are the mean and standard error of at least three determinations.

In Vivo Characterization. The writhing test was performed using male albino mice (Charles River Laboratories, CD-1/HAM/1LR) weighing between 20 and 30 g. Twenty-five minutes after sc administration of the test compound (0.1 mL/10 g body weight), 0.025% (w/v) phenylbenzoquinone was injected intraperitoneally (0.1 mL/g body weight). Five minutes later each mouse was placed in a large glass beaker, and the number of writhes that occurred in the subsequent 10 min was counted. A test compound was considered to have produced antinociception in a mouse if the number of writhes elicited by phenylbenzoquinone was equal to or less than $^1/_2$ the median number of writhes recorded for the saline-treated group that day. Each dose of test compound was administered to 10 mice, and the results were expressed as the number of mice (out of a possible 10) in which the test compound produced antinociception. The ED_{50} value, defined as the dose that inhibited writhing in 50% of the mice, and 95% confidence limits, were calculated using a maximum likelihood function.

The hot plate test was conducted using male albino mice (Charles River Laboratories, CD-1/HAM/1LR) weighing between 20 and 30 g. Hot plate latency was defined as the time that elapsed between placement of the mouse on a 55 °C surface and a lick of the hindpaw or a jump. Animals that achieved the cut-off latency of 40 s were removed from the hot plate to prevent tissue damage and assigned this value. Hot plate latency was determined before and at fixed intervals after either sc or icv administration of the test compound. One measurement was made at each time point. Two-way analyses of variance were used to determine the significance of the drug effect on response latencies as compared to saline-treated animals. The ED_{50} value was defined as the dose that produced $^1/_2$ the maximum possible increase in latency (i.e. 25 s). Calculations of ED_{50} values were based on a least squares linear regression equation computed for the data obtained at 10 min, the time of peak effect.

Acknowledgment. We are grateful to the Physical Methodology Department of G.D. Searle & Co. for spectra and analytical data and in particular to Mrs. Patricia M. Finnegan for her efforts in NMR spectra interpretation. We also acknowledge Prof. Charles B. Smith for his invaluable aid in our smooth muscle experiments.

Registry No. 1, 88373-73-3; 2, 137666-07-0; Boc-D-Pen(*p*-MeBzl)-OH, 115962-34-0.

Novel Anticancer Prodrugs of Butyric Acid. 2[1]

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The antitumor activity of novel prodrugs butyric acid was examined. The in vitro effect of the compounds on induction of cytodifferentiation and on inhibition of proliferation and clonogenicity showed that (pivaloyloxy)methyl butyrate (**1a**) (labeled AN-9) was the most active agent. SAR's suggested that its activity stemmed from hydrolytically released butyric acid. In vivo, **1a** displayed antitumor activity in B16F0 melanoma primary cancer model, manifested by a significant increase in the life span of the treated animals. Murine lung tumor burden, induced by injection of the highly metastatic melanoma cells (B16F10.9), was decreased by **1a**. It also displayed a significant therapeutic activity against spontaneous metastases which were induced by 3LL Lewis lung carcinoma cells. Moreover, **1a** has the advantage of low toxicity, with an acute $LD_{50} = 1.36 \pm 0.1$ g/kg ($n = 5$). These results suggest that **1a** is a potential antineoplastic agent.

Many well-recognized adverse effects accompany current conventional cancer chemotherapy treatment using aggressive cytotoxic agents, as a result of being nonspecific toward neoplastic cells and affecting normal cells as well. Cancer research seeks alternative methods of directly or indirectly stimulating the host's immune system, with minimal systemic toxicity and maximum specificity, and efficiency in eliminating tumor cells. Since cancer can be considered a disorder of cell differentiation, another approach which satisfies the requirements for specificity and low toxicity is induction of neoplastic cell differentiation. With the arrest of maturation, immature cells continue to proliferate, resulting in the emergence of clinically manifested cancer cells. Experimental evidence has demonstrated that tumor cells can be induced to differentiate, indicating that the malignant process can be at least partially reversed.[2] Retinoic acid, phorbol esters, DMSO, and butyric acid (BA) are among the compounds which have been shown to induce differentiation.

Butyric acid is a nontoxic natural product found in food (e.g. in butter it constitutes up to 5%). In the digestive system, it is secreted as a byproduct of microbial fermentation, and in the human colon it can reach millimolar concentrations. In a variety of human tumor cells grown in vitro, BA displayed antitumor activity, reflected in growth arrest, decreased clonogenicity, and induction of morphological and biochemical changes.[3]

Clinical trials with high doses of BA produced no detectable toxicity. In a child with acute myeloid leukemia,[4] it induced partial and temporary remission. However, no clinical activity was detected in an adult with myelomonocytic leukemia[5] or in nine adults with acute leukemia.[6]

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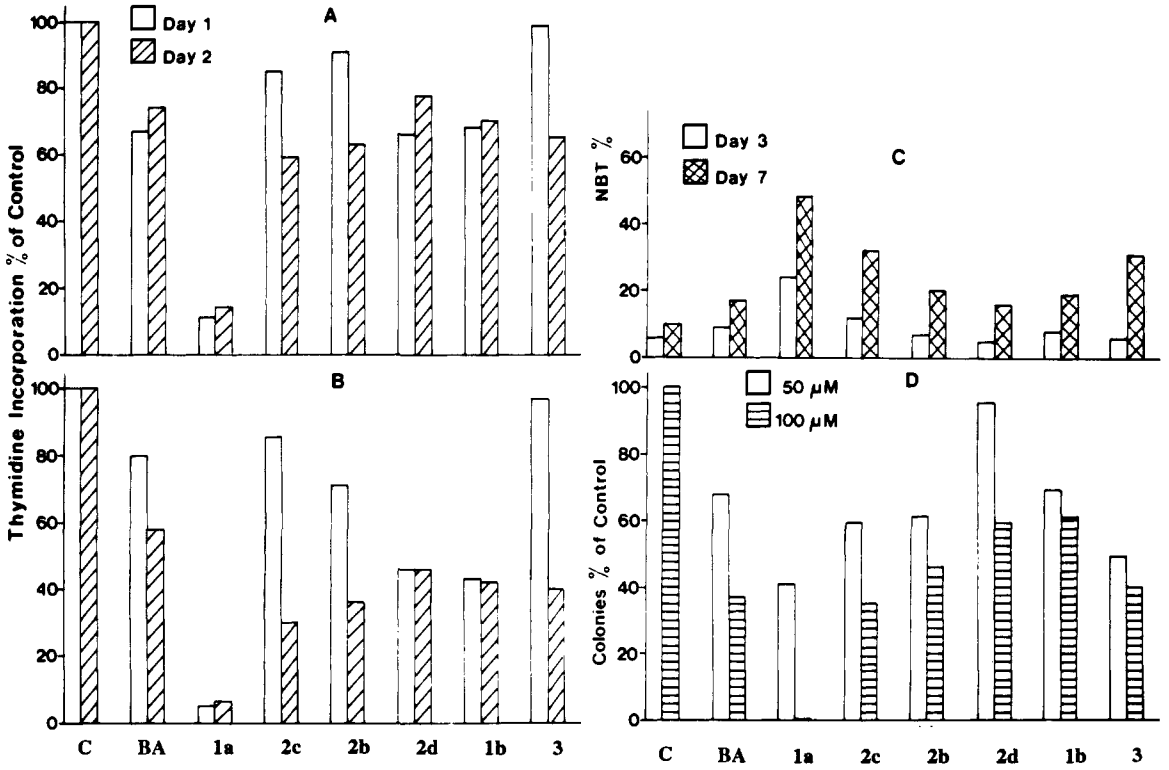


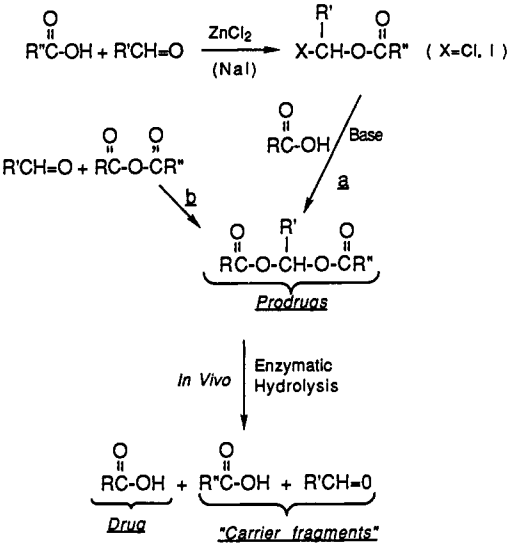
Figure 1. Effect of BA and its derivatives on proliferation, differentiation, and clonogenicity of HL-60 cells. Proliferation at 50 μM (A, 100% = 26 800 cpm) and 100 μM (B, 100% = 24 200 cpm). Differentiation (C) was assayed by the percent of cells capable of reducing NBT at 50 μM. Clonogenicity (D) at 50 and 100 μM, 100% = 106 ± 9 colonies. The data represent reproducible results obtained in at least two independent experiments.

The lack of clinical efficacy of the compound was attributed mainly to rapid metabolism, and to a lesser extent, excretion. In order to overcome these disadvantages, a search for novel prodrugs of BA which would impart reduction in clearance rates was undertaken. In this report, we describe an in vitro screen of a variety of such prodrugs and demonstrate the *in vivo* activity of the most active of these compounds, (pivaloyloxy)methyl butyrate, **1a**. Preliminary accounts of this work have appeared.[7]

Chemistry

The large number of drugs having carboxylic acid functions, i.e. penicillins, cephalosporins, nonsteroidal antiinflammatory agents, etc., prompted the development of prodrug-coupling reagents for the acidic group. The derivatives the most extensively explored belong to the family of (acyloxy)alkyl esters. They undergo intracellular esterase catalyzed hydrolysis to two acids and an aldehyde[8] and have led to clinically approved antibiotics such as

Scheme I



pivampicillin,[9a] bacampicillin,[9b] and talampicillin.[9c] Through this type of derivatization the drugs acquired increased stability and lipophilicity as well as oral activity.[10] Similar (acyloxy)alkyl prodrugs of 5-fluorouracil,

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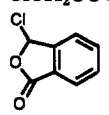
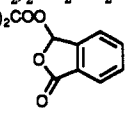
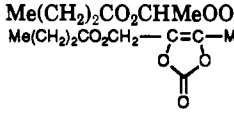
Table I. Structures of Reagents and Products

acid or anhydride	coupling reagent	no.	product
$Me(CH_2)_2CO_2H$	$ClCH_2OOCCMe_3$	**1a**	$Me(CH_2)_2CO_2CH_2OOCCMe_3$
$Me(CH_2)_2CO_2H$	$ClCH_2OOC(CH_2)_6Me$	**1b**	$Me(CH_2)_2CO_2CH_2OOC(CH_2)_6Me$
$Me(CH_2)_2CO_2H$		**1c**	
$Me(CH_2)_2CO_2H$	$ClCHMeOOCOEt$	**1d**	$Me(CH_2)_2CO_2CHMeOOCOEt$
$Me(CH_2)_2CO_2H$	#Ic	**1e**	
$Me(CH_2)_2CO_2H$	$ClCH_2OOCCHMe_2$	**1f**	$Me(CH_2)_2CO_2CH_2OOCCHMe_2$
$[Me(CH_2)_2CO]_2O$	$CH_2{=}O$	**2a**	$[Me(CH_2)_2CO_2]_2CH_2$
$[Me(CH_2)_2CO]_2O$	$MeCH{=}O$	**2b**	$[Me(CH_2)_2CO_2]_2CHMe$
$[Me(CH_2)_2CO]_2O$	$Me(CH_2)_2CH{=}O$	**2c**	$[Me(CH_2)_2CO_2]_2CH(CH_2)_2Me$
$[Me(CH_2)_2CO]_2O$	$Me_3CCH{=}O$	**2d**	$[Me(CH_2)_2CO_2]_2CHCMe_3$
$Me(CH_2)_2CO_2H$	glycerol	**3**	glyceryl tributyrate
$MeCH_2CO_2H$	$ClCH_2OOCCMe_3$	**4**[1]	$MeCH_2CO_2CH_2OOCCMe_3$
Me_2CHCO_2H	$ClCH_2OOCCMe_3$	**5a**[1]	$Me_2CHCO_2CH_2OOCCMe_3$
Me_2CHCO_2H	$ClCH_2OOCCHMe_2$	**5b**	$Me_2CHCO_2CH_2OOCCHMe_2$
$Me(CH_2)_3CO_2H$	$ClCH_2OOCCMe_3$	**6**[1]	$Me(CH_2)_3CO_2CH_2OOCCMe_3$
$BocNH(CH_2)_3CO_2H$	$ClCH_2OOC(CH_2)_2Me$	**7a**	$BocNH(CH_2)_3CO_2CH_2OOC(CH_2)_2Me$
$BocNH(CH_2)_3CO_2H$	$ClCH_2OOCCMe_3$	**7b**	$BocNH(CH_2)_3CO_2CH_2OOCCMe_3$
		8a	$NH_2(CH_2)_3CO_2CH_2OOC(CH_2)_2Me{\cdot}HCl$
		8b	$NH_2(CH_2)_3CO_2CH_2OOCCMe_3{\cdot}HCl$

which undergo intracellular hydrolysis in order to release the active drug, have been reported.[11] In an attempt to reduce the metabolic rate of BA, an analogous series of (acyloxy)alkyl butyrates was prepared (Scheme I), and their biological activity was examined in comparison to that of the free acid. In the prodrugs prepared via pathway a the R and R″ groups were different, whereas, in the symmetrical ones, prepared via pathway b, R and R″ were both butyryl groups. The methylidene[12a] (**2a**), ethylidene[12b,c] (**2b**), butylidene (**2c**), and 2,2-dimethylpropylidene (**2d**) dibutyrates were considered of special interest, since their hydrolysis would lead to the release of 2 equiv of butyrate/mole of compound. Moreover, **2c** could potentially yield 3 equiv of butyrate in vivo, provided the liberated butyraldehyde underwent rapid oxidation to the acid. In addition to the (acyloxy)alkyl derivatives, glyceryl tributyrate[13] (**3**) (Table I) was also examined.

Biology

The in vitro effects of BA and its derivatives on proliferation, clonogenicity, and differentiation of the leukemic promyelocytic cell line HL-60 (Figure 1, A–D) were studied. Proliferation and clonogenicity were evaluated by [^{3}H]thymidine incorporation and formation of colonies in semisolid agar, respectively. Compound **1a** (50 μM) brought about a rapid (after 24 h), ca. 90% inhibition in cell proliferation, whereas BA and the other compounds caused a maximum inhibition of 35%. Similar effects were seen after 48 h, both at 50 and 100 μM (A–B). At the latter concentration, **1a** completely inhibited formation of colonies (D). Cell differentiation was evaluated by the ability of the cells to reduce nitroblue tetrazolium (NBT), which correlated with subsequent morphological changes of the cells (C). Compound **1a** was the most effective differentiation inducer. Other butyrates, such as **1b**, **2a–d**, and **3**, were at most active as BA in comparison to **1a**, in spite of their similar lipophilicity and the fact that some of them were expected to release more than one butyrate unit per prodrug molecule. Unexpectedly, **1b**, **2a–d**, and **3**, which are capable of releasing more than one butyrate unit per prodrug molecule and have a lipophilicity similar to **1a**, were only as active as BA.

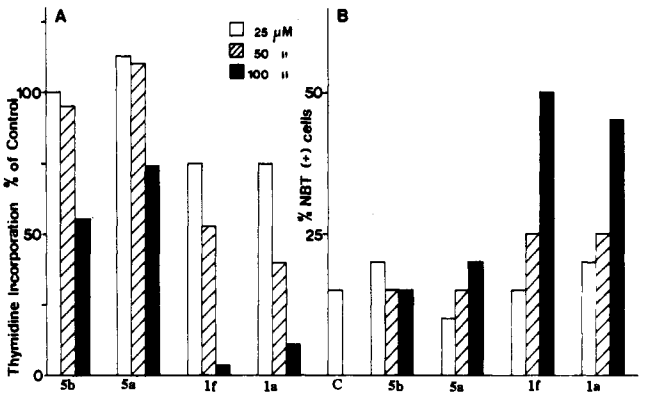


Figure 2. Proliferation (A) and differentiation (B) of HL-60 cells, after 72 h of exposure to isobutyrate. [^{3}H]Thymidine incorporation; 100% = 14477 cpm.

The ability of the various compounds to act as BA prodrugs in vitro depends on their ability to penetrate the cells and the efficiency by which intracellular esterases are capable of hydrolyzing these esters. The lack of activity of the unbranched di- and tributyrates (**2a–d** and **3**) may be due to lower penetration rates into the cells, slow hydrolysis, or a combination of both factors. Improved pharmacokinetics have been made in other prodrugs

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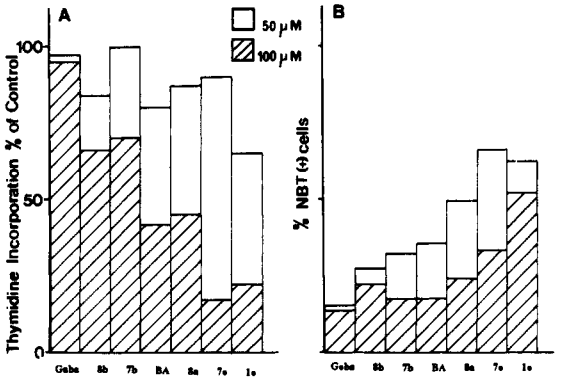

Figure 3. Comparative effect of BA, GABA, **1a**, and GABA derivatives on proliferation after 24 h (A, 100 = 18543 cpm), and differentiation (B) of HL-60 cells after 96 h of exposure to these compounds. The data represent reproducible results obtained in at least two independent experiments.

possessing pivaloyloxy groups.[9a,11]

Two acids and an aldehyde, stemming from the "drug" and the "coupling reagent" moieties, are released[8] in the course of (acyloxy)alkyl ester hydrolytic fragmentation. Therefore, it remained to be determined which of these fragments was responsible for the biological activity. The esters of propionic, isobutyric, and valeric acids (**4**, **5a**, and **6**), respectively, were inactive[1] (biological have been described), implying that neither the pivalic acid nor the formaldehyde fragments were responsible for the activity. Compound **1f**, where replacement of a pivaloyl by an isobutyroyl fragment took place, displayed activity comparable to that of **1a** (Figure 2). However, more drastic changes in the structure of the "coupling reagent" resulted in substantial reduction in the activity. In order to eliminate the possibility that the activity of **1f** stemmed from isobutyric acid, esters **5a–b** were prepared and were shown to be inactive (Figure 2). The latter, when taken together with the inactivity of **4**, **5a**, and **6**, strongly supports the notion that the biological activity of **1a** and **1f** indeed stems from the released butyric acid. A comparison of 4-aminobutyric acid (GABA) and compounds **8a–b** (Figure 3), further substantiates the dependence of the biological activity on the presence of a BA moiety. GABA, **7b**, and **8a** which lack a butyrate group were inactive. The butyrate derivatives **7a** and **8a** were more active than BA in inducing cell differentiation and displayed comparable cell proliferation inhibition. Moreover, **7a**, the more lipophilic of the two, displayed similar activity to **1a** in both tests. Compounds **1f** and **7a** which displayed in vitro activity are the subject of ongoing investigations.

All the compounds which in vitro inhibited proliferation and induced differentiation contained a butyrate fragment. However, the inverse is not necessarily true, since some compounds which contain a butyrate fragment were not more active than BA as inhibitors of proliferation and inducers of differentiation. Thus, the inclusion of a BA fragment is necessary but not sufficient for improved activity.

In spite of these results, the possibility that the intact **1a** molecule possesses intrinsic activity cannot be excluded. Furthermore, the observed activity may be the result of the combined activities of the intact **1a** and the released BA. A search for cell types or mutated cell lines which lack esterase activity, necessary for the degradation of **1a**, is being conducted. By employing such cells it may be possible to assess the intrinsic activity of **1a**.

The concentrations required for 50% proliferation inhibition (IC_{50}) of human promyelocytic HL-60 and murine myelomonocytic WEHI cell lines, were determined by [^{3}H]thymidine incorporation. The values were as follows: in HL-60, 1525 and 49.5, and in WEHI, 850 and 75 μM, for BA and **1a**, respectively (Figure 4). The inhibitory

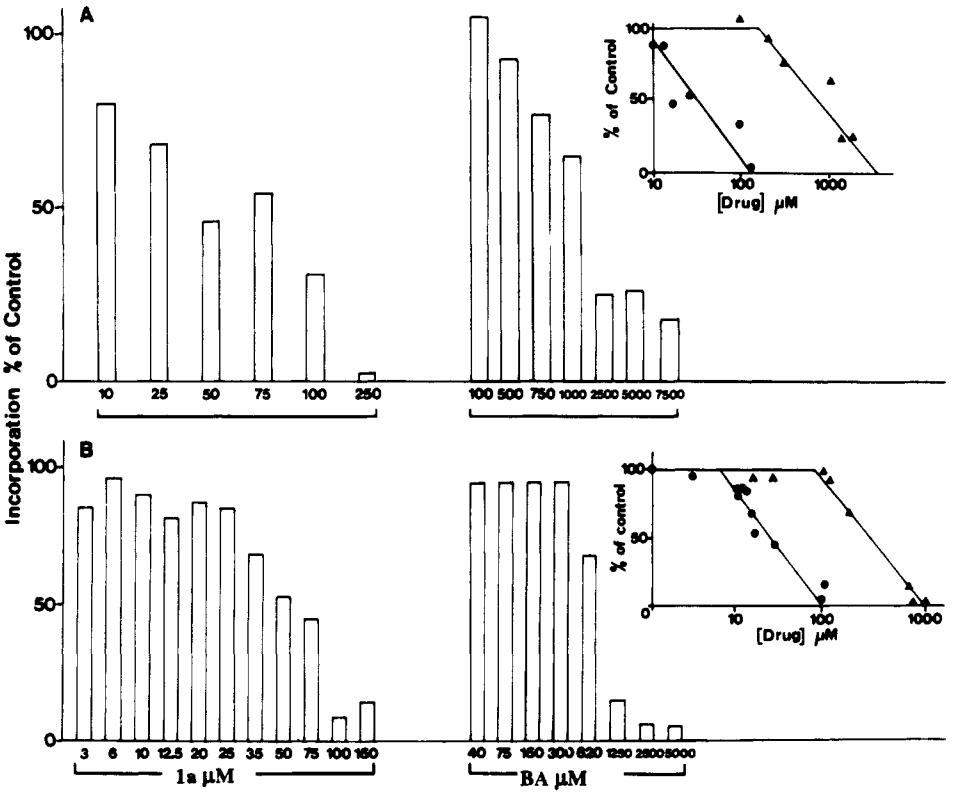

Figure 4. The effect of BA and **1a** on the proliferation of HL-60 (A) and WEHI (B) cells titration. [^{3}H]Thymidine incorporation was measured after 24 h; 100% = 9286 cpm (A), 100% = 7311 cpm (B). Inserts: percent [^{3}H]thymidine incorporation vs log of drug concentrations, **1a** (●); BA (▲). The data represent reproducible results obtained in at least two independent experiments.

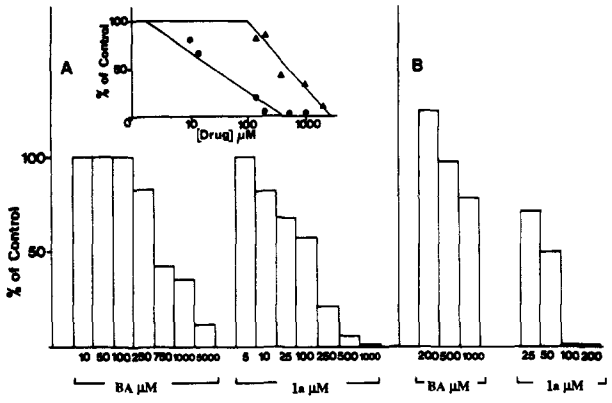

Figure 5. The effect of BA and **1a** on the growth (A, 100% = 3.5×10^5 cells/mL) and formation of colonies in semisolid agar of B16F10.9 cells (B, 100% = 39 ± 2). Insert: number of viable cells vs log of drug concentrations, **1a** (●); BA (▲). The data represent reproducible results obtained in at least two independent experiments.

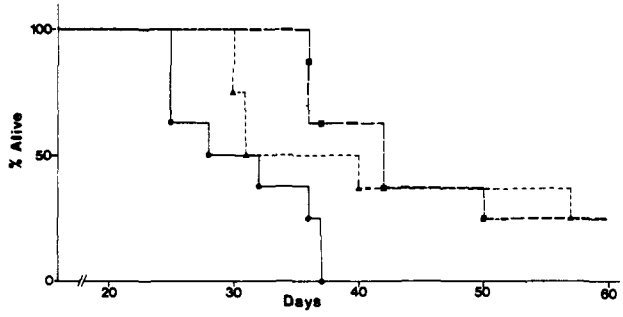

Figure 6. Effect of **1a** on the survival of $B_6C_3F_1$/OLA/HSD mice (groups of 8) implanted ip with B16F0 melanoma cells. The animals received nine consecutive ip injections of 1:1 PBS-propylene glycol (0.1 mL) for the control group, ●; cisplatin (2 mg/kg) as a positive control, ■; **1a** (250 mg/kg), ▲. The data represent reproducible results obtained in at least two independent experiments.

effect of **1a** on growth of melanoma cells (B16F10.9) was observed at lower concentrations of **1a** (IC_{50} = 82 μM) than those of BA (IC_{50} = 933 μM). The appearance of colonies in semisolid agar was inhibited by **1a** (IC_{50} = 60 μM), whereas BA at 1 mM decreased only 22% of the colonies (Figure 5). These results show that the three different cancer cell lines exhibited similar sensitivity to **1a** and were affected by it at concentrations an order of magnitude lower than that of BA. Either faster rate of intracellular penetration by the lipophilic **1a** and/or slower rate of metabolic degradation may be responsible for these differences. Consistent with our findings is the suggestion that the fragment responsible for the prodrug activity is BA. Therefore, it can be postulated that the intracellular cleavage of the prodrug takes place at a site where the released BA is "protected" from metabolic degradation and therefore becomes accessible to its cellular target. However, the intracellular target and mode of action of BA remain unknown.

The anticancer activity of **1a** in a primary model of murine B16FO melanoma[14] was examined in animals treated for nine sequential days with **1a** (ip, 250 mg/kg) or cisplatin (2 mg/kg) as a positive control. Respective mean survival times of 45 days (% T/C 148) and 49 days (% T/C 161) were obtained, indicating a significant increase in the life span of the treated animals (Figure 6). Postmortem examination did not reveal macroscopic lesions in the lungs.

(14) U.S. Department of Health and Human Services: *In Vivo* Cancer Models, NIH publication No. 84-2635, 1984.

Table II. Effect of **1a** on Experimental Metastases of B16F10.9 Melanoma

test compound	dose, mg/kg	mean	standard deviation	$P \times 10^{-3}$
		(a) Lung Weight (g)		
solvent	–	0.3600	0.18	–
cytoxan	50	0.0856	0.05	0.2
1a	12.5	0.1571	0.09	8.6
1a	25	0.1350	0.15	7.9
1a	50	0.0929	0.05	3.7
1a	100	0.2686	0.15	NS[a]
		(b) Number of Lung Lesions		
solvent	–	174.00	59.97	–
cytoxan	50	56.38	20.17	0.1
1a	12.5	97.89	57.62	10.7
1a	25	72.38	51.29	1.3
1a	50	66.60	37.81	2.2
1a	100	140.29	64.89	NS

[a] NS (not significant); $P > 0.05$%.

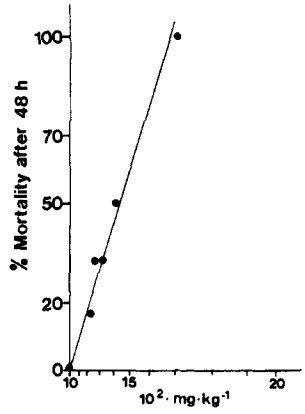

Figure 7. Acute LD_{50} of **1a** in male Balb/c mice (groups of 10). Similar results were obtained in five reproducible and independent experiments using Balb/c and C57Bl mice (male and female).

The effect of **1a** on the development of experimentally induced metastases of B16F10.9 melanoma was also evaluated. The animals were sacrificed 3 weeks after iv inoculation of tumor cells. A significant ($P < 0.0044$) decrease in the number of lung lesions and weight of the treated mice (C57B1/6) with a good correlation between these two parameters was observed (Table II). The optimal dose of **1a** was 50 mg/kg; at higher doses, a decrease in therapeutic activity was observed. The in vivo results correlate with the in vitro data presented in Figure 5.

In spontaneous metastases of 3LL Lewis lung carcinoma, **1a** also displayed significant therapeutic activity. Significant activity (% T/C = 177) was detected when **1a** (15.6 mg/kg, solubilized in pure propylene glycol) was administered ip for 5 days. At higher doses the anticancer activity decreased.

In the three animal cancer models described, **1a** displayed significant activity. However, the effective doses in each model varied. This may reflect a lack of optimization in mode and schedule of administration. Further study of these parameters is under investigation.

The low toxicity of **1a** was demonstrated in reproducible acute toxicity experiments with female and male BALB/c (3 experiments) and C57B1 mice (2 experiments). The mice (weighing 20 ± 0.5 g) were given a single injection of **1a** (0.125–2.5 g/kg) in propylene glycol (0.1 g/kg). Survival after 48 h was determined, and an LD_{50} = 1.36 ± 0.1 g/kg (n = 5) value was explained; the results of a representative

experiment are shown in Figure 7.

The renewed interest in cytodifferentiating agents such as retinoic acid,[15a] α-interferon,[15b] γ-interferon,[15c] and colony stimulating factors[15d] as potential anticancer substances has brought about significant clinical results. The in vitro activity of BA as a differentiating factor is well documented. However, in clinical trials and in vivo models, BA has been ineffective. In this report we described a novel BA prodrug which possesses in vitro as well as in vivo anticancer activity and may be potentially useful in differentiation therapy.

Experimental Section

^{1}H NMR spectra 300-MHz were obtained on a Brucker WH-300 spectrometer in $CDCl_3$ solvent. Chemical shifts were expressed in ppm downfield from Me_4Si used as internal standard. The values are given in δ scale. Mass spectra were obtained on a Varian Mat 731 spectrometer (CI = chemical ionization). Progress of the reactions was monitored by TLC on silica gel (Merck, Art. 5554) or alumina (Riedel-de Haen, Art. 37349). Flash chromatography was carried out on silica gel (Merck, Art. 9385). Chloromethyl pivalate, 3-chlorophthalide, glyceryl tributyrate (Aldrich or Janssen), and 1-chloroethyl ethyl carbonate (Tokyo Kasei) were purchased. Methylidene dibutyrate and 4-((*tert*-butoxycarbonyl)amino)butyric acid (Boc-GABA) were prepared by literature procedures.

Chloromethyl Octanoate.[16,17] To a stirred mixture of octanoic acid (9.6 g, 66 mmol), H_2O (66 mL), CH_2Cl_2 (66 mL), Na_2CO_3 (21.26 g, 25 mmol), and tetrabutylammonium hydrogen sulfate (2.26 g, 6.6 mmol) was added a solution of chloromethyl chlorosulfate (12.72 g, 76 mmol) in CH_2Cl_2 at such a rate that the temperature remained below 30 °C. Stirring was continued for 24 h. The organic phase was separated, dried over $MgSO_4$, and evaporated, and the residue was fractionally distilled to give chloromethyl octanoate[17] (10 g, 80%), bp 47–8 °C (0.2 Torr). Anal. ($C_9H_{17}ClO_2$) C, H, Cl.

Chloromethyl 2-methylpropanoate was prepared as described[18a,b,19] for chloromethyl 2,2-dimethylpropanoate,[20] bp 40–2 °C (2 Torr).

Iodomethyl Octanoate. An ice-cold solution of chloromethyl octanoate (7.9 g, 42 mmol) and NaI (18.9 g, 0.12 mol) in acetone (100 mL), stirred for 24 h in the dark, was filtered, and the filtrate was concentrated. The residue, dissolved in ether, was washed with aqueous $Na_2S_2O_3$, dried over K_2CO_3, concentrated, and used as such without further purification.

((2,2-Dimethylpropanoyl)oxy)methyl Butanoate (1a). To butyric acid (5.7 mL, 40 mmol) and chloromethyl pivalate (18 mL, 1 mmol) or their solution in acetone (10 mL) was added Et_3N (12.17 mL, 88 mmol). The mixture, stirred at room temperature for 24 h, was evaporated, and the residue was taken up in a H_2O–EtOAc mixture. The organic phase was separated, dried over K_2CO_3, filtered, and evaporated. The product was obtained by fractional distillation (4.42 g, 57%): bp 88–93 °C (2 Torr); ^{1}H NMR δ 0.95 (t, J = 8 Hz, 3 H, Me), 1.21 (s, 9 H, t-Bu), 1.67 (sextet, J = 8 Hz, 2 H, MeCH_2), 2.34 (t, J = 8 Hz, 2 H, CH_2O), 5.75 (s, 2 H, OCH_2); MS (CI) m/e 203 (MH^+). Anal. ($C_{10}H_{18}O_4$) C, H.

Compounds **1b, 1e, 1f, 4, 5a, 5b,** and **6** were prepared as described for **1a** while replacing butyric acid and chloromethyl pivalate for the respective acids and halo reagents.

(Butanoyloxy)methyl octanoate (1b) was prepared from butyric acid and chloromethyl or iodomethyl octanoate: bp 136–8 °C (1.5 Torr); ^{1}H NMR δ 0.87 (t, J = 8 Hz, 3 H, Me), 0.92 (t, J = 8 Hz, 3 H, Me), 1.1–1.3 (m, 8 H, four CH_2's), 1.6–1.7 (m, 4 H, two CH_2's), 2.11–2.38 (m, 4 H, two CH_2CO), 5.70 (s, 2 H, OCH_2); MS (CI) m/e 245 (MH^+). Anal. ($C_{13}H_{24}O_4$) C, H.

3-(Butanoyloxy)phthalide (1c). To a solution of butyric acid (1.1 g, 12.5 mmol) in DMF (13 mL) were added Et_3N (1.78 g, 17.6 mmol) and 3-chlorophthalide (2.1 g, 12.5 mmol). The mixture was stirred for 8 days, poured into EtOAc (40 mL), and filtered, and the filtrate was washed four times with H_2O (15 mL/wash), dried over $MgSO_4$, and concentrated. The residue was recrystallized from hexane–CH_2Cl_2, 1:1, to give 1c (2.28 g, 82.8%): mp 52–3 °C; ^{1}H NMR δ 0.99 (t, J = 7.4 Hz, 3 H, Me), 1.72 (sextet, J = 7.4 Hz, 2 H, CH_2Me), 2.42 (t, J = 7.4 Hz, 2 H, CH_2CO), 7.46 (s, 1 H, CH), 7.58 (dd, J = 0.7, 8.2 Hz, 1 H, H-5), 7.65 (dt, J = 1.0, 8.2 Hz, 1 H, H-4), 7.76 (dt, J = 1.1, 7.5 Hz, 1 H, H-3), 7.93 (dd, J = 0.7, 7.5 Hz, 1 H, H-2); MS (CI) m/e 221 (MH^+). Anal. ($C_{12}H_{12}O_4$) C, H.

1-(Butanoyloxy)ethyl Ethyl Carbonate (1d). To a solution of butyric acid (0.58 g, 6.55 mmol) and 1-chloroethyl ethyl carbonate (1 g, 6.55 mmol) in AcCN (15 mL) were added H_2O (2 drops), benzyltrimethylammonium hydroxide (2 drops of a 40% aqueous solution) and KOH (0.44 g, 7.86 mmol). The mixture, stirred under reflux for 4 h, was filtered, and ether was added to the filtrate. The combined organic phase was three times washed with H_2O, dried over $MgSO_4$, and filtered. The filtrate was concentrated, and the residue was distilled in a Kugelrohr apparatus (at 75 °C (0.1 Torr)), to give 8 (0.57 g, 42.7%): ^{1}H NMR δ 0.95 (t, J = 7.5 Hz, 3 H, Me), 1.318 (t, J = 7 Hz, 3 H, OCH_2Me), 1.52 (d, J = 5 Hz, 3 H, $CHMe$), 1.66 (sextet, J = 7.5 Hz, 2 H, $MeCH_2CH_2$), 2.32 (t, J = 7.5 Hz, 2 H, CH_2CO), 4.22 (q, J = 7 Hz, 2 H, MeCH_2), 6.77 (q, J = 5 Hz, 1 H, CH); MS (CI) m/e 195 (MH^+). Anal. ($C_9H_{16}O_5$) C, H.

(5-Methyl-2-oxo-1,3-dioxolan-4-yl)methyl butanoate (1e) was obtained from 4-(bromomethyl)-5-methyl-2-oxo-1,3-dioxolane[18] and butyric acid: bp 100–4 °C (3 Torr); ^{1}H NMR δ 0.94 (t, J = 6 Hz, 3 H, Me), 1.64 (sextet, J = 6 Hz, 2 H, MeCH_2), 2.15 (s, 3 H, Me), 2.31 (t, J = 6 Hz, 2 H, CH_2CO), 4.82 (s, 2 H, OCH_2); MS (CI) m/e 201 (MH^+). Anal. ($C_9H_{12}O_5$) C, H.

((2-Methylpropanoyl)oxy)methyl butanoate (1f) was prepared from butyric acid and chloromethyl isobutyrate: bp 70–2 °C (0.7 Torr); ^{1}H NMR δ 0.96 (t, J = 8 Hz, 3 H, Me), 1.19 (d, J = 8 Hz, 6 H, Me), 1.69 (sextet, J = 8 Hz, 2 H, MeCH_2), 2.36 (t,

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J = 8 Hz, 2 H, CH_2CO), 2.60 (septet, J = 8 Hz, 1 H, CH), 5.77 (s, 2 H, OCH_2); MS (CI) m/e 189 (MH^+). Anal. ($C_9H_{16}O_4$) C, H.

((2,2-Dimethylpropanoyl)oxy)methyl propanoate (4) was prepared from propionic acid and chloromethyl pivalate: bp 80–2 °C (2 Torr); ^1H NMR δ 1.08 (t, J = 10 Hz, 3 H, Me), 1.10 (s, 9 H, t-Bu), 2.18 (q, J = 10 Hz, 2 H, CH_2), 5.78 (s, 2 H, OCH_2); MS (CI) m/e 189 (MH^+). Anal. ($C_9H_{16}O_4$) C, H.

((2,2-Dimethylpropanoyl)oxy)methyl 2-methylpropanoate (5a) was prepared from isobutyric acid and chloromethyl pivalate: bp 80–3 °C (2 Torr); ^1H NMR δ 1.18 (d, J = 8 Hz, 6 H, Me), 1.20 (s, 9 H, t-Bu), 2.58 (septet, J = 8 Hz, 1 H, CH), 5.75 (s, 2 H, OCH_2); MS (CI) m/e 203 (MH^+). Anal. ($C_{10}H_{10}O_4$) C, H.

((2-Methylpropanoyl)oxy)methyl 2-methylpropanoate (5b) was prepared from isobutyric acid and chloromethyl isobutyrate: bp 70–3 °C (2 Torr); ^1H NMR δ 1.10 (d, J = 7 Hz, 12 H, four Me), (septet, J = 7 Hz, 2 H, CH), 5.77 (s, 2 H, CH_2); MS (CI) m/e 189 (MH^+). Anal. ($C_9H_{16}O_4$).

((2,2-Dimethylpropanoyl)oxy)methyl pentanoate (6) was obtained from valeric acid and chloromethyl pivalate: bp 81–3 °C (2 Torr); ^1H NMR δ 0.82 (t, J = 7 Hz, 3 H, Me), 0.90 (sextet, J = 7 Hz, 2 H, CH_2Me), 1.20 (s, 9 H, t-Bu), 1.59 (quintet, J = 7 Hz, 2 H, CH_2CH_2CO), 2.36 (t, J = 8 Hz, 2 H, CH_2CO), 5.78 (s, 2 H, OCH_2); MS (CI) m/e 217 (MH^+). Anal. ($C_{11}H_{20}O_4$) C, H.

Ethylidene Dibutanoate (2b).[12b,c] Ice-cooled boron trifluoride etherate (8.66 g, 61 mmol) was added dropwise, over 1 h, to a mixture of butyric anhydride (6.58 g, 41.6 mmol) and acetaldehyde (1.22 g, 27.7 mmol). The mixture was stirred for an additional 2 h, aqueous AcONa (10% solution, 28 mL) was added, and stirring was continued for an additional 45 min. The oily layer was extracted with ether, and the combined ethereal phase was washed with saturated aqueous Na_2CO_3 until CO_2 evolution stopped. The organic phase was washed with H_2O, dried over $MgSO_4$, and concentrated, and the residue was distilled at 8–12 Torr. The fraction which distilled up to 62 °C was collected (2.18 g, 39%): ^1H NMR δ 0.95 (t, J = 7.5 Hz, 6 H, CH_2Me), 1.47 (d, J = 5.5 Hz, 3 H, CHMe), 1.65 (sextet, J = 7.5 Hz, 4 H, $MeCH_2$), 2.30 (dt, J = 0.75, 7.5 Hz, 4 H, CH_2CO), 6.88 (q, J = 5.5 Hz, 1 H, CH).

Compounds **2c** and **2d** were prepared as described for **2b**, while replacing acetaldehyde for the corresponding aldehydes.

Butylidene dibutanoate (2c) (from butyraldehyde): bp 125–135 °C (12–15 Torr) (49%); ^1H NMR δ 0.949 and 0.953 (2 t, J = 7.5 Hz, 9 H, Me), 1.40 (q, J = 7.5 Hz, 2 H, CH_2CH_2CH), 1.65 (sextet, J = 7.5 Hz, 4 H, $MeCH_2CH_2$), 1.75 (m, 2 H, CH_2CH), 2.30 (m, 4 H, CH_2CO), 6.82 (t, J = 5.6 Hz, 1 H, CH_2CH); MS (CI) m/e 231 (MH^+). Anal. ($C_{12}H_{22}O_4$) C, H.

2,2-Dimethylpropylidene dibutanoate (2d) (from pivalaldehyde): bp 110–3 °C (5–6 Torr); ^1H NMR δ 1.49 (t, J = 7.3 Hz, 6 H, Me), 1.50 (s, 9 H, t-Bu), 2.20 (sextet, J = 7.4 Hz, 4 H, $MeCHCH_2$), 2.31 (t, J = 7.3 Hz, 1 H, CHCO), 6.59 (s, 1 H, CH); MS (CI) m/e 245 (MH^+). Anal. ($C_{13}H_{24}O_4$) C, H.

4-((*tert*-Butoxycarbonyl)amino)butanoic acid:[21] ^1H NMR δ 1.47 (s, 9 H, t-Bu), 1.80 (quintet, J = 7 Hz, 2 H, $CH_2CH_2CH_2$), 2.40 (t, J = 7 Hz, 2 H, CH_2CO), 3.18 (broad q, J = 7 Hz, 2 H, $NHCH_2$), 4.7 (br s, 1 H, NH).

((4-((*tert*-Butoxycarbonyl)amino)butanoyl)oxy)methyl butanoate (7a) (from Boc-GABA and chloromethyl butyrate): bp 150–2 °C (0.3 Torr); ^1H NMR δ 0.96 (t, J = 7 Hz, 3 H, Me), 1.45 (s, 9 H, t-Bu), 1.67 (sextet, J = 7 Hz, 2 H, $MeCH_2$), 1.83 (quintet, J = 7 Hz, 2 H, CH_2CH_2CO), 2.35 (t, J = 7 Hz, 2 H, CH_2CO), 2.40 (t, J = 7 Hz, 2 H, CH_2CO), 3.17 (q, J = 7 Hz, 2 H, NCH_2), 4.85 (br s, 1 H, NH), 5.75 (s, 2 H, OCH_2); MS (CI) m/e 304 (MH^+). Anal. ($C_{14}H_{25}NO_6$) C, H, N.

((4-((*tert*-Butoxycarbonyl)amino)butanoyl)oxy)methyl 2,2-dimethylpropanoate (7b) (from Boc-GABA and chloromethyl pivalate): bp 170–2 °C (0.2 Torr); ^1H NMR δ 1.20 (s, 9 H, t-Bu), 1.40 (s, 9 H, t-Bu), 1.80 (quintet, J = 7 Hz, 2 H, CH_2CH_2CO), 2.40 (t, J = 7 Hz, 2 H, CH_2CO), 3.14 (broad q, J = 7 Hz, 2 H, $HNCH_2$), 4.6 (br s, 1 H, NH), 5.73 (s, 2 H, OCH_2); MS (CI) m/e 318 (MH^+). Anal. ($C_{15}H_{27}NO_6$) C, H, N.

((4-Aminobutanoyl)oxy)methyl butanoate, hydrochloride (8a) was obtained from **7a** as described for **7b**: ^1H NMR δ 0.94 (t, J = 7 Hz, 3 H, Me), 1.67 (sextet, J = 7 Hz, 2 H, $MeCH_2$), 2.1 (br m, 2 H, CH_2CH_2CO), 2.34 (t, J = 7 Hz, 2 H, CH_2CO), 2.36 (t, J = 7 Hz, 2 H, CH_2CO), 3.15 (br m, 2 H, NCH_2), 5.75 (s, 2 H, OCH_2); MS (CI) m/e 204 (MH^+). Anal. ($C_9H_{17}NO_4$·HCl) C, H, N.

((4-Aminobutanoyl)oxy)methyl 2,2-Dimethylpropanoate, Hydrochloride (8b). A solution of **7b** (3.17 g, 10.1 mmol) in anisole (3 mL) was saturated with gaseous HCl. After a few minutes the solvent was evaporated under vacuum and the residue was triturated with anhydrous ether to give the product as a soft solid: ^1H NMR δ 1.20 (s, 9 H, t-Bu), 2.15 (quintet, J = 7 Hz, 2 H, CH_2CH_2CO), 2.59 (t, J = 7 Hz, 2 H, CH_2CO), 3.14 (br m, 2 H, NCH_2), 5.73 (s, 2 H, OCH_2); MS (CI) m/e 218 (MH^+). Anal. ($C_{10}H_{19}NO_4$·HCl) C, H, N.

Biology. General. Commercial products: RPMI-1640, Gibco (Grand Island NY); fetal calf serum (FCS) and all other tissue culture reagents, Beth-Haemek Cell Culture (Israel); agar, Difco (Michigan, USA); [^{3}H]thymidine, 2 Ci/mmol, Amersham (UK). $B_6C_3F_1$/OLAC/HSD mice (C57BlxBalb c), 6–8 weeks old, Olac (UK); C57Bl and BALB/c mice, 6–8 weeks old, Beilinson Medical Center animal colony (Israel); B16F0 cell line (CRL 6322), American Type Culture Collection; a highly metastatic clone of B16 cells subclone B16F10.9, HL-60, and WEHI-Moore cell lines, from the laboratories of L. Eisenbach, M. Rubinstein, and J. Lotem from The Weizmann Institute of Science, Israel, respectively. The cells were grown in RPMI and 10% fetal calf serum, supplemented with 2 mM glutamine, and incubated at 37 °C in a humidified 5% CO_2 incubator. Viability was determined by trypan blue exclusion. All the experiments described in the text were repeated at least three times. In Figure 7 is shown the result of a representative and reproducible experiment.

Cell differentiation was evaluated by nitroblue tetrazolium (NBT) reduction activity.[22] Cell cultures containing 0.1% NBT were stimulated with 400 nM 12-*O*-tetradecanoylphorbol 13-acetate (PMA). The cells were incubated for 30 min at 37 °C and examined microscopically by scoring at least 200 cells. Differentiation was also evaluated by the morphology of May Greenwald-Gimsa stained cytospan slide preparations of the cells.

Cell proliferation was measured by incorporation of [^{3}H]-thymidine into cells. During the last 14 h of incubation, cells in 96-well plates were pulsed with [^{3}H]thymidine, 1 μCi/well, and were harvested with a cell harvester (microtiter Dynatech), using glass microfiber filters (Tamar, Israel). [^{3}H]Thymidine incorporation into the acid-insoluble fraction, retained on the filter, was determined. Comparison between [^{3}H]thymidine incorporation activity and the number of viable cells in the culture, indicated that the incorporation reliably reflected the changes in cell proliferation.

Growth Inhibition of B16F10.9 Melanoma. The effect of **1a** and BA on the growth of the cells was followed by seeding 4 × 10^4 cells in 35-mm Petri dishes; 6 h later, **1a** or BA was added. On day 4 the plates were washed and the cells were suspended in PBS and 1 mM EDTA. Cells were counted using a Coulter counter.

Formation of HL60 and B16F10.9 Colonies in Semisolid Agar. The procedure used was based on the method described,[23] modified in the medium composition of the bilayer culture (total volume of 0.6 mL of RPMI). The composition (% v/v) of the layers consisted of condition medium (40):FCS (10):horse serum (15):agar (0.5) in the lower layer, and FCS (20):horse serum (20):agar (0.36) and 10^3 cells, in the upper layer, placed in 17-mm wells. BA and **1a** were added at the indicated concentrations to the upper layer. Aggregates of >20 cells, which developed on the semisolid agar bilayer after 7 days, were scored as colonies, using an inverted microscope.

Antitumor Effect of 1a on B16F0 Melanoma. The experiment was conducted with 8–9-week-old $B_6C_3F_1$/OLAC/HSD mice (groups of 8) implanted ip with 5 × 10^5 B16F0 melanoma cells.

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Treatment commenced 1 day after cell inoculation. **1a** solubilized in 1:1 PBS–propylene glycol, was administered ip for 9 consecutive days, and the evaluation was done after 60 days by the percent ratio of mean survival time of treated and untreated animals.

Experimentally Induced Melanoma Metastases. B16F10.9 cells (2×10^4) were injected to the lateral veins in the tail of C57BL/6 mice (groups of 8), 8–9 weeks old (20 g). The animals, were treated ip every other day with **1a** (solubilized in 1:1:8 ethanol–DMSO–propylene glycol) or with a single 50 mg/kg injection of cytoxan (cyclophosphamide) as positive control, given on day 1. After 3 weeks the animals were sacrificed, lungs were weighed, and lesions were counted after fixation in Bouin's fixative using a Zeiss stereomicroscope.

Antitumor Effect of 1a on Spontaneous Metastases of 3LL. Mice (groups of 19) were given sc to the left hind footpad 6×10^5 log phase 3LL cells. When the tumor reached a diameter of approximately 1 cm, the primary foot pad tumor and the regional lymph nodes were removed, and treatment commenced 1 day after surgery.

Registry No. **1a**, 122110-53-6; **1b**, 138460-00-1; **1c**, 108761-29-1; **1d**, 122110-52-5; **1e**, 138460-01-2; **1f**, 137373-55-8; **2a**, 55696-44-1; **2b**, 25572-25-2; **2c**, 117802-47-8; **2d**, 138460-02-3; **3**, 60-01-5; **4**, 137373-52-5; **5a**, 137373-53-6; **5b**, 55696-45-2; **6**, 137373-54-7; **7a**, 138460-03-4; **7b**, 138460-04-5; **8a**, 138460-05-6; **8b**, 138460-06-7; $Me(CH_2)_2CO_2H$, 107-92-6; $[Me(CH_2)_2CO]_2O$, 106-31-0; $MeCH_2CO_2H$, 79-09-4; Me_2CHCO_2H, 79-31-2; $Me(CH_2)_3CO_2H$, 109-52-4; $BocNH(CH_2)_3CO_2H$, 57294-38-9; $ClCH_2OOCCMe_3$, 18997-19-8; $ClCH_2OOC(CH_2)_6Me$, 61413-70-5; $ClCHMeOOCOEt$, 50893-36-2; $ClCH_2OOCCHMe_2$, 61644-18-6; $CH_2{=}O$, 50-00-0; $MeCH{=}O$, 75-07-0; $Me(CH_2)_2CH{=}O$, 123-72-8; $Me_3CCH{=}O$, 630-19-3; $ClCH_2OOC(CH_2)_2Me$, 33657-49-7; octanoic acid, 124-07-2; chloromethyl chlorosulfate, 49715-04-0; iodomethyl octanoate, 111013-41-3; 3-chlorophthalide, 6295-21-2; 4-(bromomethyl)-5-methyl-2-oxo-1,3-dioxolane, 80715-22-6; glycerol, 56-81-5.

Synthesis and Structure–Activity Relationships of Acyclic ω Chain Conjugated Diene Analogues of Enisoprost

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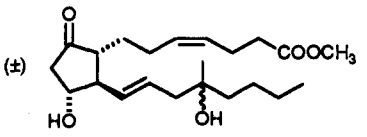
A series of acyclic ω chain conjugated diene analogues of enisoprost were synthesized and evaluated for gastric antisecretory and diarrheagenic activities in comparison to enisoprost and a previously identified cyclic dienyl analogue. Several novel approaches to the cuprate reagents involved in the synthesis of the series are described. From this SAR study, it appears that both the conjugated diene and the overall space filling characteristics of the ω chain are important components to the pharmacological profiles and selectivity of these compounds and that a cyclic structure is not required.

Introduction

In a previous paper[2] we reported that incorporation of Δ^{17} unsaturated cycloalkyl and cycloalkenyl functionality into the ω chain of enisoprost **1** produced compounds with increased separation of gastric antisecretory activity from diarrheagenic side effects. In particular the 17(*E*)-18-cyclopentenyl compound **2** displayed antisecretory activity



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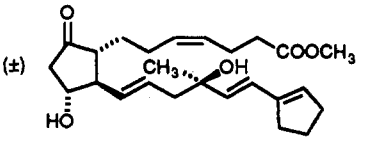

2

comparable to enisoprost but, unlike enisoprost, was virtually devoid of diarrheagenic activity. In an effort to determine the relative contributory roles of the conjugated diene system and the terminal cyclic structure to the selectivity of **2**, we decided to examine a series of acyclic conjugated dienes in which the diene system was either unsubstituted (**5f**) or methylated at C-19,20 (**5a–e,g,h**) to serve as test mimics of the cyclopentene structure of **2**. In addition the 17- and 18-methyl analogues (**5i,j**) were also prepared to complete the methyl substitution pattern along the diene system (Table I).

Chemistry

Compounds **5a–j** of Table I were prepared by conjugate addition of the respective racemic cuprate reagents **4a–j** to the racemic cyclopentenone **3**[3] followed by mild acid hydrolysis of protecting groups with pyridinium *p*-toluenesulfonate (PPTS) in aqueous acetone (Figure 1). As in previous work,[2] use of PPTS was required to avoid acid-catalyzed allylic rearrangement and elimination of the 16-hydroxyl group caused by stronger acidic conditions. Chromatographic purification on silica gel provided the desired compounds **5a–j** and their corresponding diastereomers **6a–j**. Configurational assignments of **5** and **6** were based on chromatographic elution sequence and biological activity.[2] Thus gastric antisecretory activity was observed only with the slower eluting compounds **5a–j** which were assigned the same relative stereochemistry as the bioactive isomers of misoprostol and enisoprost.

The cuprate reagents **4a–j** were accessed by four distinct routes (Figures 2–6). These routes represent an evolu-

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