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<sub>50</sub> value was defined as the dose that produced 1/2 the maximum possible increase in latency (i.e. 25 s). Calculations of ED<sub>50</sub> 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<sup>1</sup>

Abraham Nudelman,<sup>\*,†</sup> Margaretta Ruse,<sup>†</sup> Adina Aviram,<sup>‡</sup> Ester Rabizadeh,<sup>‡</sup> Matityahu Shaklai,<sup>‡</sup> Yael Zimrah,<sup>‡</sup> and Ada Rephaeli<sup>\*,‡</sup>

Chemistry Department, Bar-Ilan University, Ramat-Gan, 52910, Israel, and Hematology Division, Beilinson Hospital, Petach Tikva, 49100, Israel. Received June 17, 1991

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.<sup>2</sup> 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.<sup>3</sup>

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

(4) Novogradsky, A.; Dvir, A.; Ravid, A.; Shkolnik, T.; Stenzel, K. H.; Rubin, A. L.; Zaizov, R. Effect of Polar Organic Compounds on Leukemic Cells. *Cancer* 1983, 51, 9-14.

<sup>&</sup>lt;sup>†</sup>Bar-Ilan University.

<sup>&</sup>lt;sup>‡</sup>Beilinson Hospital.

Rephaeli, A.; Rabizadeh, E.; Aviram, A.; Shaklai, M.; Ruse, M.; Nudelman, A. Derivatives of Butyric Acid as Potential Anti-Neoplastic Agents. Int. J. Cancer 1991, 49, 66-72.

<sup>(2)</sup> Bloch, A. Induced Cell Differentiation in Cancer Therapy. Cancer Treat. Rep. 1984, 68, 199-205.

<sup>(3) (</sup>a) Prasad, K. N. Butyric Acid: A Small Fatty Acid with Diverse Biological Functions. *Life Sci.* 1980, 27, 1351-1358.
(b) Januszewicz, E.; Rabizadeh, E.; Novogradsky, A.; Shaklai, M. Inhibition of Non-Leukemic and Chronic Myeloid Leukemia, Granulocyte Macrophage Clonal Growth. *Med. Oncol. Tumor Pharmacother.* 1988, 5, 259-263.



**Figure 1.** Effect of BA and its derivatives on proliferation, differentiation, and clonogenicity of HL-60 cells. Proliferation at 50  $\mu$ M (A, 100% = 26 800 cpm) and 100  $\mu$ M (B, 100% = 24 200 cpm). Differentiation (C) was assayed by the percent of cells capable of reducing NBT at 50  $\mu$ M. Clonogenicity (D) at 50 and 100  $\mu$ M, 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.<sup>7</sup>

## 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<sup>8</sup> and have led to clinically approved antibiotics such as

- (5) Rephaeli, A.; Rabizadeh, E.; Nordenberg, J.; Kluska, A.; Prokocimer, M.; Rosenberg, I.; Novogradsky, A.; Shaklai, M. Effect of the Cell Differentiation Inducers, Butyrate, Retinoic Acid and Low Dose Cytosine Arabinoside on Leukemic Cells of Patients with Acute Nonlymphatic Leukemia. *Blood* 1986, 68, 192.
- (6) Miller, A. A.; Kurschel, E.; Osieka, R.; Schmidt, C. Clinical Pharmacology of Sodium Butyrate in Patients with Acute Leukemia. Eur. J. Cancer Clin. Oncol. 1987, 23, 1283-1287.
- (7) (a) Rephaeli, A.; Rabizadeh, E.; Aviram, A.; Shaklai, M.; Ruse, M.; Nudelman, A. Antineoplastic Activity of Butyric Acid Prodrugs. J. Cancer Res., Clin. Oncol. 15th International Cancer Congress, Hamburg, 1990, 119, 315. (b) Rabizadeh, E.; Rephaeli, A.; Aviram, A.; Nudelman, A.; Ruse, M.; Shaklai, M. Effects of Pivaloyloxymethyl Butyrate on Leukemic Cells Differentiation and Proliferation and on Mononuclear Cells Lymphokine(s) Secretion. Blood 1990, 76 suppl. 1:113a/444.
- (8) Ferres, H. Pro-drugs of β-Lactam Antibiotics. Chem. Ind. 1980, 435-440.

Scheme I



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

<sup>(9) (</sup>a) Daehne, W. v.; Fredriksen, E.; Gundersen, E.; Lund, F.; Morch, P.; Petersen, H. J.; Roholt, K.; Tybring, L.; Godtfredsen, W. O. Acyloxymethyl Esters of Ampicillin. J. Med. Chem. 1970, 13, 607-611. (b) Ehrnebo, M.; Nilsson, S. O.; Boreus, L. P. Pharmacokinetics of Ampicillin and Its Prodrugs Bacampicillin and Pivampicillin in Man. J. Pharmacokinet. Biopharm. 1979, 7, 429-451. (c) Shiobara, Y.; Tachibana, A.; Sasaki, H.; Watanabe, T.; Sado, T. Phthalydyl-D-α-aminobenzylpenicillinate Hydrochloride (PC-183). A new Orally Active Ampicillin Ester I. Absorption, Excretion and Metabolism of PC-183 and Ampicillin. J. Antibiot. 1974, 27, 665-673.

Table I. Structures of Reagents and Products

acid or anhydride	coupling reagent	no.	o. product	
Me(CH <sub>2</sub> ) <sub>2</sub> CO <sub>2</sub> H	ClCH <sub>2</sub> OOCCMe <sub>3</sub>	la	Me(CH <sub>2</sub> ) <sub>2</sub> CO <sub>2</sub> CH <sub>2</sub> OOCCMe <sub>3</sub>	
$Me(CH_2)_2CO_2H$	ClCH <sub>2</sub> OOC(CH <sub>2</sub> ) <sub>6</sub> Me	1 <b>b</b>	Me(CH <sub>2</sub> ) <sub>2</sub> CO <sub>2</sub> CH <sub>2</sub> OOC(CH <sub>2</sub> ) <sub>6</sub> Me	
$Me(CH_2)_2CO_2H$	C	1c	Me(CH <sub>2</sub> ) <sub>2</sub> COO	
	$\gamma$		y la	
Me(CHa)aCOaH	0 CICHMeOOCOEt	14	o Me(CH_s)_CO_CHMeOOCOEt	
Mo(CH) CO H	"T	16	Me(CH_2)_2CC_2CH_M2CCCCCCLt	
1416(0112/200211	#1c	10	d b	
			$\mathbb{A}$	
	CIGIL ODGGUDA			
$Me(CH_2)_2CU_2H$		11	$Me(CH_2)_2CO_2CH_2OOCCHMe_2$	
$[Me(CH_2)_2CO]_2O$	$CH_2 = 0$	28	$[Me(CH_2)_2CO_2]_2CH_2$	
$[Me(CH_2)_2CO]_2O$	MeCH=O	2b	$[Me(CH_2)_2CO_2]_2CHMe$	
$[Me(CH_2)_2CO]_2O$	$Me(CH_2)_2CH=0$	2c	$[Me(CH_2)_2CO_2]_2CH(CH_2)_2Me$	
$[Me(CH_2)_2CO]_2O$	Me <sub>3</sub> CCH=O	2d	[Me(CH <sub>2</sub> ) <sub>2</sub> CO <sub>2</sub> ] <sub>2</sub> CHCMe <sub>3</sub>	
$Me(CH_2)_2CO_2H$	glycerol	3	glyceryl tributyrate	
$MeCH_2CO_2H$	ClCH <sub>2</sub> OOCCMe <sub>3</sub>	<b>4</b> <sup>1</sup>	MeCH <sub>2</sub> CO <sub>2</sub> CH <sub>2</sub> OOCCMe <sub>3</sub>	
Me <sub>2</sub> CHCO <sub>2</sub> H	ClCH <sub>2</sub> OOCCMe <sub>3</sub>	<b>5a</b> 1	Me <sub>2</sub> CHCO <sub>2</sub> CH <sub>2</sub> OOCCMe <sub>3</sub>	
Me <sub>2</sub> CHCO <sub>2</sub> H		5b	Me <sub>2</sub> CHCO <sub>2</sub> CH <sub>2</sub> OOCCHMe <sub>2</sub>	
Me(CH <sub>2</sub> ) <sub>3</sub> CO <sub>2</sub> H	ClCH <sub>2</sub> OOCCMe <sub>3</sub>	<b>6</b> <sup>1</sup>	Me(CH <sub>2</sub> ) <sub>3</sub> CO <sub>2</sub> CH <sub>2</sub> OOCCMe <sub>3</sub>	
BocNH(CH <sub>2</sub> ) <sub>3</sub> CO <sub>2</sub> H	ClCH <sub>2</sub> OOC(CH <sub>2</sub> ) <sub>2</sub> Me	7 <b>a</b>	BocNH(CH <sub>2</sub> ) <sub>3</sub> CO <sub>2</sub> CH <sub>2</sub> OOC(CH <sub>2</sub> ) <sub>2</sub> Me	
BocNH(CH <sub>2</sub> ) <sub>3</sub> CO <sub>2</sub> H	ClCH <sub>2</sub> OOCCMe <sub>3</sub>	7b	BocNH(CH <sub>2</sub> ) <sub>3</sub> CO <sub>2</sub> CH <sub>2</sub> OOCCMe <sub>3</sub>	
	2 0	8 <b>a</b>	NH <sub>2</sub> (CH <sub>2</sub> ) <sub>3</sub> ĆÕ <sub>2</sub> CH <sub>2</sub> OÕC(CH <sub>2</sub> ) <sub>2</sub> Me•HCl	
		8b	NH2(CH2)3CO2CH2OOCCMe3.HCl	

which undergo intracellular hydrolysis in order to release the active drug, have been reported.<sup>11</sup> 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<sup>12a</sup> (2a), ethylidene<sup>12b,c</sup> (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<sup>13</sup> (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 [<sup>3</sup>H]thymidine incorporation and formation of colonies in

- (10) Wheeler, W. J.; Wright, W. E.; Line, V. D.; Frogge, J. A. Orally Active Esters of Cephalosporin Antibiotics. Synthesis and Biological Properties of Acyloxymethyl Esters of 7-(D-2-Amino-2-phenylacetamido)-3-[5-methyl-(1,3,4-thiadiazol-2yl)thiomethyl]-3-cephem-4-carboxylic Acid. J. Med. Chem. 1977, 20, 1159-1167.
- (11) Ozaki, S.; Watanabe, Y.; Hoshiko, T.; Mizuno, H.; Ishikawa, K.; Mori, H. 5-Fluorouracil Derivatives IV. Synthesis of Antitumor Active Acyloxyalkyl-5-fluorouracils. *Chem. Pharm. Bull.* 1984, 32, 733-738.
- (12) (a) Coffin, C. C.; Breazly, B. Homogeneous First-Order Gas Reactions (VI). Decomposition of Methylene Diacetate, Methylene Dipropionate and Methylene Dibutyrate. Can. J. Res. B, 1937, 15, 229-236. (b) Coffin, C. C.; Deacey, Y. R.; Parlee, N. A. D. Homogeneous First-Order Gas Reactions (VII). Decomposition of Ethylidene Dibutyrate and Heptylidene Diacetate. Can. J. Res. B 1937, 15, 247-258. (c) Mann, E. H.; Sanderson, J. J.; Hauser, C. R. Boron Fluoride Catalyzed Addition of Aliphatic Anhydrides to Aldehydes. J. Am. Chem. Soc. 1950, 72, 847-848.
- (13) Weatherby, L. S.; Ilvaine, L. M.; Maltin, D. Butyrin. J. Am. Chem. Soc. 1925, 47, 2249-2252.





semisolid agar, respectively. Compound 1a (50  $\mu$ M) 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  $\mu$ M (A–B). At the latter concentration, la 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 butvrate 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.

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



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 aldehvde, stemming from the "drug" and the "coupling reagent" moieties, are released<sup>8</sup> 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<sup>1</sup> (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<sub>50</sub>) of human promyelocytic HL-60 and murine myelomonocytic WEHI cell lines, were determined by [<sup>3</sup>H]thymidine incorporation. The values were as follows: in HL-60, 1525 and 49.5, and in WEHI, 850 and 75  $\mu$ M, 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. [<sup>3</sup>H]Thymidine incorporation was measured after 24 h; 100% = 9286 cpm (A), 100% = 7311 cpm (B). Inserts: percent [<sup>3</sup>H]thymidine incorporation vs log of drug concentrations, 1a ( $\odot$ ); BA ( $\triangle$ ). The data represent reproducible results obtained in at least two independent experiments.



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



Figure 6. Effect of 1a on the survival of  $B_eC_3F_1/OLA/HSD$  mice (groups of 8) implanted ip with B16F0 melanoma cells. The animals received nine consecutive ip injections of 1:1 PBSpropylene glycol (0.1 mL) for the control group,  $\oplus$ ; cisplatin (2 mg/kg) as a positive control,  $\blacksquare$ ; 1a (250 mg/kg),  $\blacktriangle$ . 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<sub>50</sub> =  $82 \mu$ M) than those of BA (IC<sub>50</sub> = 933  $\mu$ M). The appearance of colonies in semisolid agar was inhibited by 1a (IC<sub>50</sub> = 60  $\mu$ M), 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<sup>14</sup> 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 in-

 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			
1 <b>a</b> .	25	0.1350	0.15	7.9			
1 <b>a</b>	50	0.0929	0.05	3.7			
1 <b>a</b>	100	0.2686	0.15	NS⁰			
(b) Number of Lung Lesions							
solvent	-	174.00	59.97	-			
cytoxan	50	56.38	20.17	0.1			
la	12.5	97.89	57.62	10.7			
1 <b>a</b>	25	72.38	51.29	1.3			
1 <b>a</b>	50	66.60	37.81	2.2			
la	100	140.29	64.89	NS			

<sup>a</sup>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).

crease in the life span of the treated animals (Figure 6). Postmortem examination did not reveal macroscopic lesions in the lungs.

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  $20 \pm 0.5$  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<sub>50</sub> = 1.36 ± 0.1 g/kg (n = 5) value was explained; the results of a representative

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

## experiment are shown in Figure 7.

The renewed interest in cytodifferentiating agents such as retinoic acid, <sup>15a</sup>  $\alpha$ -interferon, <sup>15b</sup>  $\gamma$ -interferon, <sup>15c</sup> and colony stimulating factors<sup>15d</sup> 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**

<sup>1</sup>H NMR spectra 300-MHz were obtained on a Brucker WH-300 spectrometer in CDCl<sub>3</sub> solvent. Chemical shifts were expressed in ppm downfield from Me<sub>4</sub>Si used as internal standard. The values are given in  $\delta$  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.**<sup>16,17</sup> 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<sub>4</sub>, and evaporated, and the residue was fractionally distilled to give chloromethyl octanoate<sup>17</sup> (10 g, 80%), bp 47–8 °C (0.2 Torr). Anal. (C<sub>9</sub>H<sub>17</sub>ClO<sub>2</sub>) C, H, Cl.

Chloromethyl 2-methylpropanoate was prepared as described<sup>18a,b,19</sup> for chloromethyl 2,2-dimethylpropanoate,<sup>20</sup> bp 40-2

- (15) (a) Chomienne, C.; Ballerini, P.; Balitrand, N.; Amar, M.; Bernard, J. F.; Boivin, I.; Daniel, M. T.; Berger, R.; Castaigne, S.; Degos, L. Retinoic Acid - An Alternative Therapy of Promyelocytic Leukemia. Lancet 1989, 2, 746. (b) Flandrin, G.; Sigaux, F.; Castaigne, S.; Billard, C.; Aguet, M.; Boiron, M.; Falcoff, E.; Degos, J. Treatment of Hairy Cell Leukemia With Recombinant Alpha Interferon I. Quantitative Study of Bone Marrow Changes During the First Months of Treatment. Blood 1986, 67, 817. (c) Gavosto, F.; Maiolo, A. T.; Aglietta, M.; Alimenza, G.; Bernasconi, C.; Castoldi, G.; Cazzola, M.; Cortelezzi, A.; Mandelli, F.; Resegott, L.; Polli, E. Treatment with Interferon Gama in Myelodysplastic Syndromes; A Pilot Study. In The Status of Differentiation Therapy in Cancer; Warman, S., Rossi, G. B.; Takaku, F., Eds.; Raven Press: New York, 1987; p 375. (d) Ganser, A.; Vokers, B.; Greher, H.; Walther, F.; Hoelzer, D. Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor in Patients with Myelodysplastic Syndromes – A Phase I/II Trial. Onkologie 1988, 1, 53.
- (16) Binderup, E.; Hansen, E. T. Chlorosulfates as Reagents in the Synthesis of Carboxylic Acid Esters Under Phase-Transfer Conditions. Synth. Commun. 1984, 14, 857-864.
- (17) Bodor, N.; Kaminski, J. J.; Selk, S. Soft Drugs 1. Labile Quaternary Ammonium Salts as Soft Antimicrobials. J. Med. Chem. 1980, 23, 469–474.
- (18) (a) Rassmusen, M.; Leonard, N. J. The Synthesis of 3-(2'-Deoxy-D-ribofuranosyl)adenine. Application of a New Protecting Group, Pivaloyloxymethyl (POM). J. Am. Chem. Soc. 1967, 89, 5439-5445. (b) Bedford, C. D.; Harris, R. N.; Howd, R. A.; Gof, D. A.; Koolpe, G. A.; Petesch, M.; Miller, A.; Nolen, H. W.; Musallam, H. A.; Pick, R. O.; Jones, D. E.; Koplovitz, I.; Sultan, W. E. Quaternary Salts of 2-[(Hydroxyimino)-methyl]imidazole. 2. Preparation and In Vitro and In Vivo Evaluation of 1-(Alkoxymethyl)-2-[(hydroxyimino)methyl]-3-methylimidazolium Halides for Reactivation of Organophosphorus-Inhibited Acetylcholinesterases. J. Med. Chem. 1989, 32, 493-503.

°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<sub>3</sub>N (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<sub>2</sub>O-EtOAc mixture. The organic phase was separated, dried over K<sub>2</sub>CO<sub>3</sub>, filtered, and evaporated. The product was obtained by fractional distillation (4.42 g, 57%): bp 88-93 °C (2 Torr); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>), 2.34 (t, J = 8 Hz, 2 H, CH<sub>2</sub>O), 5.75 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 203 (MH<sup>+</sup>). Anal. (C<sub>10</sub>H<sub>18</sub>O<sub>4</sub>) 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); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>'s), 1.6-1.7 (m, 4 H, two CH<sub>2</sub>'s), 2.11-2.38 (m, 4 H, two CH<sub>2</sub>CO), 5.70 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 245 (MH<sup>+</sup>). Anal. (C<sub>13</sub>H<sub>24</sub>O<sub>4</sub>) 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<sub>3</sub>N (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<sub>2</sub>O (15 mL/wash), dried over MgSO<sub>4</sub>, and concentrated. The residue was recrystallized from hexane-CH<sub>2</sub>Cl<sub>2</sub>, 1:1, to give 1c (2.28 g, 82.8%): mp 52-3 °C; <sup>1</sup>H NMR  $\delta$  0.99 (t, J = 7.4 Hz, 3 H, Me), 1.72 (sextet, J = 7.4 Hz, 2 H, CH<sub>2</sub>Me), 2.42 (t, J = 7.4 Hz, 2 H, CH<sub>2</sub>CO), 7.46 (s, 1 H, CH), 7.58 (dd, J = 0.7, 8.2 Hz, 1 H, H-5), 7.65 (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<sup>+</sup>). Anal. (C<sub>12</sub>H<sub>12</sub>O<sub>4</sub>) 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<sub>2</sub>O (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<sub>2</sub>O, dried over MgSO<sub>4</sub>, 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%): <sup>1</sup>H NMR  $\delta$  0.95 (t, J = 7.5 Hz, 3 H, Me), 1.318 (t, J = 7 Hz, 3 H, OCH<sub>2</sub>Me), 1.52 (d, J = 5 Hz, 3 H, CHMe), 1.66 (sextet, J = 7.5 Hz, 2 H, MeCH<sub>2</sub>CH<sub>2</sub>), 2.32 (t, J = 7.5 Hz, 2 H, CH<sub>2</sub>CO), 4.22 (q, J = 7 Hz, 2 H, MeCH<sub>2</sub>), 6.77 (q, J = 5 Hz, 1 H, CH); MS (CI) m/e 195 (MH<sup>+</sup>). Anal. (C<sub>9</sub>H<sub>16</sub>O<sub>5</sub>) 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<sup>18</sup> and butyric acid: bp 100-4 °C (3 Torr); <sup>1</sup>H NMR  $\delta$  0.94 (t, J = 6 Hz, 3 H, Me), 1.64 (sextet, J = 6 Hz, 2 H, MeCH<sub>2</sub>), 2.15 (s, 3 H, Me), 2.31 (t, J = 6 Hz, 2 H, CH<sub>2</sub>CO), 4.82 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 201 (MH<sup>+</sup>). Anal. (C<sub>9</sub>H<sub>12</sub>O<sub>5</sub>) C, H.

((2-Methylpropanoyl)oxy)methyl butanoate (1f) was prepared from butyric acid and chloromethyl isobutyrate: bp 70–2 °C (0.7 Torr); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>), 2.36 (t,

<sup>(19)</sup> Sakamoto, F.; Ikeda, S.; Kondo, H.; Tsukamoto, J. Studies on Prodrugs II. Preparation and Characterization of (5-Substituted-2-oxo-1,3-dioxolen-4-yl)methyl Esters of Ampicillin. *Chem. Pharm. Bull.* 1984, 32, 2241-2248.

<sup>(20)</sup> Iyer, R. P.; Phillips, L. R.; Biddle, J. A.; Thakker, D. R.; Egan, W.; Aoki, S.; Mitsuya, H. Synthesis of Acyloxyalkyl Acylphosphonates as Potential Prodrugs of the Antiviral Trisodium Phosphonoformate (Foscarnet Sodium). *Tetrahedron Lett.* 1989, 30, 7141-7144.

J = 8 Hz, 2 H, CH<sub>2</sub>CO), 2.60 (septet, J = 8 Hz, 1 H, CH), 5.77 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 189 (MH<sup>+</sup>). Anal. (C<sub>9</sub>H<sub>16</sub>O<sub>4</sub>) C, H.

((2,2-Dimethylpropanoyl)oxy)methyl propanoate (4) was prepared from propionic acid and chloromethyl pivalate: bp 80–2 °C (2 Torr); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>), 5.78 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 189 (MH<sup>+</sup>). Anal. (C<sub>9</sub>H<sub>16</sub>O<sub>4</sub>) C, H.

((2,2-Dimethylpropanoyl)oxy)methyl 2-methylpropanoate (5a) was prepared from isobutyric acid and chloromethyl pivalate: bp 80-3 °C (2 Torr); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>); MS (CI) m/e 203 (MH<sup>+</sup>). Anal. (C<sub>10</sub>H<sub>10</sub>O<sub>4</sub>) C, H.

((2-Methylpropanoyl)oxy)methyl 2-methylpropanoate (5b) was prepared from isobutyric acid and chloromethyl isobutyrate: bp 70-3 °C (2 Torr); <sup>1</sup>H NMR  $\delta$  1.10 (d, J = 7 Hz, 12 H, four Me), (septet, J = 7 Hz, 2 H, CH), 5.77 (s, 2 H, CH<sub>2</sub>); MS (CI) m/e 189 (MH<sup>+</sup>). Anal. (C<sub>9</sub>H<sub>16</sub>O<sub>4</sub>).

((2,2-Dimethylpropanoyl)oxy)methyl pentanoate (6) was obtained from valeric acid and chloromethyl pivalate: bp 81-3 °C (2 Torr); <sup>1</sup>H NMR  $\delta$  0.82 (t, J = 7 Hz, 3 H, Me), 0.90 (sextet, J = 7 Hz, 2 H, CH<sub>2</sub>Me), 1.20 (s, 9 H, t-Bu), 1.59 (quintet, J =7 Hz, 2 H, CH<sub>2</sub>CH<sub>2</sub>CO), 2.36 (t, J = 8 Hz, 2 H, CH<sub>2</sub>CO), 5.78 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 217 (MH<sup>+</sup>). Anal. (C<sub>11</sub>H<sub>20</sub>O<sub>4</sub>) C, H.

Ethylidene Dibutanoate (2b).<sup>12b.</sup> 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<sub>2</sub>CO<sub>3</sub> until CO<sub>2</sub> evolution stopped. The organic phase was washed with H<sub>2</sub>O, dried over MgSO<sub>4</sub>, 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%): <sup>1</sup>H NMR  $\delta$  0.95 (t, J = 7.5 Hz, 6 H, CH<sub>2</sub>Me), 1.47 (d, J = 5.5 Hz, 3 H, CHMe), 1.65 (sextet, J = 7.5 Hz, 4 H, MeCH<sub>2</sub>), 2.30 (dt, J = 0.75, 7.5 Hz, 4 H, CH<sub>2</sub>CO), 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%); <sup>1</sup>H NMR  $\delta$  0.949 and 0.953 (2 t, J = 7.5 Hz, 9 H, Me), 1.40 (q, J = 7.5 Hz, 2 H, CH<sub>2</sub>CH<sub>2</sub>CH), 1.65 (sextet, J = 7.5 Hz, 4 H, MeCH<sub>2</sub>CH<sub>2</sub>), 1.75 (m, 2 H, CH<sub>2</sub>CH), 2.30 (m, 4 H, CH<sub>2</sub>CO), 6.82 (t, J = 5.6 Hz, 1 H, CH<sub>2</sub>CH); MS (CI) m/e 231 (MH<sup>+</sup>). Anal. (C<sub>12</sub>H<sub>22</sub>O<sub>4</sub>) C, H.

**2,2-Dimethylpropylidene dibutanoate (2d)** (from pivalaldehyde): bp 110–3 °C (5–6 Torr); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>), 2.31 (t, J = 7.3 Hz, 1 H, CHCO), 6.59 (s, 1 H, CH); MS (CI) m/e 245 (MH<sup>+</sup>). Anal. (C<sub>13</sub>H<sub>24</sub>O<sub>4</sub>) C, H.

4-((tert-Butoxycarbonyl)amino)butanoic acid:<sup>21</sup> <sup>1</sup>H NMR  $\delta$  1.47 (s, 9 H, t-Bu), 1.80 (quintet, J = 7 Hz, 2 H, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>), 2.40 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 3.18 (broad q, J = 7 Hz, 2 H, NHCH<sub>2</sub>), 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); <sup>1</sup>H NMR  $\delta$  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<sub>2</sub>), 1.83 (quintet, J = 7 Hz, 2 H, CH<sub>2</sub>CH<sub>2</sub>CO), 2.35 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 2.40 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 3.17 (q, J = 7 Hz, 2 H, NCH<sub>2</sub>), 4.85 (br s, 1 H, NH), 5.75 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e304 (MH<sup>+</sup>). Anal. (C<sub>14</sub>H<sub>25</sub>NO<sub>6</sub>) 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); <sup>1</sup>H NMR  $\delta$  1.20 (s, 9 H, t-Bu), 1.40 (s, 9 H, t-Bu), 1.80 (quintet, J = 7 Hz, 2 H, CH<sub>2</sub>CH<sub>2</sub>CO), 2.40 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 3.14 (broad q, J = 7 Hz, 2 H, HNCH<sub>2</sub>), 4.6 (br s, 1 H, NH), 5.73 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 318 (MH<sup>+</sup>). Anal. (C<sub>16</sub>H<sub>27</sub>NO<sub>6</sub>) C, H, N. ((4-Aminobutanoyl)oxy)methyl butanoate, hydrochloride (8a) was obtained from 7a as described for 7b: <sup>1</sup>H NMR  $\delta$  0.94 (t, J = 7 Hz, 3 H, Me), 1.67 (sextet, J = 7 Hz, 2 H, MeCH<sub>2</sub>), 2.1 (br m, 2 H, CH<sub>2</sub>CH<sub>2</sub>CO), 2.34 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 2.36 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 3.15 (br m, 2 H, NCH<sub>2</sub>), 5.75 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 204 (MH<sup>+</sup>). Anal. (C<sub>9</sub>H<sub>17</sub>NO<sub>4</sub>·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: <sup>1</sup>H NMR  $\delta$  1.20 (s, 9 H, t-Bu), 2.15 (quintet, J = 7 Hz, 2 H, CH<sub>2</sub>CH<sub>2</sub>CO), 2.59 (t, J = 7 Hz, 2 H, CH<sub>2</sub>CO), 3.14 (br m, 2 H, NCH<sub>2</sub>), 5.73 (s, 2 H, OCH<sub>2</sub>); MS (CI) m/e 218 (MH<sup>+</sup>). Anal. (C<sub>10</sub>H<sub>19</sub>NO<sub>4</sub>·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); [<sup>3</sup>H]thymidine, 2 Ci/mmol, Amersham (UK). B<sub>6</sub>C<sub>3</sub>F<sub>1</sub>/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.<sup>22</sup> 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  $\mu$ Ci/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  $4 \times 10^4$  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,<sup>23</sup> 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<sup>3</sup> 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<sup>5</sup> B16F0 melanoma cells.

<sup>(21)</sup> Schnabel, E. Improved Synthesis of tert-Butoxycarbonyl Amino Acids by a Constant pH Reaction. Justus Liebigs Ann. Chem. 1967, 702, 188–196.

<sup>(22)</sup> Koeffler, H. P. Induction of Differentiation of Human Acute Myelogenous Leukemia Cells: Therapeutic Implications. Blood 1983, 62, 709-721.

<sup>(23)</sup> Hamburger, A. W.; Salmon, S. E. Primary Bioassay of Human Tumor Stem Cells. Science 1977, 197, 461-463.

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  $(2 \times 10^4)$  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  $6 \times 10^5$  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<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>H, 107-92-6; [Me(CH<sub>2</sub>)<sub>2</sub>CO]<sub>2</sub>O, 106-31-0; MeCH2CO2H, 79-09-4; Me2CHCO2H, 79-31-2; Me(CH2)2CO2H, 109-52-4; BocNH(CH<sub>2</sub>)<sub>3</sub>CO<sub>2</sub>H, 57294-38-9; ClCH<sub>2</sub>OOCCMe<sub>3</sub>, 18997-19-8; ClCH2OOC(CH2)6Me, 61413-70-5; ClCHMeOOCOEt, 50893-36-2; ClCH<sub>2</sub>OOCCHMe<sub>2</sub>, 61644-18-6; CH<sub>2</sub>=O, 50-00-0; MeCH=O, 75-07-0; Me(CH<sub>2</sub>)<sub>2</sub>CH=O, 123-72-8; Me<sub>3</sub>CCH=O, 630-19-3; ClCH<sub>2</sub>OOC(CH<sub>2</sub>)<sub>2</sub>Me, 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 $\omega$ Chain Conjugated Diene Analogues of Enisoprost

P. W. Collins,\* R. L. Shone, W. E. Perkins, A. F. Gasiecki, V. J. Kalish,<sup>1</sup> S. W. Kramer, and R. G. Bianchi

Gastrointestinal Diseases Research, G.D. Searle & Co., Skokie, Illinois 60077. Received August 12, 1991

A series of acyclic  $\omega$  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  $\omega$  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<sup>2</sup> we reported that incorporation of  $\Delta^{17}$  unsaturated cycloalkyl and cycloalkenyl functionality into the  $\omega$  chain of enisoprost 1 produced compounds with increased separation of gastric antisecretory activity from diarrheagenic side effects. In particular the 17(*E*)-18cyclopentenyl compound 2 displayed antisecretory activity



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 ptoluenesulfonate (PPTS) in aqueous acetone (Figure 1). As in previous work,<sup>2</sup> 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.<sup>2</sup> 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-

Present address: Agouron Pharmaceuticals, Inc. La Jolla, CA 92037.

<sup>(2)</sup> Collins, P. W.; Gasiecki, A. F.; Perkins, W. E.; Gullikson, G. W.; Bianchi, R. G.; Kramer, S. W.; Ng, J. S.; Yonan, E. E.; Swenton, L.; Jones, P. H.; Bauer, R. F. Chemistry and Structure-Activity Relationships of C-17 Unsaturated 18-Cycloalkyl and Cycloalkenyl Analogues of Enisoprost. Identification of a Promising New Antiulcer Prostaglandin. J. Med. Chem. 1990, 33, 2784-2793.

<sup>(3)</sup> Collins, P. W.; Dajani, E. Z.; Pappo, R.; Gasiecki, A. F.; Bianchi, R. G.; Woods, E. M. Synthesis and Gastric Antisecretory Properties of 4,5-Unsaturated Derivatives of 15-Deoxy-16hydroxy-16-methylprostaglandin E<sub>1</sub>. J. Med. Chem. 1983, 36, 786-790.