Clavaric Acid and Steroidal Analogues as Ras- and FPP-Directed Inhibitors of Human Farnesyl-Protein Transferase

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We have identified a novel fungal metabolite that is an inhibitor of human farnesyl-protein transferase (FPTase) by randomly screening natural product extracts using a high-throughput biochemical assay. Clavaric acid [24,25-dihydroxy-2-(3-hydroxy-3-methylglutaryl)lanostan-3-one] was isolated from *Clavariadelphus truncatus*; it specifically inhibits human FPTase (IC_{50} = 1.3 μM) and does not inhibit geranylgeranyl-protein transferase-I (GGPTase-I) or squalene synthase activity. It is competitive with respect to Ras and is a reversible inhibitor of FPTase. An alkaline hydrolysis product of clavaric acid, clavarinone [2,24,25-trihydroxylanostan-3-one], lacking the 3-hydroxy-3-methylglutaric acid side chain is less active as a FPTase inhibitor. Similarly, a methyl ester derivative of clavaric acid is also inactive. In Rat1 *ras*-transformed cells clavaric acid and lovastatin inhibited Ras processing without being overtly cytotoxic. Excess mevalonate reversed the effects of lovastatin but not of clavaric acid suggesting that the block on Ras processing by clavaric acid was due to inhibition of FPTase and not due to inhibition of HMG-CoA reductase. Despite these results, the possibility existed that clavaric acid inhibited Ras processing by directly inhibiting HMG-CoA reductase. To directly examine the effects of clavaric acid and clavarinone on HMG-CoA reductase, cholesterol synthesis was measured in HepG2 cells. No inhibition of HMG-CoA reductase was observed indicating that the inhibition of Ras processing by this class of compounds is due to inhibition of FPTase. To date, clavaric acid is the second reported nitrogen-free compound that competes with Ras to inhibit FPTase activity. A series of related compounds derived from computer-based similarity searches and subsequent rational chemical synthetic design provided compounds that exhibited a range of activity (0.04 → 100 μM) against FPTase. Modest changes in the structures of these inhibitors dramatically change the inhibitory activity of these inhibitors.

Introduction

The development and progression of cancer is a multifactorial and, to a large degree, still an unknown process. Various genetic and environmental factors may have an impact on the development of cancer. One prevalent cancer-causing mutation involves a mutated form of the *ras* protooncogene that is associated with approximately 25% of all human cancers. Mutated forms of *ras* occur at higher rates in colon (>50%) and pancreatic (>90%) tumors.[1−4] The *ras* oncogenes encode Ras proteins that function, in part, by transmitting signals involved in cellular proliferation and differentiation. Ras, a guanine nucleotide (GTP) binding protein, links activated growth factor receptor tyrosine kinases to intracellular signal transduction pathways. In normal cells, an intrinsic GTPase regulates Ras activity promoting formation of a Ras−GDP complex that is functionally silent. Oncogenic forms of Ras, deficient in GTPase activity, are complexed to GTP. Mutant Ras is constitutively active and steadfastly transmits mitogenic signals inside the cell. Malignant transformation, in part, results from the uncontrolled stimulation of intracellular signal transduction pathways. Continued expression of oncogenic *ras* is crucial to maintain the transformed phenotype.

Normal and oncogenic forms of Ras are processed posttranslationally (for example, by farnesylation, proteolysis, methylation, and palmitoylation) before membrane association. Following prenylation by FPTase, Ras is anchored within the inner leaflet of the cell membrane. Farnesylation occurs on the carboxyl terminal cysteine residue of Ras, which is part of the CAAX motif, and precedes proteolysis, methylation, and palmitoylation. Inhibition of Ras farnesylation prevents Ras membrane localization and blocks Ras cell-transforming activity, thereby establishing FPTase as a valid and attractive target for anticancer chemotherapeutics.[3−13] Several FPTase inhibitors selectively inhibit Ras processing in cell lines,[14−20] prevent tumorogenesis in nude mice,[20,21] and promote regression of mammary and salivary gland carcinomas in *ras*-transformed transgenic mice.[22]

To identify unique chemical templates on which to design inhibitors of FPTase, we initiated a program to identify inhibitors of FPTase from natural product sources. We discovered chaetomellic acids,[5,23,24] fusidienol A,[25] preussomerins and deoxypreussomerins,[26]

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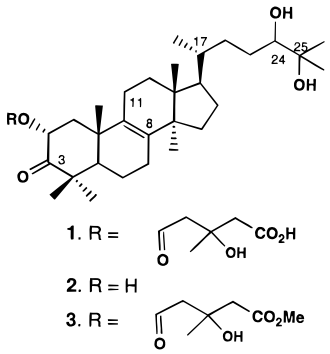


Figure 1. Structures of clavaric acid (**1**), clavarinone (**2**), and a methyl ester derivative (**3**).

actinoplanic acids,[27−29] barceloneic acid,[30] cylindrols,[31] and oreganic acid,[32,33] which are structurally diverse, selective, and potent natural product inhibitors of FPTase. Three of these inhibitors (chaetomellic acids, actinoplanic acids, and oreganic acid) were competitive with the farnesyl diphosphate substrate, while the remainder were noncompetitive with either substrate. Our extended search for unique inhibitors of FPTase from natural product sources resulted in the discovery of clavaric acid, a novel, specific, and reversible inhibitor of FPTase. Clavaric acid inhibits FPTase by competing with Ras and is the second reported nitrogen-free compound, the first being cembranolide,[34] to do so. The isolation and structural elucidation of clavaric acid were reported by Jayasuriya et al.[35] A series of related compounds derived from computer-based similarity searches and subsequent rational chemical synthetic design provided compounds that exhibited a range of activity (0.04 → 100 μM) against FPTase. Modest changes in the structures of these inhibitors dramatically change the inhibitory activity of these compounds. The biological characterizations of clavaric acid and a series of related compounds derived from computer-based similarity searches of internal databases and targeted chemical syntheses are described in this manuscript.

Results and Discussion

Our extended search for novel inhibitors of FPTase from random and targeted screening of natural product extracts resulted in the discovery of clavaric acid (**1**; Figure 1). The structures of clavarinone (**2**; a hydrolytic product of **1**) as well as a methyl ester derivative (**3**) are presented in Figure 1. The isolation and structure determination of clavaric acid have been described by Jayasuriya et al.[35] Clavaric acid has a molecular weight of 618 and an empirical formula of $C_{36}H_{58}O_8$.

Clavaric acid is an inhibitor of human FPTase activity exhibiting IC_{50} values of 1.3 or 0.35 μM when Ras-CVIM or Ras-CVLS is used as peptide substrate, respectively (Table 1). Clavarinone is a weaker inhibitor of FPTase activity with IC_{50} values of 15 μM (12 times less active than clavaric acid) or 8 μM (23 times less active than clavaric acid) when Ras-CVIM or Ras-CVLS is used as peptide substrate, respectively. Clavaric acid weakly inhibits human GGPTase-I activity with IC_{50} values of 21 or >50 μM when Ras-CAIL or Ras-CVIM is used as peptide substrate, respectively. Clavaric acid is 16 times more active against FPTase using Ras-CVIM than GGPTase-I using Ras-CAIL. Furthermore, clavaric acid inhibited rat liver squalene synthase with an IC_{50} value of 250 μM, reinforcing the conclusion that clavaric acid is a specific inhibitor of FPTase. Chaetomellic acid A and oreganic acid, compounds that are competitive with FPP,[5,23,33] when tested using either peptide substrate, inhibited FPTase activity with similar IC_{50} values. These compounds are also very poor inhibitors of human GGPTase-I. As previously reported these compounds were inactive against bovine GGPTase-I.[5,23,33]

Since the inhibitory activity of clavaric acid was dependent upon which peptide substrate was used, this implied that it may be interacting at the Ras binding site on the enzyme. To examine this directly, a kinetic analysis was performed, and the results are presented in Figure 2. Clavaric acid is competitive with respect to Ras-CVIM exhibiting an apparent inhibition constant (K_i) of 1.4 μM (Figure 2) and displays a complex kinetic pattern of mixed noncompetitive or uncompetitive inhibition with respect to the isoprenoid substrate (data not shown). Clavaric acid binds reversibly to FPTase (Table 2) and is not a time-dependent inhibitor of FPTase (data not shown). These data indicate that,

Table 1. Activity of Clavaric Acid against Prenyl-Protein Transferases Using Different Ras Peptides[a]

	IC_{50} (μM)			
	human FPTase		human GGPTase	
compd	Ras-CVIM	Ras-CVLS	Ras-CAIL	Ras-CVIM
clavaric acid	1.3	0.35	21	>50
clavarinone	15	8	35	>50
chaetomellic acid A	0.055[b]	0.035[b]	38	62
oreganic acid	0.014[c]	0.014[c]	60[c]	NT[d]

[a] Farnesyl-protein and geranylgeranyl-protein transferase assays were performed as described in Experimental Procedures. For FPTase assays, Ras peptides were used at either 100 nM (Ras-CVIM) or 400 nM (Ras-CVLS). For GGPTase-I assays, Ras peptides were used at either 500 nM (Ras-CAIL) or 1600 nM (Ras-CVIM). Compounds were dissolved in 100% DMSO and diluted 20-fold into the assay to give a final solvent concentration of 5%. [b] Data from Lingham et al.[23] [c] Data from Silverman et al.[33] [d] NT, not tested.

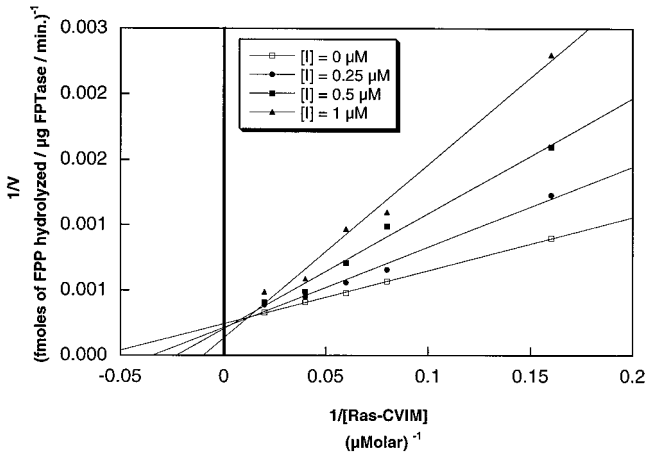


Figure 2. Kinetic constants were determined as previously described by Gibbs et al.[5] Ras-CVIM concentrations were varied as the concentrations of [^{3}H]FPP and human FPTase were held constant at 400 and 2 nM, respectively. The concentrations of clavaric acid used were 0, 0.25, 0.5, and 1 μM. Kinetic analysis was performed using k_{cat} enzyme kinetics software from Biometallics, Inc., Princeton, NJ.

Table 2. Reversible Nature of Clavaric Acid as an Inhibitor of Farnesyl-Protein Transferase[a]

	conditions	FPTase activity (fmol/μg/min)	percent inhibition (%)
	I. Initial Incubation and No Centrifugation		
1	FPTase + 5% DMSO	7342	
2	FPTase + 3 μM clavaric acid	2392	70
	II. Inhibitor + Centrifugation		
3	FPTase + 5% DMSO + centrifugation + 5% DMSO added back in the assay	3331	
4	FPTase + 5% DMSO + centrifugation + 3 μM clavaric acid added back in the assay	592	84
5	FPTase + 3 μM clavaric acid + centrifugation + 5% DMSO added back in the assay	3340	
6	FPTase + 3 μM clavaric acid + centrifugation + 3 μM clavaric acid added back in the assay	694	81

[a] Tubes containing 20 nM human FPTase were incubated with either (i) DMSO or (ii) 3 μM clavaric acid for 20 min at 31 °C. Aliquots (10 μL) were assayed directly (see condition I of the table). The remaining volumes were gel-filtered through 2.2-mL Sephadex G-25 columns equilibrated with 100 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, and 0.2% (w/v) *n*-octyl β-D-glucopyranoside. The column eluates (25 μL), in column equilibration buffer, were tested for FPTase activity as described.[23] DMSO or clavaric acid was added back to the assay tubes as shown above (see condition II of the table). Data were calculated relative to the DMSO control.

while clavaric acid binds reversibly to FPTase, the specific site of interaction of clavaric acid with FPTase is not known. It is most likely that clavaric acid binds at or close to the Ras binding site on FPTase to prevent Ras from associating with the enzyme. The data in Figure 2 is consistent with this conclusion.

The difference in the activity of clavaric acid against FPTase when different peptide substrates are used is likely due to the 20-fold higher affinity FPTase has for Ras-CVIM than for Ras-CVLS.[36−38] Clavaric acid is more potent when Ras-CVLS is the acceptor peptide suggesting that it is better able to displace Ras-CVLS from the enzyme. These data are consistent with those of Zhang et al.[38] who reported that 10−20-fold higher levels of CAAX-competitive compounds were required to inhibit Ki-Ras4A farnesylation. Clavaric acid is also active when the relevant peptide substrate (Ki-Ras-CVIM) is used and that the difference in potency can be attributed to the tighter association of Ras-CVIM with the enzyme.

GGPTase-I prenylates Ras-CAIL peptides and is, under certain conditions, also able to prenylate Ras-CVIM peptides.[38,39] Several groups have shown that cellular disruption of farnesylation of Ki-Ras results in geranylgeranylation of Ki-Ras to give a presumably active protein.[40,41] Furthermore, specific GGPTase-I inhibitors[18] disrupt processing of Ras in cells.[17] Since Ki-Ras is the major form of Ras involved in human malignancies, the ability to suppress both forms of prenylation may be useful. While clavaric acid is 16 times more active against FPTase, it is a very weak inhibitor of GGPTase activity (Table 1). A focused medicinal chemistry effort, with clavaric acid as a template, has the potential to produce dual inhibitors that may be more effective therapeutically.

In NIH3T3 or Rat1 cells transformed with viral Ha-*ras*, it is possible to monitor the effects of compounds that influence posttranslational processing of Ras. Transformed cells are incubated in the presence of compounds for 24 h and labeled with [^{35}S]methionine for the last 20 h. Ras is immunoprecipitated from detergent lysates of cell extracts using a monoclonal antibody to Ras. Farnesylation affects the mobility of Ras that is visualized on denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Clavaric acid was tested in this assay to determine whether it could modulate posttranslational processing of Ras in cells, and the results are presented in Figure 3. In the presence of 0.1% methanol (Figure 3, lane 1), or 200 μM

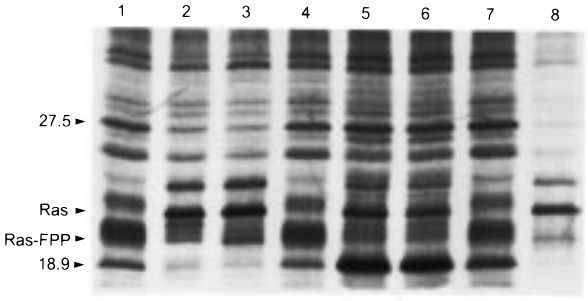
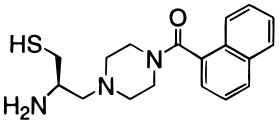


Figure 3. Rat1 cells, transformed with viral Ha-*ras*,[16] were incubated with methanol (0.1%, lane 1), lovastatin (15 μM, lane 2), **4** (10 μM, lane 3), mevalonate acid lactone (MVA; Aldrich; 200 μM, lane 4), clavaric acid (50 μM, lane 5), clavaric acid plus MVA (lane 6), lovastatin plus MVA (lane 7), and **4** plus MVA (lane 8) for 4 h, at which time fresh compound was added together with [^{35}S]methionine (133 μC/mL; Amersham). After incubation for an additional 20 h, cells were lysed, and Ras protein was immunoprecipitated, resolved by SDS−PAGE (15% gels), and detected by fluorography as described.[16] Ras-FPP, processed Ras; Ras, unprocessed Ras. Molecular sizes of protein standards are indicated on the left in kDa.

Chart 1. Structure of Synthetic FPTase Inhibitor



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mevalonate (Figure 3, lane 4), prenylated Ras migrates rapidly through the gel. In the presence of lovastatin, Ras migrated more slowly indicating that prenylation and posttranslational processing had not occurred (Figure 3, lane 2). These effects were reversed in the presence of an excess of mevalonate (Figure 3, lane 7). In the presence of clavaric acid (Figure 3, lane 5, 50 μM) or **4** (Chart 1; Figure 3, lane 3, 10 μM), Ras migrated slower through the gel. (**4** is a synthetic inhibitor of FPTase that is competitive with the Ras peptide substrate.[42]) This indicated the lack of prenylation and posttranslational processing that was not reversed by excess mevalonate (Figure 3, lanes 6 and 8).

These data, while indicating that the effects of clavaric acid were independent of inhibition of HMG-CoA reductase, did not eliminate the possibility that direct inhibition of HMG-CoA reductase by clavaric acid was occurring. To examine this directly, clavaric acid and clavarinone were tested in an assay to measure cholesterol production. HepG2 cells, when incubated with

Table 3. Effect of Clavaric Acid and Clavarinone on Cholesterol Biosynthesis[a]

compd	concn (μM)	[^{14}C]acetate incorporation into cholesterol (percent inhibition, %)
25-hydroxycholesterol	2.5	87
	25	90
clavaric acid	16	0
	32	0
	81	69
clavarinone	21	59
	42	74
	106	97

[a] HepG2 cells were seed and treated as described in Experimental Procedures. Compounds, dissolved in 100% DMSO, were added and incubated for 4 h. Cells were labeled with [^{14}C]acetate (3 μCi/mL) for 2 h, washed, saponified, acidified, and extracted twice with petroleum ether. Extracts were dried down, and 200 μL was spotted onto silica plates and developed in petroleum ether−ethyl ether−acetic acid (75:25:1). Plates were exposed to an intensifying phosphor screen overnight and read the next day on a phosphor imager (Molecular Devices).

labeled [^{14}C]acetate and extracted and processed as described in Experimental Procedures, incorporate labeled [^{14}C]acetate into cholesterol. 25-Hydroxycholesterol, a known inhibitor of HMG-CoA reductase, at 2.5 and 25 μM, decreased labeled [^{14}C]acetate incorporation into cholesterol by 87% and 90%, respectively. Clavaric acid was inactive at 16 and 32 μM. At 81 μM, clavaric acid decreased labeled [^{14}C]acetate incorporation into cholesterol by 69%. In contrast, clavarinone was a better inhibitor of cholesterol biosynthesis. At 21, 42, and 106 μM, clavarinone inhibited [^{14}C]acetate incorporation into cholesterol by 59%, 74%, and 97%, respectively (Table 3). Although at higher concentrations clavaric acid can suppress HMG-CoA reductase expression, clavarinone is more potent at suppressing reductase expression but less potent at inhibiting Ras processing thus dissociating inhibition of reductase and Ras processing by this class of compounds. Furthermore, addition of excess mevalonate to cells blocks the inhibition of Ras processing by lovastatin but not by clavaric acid or by other FPTase inhibitors. These data are consistent with the conclusion that the major intracellular target for clavaric acid is FPTase.

To examine the structural requirements for the inhibitory effects of clavaric acid and clavarinone, a stepwise computer-based two-dimensional topological similarity search was performed. The first search made use of the structural topology of clavaric acid and resulted in the identification of compounds exemplified by structures **5**−**10** shown in Chart 2. Like clavaric acid, all these structures contain keto-steroid ester moieties with one or more free carboxyl groups. A second similarity search based on the structure of **2** and subsequent refinement of these structural searches resulted in the structures shown in Chart 3. The latter search resulted in structures possessing one or more keto groups and falling in several distinct steroidal and triterpenoidal structural classes with or without free carboxyl groups. The representative structures include corticosteroids (**11**−**18**), lanostanes and related compounds including bile acids (**19**−**21**), and des-A steroid acids (**22**−**27**).

(A) Corticosteroids. The corticosteroid class of inhibitors can be classified into a negatively charged group exemplified by compounds **5**−**10** and a neutral group as illustrated by structures **11**−**18**. Cortisone 21-hemisuccinate (**5**) was the most active compound in the charged group (IC_{50} = 24 μM). Introduction of an additional negative charge as in **6** or reduction of the 11-keto group as in **7**−**10** reduced the inhibitory activity. The compounds in the neutral group also revealed a similar structure−activity relationship. Cortisone acetate (**11**) was the most active (IC_{50} = 12 μM) in this series with the exception of the dienone aldehyde **17**, which was 10 times more active (IC_{50} = 1.2 μM). The inhibitory activity of **17** was attributed to the presence of a conjugated aldehyde group at C-2, and this was confirmed when the aldehyde group was blocked as an hemiacetal (**18**) with an attendant decrease in potency (IC_{50} = 38 μM). A reduction in the inhibitory activity was also observed by either reduction or elimination of the 11-keto group (**12**−**14**) or introduction of an additional olefin in the A ring (**14**−**18**).

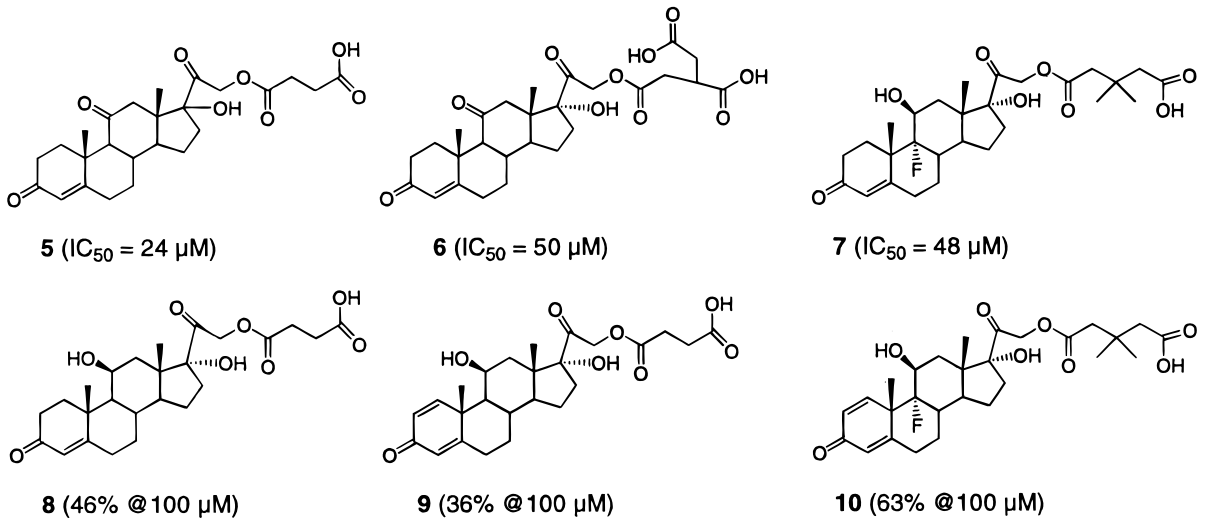
(B) Triterpenoids, Cholestanone, and Bile Acids. Lanostan-3-one (**19**) inhibited FPTase activity (IC_{50} = 12 μM), while related compounds including a triterpenoid (**20**), cholestan-3-one, cholesterol, 25-hydroxycholesterol, and bile acids as exemplified by **21** did not display any inhibitory activity at levels > 100 μM.

(C) Des-A Steroid Acids. The 7β-methyl des-A steroid acid **22a** was active against FPTase (IC_{50} = 0.9 μM). The compound lacking a 7β-methyl group, **23**, was 40 times less active, whereas the compound with 19-nor, **24,** was only slightly less active (IC_{50} = 1.2 μM) when compared to the activity of **22a**. Modification of the C-17 side chain reduced activity by 31-fold (**25**, IC_{50} = 31 μM). Replacement of the C-5 keto with a carboxyl group (**26** and **27**) increased the inhibitory activity by 2- and 5-fold, respectively, when compared with **23**. However, these compounds were less active than the 7β-methyl des-A steroid acid **22a**. Esterification of **22a** gave the methyl ester **22b** that was completely inactive at 100 μM.

Four essential features emerged from the analysis of the structure−activity relationship of the compounds described above. (1) A free alkyl carboxyl substituent in the B-ring of des-A steroid is required for inhibitory activity. (2) A 7β-alkyl substitution in the B-ring of the des-A steroid may contribute toward the potency. (3) The 11-keto group in the corticosteroids is essential for FPTase activity. (4) The inhibitory activity is sensitive to the functionality of the side chain at C-17. For example, compounds containing side chains as those in cholesterol are more active. These distinct structural requirements raised an important question, which is: are these features additive? To address these issues, a number of mixed and matched compounds were prepared and their inhibitory potential was evaluated as described below.

(I) 11-Keto Compounds with the B-Ring Possessing a Free Carboxyl Group. Oxidation of BMD-protected prednisone **28** provided the acid **29** (Scheme 1). Similar oxidations of 11-ketoprogesterone, **30** BMD protection, and subsequent oxidation of cortisone (**32**) gave acids **31** and **33**, respectively. Methylation of acid **33** with (trimethylsilyl)diazomethane afforded the methyl ester **34**. Acids **29** and **31** did not show any inhibitory activity at 100 μM; however, the acid **33** (IC_{50} = 29 μM)

Chart 2. Topological Search Results Using Clavaric Acid with Activity (FPTase IC_{50} or % Inhibition) in Parentheses



and the ester **34** (IC_{50} = 26 μM) inhibited FPTase with almost equal potency. These data indicate that, like clavaric acid, the latter compounds may be binding at or close to the Ras-peptide site on FPTase although the lack of inhibitory activity of **3** is an inexplicable contradiction.

Reaction of 11-dehydrocorticosterone (**35**) with isobutyl chloride in the presence of (dimethylamino)pyridine furnished the isobutyl ester **36**. Expectations were that the ester side chain would mimic the C-17 cholesterol side chain found in **22a**. Indeed, esterification did increase the activity significantly (IC_{50} value of 6.8 μM) as compared to the parent compound **35** (IC_{50} > 100 μM). Although the activity of cortisone acetate, **11,** and the isobutyl ester **38** was almost identical, a cortisone ester with an extended side chain, **39,** was 4 times less potent than the isobutyl ester **38**. Contrary to expectations, the oxidation of the enone group of **36** gave the des-A steroid acid **37**, the activity of which was decreased to 40 μM. Therefore, neither the introduction of the topologically similar side chain nor the introduction of the acid substituent in the B-ring, making the compounds resemble the des-A steroid system while keeping the 11-keto group, showed any additive effect toward the inhibitory activity of these compounds.

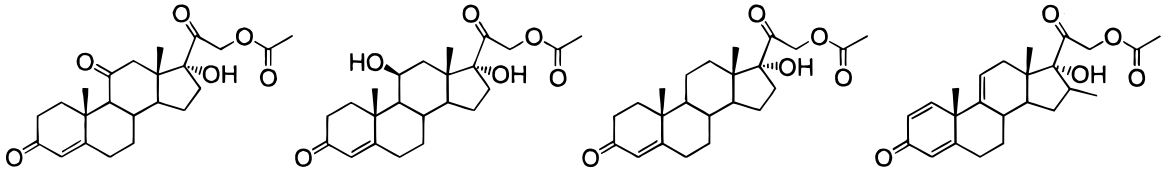
(II) Succinic Acid Analogues of Des-A Steroids. The lack of activity of **22b**, a methyl ester of **22a** (IC_{50} = 0.9 μM), led us to suspect that this class of inhibitors, like chaetomellic[5,23] and oreganic acids,[33] might have affinity for the FPP binding site of FPTase. To test this hypothesis, des-A steroids containing a succinic acid derivative were prepared (Scheme 2). Deprotonation of 4-cholesten-3-one (**41**) using LHMDS followed by reaction with ethyl bromoacetate provided the alkylated product **42**. Oxidative cleavage followed by alkaline hydrolysis yielded the diacid **45** that exhibited an IC_{50} value of 0.19 μM, a 5- and 210-fold increase in potency when compared to **22a** and **23**, respectively. Based on the structure−activity relationships described for the des-A steroids, a potency improvement would be expected from a 7β-alkyl substitution in the succinic acid analogue **45**. To test this hypothesis the 7β-methyl and 7β-ethyl analogues **48** and **51** were prepared. Compound **48** was prepared from **22a** via compounds **46** and **47** by enol lactonization and alkylation followed by alkaline hydrolysis (Scheme 3). Compound **51** was prepared via compounds **49** and **50** (Chart 4) following the protocol described in Scheme 2. The 7β-methyl analogue **48** exhibited an IC_{50} of 40 nM that, as expected, was a 5-fold potency improvement over compound **45**. Furthermore, **48** did not inhibit rat liver squalene synthase activity at levels up to 400 μM. However, the 7β-ethyl analogue **51** was 17 times less active (IC_{50} = 3 μM) than **45** and 75 times less active than the methyl analogue **48**. These results led to the preparation of the des-A 7β-ethyl analogue **52** that was compared directly with the methyl analogue **22a**. Similar to the succinate series (compounds **45**, **48**, **51**), the ethyl substitution at C-7 produced a 67-fold (IC_{50} = 60 μM) decrease in the inhibitory activity of **52** compared to the corresponding 7β-methyl analogue **22a** (IC_{50} = 0.9 μM) but only a 1.5-fold reduction in activity compared to **23**, the C-7-unsubstituted analogue. These data suggest that the hydrophobic interactions that occur with the methyl group at the C-7 position are optimal, while those afforded by the hydrogen or ethyl groups are not. This is likely due to either the smaller size of the hydrogen or the steric bulk of the ethyl group.

From close inspection of all the data, it becomes clear that structure−activity relationships are not additive within or between the compound series except in the des-A steroid series. The 11-keto group present in corticosteroids and the Δ^8 in the lanostan-3-one (**19**) series including clavaric acid play a significant role in its affinity to FPTase. The compound may interact with the CAAX binding site even in the presence of a free carboxyl group. A des-A steroid, for example **22a**, which lacks these two critical features, is competitive with FPP (Figure 4a) displaying a K_i of 0.325 μM and is uncompetitive with respect to Ras-CVIM (Figure 4b). Simple mono long-chain esters of 3-hydroxy-3-methylglutaric acids were inactive up to 500 μM. In addition, like clavaric acid, compound **14** partially inhibited Ras processing in Rat1 cells at 100 μM.

Steroidal compounds that structurally resemble clavaric acid have recently been reported to be FPTase inhibitors.[43] Andrastin C, the most active of the series, was a modest inhibitor of in vitro FPTase activity (IC_{50} = 13 μM) and was not reported to exhibit any Ras processing activity in cells.

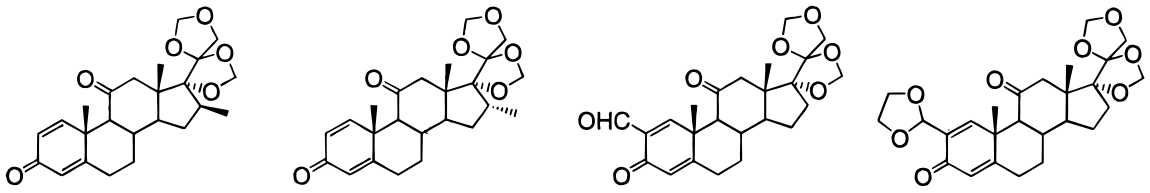
Chart 3. Topological Search Results Using Clavarinone with Activity (FPTase IC_{50} or % Inhibition) in Parentheses

A: (a) Corticosteroids neutral ester series



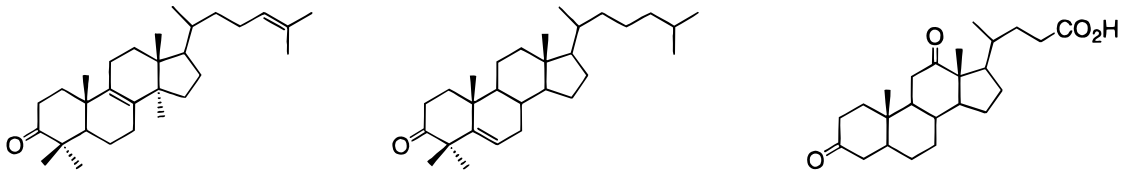
11 (IC_{50} = 14 μM) **12** (6% @100 μM) **13** (6% @100 μM) **14** (IC_{50} = 40 μM)

(b) BMD protected corticosteroids



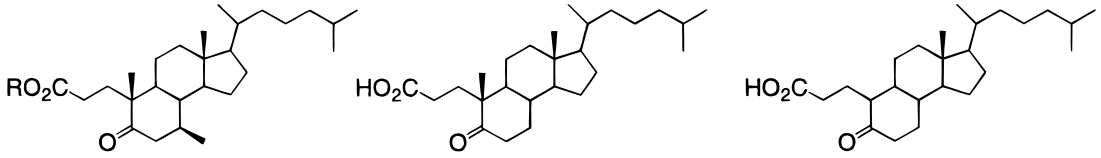
15 (IC_{50} = 96 μM) **16** (IC_{50} = >100 μM) **17** (IC_{50} = 1.2 μM) **18** (IC_{50} = 38 μM)

B: Lanostane, Cholestane and bile acid series:

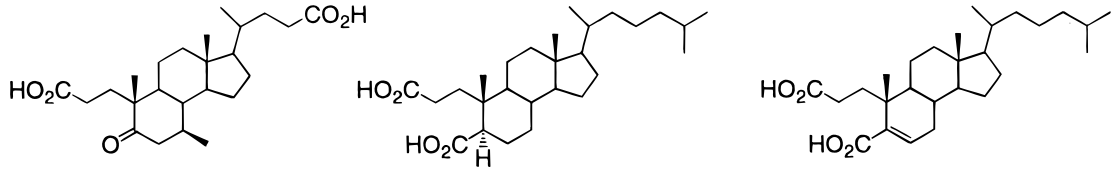


19 (IC_{50} = 12 μM) **20** (inactive @100 μM) **21** (33% @ 100 μM)

C: Des-A Steroid acids:



22a R = H, (IC_{50} = 0.9 μM)
22b R = Me, (IC_{50} = > 100 μM)
23 (IC_{50} = 40 μM) **24** (IC_{50} = 1.2 μM)



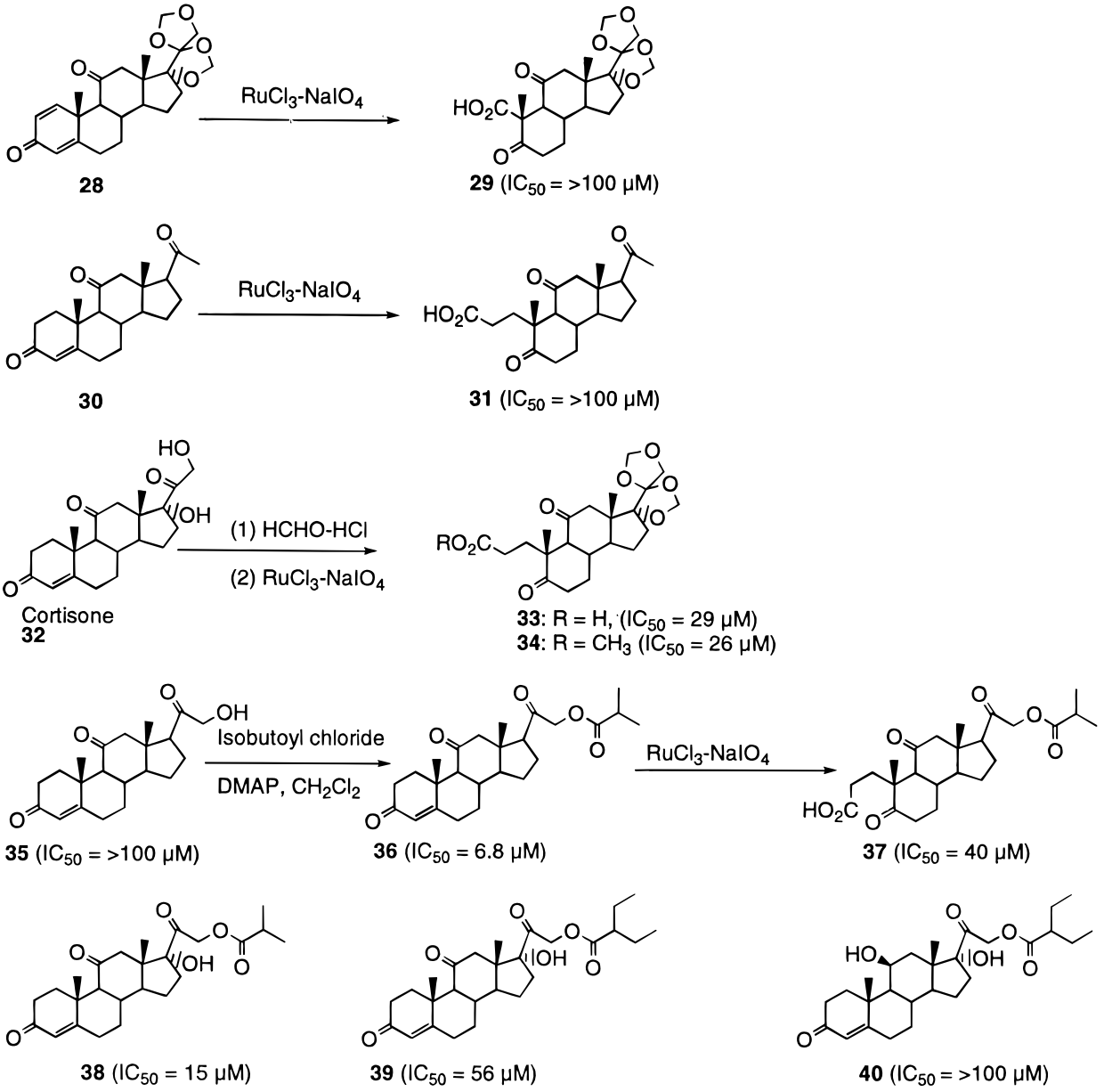
25 (IC_{50} = 31 μM) **26** (IC_{50} = 21 μM) **27** (IC_{50} = 8 μM)

In summary, clavaric acid is the second non-nitrogen-containing FPTase inhibitor described to inhibit Ras processing in whole cell assays; it is specific for FPTase and is competitive with respect to Ras. Furthermore, clavaric acid also provided valuable chemical insights that led to the design and synthesis of more potent compounds that can be selectively directed to either substrate binding site on the enzyme. This insight also resulted in the discovery of additional steroid- and terpenoid-based inhibitors. Modest structure−activity relationships within and between the different classes of compounds were also demonstrated. The discovery of clavaric acid is encouraging in that it reaffirms that natural products remain a vast and still unexplored resource from which to identify unique leads, and potentially drugs, for FPTase and other enzyme targets. The current challenge is to find more potent inhibitors of FPTase that exhibit in vivo activity.

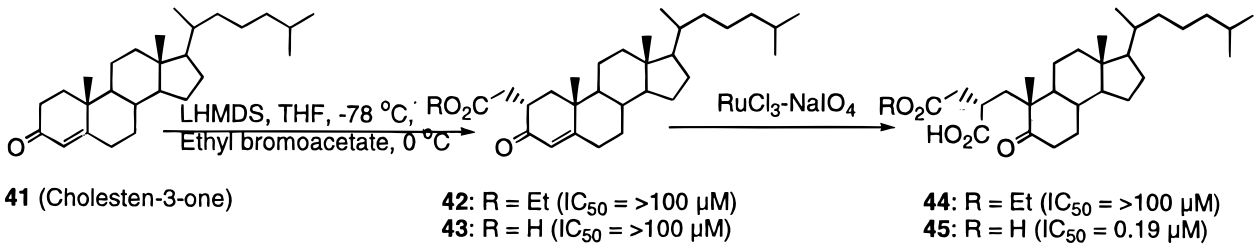
Experimental Procedures

Enzyme Assays. Recombinant human FPTase and GGPTase-I were prepared as described.[47] FPTase and GGPTase-I assays were performed as described[23] and contained the following: for FPTase, 2 nM FPTase, 50 nM [^{3}H]-farnesyl diphosphate (FPP), and 100 nM Ras-CVIM or 400 nM Ras-CVLS; for GGPTase, 2 nM human GGPTase-I, 100 nM [^{3}H]geranylgeranyl diphosphate (GGPP), and 500 nM Ras-CAIL or 1600 nM Ras-CVIM. All substrates were used at concentrations that corresponded to K_m levels. Kinetic constants were determined and reversibility studies were performed as described.[5,23,33] Fermentation extracts or pure

Scheme 1. Preparation of Esters and Des-A Derivatives of Corticosteroids



Scheme 2. Succinate Derivatives of Des-A Steroids



compounds were dissolved in 100% DMSO and diluted 20-fold into the assay to give a final solvent concentration of 5%. All FPTase IC_{50} data reported in this manuscript were determined by using Ras-CVIM peptide as a substrate and recombinant human enzyme unless reported otherwise.

Cell-Based Ras Processing Assay. The effect of compounds on cellular Ras processing was measured as described.[16] Briefly, Rat1 cells transformed with viral Ha-*ras* were incubated with compounds for 4 h at which time fresh compound was added together with [^{35}S]methionine (133 μC/mL; Amersham). After incubation for an additional 20 h, cells were lysed, and Ras protein was immunoprecipitated, resolved by SDS−PAGE (15% gels), and detected by fluorography as described.[16]

HepG2 Cell-Based Assay To Measure Cholesterol Production. HepG2 cells were seeded at 2×10^5/well in 6-well plates on day 0. On day 3, fresh media containing delipidated serum was added. On day 5, 1 mL of fresh media containing delipidated serum was added and test compounds dissolved in 100% DMSO were added and incubated for 4 h. Cells were labeled with [^{14}C]acetate (3 μCi/mL) for 2 h, washed, and saponified. The saponified material was acidified with HCl

Scheme 3. Succinate Derivatives of Des-A 7β-Methyl Steroids

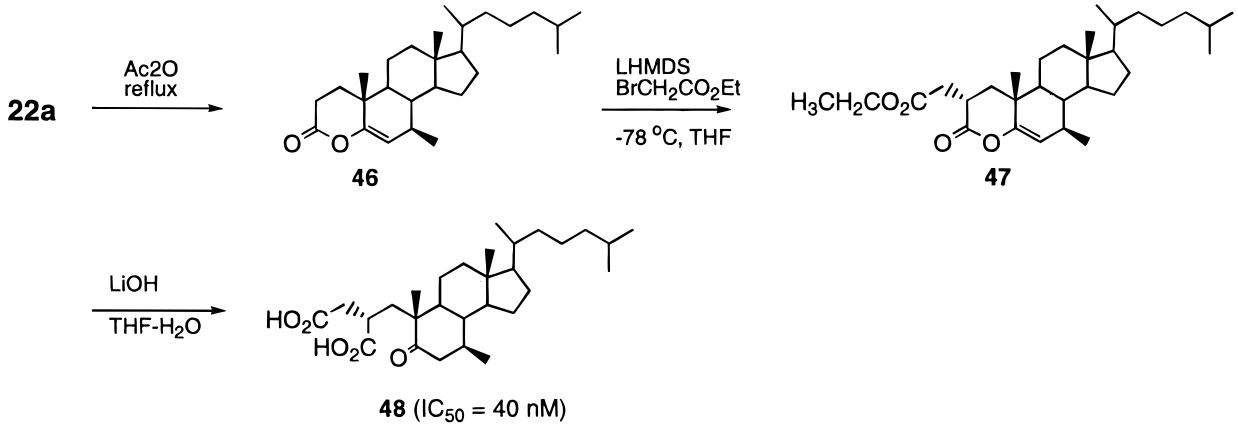
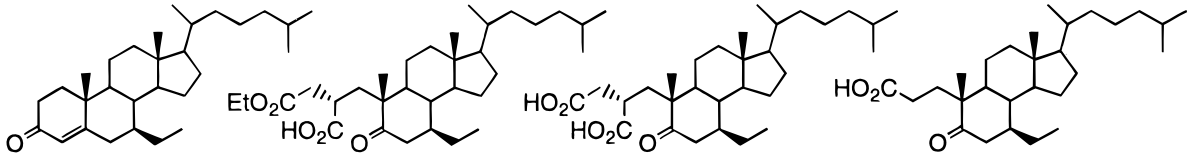


Chart 4. Ethyl-Substituted Des-A Steroids and Succinate Derivatives



and extracted twice with 4 mL of petroleum ether. Extracts were dried down, and 200 μL was spotted onto silica plates and developed in petroleum ether−ethyl ether−acetic acid (75:25:1). Plates were exposed to an intensifying phosphor screen overnight and read the next day on a phosphor imager (Molecular Dynamics).

Clavaric Acid (1) and Derivatives 2 and 3. The fermentation, isolation, and structure elucidation of clavaric acid (**1**) and the preparation of clavarinone (**2**) and a methyl ester derivative (**3**) have been described by Jayasuriya et al.[35]

Steroidal Inhibitors 5−52. A large number of steroidal inhibitors (**5**−**28**, **30**, **32**, **35**) reported in this study were prepared for other research programs at Merck and were obtained from the Merck sample collection. Cortisone 21-hemisuccinate (**5**) was also obtained from Research Corporation, while hydrocortisone 21-hemisuccinate (**8**), prednisolone 21-hemisuccinate (**9**), cortisone acetate (**11**), corticosterone 21-acetate (**12**), 11-deoxycortisone acetate (**13**), 11-ketoprogesterone (**30**), cortisone (**32**), prednisone, cholesterol, 25-hydroxycholesterol, and 4-cholesten-3-one were obtained from Sigma-Aldrich. Compounds **15** (RN 124113-90-2), **16** (RN 110428-41-6), **17** (RN 3903-93-3), **22a**,[44] **23**,[44] **29** (RN 100625-58-9), **33** (RN 62206-69-3), and **52**[44] are known in the literature.

Preparation of 31. 11-Ketoprogesterone was converted to the keto acid **31** in 16% yield as described below for the conversion of **42** to **44**. Anal. Calcd for $C_{20}H_{28}O_5$: C, 68.94; H, 8.10. Found: C, 69.32; H, 8.03.

Preparation of 37. The isobutyrate ester of cortisone was converted to the keto acid **37** in 53% yield as described below for the conversion of **42** to **44**. Anal. Calcd for $C_{24}H_{34}O_7 \cdot 0.3CHCl_3$: C, 62.05; H, 7.35. Found: C, 61.79; H, 7.49.

Preparation of 44. Cholestenone (**41**; 770 mg, 2.0 mmol) was dissolved in 8 mL of THF and cooled to −78 °C. A 1 M solution of lithium hexamethyldisilazide (2.4 mL) was added dropwise. The solution was warmed to 0 °C and stirred for 1 h. Ethyl bromoacetate (265 μL, 2.4 mmol) was added dropwise by syringe, and the cooling bath was removed. After 4 h, the solution was partitioned between EtOAc and saturated ammonium chloride. The organic phase was washed with saturated $NaHCO_3$ and brine. The organic solution was dried and evaporated. The residue was chromatographed on silica gel (EtOAc/hexane) to furnish 60 mg of **41**, 244 mg of a 1:3 mixture of **41** and **42**, and 418 mg (49%) of pure **42** as an oil. FAB mass spectrum: *m*/*z* 471 (M + H).

Compound **42** (240 mg, 0.51 mmol) was dissolved in a mixture of carbon tetrachloride (6 mL), acetonitrile (6 mL), and water (9 mL). $RuCl_3 \cdot H_2O$ (10 mg) and sodium periodate (656 mg, 3.08 mmol)[45,46] were added, and the mixture was stirred for 45 min. Diethyl ether was added, and the phases were separated. The ether solution was washed with dilute HCl and brine. After drying, the solution was evaporated, and the residue was chromatographed on silica gel (30% EtOAc/hexane) to give 114 mg (45%) of **44**. Anal. Calcd for $C_{30}H_{50}O_5$: C, 73.43; H, 10.27. Found: C, 73.67; H, 10.18.

Preparation of 43. A solution of **42** (60 mg, 0.13 mmol) was prepared in 4 mL of DME. Water (1 mL) and LiOH (53 mg, 1.3 mmol) were added, and the mixture was stirred overnight. After addition of 5 mL of 1 N HCl, the mixture was extracted with EtOAc. The extract was washed with brine, dried, and evaporated. The residue was chromatographed on silica gel (3−10% MeOH/$CHCl_3$) to give 26 mg (46%) of **43**. HRMS: *m*/*z* calcd for $C_{29}H_{47}O_3$ (M + H), 443.3525; observed 443.3521. Anal. Calcd for $C_{29}H_{46}O_3 \cdot 0.1CHCl_3$: C, 76.87; H, 10.22. Found: C, 77.07; H, 10.06.

Preparation of 45. Using the procedure described for the preparation of **43**, **44** was converted to the diacid **45** in 19% yield after chromatography. HRMS: *m*/*z* calcd for $C_{28}H_{47}O_5$ (M + H), 463.3424; observed 463.3437. Anal. Calcd for $C_{28}H_{46}O_5 \cdot 0.5CHCl_3$: C, 65.53; H, 8.97. Found: C, 65.38; H, 8.74.

Preparation of 48. The succinate derivative **48** was prepared as described in Scheme 3. A solution of **22a** (500 mg, 1.2 mmol) in acetic anhydride (7 mL) was refluxed for 16 h. Acetic anhydride was removed by distillation, and the product was purified on silica gel (2−4% EtOAc/hexane) to afford **46** (340 mg, 71%) as a gum. HRMS: *m*/*z* calcd for $C_{27}H_{44}O_2$ (M^+), 400.3341; observed 400.3254.

To a cooled (−78 °C) solution of **46** (200 mg, 0.5 mmol) in THF (5 mL) was added LHMDS (0.52 mmol). The yellow solution was stirred for 30 min followed by addition of bromoethyl acetate (100 mg, 0.6 mmol). After 20 min the reaction mixture was slowly allowed to warm to room temperature. The color of the solution changed to faint yellow, and the reaction was complete. The mixture was cooled to −78 °C and quenched with water and 10% aqueous citric acid. The product was extracted with EtOAc (50 mL), washed once each with 50 mL of 10% aqueous citric acid and water, and dried (Na_2SO_4). Solvent was removed under reduced pressure

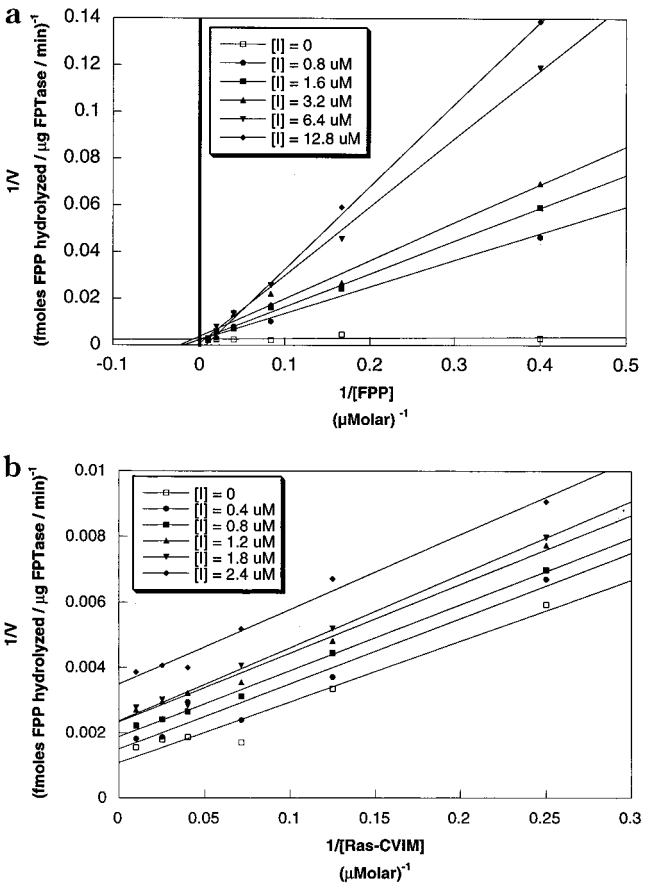


Figure 4. (a) Kinetic constants were determined as previously described by Gibbs et al.[5] [^{3}H]FPP concentrations were varied as the concentrations of Ras-CVIM and human FPTase were held constant at 100 and 1 nM, respectively. The concentrations of **22a** used were 0, 0.8, 1.6, 3.2, 6.4, and 12.8 μM. Kinetic analysis was performed using k_{cat} enzyme kinetics software from Biometallics, Inc., Princeton, NJ. (b) Kinetic constants were determined as previously described by Gibbs et al.[5] Ras-CVIM concentrations were varied as the concentrations of [^{3}H]-FPP and human FPTase were held constant at 100 and 1 nM, respectively. The concentrations of **22a** used were 0, 0.8, 1.6, 3.2, 6.4, and 12.8 μM. Kinetic analysis was performed using k_{cat} enzyme kinetics software from Biometallics, Inc., Princeton, NJ.

to give ester **47** which was used without purification. HRMS: *m/z* calcd for $C_{31}H_{50}O_4$ (M^+), 486.3709; observed, 486.3682.

A solution of LiOH (147 mg) in water (4 mL) was added to a solution of **47** in THF (4 mL). The solution was stirred for 2 h and acidified to pH 2.0 by dropwise addition of 4 N HCl. The desired diacid **48** was extracted with EtOAc (50 mL), and the organic layer was washed with water (50 mL) and dried (Na_2SO_4). The solvent was removed under reduced pressure, and the product was chromatographed on a reverse-phase HPLC (Zorbax RX C-8, 22 × 250 mm). Gradient elution with 60−90% aqueous CH_3CN containing 0.1% TFA followed by lyophilization of the fractions gave pure diacid **48** (105 mg, 44%) as an amorphous powder. HR-FAB: *m/z* calcd for $C_{29}H_{48}O_5Na$ (M + Na), 499.3399; observed, 499.3386.

Preparation of 50. The 7β-ethyl derivative **49** of cholestenone was prepared as described in the literature. Carboxymethylation and ring-A scission were performed as described in the preparation of **44**. Compound **50** was obtained in 19% overall yield. HRMS: *m/z* calcd for $C_{32}H_{55}O_5$ (M + H), 519.4050; observed, 519.4049.

Preparation of 51. Compound **50** was hydrolyzed as described in the preparation of **43** to furnish the diacid **51** in 30% yield. HRMS: *m/z* calcd for $C_{30}H_{51}O_5$ (M + H), 491.3736; observed, 491.3737.

Acknowledgment. The authors are thankful to Dr. R. K. Bakshi for helpful discussions on the synthesis of some compounds.

Supporting Information Available: Copies of ^{1}H NMR spectra of representative compounds **31**, **37**, **42**, **43**, **44**, **48**, and **50** (7 pages). Ordering information is given on any current masthead page.

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