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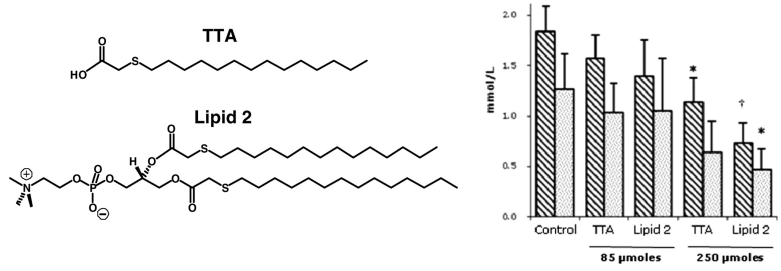

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Synthesis and Analysis of Novel Glycerolipids for the Treatment of Metabolic Syndrome

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Tetradecylthioacetic acid (TTA) **1** is a peroxisome proliferator-activated receptor (PPAR) agonist found to improve insulin sensitivity, lower blood lipid levels, enhance fatty acid oxidation, and promote anti-inflammation in vivo. In an attempt to enhance these properties, two key thioether fatty acid (Thefa) lipids, ditetradecylthioacetyl phosphatidylcholine **2** and tritetradecylthioacetyl glycerol **3**, are synthesized and administered po to male Wistar rats at two different doses to study and compare metabolic outcomes relative to the administration of **1** alone after 6 days. Liposomal formulations of **1** and **2** are also prepared to evaluate acute metabolic responses (at 3 h) post iv injection. Across all metrics measured, **1**-induced responses post po administration are in line with previous data. Responses induced from **3** are mostly equivalent to **1**-induced responses. By contrast, **2**-induced responses almost always outperform those of **1** and **3**. Therefore, **2** may represent a new lead for the treatment of metabolic syndrome.

Introduction

Disorders of lipid metabolism are connected to many common, life-style related diseases. Obesity and obesity-related disorders, often referred to as the metabolic syndrome, affect an increasing part of the population in Europe, the USA, and even several developing countries.[1−3] The key pathophysiological features of the metabolic syndrome include insulin resistance (impaired glucose tolerance, insulin insensitivity, type 2 diabetes), dyslipidemia (elevated triacylglycerols and low density lipoprotein (LDL[a]) with reduced high density lipoprotein (HDL) cholesterol), abdominal obesity, hypertension, and endothelial dysfunction. Whereas the pathogenesis of the metabolic syndrome is complex and not well understood, obesity is acknowledged as an important causative factor. The presence of the metabolic syndrome predicts increased risk of developing diabetes type 2 and cardiovascular disease with fatal consequences.[4−7]

Over the past 10 years, peroxisome proliferator-activated receptors (PPARs) have been targeted in drug discovery for the treatment of metabolic diseases because of their critical role in the regulation of lipid metabolism and fat cell differentiation. Indeed, these nuclear receptors are transcription factors that play an important role in the regulation of gene expression by binding to specific peroxisome proliferator response elements (PPREs) within promoters.[8,9] There are three subtypes of PPARs: α, γ, and δ.[10−17] Natural ligands of the PPARs include a wide variety of saturated and unsaturated fatty acids plus eicosanoid derivatives.[18] It has been well documented that hypolipidemic fibrates and antidiabetic thiazolidinediones (TZDs) are synthetic ligands for PPARα and PPARγ, respectively. The thiazolidinediones improve insulin sensitivity, decrease hepatic glucose output, and improve nonglycemic effects.[19] However, use of thiazolidinediones is known to have attendant side effects such as weight gain, edema, and anemia with possible liver dysfunction.[20] Accordingly, there have been attempts to develop dual or even pan PPAR agonists in the hope of achieving complementary and synergistic actions on lipid homeostasis and insulin sensitivity with minimal attendant side effects.[21]

In the past decade, it has been found that structurally modified fatty acids show an enhanced potency compared to natural fatty acids in modulating critical steps in the regulation of lipid metabolism. One such molecule is tetradecylthioacetic acid (TTA) **1** (Scheme 1). Fatty acid **1** is a modified 16-carbon saturated fatty acid with a thioether bridge inserted between carbon atoms 2 and 3. This thioether bridge is considered to stabilize the compound with respect to β-oxidation in vivo.[22] In other aspects, the chemical properties of such thioether fatty acids align with those of normal fatty acids. The pleiotropic effects of **1** have been revealed over the past decade. The biological responses include increased hepatic and muscle mitochondrial fatty acid oxidation in mammals, reduced body fat (as seen in rats), decreased plasma lipid levels, modification of plasma and tissue fatty acid profiles, improved insulin sensitivity, potent antioxidant effects, and anti-inflammatory actions.[23,24] There are also effects involving increased mitochondrial β-oxidation rates that associate with a lowered proton electrochemical potential and enhanced expression of uncoupling protein 2 (UCP-2).[25] This multitude of biological responses suggests that fatty acid **1** should be a potent antiatherogenic compound. Indeed, recent clinical trials have indicated that **1** could have significant effects on lipid metabolism in humans.[26] As a pan PPAR ligand, fatty acid **1** regulates the expression of those particular lipid-metabolizing enzymes involved in catabolic metabolic pathways in a PPAR dependent manner. Indeed, **1**

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[a] Abbreviations: BSA, bovine serum albumin; CDI, *N*,*N*′-carbonyldiimidazole; CPT-II, carnitine palmitoyl transferase-II; DBU, 1,8-diazabicyclo(5.4.0)undec-1-ene; DMAP, 4-dimethylaminopyridine; DMPC, dimyristoyl-L-α-phosphatidylcholine; DMSO, dimethylsulfoxide; EI, electron ionization; FAO, fatty acyl-CoA oxidase; GPC, L-α-glycero-phosphocholine; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MUFA, monounsaturated fatty acid; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; SA, stearic acid; SFA, saturated fatty acid; Thefa, thioether fatty acid; TTA, tetradecylthioacetic acid; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

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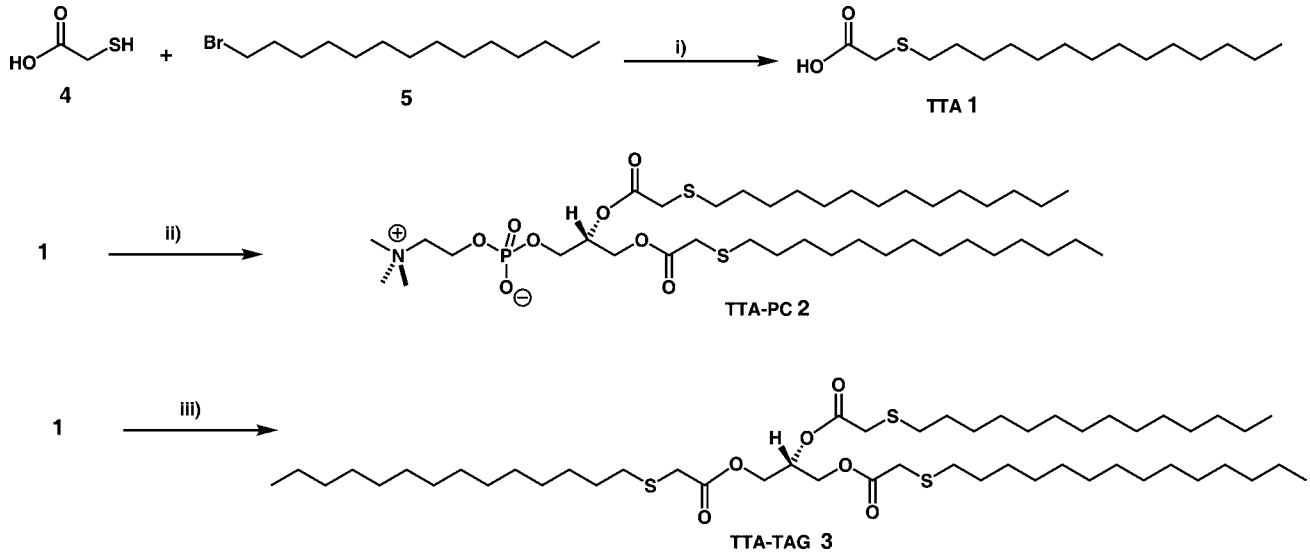
Scheme 1. Structures and Syntheses of **1**, **2**, and **3**[a]



[a] (i) NaOH/MeOH, ambient temperature, 91%; (ii) L-α-glycerophosphocholine, CDI, DBU, CH_2Cl_2, DMSO, 70%; (iii) glycerol, HBTU, DMAP, CH_2Cl_2, 83%.

appears to activate human PPARs in the following order: $\delta > \alpha > \gamma$.[27] Alternatively, fatty acid **1** has also been found to activate murine PPARs in cells post transfection but in the following rank order $\alpha > \delta \gg \gamma$.[28,29] In addition, PPAR independent metabolic pathways appear to be important for other metabolic effects of **1** and nonmetabolic effects such as the anti-inflammatory activities of **1**. Indeed **1**, as an active lipid metabolite, may affect specific signal transduction pathways.[29]

In terms of metabolism, data to date suggest that fatty acid **1** is incorporated into glycerolipids in vivo, with a preference for phospholipids over acylglycerols.[30,31] Both phospholipids and acylglycerols are partially degraded by pancreatic lipases in the gut releasing fatty acids from the *sn*-1 and *sn*-3 positions. Micelles are then generated from 2-monoacylglycerols, *lyso*-phospholipids, cholesterol, bile salts, and free acids prior to absorption into enterocytes by simple diffusion across plasma membrane. Once there, lipids are resynthesized and subsequently assembled into large lipoprotein particles known as chylomicrons. These pass into the general circulation via the thoracic duct and are transported in the bloodstream to the liver. Given this uptake mechanism, we were curious to establish if the properties of fatty acid **1** could be enhanced and/or indeed modified by the substitution of **1** into phospholipid or triacylglycerol structures. Here we describe the testing of this hypothesis and observe that administration of a TTA-containing phospholipid does indeed lead to fatty acid **1** like behavior that is much enhanced in many instances. By contrast, administration of a TTA-containing triacylglycerol structure does not appear typically to result in any enhanced fatty acid **1** effects.

Results

Chemistry. The chosen phospholipid and triacylglycerol targets for "incorporation" of **1** were ditetradecylthioacetyl-L-α-phosphatidylcholine **2** and tritetradecylthioacetyl triglycerol **3** (Scheme 1).[32] Fatty acid **1** was synthesized by a nucleophilic substitution between thioglycolic acid **4** and tetradecylbromide **5** under basic conditions. Thereafter, **1** was activated with *N*,*N*′-carbonyldiimidazole (CDI) and reacted with L-α-glycerophosphocholine (GPC) to give lipid **2**. The final coupling reaction, adapted from Warner et al., was optimized using a dichloromethane−dimethylsulfoxide (DMSO) solvent system (Scheme 1).[33] The triacylglycerol analogue, lipid **3**, was synthesized by coupling **1** with glycerol using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluoro-phosphate (HBTU) as coupling agent and 4-dimethylaminopyridine (DMAP) as a nucleophilic catalyst (Scheme 1).

Decrease in Plasma and Hepatic Lipids. Male Wistar rats were fed fatty acid **1** or lipids **2** or **3** at doses equivalent to 85 μmol or 250 μmol of TTA/animal, resulting in a general dose dependent decrease in plasma cholesterol and triacylglycerols levels (Figure 1A). Administration of fatty acid **1** or lipids **2** or **3** at the lower dose all resulted in broadly similar decreased levels of cholesterol and triacylglycerols in plasma. By contrast, administration of lipid **2** at the higher dose resulted in a more enhanced lipid lowering effect than was observed with either fatty acid **1** or lipid **3**. Overall, administration of **2** was responsible for a drop of approximately 60% in both plasma cholesterol and triacylglycerol levels relative to control (Figure 1A). Similar but less emphatic trends were also observed with liver cholesterol and triacylglycerols levels (Figure 1B). In the liver, administration of lipid **2** was found to be responsible for a drop of approximately 20% and 25%, respectively, in liver cholesterol and triacylglycerol levels relative to control (Figure 1B).

Increased Expression of PPARα Regulated Genes. Fatty acid **1** is an agonist for the different PPARs and is a reasonably potent PPARα agonist.[27] Hence, the activities of three important products of PPARα-regulated genes that are all involved in fatty acid catabolism were examined by different assays on biopsy tissue. These three gene products were 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, fatty acyl-CoA oxidase (FAO), and carnitine palmitoyl transferase-II (CPT-II). HMG-CoA synthase is a mitochondrial protein that typically acts as the rate-determining enzyme for ketone body formation. FAO is the rate-determining enzyme peroxisomal fatty acid oxidation. Both were found to increase in a dose dependent manner at 6 days post po administration of fatty acid **1** or lipids **2** or **3** (Figure 2). However, administration of lipid **2** at the higher dose resulted in, by far, the largest increases in mitochondrial HMG-CoA synthase and FAO activities above control by approximately 4-, and 5- to 6-fold, respectively.

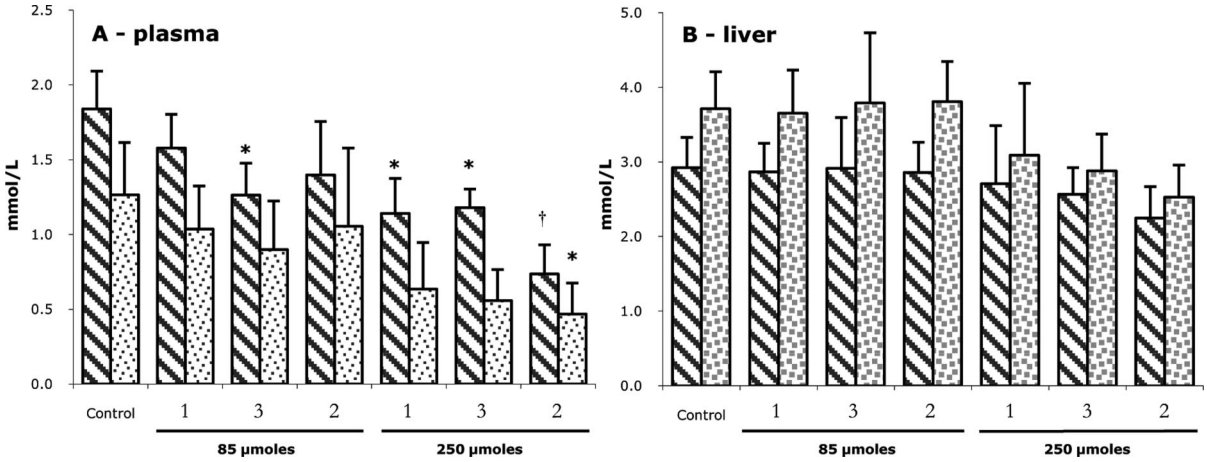


Figure 1. Effects of low (85 μmol) and high (250 μmol) equimolar doses of TTA, fed as unesterified fatty acid **1** or lipids **2** or **3** on levels of cholesterol (dark striped bars) and triacylglycerol (light speckled bars) in plasma (A) and liver (B). Male Wistar rats were fed the different compounds by gastric intubation for 6 days. The values represent the means ± SD from 5 rats in each group. *: significant difference from control animals ($P < 0.05$). †: significant difference from control and the situations where animals were treated at same dose level with either **1** or lipid **3** ($P < 0.05$).

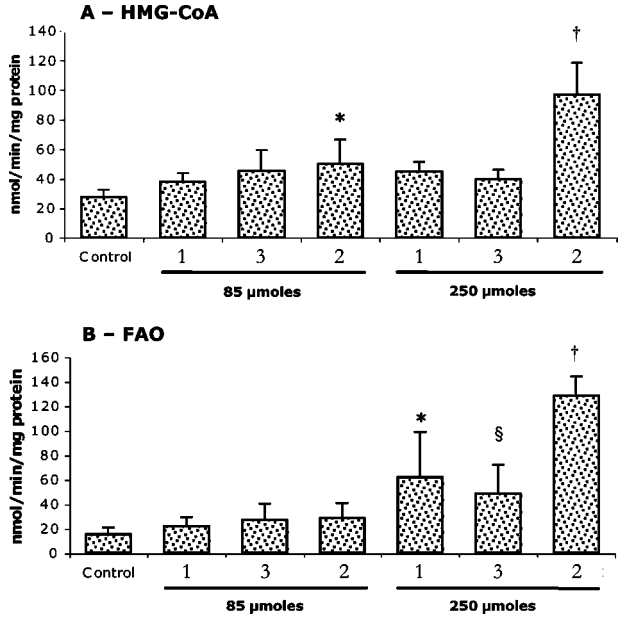


Figure 2. Effect of low (85 μmol) and high (250 μmol) equimolar doses of TTA, fed as unesterified fatty acid **1** or lipids **2** or **3** on the enzyme activities of HMG-CoA synthase (A) and FAO (B). Male Wistar rats were fed the different compounds by gastric intubation for 6 days. The values represent the means ± SD from 5 rats in each group. *: significant difference from control animals ($P < 0.05$). §: significant difference from control and **1** at the lower dose ($P < 0.05$). †: significant difference from control, plus **1** and **3** treated animals at same dose level, and also from **1** at the lower dose ($P < 0.05$).

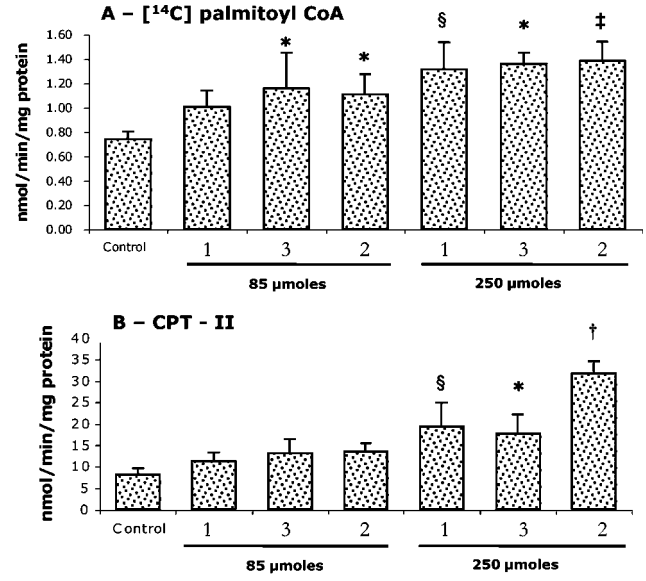


Figure 3. Effect of low (85 μmol) and high (250 μmol) equimolar doses of TTA fed as unesterified fatty acid **1** or lipids **2** or **3** on fatty acid oxidation of [1-^{14}C]-palmitoyl-CoA in liver homogenates (A) and on the mitochondrial CPT-II enzyme activity (B). Male Wistar rats were fed the different compounds by gastric intubation for 6 days. The values represent the means ± SD from 5 rats in each group. *: significant difference from control animals ($P < 0.05$). §: significant difference from control and **1** treated animals at the lower dose ($P < 0.05$). ‡: significant difference from control, plus **1** and **2** treated animals at the lower dose ($P < 0.05$). †: significant difference from control, plus **1** and **3** treated animals at same dose level, also from **1**, **2**, and **3** treated animals at the lower dose ($P < 0.05$).

In a similar way, the activity of CPT-II, the protein system responsible for the translocation of fatty acids over the inner mitochondrial membrane was found to increase in a dose dependent manner at 6 days post po administration of fatty acid **1** or lipids **2** or **3** (Figure 3B), with the largest increase of approximately 3-fold above control after administration of **2** at the higher dose. By contrast, when mitochondrial β-oxidation levels were measured as a function of [^{14}C]-palmitoyl CoA isolated from liver homogenates, levels were found to increase in a dose dependent manner at 6 days post po administration of fatty acid **1** or lipids **2** or **3** (Figure 3A), but in this case, administration of lipid **2** at the higher dose was no more effective than administration of fatty acid **1** or lipid **3** at the same higher dose.

Modification of Fatty Acid Composition. The metabolism of fatty acid **1** involves coenzyme A activation and incorporation into esterified lipids, mostly phospholipids more than triacylglycerols. Only minor amounts of free fatty acids can be detected in plasma and liver.[16,30] As a result of feeding fatty acid **1**, the fatty acid composition is known to be changed in plasma and tissues.[17,31] In our case, analysis of the fatty acid composition in plasma and liver revealed that the levels of **1** became

Table 1. Fatty Acid Composition of Plasma and Liver in Male Wistar Rats Fed 85 μmol or 250 μmol of **1** for 6 Days as Fatty Acid **1** or Lipids **2** or **3**[a]

daily dose/rat	1	SFA	MUFA	PUFA, n-3	18:2, n-6	LCPUFA, n-6	LCn-6/18:2, n-6
			A. Plasma FA wt %				
control	0.00	35.60 ± 1.56	21.47 ± 3.51	2.85 ± 0.39	20.06 ± 2.50	19.38 ± 4.1	0.98 ± 0.26
1 85 μmol	0.32 ± 0.20	36.02 ± 0.38	23.49 ± 2.35	2.47 ± 0.12	18.46 ± 1.91	18.75 ± 2.0	1.03 ± 0.19
3 (equiv 85 μmol of **1**)	0.51 ± 0.26	36.21 ± 1.26	21.80 ± 2.96	2.35 ± 0.16	19.73 ± 2.32	19.08 ± 1.22	0.98 ± 0.12
2 (equiv 85 μmol of **1**)	0.90 ± 0.55	35.65 ± 1.27	22.53 ± 4.54	2.42 ± 0.26	19.52 ± 0.98	19.06 ± 3.66	0.97 ± 0.14
1 250 μmol	1.60 ± 0.76[b]	35.00 ± 0.54	21.32 ± 2.48	2.24 ± 0.20[b]	19.68 ± 1.35	20.82 ± 1.72	1.06 ± 0.11
3 (equiv 250 μmol of **1**)	1.49 ± 0.68[b]	34.67 ± 1.05	21.63 ± 4.01	2.29 ± 0.18[b]	18.25 ± 0.92	22.24 ± 2.13	1.22 ± 0.09
2 (equiv 250 μmol of **1**)	1.95 ± 0.75[b]	35.25 ± 0.97	21.62 ± 4.55	1.86 ± 0.41[b]	17.97 ± 2.06	22.07 ± 4.88	1.25 ± 0.34
			B. Hepatic FA wt %				
control	0.00	39.56 ± 1.35	14.68 ± 2.00	6.06 ± 0.29	13.11 ± 1.86	26.09 ± 1.51	2.02 ± 0.26
1 85 μmol	0.36 ± 0.20	39.67 ± 0.90	17.35 ± 1.87	5.44 ± 0.40[b]	11.15 ± 1.39	25.70 ± 0.64	2.34 ± 0.33
3 (equiv 85 μmol of **1**)	0.69 ± 0.35	39.82 ± 1.12	17.27 ± 2.41	5.18 ± 0.42[b]	11.28 ± 1.07	25.81 ± 1.75	2.30 ± 0.25
2 (equiv 85 μmol of **1**)	1.28 ± 1.07	39.67 ± 1.21	16.66 ± 3.20	4.91 ± 0.52[b]	11.99 ± 1.31	26.13 ± 1.48	2.19 ± 0.13
1 250 μmol	2.03 ± 1.02[b]	38.71 ± 0.65	16.86 ± 2.01	4.94 ± 0.48[b]	11.02 ± 1.80	27.66 ± 0.54	2.56 ± 0.41
3 (equiv 250 μmol of **1**)	2.04 ± 0.8[b]	38.98 ± 0.49	15.91 ± 1.33	4.97 ± 0.34[b]	10.67 ± 0.76	28.73 ± 1.09[e]	2.70 ± 0.21[b]
2 (equiv 250 μmol of **1**)	3.34 ± 1.55[b,e]	39.22 ± 0.56	16.33 ± 1.40	4.05 ± 0.44[b]	8.79 ± 1.08[b,e]	30.30 ± 1.60[b,e]	3.49 ± 0.46[b,c,d,e]

[a] The values represent means ± SD from 5 rats in each group. [b] Significant difference ($P < 0.05$) from control animals. [c] Significant difference ($P < 0.05$) from **1** at same dose level. [d] Significant difference ($P < 0.05$) from **3** at same dose level. [e] Significant difference ($P < 0.05$) from **1** at the low dose.

Table 2. Liposomal Formulations of Fatty Acid **1** and Lipid **2**[a]

liposome	[**1**] (mg/mL)	[total lipid] (mg/mL)	ζ potential (mV)	T_C (°C)	diameter (nm)
1/DMPC (1:1)	8.9	28.6	−36	36	265.3 ± 114.3
2/SA (2:1)	12.5	14.7	−10	44	209.1 ± 84.6

[a] ζ potentials are estimates.

substantial post po administration of fatty acid **1** or lipids **2** or **3** (Table 1). Saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) levels were unchanged relative to control post po administration of fatty acid **1** or lipids **2** or **3** (Table 1). In contrast, plasma and liver levels of polyunsaturated fatty acids (PUFA, n-3), and 18:2, n-6 fatty acids were obviously decreased in a dose dependent manner after administration of the three compounds (this decrease was seen for 22:6, n-3, 20:5, n-3 fatty acids as well, data not shown). Administration of lipid **2** at the highest dose resulted unexpectedly in the most significant decline of both PUFA, n-3 and 18:2, n-6 fatty acid levels, particularly in liver tissue, relative to controls. Conversely, plasma and liver levels of long-chain PUFAs (LCPUFA, n-6; LCn-6/18:2, n-6) were increased in a dose dependent manner by administration of fatty acid **1** or lipids **2** or **3**, and once again administration of lipid **2** at the highest dose resulted in the most significant increase in LCPUFA, n-6 and LCn-6/18:2, n-6 levels, particularly in liver tissue, relative to controls (Table 1).

Liposomal Delivery of 1 and 2. To examine the potential role of intestinal absorption in the enhanced effects of lipid **2** compared to the effects of fatty acid **1** after po administration, fatty acid **1** and lipid **2** were formulated into liposomes with dimyristoyl-L-α-phosphatidylcholine (DMPC) (1:1, m/m) or stearic acid (SA) (2:1, m/m), respectively (Table 2). Free fatty acid **1** was also prepared in bovine serum albumin (BSA) solution to ensure solubility. The effects of iv administration of liposomes **1**/DMPC (1:1, m/m), **2**/SA (2:1, m/m), or DMPC control liposomes in PBS to male Wistar rats (tail vein injection) were compared with the effects of the iv administration of BSA-associated free fatty acid **1** in the same animal model. The **2**/SA liposomes (liposomal **2**) were administered at a total lipid **2** dose of 9 or 46 μmol (that were equivalent with fatty acid **1** doses of 12 or 62 μmol), **1**/DMPC liposome formulations (liposomal **1**) were administered with total fatty acid **1** doses of 12 or 62 μmol. Our expectation was that iv administration of these liposomes might reveal evidence for an acute fatty acid **1** response in a matter of 2−4 h as opposed to the 6 day effects studied post po administration. Indeed, the data concerning triacylglycerol and cholesterol levels in plasma appeared to support the possibility that there are acute **1**/DMPC and **2**/SA liposome mediated lipid lowering effects compared to the control, but BSA-associated free fatty acid **1** administration was at least as effective, if not more so, especially where the lowering of cholesterol (Chol) levels were concerned (Table 3). A dose response effect was not strongly evident on either plasma lipids or the hepatic content of fatty acid **1**. Only a few animals were available for analysis of the hepatic content of fatty acid **1**, so these data have not been treated statistically. These data also seem to indicate a nondose response except where the liver fatty acid **1** content was determined after administration of the **2**/SA liposomes at the highest total lipid dose (Table 3B).

Discussion

The pleiotropic effects of fatty acid **1** are well documented, and significant benefits are conferred on lipid metabolism, immune response, and oxidative stress post administration of **1**.[23,24] In this study, it has now been demonstrated that oral feeding with esterified or unesterified **1** or intravenous injection of the fatty acid will differentially affect the absorption and distribution of this potent fatty acid. Across all metrics measured, the responses to fatty acid **1** and lipid **3** were mostly equivalent. By contrast, the responses to lipid **2** almost always outperformed the responses to **1** and **3**. Compared to administration of fatty acid **1** or lipid **3**, administration of lipid **2** appeared to result in significant increases in the enzyme activities of PPARα regulated gene products and mitochondrial β-oxidation in addition to having the largest impact on plasma and hepatic fatty acid composition (Figures 1, 2, and 3). Most of these effects were dose dependent, although not to the same extent. Especially with regard to enzyme activities, the changes observed with the highest dose used were substantially greater (4−6 fold) than the decrease in plasma and hepatic lipid levels (Figures 1 and 2). This indicates that the changes in lipid levels are not simply a reflection of the upregulation of the PPAR regulated genes, but genes of other regulatory pathways involved in lipid metabolism are also affected. Regarding the incorporation of fatty acid **1** into lipids, the highest level of incorporation of **1** was attained in plasma and liver after feeding lipid **2** in a dose dependent manner (Table 1). The hepatic and plasma fatty acid composition was to a large extent also changed in a dose dependent manner in line with changes to the hepatic content of fatty acid **1** across all the different formulations tested. These modifications have been observed previously in studies with

Table 3. Plasma Triacylglycerol (TAG) or Cholesterol (Chol) Levels (A) and Hepatic Content of Fatty Acid **1** (B) in Rats Injected Intravenously with Either DMPC Control Liposomes (DMPC), a BSA-Associated Solution of Free Fatty Acid **1** (Free **1**), **1**/DMPC (1:1) Liposomes (Liposomal **1**), or **2**/SA (2:1) Liposomes (Liposomal **2**) (see Table 2)[a]

	DMPC	free **1**	liposomal **1** [**1** /DMPC (1:1)]		liposomal **2** [**2**/SA (2:1)]	
at 3 h	liposomes	[**1**] 12 μmol	[**1**] 12 μmol	[**1**] 62 μmol	[total] 9 μmol	[total] 46 μmol
			A. Plasma Lipid Levels (mmol/L)			
TAG	1.97 ± 0.28	1.51 ± 0.27^b	1.67 ± 0.32	1.38 ± 0.28^b	1.65 ± 0.63	1.69 ± 0.6
Chol	1.80 ± 0.24	1.43 ± 0.34^b	1.64 ± 0.16	1.72 ± 0.24^b	1.73 ± 0.25	1.52 ± 0.29
			B. Hepatic Content wt % of Fatty Acid **1**			
wt % of **1**	0.0	0.24	0.18	0.30	0.13	0.55

[a] In (A), the values represent means $\pm$ SD from 4−7 rats in each group. In (B), the values are representative average data from 3 rats in each group. [b] Significantly different ($P < 0.05$) from control animals.

1,[23] the main features being the decrease in PUFA, n-3 fatty acids and an apparent increase in the long chain (20C and more) n-6 fatty acids.

The simplest explanation for the better performance of lipid **2** is that this compound has a higher intestinal absorption than fatty acid **1**, thereby facilitating incorporation into plasma and hepatic lipids post absorption. This is supported by the fact that the quantity of **1** recovered from liver post intravenous administration of free **1** is at least equivalent to if not more than the quantity of **1** recovered post intravenous administration of liposomal **2** (administered in such an amount as to correspond to an approximately equimolar dose of **1**) (see Table 3). In fact, it has been demonstrated that approximately 90% of **1** absorbed actually becomes incorporated into phospholipids post po administration,[30,31] and this rather uncommon distribution for a fatty acid appears to be a central component of effects mediated by **1** on metabolic responses. Therefore, the promotional influence on metabolic responses of feeding **2** (Figures 1, 2, and 3) may well be consistent with these metabolic observations. Accordingly, lipid **2** could just be regarded as a more suitable "means of carriage" to enhance the bioavailability of **1** to influence metabolic responses. However, we do not necessarily believe that the enhancing effects of lipid **2** after po administration can be due to such proposed fatty acid **1**-prodrug-like behavior alone. Indeed, the observed capacity of lipid **2** to enhance metabolic responses, over and above the effects of **1** and **3** post po administration, may also be associated with other unique properties and characteristics that are not necessarily linked with the release of free **1** in a timely fashion such as membrane active functions. We shall be carrying out further investigations into this area of research in order to determine if lipid **2** has such potentially unique properties that could contribute to the control of metabolic responses in a manner independent of fatty acid **1**.

The liposome data are also interesting. A comparison can be made between the extent of accumulation of **1** into liver post po administration of **1** or lipid **2**, and the contrasituation post iv administration of either free **1**, injected in association with BSA, liposomal **1**, or liposomal **2**. In the first instance, po administration of lipid **2** leads to the largest increase in **1** found in the liver (Table 1A). In the second instance, administration of free **1** leads to the highest levels of accumulation of **1** in liver, followed by the administration of liposomal **1** (Table 3B). The administration of free **1** also resulted in the most significant decrease in plasma triacylglycerols and cholesterol levels at 3 h post injection. Certainly, such data could be considered consistent with the argument above that lipid **2** is primarily a "means of carriage" for fatty acid **1** post po administration to enhance the bioavailability of this fatty acid **1** to influence metabolic responses. However, these data more properly demonstrate that acute effects of fatty acid **1** might be able to take advantage of iv compliant formulations to accelerate the bioavailability of **1** to the liver. Indeed iv administration of liposomal **1** leads to an equivalent level of **1** in the liver compared to the amount observed post po administration even though the dose of iv administered liposomal **1** is almost an order of magnitude lower (Tables 1B and 3B). Having said this, some caution is necessary because there is the possibility that the metabolic effects of fatty acid **1** may also vary according to whether or not **1** is administered in free form or in a liposome associated form, owing to potential differences in physical properties between liposomal **1** and free fatty acid **1** (Table 3). Nevertheless, iv administration of liposomal **1** may represent a useful alternative to po administration for specific purposes such as the targeted delivery of modified fatty acids and similar compounds. Overall, less **1** seems to be required for a biological responses post administration by iv injection compared with the amount of **1** required to provoke responses post po administration, a characteristic which is characterized by the fact that the lowest ratio between the dose administered and the hepatic content of **1** is obtained after iv injection (compare Tables 1 and 3).

In conclusion, we are of the opinion that the biological responses of **1**, **3**, and **2** are mediated by effects on gene expression and by direct interference in the metabolism of hepatic and plasma lipoproteins. Particularly at the higher dose, **2** is the most profound agent of these changes. Overall, po administration of **2** gave better results than **1** and **3**. Given this promising data, other novel lipid analogues can now be developed as potential therapeutics for metabolic syndrome related conditions such as hyperlipidaemia and atherosclerosis.

Experimental Section

Synthesis. All reactions were carried out under an atmosphere of nitrogen or argon, in oven-dried glassware, unless otherwise stated. Flash column chromatography was performed with Merck silica gel 60 (230−240 mesh) according to the method of W. C. Still.[34] TLC refers to thin layer chromatography performed on precoated Merck silica gel 60 F_{254} aluminum-backed plates and visualized with a UV lamp (254 nm) and/or stained with acidic ammonium molybdate(IV), basic potassium manganate(VII; $KMnO_4$), iodine, or phosphomolybdic acid. CH_2Cl_2 was distilled over P_2O_5, and other solvents were bought and predried as required. Hipersolv $CHCl_3$ was used for some reactions. All chemicals were purchased from Sigma-Aldrich, Lancaster and Merck Biosciences. Special solvent mixtures are: solvent A, CH_2Cl_2/MeOH/H_2O 345:90:10; solvent B, CH_2Cl_2/MeOH/H_2O 65:25:4; solvent C, CH_2Cl_2/MeOH/AcOH 92:7:1. ^{1}H NMR spectra were recorded on either a Bruker DRX300 or Avance 400 using residual isotopic solvent ($CDCl_3$, $\delta_H = 7.27$ ppm; CD_3OD, $\delta_H = 3.84$ ppm) as an internal reference. Data is reported as follows: (br = broad; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; coupling constant(s) in Hz; integration; assignment). Mass spectra were recorded using VG-070B, Joel SX-10,2 or Bruker Esquire 3000 ESI instruments.

Tetradecylthioacetic Acid 1. Tetradecyl bromide (5.36 mL, 18.0 mmol) was added to a solution of thioglycolic acid (1.25 mL, 18.0 mmol) in 25% NaOH in MeOH (6.03 g in 24.0 mL), and the mixture was stirred vigorously at room temperature for 72 h. After dilution with water, the mixture was acidified to pH 1 with concentrated HCl and the aqueous phase was extracted with diethyl ether. After drying over $MgSO_4$ and concentration in vacuo, this residue was then purified by flash column chromatography on silica gel (hexane/EtOAc 3:1) to yield 4.73 g (91%) of **1** as a hygroscopic white gum. δ_H ($CDCl_3$) 3.27 (s, 2H, SCH_2COOH), 2.68−2.65 (t, J = 7.6 Hz, 2H, SCH_2), 1.66−1.58 (m, 2H), 1.41−1.36 (m, 2H), 1.27 (s, 20H), 0.908−0.874 (t, J = 6.8 Hz, 3H); m/z (EI^+) 288; found $[M]^+$, 288.2121, $C_{16}H_{32}O_2S$ requires $[M]^+$, 288.2123.

Ditetradecylthioacetyl-*sn*-glycero-3-phosphocholine 2. To a solution of **1** (261 mg, 0.905 mmol) in CH_2Cl_2 (5.00 mL) was added CDI (190 mg, 1.17 mmol) and the solution stirred for 2 h 45 min at RT under argon. In the meantime, DBU (135 μL, 0.905 mmol) was added to a solution of L-α-GPC (133 mg, 0.301 mmol) in DMSO (5.00 mL) and stirred at RT under argon for 2 h 45 min. The first solution was then transferred to the second and the mixture stirred at RT for 16 h. The mixture was acidified with acetic acid 0.1 M (12.0 mL) and washed with $CHCl_3$/MeOH 2:1 (5 × 10.0 mL). The organic phase was then washed with H_2O/MeOH 1:1 (5 × 50 mL) and the aqueous phase extracted with $CHCl_3$/MeOH 2:1 (3 × 100 mL). The combined phases were concentrated in vacuo to give a brownish oil (2.78 g), which was purified by flash column chromatography on silica gel (CH_2Cl_2, solvent A in CH_2Cl_2: 10%, 20%, 80%, 90%, solvent A, solvent B, methanol) to yield 168 mg (70%) of **2** as a hygroscopic gum; δ_H ($CDCl_3$) 5.25 (br, 1H, OCH_2CHOCH_2), 4.45−4.41 (dd, J = 12 Hz, J = 2.8 Hz, 2H, $COOCH_2CH$), 4.28−4.23 (m, 2H, $CHCH_2OP$), 4.00−3.97 (t, J = 6.2 Hz, 2H, $POCH_2$), 3.85−3.74 (m, 2H, $CH_2N(CH_3)_3$), 3.31−3.28 (br, 13H, 3 × NCH_3, 2 × SCH_2COOH), 2.63−2.58 (m, 4H, Hb), 1.61−1.54 (m, 4H), 1.38−1.35 (m, 2H), 1.26 (s, 40H), 0.85−0.82 (t, J = 6.8 Hz, 6H); m/z (FAB^+) 798 ($[M + H]^+$; found $[M + H]^+$, 798.5156, $C_{40}H_{81}NO_8PS_2$ requires $[M + H]^+$, 798.5141.

Tritetradecylthioacetyl Glycerol 3. HBTU (1.16 g, 3.05 mmol) and DMAP (1.09 g, 8.95 mmol) were added to a solution of **1** (834.0 mg, 2.89 mmol) and glycerol (85.5 mg, 0.928 mmol) in dry CH_2Cl_2 under argon. The mixture was stirred at room temperature for 19 h. The reaction had not gone to completion, so some more TTA (93.1 mg, 0.323 mmol) was added and the mixture stirred for a further 4 h. Citric acid solution (7%, 15.0 mL) was added to the reaction mixture. The phases were separated and extracted with CH_2Cl_2 (4 × 25.0 mL). The organic phases were combined, dried over $MgSO_4$, and concentrated in vacuo before purification by flash column chromatography on silica gel (hexane, hexane/EtOAc 9:1, 8:2, 6:4, EtOAc) to yield 698 mg (83%) of **3** as a hygroscopic white gum; δ_H ($CDCl_3$/MeOD 2:1) 5.32−5.26 (m, 1H, OCH_2CHOCH_2O), 4.39−4.35 (dd, J = 12.0 Hz, J = 4.0 Hz, 2H, OCH_2CHOCH_2O), 4.26−4.21 (dd, J = 11.8 Hz, J = 6.2 Hz, 2H, OCH_2CHOCH_2O), 3.20 (s, 6H, 3 × $COCH_2S$), 2.61−2.57 (m, 6H, 3 × $COCH_2SCH_2$), 1.59−1.52 (m, 6H), 1.36−1.31 (m, 6H), 1.22 (br, 60H), 0.86−0.82 (t, J = 6.8 Hz, 9H); m/z (FAB^+) 901 ($[M]^+$); found $[M + H]^+$, 901.5948, $C_{51}H_{98}O_6S_3$ requires $[M + H]^+$, 901.5906.

Purity Evaluation. Analytical HPLC was conducted on a Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories PL-ELS 1000 evaporative light scattering detector and a C-4 peptide column. HPLC solvent mixes assigned as follows: mix A = H_2O/0.1% TFA; mix B = MeCN/0.1% TFA; mix C = MeOH. The solvent gradient used was: 0.0 min [100% A], 15−25.0 min [100% B], 25.1−45.0 min [100% C], 45.1−55.0 min [100% A]; at a flow rate of 1 mL/min. Purities were estimated from peak areas as **1**, 99.7 ± 0.1%; **2**, 99.6 ± 0.1%; and **3**, 95.3 ± 0.1%.

Animals and Treatments. Male Wistar rats (Mol:Wist) from Møllegard Breeding Laboratorium (Ejby, Denmark), weighing 170−210 g, were housed in pairs, both receiving the same treatment, in metal wire cages in a room maintained at 12 h light−dark cycles, at a temperature of 20 ± 3 °C and relative humidity of 65 ± 15%. Rats were acclimatized for at least a week under these conditions before the start of the experiments.[35] They had free access to tap water and a grower rat maintenance chow: Standard Laboratory Rat Chow R-34-EWOS-ALAB (EWOS Sweden). Two rats were housed per cage. The animals were acclimatized for at least 5 days before the start of the experiment. Weight gain and food intake were recorded daily. Fatty acid **1** and lipids **2** and **3** were suspended in 0.5% (w/v) carboxymethylcellulose (CMC) and administered by gastric intubation in a final volume of 0.8−1.2 mL once a day for 6 days. Control rats received palmitic acid suspended in CMC. At the end of the experiment, there was no difference in weight gain between the groups (data not shown). At the end of the feeding period, the animals were fasted for 12 h and anaesthetized subcutaneously with a 1:1 mixture of Hypnorm (fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL, Janssen Animal Health) and Dormicum (midazolam 5 mg/mL, Hoffmann-La Roche), 0.2 mL/100 g body weight. Cardiac puncture was performed to collect blood samples, and livers and hearts were dissected. Plasma was prepared, and triacylglycerol and cholesterol levels were measured using the Monotest enzymatic kit (Boehringer Mannheim, Germany) at a dose of 300 mg/day/kg body weight. Parts of the liver were immediately frozen in liquid nitrogen, while the rest of the liver was chilled on ice for homogenization. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Homogenization and Preparation of Subcellular Fractions. Livers and hearts from the rats were homogenized individually in ice-cold sucrose solution (0.25 M sucrose in 10 mM HEPES buffer pH 7.4 and 1 mM EDTA) using a Potter−Elvehjem homogenizer. Preparation of subcellular fractions was performed as previously described.[36] The procedure was performed at 0−4 °C, and the fractions were stored at −80 °C. Protein was assayed using the BioRad protein assay kit (BioRad, Heraules, CA) and bovine serum albumin as standard. To measure the hepatic fatty acid composition, fatty acids were extracted from subcellular fractions with 2:1 chloroform:methanol.[37] To the lipid extracts were added heneicosanoic acid (21:0) as internal standard and transesterified in 12% BF_3 in methanol (v/v). To remove neutral sterols and nonsaponifiable material, extracts of fatty methyl esters were heated in 0.5 mol/L of KOH in ethanol/water (9:1, v/v). Recovered fatty acids were re-esterified using BF_3-methanol. The methyl esters were quantified as previously described.[38]

Enzyme Assays. Palmitoyl-CoA oxidation was measured in the postnuclear fraction as acid-soluble products, using [1-^{14}C]-palmitoyl-CoA (Radiochemical Centre, Amersham, England) as substrate as described previously.[39] Carnitine palmitoyl transferase-II activities were measured in the mitochondrial fraction as described, and 3-hydroxy-3-methylglutaryl-CoA synthase activities were measured according to the method of Clinkenbeard et al. in the postnuclear fraction.[40,41] Finally, fatty acyl-CoA oxidase was measured in the postnuclear fraction as well by the coupled assay according to the protocols of Small et al.[42] The production of hydrogen peroxide was measured by monitoring the increase in dichlorofluorescein absorbance in the presence of palmitoyl-CoA.

Preparation, Characterization, and Effectiveness of Liposomal Formulations. General Procedures. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and stearic acid (SA) were purchased from Sigma-Aldrich. Stock solutions of the lipids (5 mg/mL) were prepared in CH_2Cl_2 and stored at −20 °C. Freshly distilled CH_2Cl_2 and PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) were used for liposome hydration.

Preparation of Liposomes. The relevant lipid mixture in CH_2Cl_2 was dried as a thin layer in a 50 mL round-bottomed flask. PBS was added to the lipid film and the mixture was then shaken for 10 min in a water bath at 40−50 °C. After this, the liposome suspension obtained was sonicated for 5 min.

Differential Scanning Calorimetry (DSC). Thermograms were obtained using a VP-DSC microcalorimeter (MicroCal Incorporated) at a heating rate of 1 °C/min from 5 to 60 °C. The phase transition temperature (T_c) was determined based on the peak top temperature.

Photon Correlation Spectroscopy (PCS). Particle sizes were measured on a N4 plus MD submicron particle analyzer (Beckman

Coulter, High Wycombe, Buckinghamshire, UK). All measurements were performed at 20 °C and recorded at 90 °C, with an equilibrium time of 1 min and individual run times of 300 s. The refractive index of the buffer was set to 1.333. Unimodal analysis was used throughout to calculate the mean particle size and standard distribution.

Zeta Potential (ZP). Zeta potentials were measured using surface charge electrophoresis on a Delsa 440 SX (Beckman Coulter, High Wycombe UK).

Liposomal 1 Formulation. Fatty acid **1** was formulated by combining **1** with DMPC. Different molar ratios of **1** to DMPC liposomes were analyzed and characterized. The final formulation used for the iv injection of **1** was **1**/DMPC (1:1, m/m) in PBS.

Liposomal 2 Formulation. Liposomal **2** was initially formulated from **2** alone, but it was found that the highest concentration that could be achieved was only 2.5 mg/mL of lipid. For higher concentrations, liposomal **2** formulations were prepared with SA. The final formulation used for the iv injection of **2** was **2**/SA (2:1, m/m) in PBS. The doses injected were calculated in relation to the amount of latent **1** present in **2**. The liposomes were suspended in phosphate buffered saline in the concentrations indicated and the solution sonicated before use. Nonesterified **1** (12 mM) was dissolved in 200 mg/mL albumin injection solution ("Octapharma") after dissolution in equimolar amounts of NaOH. Liposomes (Table 3) or free **1** in solution with BSA (1 mL) were injected at pH 7.0 through the tail vein. Following this, rats were anaesthetized with Fluorane (0.4 mL/100 g body weight), and sacrificed as described above.

Statistical Analysis. Statistical analysis was carried out in GraphPad Prism version 5.0a. All P values were two-sided and values <0.05 were considered statistically significant. Values are given as means with standard deviations (SD). One-way analysis of variance and Tukey's multiple comparison test were used to demonstrate significant differences.

Supporting Information Available: HPLC data collected to demonstrate the homogeneity and purity of the synthesized compounds fatty acid **1** and lipids **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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