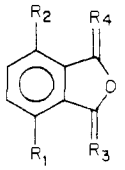
Use of the chemical shift of the lactone methylene protons of **8** and **9** in the NMR as an aid to assigning structure, as has been done for naphthalides,[35,36] was found to be misleading initially. Whereas lactones of type A shown below display lactone methylene absorption downfield from those found in type B caused by shielding by the pendant aromatic ring, deshielding of the lactone methylene protons was found in phthalides **8** (Table II). Compare also the values for phthalide and 3,6-dimethyl phthalide in Table II. This we feel implies a more planar arrangement of the aromatic rings in the phthalide system thus leading to deshielding.



A B

The stereochemistry of the lactone ring fusion is assigned to be cis in both **5** and **7** based upon the adsorption position of the carbonyl in the ir and the all-cis stereochemistry of the starting anhydride.[31] The aryl group and

Table II



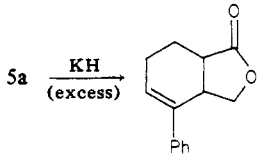
Compd	R_1	R_2	R_3	R_4	Lactone methylene, δ	Mp, °C	Yield,[a] %	Formula[b]
8a	Ar	H	H_2	O	5.38	114-116	43.4	$C_{14}H_{10}O_2$
8b	Ar	H	H_2	O	5.38	174-176	37.6	$C_{15}H_{10}H_4$
8c	Ar	H	H_2	O	5.43	187-189	50.6	$C_{17}H_{16}O_5$
9a	Ar	H	O	H_2	5.27	140-142	55.2	$C_{14}H_{10}O_2$
9b	Ar	H	O	H_2	5.27	160-162	42.1	$C_{15}H_{10}O_4$
9c	Ar	H	O	H_2	5.31	152-154	41.2	
	H	H	O	H_2	5.32	c	c	
	Ph	Ph	O	H_2	5.36	167-169	44.2	$C_{20}H_{14}O_2$

[a,b] See corresponding footnotes in Table I. [c] Literature melting point, obtained from Aldrich Chemical Co., Milwaukee, Wis.

lactone carbonyl function are likewise assumed to be cis since sodium borohydride reduction of the anhydride would not be expected to give isomerization.

The final step in the synthetic pathway to the all-trans lactones involved the epimerization of the all-cis lactones. This was accomplished in much the same manner as Gensler had performed his picropodophyllotoxin–podophyllotoxin interconversion.[37] Treatment of cis lactones **5** and **7** with KH–THF to generate the enolate anion followed by acidic quenching with HOAc afforded a mixture from which the requisite trans lactones **11** and **12**, respectively, were isolated (Scheme III). Each of these lactones exhibited a higher energy carbonyl adsorption than the starting cis lactones (see Table I) reflecting the more highly strained trans ring juncture in the six-five ring system.[38]

Although no evidence (NMR, uv) of double bond isomerization by using an excess of KH was encountered with lactones **5b** and **5c** and **7a–c**, it was found essential to use only 1 equiv of KH when **5a** was epimerized; if an excess of KH was employed the isomeric lactone **13** was formed in near quantitative yield apparently due to the acidity of the benzylic H in the unsubstituted phenyl compound.



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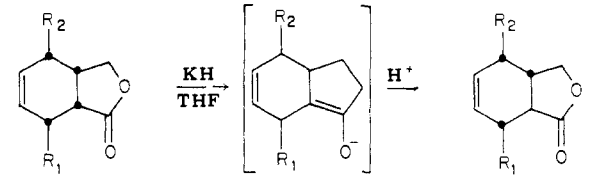
The epimerization reaction itself was found to proceed to a much greater degree in compounds **7**; whereas compounds **5** were found to be only epimerized to the extent of about 50% (by TLC), compounds **7** were epimerized in nearly quantitative yield, implying a more preferential proton attack on the enolate in the latter case due to steric reasons.

Biological Results. The antimitotic activity of the synthetic lactones and anhydrides was measured both in living cells and in vitro. The in vitro assay was that of inhibition of colchicine binding to isolated and purified tubulin. This followed the general method outlined by Borisy.[39] The living cell assay employed the technique of inhibition of colony formation by bone marrow cells or mastocytoma cells (P-815) as described by Pazdernik and Uyeki.[40] The testing procedures are reported in detail in the Experimental Section.

The in vitro test for activity involves the ability of the

Scheme III



5, R_1 = H; R_2 = Ar
7, R_1 = Ar; R_2 = H

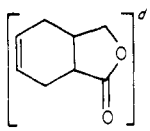
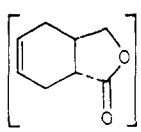
11, R_1 = H; R_2 = Ar
12, R_1 = Ar; R_2 = H

compounds to prevent the binding of [^{3}H]colchicine to tubulin and, therefore, to reduce the number of counts bound to the filter pad. A concentration of 10 μM [^{3}H]colchicine was chosen to use for these experiments since an adequate number of counts was bound to the filter pad (Table III). This concentration would still give poorly binding compounds a reasonable chance to compete. Podophyllotoxin is able to compete very efficiently and reduces the counts per minute by about 50% at a concentration of 10 μM. Podophyllotoxin has a K_{dis} of 5–8 μM. A concentration of 1% acetone reduces colchicine binding by about 20%. Nevertheless, because of the solubility characteristics of the compounds, it was necessary to use 1% acetone throughout.

The cell tests measure the ability of the analogs to suppress colony formation of P-815 mastocytoma tumor cells and hematopoietic bone marrow cells. The effects of these agents on the P-815 and bone marrow colony-forming cells are illustrated in Table III. Podophyllotoxin suppressed colony formation by both P-815 and bone marrow cells at concentrations as low as 5×10^{-8} M.

The previous studies of structure–activity relationship in the podophyllotoxin lactone series suggested that for good activity the lactones must be trans fused and the lactone carbonyl cis to the pendant aromatic ring.[41] It was also demonstrated that deoxypodophyllotoxin was nearly equipotent to podophyllotoxin. The lack of significant biological activity in the γ-lactones prepared in this study is in agreement with those previous studies. However, this inactivity may only reflect insufficient structural similarity to podophyllotoxin for these agents which does not allow them to bind to the tubulin protein, binding being responsible for the antimitotic activity of podophyllotoxin. The slight stimulation of colony formation observed with some agents, especially at 5×10^{-5} M, may be due to a nonspecific antioxidant effect similar to that observed with sulfhydryl agents. The syntheses of the trans-fused lactones with the carbonyl cis to the pendant aromatic ring are currently under way and will be the subject of a future paper.

Table III. Effects of Podophyllotoxin and Analogs on Colchicine Microtubule Binding and on Colony Formation from Bone Marrow Cells and from P-815 Mastocytoma Tumor Cells Cultured in Vitro

Compd	[^{3}H]Colchicine[a] Concn, μM	[^{3}H]Colchicine[a] cpm	Colony formation Concn, μM	Colony formation % of bone marrow CFC control	Colony formation % of P-815 CFC control
1	2[b]	14971	5[e]	95	91
	4	13709	50[e]	1	0
	8	10316	0.5	0	0
	16	3781			
	20	2376			
5a	10	15831			
	40	14828			
	60	5382			
	100	13148			
5b	10	18977	0.5	103	71
	40	16747	5	105	98
	60	16970	50	121	106
	100	17035			
5c	10	18469	0.5	75	81
	40	11563	5	73	89
	60	18358	50	55	178
	100	18231			
7a	10	1315			
	40	12287			
	60	13893			
	100	13627			
7b	10	17625	0.5	97	89
	40	17642	5	105	112
	60	17340	50	115	138
	100	12450			
7c	No data available				
11a	No data available				
11b	10	17379	0.5	104	100
	40	16728	5	135	61
	60	15588	50	166	169
	100	16692			
11c	20[c]	9066	0.5	90	89
	50	11880	5	74	95
	100	11500	50	135	109
	200	8690			
12a	10	12172	0.5	96	74
	40	15572	5	95	75
	60	18543	50	107	93
	100	15510			
12b	10	16011	0.5	87	101
	40	20096	5	143	86
	60	18213	50	170	143
	100	19576			
12c	10	17046	0.5	35	84
	40	15460	5	105	42
	60	14602	50	75	9
	100	15236			
 7, R = H [d]	10	15758	0.5	106	93
	40	16779	5	103	69
	60	15851	50	114	111
	100	14128			
 12, R = H [d]	10	15474	0.5	102	102
	40	14876	5	91	95
	60	15206	50	106	112
	100	14699			

[a] [^{3}H]Colchicine concentration is 10 μM unless otherwise noted. [b] [^{3}H]Colchicine concentration is 20 μM. [c] [^{3}H]Colchicine concentration is 40 μM. [d] J. J. Bloomfield and S. L. Lee, *J. Org. Chem.*, 32, 3919 (1967). [e] nM.

Experimental Section

Boiling points are uncorrected. Melting points were obtained on a Thomas-Hoover Unimelt and are corrected. Ir data were recorded on a Beckman IR-33 spectrophotometer, uv data on a Cary Model 14 spectrophotometer, and NMR data on a Varian Associates Model T-60 spectrometer (Me_4Si). Microanalyses were performed on a F & M Model 185 C, H, N analyzer, University of Kansas, Lawrence, Kan. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. Representative procedures for the preparation of compounds are illustrated below.

Arylallylcarbinols 3a–c. Compounds **3a** and **3b** were prepared by literature procedure[31] in 85.0 and 88.0% yield, respectively. Compound **3c** was prepared in like manner in 65.9% yield: bp 150–152° (0.4 mm). Anal. C, H.

Anhydrides 4a–c. ***cis*-3-(3,4,5-Trimethoxyphenyl)-4-cyclohexene-*cis*-1,2-dicarboxylic Acid Anhydride (4c).** To 7.0 g (0.0294 mol) of allylcarbinol (**3c**) dissolved in 50 ml of dry PhH was added 0.1 g of TsOH and the resulting solution heated at reflux under Ar with provision made for the removal of H_2O as formed (Dean Stark) for 1.5 hr. The solution was then transferred to a steel autoclave which had previously been charged with 2.9 g (0.0295 mol) of maleic anhydride and the mixture heated at 120° for 24 hr.

The yellow solution which resulted was evaporated at reduced pressure to afford a viscous oil which crystallized on trituration with Et_2O–PhH: yield 5.1 g (54.5%); mp 128–130°. Recrystallization from PhH gave the analytical sample: mp 131–133°. Anal. C, H.

Cis Lactones 5a–c and 7a–c. (a) Via Reduction of Anhydrides 4a–c with Red-Al. *cis*-5-Phenyl-*cis*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactone (5a) and *cis*-2-Phenyl-*cis*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactone (7a). To 4.0 g (0.0176 mol) of anhydride **4a** dissolved in 100 ml of PhH was added dropwise over 10 min at room temperature a solution of 4.8 g (0.0176 mol) of Red-Al (70% solution in PhH) in 50 ml of PhH. After the addition was complete, the clear solution was stirred at room temperature for an additional 0.5 hr and then poured into 200 ml of cold 3 N HCl and stirred for 0.5 hr. Et_2O (200 ml) was then added and the organic phase separated; the aqueous phase was washed with 100 ml of Et_2O. The combined organic layers were washed with H_2O (2 × 200 ml), dried (Na_2SO_4), and evaporated to yield 4.0 g of a semisolid. Trituration with Et_2O and filtration gave 1.0 g (20.6%) of **6a** as a colorless solid: mp 180–185°. Anal. ($C_{14}H_{16}O_3$) C, H; C: calcd, 72.41; found, 71.85. The filtrate was evaporated and the resulting oil (2.5 g) chromatographed on 40 g of Florisil, eluent PhH–EtOAc (8:2), to yield 0.81 g (21.6%) of **5a** as colorless crystals: mp 78–80°.

Hydroxy acid **6a** (1.0 g, 0.0036 mol) was dissolved in 75 ml of dioxane, 0.88 g (0.0042 mol) of DCC was added, and the mixture was allowed to stir at room temperature for 2 hr. The precipitated DCU was filtered off, the solvent evaporated at reduced pressure, the semisolid residue treated with 20 ml of cold acetone, the mixture filtered, and the solvent again evaporated to yield 0.9 g of a colorless oil. Chromatography on 15 g of Florisil, eluent PhH–EtOAc (8:2), afforded 0.585 g (63.6%) of **7a** as a colorless oil which could be crystallized with difficulty from Et_2O–Skelly B: mp 47–49°.

(b) Via Reduction of Anhydrides 4a–c with $NaBH_4$. *cis*-5-(3,4,5-Trimethoxyphenyl)-*cis*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactone (5c) and *cis*-2-(3,4,5-Trimethoxyphenyl)-*cis*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactone (7c). To 0.37 g (0.0097 mol) of $NaBH_4$ suspended in 20 ml of dry THF and cooled in an ice bath was added dropwise over 5 min a solution containing 3.1 g (0.0097 mol) of anhydride **5c** in 50 ml of dry THF. After the addition was complete, the ice bath was removed and the reaction mixture stirred at room temperature for 0.5 hr. The excess borohydride was then decomposed by the dropwise addition of 6 N HCl (~2 ml) to the cooled reaction mixture. The solvent was removed at reduced pressure and the resulting semisolid partitioned between Et_2O–H_2O. The Et_2O solution was washed with 5% $NaHCO_3$ (2 × 50 ml), dried (Na_2SO_4), and evaporated to yield 1.2 g of crude **5c**. Recrystallization from Et_2O gave 0.60

g (20.6%) of pure **5c** as colorless needles: mp 136–138°. Anal. C, H.

The bicarbonate wash above was acidified with concentrated HCl and extracted with Et_2O; the Et_2O was dried (Na_2SO_4) and evaporated to yield 1.5 g of crude **6c** as a semisolid. Treatment of this with 1.0 g (0.0048 mol) of DCC in 125 ml of dioxane and work-up as in (a) above gave 0.100 g (10.1%) of **7c** as iridescent plates: mp 128–130°. Anal. C, H.

(c) Via Reduction of Anhydrides 4a–c with $LiAlH_4$ or LTBA. The procedure as outlined in the literature[32] for the reduction of anhydrides with $LiAlH_4$ was followed. The same general procedure was likewise used for the reductions with LTBA, except that the reductions were carried out at 0° instead of −55°. Both methods were found to be more troublesome and inferior to the two methods outlined in detail above.

Phthalides 8a–c and 9a–c. 7-Phenyl Phthalide (9a). To 0.185 g (0.00072 mol) of **7a** dissolved in 60 ml of mesitylene was added 0.090 g of 10% Pd/C and the mixture was refluxed for 16 hr with vigorous stirring. The reaction mixture was then cooled, the catalyst filtered off, and the colorless solution evaporated at reduced pressure without heating. The resulting pale yellow solid was recrystallized from Et_2O to yield 0.10 g (55.2%) of **9a** as colorless plates: mp 140–142°. Anal. C, H.

Trans Lactones 11a–c and 12a–c. *trans*-2-(3,4,5-Trimethoxyphenyl)-*trans*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactone (12c). To 0.050 g (0.00125 mol) of KH suspended in 20 ml of dry THF, under Ar, was added a solution of 0.032 g (0.00012 mol) of cis lactone **9c** in 25 ml of dry THF all at once. After stirring at room temperature for 1 hr the reaction mixture was cooled in an ice bath and quenched by the rapid addition of 4 ml of HOAc. The colorless solution was poured into 100 ml of H_2O and extracted with Et_2O (2 × 75 ml). The ethereal extracts were then washed with H_2O (2 × 50 ml) and saturated NaCl solution (1 × 50 ml), dried (Na_2SO_4), and evaporated to yield 35 mg of a semisolid. Chromatography on 4 g of Florisil, eluent PhH–EtOAc (8:2), yielded 15 mg (46.9%) of pure **12c** as microneedles: mp 136–138°. Recrystallization from Et_2O–Skelly B gave the analytical sample: mp 137–138°. Anal. C, H.

Preparation of *cis*-2-Phenyl-*cis*-6-hydroxymethyl-3-cyclohexenecarboxylic Acid γ-Lactone (7a) via Reduction of Methyl *cis*-2-Carboxy-*cis*-3-phenyl-4-cyclohexenecarboxylate (12) with $LiBH_4$. To a solution of 0.475 g (0.0125 mol) of $NaBH_4$ in 25 ml of diglyme was added 1.08 g (0.0125 mol) of LiBr. After stirring at room temperature for 30 min, 0.52 g (0.002 mol) of half-acid ester **12** was added and the mixture heated on a steam bath for 3 hr. The solution was then poured onto 50 g of ice and 5 ml of concentrated HCl. Et_2O (50 ml) was added and the layers were separated. The Et_2O was dried (Na_2SO_4) and evaporated to yield 0.50 g of a semisolid. Treatment with DCC and work-up in the usual manner afforded 0.175 g (33.9%) of **7a** as a mobile oil, identical in all respects (ir, NMR, TLC) with lactone **7a** described above.

Biological Procedures. Materials. Tubulin Preparation. Tubulin was prepared from fresh beef brains using the method of Lee et al.[42] and stored for up to 1 month at −80°C. The preparation gave a single major band at 55000 molecular weight in polyacrylamide gel electrophoresis in sodium dodecyl sulfate and a high-molecular-weight minor band near the top of the gel. [^{3}H]Colchicine was purchased from New England Nuclear and diluted with nonradioactive colchicine to a concentration of 4 μ*M* and a specific activity of 24.8 Ci/mol. This was further diluted before use to 13.4 m*M* [^{3}H]colchicine (4.2 Ci/mol) in 0.2 *M* sodium phosphate, pH 6.8.

Animals. Female BDF_1 mice ($C57Bl/6 \times DBA/2$), 8–12 weeks old, were purchased from Jackson Laboratories, Bar Harbor, Me.

Tumor Cells. Mastocytoma tumor cells (P-815) were obtained from N. Tolson at Microbiological Associates, Bethesda, Md. P-815 tumor cells were continuously passaged in vitro.

Tissue-Culture Media. Fetal calf serum and horse serum were obtained from Kam Laboratories, St. Joseph, Mo. Concentrates of minimum Eagle's medium (MEM), McCoy's 5A medium (modified), and Hank's balanced salt solution medium were obtained from Grand Island Biological Co., Grand Island, N.Y. Bacto-agar (Difco-0140-02) was obtained from Difco Laboratories, Detroit, Mich. Glutamine, sodium pyruvate, serine, asparagine, and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo.

Methods. The assay procedure for colchicine binding follows the general method outlined by Borisy. Each assay contained 0.58 mg of purified beef brain tubulin added in a volume of 75–375 μl of 13.4 μ*M* [^{3}H]colchicine in 0.2 *M* sodium phosphate, pH 6.8, and either 5, 20, 30, or 50 μl of the solution of the compound to be tested. The compounds to be tested were dissolved in 5% acetone concentration of 1 m*M* by first adding the acetone and then the water. Additional volumes of 5% acetone were added to each tube so that all contained 50 μl of 5% acetone (1% final concentration). All compounds except one were initially soluble in 5% acetone, but some crystallized out within 2 hr while others required 3 days. After addition of tubulin to the [^{3}H]colchicine–"compound" buffer solution (equilibrated to 37°C) further incubation at 37°C was carried out for 90 min after which the entire contents of the tube was filtered through a pad of three DEAE-cellulose filters to trap the [^{3}H]colchicine–tubulin complex. The filter pad was washed with three 10-ml portions of 10 m*M* sodium phosphate, pH 6.8, and counted by liquid scintillation in Bray's solution. The binding of [^{3}H]colchicine shows a linear dependence upon the concentration of tubulin over the range tested, up to 1.5 mg/ml.

Culture Technique. All cultures were performed in 35-mm Falcon plastic tissue culture dishes, obtained from Falcon Plastics, Los Angeles, Calif. A modification of the technique of Metcalf and Moore[43] was used for culturing colony-forming cells (CFC) in vitro as reported by Pazdernik and Uyeki.[40] Briefly, this involved the extrusion of bone marrow from femurs and gentle trituration of marrow plugs to obtain dispersed bone marrow cell suspensions. Using McCoy's 5A tissue culture media (modified) supplemented with *l*-asparagine, glutamine, sodium pyruvate, serine, sodium bicarbonate, 1×10^{-4} *M* dithiothreitol, 10% fetal calf serum and 5% horse serum, 1×10^5 bone marrow cells, or 1×10^3 P-815 mastocytoma tumor cells were cultured in 1.0 ml of semisolid agar (final concentration was 0.3% of bacto-agar). Colony-stimulating factor (CSF) obtained from conditioned media of L-cells was added to each of the bone marrow cultures. Compounds to be tested were dissolved in a 10% Me_2SO–50% EtOH saline solution at a concentration of 1×10^{-3} *M*. Serial dilutions (1:10) were made with saline and 50 μl of the appropriate drug solutions was added to the designated dishes containing either 1×10^5 bone marrow cells or 1×10^3 P-815 tumor cells in 1.0 ml of semisolid media. The cultures were placed in humidified air-tight boxes and briefly gassed with a 12% carbon dioxide–8% oxygen–80% nitrogen gas mixture. After 7 days of incubation at 37°C, culture dishes were examined for colony formation at 20 magnification, using an inverted Unitron microscope.

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References and Notes

(1) Presented in part at the Midwest Regional Meeting of the American Chemical Society, Iowa City, Iowa, Nov 1974.
(2) (a) M. Belkin, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **6**, 308 (1947); (b) J. C. Hartwell and M. J. Shear, *Cancer Res.*, **7**, 716 (1947).
(3) J. Leiter, V. Downing, J. L. Hartwell, and M. J. Shear, *J. Natl. Cancer Inst.*, **10**, 1773 (1950).
(4) J. L. Hartwell, A. W. Schrecker, and J. Leiter, *Proc. Am. Assoc. Cancer Res.*, 1, 19 (1954).
(5) H. Savel, *Proc. Am. Assoc. Cancer Res.*, **5**, 56 (1964).
(6) H. Savel, *Prog. Exp. Tumor Res.*, **8**, 189 (1966).
(7) W. Reeve and W. M. Eareckson, *J. Am. Chem. Soc.*, **72**, 5195 (1950).
(8) K. N. Campbell, J. A. Cella, and B. K. Campbell, *J. Am. Chem. Soc.*, **75**, 4683 (1953).

(9) W. Reeve and H. Myers, *J. Am. Chem. Soc.*, **75**, 4957 (1953).
(10) G. N. Walker, *J. Am. Chem. Soc.*, **78**, 2316 (1956).
(11) E. A. Fehnel and J. A. Stuber, *J. Org. Chem.*, **24**, 1219 (1959).
(12) G. R. Petitt and D. S. Alkalay, *J. Org. Chem.*, **25**, 1363 (1960).
(13) W. J. Gensler, F. Johnson, and D. B. Sloan, *J. Am. Chem. Soc.*, **82**, 6074 (1960).
(14) G. R. Petitt, M. F. Bauman, and K. N. Rangammal, *J. Med. Chem.*, **5**, 800 (1962).
(15) E. Schreier, *Helv. Chim. Acta*, **46**, 75 (1963).
(16) E. Schreier, *Helv. Chim. Acta*, **46**, 2940 (1963).
(17) W. J. Gensler, *J. Am. Chem. Soc.*, **85**, 3670 (1963).
(18) S. N. Lewis and T. L. Popper, *Tetrahedron*, **23**, 4197 (1968).
(19) A. Von Wartburg, M. Kuhn, C. Keller, and J. Renz, S. African Patent 66017585; *Chem. Abstr.*, **70**, 78340 (1968).
(20) R. K. Vaitkevicius and M. L. Reed, *Cancer Chemother. Rep.*, **50**, 565 (1966).
(21) R. C. Chakravorty, S. K. Sarkar, S. Sen, and B. Mukerji, *Br. J. Cancer*, **21**, 33 (1967).
(22) T. Koike, *Nippon Kagaku Ryohogakukai Zasshi*, **18**, 189 (1970); *Chem. Abstr.*, **73**, 54553 (1970).
(23) H. Stahelin, *Proc. Am. Assoc. Cancer Res.*, **10**, 86 (1969).
(24) F. M. Muggia, O. S. Selawry, and H. H. Hansen, *Cancer Chemother. Rep.*, **55**, 575 (1971).
(25) P. Dombernowsky, N. I. Nissen, and V. Larsen, *Cancer Chemother. Rep.*, **56**, 71 (1972).
(26) *Br. Med. J.*, **2**, 747 (1972).
(27) S. M. Kupchan, R. J. Hemingway, and J. C. Hemingway, *J. Pharm. Sci.*, **56**, 408 (1967).
(28) E. Bianchi, M. E. Caldwell, and J. R. Cole, *J. Pharm. Sci.*, **57**, 696 (1968).
(29) Y. Aynehchi, *J. Pharm. Sci.*, **60**, 121 (1971).
(30) A. Akahori, F. Yasuda, M. Ando, K. Hori, and T. Okanishi, *Chem. Pharm. Bull.*, **20**, 1150 (1972).
(31) R. T. Arnold and E. C. Coyner, *J. Am. Chem. Soc.*, **66**, 1542 (1944).
(32) J. J. Bloomfield and S. L. Lee, *J. Org. Chem.*, **32**, 3919 (1967).
(33) D. M. Bailey and R. E. Johnson, *J. Org. Chem.*, **35**, 3574 (1970).
(34) J. Klein and E. Dunkelblum, *J. Org. Chem.*, **36**, 142 (1971).
(35) Z. Horii, M. Tsujiuchi, and T. Momose, *Tetrahedron Lett.*, 1079 (1969).
(36) T. L. Holmes and R. Stevenson, *J. Chem. Soc. C*, 2091 (1971).
(37) W. J. Gensler and C. D. Gatsonis, *J. Org. Chem.*, **31**, 4004 (1966).
(38) E. L. Eliel and C. Pillar, *J. Am. Chem. Soc.*, **77**, 3600 (1955).
(39) G. G. Borisy, *Anal. Biochem.*, **50**, 373 (1972).
(40) T. L. Pazdernik and E. M. Uyeki, *Int. J. Radiat. Biol.*, **26**, 331 (1974).
(41) J. L. Hartwell and A. W. Schrecker, *Fortschr. Chem. Org. Naturst.*, **15**, 83 (1958).
(42) Y. C. Lee, F. E. Samson, L. L. Houston, and R. H. Himes, *J. Neurobiol.*, **5**, 317 (1974).
(43) D. Metcalf and M. A. S. Moore, "Haemopoietic Cells", A. Neuberger and E. L. Tatum, Ed., American Elsevier, New York, N.Y., 1971, p 35.

Stereochemical Analogs of a Muscarinic, Ganglionic Stimulant. 2. Cis and Trans Olefinic, Epoxide, and Cyclopropane Analogs Related to 4-[*N*-(3-Chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium Chloride (McN-A-343)†,‡,[1,2]

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Preparation of analogs of 4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride [1 (McN-A-343)], *cis*- and *trans*-4-[*N*-(4-chlorophenyl)carbamoyloxy]-2-butenyltrimethylammonium iodides (**5** and **6**), and the corresponding epoxides and cyclopropanes is reported. Pharmacological testing for ganglion-stimulating activity demonstrated that the trans olefin **6** and trans epoxide **8** have properties similar to **1**, while the trans cyclopropane analog **10** was inactive. All cis compounds were inactive. The muscarinic ganglion-stimulating properties of the active compounds are interpreted in terms of similar fit at the receptor level by the alkyltrimethylammonium ion and the ether oxygen 5.7 Å distant, as well as an electron-rich center midway between these groups in the form of a double bond or unshared electron pairs. Comparison of smooth muscle and ganglion-stimulating properties of the compounds showed that trans epoxide **8** was the most selective for muscarinic ganglionic sites.

A detailed pharmacological study by Roszkowski[3] of 4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride [**1** (McN-A-343)] demonstrated that this compound possessed unique ganglionic stimulant properties, exciting sympathetic ganglia at muscarinic (atropine sensitive) sites to produce an increase in blood pressure, after a short initial depressor effect. Other less important effects were noted including some classical muscarinic effects of vasodilitation and stimulation of intestinal smooth muscle. Recently, parasympathetic ganglion-stimulating effects have been noted[4,5] as well as nonmuscarinic effects including antagonism of the amine uptake pump of the sympathetic nerve terminal[6,7] and local anesthetic effects.[8]

In an earlier study[1] we demonstrated that the trans olefinic analog **4** possessed very similar muscarinic ganglion-stimulant properties, being about one-third as potent as **1**, in elevating blood pressure in anesthetized cats. Cis compound **3** was much less active. Neither olefinic analog showed significant muscarinic effects on smooth muscle similar to **1**. These results suggested that extended conformations of **1** and **4** are responsible for ganglion-stimulant activity, where the ether oxygen and quaternary nitrogen are 5.7 Å apart. Only in these conformations do **1** and **4** approximate each other in a spatial

† Dedicated to the memory of Edward E. Smissman.

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