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Toxicity of myristic acid analogs toward African trypanosomes

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ABSTRACT **New drugs are needed for treatment of diseases caused by African trypanosomes. One possible target for chemotherapy is the biosynthesis of the glycosyl phosphatidylinositol (GPI) of this parasite's variant surface glycoprotein (VSG). Unlike mammalian GPIs, the diacylglycerol moiety of the VSG anchor contains only myristate (tetradecanoate), added in unique remodeling reactions. We previously found that 11-oxatetradecanoic acid [i.e., 10-(propoxy)decanoic acid] is selectively toxic to trypanosomes. We have now assayed 244 different fatty acid analogs, most with chain lengths comparable to that of myristate, for trypanocidal effects. In these assays we surveyed the effects on toxicity of systematic alterations in the analogs' steric, conformational, and hydrophobic properties. We also used three ^{3}H-labeled oxatetradecanoic acids to explore the mechanism of analog action. Their incorporation into VSG correlated roughly with toxicity, although they also were incorporated into phospholipids and other proteins. Myristate analogs are useful for studying the mechanism of GPI myristoylation, and they are candidates for antitrypanosomal chemotherapy.**

The glycosyl phosphatidylinositol (GPI) membrane anchor of the *Trypanosoma brucei* variant surface glycoprotein (VSG) is unusual in that its lipid moiety is a diacylglycerol containing exclusively myristate (1). GPI anchors from other eukaryotes usually contain a diacylglycerol or alkyl/acylglycerol in which the fatty acyl or alkyl groups are a heterogeneous mixture of species differing in chain length and degree of unsaturation (see refs. 2 and 3 for review). In trypanosomes myristate is incorporated into a precursor GPI, glycolipid A, either through fatty acid remodeling, in which other fatty acids are replaced by myristate (4), or by a myristate exchange reaction, which acts on free GPIs as well as on anchors linked to protein (L. Buxbaum and P.T.E., unpublished work). The presence of two GPI myristoylation processes suggests a critical structural and/or functional requirement for this fatty acid in trypanosome VSG anchors.

GPI myristoylation is an attractive target for antitrypanosomal drugs because it does not occur in mammalian cells and because the parasite must scavenge its entire supply of myristate from its mammalian host. We previously tested three myristic acid analogs, containing an oxygen substituted for a methylene group, for their effects on trypanosomes (5). We found that 11-oxatetradecanoic acid was the most toxic to these parasites, with an LD_{50} of 1 μM. At 100 μM this compound is not toxic to cultured mammalian cells, and at 10 μM it had no effect on procyclic trypanosomes, a form in the tsetse fly vector whose GPI protein anchors are not myristoylated. We also found that [^{3}H]11-oxatetradecanoic acid was incorporated into VSG and glycolipid A.

We have now examined the effects on trypanosomes of an extensive panel of myristate analogs, originally synthesized to study another myristate-specific reaction, protein N-myristoylation (reviewed in ref. 6). This set of 244 compounds (Table 1) was designed to systematically alter the physical-chemical properties of myristate. Some modifications have complex effects on both conformation and stereoelectronic properties (e.g., introducing an ester group into the alkyl chain), whereas others predominantly affect only one of these properties (e.g., conformation but not polarity in the case of olefins). To explore the mechanism by which myristate analogs exert their toxic effects, we also studied the incorporation of three radiolabeled oxatetradecanoic acids into *T. brucei* protein and lipid. Our goals are to use myristate analogs as probes of the specificity and mechanism of GPI myristoylation, to study correlations between their physical-chemical properties and their toxic effects, and to identify analogs which may be useful as antitrypanosomal agents in mammals.

EXPERIMENTAL PROCEDURES

Toxicity Assay. Parasite growth was measured by the color change of the phenol red indicator in the culture medium. A similar assay has been published (13). Stocks of analogs (10 mM in absolute ethanol, identified only by code) were diluted in culture medium to twice the concentration to be tested (usually 10 μM). To evaluate reproducibility, several analogs were coded twice with different numbers. Quadruplicate aliquots of 100 μl were dispensed into a microtiter plate and warmed to 37°C in a 5% CO_2 incubator before the addition of an equal volume of cell suspension. Mouse blood containing *T. brucei* (strain 221, from G. A. M. Cross, Rockefeller University) at 2–5 $\times$ 10^8 per ml was centrifuged at 430 $\times$ *g* for 8 min at 4°C, the upper portion of the buffy coat was washed in BBS (50 mM bicine/70 mM glucose/5 mM KCl/50 mM NaCl, pH 8.0) containing fatty acid-free bovine serum albumin at 1 mg/ml, and the cell pellet was suspended (1.5 $\times$ 10^7 cells per ml) in supplemented minimal essential medium α (14). Control wells included appropriately diluted ethanol (which did not affect cell growth) and 10 μM 11-oxatetradecanoic acid, a known toxic analog (5). The plate was incubated for 36 hr at 37°C and then stored at 4°C for 12 hr to allow equilibration of CO_2 in the medium with that in air. The absorbance of each sample at 550 nm and 405 nm (values chosen from absorption spectra of fresh and acidified media) was read in a microplate reader (Molecular Devices). To control for variation in sample volume, the ratio A_{550}/A_{405} was averaged for each quadruplicate set; the four wells were generally within 5% of the mean. A_{550}/A_{405} for 11-

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Abbreviations: VSG, variant surface glycoprotein; GPI, glycosyl phosphatidylinositol.

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Table 1. Toxicity to *T. brucei* of myristate analogs tested at 10 μM

Oxatetradecanoic acids (7)	
$CH_3O(CH_2)_{11}COOH$	2
$CH_3CH_2O(CH_2)_{10}COOH$	1
$CH_3(CH_2)_2O(CH_2)_9COOH$	3
$CH_3(CH_2)_3O(CH_2)_8COOH$	3
$CH_3(CH_2)_5O(CH_2)_6COOH$	3b
$CH_3(CH_2)_6O(CH_2)_5COOH$	3
$CH_3(CH_2)_7O(CH_2)_4COOH$	1
$CH_3(CH_2)_8O(CH_2)_3COOH$	3
$CH_3(CH_2)_9O(CH_2)_2COOH$	2
$CH_3(CH_2)_{10}OCH_2COOH$	1
Analogs with oxygen/sulfur (7)	
$CH_3CH_2S(CH_2)_5S(CH_2)_4COOH$	1
$CH_3CH_2S(CH_2)_2O(CH_2)_7COOH$	2
$CH_3CH_2O(CH_2)_2S(CH_2)_7COOH$	1
$CH_3CH_2O(CH_2)_5S(CH_2)_4COOH$	1
$CH_3O(CH_2)_2O(CH_2)_2O(CH_2)_5COOH$	1
Analogs with ester groups (9)	
$CH_3OCO(CH_2)_{10}COOH$	2
$CH_3CH_2OCO(CH_2)_9COOH$	1
$CH_3(CH_2)_2OCO(CH_2)_8COOH$	2
$CH_3(CH_2)_3OCO(CH_2)_7COOH$	2a
$CH_3(CH_2)_4OCO(CH_2)_6COOH$	1a
$CH_3(CH_2)_5OCO(CH_2)_5COOH$	2b
$CH_3(CH_2)_6OCO(CH_2)_4COOH$	2
$CH_3(CH_2)_7OCO(CH_2)_3COOH$	3
$CH_3(CH_2)_8OCO(CH_2)_2COOH$	3b
$CH_3(CH_2)_9OCOCH_2COOH$	1
$CH_3CH_2OCO(CH_2)_{10}COOH$	1
Nitroalkylcarboxylic acids (10)	
$O_2N(CH_2)_9COOH$	1
$O_2N(CH_2)_{10}COOH$	2a
$O_2N(CH_2)_{12}COOH$	3
Tetradecynoic acids (7)	
$HC{\equiv}C(CH_2)_{11}COOH$	2b
$CH_3C{\equiv}C(CH_2)_{10}COOH$	1
$CH_3CH_2C{\equiv}C(CH_2)_9COOH$	1
$CH_3(CH_2)_2C{\equiv}C(CH_2)_8COOH$	2
$CH_3(CH_2)_3C{\equiv}C(CH_2)_7COOH$	3a
$CH_3(CH_2)_4C{\equiv}C(CH_2)_6COOH$	3
$CH_3(CH_2)_5C{\equiv}C(CH_2)_5COOH$	2
$CH_3(CH_2)_6C{\equiv}C(CH_2)_4COOH$	2
$CH_3(CH_2)_7C{\equiv}C(CH_2)_3COOH$	2
$CH_3(CH_2)_8C{\equiv}C(CH_2)_2COOH$	1
$CH_3(CH_2)_9C{\equiv}CCH_2COOH$	1
$CH_3(CH_2)_{10}C{\equiv}CCOOH$	1
Alkynoic acids (C13,C15,C16)(12)	
$CH_3(CH_2)_7C{\equiv}C(CH_2)_2COOH$	1
$CH_3(CH_2)_6C{\equiv}C(CH_2)_3COOH$	1
$CH_3(CH_2)_5C{\equiv}C(CH_2)_4COOH$	2b
$CH_3(CH_2)_9C{\equiv}C(CH_2)_2COOH$	1
$CH_3(CH_2)_8C{\equiv}C(CH_2)_3COOH$	1
$CH_3(CH_2)_7C{\equiv}C(CH_2)_4COOH$	1
$CH_3(CH_2)_{10}C{\equiv}C(CH_2)_2COOH$	1
$CH_3(CH_2)_9C{\equiv}C(CH_2)_3COOH$	1
$CH_3(CH_2)_8C{\equiv}C(CH_2)_4COOH$	1
2,5-Furyl (Fu) analogs	
$CH_3(CH_2)_7$-Fu-$(CH_2)_2COOH$ (12)	1
$CH_3(CH_2)_6$-Fu-$(CH_2)_3COOH$ (12)	2
$CH_3(CH_2)_5$-Fu-$(CH_2)_4COOH$ (12)	3
$CH_3(CH_2)_4$-Fu-$(CH_2)_5COOH$ (12)	2b
2-Fu-$(CH_2)_8COOH$ (†)	1
[2-(5-CH_3Fu)]$(CH_2)_8COOH$ (†)	1
2-Fu-$(CH_2)_{10}COOH$ (†)	2b
2-Fu-CH=CH$(CH_2)_6COOH$ (†)	1
[2-(5-CH_3-Fu)]CH=CH$(CH_2)_6COOH$ (†)	1
2-Fu-CH=CH$(CH_2)_8COOH$ (†)	1
2-Fu-CH=CH$(CH_2)_9COOH$ (†)	1
Thiatetradecanoic acids (7)	
$CH_3S(CH_2)_{11}COOH$	3
$CH_3CH_2S(CH_2)_{10}COOH$	2
$CH_3(CH_2)_3S(CH_2)_8COOH$	2
$CH_3(CH_2)_4S(CH_2)_7COOH$	3b
$CH_3(CH_2)_5S(CH_2)_6COOH$	2
$CH_3(CH_2)_6S(CH_2)_5COOH$	3
$CH_3(CH_2)_7S(CH_2)_4COOH$	2a
$CH_3(CH_2)_8S(CH_2)_3COOH$	3
$CH_3(CH_2)_9S(CH_2)_2COOH$	1
$CH_3(CH_2)_{10}SCH_2COOH$	1
Oxotetradecanoic acids (9)	
$CH_3CO(CH_2)_{11}COOH$	2
$CH_3CH_2CO(CH_2)_{10}COOH$	1
$CH_3(CH_2)_2CO(CH_2)_9COOH$	2a
$CH_3(CH_2)_3CO(CH_2)_8COOH$	2b
$CH_3(CH_2)_4CO(CH_2)_7COOH$	2a
$CH_3(CH_2)_5CO(CH_2)_6COOH$	1
$CH_3(CH_2)_6CO(CH_2)_5COOH$	3
$CH_3(CH_2)_7CO(CH_2)_4COOH$	1
$CH_3(CH_2)_8CO(CH_2)_3COOH$	3b
$CH_3(CH_2)_9CO(CH_2)_2COOH$	2
$CH_3(CH_2)_{10}COCH_2COOH$	1
Tetradecadienoic acids (†)*	
$CH_3(CH_2)_2CH{=}CHCH{=}CH(CH_2)_6COOH$	1
$CH_3(CH_2)_4CH{=}CHCH{=}CH(CH_2)_4COOH$	2b
$CH_3(CH_2)_5CH{=}CHCH{=}CH(CH_2)_3COOH$	3
Halogenated analogs (10)	
$Br(CH_2)_{12}COOH$	2
$Br(CH_2)_{13}COOH$	2
$F_3C(CH_2)_{12}COOH$	1
$F_3CCH{=}CH(CH_2)_{10}COOH$	1
Tetradecenoic acids (7)	
$CH_2{=}CH(CH_2)_{11}COOH$	1
$CH_3CH{=}CH(CH_2)_{10}COOH$ Z	1
$CH_3CH_2CH{=}CH(CH_2)_9COOH$ Z	2b
$CH_3(CH_2)_2CH{=}CH(CH_2)_8COOH$ Z	1
$CH_3(CH_2)_4CH{=}CH(CH_2)_6COOH$ Z	1a
$CH_3(CH_2)_5CH{=}CH(CH_2)_5COOH$ Z	2b
$CH_3(CH_2)_5CH{=}CH(CH_2)_5COOH$ E	1
$CH_3(CH_2)_6CH{=}CH(CH_2)_4COOH$ E	2b
$CH_3(CH_2)_6CH{=}CH(CH_2)_4COOH$ Z	1
$CH_3(CH_2)_7CH{=}CH(CH_2)_3COOH$ Z	1
$CH_3(CH_2)_7CH{=}CH(CH_2)_3COOH$ E	1
$CH_3(CH_2)_8CH{=}CH(CH_2)_2COOH$ Z	1
$CH_3(CH_2)_8CH{=}CH(CH_2)_2COOH$ E	1
$CH_3(CH_2)_9CH{=}CHCH_2COOH$ Z	1
$CH_3(CH_2)_{10}CH{=}CHCOOH$ E	1
$CH_3(CH_2)_{10}CH{=}CHCOOH$ Z	1
2,5-Thienyl (Th) analogs	
2-Th-$(CH_2)_8COOH$ (†)	1a
2-Th-$(CH_2)_9COOH$ (7)	1
[2-(5-CH_3-Th)]$(CH_2)_8COOH$ (†)	1
2-Th-$(CH_2)_{10}COOH$ (†)	2b
2-Th-CH=CH$(CH_2)_6COOH$ (†)	1
[2-(5-CH_3-Th)]CH=CH$(CH_2)_6COOH$ (†)	1
2-Th-CH=CH$(CH_2)_8COOH$ (†)	1
Aromatic analogs‡	
11 Carbon Equivalent Length (7)	
$C_6H_5(CH_2)_7COOH$	2b
$C_6H_5(CH_2)_2CH{=}CH(CH_2)_3COOH$	1
12 Carbon Equivalent Length	
$C_6H_5(CH_2)_8COOH$ (13)	1
$C_6H_5CH{=}CH(CH_2)_6COOH$ (7)	1a
$C_6H_5(CH_2)_2CH{=}CH(CH_2)_4COOH$ (7)	1
$C_6H_5O(CH_2)_7COOH$ (†)	1
Aromatic analogs‡	
13 Carbon Equivalent Length	
$C_6H_5(CH_2)_9COOH$ (7)	2b
$CH_3Ph(CH_2)_8COOH$ (7)	1
$CH_3CH_2Ph(CH_2)_7COOH$ (7)	2b
$CH_3(CH_2)_2Ph(CH_2)_6COOH$ (7)	1
$CH_3(CH_2)_3Ph(CH_2)_5COOH$ (7)	1
$CH_3(CH_2)_4Ph(CH_2)_4COOH$ (8)	1
$CH_3(CH_2)_5Ph(CH_2)_3COOH$ (8)	1a
$CH_3(CH_2)_6Ph(CH_2)_2COOH$ (8)	1
$C_6H_5CH_2CH{=}CH(CH_2)_6COOH$ (7)	1
$CH_3PhCH{=}CH(CH_2)_6COOH$ (7)	1
$CH_3CH_2PhCH{=}CH(CH_2)_5COOH$ (7)	1
$CH_3(CH_2)_2PhCH{=}CH(CH_2)_4COOH$ (7)	1
$CH_3(CH_2)_3PhCH{=}CH(CH_2)_3COOH$ (7)	1
$CH_3(CH_2)_4PhCH{=}CH(CH_2)_2COOH$ (8)	1
$CH_3(CH_2)_5PhCH{=}CHCH_2COOH$ (8)	1
$CH_3(CH_2)_6PhCH{=}CHCOOH$ (8)	1
$CH_3OPh(CH_2)_7COOH$ (†)	1
$CH_3CH_2OPh(CH_2)_6COOH$ (†)	1
$CH_3(CH_2)_2OPh(CH_2)_5COOH$ (†)	1
$CH_3(CH_2)_3OPh(CH_2)_4COOH$ (8)	3
$CH_3(CH_2)_4OPh(CH_2)_3COOH$ (8)	1
$CH_3(CH_2)_5OPh(CH_2)_2COOH$ (8)	1
$CH_3OPhCH{=}CH(CH_2)_5COOH$ (†)	1
$CH_3CH_2OPhCH{=}CH(CH_2)_4COOH$ (†)	1
$CH_3(CH_2)_2OPhCH{=}CH(CH_2)_3COOH$ (†)	1
$CH_3(CH_2)_3OPhCH{=}CH(CH_2)_2COOH$ (8)	1
$CH_3(CH_2)_4OPhCH{=}CHCH_2COOH$ (8)	1
$CH_3(CH_2)_5OPhCH{=}CHCOOH$ (8)	1
$C_6H_5O(CH_2)_8COOH$ (†)	1
$C_6H_5S(CH_2)_8COOH$ (†)	1
$C_6H_5CH_2O(CH_2)_7COOH$ (†)	1
$C_6H_5CH_2S(CH_2)_7COOH$ (†)	1
14 Carbon Equivalent Length	
$C_6H_5(CH_2)_{10}COOH$ (11)	2b
$CH_3Ph(CH_2)_9COOH$ (7)	1
$CH_3CH_2Ph(CH_2)_8COOH$ (7)	1a
$CH_3(CH_2)_2Ph(CH_2)_7COOH$ (†)	1
$CH_3(CH_2)_3Ph(CH_2)_6COOH$ (†)	1
$CH_3(CH_2)_4Ph(CH_2)_5COOH$ (†)	1
$CH_3(CH_2)_5Ph(CH_2)_4COOH$ (8)	1
$CH_3(CH_2)_6Ph(CH_2)_3COOH$ (8)	1
$C_6H_5(CH_2)_2CH{=}CH(CH_2)_6COOH$ (11)	1
$CH_3PhCH{=}CH(CH_2)_7COOH$ (7)	1
$CH_3CH_2PhCH{=}CH(CH_2)_6COOH$ (7)	1
$CH_3(CH_2)_2PhCH{=}CH(CH_2)_5COOH$ (†)	1
$CH_3(CH_2)_3PhCH{=}CH(CH_2)_4COOH$ (†)	1
$CH_3(CH_2)_4PhCH{=}CH(CH_2)_3COOH$ (†)	1
$CH_3(CH_2)_5PhCH{=}CH(CH_2)_2COOH$ (8)	1
$CH_3(CH_2)_6PhCH{=}CHCH_2COOH$ (8)	1
$CH_3OPh(CH_2)_8COOH$ (7)	1a
$CH_3CH_2OPh(CH_2)_7COOH$ (7)	1a
$CH_3(CH_2)_2OPh(CH_2)_6COOH$ (†)	1
$CH_3(CH_2)_3OPh(CH_2)_5COOH$ (†)	1
$CH_3(CH_2)_4OPh(CH_2)_4COOH$ (8)	3
$CH_3(CH_2)_5OPh(CH_2)_3COOH$ (8)	1
$CH_3(CH_2)_6OPh(CH_2)_2COOH$ (8)	1
$CH_3OPhCH{=}CH(CH_2)_6COOH$ (7)	1
$CH_3CH_2OPhCH{=}CH(CH_2)_5COOH$ (7)	1a
$CH_3(CH_2)_2OPhCH{=}CH(CH_2)_4COOH$ (†)	1
$CH_3(CH_2)_3OPhCH{=}CH(CH_2)_3COOH$ (†)	2a
$CH_3(CH_2)_4OPhCH{=}CH(CH_2)_2COOH$ (8)	2
$CH_3(CH_2)_5OPhCH{=}CHCH_2COOH$ (8)	1
$CH_3(CH_2)_6OPhCH{=}CHCOOH$ (8)	1
$C_6H_5O(CH_2)_9COOH$ (7)	1
$C_6H_5S(CH_2)_9COOH$ (7)	1
$CH_3PhO(CH_2)_8COOH$ (7)	1
$CH_3PhS(CH_2)_8COOH$ (7)	1
$CH_3SPh(CH_2)_8COOH$ (7)	1
$CH_3SPhCH{=}CH(CH_2)_6COOH$ (7)	1
$CH_3CH_2PhO(CH_2)_7COOH$ (†)	1

The efficacy group for each compound is shown, with 3 being most toxic (highlighted in bold) and 1 being least toxic; "a" indicates a compound at the upper boundary of an efficacy group and "b" indicates a compound at the lower boundary. Other compounds tested had little or no toxicity

oxatetradecanoic acid was 1.12 ± 0.04 for 100 determinations during a 6-month period. The ratio for each compound was normalized to that for 11-oxatetradecanoic acid, yielding an "efficacy value." We defined the efficacy of 10 μM 11-oxatetradecanoic acid (which was 1.0 after normalization) as the center of efficacy group 3 and the ethanol control as the center of efficacy group 1 (see Fig. 1). To control for differences in medium or cell growth, we defined the interval ranges of each group independently for each experiment, based on the 11-oxatetradecanoic acid and ethanol values. All analogs were tested at least twice, and we tested all compounds near the border between two groups in at least three assays.

RESULTS

Screening of Myristate Analogs for Toxicity Against Trypanosomes. We screened 244 analogs which are subdivided into groups as shown in Table 1. The analogs vary with respect to polarity, steric bulk, conformation, and, to a limited degree, chain length. In many groups, the effects of the substituent(s) have been assessed at nearly every position in the chain.

For this large-scale screen, we needed a reproducible and rapid assay for parasite growth. We developed an assay in which small cultures, in wells of a microtiter plate, were monitored by the color change of a phenol red indicator. This color change is produced by acidification of the medium due to secretion of pyruvic acid by growing trypanosomes. After a standard growth period, an efficacy value was calculated from the ratio A_{550}/A_{405}. Fig. 1 shows a typical screen. This set of compounds included samples of ethanol and 11-oxatetradecanoic acid as both controls and coded samples (compounds E and 24 and O-11 and 27, respectively); there is excellent agreement between these samples and controls. After screening and categorizing all analogs in this manner, we broke the code and matched the structures of the compounds with their efficacy. Note that 11-oxa-, 13-oxa-, and 6-oxatetradecanoic acids fall in groups 3, 2, and 1, correlating well with their effects on trypanosome growth assessed by cell counts (5). Fig. 2 shows the structures of the 20 most active compounds (group 3), grouped by chemical class.

Uptake and Metabolic Processing of [^{3}H]Oxatetradecanoic Acids. We could not directly assess the mechanism of toxicity of most of the compounds shown in Fig. 2 because they were not available in radiolabeled form. However, we did examine the incorporation into cellular lipid and proteins of three [^{3}H]oxatetradecanoic acids, which we had shown previously to differ in toxicity. We had found that, at 10 μM, 11-oxatetradecanoic acid killed >99% of cultured trypanosomes within 24 hr, 13-oxatetradecanoic acid inhibited the growth rate by 40%, and 6-oxatetradecanoic acid inhibited the growth rate by 10% (5). We incubated cultures of trypanosomes with [^{3}H]myristic acid or with one of the three [^{3}H]oxatetradecanoic acids. The TLC in Fig. 3*A* shows incorporation into GPIs and other lipid species. Although the amount of free fatty acid associated with the washed cells was comparable for myristate and the three analogs (compare lanes 2–5), glycolipid A (the VSG anchor precursor), and glycolipid C (an identical GPI, except with an acylated inositol) were

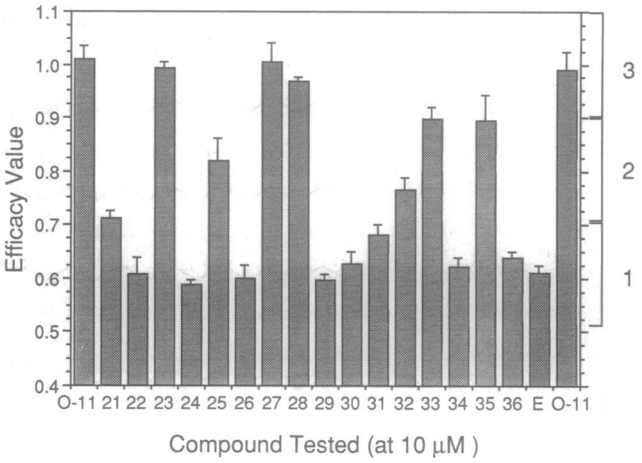

FIG. 1. Assay of analog toxicity. The toxicity of 16 compounds (10 μM), identified by code number, was assessed relative to 11-oxatetradecanoic acid (O-11) and ethanol (E) controls. Each bar shows the mean and standard deviation of quadruplicate assays. Brackets at right indicate the efficacy group designations for this experiment. Compounds evaluated: 21, $CH_3(CH_2)_3CO(CH_2)_8COOH$; 22, $CH_3(CH_2)_7NHCO(CH_2)_3COOH$; 23, $CH_3(CH_2)_6CO(CH_2)_5COOH$; 24, ethanol; 25, $CH_3(CH_2)_4CO(CH_2)_7COOH$; 26, $CH_3(CH_2)_{10}COCH_2COOH$; 27, $CH_3(CH_2)_2O(CH_2)_9COOH$ (O-11); 28, $CH_3(CH_2)_8CO(CH_2)_3COOH$; 29, $CH_3(CH_2)_6NHCO(CH_2)_4COOH$; 30, $CH_3CH_2CO(CH_2)_{10}COOH$; 31, $CH_3(CH_2)_5CO(CH_2)_6COOH$; 32, $CH_3(CH_2)_9CO(CH_2)_2COOH$; 33, $CH_3(CH_2)_2CO(CH_2)_9COOH$; 34, $CH_3(CH_2)_8NHCO(CH_2)_2COOH$; 35, $CH_3CO(CH_2)_{11}COOH$; 36, $CH_3(CH_2)_7CO(CH_2)_4COOH$.

labeled less efficiently by the three analogs than by myristic acid. Quantitation using a Bioscan System 2000 TLC scanner indicated that [^{3}H]11-oxatetradecanoic acid labeled GPIs 8% as well as [^{3}H]myristate (compare lanes 2 and 3), and [^{3}H]6-oxatetradecanoic acid labeled 2–3% as well (compare lanes 2 and 5). [^{3}H]13-Oxatetradecanoic acid labeled glycolipid C at only 1% the level obtained with [^{3}H]myristate (lane 4) and labeled glycolipid A even more poorly (the latter is not even visible in the exposure used for Fig. 3*A*). We also noted differences in labeling of cellular phospholipids. For example, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine accounted for 75% of the total cellular lipid species labeled with [^{3}H]myristate. The comparable values for 11-oxa-, 6-oxa-, and 13-oxatetradecanoic acids were 35%, 15%, and 29%. The relative distribution of label into phospholipids was similar with ^{3}H-labeled myristic acid, 11-oxatetradecanoic acid, and 13-oxatetradecanoic acid (compare lanes 2–4 in Fig. 3*A*). However, with [^{3}H]6-oxatetradecanoic acid the pattern was altered, with efficient labeling of phosphatidylcholine but extremely poor radiolabeling of phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin (Fig. 3*A*, lane 5).

All of the analogs were incorporated into cellular proteins, and in each case, the predominant radiolabeled species was VSG (Fig. 3*B*). However, the efficiency of labeling of VSG was significantly lower with each of the tritiated analogs (lanes 1–3) than with [^{3}H]myristate (lane 4; note that the number of cell equivalents varies in the different lanes in Fig.

when tested at 10 μM. These included saturated fatty acids (C_8, C_{10}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{18}, and C_{20}), a series of nine myristate analogs containing amide groups (9), eight containing acylamino amide groups (9), three 15-carbon-equivalent analogs ($C_6H_5(CH_2)_{11}COOH$, $C_6H_5CH{=}CH(CH_2)_9COOH$, and $C_6H_5O(CH_2)_{10}COOH$), eleven azidoaromatic analogs (10), five nitroaromatic analogs (10), and twelve haloaromatic analogs (10). References describing synthesis and characterization of all compounds are given in parentheses. Details of synthesis and characterization of compounds marked (†) are available from the authors. Z, cis double bond; E, trans double bond; Fu, furyl; Th, thienyl; Ph, phenyl.

*Stereochemistry of double bonds is uncertain.

‡Corey–Pauling–Koltun (CPK) space-filling atomic models indicate that the width of an aromatic ring is equivalent to three methylenes.

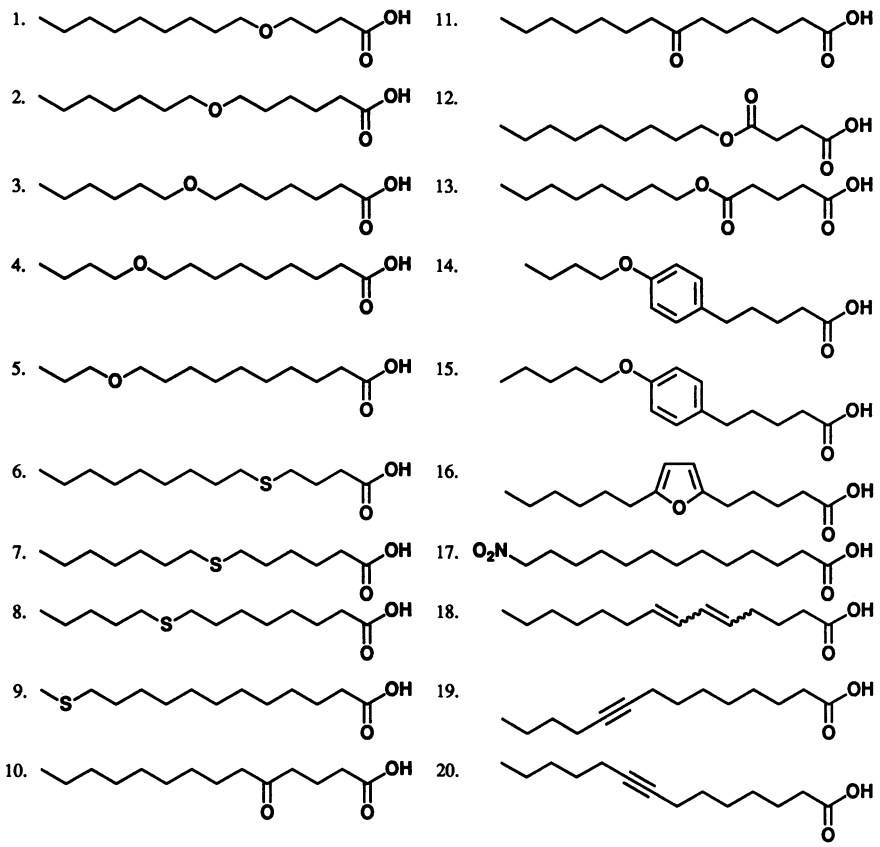

FIG. 2. The 20 most toxic fatty acid analogs. Compound 19 was consistently at the upper boundary of group 3; compounds 3, 8, 10, and 12 were always near the lower boundary. Compounds 2, 6, 7, 11, and 13 were in group 3 even when tested at 2 μM; compounds 6 and 7 were still toxic (group 2) at 0.4 μM. All 20 compounds were toxic at 10 μM when tested by cell counts as in ref. 5; morphology of the cells after drug treatment, as viewed by light microscopy, was identical to that produced by 11-oxatetradecanoic acid (5).

3*B*). Less prominently labeled protein species were also evident, especially in lanes 2 and 4. These species are probably *N*-myristoylproteins because, in contrast to VSG, their radiolabel was not released when the gel was incubated in 0.2 M KOH (data not shown). This treatment cleaves esters such as those in the VSG GPI, but not the amide bond linking myristate to the N-terminal glycine of *N*-myristoylproteins.

DISCUSSION

Our screen of 244 myristate analogs has revealed 20 compounds which are strongly toxic to *T. brucei* in culture (Table 1). Of the 20 most toxic compounds (Fig. 2), 9 are either thiatetradecanoic or oxatetradecanoic acids. The ether functional group is present in conjunction with an aromatic residue in two other compounds and an additional four analogs contain oxygen, in the form of either a ketocarbonyl or an ester group. The remaining structures include 13-nitrotridecanoic acid, a furyl derivative, and three unsaturated tetradecanoic acids.

The toxic compounds might interfere with several cellular processes, any of which could account for their toxicity. Possibilities related to GPIs include (*i*) incorporation of analogs in lieu of myristate into the GPI diacylglycerol, (*ii*) inhibition of any of the enzymes responsible for GPI myristoylation, or (*iii*) alteration in the interactions of the VSG with membranes due to analog incorporation into GPIs or into membrane phospholipids. Processes not directly related to GPIs include (*i*) effects on uptake or intracellular transport of exogenous fatty acids, (*ii*) alterations in the activities of acylCoA synthases, (*iii*) alterations in composition of cellular phospholipids by entry into, or inhibition of, phospholipid biosynthetic pathways, (*iv*) inhibition of myristoyl-CoA:protein N-myristoyltransferase, or (*v*) disruption of the function of *N*-myristoylproteins through incorporation of the analog.

As mentioned above, the toxicity of the analogs could result from interactions with proteins involved in GPI synthesis or remodeling, lipid synthesis, or protein acylation. There are several examples which provide insight into the mode of interaction of fatty acids with protein, all of which indicate that the fatty acid is bound in a bent conformation. In one example, the methylene chain of myristate is bound to rat intestinal fatty acid-binding protein in a bent conformation with the bend in the vicinity of C-9 (15). Similarly, myristate binds to both *Homo sapiens* and *Saccharomyces cerevisiae* N-myristoyltransferase in a bent conformation, with the principal bend in the vicinity of C-5 to C-7 (reviewed in ref. 6). Also, palmitoyl-CoA is bound to liver acyl-CoA-binding protein in a bent conformation with the principal bend extending from C-6 to C-9 (16). We have built CPK models of the 20 most toxic compounds, and most can accommodate a bend in the C-6 to C-7 region. Inspection of CPK models of 5-(4-butoxyphenyl)pentanoic acid and 5-(5-pentoxyphenyl)-pentanoic acid suggests that the distance between the carboxyl (C-1) and the bend may be important among the active analogs. An analogous situation occurs with N-myristoyltransferase, where variations in chain length are better tolerated from the bend to the terminal methyl than from the bend to carboxyl (6).

We initiated a study of the mechanism of analog toxicity by examining three radiolabeled oxatetradecanoic acids. On incubation of these analogs with cultured trypanosomes, we found that the amount of labeled free fatty acids associated with washed cells was similar whether the organism was

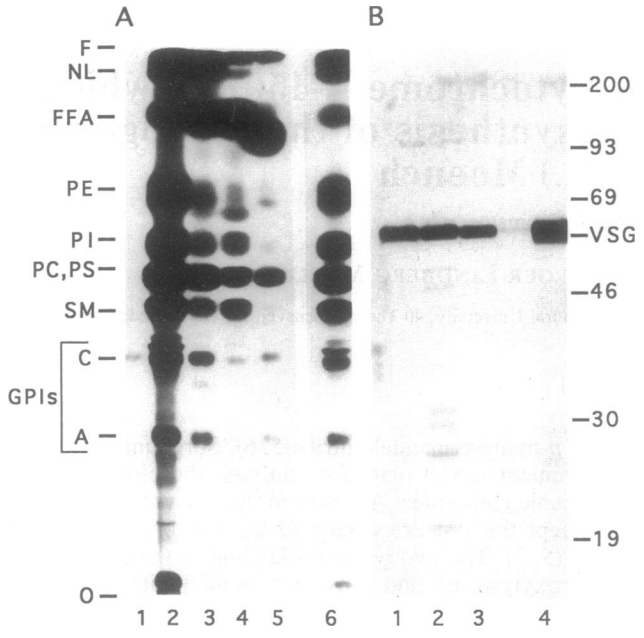

FIG. 3. Radiolabeling of lipids and protein with [^{3}H]myristic acid or [^{3}H]oxatetradecanoic acids. Trypanosomes (ILTat 1.3) were resuspended (5×10^7 cells per ml) in supplemented minimal essential medium α (14) containing either [^{3}H]myristate (NEN) or a [^{3}H]-oxatetradecanoic acid (5) at 100 μCi/ml (specific activity, 30 Ci/mmol; 1 Ci = 37 GBq); the radiolabeled compounds were dried and then dissolved in a small volume of ethanol, <0.5% of final volume. Cells were incubated under 5% CO_2/95% air for 1 hr at 37°C and washed (14). The radiolabeling patterns of proteins and lipids were identical between strains ILTat 1.3 and 221 (data not shown). (*A*) Total lipids were extracted from 10^7 cells and analyzed (14). Lane 1, glycolipid C marker (C is a trypanosome GPI with an acylated inositol); lanes 2–5, lipids radiolabeled with [^{3}H]myristic acid, [^{3}H]11-oxa-, [^{3}H]13-oxa-, or [^{3}H]6-oxatetradecanoic acid, respectively (4-day exposure for lanes 1–5); lane 6, a 10-hr exposure of lane 1. Glycolipids A and C had the appropriate sensitivity to phosphatidylinositol-specific phospholipase C and GPI-specific phospholipases C and D. A, glycolipid A; C, glycolipid C; O, origin; F, front; NL, neutral lipids; FFA, free fatty acids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin. (*B*) Cells were boiled for 5 min in sample buffer and proteins were fractionated by SDS/PAGE (5) (a 2-week autoradiographic exposure is shown). Lane 1, 5×10^6 cells labeled with [^{3}H]11-oxatetradecanoic acid; lanes 2 and 3, 10^7 cells labeled with [^{3}H]13-oxa- or [^{3}H]6-oxatetradecanoic acid, respectively; lane 4, 10^6 cells labeled with [^{3}H]myristic acid. Molecular masses (kDa) of standard proteins are at right.

incubated with [^{3}H]myristic acid or any of the three [^{3}H]oxatetradecanoic acids (compare lanes 2–5 in Fig. 3*A*). This suggests that substitution of oxygen at C-6, C-11, or C-13 does not affect the recognition of these analogs by the trypanosome's myristic acid import apparatus. Therefore, differences in labeling of GPIs *in vivo* could be due to differences in their recognition by the various enzymes or proteins involved in processes such as fatty acid activation or transport. Not surprisingly, we found that 10 μM myristate slightly reduced the toxicity of 10 μM 11-oxatetradecanoic acid; 30 μM myristate essentially abolished the toxicity (data not shown).

The toxicity of the oxatetradecanoic acids (5) correlated roughly with their incorporation into VSG (Fig. 3*B*). Nevertheless, we cannot conclude that toxicity was due solely to incorporation into the VSG anchor, as there were also changes in labeling of phospholipids (Fig. 3*A*) and of proteins which are probably N-myristoylated (Fig. 3*B*). In the latter case, it is interesting that proteins labeled in the presence of [^{3}H]13-oxatetradecanoic acid exhibit increased radioactivity and altered mobility compared with proteins labeled in the presence of [^{3}H]myristic acid (compare lanes 2 and 4 in Fig. 3*B*). Similar effects have been observed previously (e.g., ref. 17).

Although it is clear that the 20 most toxic analogs (Fig. 2) may have diverse mechanisms of toxicity, we are encouraged to pursue these mechanisms. These analyses will be facilitated by the fact that analog effects on GPI biosynthesis can be studied in cell-free systems capable of catalyzing remodeling or myristate exchange into GPIs. Furthermore, we have identified a number of analogs with toxicity comparable to that of 11-oxatetradecanoic acid studied earlier, which we believe may be candidate antitrypanosomal drugs.

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1. Ferguson, M. A. & Cross, G. A. M. (1984) *J. Biol. Chem.* **259,** 3011–3015.
2. Englund, P. T. (1993) *Annu. Rev. Biochem.* **62,** 121–138.
3. McConville, M. J. & Ferguson, M. A. J. (1993) *Biochem. J.* **294,** 305–324.
4. Masterson, W. J., Raper, J., Doering, T. L., Hart, G. W. & Englund, P. T. (1990) *Cell* **62,** 73–80.
5. Doering, T. L., Raper, J., Buxbaum, L. U., Adams, S. P., Gordon, J. I., Hart, G. W. & Englund, P. T. (1991) *Science* **252,** 1851–1854.
6. Johnson, R., Bhatnager, R. S., Knoll, L. J. & Gordon, J. I. (1994) *Annu. Rev. Biochem.* **63,** 869–914.
7. Kishore, N. S., Lu, T., Knoll, L. J., Katoh, A., Rudnick, D. A., Mehta, P. P., Devadas, B., Huhn, M., Atwood, J. L., Adams, S. P., Gokel, G. W. & Gordon, J. I. (1991) *J. Biol. Chem.* **266,** 8835–8855.
8. Gokel, G. W., Lu, T., Rudnick, D. A., Jackson-Machelski, E. & Gordon, J. I. (1992) *Israel J. Chem.* **32,** 127–133.
9. Devadas, B., Lu, T., Katoh, A., Kishore, N. S., Wade, A. C., Mehta, P. P., Rudnick, D. A., Bryant, M. L., Adams, S. P., Li, Q., Gokel, G. W. & Gordon, J. I. (1992) *J. Biol. Chem.* **267,** 7224–7239.
10. Lu, T., Li, Q., Akira, K., Hernandez, J., Duffin, K., Jackson-Machelski, E., Knoll, L. J., Gokel, G. W. & Gordon, J. I. (1994) *J. Biol. Chem.* **269,** 5346–5357.
11. Heuckeroth, R. O., Jackson-Machelski, E., Adams, S. P., Kishore, N. S., Huhn, M., Katoh, A., Lu, T., Gokel, G. W. & Gordon, J. I. (1990) *J. Lipid Res.* **31,** 1121–1129.
12. Rudnick, D. A., Lu, T., Jackson-Machelski, E., Hernandez, J. C., Li, Q., Gokel, G. W. & Gordon, J. I. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10507–10511.
13. Zinsstag, J., Brun, R. & Gessler, M. (1991) *Parasitol. Res.* **77,** 33–38.
14. Doering, T. L., Raper, J., Buxbaum, L. U., Hart, G. W. & Englund, P. T. (1990) *Methods Companion Methods Enzymol.* **1,** 288–296.
15. Eads, J., Sacchettini, J. C., Kromminga, A. & Gordon, J. I. (1994) *J. Biol. Chem.* **268,** 26375–26385.
16. Kragelund, B. B., Andersen, K. V., Madsen, J. C., Knudsen, J. & Poulsen, F. M. (1993) *J. Mol. Biol.* **230,** 1260–1277.
17. Heuckeroth, R. O. & Gordon, J. I. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5262–5266.